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sVCAM-1 levels in patients with type 2 diabetes mellitus with micro- and macrovascular complications

Poziomy sVCAM-1 u pacjentów z cukrzycą typu 2 z powikłaniami mikro- i makronaczyniowymi

INTRODUCTION

Type 2 diabetes is a disease with a long latency period and for this reason it is often not diagnosed at early stage. In most cases, the is detected at the time of it first complications occurrence. Complications may also result from poorly treated diabetes. Late complications of diabetes are a major problem in diabetes care. They are a major mortality factor. Currently, 75% of diabetic patients die of cardiovascular disease. Late complications include micro and macroangiopathy, among them the most important being: diabetic nephro- and neuro-pathy, retinopathy, diabetic foot, hypertension, atherosclerosis, stroke, ischemic heart disease, myocardial infarction.

In the course of prolonged hyperglycemia changes similar to chronic inflammation occur in the organism, with increased phagocytic cell transition (neutrophils, monocytes) by the endothelium towards the sites of inflammation [16]. An important role in these processes is played by some of the protein structures found on the cell surface which serve to allow interaction among cells and between cells and the extracellular matrix. Those molecules are called adhesion molecules (cell adhesion molecules – CAM) [14]. So far, the known cell adhesion molecules have been classified into the following groups: integrins, immunoglobulin molecules (immunoglobulin supergene family), selectins, cadherins, and unclassified molecules- antigen CD44 [4,10].

Vascular cell adhesion molecule, or immunoglobulin related molecules, plays a special role in the controlling of oriented cell migration process (chemotaxis) to the extravasular space (outside the intravascular space) [14]. Vascular adhesion molecule-1 VCAM-1 (CD106) included into this group is a glycoprotein with a molecular weight of 110 kDa. VCAM is involved particularly in monocytes and endothelial cells adhesion and their passage through the endothelial barrier [14].

The soluble form of VCAM-1 (sVCAM-1) is considered an indicator of endothelial activation, as an early marker of immune activation and inflammation. Elevated sVCAM-1 concentrations were

found in the course of many neoplasms, including ovarian, stomach, intestines, bladder cancers, immune diseases: multiple sclerosis, lupus erythematosus, as well as in patients with type 1 and 2 diabetes mellitus [9]. Therefore, the aim of this study was to evaluate the concentration of soluble vascular cell adhesion molecules (sVCAM) in serum of patients with type 2 diabetes with associated micro-and macro-vascular complications.

MATERIAL AND METHODS

The study was conducted in 51 patients with type 2 diabetes (mean age 62.3 ± 9.3 years). Among the enrolled subjects were: 26 women (51%) with the mean age 40.3 ± 17.3 years and 25 men (49%) with the mean age 45.8 ± 18.0 years. The patients were treated in the Endocrinology Clinic of the Independent Public Clinical Hospital No. 4 (SPSK 4) in Lublin The average *disease* duration *since diagnosis was* 133.4 ± 84.2 months.

In the medical history records of the studied diabetic patients macro - (58.8%), micro-vascular (37.3%) and concomitantly (micro + macro) (76.5%) complications were found; mainly arterial hypertension (80.4%), coronary artery disease (53%), myocardial infarction (25.5 %), heart failure (17.7%), stroke (9.8%), diabetic nephropathy (9.8%), retinopathy (13.7%), polineuropathy (9.6%), diabetic foot syndrome (2%), metabolic syndrome (63.3%), and overweight based on BMI (65.3 %).

The control group was composed of healthy subjects (n=30) with the mean age of 55.1 13.2 years, attending the periodic health checks at the Department of Laboratory Diagnostics of the Independent Public Clinical Hospital No. 1 in Lublin.

The biochemical parameters were measured using a biochemical analyser Konelab (B*ioMérieux)* with dedicated reagents from the same company based on methods routinely used in clinical laboratories. The concentration of sVCAM was measured with immunoenzymatic ELISA (*enzym linked immunosorbent assay*) from DIACLONE. The reference range was 80–1502 ng/ml.

The clinical data and biochemical determinations were expressed with the use of descriptive statistics (mean – X, standard deviation – SD, median – Me). Distributions of the analysed variables were tested using the Shapiro-Wilk test. For a comparison of independent variables between patients with and without diabetes the Student t or U Mann-Whitney tests were used. Correlations between variables were investigated using Pearson's or Spearman's test. A p value ≤ 0.05 was considered as statistically significant in all analyses.

RESULTS

Table 1 shows the results the selected biochemical parameters in patients with diabetes mellitus and healthy controls. The median of sVCAM-1 concentrations in the group with diabetes was 847.8 ng/ml and was significantly higher than in control group (572 ng/ml).

Table 1. The concentrations of chosen biochemical parameters in the diabetic patients and in the control group

	Control Group	Study Group	P
sVCAM-1	604.0 ± 170.0 572.0 (334.7 – 996.8)	939.4 ± 307.4 847.8 (508.5 – 1750.0)	< 0.001
Glucose	89.0 ± 11.5 89.0 (70.0 – 113.0)	149.2 ± 41.6 137.0 (94.0 – 246.0)	< 0.001
HbA1c	-	8.72 ± 1.86 8.60 (5.10 – 14.00)	-
Urea	36.8 ± 11.5 35.0 (19.2 – 73.8)	41.3 ± 16.6 39.8 (10.6 – 107.4)	NS
Creatinine	0.85 ± 0.20 0.81 (0.60 – 1.30)	1.10 ± 0.28 1.05 (0.7 – 2.2)	< 0.001
Total protein	6.9 ± 0.7 7.0 (5.2 – 8.5)	6.9 ± 0.6 7.1 (5.7 – 8.0)	NS
Fibrynogen	3.41 ± 0.42 3.36 (2.2 – 4.8)	4.19 ± 0.94 4.12 (2.68 – 7.05)	< 0.05
CRP	3.25 ± 0.57 3.00 (2.99 – 4.10)	9.96 ± 16.7 4.41 (0.40 – 71.80)	NS
Total cholesterol	207.2 ± 38.5 196.5 (130.0 – 300.0)	194.1 ± 53.5 193.0 (88.0 – 344.0)	NS
HDL cholesterol	60.3 ± 17.4 56.5 (30.0 – 105.0)	40.9 ± 10.2 40.0 (25.0 – 77.9)	< 0.001
LDL cholesterol	124.6 ± 30.5 121.0 (64.0 – 181.0)	119.9 ± 46.4 117.0 (54.0 – 293.8)	NS
Triglycerides	112.1 ± 46.9 108.5 (26.0 – 203.0)	173.8 ± 119.3 149.5 (42.0 – 796.0)	< 0.01

Moreover, in diabetic patients we also found significantly higher levels of blood glucose (149.2 ± 41.6 mg/dl), creatinine (1.10 ± 0.28 mg/dl), fibrinogen (4.19 ± 0.94 g/l) and triglycerides (173.8 ± 119.3 mg/dl) in comparison with control subjects (respectively: 89.0 ± 11.5 mg/dl, 0.85 ± 0.20 mg/dl, 3.41 ± 0.42 g/L and 112.1 ± 46.9 mg/dl). However, the level of HDL cholesterol in the study group was significantly lower than in the control group (40.9 ± 10.2 mg/dl vs. 60.3 ± 17.4 mg/dl).

Table 2. sVCAM-1 concentrations in the study group with and without complications of diabetes

	Diabetic complications	
	present	absent
hypertension	933.2 ± 317.8 787.1 (508.5 – 1507.0)	964.7 ± 274.2 936.1 (599.5 – 1750.0)
Ischemic heart disease	899.9 ± 283.8 787.1 (508.5 – 1471.0)	983.8 ± 332.3 907.2 (547.1 – 1750.0)
Heart infarct	843.3 ± 250.2 784.4 (514.0 – 1416.0)	972.2 ± 321.0 919.6 (508.5 – 1750.0)
Stroke	862.1 ± 274.1 847.8 (514.0 – 1179.0)	947.8 ± 312.4 856.1 (508.5 – 1750.0)
Heart failure	986.9 ± 328.5 894.7 (514.0 – 1507.0)	929.2 ± 305.9 845.1 (508.5 – 1750.0)
Retinopathy	810.5 ± 182.3 762.3 (643.7 – 1160.0)	959.9 ± 319.5 882.3 (508.5 – 1750.0)
Nephropaty	899.1 ± 323.2 787.1 (514.0 – 1286.0)	943.8 ± 309.0 858.9 (508.5 – 1750.0)
Metabolic syndrome	940.2 ± 324.9 784.4 (508.5 – 1507.0)	948.3 ± 278.4 922.3 (552.6 – 750.0)
Microangipathic complications	876.7 ± 229.6 787.1 (514.0 – 1286.0)	976.6 ± 343.4 924.2 (508.5 – 1750.0)
Macroangiopayhic complications	831.3 ± 293.0 786.3 (508.5 – 1507.0)	991.1 ± 333.9 942.3 (547.1 – 1750.0)

The highest median of sVCAM-1 levels were found in patients with diabetes who showed macrovascular complications (Table 2). In these patients, the median level of sVCAM-1 was 942.3 ng/ml and it was significantly higher than that observed in patients without these complications (786.3 ng/ml). In this group, patients with myocardial infarct (919.6 ng/ml) and coronary heart disease (907.2 ng/ml) had significantly higher levels of sVCAM-1 compared with the group without complications. In patients with stroke and congestive heart failure sVACM-1 levels were comparable with the levels found in the group without complications.

In patients with microvascular complications, the median of sVCAM-1 concentration was 922.3 ng/ml and was also significantly higher than in patients without these complications (787.1 ng/ml). In this group diabetic retinopathy was the dominant type of diabetes complications and the concentrations of sVCAM-1 were 882.3 ng/ml.

Hypertension was the most common among the other complications of diabetes. In the patients group with hypertension the median level of sVCAM was 936.1 ng/ml and was significantly higher than in the control group (787.1 ng/ml).

The concomitant presence of type 2 diabetes and metabolic syndrome also significantly increased the concentrations of sVCAM-1. These patients had a significantly higher median concentration of sVCAM in comparison to the control group (784.4 ng/ml and 922.3 ng/ml; respectively).

In the study group a significant correlation between the concentration of sVCAM-1 and glycated hemoglobin was found (Figure 1). However, no significant relationships were found among sVCAM-1 concentrations and other parameters (age, height, weight, BMI, blood urea, creatinine, CRP, fibrinogen, total cholesterol, HDL cholesterol and triglycerides).

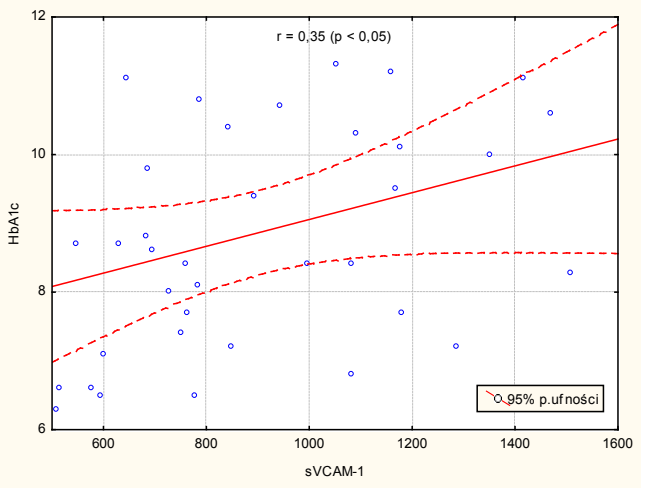

Fig. 1. Correlation of sVCAM-1 concentration with glycated hemoglobin in patients with type2 diabetes mellitus

DISCUSSION

Recently, a huge interest in adhesion molecules has been observed. It is related to the growing knowledge about their role in the pathogenesis of inflammatory and immunological diseases as well as in the development of type 2 diabetes complications [6].

Nowadays it is believed that endothelial cell dysfunction is not only a marker of vascular injury, but it plays an important role in the initiation, progression and the occurrence of vascular disease clinical symptoms due to atherosclerosis. The factors that stimulate endothelial cell dysfunction in atherosclerosis include: diabetes, oxygen free radicals from cigarette smoke, hemodynamic disturbances, as well as elevated LDL-cholesterol and homocysteine levels [17]. A high circulating level of serum adhesion molecules reflects their own expression on the surface of endothelial cells and may be an early marker of vascular lesions [17].

Results of this study showed an increase of soluble vascular cell adhesion molecules concentrations in patients with type 2 diabetes. This may indicate that in patients with macrovacular complications, hyperglycemia is a significant impact on these molecules expression and the presence of atherosclerotic lesions. Many other researchers confirmed elevated levels of sVCAM-1 in the blood of patients with atherosclerosis [13,17]; additionally, O'Brien et al. [12] showed a correlation of blood sVCAM-1 levels with the severity of atherosclerosis.

Results of the study by Otsuki et al. [11] conducted in patients with atherosclerosis with and without concomitant diabetes, revealed higher sVCAM-1 levels in patients with simultaneous diseases presence. Otsuki et al. [11] in their study conducted in patients with atherosclerosis with and without diabetes, noted higher levels of sVCAM-1 in patients with simultaneous diseases presence. Therefore, our results as well as numerous literature reports confirm that atherosclerosis is blood vessels' chronic inflammatory process, during which damage of endothelium and expression of adhesion molecules occurs.

Moreover, we found significantly higher levels of sVCAM-1(> 900 ng/ml) in patients with macrovascular complications compared with the group without these complications within the group of patients with myocardial infarction and coronary heart disease. Our results are accordance with other authors reports.

Jarosz and Nowicki [7] in their study compared the levels of sVCAM-1 between groups of men with and without coronary artery disease. They found very high levels of serum sVCAM-l which exceeded 800 ng/ml in patients with ischemic heart disease. These results may suggest that molecules as VCAM-1 could be a helpful parameter in assessing the probability of sudden vascular events in patients with coronary heart disease. Furthermore, they may be useful in the decision making in regards to therapy intensification in patients with high VCAM-1 concentrations.

A similar study was conducted by Drobniak-Hełdak et al. [3], who assessed the levels of sVCAM-1 in the group of 40 males with stable coronary artery disease without diabetes. These results showed increased levels of sVCAM-1 in patients with coronary artery disease compared with healthy subjects.

Furthermore, it was found that in the course of myocardial infarct (MI), due to muscle cells ischaemia (hypoxia), increased expression of adhesion molecules on the surface of endothelial cells in contact with the changed necrotic cardiomyocytes is observed. As a result, the number of molecules both on the surface of endothelial cells as well as their soluble forms in plasma increase. In patients with acute cardiac events an increased expression of sVCAM-1 soluble forms was detected [19].

Thus, on the basis of these results and available literature it could be concluded that both patients with type 2 diabetes and myocardial infarct, as well as patients with MI without diabetes demonstrate changes in the vascular endothelium, increased levels of adhesion molecules and acute phase proteins such as CRP and fibrinogen. This may indicate the presence of inflammatory endothelial changes in these patients.

In our study conducted in patients with type 2 diabetes we showed increased concentrations of adhesion molecules as a result of the history of stroke. In a stroke accompanied by inflammatory response, locally synthesized pro-inflammatory cytokines induce expression of adhesion molecules such as immunoglobulin like molecules, i.e. VCAM-1 at the surface of endothelial cells, increasing their concentration in the blood. This was confirmed in other studies, i.e. by Zareba and Losy [18]. Similarly, Fedorowicz and Chlopicki [5] demonstrated endothelial cells failure in terms of PGI, NO production and activation of endothelial inflammation in patients with hypertension, which manifests itself in increased soluble VCAM-1 concentrations.

In our study we found high levels of sVCAM-1 in the blood of patients with microvascular complications in whom the dominant pathology was diabetic retinopathy. Similar results were

obtained by Adamiec and Oficjalska-Mlynczak [1,2], in the group of 46 subjects with type 2 diabetes mellitus. They showed that the serum and ocular levels of sVCAM-1 and proinflammatory cytokines (IL-6, TNF-α) in diabetic patients significantly exceeded the values observed in the control group. At the same time, the obtained results confirmed the correlation of HbA1c with intraocular levels of VCAM-1. These results revealed directly proportional increase in the concentration of VCAM-1 and TNFα in the vitreous depending on the degree of compensation (evaluated by HbA1c)

Total cholesterol and LDL cholesterol concentrations remained at a comparable level between patients with diabetes and the control group, while triglyceride levels were higher, and HDL cholesterol lower in patients with type 2 diabetes. Similar results were found by Winiarska et al. [15]. Moreover, in the group of patients with type 2 diabetes we also found a significantly increased plasma fibrinogen. In contrast, Kolcowa et al. [33] found elevated levels of fibrinogen in patients with type 2 diabetes. Additionally, they found a positive correlation between concentrations of adhesion molecules and the concentration of fibrinogen. Since both proteins are markers of inflammation these results may indicate the presence of inflammation in these group of patients. They also found a positive correlation of adhesion molecules with LDL cholesterol concentration, and a negative one with HDL cholesterol is serum of patients with diabetes mellitus type 2.

CONCLUSIONS

Taking into account the available literature reports as well as the results obtained during this study we can conclude that the metabolic abnormalities characteristic of type 2 diabetes, i.e. hyperglycemia, are the results of inflammation and may be responsible for their intensification.

Markers of inflammation as adhesion molecules may be useful predictive indicators of cardiovascular complications of diabetes. More studies are needed to understand the sequence of events leading to abnormal vascular function in subjects with type 2 diabetes.

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SUMMARY

In the course of prolonged hyperglycemia, changes similar to chronic inflammation occur in the organism, with increased phagocytic cell transition (neutrophils, monocytes) by the endothelium towards sites of inflammation. An important role in these processes is played by some of the protein structures found on the cell surface which serve to allow interaction among cells and between cells and the extracellular matrix. Those molecules are called adhesion molecules (cell adhesion molecules – CAM). Therefore, the aim of this study was to evaluate the concentration of soluble vascular cell adhesion molecules (sVCAM) in serum of patients with type 2 diabetes with associated micro-and macro-vascular complications. The highest median sVCAM-1 levels were found in patients with diabetes who showed macrovascular complications. In this group, patients with myocardial infarct (919.6 ng/ml) and coronary heart disease (907.2 ng/ml) had significantly higher levels of sVCAM-1 compared with the group without complications. Taking into account the available literature reports

as well as the results obtained during this study we can conclude that the metabolic abnormalities characteristic of type 2 diabetes i.e. hyperglycemia, are the results of inflammation and may be responsible for their intensification. Markers of inflammation as adhesion molecules may be useful predictive indicators of cardiovascular complications of diabetes. More studies are needed to understand the sequence of events leading to abnormal vascular function in subjects with type 2 diabetes.

Keywords: diabetes, microangiopathy, macroangiopathy, sVCAM-1, cardiovascular complications

STRESZCZENIE

Zmiany zachodzące w organizmie człowieka w warunkach długotrwałej hiperglikemii przypominają przewlekły odczyn zapalny z nasilonym przechodzeniem komórek fagocytujących (granulocyty obojętnochłonne, monocyty) przez śródbłonek naczyniowy w kierunku miejsca zapalenia. Istotną rolę w tych procesach odgrywają struktury białkowe znajdujące się zwykle na powierzchni błony komórkowej i umożliwiające interakcje pomiędzy komórkami oraz komórkami i macierzą pozakomórkową, nazywane cząsteczkami adhezyjnymi (*cell adhesion molecules* – CAM). Celem niniejszej pracy była ocena stężenia rozpuszczalnych cząstek adhezji komórkowej naczyń (sVCAM) w surowicy krwi chorych na cukrzycę typu 2 ze współistniejącymi powikłaniami mikro- i mikronaczyniowymi. Najwyższe stężenie cząstek sVCAM-1 stwierdzono u osób chorych na cukrzycę, u których wykazano występowanie powikłań makronaczyniowych. W tej grupie chorych istotnie wyższe stężenia sVCAM-1 w porównaniu z grupą bez powikłań zaobserwowano u osób z zawałem mięśnia sercowego (919,6 ng/ml) oraz chorobą niedokrwienną serca (907,2 ng/ml). Biorąc pod uwagę doniesienia literatury jak również wyniki tej pracy można stwierdzić, iż zaburzenia metaboliczne, charakterystyczne dla cukrzycy typu 2, takie jak hiperglikemia, zarówno nasilają toczący się proces zapalny, jak i są jego rezultatem. Markery reakcji zapalnej, takie jak molekuły adhezyjne, mogą służyć jako predykatory powikłań sercowo-naczyniowych.

Słowa kluczowe: cukrzyca, mikroangiopatia, makroangiopatia, sVCAM-1, powikłania sercowo-naczyniowe

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URSZULA KOSIKOWSKA, MAREK JUDA, AGNIESZKA GRZEGORCZYK, ANNA BIERNASIUK, ANNA MALM

A comparison of colonization of the upper respiratory tract by Haemophilus influenzae and Staphylococcus aureus in healthy pre-school children exposed and unexposed to tobacco smoke

Porównanie kolonizacji górnych dróg oddechowych przez *Haemophilus influenzae* i *Staphylococcus aureus* u zdrowych dzieci w wieku przedszkolnym eksponowanych i nieeksponowanych na dym tytoniowy

INTRODUCTION

The ecosystem of the upper respiratory tract microflora may be a reservoir of bacterial pathogens, e.g. *Haemophilus influenzae* or *Staphylococcus aureus*, responsible for the community-acquired infections, especially in young children [11, 14]. The carriage of potentially pathogenic microorganisms within upper airways in children during the first years of life depends on various epidemiological and socioeconomic factors. There are controversial literature data concerning passive smoking as a risk factor predisposing to colonization of the upper respiratory tract by some bacteria [1, 2, 5, 7, 9].

The aim of our studies was to investigate if exposure to tobacco smoke had an effect on the rate of the upper respiratory tract colonization by *H. influenzae* and *S. aureus* in healthy pre-school children.

MATERIAL AND METHODS

During our experiments pre-school children (3–5 years old) were divided into two groups: 268 children attending a day care center (DCC group) and 76 staying at home (control group), were included. According to the parents' information, 159 (46.2%) children were exposed to tobacco smoke (parents or household members smoke in the presence of a child 2 or more cigarettes a day), whereas 185 individuals did not have contact with tobacco smoke.

Isolates of *Haemophilus influenzae* and *Staphylococcus aureus* were selected from swabs taken from throat and both nostrils (three samples from one child). The specimens were immediately

placed onto the selective media (*Haemophilus* chocolate agar and Chapman agar in the direction of *H. influenzae* and *S. aure*us, respectively) and then incubated in the appropriate atmosphere (with or without the increased CO_2 concentration) for 18–48 hrs at 35°C. The isolated microorganisms were identified on the basis of routine diagnostic methods (macroscopic, microscopic or biochemical assays) or by rapid commercial latex test – Slidex Staph-Kit (bioMérieux).

Statistical analyses included Fisher exact test and determination of relative risk (RR) was done using GraphPad InStad version 3 software.

RESULTS

It was found that the overall prevalence of *H. influenzae* in the upper respiratory tract of the assayed children was 11.3%, similarly in DCC group and in control group – 11.9% and 9.2%, respectively. The prevalence of *S. aureus* was 37.2%, also similarly in DCC group and in control group – 35.1% and 44.7%, respectively.

As presented in Table 1, in case of colonization of the upper respiratory tract by *H. influenzae*, the statistically significant difference among children exposed and unexposed to tobacco smoke was observed, both in the total population (16.3% vs. 7%) and in DCC group (17.2% vs. 7.1%). No statistically significant difference between passive smoking and nonsmoking children in the home group (12.9% vs. 6.7%), was found despite relatively high RR > 1.94, most probably due to the low number of children colonized by *H. influenzae*. There was no statistically significant difference in the prevalence of *S. aureus* within the upper airways in passive smoking children compared to those unexposed to tobacco smoke (Table 2).

Table 1. Influence of exposure to tobacco smoke on colonization of the upper respiratory tract by *Haemophilus influenzae* in healthy preschool children

Group of children	No. (%) of exposed children	No. (%) of unexposed children	*p* (RR)
DCC group (n = 268)	22 (17.2)	10 (7.1)	0.014 (2.41)
Control group (n = 76)	4 (12.9)	3 (6.7)	0.43 (1.94)
Total (n = 344)	26 (16.3)	13 (7.0)	0.0098 (2.33)

Table 2. Influence of exposure to tobacco smoke on colonization of the upper respiratory tract by *Staphylococcus aureus* in healthy preschool children

Group of children	No. (%) of exposed children	No. (%) of unexposed children	*p* (RR)
DCC group (n = 268)	43 (33.6)	51 (36.4)	0.7 (0.92)
Control group (n = 76)	18 (58.1)	16 (35.5)	0.06 (1.63)
Total (n = 344)	61 (38.4)	67 (36.2)	0.73 (1.06)

DISCUSSION

Several studies have been undertaken to assess risk factors for the prevalence of the main respiratory pathogens, including *H. influenzae* and *S. aureus* within the upper respiratory tract [2, 5]. An important factor appears to be exposure to tobacco smoke, resulting in damage of respiratory mucous membranes, facilitating bacterial adherence to airways epithelial cells and disturbance of host defense mechanisms [14].

Our data showed that exposure to tobacco smoke may predispose to increased colonization of the upper respiratory tract by *H. influenzae* in healthy pre-school children aged 3–5 years old, especially those attending day care centers. However, data of Greenberg *et al.* [5] and Principi et al. [13] indicate that exposure to tobacco smoke did not influence the *H. influenzae* carriage in the upper respiratory tract in children aged < 5 years. According to Pereiró et al. [12], children exposed to tobacco smoke had an increased risk for diseases caused by serotype b of *H. inf luenzae*. It should be noted that *H. influenzae* in one of gram-negative rods comprising the commensal flora of the upper respiratory tract [8, 10]. In young children it is the most common bacterial pathogen to cause respiratory infections (e.g. acute respiratory infection, pneumonia, otitis media), or invasive infections (e.g. bacteremia, meningitis) [8, 10].

According to our data, exposure to tobacco smoke had no noticeable effect on *S. aureus* prevalence in the upper respiratory tract. However, Durmaz et al. [6] found significant differences in *S. aureus* nasal carriage in smoking and nonsmoking people but in the adult population.

Discrepancies between our observations and literature data suggest that the effect of exposure to tobacco smoke on the *H. influenzae* or *S. aureus* prevalence in the upper respiratory tract may depend on various individual or socioeconomic factors, e.g. viral or bacterial respiratory infections, previous antibiotic treatment, age, being in large group (e.g. attending day care center), geographic area [4, 6, 11, 15].

CONCLUSIONS

The obtained data suggest that in young children, especially those attending day care centers, passive smoking may predispose to colonization of the upper airways by *H. influenzae* but not by *S. aureus*.

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SUMMARY

The effect of tobacco smoke exposure on colonization of the upper respiratory tract by *Haemophilus influenzae* and *Staphylococcus aureus* was assessed in healthy pre-school children (3–5 years old). A total of 344 children, including 268 attending a day care center and 76 staying at home were assayed. For identification of the bacterial isolates, routine diagnostic methods were used. Exposure to tobacco smoke statistically significantly increased the colonization of the upper respiratory tract by *H. influenzae*, but not by *S. aureus*. The rate of *H. influenzae* colonization in exposed and unexposed children in the total population was 16.3% vs. 7.0% ($p = 0.0098$, RR = 2.327) and in children attending day care centers – 17.2% vs. 7.1% ($p = 0.014$, RR = 2.41). The obtained data suggest that in young children, especially those attending day care centers, passive smoking may predispose to colonization of the upper airways by *H. influenzae*.

Keywords: Haemophilus influenzae, *Staphylococcus aureus*, upper airways colonization, passive smoking, healthy pre-school children

STRESZCZENIE

W badaniach oceniano wpływ narażenia na dym tytoniowy na kolonizację górnych dróg oddechowych przez *Haemophilus influenzae* oraz *Staphylococcus aureus* u zdrowych dzieci w wieku przedszkolnym (3–5 lat). Do badań włączono 344 dzieci, w tym 268 uczęszczających do przedszkola i 76 przebywających w domu. Do identyfikacji wyizolowanych bakterii stosowano rutynowe metody diagnostyczne. Narażenie na dym tytoniowy istotnie statystycznie zwiększyło częstość kolonizacji górnych dróg oddechowych przez *H. influenzae* w porównaniu z kolonizacją u dzieci bez ekspozycji, bez wpływu na częstość kolonizacji przez *S. aureus*. Wzrost częstości kolonizacji przez *H. influenzae* stwierdzono zarówno w ogólnej populacji – z 7,0% na 16,3% ($p = 0{,}0098$, RR = 2,327), jak i u dzieci przebywających w przedszkolach – z 7,1% na 17,2% ($p = 0{,}014$, RR = 2,41). Uzyskane dane sugerują, że u małych dzieci, zwłaszcza przebywających w przedszkolach, bierne palenie może być czynnikiem predysponującym do kolonizacji górnych dróg oddechowych przez *H. influenzae*.

Słowa kluczowe: Haemophilus influenzae, *Staphylococcus aureus*, kolonizacja górnych dróg oddechowych, bierne palenie, zdrowe dzieci przedszkolne

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Paclitaxel and apoptosis in breast cancer cells

Paklitaksel i apoptoza w komórkach raka gruczołu piersiowego

INTRODUCTION

In neoplasm cells alterations are observed in the function of genes which are responsible for regulation of cell cycle concerned with the increase of expression of genes participating in neoplasm processes. These changes can be connected with the influence on the activity of genes controlling growth, division and transcription processes in the cells.

Genes concerned with apoptosis belong to a group which can inhibit the process of transformation (Bcl-2, Bcl-XL, BclW, MCL1, BFL-1/A1, BCL-W, BCL-G) or can activate this process (BAX, BCLX, BAK, BOK, BAD, BIK, BID, BIM, BCL-XS, KRK, MTD, NIP3, NOXA, BCL-B) [5]. Proapoptotic genes of Bcl-2 family are proteins which are a part of intracellular organella, such as mitochondria, reticuloendothelial system, cytosol and cytoskeletal system. In the case of initiation of signal to apoptosis this protein passes through mitochondrial membranes and cause programmed cell death (BAX, BCLX, BAK, BOK, BAD, BIK, BID, BIM, BCL-XS, KRK, MTD, NIP3, NOXA, BCL-B). A gene which inhibits apoptotic process BCL-XL can be joined to APAF-1, and cause inactivation of caspases [2-4,9,16]. In the case of increasing the products of genes activating apoptotic process it causes increase in the permeability of mitochondrial membrane, which can lead do the release of cytochrom c to the cytoplasm.This factor binds with APAF1 and can activate caspases 8 and 9. Caspases 8 and 10 can be activated through TNF. Apoptosis can be induced by passing a signal from some receptors: APO1, IGF1R, TNFR1 inside the cell. This process activates caspases. P-53 protein, which leads to the increase of products connected with apoptosis (BAX, BCLX), can release c cyclin. This process is connected with activation of sphingomielin and release of ceramids. Ceramids inhibit the activity of genes which enable the survival of the cells. In the process of activation of apoptosis transcriptor factor NF-kB, MAPK family, ERK kinases, SAPK/JNK and caspases participate. This phenomenon can be induced through the increase of the activity of membrane receptors connected with FASL ligands. These factors bind with signal proteins FAS and cause degradation of the cells through activation of caspase 8.

Lately, multilevel analysis of gene expression performed by microarray technique has broadened the knowledge about proliferation of neoplasm. Microarray consists of stable plastic or glass grunt of small size on which are localized small points for genes [8].

Our analysis was connected with estimation of changes in the activity of genes participating in apoptotic and neoplasm processes under the influence of paclitaxel applied in the treatment of breast cancer.

Paclitaxel belongs to taxanes, which are a group of drugs binding with units of beta tubulin. Microtubules are structures which have the ability of polimerysation and depolimerysation. Drugs from the group of taxanes cause inhibition of this process. It leads to the inhibition of cell division in G2/M phase. The mechanism of the action of paclitaxel causes inhibition in forming the mitotic spindle. It increases the inhibition of neoplasm through the activation of apoptotic process and the inhibition of angiogenesis. It influences phosphorylation of BCL-2, and causes acceleration of apoptosis in neoplasm cells [2, 4, 6, 14,18].

MATERIAL AND METHODS

In research *in vitro* breast cancer cell line (T47D ECACC 85012201) was used. These cultures were incubated in standard conditions (RPMI 1640, 10% FBS, antibiotics, 37°C, 5% CO_2, 90% humidity of the air). Paclitaxel was administered to such cultures in the following doses: 60 ng/ml (P60 group) and 300 ng/ml (P300 group). These concentrations are equivalent to standard polytherapeutic and monotherapeutic doses. The control group was breast cancer cells *in vitro* incubated without paclitaxel.

TRNA from the cells was isolated and RT-PCR reaction was performed to estimate cDNA. The samples were exposed to specific human gene cancer primers (Human Cancer cDNA Labeling Primers – SIGMA). By exposing a reverse transcription reaction in buffor nucleotides labeled 32P cDNA were estimated. This product was purified in Sephadex column (Sephadex G-25) by centrifugation. Such cDNA were hybridized on the soil in a hybridization chamber during 18 hours. The matrix was washed after this process and stored in a special cassette containing radiosensitive soil. Then the soil of the matrix was scanned on a high resolution scanner detecting 50 microne points. The number of activities from points which indicated the level of expression following the genes was compared with a the model level of activity of E. coli-B1444 gene located on the matrix.

The next step was counting the activity from points of the matrix including the activity of individual genes in a control group and in groups where paclitaxel was administered (15).

Genes participating in apoptotic process were divided into the groups: apoptotic (BAD, BID, BIK, DEDD, BOK, CIDEP, CRADD) and antiapoptotic (BCL1,2,2A1,3,6,7A,9, BAX, BAG-1, DED,) caspases 1-10, 13-14). After estimation of the view of activity it was put in the computer created network.

Genes	Number	Example of Genes
Apoptotic genes	37	BAD, BID, BIK, DEDD, BOK, CIDEP, CRADD
Antiapoptotic genes	12	BCL1,2,2A1,3,6,7A,9, BAX, BAG-1, DED, DAD1,
Caspases genes	13	CASP1-10, 13-14,

RESULTS

Table 1. Expression of apoptotic and caspase genes in breast cancer cell line (T47D) after treatment of paclitaxel

	Control	Dose of paclitaxel 60 ng/ml	Dose of paclitaxel 300 ng/ml	P
Proapoptotic genes N=37	6.05	19.20*	4.15*	< 0.001
Antiapoptotic genes N=12	5.02	15.25*	4.95	< 0.001
Caspase genes N=13	4.85	14.92*	3.55*	< 0.001

The analysis showed that in a group of cells where paclitaxel was administered in a lower do 60 ng/ml it caused a statistically significant increase into the expression of pro and antiapoptotic genes and genes coding caspases in comparison to the control group.

A statistically significant correlation was noted between the control group and the first group for apoptotic genes, and a statistically significant correlation between the control group and the second group for the mentioned genes.

CONCLUSIONS

Results of the study showed that in a group of cells where paclitaxel was administered in a lower dose – 60 ng/ml it caused a statistically significant increase expression of pro and anti- apoptotic genes and genes coding caspases in comparison to the control group

High levels of expression in many genes connected with acceleration of proliferation can be related with apoptotsis of cancer cells which induces sensitivity of this cells to paclitaxel.

A significant decrease of caspases and apoptotic gene levels after administration of paclitaxel in a dose 300 ng/ml indicates the evident cytotoxic effect, which leads to the inhibition of the majority of cellular processes.

Activation of many ways connected with processes preceding G2/M phase can indicate that paclitaxel influences earlier control of cancer cell cycle phases.

DISCUSSION

In our investigation we found an increase of antiapoptotic gene expression derived from Bcl2 family and also a similar increase in the level of proapoptotic genes. It is strange that a similar level in these two opposite group of genes induces only apoptosis processes. Inhibition of cell growth and death of the cells was seen after administration of paclitaxel in a dose 60 ng/ml. There are different explanations of this process. One of them can be connected with some domination of the expression of apoptotic genes. This hypothesis dose not explain entirely why the processes of apoptosis are so intensive in the breast cancer *in vitro*. Another explanation can be connected with a probably greater intensity of metabolic pathways in these cultures (bigger

number of this processes in comparison to antiapoptotic). This suggestion could be supported by described in many reports possibility of phosphorylation of products of Bcl-2 by many proteins, which leads to inhibition of Bcl-2 activity. Such proceses are seen when MAPK is activated. Another kinase which is responsible for the mentioned phosphorylation BCL-2 is PKA (activated by microtubuls fractures) [8] and is also connected with the activation of cRAF kinase which leads to the activation of kinase A and is dependent on the degree of polimerysation of microtubules. In addition, phosphorylated form of BCL-2 can activate caspase 3 which can lead to an irreversible apoptotic process. Apoptotic processes have prevalence because in the cells a very intense process of phosphorylation of BCL-2 products is seen. It leads to inhibition of antiapoptotic products and escalates apoptosis processes in the mechanism of activation of many caspases. Selective activation of apoptosis in T47D cell line should be consider under influence of paclitaxel and sensitivity to this drug. In reports information appears that paclitaxel can activate inhibition of mitotic process [13]. One of the mentioned mechanisms is concerned with serine-threonine kinase cMOS. It was noticed this protein can be stored during mitotic division of the cells and is responsible for phosphorylation of tubulin protein.

Chang et all. [4] noticed an increased level of 78 of 92 genes coding apoptotic and thermal shock proteins in 48% of 24 women with breast cancer treated with paclitaxel. It indicates that this drug influences the activation of products concerned with apoptosis. It can explain why apoptotic processes have so strong manifestation in the treated lines of the cells. In the activation of apoptosis by paclitaxel we can find at least 3 ways: phosphorylation BCL-2, activation of apoptosis during inhibition of cell division and direct activation of apoptotic products. Another explanationof the domination of apoptosis process in these cultures is possible. Research conducted on T47D line reveals that increased expression of BIC product and ER-2 receptors is observed after administration of beta estradiol. In cultures we also observed increased expression of Bic. It is known that BIC can bind with BCL-2 protein and inhibit its activity. Perhaps this way of activation is significant and has strong influence on the inhibition of BCl-2 product. Additionally, another raport [7] described protein DAXX which belongs to apoptotic products. This protein interacts with repressors of transcription such as PAX5, ETS-1 and reduces their expression. DAXX activates many ways concerned with apoptosis [9,10]. They are connected with increased expression of genes coding caspases, transcriptor factors as well histone proteins. In connection with activation of ASK-1 it is a very important way of apoptosis. A high level of this protein induces the activity of caspases 8 and 9, NFk-B and E2 F1. It can prove that in cell cultures irreversible apoptotic processes dominate impossible to inhibition even if the level of BCL-2 is high. These processes are induced by irreversible degradation for cell organella by caspases. It is the reason for advantageous apoptotic process despite the high level of antiapoptotic products even in active forms. Activation of apoptosis is induced by caspase 3. It interacts with ICAD which is the reason for the lower activity of CAD and leads to activation of DNA-se. In this process CIDE participates which, after connection with DFF45, activates DNA-se in the same way. Another pro-apoptotic protein DAP-3 binds with the inner mitochondrial membrane. It activates apoptosis by binding to DISC complex through TRAIL, FAS and FADD [1,3,12]. It probably stops translation process in the matrix of mitochondrium by replacing the active product of translation in the site of initiation.

The increase of the level of apoptotic genes has clinical significance. The statement of percentage of activity pro and antiapoptotic genes in relation to all group of activated genes and also estimation of the degree of their expression and gene classification can estimate the degree of malignancy of the neoplasm, sensibility to treatment. In breast cell cancer MCF-7M increased expression of Bcl-2 an low p-53 and Bax was observed [9,11,17].

The increase of the expression of antiapoptotic genes after administration of paclitaxel in a dose 60 ng/ml requires explanation In spite of the fact that the examined cell showed intense division. These observations suggest that expression of antiapoptotic genes should not be so intense. It is possible that expression of these genes is less that proapoptotic. Another explanation suggests the levels of both group are similar, but because of an unknown cell mechanism prevalence is achieved by proapototic genes.

It is possible that in this process caspase activation has significance, which leads to an irreversible reaction causing degradation of cell structures.

Paclitaxel in a dose 60 ng/ml is activated opposing by groups of pro and antiapoptotic genes. The probable reason for the advantage of apoptotic processes over antiapoptotic processes in these cancer cells may be explained by activation of a significant numbers of ways connected with phosphorylation of Bcl-2 gene products.

It was proved that activation of apoptotic process is connecteded with the proper relation between pro and antiapoptotic genes. It should be examined whether such ways of activation are connected with the influence of paclitaxel, or the type of activity of the following cancer cell lines. It was stated that beginning of apoptosis process is connected with a lasting proper relation the expression of Bax/Bcl-2, which influence the mitochondrial potential and increase of permeability of mitochondrial membrane. The proper relation of these group of genes influence the activation or inactivation of apoptosis. This information indicates that induction of apoptosis is concerned with the proper relation both of these groups.

Chang and al. [4] conducted the analysis of the expression of genes treated with docetaxel in the base of RNA extracted after biopsy conducted in 24 women with breast cancer. Reports showed overexpression of 14 from 92 antiapoptotic genes in 54% patients. It is possible that the expression level is responsible for the difference in the reaction after treatment with paclitaxel. Perhaps this reaction is dependent on the relation between the level of many groups of genes.

Possible reason high activity of antiapoptotic genes, and their simultaneous weak influence on inhibition of apoptotic process can be an effect of higher expression mentioned genes connected with phosphorylation their protein products inducted by paclitaxel.

The reasons for the decrease in the antiapoptotic activity of Bcl-2 can be different. It is possible the an increase of resistance of the cell to this drug caused by the increase of the activity of antiapoptotic genes. Probably it activates enough of these genes and their product sand leads to anti-apoptotic process. In this activity opposite mechanisms can be activated. For instance, BAX can influence Bcl-2 and induce inactivation of this gene. It can activate dimerisation and translocation in mitochondria which leads to programmed cell death.

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SUMMARY

Our analysis was connected with estimation of changes in the expression of genes participating in the apoptotic process under the influence of paclitaxel applied in breast cancer cells *in vitro*. Increase of the expression of antiapototic genes after administration of paclitaxel in a dose 60 ng/ml requires

explanation. These observations suggest that the expression of antiapoptotic genes should not be so intense. It is possible that the expression of antiapoptotic genes is less that proapoptotic. Another explanation suggests the levels of both group are similar, but because of an unknown cell mechanism the prevalence is achieved by proapototic genes. It is possible that in this process caspase activation has significance, which leads to a irreversible reaction causing degradation of cell structures.

Paclitaxel in a dose 60 ng/ml is activated by opposing groups of pro and antiapoptotic genes. The probable reason of the advantage of apoptotic processes over antiapoptotic processes in breast cancer cells may be explained by activation of a significant number of ways connected with phosphorylation of Bcl-2 gene products.

Keywords: paclitaxel, apoptosis, breast cancer, caspases, microarray method

STRESZCZENIE

Badania nasze miały na celu określenie zmian ekspresji genów uczestniczących w procesie apoptozy pod wpływem paklitakselu dodawanego do komórek raka piersi *in vitro*. Wzrost ekspresji antyapoptotycznych genów po podaniu paklitakselu w dawce 60 ng/ml wymaga wyjaśnienia. Badania sugerują, że ekspresja tych genów nie powinna być tak intensywna. Możliwe jest, że ekspresja antyapoptotycznych genów jest niższa niż genów proapoptotycznych. Inne tłumaczenie to podobne poziomy ekspresji obu grup genów z nieznanym mechanizmem komórkowej regulacji genów proapoptotycznych. W procesie tym dużą rolę może odgrywać aktywacja kaspaz, która prowadzi do nieodwracalnej reakcji powodującej uszkodzenie organelli komórkowych. Paklitaksel w dawce 60 ng/ml aktywuje dwie antagonistycznie działające grupy genów: pro- i anty- apoptotyczne. Przewagę proapoptotycznego procesu nad antyapoptotycznym w komórkach nowotworowych piersi można tłumaczyć aktywacją wielu szlaków związanych z fosforylacją białek kodowanych przez gen Bcl-2.

Słowa kluczowe: paklitaksel, apoptoza, rak piersi, kaspazy, metoda microarray

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Amikacin-modified hybrid biomaterial biological properties

Właściwości biologiczne hybrydowego biomateriału modyfikowanego amikacyną

INTRODUCTION

Bone defects represent major clinical problems in the practice of reconstructive orthopaedic and craniofacial surgery. Treatment for these applications, such as autogenous or allogenous bone grafting have some limitations, so new approaches for bone tissue repair are required. Calcium and phosphate ceramics are being increasingly used as bone substitutes in orthopaedic, oral and maxillo-facial surgery. Such ceramics are biocompatible and lead to local osteogenesis in anosseous site. However, in cases involving large bone defects, there is still a need to reduce the time necessary to establish the ceramic-bone interface and bony ingrowth [1,7]. Recently, many biological substances such as bone morphogenetic proteins (BMPs) [2], transforming growth factor-*beta* (∃-TGF) [3] or keratin [10] have been investigated for their bone-inducing properties. Some antimicrobial substances combined with hydroxyapatite (HAp) influence on human bone were also studied [9]. However, this idea of connection HAp with various biological substances needs to answer such questions as the dose, the suitable carrier acting as a slow release delivery system, the mode of impregnation and the activity *in situ* of these substances. Lack of control of one of these parameters might lead to cytotoxicity, osteosarcoma formation or ectopic bone formation [1].

Experimental verification of amikacin-modified hybrid biomaterial influence on osteoblast cell culture was the main aim of this study.

MATERIALS AND METHODS

Biomaterials

Hydroxyapatite (HAp) was made in Department of Technology of Ceramics and Refractories, AGH-University of Science and Technology, Cracow, Poland. Hydroxyapatite parameters were: diameter: 0.3-0.5 mm, open porosity: 67%, sintering temperature: 800ºC.

Immobilization process

Portion of HAp was covered by γ-aminopropyltriethoxysilane and divide into three parts. Two parts of silanized-HAp were chemically modified by two kinds of protein (porcine gelatin or keratin derived from human hair) according to Weetall [11] procedure in authors own modification. So, three types of matrix were obtained (silanized-HAp, gelatin-HAp and keratin-HAp). Amikacin (Biodacyna®, Bioton, Poland; 250 mg/ml) was immobilized according to the Polish Patent [4] and its concentration was estimated spectrophotometrically according Ginalska et al. method [5].

Cell culture

Human fetal osteoblasts cell line hFOB 1.19 from ATTC (American Type Culture Collection) was used in our *in vitro* research. Cell cultures were incubated in 34°C in a humidified atmosphere consists of 95% air and 5% CO_2. Day before the beginning of experiment HAp granules were stabilized in culture medium. Next HAp granules were inoculated with hFOB cells (5×10^4) suspended in fresh culture medium. Osteoblasts growth and division in presence of HAp granules were observed during this experiment. Cell growth was estimated after 72, 120, 168, 216, 264, 312, 360 and 408 hours. The growth medium was replaced every two days. Cell viability was determined by 0.4% Trypan Blue exclusion.

LDH activity test

Substances, which have toxic influence on cells can caused their membrane damage. It leads to some enzymes release. Lactic dehydrogenase (LDH) is one of them. LDH concentration in medium samples was assayed to define whereas HAp granules indicate cytotoxity against human osteoblasts. The more toxic biomaterial is, the higher level of LDH in medium samples is detected. As a control we used osteoblasts growing in culture medium directly on polystyrene plates. LDH activity was measured after 24h of hFOB growth on HAp granules using LDH Cytotoxicity Detection Kit (Roche Diagnostic, Switzerland).

Confocal microscopy

Firstly, hFOB cells were cultured on HAp granules in LabTek chambers (Nunc, Denmark) during 360 hours. Next granules covered by hFOB cells were washed twice in PBS and then stained with 3,3'-dihexyloxacarbo-cyanine iodine ($DIO_{3(6)}$) for 10 minutes in dark. Samples were analyzed using confocal microscope (LSM-5, Zeiss, Germany) at 514 nm.

RESULTS

Cell viability

New hybrid biomaterial influence on growth and division of human osteoblasts was tested. Data presented on FIGURE 1 showed that during the experiment, cell number increased with time reaching the highest value at 312 h. HAp granules did not inhibit osteoblasts growth. Cell viability exceeded 95% what indicates that modified carriers were non toxic to osteoblasts. These results proved that our biomaterials modified by amikacin could be used as implants in orthopaedic, oral and maxillo-facial surgery in the future.

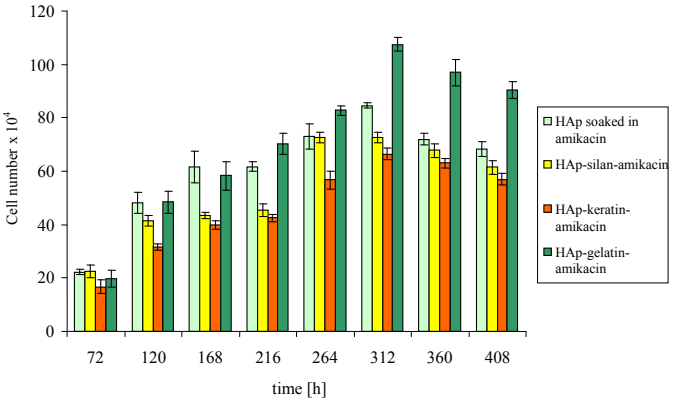

Fig. 1. Osteoblasts' growth on HAp granules depending on cell culture duration

LDH activity test

Assessment of HAp granules modified by amikacin potential cytotoxicity was the main purpose of experiment. To reach this aim LDH activity was measured in extracellular medium. Osteoblasts cell culture was a control in our study. Results presented in FIGURE 2 showed that none of HAp granules revealed cytotoxicity features. LDH activity was similar to control LDH activity (osteoblasts cell culture without any HAp granules type presence) for all types HAp granules. It was also observed that HAp granules with amikacin influenced on LDH activity reduction and gave evidence that smaller number of osteoblasts was damaged.

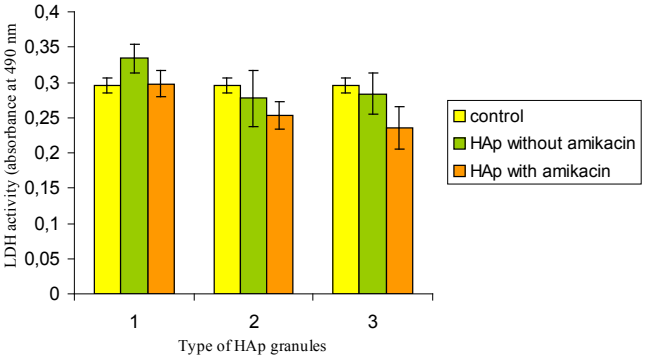

Fig. 2. Various HAp types influence on LDH activity.

Confocal microscopy

New hybrid biomaterial HAp-amikacin microphotographs were made using confocal microscope to visualize osteoblasts cells presence on amikacin modified HAp-granules. FIGURE 3 presented

HAp granules surface completely covered by osteoblasts what confirmed our previous experiments and proved that examined biomaterial did not show toxic effect towards human osteoblasts.

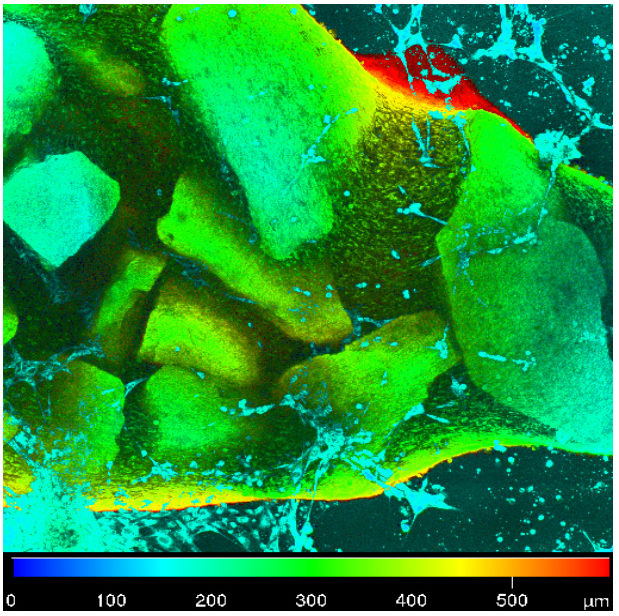

Fig 3. Micrograph (confocal microscopy) showing HAp-keratin-amikacin granules covered with hFOB cells (photograph with DepthCod option).
Magnification x 50

DISSCUSION

Hydroxyapatite bioceramics shows neither cytotoxicity nor carcinogenic effects after implantation because its chemical and mineralogical composition is similar to bones and teeth building substance. Bioceramics has also two main features: great biocompatibility towards soft and bone tissue and bioactivity responsible for direct connection with bone. Its porous structure gives possibility to use HAp as a various biological and chemical substances local delivery system. Such systems have to be investigated by scientists in order to check their potential cytotoxicity towards tissues.

Krisanapiboon et al. [6] investigated the biocompatibility of hydroxyapatite composite impregnated with gentamicin, fosfomycin, imipenem and amphotericin B. Extracts from all drugs showed good biocompatibility and no osteoblasts morphological changes were observed in all drug tests at any concentrations.

The purpose of study made by Rauschmann et al. [8] was to assess sulphate nanoparticulate HA composite material properties and to analyze its *in vitro* uptake and release of vancomycin and gentamicin. Furthermore, *in vitro* cytotoxic properties were also investigated – direct growth and adhesion of human osteoblasts onto the surface. This calcium sulphate nanoparticulate HA composite

material did not show any in vitro cytotoxicity and exhibited good biocompatibility compared. Loading with antibiotics was performed after hardening and sterilization of the pellets to prevent inactivation of atibiotics by these procedures. Loading is not limited to one specific antibiotic (e.g. tobramycin or gentamicin) but can be done according to antibiograms offering individual treatment options. The high porosity of this composite material revealed initial high antibiotic release with subsequent decline ensuring concentrations above MICs. Our studies confirmed these experiments. HAp granules did not affect osteoblast proliferation or cell morphology.

To increased cells adhesion to biomaterial some researchers used keratin which showes advantageous influence on osteoblasts growth and proliferation. Keratins are fibrillar proteins building hair, wool and nails. Cystein residues presence in keratin gives possibility to create disulfide bonds. It is important feature, because keratin modification leads to bioactive substances attachement to cystein residues of keratin. Moreover keratins possess sequences which facilitate cells adhesion: RGD (Arg-Gly-Asn) and LVD (Leu-Wal-Asn). Tachinaba et al. [10] investigated influence keratin-HAp sponge on osteoblast's proliferation. It was showed that preosteoblasts MC3T3–E1 grew correctly both on keratin-HAp sponge and control sponge (without keratin) but proliferation process began earlier on keratin sponge. This experiment confirmed profitable influence of keratin on osteoblasts growth and proliferation. In our research keratin also did not influenced negatively on cell growth and proliferation process.

CONCLUSION

Antibiotic-modified hybrid biomaterial do not show any cytotoxicity features towards hFOB in our *in vitro* experiments. Therefore, this biomaterial could function as scaffolds for bone regeneration and eradicate infection at the same time.

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SUMMARY

Hydroxyapatite is used in bone reconstruction because of its similar chemical structure compared to the inorganic composition of human bone. Bone is a complex material composed of proteins, mainly collagen, with hydroxyapatite. Therefore, now many investigations are focused on the hybrid biomaterials of hydroxyapatite with proteins and other synthetic polymers. We investigated HAp granules covered by two types of proteins (keratin or gelatin) in our study. This biomaterial was also modified by amikacin. In our previous paper [12] we proved that such hybrid biomaterial had antimicrobial properties. This paper gives evidence that HAp-modified granules did not influence on growth and proliferation of osteoblasts. So this new biomaterial could be used as an implant on orthopaedic, oral and maxillo-facial surgery in the future.

Keywords: hydroxyapatite, amikacin, antibiotic immobilization, implant infection, cytotoxicity

STRESZCZENIE

Hydroksyapatyt jest używany w rekonstrukcji kości z powodu jego struktury chemicznej podobnej do nieorganicznych składników kości ludzkich. Kość jest kompleksem złożonym z białek (głównie kolagenu) i hydroksyapatytu. Dlatego też obecnie wielu badaczy jest skupionych na tworzeniu materiałów hybrydowych. W naszych badaniach testowano granule HAp pokryte dwoma rodzajami białka (keratyną lub żelatyną). Biomateriał ten był też modyfikowany amikacyną. W poprzedniej publikacji [12] wykazano, że ten typ biomateriału hybrydowego posiada właściwości antybakteryjne. W obecnych badaniach wykazano, że modyfikowane granule HAp nie wpływają negatywnie na wzrost i różnicowanie osteoblastów. Tak więc nowy typ biomateriału w przyszłości może znaleźć zastosowanie jako materiał implantacyjny w chirurgii ortopedycznej, stomatologicznej i twarzoczaszki.

Słowa kluczowe: hydroksyapatyt, amikacyna, immobilizacja antybiotyków, zakażenia implantów, cytotoksyczność

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Breast cancer – an epidemiological and social problem in Poland

Rak gruczołu piersiowego – problem epidemiologiczny i społeczny w Polsce

INTRODUCTION

Breast cancer is the most common female malignant neoplasm. In recent years research was undertaken into the aetiology and development of cancer which has changed the views on the recommended forms of treatment. The perception of the illness in the constitutional aspect, not only locoregional, is reflected in modifications in surgical methods and a change in chemotherapy schemes. Implementation of the operational techniques of plastic surgery in breast cancer surgery has fulfilled the aesthetic expectations of female patients. However, the formation of distant metastases, which are the main reason for failure in treatment, is still a problem that remains unsolved. An early diagnostics in the detection of metastases and prevention of their formation started a new direction in the research on breast cancer. One thing that seems to be a comfort is the fact that despite the increase of the incidence of this cancer, mortality rates begin to indicate a decrease in the tendency.

EPIDEMIOLOGY OF BREAST CANCER IN POLAND AND WORLDWIDE

The incidence of breast cancer constitutes approx. 23% of the incidence of female malignant neoplasm worldwide [1, 2]. They are among the major risk factors in female mortality in the highly-developed countries. The risk of the incidence of this type of cancer increases each year [2, 3, 4]. Diagnostics, treatment and preventive programmes for breast cancer play a decisive part in the oncological policy of many countries. Due to a high incidence of breast cancer among American women, the analysis of the epidemiological situation in this country facilitates world preventive actions. According to Chu and associates [5], due to the progress in the diagnostics and treatment

of breast cancer, the mortality rate in USA decreased between 1989 and 1993 by 6.8%. According to Jansen and associates [6], based on the Scandinavian countries, this form of action may reduce the mortality rate even by 20–30%. Currently, the 5-year-survival predictor for different European countries is about 79%. In Sweden, however, it is as high as 86% [2]. In Poland, the incidence of breast cancer constitutes 19.7% of malignant tumour incidence among women. Deaths caused by this cancer is estimated to be approx. 13% of all cancer deaths [7] (Fig. 1).

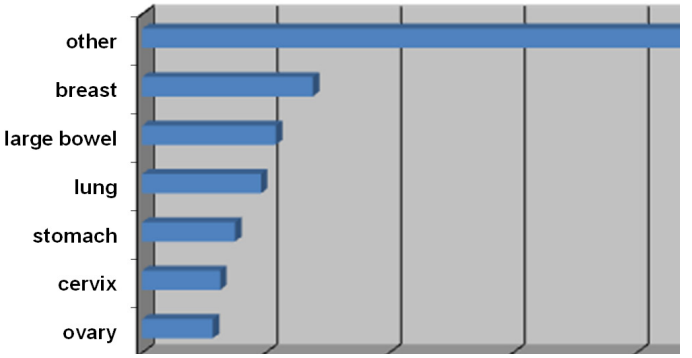

Fig. 1. Annually frequency [thousands] of malignant neoplasm cases among women in Poland [7]

It is estimated that the number of new breast cancer cases reaches about 13500 women each year in Poland [8]. The study of incidence in patients diagnosed between 1996–1999 reported an increase by almost 4% [7]. However, since the beginning of the 80's, there has been an increase in the incidence from about 25 to 42 people in 100000 residents. The majority of incidences concerned the group of women aged between 45 and 69 [8]. Over 50% of all incidences occurred in the 50–69 age group. Incidence rates increase with age between 40 and 59 and stabilise and decrease after the age of 70 [9]. It is estimated that the problem of breast cancer currently concerns about 55000 Polish women.

Fortunately, mortality rates seems to be different. Over the thirty year period 1963–1992, the risk of death increased by 1.6% annually. This increase concerned mainly women aged 50 or over, while in the younger groups there was a stabilisation in mortality, particularly after the 1980s. The mortality rate in 1963 was 9 people per 100,000. In the following years, there was a major increase in the mortality rate to up to 15/100000 and this number is rather constant, with a slight tendency to decrease [9, 10]. The mortality rates increase linearly with women aged from 35 to 80.

It is also important to trace the formation of incidence and mortality trends in the last 50 years. Until the end of the 70's, the mortality and incidence rates were increasing. In the 90's, there was a slowdown in mortality with an increase in its incidence since the 80's.

Currently, the incidence is increased, while the mortality rates are stable. The incidence and mortality trends "diverged". It seems that the undisputed reason for this situation is an improvement in diagnostics and treatment. The major role in this process is ascribed to the national screening

programmes. In Poland, this programme was started in 2006. The details of the benefits are not prepared yet. However, it is clear, that only 31% of females take part in this programme, which according to the estimates, will not ensure the expected populational advantages. Currently, the 5-year-survival predictor for the period of time before 2006 is 72% for Poland.

The mortality rate is higher in the major Polish cities. The Eastern regions of Poland and the villages are characterised by a lower mortality rate from breast cancer. The incidence of a malignant breast cancer is 1.5 times higher for the population of the cities than of villages. Men have this cancer 100-times less often than women and constitute approx. 0.5% of the ill [11].

THE RISK FACTORS OF BREAST CANCER

Defining the factors that increase of the risk of being affected by breast cancer made it possible to isolate the group of women who were especially in the risk of cancer. This group of patients should be included in the special programmes for early breast cancer detection or recently developed and controversial preventive programmes.

Among the major factors increasing the risk of breast cancer are: genetic factors, age, past breast cancer, family history of breast cancer, minor proliferative diseases of the breast, exogenous and endogenous sex hormones, ionizing radiation, obesity, alcohol.

Genetic factors. It is estimated, that about 5–10% of breast cancer are caused by a hereditary mutation of the embryonic stem-cell line of breast cancer susceptible genes - *BRCA1* and *BRCA2*. The mutation is inherited in an autosomatic, dominating way with varying penetration. The woman that inherits the mutated gene *BRCA1* is in the 56–85% risk of being affected by breast cancer and 15–45% by ovary cancer [1, 12].

Breast cancer developing from the *BRCA1* mutation is most often low diverse. It is characterised by aneuploid and cells devoid of hormonal receptors, a huge percentage of which is in the S phase of the cell cycle. The *BRCA1* and *BRCA2* genes are the suppressors, inhibiting the development of cancers. As a result of the mutation within these genes, the carcinogenesis is initiated. The proteins coded by these genes take part in the response processes of a cell to DNA damage [13].

Currently-employed preventive actions concerning inheritors of the mutated *BRCA* gene include: 1) preventive mastectomy with simultaneous reconstructive surgery, 2) preventive ovary resection and hormonal supplementary therapy, 3) regular health examinations, 4) chemoprevention. The recently-published results from the randomised, multicentre clinical examinations run by *the National Surgical Adjuvant Breast and the Bowel Project* (NSABP) under the leadership of Bernard Fisher, MD, as a *Breast Cancer Prevention Trial* (BCPT) revealed a substantial 49% decrease in the frequency of the incidence of breast cancer among women from the risk group taking tamoxifen [13].

Age. The rate of the incidence of breast cancer increases with age. In Poland, 80% of women with cancer are over 50 [9]. The phenomenon is explained by the aging processes of cells and apoptosis disorders.

Past breast cancer. The risk of developing cancer in the other breast is approx. 1% [14].

The family history of breast cancer. 25% of breast cancer incidence is with a family background [15]. The major risk of developing cancer is among women whose mother or

sister had this cancer and is 14 times higher than the risk of the women without a family history of cancer. The risk of breast cancer among the women whose closest relative developed cancer before menopause increases 3 times. In the case when relatives are affected by cancer after menopause the risk increases 1.5 times [14].

Minor proliferative diseases of the breast. The presence of duct hyperplasia increases the risk by a factor of 2. The presence of atypical duct hyperplasia means a 4- to 5-fold higher risk. Diagnosis of *ca ductale in situ* is connected, according to Pieńkowski [14], with an 8–11 times increase in the risk of developing an invasive cancer.

According to other authors, the presented values differ significantly and depend on the type of cancer [16, 17, 18]. 80 patients with *ca ductale in situ* were observed for 5–28 months. 11 of them (14%) developed an infiltrating cancer.

Exogenous sex hormones. Long-term, 5–7-year hormone-replacement therapy (HRT) is connected with an increase in breast cancer risk. Multicentre research on hormonal factors carried out in recent years has shown that in women using HRT for a minimum of 5 years, the breast cancer incidence has increased by 35% [19]. The increase of its incidence in this group of women is probably caused by the mitogenic influence of estrogens and progesterone. These hormones intensify gland cell division. The cells in the proliferative phase are more prone to the influence of mutagenic factors [20. 21]. Long-term oestrogen-progestagen therapy may result in a higher breast cancer risk than using estrogens alone [22]. There are various ambiguous opinions on the influence of contraceptives with low hormone levels on the increase in breast cancer risk [23]. *Population case-control* research run by Marchbanks and associates [24] (approx. 9 thousands participants) indicated that the use of orally administered contraceptives does not increase breast cancer risk in older age.

Physiological hormones activity. First menstruation under the age of 12 and menopause over the age of 55 are connected with an increased risk of breast cancer. Having the first child under the age of 25 and breastfeeding are the factors that minimise the risk [25].

Ionising radiation. A long term exposition to the ionising radiation increases the risk of breast cancer [14].

Obesity. The consumption of considerable amounts of animal fats is connected with the increase in breast cancer risk. Most detrimental is the presence of saturated fats in the diet [26].

Alcohol. Regular, moderate consumption of alcohol increases the risk of breast cancer [25]. The reason may be an increased concentration of estrogens in blood serum caused by alcohol [27].

Finally, it is worth emphasising that only 4–8% of cases of breast cancer result from hereditary mutations. The majority of cases result from sporadic somatic mutations, influenced mainly by environmental factors. The elimination of common carcinogenic factors through the implementation of a diet, a healthy lifestyle, or ending a long-term hormone therapy can decrease the incidence rate.

Wider participation of women in preventive screening creates a real chance of decreasing the mortality rate by almost 40%.

It seems that effective preventive actions in social policy can solve this significant health problem in Poland.

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SUMMARY

Breast cancer is the most common female malignant neoplasm. Early diagnostics and effective preventive treatment help decrease the mortality rates by 30%. An estimated 55000 Polish women suffer from breast cancer. Annually, about 13500 new cases are diagnosed (42/100000 residents). Mortality rate is currently 15/100000. In recent years a stabilisation in the mortality rate despite its increasing incidence, which is thought to be the result of early diagnosis and treatment of this type of cancer, has been observed. However, the number of woman participating in population preventive screening tests is still too low and five-year survival rates are around 72% for woman in Poland based on diagnoses up to the end of 2006.

Keywords: breast cancer, epidemiology, risk factors

STRESZCZENIE

Rak gruczołu piersiowego jest najczęściej występującym u kobiet nowotworem złośliwym. Wczesna diagnostyka i efektywne działania profilaktyczne umożliwiają obniżenie wskaźników umieralności nawet o 30%. Problem raka gruczołu piersiowego w Polsce dotyczy obecnie 55000 kobiet. Rocznie stwierdza się w kraju około 13500 nowych zachorowań (42/100000 mieszkańców). Współczynnik umieralności wynosi obecnie 15/100000. W ostatnich latach obserwuje się stabilizację współczynnika umieralności pomimo wzrostu liczby zachorowań, co jak się przypuszcza jest wynikiem znacznej poprawy wczesnej diagnostyki i leczenia tego typu nowotworu. Wciąż jednak zbyt mała liczba kobiet zgłasza się na populacyjne badania profilaktyczne (31%), a wyliczony za okres przed rokiem 2006 wskaźnik 5-letniego przeżycia wynosi w Polsce zaledwie 72%. W pracy przedstawiono również poznane dotychczas główne czynniki ryzyka zachorowania na raka piersi.

Słowa kluczowe: rak piersi, epidemiologia, wskaźniki ryzyka

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Influence of the preliminary sample preparation on the tannins content in the extracts obtained from Mutellina purpurea Poir

Wpływ przygotowania próbki na zawartość garbników w ekstraktach z *Mutellina purpurea* Poir.

INTRODUCTION

Tannins (commonly referred to as tannic acid) are water-soluble, polyphenolic compounds naturally occurring in higher plants. The primary definition of this class of secondary metabolites was established by Bate-Smith and Swain. They classified tannins as phenolic compounds with a molar mass between 300 and 3000, showing the usual phenol reactions and precipitating alkaloids and other proteins [1]. However molecules with a molar mass of up to 20000 D have also been described and their molecular structures indicate that they should be classified as tannins. Since those tannins are defined as "macromolecular phenolic substances", they are divided into two major groups: the 'hydrolysable' and 'condensed' tannins [4]. Hydrolysable tannins include glucosides either from gallic acid, i.e. gallotannins, or from ellagic acid, i.e. ellagitannins or complex tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit. Condensed tannins are all oligomeric and polymeric proanthocyanidins composed of flavan-3-ol monomer subunits, such as catechin, epicatechin and their gallates [6]. Tannins play varied biological roles such as protein precipitating agents, biological antioxidants, metal ion chelators, or antymicrobial agents. It is possible because of structural variation among this group of natural products [20].

Antioxidants are important in protecting cellular oxidative damage, including lipid peroxidation and damage of DNA chain. Biological antioxidants are classified into enzymes, inhibitors of radical formation and free radical neutralizing agents [16]. Polyphenoles including tannins neutralise oxygen-free radicals, which are unstable and highly reactive molecules that contain unpaired electrons. Free radical molecules are produced via normal metabolic processes and oxidative stress [6]. Tannins interacting with free radicals are known as protectors of cells against aging, cancer and cardiovascular disease. Many tannin molecules have also been shown to reduce the mutagenic activity of a number of mutagens [5].

The Folin–Ciocalteu (F–C) reaction as a standard method for quantitative determination of phenolic compounds is used. This reaction is based on oxidation in alkaline solution of phenols by the yellow molybdotungstophosphoric heteropolyanion reagent and colorimetric measurement of the resultant molybdotungstophosphate blue [14]. It is a determination of total phenolics, which are reducing agents, and the content of tannins and other compounds such as flavonoids, phenolic acids or anthocyanes. Determination of total tannins is partly a chemical reaction and partly a physical interaction. Tannins are measured as the reduction in phenolics that occurs when a binding agent (polyvinyl polypyrrolidone) is added to the extract [17].

Mutellina purpurea (Poir.) Thell. is a perennial herb growing on Alpine pastures, among mountain pines or on the glades. It is the herb typical of the Carpathian Mountains and of the Polish Tatra Mountains [2]. *M. purpurea* grows up to 50cm and like other *Apiaceae* plants produces umbels with seeds ripening at summer [12]. In alternative medicine, *M. purpurea* is used for substitution of calcium and potassium. Since mineral balance is disturbed during the cancer preventing diet excluding proteins, tea made from *M. mutellina* herb supplements lack of calcium and potassium [3]. Methanolic extracts from *M. purpurea* herb are active against some *Staphylococcus* and *Pseudomonas* species [Sieniawska E. et al. article under revision]. The essential oil obtained from roots of different collections of *M. purpuraa* contains ligustylid as one of the major constituents [13]. Ligustilide suppreses reactive oxygen species production and extracellular signal-related kinases. Thus, ligustilide contributes to be an effective agent in preventing cardiovascular diseases and cancer[10].

MATERIAL AND METHODS

E x p e r i m e n t a l d e s i g n . Chemical reagents of high purity were purchased from Sigma–Aldrich Chemie GmbH (Munich, Germany) and Merck (Darmstadt, Germany). The plant material was collected in the Botanical Garden of the Medical University in Lublin in June 2010. Plants were dried at room temperature, powdered and extracted.

EXTRACT PREPARATION OBTAINED BY POLISH PHARMACOPOEIA VIII METHOD [12]

Pulverized herb (1 g) of *M. purpurea* was subjected to 30 min extraction on a water bath under reflux with 150 ml of deionised water. The cooled extract was left for precipitation of a sediment. The extract was filtrated with filter paper and 50 ml of filtrate was discarded. The obtained solution (solution I) was filled with deionised water up to 250 ml.

EXTRACTION WITH PRELIMINARY SAMPLE PREPARATION

H o m o g e n i z a t i o n . The first portion of pulverized herb (1 g) of *M. purpurea* was subjected to 30 min. homogenization with 150 ml of deionised water in the mechanical homogenizer (Homogenizer type 302, Mechanika Precyzyjna, Poland) with 5000 rpm speed. The obtained blend was extracted for 30 min. on a water bath under reflux as described in the Pharmacopoeia method (H-FPVIII).

M i x i n g . The second portion of pulverized herb (1 g) of *M. purpurea* was subjected to 60 min maceration with 150 ml of deionised water in the orbital shaker (RS10 Control, IKA Werke GmbH,

Germany), speed of shaking 180 min^{-1}. Subsequently, the obtained mixture was extracted for 30 min on a water bath under reflux (M-FPVIII).

Ultrasonic. The third portion of pulverized herb (1 g) of *M. purpurea* was subjected to 30 min sonification with 150 ml of deionised water in the ultrasonic water bath (Sonorex Digitral 10P, Bandelin, Germany), extraction temperature was approximately 60ºC, with 100% of sonifcation power. Subsequently, the obtained mixture was extracted for 30 min. on the water bath under reflux (U-FPVIII).

DETERMINATION OF TOTAL PHENOLICS

5 ml of solution I was taken and filled with deionised water up to 25 ml (solution II) . Subsequently, 2 ml volume of solution II was added to 1 ml of molybdotungstophosphoric heteropolyanion reagent, 10 ml of water and filled up to 25 ml with sodium carbonate. Spectrophotometric measurement was done after 30 min time in the λ=760 nm (A_1) (Cary 50 UV-Vis Spectrophotometer, Varian INC, USA). Kinetics of reaction and maximum absorbance were obtained by using Cary WinUV Software.

DETERMINATION OF CONDENSED TANNINS (PHENOLICS NOT INTRACTING WITH HIDE POWDER)

10 ml of solution I was taken, 100 mg of *hide powder* was added and the mixture was shaken for 60 min (speed 180 min^{-1}). Then the extract was filtrated, 5 ml was taken and filled with deionised water up to 25 ml (solution III). Subsequently, 2 ml volume of solution II was added to 1 ml of molybdotungstophosphoric heteropolyanion reagent, 10 ml of water and filled up to 25 ml with sodium carbonate. Spectrophotometric measurement was done after 30 min. time in the λ=760 nm (A_2).

REFERENCE SOLUTION SPECTROPHOTOMETRIC MEASUREMENT

50 mg of pirogallol was suspended in deionised water and filled up to 100 ml volume, *ex tempore* (standard solution I). 5 ml of standard solution I was taken and filled with deionised water up to 100 ml (standard solution II). Subsequently, 2 ml volume of solution II was added to 1 ml of molybdotungstophosphoric heteropolyanion reagent, 10 ml of deionised water and filled up to 25 ml with sodium carbonate. Spectrophotometric measurement was done after 30 min. time in the λ=760 nm (A_3). The percentage of tannin content in the plant material on the basis of the following equation was calculated.

$$x_i(\%) = 62{,}5\ (A_1 - A_2)\ m_2 / A_3 \times m_1$$

x_i = tannins in the plant material, m_1 = plant material weight [g]; m_2 = pirogallol weight [g]

Statistical analysis, according the Pawlaczyk et al. [14] on the basis of Q-Dixona test was conducted. A the confidence level α = 0.05% (P = 95%) it was verified that the results in the particular preliminary extraction method belong to the same test population [14]. Statistical parameters: arithmetic means, standard deviation of the individual value and arithmetic means and relative standard deviation are given in Table 1.

Table 1. Total tannins content in the aerial parts of *M. purpurea* calculated on pirogallol. Tannins amounts coresponding to the preliminary sample preparation and not modified FP VIII method of extraction. Statistical analysis of percent tannins content

Polish Pharmacopoeia method				
n	A1	A2	Percentage content	Statistical parameters
1	0.1314	0.1098	0.2368	$n = 10.\ t_{\alpha,f}\ (\alpha = 0.05.\ f = 9) = 2.262$
2	0.1300	0.1100	0.2193	
3	0.1301	0.1095	0.2259	$\bar{x} = 0.2241\%;\ S_1 = 5.3173 \times 10^{-3};\ S_1^2 = 2.8273 \times 10^{-5};$
4	0.1300	0.1096	0.2237	
5	0.1302	0.1096	0.2259	$S\bar{x} = 1.603 \times 10^{-4}$
6	0.1301	0.1096	0.2248	
7	0.1301	0.1097	0.2237	$\mu = 0.2241\% \pm 0.0038;\ RSD = 0.0237$
8	0.1303	0.1099	0.2237	
9	0.1302	0.1104	0.2171	
10	0.1299	0.1098	0.2204	
H-FPVIII				
1	0.0987	0.0864	0.1349	$\bar{x} = 0.1297\%;\ S_1 = 3.9847 \times 10^{-3};\ S_1^2 = 1.5878 \times 10^{-5};$
2	0.0984	0.0866	0.1294	
3	0.0983	0.0867	0.1272	$S\bar{x} = 1.260 \times 10^{-3}$
4	0.0983	0.0863	0.1316	
5	0.0987	0.0865	0.1338	$\mu = 0.1297\% \pm 0.0028;\ RSD = 0.0307$
6	0.0990	0.0874	0.1272	
7	0.0980	0.0867	0.1239	
8	0.0981	0.0863	0.1294	
9	0.0978	0.0864	0.1250	
10	0.0988	0.0865	0.1349	
M-FPVIII				
1	0.1010	0.0795	0.2357	$\bar{x} = 0.2387\%;\ S_1 = 8.2901 \times 10^{-3};\ S_1^2 = 6.8727 \times 10^{-5};$
2	0.1007	0.0799	0.2281	
3	0.1012	0.0797	0.2357	$S\bar{x} = 2.6215 \times 10^{-3}$
4	0.1017	0.0796	0.2423	
5	0.1022	0.0794	0.2500	$\mu = 0.2387\% \pm 0.0059;\ RSD = 0.0347$
6	0.1019	0.0795	0.2456	
7	0.1023	0.0798	0.2467	
8	0.1022	0.0799	0.2445	
9	0.1015	0.0803	0.2325	
10	0.1007	0.0801	0.2259	
U-FPVIII				
1	0.1339	0.1075	0.2895	$\bar{x} = 0.3016\%;\ S_1 = 7.6735 \times 10^{-3};\ S_1^2 = 5.8882 \times 10^{-5};$
2	0.1344	0.1069	0.3015	
3	0.136	0.1069	0.3191	$S\bar{x} = 2.4266 \times 10^{-3}$
4	0.1344	0.1067	0.3037	
5	0.1345	0.1066	0.3059	$\mu = 0.3016\% \pm 0.0055;\ RSD = 0.0254$
6	0.1342	0.1066	0.3026	
7	0.1340	0.1066	0.3004	
8	0.1338	0.1064	0.3004	
9	0.1337	0.1067	0.2961	
10	0.1339	0.1068	0.2971	

A1 – absorbance of total phenolics; A2 – absorbance of phenolics not intracting with *hide powder*; A3 = 0.285

RESULTS AND DISCUSSION

On the basis of UV spectrum of *M. purpurea* extract with proper reagents (molybdotungstophosphoric heteropolyanion reagent, deionised water, sodium carbonate), the maximum of absorbance for this complex was estimated as λ=760 nm (Fig. 1). In this wave length, the kinetics of the spectrophotometrical reaction was determined. It is shown in Fig. 2 that the reaction became stable after 25 min. The result concerning the tannins content in the aerial parts of *M. purpurea* in the Table 1 was shown.

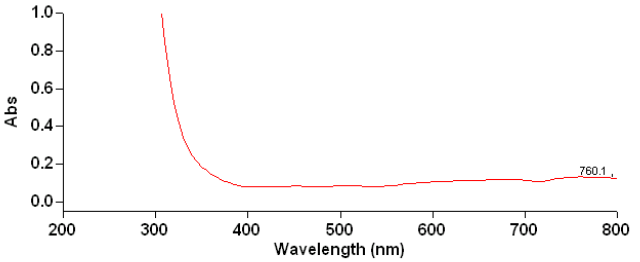

Fig. 1. UV spectrum for the *M. purpurea* extract with added reagents (molybdotungstophosphoric heteropolyanion reagent, deionised water, sodium carbonate)

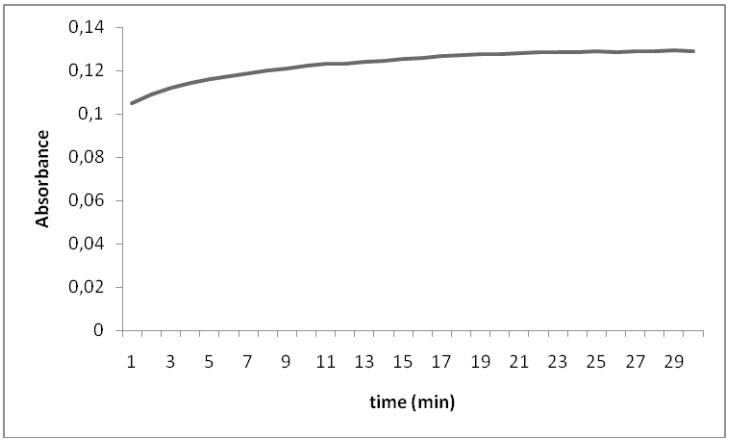

Fig. 2. Kinetics of tannins estimation reaction in *M. purpurea*, λ = 760

As results from Table 1, the highest percent content of tannins in the investigated plant material was achieved applying preliminary ultrasound assisted extraction followed by classical extraction the FP VIII 0.30%. Somewhat lower tannins content was after Polish Pharmacopoeia VIII method for isolation of tannins, 0.22%. The lowest stated amount of tannins in *M. purpurea* was after previous

homogenisation of plant material, 0.13%. The percent amount after preliminary maceration was slightly higher than for FP VIII method, 0.24%.

According to Polish Pharmacopoeia VIII, standard tannins extraction procedure involves the boiling of plant material with hot water [15]. This method reduces the number of possible solvent interaction with water. Recently, many authors have applied different extraction methods to find the most efficient one [7–9, 19]. These researches focus on comparing individual extraction techniques. On the contrary, the present authors' present pre-extraction procedures coupled with proper extraction, because preliminary sample preparation gives a possibility to enhance solvent penetration to plant tissue. This is the first time when the influence of sample preparation on tannins content is investigated.

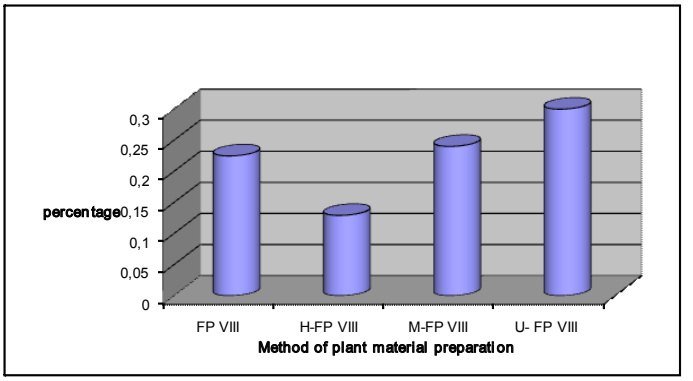

Fig. 3. Influence of the preliminary sample preparation on tannins content in the *M. purpurea.* FP VIII- Polish Pharmacopoeia VIII extraction procedure, H- FP VIII – homogenisation + FP VIII, M-FP VIII – maceration + FP VIII, U-FP VIII – ultrasound assisted extraction + FP VIII

Among the investigated methods of preextraction, compared with standard tannins extraction FPVIII, only U-FPVIII gave an increase in the amount of the extracted tannins (Fig. 3). Recently, ultrasound assisted extraction (UAE) has been used quite often on the phytochemical field however, it gives no information about using ultrasounds in the tannins extraction. It is difficult to apply UAE methods for tannins extraction because even modern ultrasound water baths have a maximum temperature of approximately 80°C. However ultrasounds joined with the following classical extraction (U-FPVIII) give good results (Table 1). Unexpectedly, using homogenisation (H-FPVIII) as a sample preparation technique, the lowest quantity of tannins was obtained (Tab. 1). This result can be caused by drastic, mechanical mincing of plant tissue effecting tannin compounds hydrolysis in the water medium. Two of the compared methods of preliminary sample preparation involve a kinetic energy increase. In the U-FPVIII and H-FPVIII solvent-substance system higher temperature should enhance the extraction process. However, concerning homogenisation, the temperature increase in the solvent-substance system was from ambient temperature to 55°C, which could cause decrease in the tannins content. On the contrary, ultrasound energy is strong, but does not rip the plant structures, only improves solvent penetration into the cells and the isolation of biologically active compounds. One hour maceration with constant shaking did not affect extraction improvement (Table 1). This

suggests that the FPVIII extraction method is sufficient for tannins isolation from the plant material. It is the first scientific report about tannins content in the *M. purpurea*. The tannins quantity in this herb is comparable to the tannins content in Black YUNNAN Golden Leaf Tea, Green Oryginal „Celmar" Tea and Black BROOKE BOND Tea [18].

CONCLUSIONS

In the presented work the joined techniques of preliminary sample preparation and extraction were applied. The authors indicate that preliminary ultrasound assisted extraction increases the amount of tannins extracted from *M. purpurea* compared to classical methods described by the Polish Pharmacopoeia VIII. On this plant material it was revealed that one hour of preliminary maceration did not influence the efficiency of tannins extraction, whereas mechanical homogenization reduced the tannin content by approximately 50%. On the basis of this research, it seems that preliminary ultrasound assisted extraction joined with classical extraction FPVIII is a simple way to improve tannins isolation from plant material. It is the first time the tannins content in the aerial parts of *Mutellina purpurea* was assigned.

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SUMMARY

There is no information in the current literature about scientific research describing tannins in the *Mutellina purpurea* Therefore, this work presents investigation of total tannins in the aerial parts of *M. purpurea*. Determination of tannins was done according to the method described in the Polish Pharmacopoeia VIII, with the molybdotungstophosphoric heteropolyanion reagent. The authors applied the preliminary sample preparation procedure before the classic extraction. Maceration of plant material, homogenization and ultrasonication were tested. The best result with the preextraction in the ultrasounds was obtained (0.30%).

Keywords: Mutellina purpurea, tannins, extraction, homogenization, maceration, ultrasonic

STRESZCZENIE

W danych literatury brak jest doniesień dotyczących zawartości związków garbnikowych w *Mutellina purpurea*, dlatego też autorzy pracy przeprowadzili analizę sumy garbników w nadziemnych częściach tej rośliny. Oznaczenie prowadzono metodą farmakopealną (FP VIII) z odczynnikiem fosforomolibdenowolframowym. W pracy zbadano także wpływ przygotowania próbki na zawartość oznaczanej grupy związków. Przed klasycznym procesem ekstrakcyjnym (FP VIII) substancja roślinna była poddana homogenizacji, maceracji lub działaniu ultradźwięków. Z uzyskanych danych wynika, iż po wstępnej ekstrakcji ultradźwiękowej udało się uzyskać najwyższą zawartość związków garbnikowych w badanej substancji roślinnej (0,30%). Wpływ pozostałych metod wstępnej ekstrakcji był nieznaczny.

Słowa kluczowe: Mutellina purpurea, garbniki, maceracja, homogenizacja, ultradźwięki

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Chemical composition and antioxidant activity of the essentials oil of hyssop (Hyssopus officinalis L. ssp. officinalis)

Skład chemiczny oraz aktywność antyoksydacyjna olejku eterycznego z hyzopu lekarskiego *(Hyssopus officinalis* L. ssp. *officinalis)*

INTRODUCTION

Hyssop (*Hyssopus officinalis* L*,* F. *Lamiaceae*) probably originates from south-west Asia and south Europe. It can be found on lowlands and foothills, seldom appearing in mountain areas. Hyssop grows on dry and insolated slopes and meadows with calcareous soil, and sometimes in gardens commonly in old monastic gardens [22]. This herb is cultivated in eastern and central Europe, in France, Italy, the Balkans, Ukraine (Crimea) and in Asia [8]. Essential oil obtained form hyssop is used in food, cosmetics and within the pharmaceutical industry. It also possesses antibacterial, antiviral, antifungal and expectorant properties [4, 5, 16]. Recent research suggests that essential oil present in hyssop shows some antiplatelet activity [19]. What is more, spasmolytic action of this oil was described [10].

Essential oil is the main physiologically active constituent of hyssop. In leaves the content of the oil oscillates between 0.3% and 1.5%, in flowers between 0.9% and 2.0% whilst in stems, it is only present in trace amounts. It is obtained during steam distillation of dried or fresh herb (efficiency is about 0.15–0.3% and 0.3–0.8, respectively). On a large scale, it is produced in Mediterranean countries – in France and Italy and also in former Yugoslavia and the Ukraine [6]. Hyssop oil is a light green or light yellow liquid with a sweet camphoric scent. The amount and composition of essential oil from hyssop depends on many external factors (e.g. climatic conditions, type of soil), on the origin of the plant and harvesting time [22,23]. In previous studies, 31 chemical compounds were described in oils from different subspecies of hyssop. The main compounds usually found were *cis*-pinocamphone and *trans*-pinocamphone. Furthermore *β*-pinene, pinocarvone, limonene, linalool, *β*-caryophyllene, germacrene D, thujones and myrtenol were also often present [6, 7, 11, 12, 14].

The purpose of this work was to examine the composition of essential oil obtained from aerial parts of *Hyssopus officinalis L*. ssp. *officinalis* grown in Lublin (Eastern Poland). The full GC/MS and GC/FID analysis of essential oil,obtained from hyssop grown in Poland was performed for the first time. Due to the fact that numerous studies conducted have described that toxic compounds like methyl eugenol, a compound with confirmed carcinogenic activity, or monoterpenic ketones with strong epileptogenic properties can be present in essential oil isolated from hyssop it was necessary to perform the analysis of this oil [3, 18, 21].

MATERIAL AND METHODS

Plant material. Hyssop (*Hyssopus officinalis* L. ssp. *officinalis*) was grown in the herb garden at the Faculty of Pharmacy, Medical University of Lublin, Poland (N 51°16' E 22°34'). Aerial parts of hyssop were harvested during the flowering stage in August 2005. The taxonomic identification was confirmed by the plant taxonomist, Stanisław Kwiatkowski, in the Dept. of Pharmacognosy within the Medicinal Plant Laboratory of the Faculty of Pharmacy (Medical University of Lublin, Poland). After identification the plant material was dried at 35ºC and then ground. The voucher specimen was deposited at the Herbarium of the Department of Pharmacy, Medical University of Lublin, Poland (No. 0501).

Isolation of essential oil. Essential oil was isolated using steam distillation in the Deryng-type apparatus – 50 g of dried hyssop was distillated with 400 ml of water for three hours. This method of obtaining essential oil is recommended by Polish Pharmacopoeia [15]. The obtained oil was dried with anhydrous sodium sulphate and stored at 4°C until tested and analysed.

GC/MS and GC/FID analysis conditions. The qualitative and quantitative analysis of particular components of the essential oil was made by means of gas chromatography techniques: GC/MS and GC/FID. For GC/MS analysis ITMS Varian 4000 GC/MS/MS (Varian, USA) apparatus equipped with a CP-8410 autoinjector and a VF-5ms column (column size: 30 m x 0.25 mm, film thickness: 0.25 μm Varian, USA) was used. Operating conditions were as follows: injector temperature – 220°C, detector temperature – 200°C, carrier gas: Helium at the flow rate of 1 ml/min, split injection with split ratio 1:20 and inject volume 1 μl. The temperature gradient was applied – initially 60°C for 5 minutes, then raised to 246°C at the rate of 3°C/min and finally held at that temperature for 10 minutes. For GC/FID analysis Varian 3800 with DB-5 column (J&W, USA) was used. Operating conditions were similar to GC/MS. The FID detector's temperature was 256°C.

The qualitative analysis was carried out on the basis of MS spectra which were compared with the spectra of the NIST library [13], and data available in literature- Kováts' retention [1, 9]. Identity of the compounds was confirmed by their retention indices taken from literature and own data [1, 9]. The composition of the essential oil was determined by GC/FID, by assuming the totality of all the particular oil to be 100% pure.

RESULTS AND DISCUSSION

The amount of the essential oil distillated from hyssop was 0.18% (v/w) on dry weight basis of herbage. The GC chromatogram of compounds present in the tested essential oil is shown in Figure 1.

Table 1 presents the percentage composition of this oil. Components are listed in order of their elution from the DB-5 capillary column.

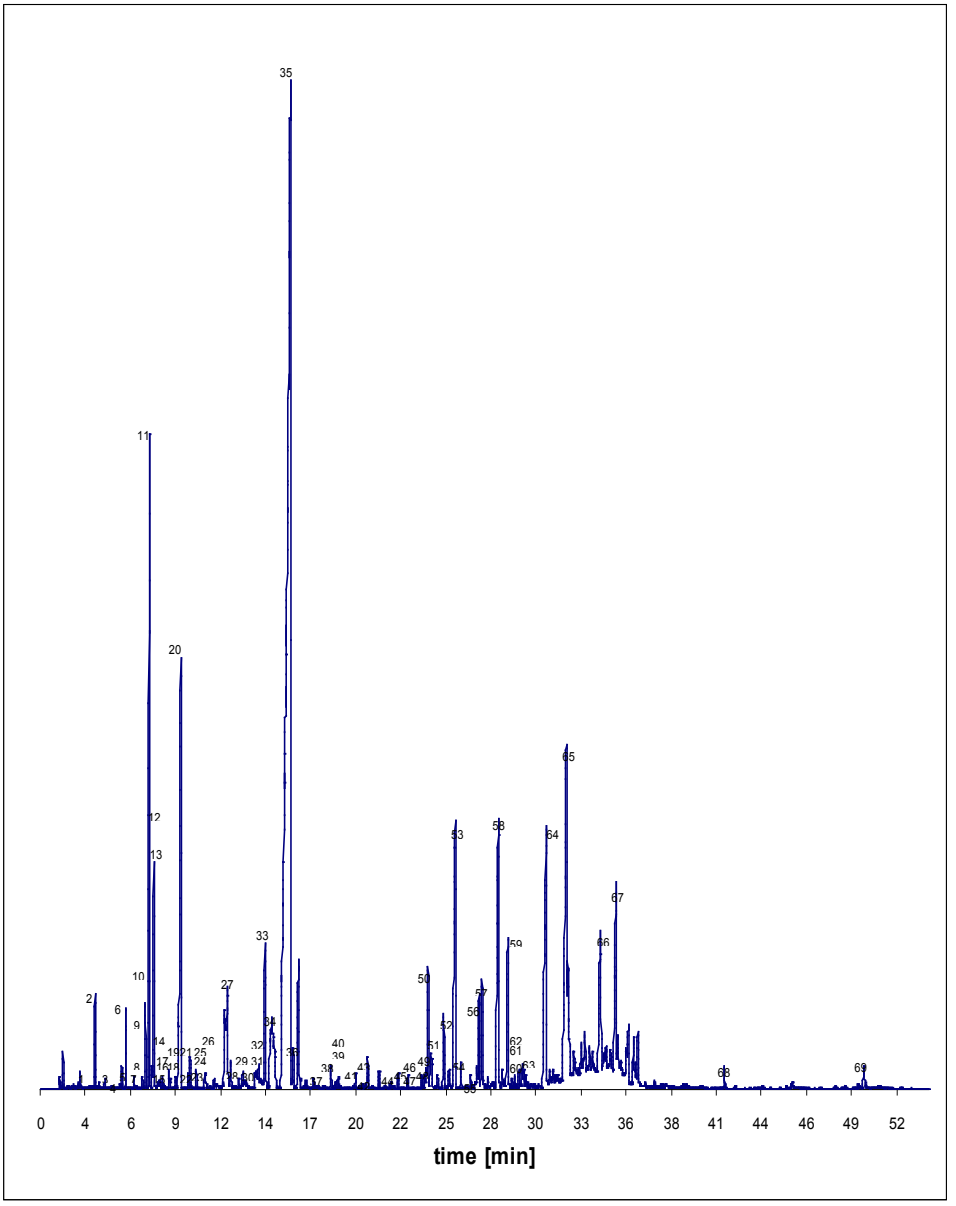

Fig. 1. GC chromatogram of chemical compounds present in essential oil of *Hyssopus officinalis L.* – compounds are marked in accordance with Table 1

Table 1. Percentage composition of essential oil obtained from *Hyssopus officinalis L.*

No	Compound	KI	Percentage	Method of identification	References
1	Hexanal	794	0.11	MS, RI	[1, 13]
2	*2(E)*-Hexenal	844	0.46	MS, RI	[1, 13]
3	*n*-Hexanol	858	tr	MS, RI	[1, 13]
4	Heptanal	903	tr	MS, RI	[1, 13]
5	*α*-Thujene	926	0.09	MS, RI	[1, 13]
6	*α*-Pinene	934	0.32	MS, RI, Co	[1, 13]
7	Camphene	949	0.07	MS, RI	[1, 13]
8	Thuja-2,4(10)-diene	956	0.11	MS, RI	[1, 13]
9	Benzaldehyde	969	tr	MS, RI	[1, 13]
10	Sabinene	974	0.48	MS, RI	[1, 13]
11	*β*-Pinene	981	6.14	MS, RI, Co	[1, 13]
12	3-Octanone	983	0.09	MS, RI	[1, 13]
13	Myrcene	991	1.26	MS, RI	[1, 13]
14	dehydro-1,8-Cineole	993	tr	MS, RI	[1, 13]
15	*p*-Mentha-1(7),8-diene	1008	0.12	MS, RI	[1, 13]
16	*α*-Phellandrene	1008	tr	MS, RI	[1, 13]
17	*α*-Terpinene	1017	tr	MS, RI	[1, 13]
21	*p*-Cymene	1025	tr	MS, RI	[1, 13]
19	Limonene	1030	0.71	MS, RI, Co	[1, 13]
20	1,8-Cineole	1033	5.78	MS, RI	[1, 13]
21	*Z-β*-Ocimene	1036	tr	MS, RI	[1, 13]
22	Benzene acetaldehyde	1041	0.17	MS, RI	[1, 13]
23	*γ*-Terpinene	1058	0.08	MS, RI	[1, 13]
24	n.i.*	1068	tr	–	–
25	Terpinolene	1089	tr	MS, RI	[1, 13]
26	*cis*-Linalool oxide	1091	tr	MS, RI	[1, 13]
27	Linalool	1107	1.33	MS, RI, Co	[1, 13]
28	*α*-Thujone	1117	0.07	MS, RI	[1, 13]
29	*β*-Thujone	1122	tr	MS, RI	[1, 13]
30	*cis-p*-Menth-2en-1-ol	1129	tr	MS, RI	[1, 13]
31	*trans*-Pinocarveol	1149	0.29	MS, RI	[1, 13]
32	*trans-p*-Menth-2-en-1-ol	1151	tr	MS, RI	[1, 13]
33	n.i.*	1158	2.21	–	–
34	*trans*-Pinocamphone	1161	1.90	MS, RI	[1, 13]
35	*cis*-Pinocamphone	1219	48.56	MS, RI	[1, 13]
36	*cis*-Piperitol	1221	tr	MS, RI	[1, 13]
37	n.i.*	1223	tr	–	–
38	Carvone	1241	0.14	MS, RI	[1, 13]
39	Carvotanacetone	1250	0.07	MS, RI	[1, 13]
40	*trans*-2-hydroxy-Pinocamphone	1255	tr	MS, RI	[1, 13]
41	*p*-Menth-1-en-7-al	1284	tr	MS, RI	[1, 13]
42	*α*-Terpinen-7-al	1292	tr	MS, RI	[1, 13]
43	*p*-Cymen-7-ol	1298	0.22	MS, RI	[1, 13]
44	*m*-Acetanisole	1313	0.12	MS, RI	[1, 13]
45	Eugenol	1357	0.11	MS, RI	[1, 13]

No	Compound	KI	Percentage	Method of identification	References
46	n.i.*	1340	0.21	–	–
47	*α*-Copaene	1373	0.07	MS, RI	[1, 9, 13]
48	Menthyl *p*-anisate	1380	0.08	MS, RI	[1, 13]
49	*(E)-β*-Damscenone	1386	0.10	MS, RI	[1, 13]
50	*β*-Bourbonene	1390	1.06	MS, RI	[1, 9, 13]
51	*(E)*-Jasmone	1395	0.14	MS, RI	[1, 13]
52	Methyl eugenol	1405	0.38	MS, RI	[1, 13]
53	*(E)*-Caryophyllene	1428	3.99	MS, RI	[1, 9, 13]
54	*β*-Copaene	1434	0.17	MS, RI	[1, 9, 13]
55	n.i.*	1449	0.07	–	–
56	*α*-Humulene	1459	0.82	MS, RI	[1, 9, 13]
57	allo-Aromadendrene	1467	0.84	MS, RI	[1, 9, 13]
58	Germacrene D	1489	3.37	MS, RI	[1, 9, 13]
59	Bicyclogermacrene	1503	1.34	MS, RI	[1, 9, 13]
60	*β*-Bisabolene	1511	0.06	MS, RI	[1, 9, 13]
61	*γ*-Cadinene	1521	0.09	MS, RI	[1, 9, 13]
62	*δ*-Cadinene	1521	tr	MS, RI	[1, 9, 13]
63	*β*-Sesquiphellandrene	1526	0.38	MS, RI	[1, 9, 13]
64	Elemol	1561	7.43	MS, RI	[1, 13]
65	Caryophyllene oxide	1590	5.17	MS, RI	[1, 13]
66	*γ*-Eudesmol	1639	1.25	MS, RI	[1, 13]
67	*α*−Eudesmol	1662	1.39	MS, RI	[1, 13]
68	n.i.*	2145	0.08	–	–
69	Phytol acetate	2219	0.17	MS, RI	[1, 13]
Total			99.67		

*GC-MS 70eV, 200°C *m/z* (rel. int.): compound **24**: no M+, 43(100), 95(79), 79 (70), 81(50) 41(37), 39(36), 93(32), 67(30), 71(29), 53(27). Compound **33**: no M+, 91(100), 45(89), 93(64), 79(55), 92(51), 41(48), 39(32), 77(27), 119(26), 67(24). Compound **37**: no M+, 43(100), 79(97), 39(84), , 41(81), 94(64), 67(59), 91(56), 109(56), 99(48), 152(42). Compound **46**: no M+, 41(100), 39(85), 69(73), 83(67), 81(52), 96(38), 42(27), 53(25), 67(23), 95(19). Compound **55**: no M+, 41(100), 161(100), 91(83), 105(81), 93(70), 77(69), 79(60), 39(60), 81(58), 133(44). Compound **68**: no M+, 43(100), 41(58), 58(44), 110(39), 95(36), 55(33), 71(31), 59(28), 57(24), 39(23). Explanations: n.i., not identified; tr, trace amount (< 0.05%); KI, Kováts indices; MS, mass fragmentation; RI, comparison of Kováts indices with literature values; Co, Co-chromatography with authentic sample

In total 69 chemical compounds were found accounting for 99.7% of the sample and 63 were identified (97.1% of the sample). The main constituent was *cis*-pinocamphone (48.6%), followed by elemol (7.4%), *β*-pinene (6.1%), *1,8*-cineole (5.8%) and caryophyllene oxide (5.2%). A comparison of those results with the review of the published literature indicates that the composition of the essential oil isolated from Polish hyssop is similar to those obtained from hyssop in Serbia [12]. Furthermore, the level of *cis*-pinocamphone corresponded with ISO 984 Standard (1991 E) which recommends 34.5–50% for *cis*-pinocamphone. The level of the second isomer of pinocamphone (*trans*-pinocamphone) and the level of *β*-pinene are below the ISO Standard that demands 5.5–17.5% of *trans*-pinocamphone and 13.5–23% of *β*-pinene [11]. Investigations of essential oils obtained from hyssop plants that originate from

different locations are necessary because of their great diversity in chemical composition. For example, in essential oil obtained from hyssop grown in India, the main compound is *trans*-pinocamphone (49.1%); also, high levels of *β*-pinene (18.4%) and *cis*-pinocamphone (9.7%) are present [5]. The predominant compound in essential oil from *Hyssopus officinalis L. subsp. angustifolius (Bieb.) Arcangeli* (from Turkey) is pinocarvone. Moreover, high levels of *trans*-pinocamphone (19.6%), *β*-pinene (10.6%) and *1,8*-cineole (7.2%) are present, whilst the amount of *cis*-pinocamphone (5.3%) is relatively low [9]. The chemotype of hyssop from Spain has a unique composition, because of high amounts of *1,8*-cineole (52.89%) [20], whereas in essential oil from *Hyssopus officinalis var. decumbens* (from France) linalool (49.6%) predominates over other compounds. The characteristic feature of this oil is a very low level of monoterpenic ketones: *trans*-pinocamphone and *cis*-pinocamphone [17]. In addition to this, *Hyssopus officinalis L. subsp. aristatus*, an endemic plant growing in three regions of Italy – Popoli, Avezzano and Assergi – has low contents of these compounds. Furthermore, the chemotype from every region mentioned above has a unique chemical composition [22, 23].

CONCLUSIONS

Polish hyssop that was the subject of our research belongs to the group of chemotypes rich in bicyclic monoterpenic ketones – *cis*-pinocamphone and *trans*-pinocamphone. The amount of essential oil obtained from air dried hyssop harvested during the flowering stage – 0.18% (v/w) – is relatively low in comparison with data published in literature – 1.18% (v/w) [5] and 1.13% (v/w) [14].

The quality of essential oil obtained from Polish hyssop does not come up to expectations of ISO 984 Standard (1991 E) [11], because of low content of *trans*-pinocamphone and *β*-pinene.

High levels of *cis*-pinocamphone suggests strong epileptogenic properties of oil obtained from Polish hyssop which were proven for several other rich in cis-pinocamphon chemotypes of hyssop [3, 4, 18]. That is why hyssop and the remedies that contain hyssop or its essential oil should not be used by children and those patients that suffer from epilepsy. What is more, allergic reactions to hyssop, as well as to the other species belonging to the *Labiatae* family, should also be taken under consideration while using hyssop [2]. Our experiment shows that Polish hyssop is poor in methyl eugenol (0.379%) that possesses carcinogenic properties [21].

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SUMMARY

The plants of *Hyssopus officinalis L*. were grown in the herb garden at the Faculty of Pharmacy, Medical University of Lublin, Poland. The oil obtained with the use of steam distillation from the air-dried, aerial parts of hyssop was analysed by means of GC/MS and GC/FID techniques. Sixty-nine components were found, representing 99.7% of the essential oil and sixty-three of them were identified. The major constituents were identified as *cis*-pinocamphone (48.6%), elemol (7.4%), *β*-pinene (6.1%), *1,8*-cineole (5.8%) and caryophyllene oxide (5.2%).

Keywords: Hyssopus officinalis L., hyssop, essential oil, cis-pinocamphone, GC/MS, GC/FID

STRESZCZENIE

Surowiec do badań stanowiło ziele hyzopu lekarskiego *Hyssopus officinalis L.*, uprawianego w Pracowni Roślin Leczniczych, Katedry i Zakładu Farmakognozji UM w Lublinie. Olejek eteryczny otrzymano poprzez destylację z parą wodną, a następnie analizowano metodą GC/MS oraz GC/FID. Oznaczono udział 69 składników olejku eterycznego (99,7%), z czego 63 zidentyfikowano. Głównymi składnikami olejku były *cis*-pinokamfon (48,6%), elemol (7,4%), *β*-pinen (6,1%), *1,8*-cineol (5,8%) oraz tlenek kariofilenu (5,2%).

Słowa kluczowe: Hyssopus officinalis L., hyzop, olejek eteryczny, cis-pinokamfon, GC/MS, GC/FID

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Acute complications of diabetes – the present state of knowledge. Part I. Diabetic ketoacidosis

Ostre powikłania cukrzycy – stan wiedzy. Cz. I . Cukrzycowa kwasica ketonowa

INTRODUCTION

Acute complications of diabetes comprise clinical conditions in the course of hyperglycemia: diabetic ketoacidosis (DKA), non-ketotic hyperosmolar hyperglycemia (HHNK syndrome), lactic acidosis (lactic coma) and hypoglycemia (hypoglycemic coma). The most commonly occurring acute complication of diabetes is DKA as a complication of mostly type 1 diabetes, sporadically type 2; less frequent is HHNK and very rare lactic acidosis. Hypoglycemic coma occurs rarely, more frequent are hypoglycemias as complications of treatment [1,4,6,10]. Mortality in acute complications of diabetes ranges at present from approximately 5% in DKA to approximately 15% in HHNK, in lactic acidosis it exceeds 50% . Before the discovery of insulin, mortality in DKA was 100%; it was significantly reduced together with progress in treatment and increasingly greater access to insulin preparations, although it still remains a serious, even life-threatening complication in the population of patients with diabetes [2,7,12].

EPIDEMIOLOGY

Epidemiological data concerning DKA indicate that this complication occurs with the frequency of 4.6–8.0 / 1000 people / year in the population of patients with diabetes. In children and adolescents at the first diagnosis of type 1 diabetes DKA occurs with the frequency of 25%–40%. Mortality in DKA according to the data of Chiasson et al. is from 4% to 10%, and according to Efstsathiou et al. it ranges from 5% to 15% depending on the clinical condition, complications and the place of hospitalization, reaching even 30% in intensive care units [1,2]. Epidemiological data concerning the HHNK syndrome are more varied and they are 5%–15% of all acute complications of diabetes in adult and child populations. The incidence of HHNK in diabetic adults is 17.5 cases / 100 000 people / year however, American data from the year 2003 give the incidence of < 1 case /1000 people / year in the population of people with diabetes, but aged > 60 years [3,7,11]. Interesting data concerning the

incidence of HHNK in children *de novo* diagnosed with type 2 diabetes were presented by Fourtner et al. in 2003 [3]. These authors observed the syndrome in approximately 4% of children and they associated the fact with an increasing trend in incidence of obesity among children. Lactic acidosis is a severe metabolic disorder which more commonly concerns older people and it occurs in 1% of general hospitalized population [6].

DIABETIC KETOACIDOSIS

D e f i n i t i o n . DKA is a state of acute and absolute insulin deficiency causing complex disturbances in metabolism of carbohydrates, fats, proteins, in water-electrolyte equilibrium and acid-base balance. This condition is characterized by hyperglycemia, ketonemia with ketonuria and metabolic acidosis.

P a t h o g e n e s i s . Important factors in DKA pathogenesis include: insulin deficiency, dehydration and electrolyte disturbances, an increase in the concentration of counter-regulating hormones against insulin, ketonemia and metabolic acidosis. Insulin deficiency decreases utilization of glucose in tissues, leading to hyperglycemia, glucosuria and osmotic dieresis. Moreover, it increases protein catabolism and disturbs fat metabolism. Disturbances in protein metabolism lead to hyperaminoacidemia, an increase in gluconeogenesis and hyperglycemia, and also to cellular dehydration and tissue hypoxia. An increase in lipolysis leads to hyperlipemia, hepatic ketogenesis, i.e. an increase in hepatic production of ketone bodies from free fatty acids (acetoacetic acid and β-hydroxybutyric acid), ketonemia and ketonuria, causing a reduction in alkaline reserve of the organism and development of metabolic acidosis. As a result, these metabolic disturbances lead to a complete clinical picture of diabetic ketoacidosis, and in the case of lack of proper treatment to development of hypovolemic shock, anuria, coma and a direct threat to life. An increase in the concentration of counter-regulating hormones, mainly glucagon and cortisol, aggravate already existing hyperglycemia through stimulation of gluconeogenesis and lipolysis. Growth hormone and katecholamines also participate in the last process.

C a u s e s . In cases of type 1 diabetes DKA is in 10% to 20% of cases connected with lack of or delay in diagnosis of diabetes. Bacterial infections, especially purulent, constitute from 40% to 50% of causes in type 2 diabetes, and in type 1 up to 20%. Pancreatitis should also be mentioned here together with viral infections (influenza and its complications, hepatitis).

Mistakes in insulin therapy, such as interruption in the use of insulin, missing one or several doses of insulin or oral medication, using insulin after expiry date or improperly stored (frozen) insulin and interruption in subcutaneous infusion of insulin (personal pump) due to technical problems constitute another group of causes of DKA.

Acute non-infectious diseases, such as myocardial infarction, cerebral stroke, the alimentary tract obstruction, hypertensive crisis and increased hyperthyroidism, are another group of causes. Other possible causes of DKA include pregnancy, injuries, surgical procedures in people with undiagnosed diabetes, alcohol and stress. It should also be emphasized that in a group of approximately 20% of cases the cause of acidosis remains unknown.

High risk factors in DKA include concomitant renal failure, myocardial infarction, cerebral stroke, pregnancy, old age and hyperglycemia > 600 mg/dl (>33.3 mmol/l).

Clinical picture. Major clinical symptoms in DKA are increased thirst and polyuria reaching 5–7 l/24 hours, loss of body mass linked to dehydration, nausea and vomiting, muscular weakness and cramps. Prodromal symptoms can include tiredness, disturbed vision, vertigo, disturbances in balance, difficulty in concentrating and increasing drowsiness. In the period of advanced acidosis hyperventilation develops with Kussmaul breath and the presence of acetone smell in exhaled air (the smell of fruit compote), chest pains aggravated during respiration may occur and also abdominal pains with marked muscular defence suggesting the so-called "acute abdomen" resulting from severe dehydration. Typically, hypotonia and tachycardia leading to hypovolemic shock appear. Leukocytosis, which is an expression of acidosis, not infection, is frequently observed. Worrying symptoms which may indicate poor prognosis include increasing drowsiness, dementia and coma, which occur in 10% of patients with DKA. Additionally, coma with lack of reactions to pain stimuli and removal of deep reflexes indicate bad prognosis [9,10]. Degrees of severity of DKA are presented in table 1.

Table 1. Degrees of severity of diabetic ketoacidosis

MILD DIABETIC KETOACIDOSIS
Conscious patient, glycemia > 250 mg/dl (> 14.0 mmol/l), ketonuria,
HCO_3^- 15–18 mmol/l, blood pH 7.25–7.3; anion gap >10
MODERATE DIABETIC KETOACIDOSIS
Patient with disturbed orientation,drowsiness, glycemia > 250 mg/dl (> 14.0 mmol/l), ketonuria,
HCO_3^- 10–15 mmol/l, blood pH 7.0–7.25; anion gap > 12
SEVERE DIABETIC KETOACIDOSIS (COMA)
Patient in coma, glycemia > 400 mg/dl (> 22.0 mmol/l), ketonuria,
HCO_3^- < 10 mmol/l, blood pH < 7.0; anion gap > 12

Diagnosis. The basis for diagnosis is hyperglycemia > 250 mg/dl (>13.9 mmol/l), ketonemia and /or ketonuria, blood pH < 7.3, bicarbonate content < 15 mmol/l and an anion gap > 10 [5].

Differential diagnosis. DKA should be differentiated from other diabetic comas, metabolic acidosis (uremic, hepatic), CNS disorders (cerebral stroke, tumours, inflammations), myocardial infarction, thyroid, adrenal, hypercalcemic crises, poisonings (glycol, methanol, salicylates), psychiatric disorders and starvation ketosis.

Treatment. The aim of treatment of DKA is to control water electrolyte disturbances, reduce hyperglycemia and return acid base balance. Additionally, the cause of the condition should be found and possibly eliminated, and preventive measures taken [5,8,9].

Hydration of the patient is achieved through infusion of 2l of 0.9% NaCl in the first two hours of treatment, another 2l are given during the next 2–4 hours, and in further treatment the infusion is continued at the rate of 250 ml/h until water electrolyte balance is achieved. In total, during 24 hours of treatment the patient should receive 5–6.5 l of fluid, but it should be stressed that the patient's condition must be taken into account, i.e. functioning of circulation, kidneys (hourly dieresis) and arterial pressure values. Before hydration of the patient is begun, effective plasma

molality and corrected (real) sodium concentration should be calculated. It is connected with the fact that during hyperglycemia sodium concentration is higher than laboratory indications and then infusions of 0.45% NaCl should be administered until proper natremia is achieved. In the case of existing hypernatremia with the values of Na > 150 mmol/l and effective plasma osmolality > 300 mOsm/kg H_2O, 0.45% NaCl solution should be infused as was mentioned above. Proper hydration can cause a decrease in glycemia even by 30%, but it does not exclude the application of the next stage of treatment, i.e. reduction in hyperglycemia involving insulin therapy.

Compensation of electrolyte deficiency. The greatest loss of electrolytes concerns potassium, sodium and chlorine. It is compensated for by administration of 0.9% NaCl correcting deficiency of mainly sodium and chlorine, and administration of potassium chloride (KCl) corrects deficiency of potassium. Supplementation of potassium is given depending on the concentration of potassium in blood serum. So KCl is not administered at the concentrations > 6 mmol/l, at the concentrations of 5–6 mmol/l we administer 5–10 mmol/h, at 4–5 mmol/l 10–15 mmol/h, at 3–4 mmol/l 15–20 mmol/h, below 3 mmol/l 25 mmol/h (1 ampoule of 10 ml KCl contains 40 mmol of potassium).

Insulin therapy is conducted using short-action insulin (Actrapid, Gensulin R, Humulin R, Polhumin R) in the form of intravenous infusion with an infusion pump. Appropriately 20 or 50 units of short-action insulin are added to 20 or 50 ml of 0.9% NaCl, obtaining 1 unit of insulin in 1 ml of solution, then insulin is administered at the dose of 0.1 unit/kg body mass/h. Insulin infusion is preceded by intravenous administration of a single dose of the so-called bolus of short-action insulin at the dose of 0.1 unit / kg body mass /h, that is from administration of 7–10 units. Then the rate of insulin infusion should be controlled and related to the present state of glycemia, and an hourly decrease in glycemia should not exceed 100 mg/dl (5.6 mmol/l). Glycemia must be decreased gradually in order to prevent blood osmolarity. When it reaches the value < 250 mg/dl (14.0 mmol/l) or the value of starting glycemia is reduced by half, the rate of insulin infusion is decreased by half and infusion of 5% glucose is added to the fluids to prevent possible hypoglycemia. The rate of insulin infusion should be adequate to present glycemia in order to ensure stable glycemia within the range of 120–140 mg/dl (6.7–7.8 mmol/l) and then intravenous insulin infusion should be replaced by subcutaneous insulin therapy, but only within 30–60 minutes after the first dose of subcutaneous insulin [13].

Controlling metabolic acidosis. In most cases, proper hydration, insulin therapy and compensation of electrolyte disturbances are sufficient to control ketoacidosis without the necessity of administration of bicarbonates. An indication for using bicarbonates is the situation when the value of arterial blood pH found in a patient with diabetic coma is less than 7.0 (< 6.9) or when ketoacidosis coincides with chronic renal failure. Then 8.4% sodium bicarbonate (1 ml = 1 mmol) is administered in the maximum dose of 1 mmol/kg of body mass, i.e. 50–100 ml in infusion of 0.9% NaCl or 5% glucose. A counterindication for administration of sodium bicarbonate is hypernatremia > 150 mmol/l, as it poses a risk of complications – pulmonary and/or cerebral edema [9].

Complications. Serious complications in DKA include: hypovolemic shock, pulmonary edema, cerebral edema, heart rhythm disturbances (ventricular), ARDS syndrome. Some complications can result from careless treatment, for example too rapid decrease in glycemia or inappropriate use of bicarbonates.

Monitoring of treatment includes control of glycemia and diuresis every hour, control of Na and K electrolytes, fluid balance and state of consciousness every 2 hours. Gasometry should be checked every 4 hours, the presence of ketone bodies in urine every 8 hours. Additionally, arterial pressure, pulse, respiration rate and general condition of the patient should be continuously controlled. After successful management of DKA, the previously applied model of treatment (insulin therapy) should be resumed or modified [13,14].

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SUMMARY

Diabetic ketoacidosis (DKA) is the most common acute hyperglycemic complication, which poses problems mostly in type 1 diabetes, sporadically in type 2. The most frequent cause of developing

ketoacidosis in patients with type 1 diabetes is withdrawal of or missing an insulin dose or delayed diagnosis; in patients with type 2 diabetes it is severe infection or stress with accompanying relative insulin deficiency. The basis for diagnosis is hyperglycemia, ketonemia and/or ketonuria and features of metabolic acidosis. Mortality in the pathology reaches 5%. The study presents the current state of knowledge about DKA concerning epidemiology, pathogenesis, clinical picture and particularly current standards of treatment.

Keywords: diabetic ketoacidosis, type 1 diabetes, type 2 diabetes, epidemiology, pathogenesis, clinical picture, standards of treatment

STRESZCZENIE

Cukrzycowa kwasica ketonowa to najczęściej występujące ostre, hiperglikemiczne powikłanie, które komplikuje przebieg przede wszystkim cukrzycy typu 1, sporadycznie typu 2. Najczęstszą przyczyną wystąpienia kwasicy ketonowej u chorych na cukrzycę typu 1 jest odstawienie lub pominięcie dawki insuliny bądź opóźnione rozpoznanie, natomiast u chorych na cukrzycę typu 2 ciężka infekcja lub silny stres, w których przebiegu dochodzi do względnego niedoboru insuliny. Podstawą rozpoznania jest stwierdzenie hiperglikemii, ketonemii i/lub ketonurii i cech kwasicy metabolicznej. Śmiertelność tej patologii sięga 5%. Praca przedstawia stan wiedzy na temat DKA w zakresie epidemiologii, patogenezy, obrazu klinicznego, a szczególnie aktualnych standardów leczenia.

Słowa kluczowe: cukrzycowa kwasica ketonowa, cukrzyca typu 1, cukrzyca typu 2, epidemiologia, patogeneza, obraz kliniczny, standardy leczenia

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Comparison of the course of type 2 diabetes in village and town inhabitants in the Lublin region

Porównanie przebiegu cukrzycy typu 2 u mieszkańców wsi i miast regionu lubelskiego

INTRODUCTION

Diabetes type 2 is a chronic metabolic disorder with frequent latent onset, in the course of which chronic vascular complications such as micro- and macroangiopathy develop. The development of microangiopathy leads to disabilities and worse life quality of patients with diabetes. Even though cardiovascular disorders are not typical of diabetes, they occur more often in diabetic patients and frequently contribute to a higher mortality rate. It must be noted that at diagnosis a prominent number of patients chronic complications are mainly connected with the cardiovascular system. This type of diabetes comprises 85–95% of diabetes cases and its occurrence increases systematically and dramatically, which is an important and still unsolved problem of public health [10]. Diabetes type 2, recognized as a cardiovascular disorder, is one of the most frequent causes of death and disabilities of the present day [1]. A total of 5-7% of the world's population suffers from this disease and the number of the sick is rising more quickly than it was expected [6]. It is said that by 2030 the number of people suffering from diabetes will have risen up to 366 million in the world [18].

A lot of data show that the compensation level in most of the diabetes type 2 patients is still insufficient, which considerably contributes to the development of complications and leads to the rise in costs of its treatment. Many clinical trials have provided data which show that decreasing the risk of development and progress of cardiovascular complications of diabetes is possible. To reach this goal, however, multifactorial treatment must be employed not only with the intention to reach a good parameter of carbohydrate metabolism but also to effectively treat hypertension, lipid disorders and obesity [3].

So far there has been little research to compare the course of diabetes type 2 in town and village dwellers. Available literature shows that patients from the countryside are usually worse compensated than those living in towns [17,14].

The aim of the study was to compare the course of diabetes type 2 in village and town inhabitants in the Lublin region hospitalized in the Endocrinology Clinic for 3 years.

MATERIAL AND METHODS

The material of the analysis included 703 patients with diabetes type 2 who were hospitalized in the Endocrinology Clinic of Medical University of Lublin for three years, i.e. from November 2006 to October 2009. At first the patients were divided into two groups depending on their place of abode. Group I included town inhabitants and group II subjects from the countryside. Each patient from the region of over 10,000 inhabitants was admitted to group I, whilst patients living in the country (up to 1,999 inhabitants) and in the suburbs (from 2,000 to 9,999 inhabitants) were included into group II.

Type 2 diabetes was diagnosed in compliance with the WHO standards and the Polish Diabetes Association (PDA) with oral glucose tolerance test (OGTT) or with twice acquired fasting glucose level >126mg% or observed incidental glycemia >200mg% together with typical diabetes type 2 symptoms [13,20]. The date of the test was recognized as the date of diagnosis. If a patient was hospitalized a few times during the trial, only the first visit in the Clinic was taken into consideration.

The method used in the study was the retrospective and comparative evaluation of the results of examination of diabetic patients from town or village areas. Patients' primary data were analyzed in relation to the reason for hospitalization (diabetic cause or other). While analyzing patients' history, the age at which the disease appeared and the present age was assessed, thanks to which accurate disease duration was calculated in years. Next, anthropometric parameters were evaluated and BMI was calculated. Among biochemical parameters the following were assessed: fasting glucose level, metabolic compensation by glycated hemoglobin concentration (HbA1c) and lipidogram. Another step included the comparison of the incidence of microangiopathic complications (retinopathy, nephropathy, polyneuropathy) and macroangiopathic complications (ischemic heart disease, heart failure, hypertension, diabetic foot). Finally, the type of treatment of both the primary disease (oral treatment, insulinotherapy, mixed treatment) as well as the coexisting ones was checked.

Statistical analysis. The results underwent statistical analysis. The values of the analyzed parameters measured in the nominal scale are characterized by size and percentage, whilst quantitative variables are shown as mean values and standard deviations. Mean values of the examined parameters in both groups were compared with Student's t-test for independent variables. The non-parametric Mann-Whitney U test was employed to analyze the results with abnormal distribution compared in the two groups. The observed differences were recognized as statistically significant with $p<0.05$.

Statistical analysis was carried out with "Statistica 8" software.

RESULTS

A total of 703 patients with diabetes type 2 were examined with 499 village inhabitants aged 63.3±12.2 years and 204 town inhabitants aged 62.3 ±12.3 years. Clinical and biochemical

characteristics are shown in Table 1. Analyzing the patients' history we calculated the disease duration which was 11.2 years (±8.6) in town dwellers and did not differ from the country dwellers 10.4 years (±8.2). Next, anthropometric parameters were taken (weight, height) and BMI was assessed. Significant differences were found in BMI of town inhabitants as compared to the country ones: weight 83.6±16.2kg vs. 87.3±17.8kg, $p<0.05$, respectively; BMI 30.7±10.7kg/m^2 vs. 34.3±19.6kg/m^2, $p<0.05$, respectively. The analysis of the diabetes metabolic compensation started with fasting glucose level which differed between the groups considerably. Mean fasting glucose levels were notably higher in group I than in group II, i.e. 141.8±42.7mg/dL vs 134.2±26.0 mg/dL, $p<0.05$, respectively, which is shown in Table 1. However, glycemia <110mg/dL was reached by a comparable number of patients from town and village inhabitants, i.e. 19.4% and 19.6%, respectively. Another biochemical parameter that was analyzed was HbA1c concentration which was almost identical in both groups (Table 1), and the HbA1c percentage <7% was equally frequent in both groups i.e. 42.1% and 44.1%, respectively.

Table 1. Clinical and biochemical characteristics

Examined parameter	Whole group (N=703)		Town (N=499)		Village (N=204)		p
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	
Patient's age (years)	62.9	12.2	63.3	12.2	62.3	12.3	0.41
Age of diabetes (years)	11.0	8.5	11.3	8.6	10.4	8.5	0.35
Weight (kg)	84.7	16.7	83.6	16.2	87.3	17.8	<0.05
Height (m)	1.6	11.2	2.7	13.4	1.6	0.2	0.11
BMI (kg/m^2)	31.8	14.1	30.7	10.7	34.3	19.6	<0.05
$HbA_{1c\,(\%)}$	8.1	2.1	8.2	2.1	8.1	1.9	0.66
Fasting glucose level (mg/dL)	139.6	38.7	141.8	42.7	134.2	26.0	<0.05
Total cholesterol (mg/dL)	181.6	48.5	180.5	48.5	184.4	49.2	0.09
HDL(mg/dL)	48.3	18.7	48.2	19.4	48.8	17.1	0.35
LDL(mg/dL)	105.4	37.8	103.1	37.4	111.0	38.2	<0.05
Triglycerides (mg/dL)	165.0	115.9	165.5	117.1	163.9	113.4	0.75
Creatinine (mg/dL)	0.9	0.5	1.0	0.5	0.9	0.3	0.55
Systolic (mmHg)	133.2	15.1	132.7	14.6	134.3	16.1	0.08
Diastolic (mmHg)	82.6	8.6	82.4	8.9	83.2	7.9	0.26
Microalbuminuria (mg/24 hrs)	72.47	248.2	84.5	284.5	38.8	78.8	0.28

Parameters of lipid metabolism compensation were also analyzed, where total cholesterol and its fraction in plasma were assessed. Mean LDL cholesterol fraction concentrations were significantly lower in town inhabitants as compared to those living in the country, i.e. 103.1±37.4mg/dL vs. 111.0±38.2mg/dL, $p<0.05$, respectively, without any difference in other lipid fractions. Metabolic compensation criterion <175mg/dL was present in 52.7% of village dwellers and 12.2% in town ones, which is a significant value (Fig. 1). Table 2 shows the incidence of diabetes complications and the type of treatment of the examined patients. The frequency of microangiopathic complications

(retinopathy, nephropathy and diabetic polyneuropathy) was not significant. Retinopathy was diagnosed in 7.3% of patients in group I and 5.6% of patients in group II. Diabetic polyneuropathy was present with comparable frequency in both group I and II, 9.7% and 7.5%, respectively.

Table 2. Complications of diabetes and types of treatment in both groups

Examined parameter	Town (N=499)		Village (N=204)		p
	Yes	No	Yes	No	
Reason for hospitalization - diabetes	85.3%	14.7%	78.8%	21.2%	p<0.05
Hypertension	82.8%	17.2%	86.4%	13.6%	NS
Dyslipidemia	66.5%	33.5%	67.7%	32.2%	NS
Obesity	54.7%	45.3%	72.0%	28.0%	p<0.001
Ischemic heart disease	26.2%	73.8%	29.3%	70.7%	NS
Retinopathy	7.3%	92.7%	5.6%	94.4%	NS
Polyneuropathy	9.7%	90.3%	7.5%	92.5%	NS
Diabetic foot	4.4%	95.6%	4.0%	96.0%	NS
Insulinotherapy	61.5%	38.5%	53.3%	46.7%	p<0.05
Intensive insulinotherapy	20.5%	79.5%	17.6%	82.4%	NS
Conventional insulinotherapy	35.8%	64.2%	33.7%	66.3%	NS
NPH insulin at night	5.9%	94.1%	3.0%	97.0%	NS
Metphormin	50.3%	49.7%	56.8%	43.2%	NS
Sulphonylurea	24.8%	75.2%	28.1%	71.9%	NS
Acarbose	7.3%	92.7%	5.5%	94.5%	NS
Converting inhibitors	66.4%	33.6%	66.3%	33.7%	NS
Beta blokers	44.9%	55.1%	44.3%	55.7%	NS
Statins	53.9%	46.1%	56.8%	43.2%	NS
ASA	50.2%	49.8%	57.8%	42.2%	NS

Hypertension was among the most frequently reported macroangiopathic complications in both groups; taking into consideration higher values of systolic pressure (<130mmHg) – the percentage of patients in group I (51.5%, mean 132.75mmHg ± 14.6) as compared to group II (134.29mmHg ± 16.1) was not significant. Analyzing diastolic pressure (>80mmHg); no significant differences were noted between the two groups (town 44.3%, mean 82.4mmHg ± 8.9, village 49.1%, mean 83.2mmHg ± 7.9).

The next step concerned the type of treatment, pointing towards insulinotherapy, 61.5% and 53.3% group I and group II, respectively. No significant difference was noted as regards the oral pharmacological treatment. Over 50% of patients in both groups were treated with metphormin, a small number with sulphonylurea, and the fewest number of patients with acarbose.

Pharmacotherapy of coexisting diseases was also evaluated, i.e., hypertension and dyslipidemia. The fact worth noticing is the frequency of using antihypertensives from group of angiotensin-converting enzyme inhibitors as compared to beta blockers or calcium channel inhibitors which was similar in both groups. Hypoglycemic type of treatment was used in a similar number of patients in both groups.

As for taking acetylsalicylic acid derivatives (ASA), generally a low percentage of patients with type 2 diabetes in our study group (52.4%) was treated with this medicine as a preventive measure against cardiovascular diseases. The respective percentage of patients taking ASA in group I was 57.8% and group II 50.2%. In both groups, the primary cause of hospitalization in the Endocrinology Clinic was insufficient metabolic compensation, which was present in 85.3% of patients in group I and 78.8% in group II; however, subjects from the latter group were hospitalized more often because of other causes, 21.2% vs. 14.7%, $p<0.05$.

DISCUSSION

In current literature on diabetology the fact worth noting is that there has been little research about the degree of metabolic compensation in diabetic patients living outside cities, i.e. in towns and in the country. Szurkowska et al. in their analysis show that compensation of diabetes in village inhabitants was worse than in their town equivalents [17]. Malec et al. reached similar conclusions, indicating that patients with type 2 diabetes living in the country had insufficient metabolic compensation which seemed worse than of patients living in towns. The possible reason behind it may be the higher level of obesity and overweight of village dwellers together with worse access to specialized medical care [8]. Undoubtedly, this aspect should be investigated further with cross-sectional research of both populations.

In both examined groups of patients with diabetes type 2, the parameters of carbohydrate metabolism were insufficient according to the PDA guidelines regardless to the place of abode [20]. Fasting glucose level was significantly higher in town subjects as compared to the village ones, however, there was no differentiation in HbA1c values. Target fasting glucose <110mg/dL was reached by a small, but similar in both groups, percentage of subjects, i.e. 19.4% in towns and 19.6% in villages, which seems to result from patients inconsistency in following diabetic recommendations. Another evaluated biochemical parameter was the HbA1c concentration which was a lot above the recommendations of the PDA, whilst its mean value was almost identical in both groups i.e. group I – 8.1% and group II – 8.2%, which indicates that mean glycemia was >154mg% (8.6 mol/L) [20,11]. This parameter was higher than the mean value of the Polish national study "DINAMIC 2": in 2488 patients it was 7.37% [16]. Unfortunately, it was also a lot higher than the mean of the Lublin Province which was 7.27% shown in this study [4]. The most probable explanation for this is the fact that the patients examined in our study were not randomized but they were hospitalized because of the lack of metabolic compensation. However, the percentage of patients that reached the HbA1c threshold <7% was distributed equally in both groups of our study, 42.1%; and 44.1% respectively as well as in the "DINAMIC 2" study. As compared to the results of another national study evaluating the degree of carbohydrate metabolism called "PolDiab", our findings were the same. Mean HbA1c in this study was 8.07%, but the percentage of patients which reached HbA1c <7% was twice as low (23.3%) as the percentage found in our study [15].

Body weight control in patients with diabetes type 2 is one of the criteria of the compensation of the disease according to the PDA and the ADA [7,20]. Unfortunately, most of our patients did not meet this criterion since obesity was present in 54.7% of subjects in group I and in as many as

72.0% in group II. However, this fact did not explicitly affect the remaining parameters of metabolic compensation, which is consistent with our previous study [9]. However, among more obese village population the compensation criterion for total cholesterol <175mg/dL was reached only by 12.2% of diabetic patients as compared to 52.7% of town population. Undoubtedly, it is connected with a different lifestyle of those patients who live on a high-calorie diet and undertake hard physical work. It should be noted that obesity and dyslipidemia are independent cardiovascular risk factors, therefore, a corrective therapy (reduction of body weight and lipid disorders) should be an integral part of the multifactorial treatment of diabetes [12].

With no regard to the place of abode, the primary treatment in our study was insulinotherapy which was used in >50% of the patients with preferential treatment of conventional insulinotherapy. Taking into consideration disease duration, which for the whole studied population was 11 years, it was probably the result of acquired drug resistance to oral medications for glycemic control. According to the PDA; insulinotherapy should be provided when the HbA1c percentage exceeds 7% while using the maximum dosage of oral drugs (if removable causes of hiperglycemia have been excluded and there are no recommendations for an earlier use of insulin) [20]. Insulinotherapy as treatment for diabetes type 2 should not be provided too early because of the potential weight gain and a possibility of iatrogenic metabolic syndrome, and probably an increased risk of colorectal cancer; however, it should not be provided too late either as glucotoxicity, adverse effects of proinsulin and chronic hyperglycemia lead to vascular complications [2,5].

CONCLUSIONS

In the population of patients with type 2 diabetes in the Lublin region, the place of abode does not affect the course of diabetes type 2, though BMI was significantly higher in village than in town inhabitants. Despite higher fasting glucose levels in patients from towns, no significant HbA1c values were noted as compared to those of the country dwellers. Both groups did not meet the metabolic compensation criteria of the PDA to a similar extent and required absolute intensification treatment.

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SUMMARY

Diabetes type 2, a chronic metabolic disorder and frequent lifestyle disease is, according to WHO, the biggest unsolved problem of public health. The aim of the study was to compare the course of diabetes type 2 in village and town inhabitants in the Lublin region hospitalized in the Endocrinology Clinic for 3 years. The analysis was based on retrospective evaluation of medical history of 703

patients with diabetes type 2 hospitalized in the Endocrinology Clinic November 2006–October 2009, including 204 country dwellers (29%) and 499 town dwellers (71%) with mean disease duration 11±8.5 years. The following parameters were analyzed: reasons of hospitalization, disease duration, anthropometric features, metabolic compensation criteria, presence of chronic complications and types of hypoglycemic treatment. In both groups, the main reason of hospitalization was the lack of metabolic compensation. Mean fasting glucose levels were considerably higher in patients from towns than those of from villages, though, this fact was not significantly reflected in HbA1c values. No significant difference between the groups was found with regard to the patients' age, disease duration, total cholesterol, HDL cholesterol, triglycerides, creatinine, microalbuminuria, blood pressure or the presence and types of chronic vascular complications. Mean BMI was considerably higher for inhabitants of the country than in the town, respectively: 34.3±19.6 vs. 30.7±10.7, $p<0.001$. The tested groups did not meet the metabolic compensation criteria of the PDA to a similar extent. Both groups were treated mainly with insulinotherapy. In the population of patients with type 2 diabetes in the Lublin region, the place of abode does not affect the course of diabetes type 2, though BMI was higher in village than in town inhabitants. Despite higher fasting glucose levels in patients from towns, no significant HbA1c values were noted as compared to those of the country dwellers.

Keywords: diabetes type 2, villages, town, metabolic compensation, obesity

STRESZCZENIE

Cukrzyca typu 2, przewlekła choroba metaboliczna i jedna z głównych chorób cywilizacyjnych, stanowi obecnie wg WHO największy nierozwiązany problem zdrowia publicznego. Celem pracy było porównanie przebiegu cukrzycy typu 2 u mieszkańców wsi i miast regionu lubelskiego, hospitalizowanych w Klinice Endokrynologii w okresie 3-letnim. Analizę przeprowadzono w oparciu o retrospektywną ocenę dokumentacji medycznej 703 chorych z cukrzycą t. 2 hospitalizowanych w Klinice Endokrynologii od listopada 2006 r. do października 2009 r., w tym 204 chorych (29%) zamieszkujących obszary wiejskie i 499 pacjentów (71%) z miasta, o średnim czasie trwania choroby 11±8,5 roku. Analizie poddano następujące parametry: przyczyny hospitalizacji, czas trwania cukrzycy, cechy antropometryczne, kryteria wyrównania metabolicznego, obecność przewlekłych powikłań choroby, rodzaj leczenia hipoglikemizującego. Zarówno wśród mieszkańców miast, jak i wsi głównym powodem hospitalizacji był brak wyrównania metabolicznego choroby. Średnie wartości glikemii na czczo były istotnie wyższe u pacjentów w populacji miejskiej w porównaniu z populacją wiejską, chociaż nie odzwierciedlało się to istotnie w wartości HbA1c.Nie obserwowano istotnych różnic w zakresie wieku chorych, czasu trwania choroby, cholesterolu całkowitego, frakcji HDL, triglicerydów, kreatyniny, mikroalbuminurii, wartości ciśnienia tętniczego, jak również obecności i rodzaju przewlekłych powikłań naczyniowych w zależności od miejsca zamieszkania. Średnie wartości BMI były istotnie wyższe u pacjentów z rejonów wiejskich w porównaniu z mieszkańcami miast, odpowiednio: 34,3±19,6 vs 30,7±10,7, $p<0,001$. Badane grupy pacjentów nie spełniały kryteriów wyrównania metabolicznego PTD w porównywalnym stopniu. W obydwu grupach pacjentów dominującym sposobem leczenia była insulinoterapia. W populacji pacjentów z cukrzycą typu 2 regionu lubelskiego miejsce zamieszkania nie wpływało na przebieg cukrzycy

typu 2, chociaż wartości BMI były istotnie wyższe u pacjentów z rejonów wiejskich niż u pacjentów z miasta. Pomimo istotnie wyższej glikemii na czczo u pacjentów z rejonów miejskich nie obserwowano istotnego zwiększenia wartości HbA1c w porównaniu z pacjentami ze wsi. Badane grupy pacjentów nie spełniały kryteriów wyrównania metabolicznego PTD w porównywalnym stopniu.

Słowa kluczowe: cukrzyca typu 2, tereny wiejskie, miasto, wyrównanie metaboliczne, otyłość

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Phosphoglucose isomerase – "portrait of the protein with many faces"

Izomeraza fosfoglukozowa – „portret białka o wielu twarzach"

Phosphoglucose isomerase (D-glucose-6-phosphate aldose-ketose-isomerase; glucose-6-phosphate isomerase; phosphohexose isomerase; PGI; EC 5.3.1.9) catalyzes the reversible isomerization of glucose-6-phosphate (G6P) to D-fructose-6-phosphate ester (F6P).

BIOLOGICAL FUNCTIONS OF PGI

PGI plays a crucial role in the phosphorylated sugars metabolism: glycolysis, phosphate pentose pathway and gluconeogenesis. Hence, phosphoglucose isomerase has been sometimes described as a "workhouse" enzyme of sugar metabolism [10]. PGI is involved in biosynthesis of arabinogalactan (component of mycobacterial cell wall) [1, 25]. This enzyme performs the key role in the governing carbon availability for the synthesis extracellular polysaccharides (EPS) of bacterial biofilms *in vivo* [37].

PGI has a number of other "moonlighting" functions [19, 20]. Jeffery explained that "moonlighting" refers to a single protein that has multiple independent functions, which are not the effects of gene fusion, splice variants or the presence of multiple proteolytic fragments [20]. The function of these type proteins may be a consequence of changes in cellular localization, cell type, oligomeric state, concentration of a ligand, substrate, cofactor or product. In many cases, the protein uses a combination of these methods to switch between functions [19].

PGI with novel lysyl aminopeptidase activity (PGI-LysAP) was characterized from the human pathogen *Vibrio* (*V.) vulnificus* [28, 29] and was also detected in *V. cholerae* and *V. parahemolyticus* [28].

PGI revealed specific inhibitory activity towards a myofibril-bound serine proteinase (MBSP) from crucian carp (*Carassius auratus*) [33] and white croacker (*Argyrosomus argentatus*) [8].

In mammals, it was shown that several proteins such as neuroleukin (NLK), autocrine motility factor (AMF), and differentiation and maturation factor (DMF) are closely related, supposedly even identical to PGI [34]. GPI as NLK is a neutrophic factor for sensory and specific embryogenic spinal neurons [9, 12, 13]. NLK is secreted by lectin-activated T lymphocytes and it promotes secretion of immunoglobulins by cultured human peripheral blood mononuclear cells [12, 13, 39]. PGI was also identified as the SA-36 antigen involved in sperm agglutination. It was found that SA-36 cDNA displayed > 99% homology

to human GPI/NLK [39]. In fact, only three SA-36 amino acids (aa) at positions 158, 426 and 436 were not identical to 558 aa of human GPI/NLK [39]. AMF is secreted by tumor cells to promote cell motility and proliferation [42]. An additional biological function of GPI as a DMF is the ability to mediate the differentiation of human myeloid leucemic HL-60 cells to terminal monocytic cells [38]. A positive correlation between anti-GPI autoantibody and the arthritis disease in humans have been suggested [31]. Inherited deficiency of human GPI affects mostly erythrocytes causing hereditary nonspherocytic hemolytic anemia (HNSHA) in humans. A severe deficiency was associated with hydrops fetalis, immediate neonatal death and neurological impairment [24]. Approximately 50 clinical cases of HNSHA associated with GPI deficiency have been reported [22]. By 2009, 29 mutations including 24 missense, 3 nonsense and 2 splice site were documented [24].

CLASSIFICATION AND STRUCTURE OF DIFFERENT TYPES OF PGI

Two different PGI superfamilies have been recently identified. A conventional PGI superfamily comprises a PGI family and PGI/PMI family. Two regions of conserved aa [DENS]-X-[LIVM]-G-G-R-[FY]-S-[LIVMT-X-[STA]-[PSAC]-LIVMA]-G- and [GS]-X-[LIVM]-[LIVMFYW]-X_4-[FY]-[DN]-Q-X-G-V-E-X_2-K were documented as specific motifs for the PGI superfamily [4]. The aa sequence S-Y-S-G-[NT]-T-[ESTIL]-E-T-[LIV] was described as a specific motif for PGI/PMI. PGIs contain a Lys or an Arg at 2nd position of this pattern [16].

PGI family has been divided into three major subfamilies: I – eukaryotic, II – cyanobacterial/chloroplasts and III – bacterial. Subfamily I also includes several eubacterial clades: *Zymonas mobilis*, *Xanthomonas campestris*, *E. coli* and *Haemophilus influenzae*. PGI subfamily II comprises phosphoglucose isomerases from chloroplasts, cyanobacteria and amitochondriate protists. Interestingly, PGIs from *Methanococcus jannaschii*, *Halobacterium* NRC1 and *Haloarcula marismortui* (all three strains are euryarcheota) were included into III subfamilies of PGI [16].

Putative homologs of the bifunctional *Aeropyrum pernix* PGI/PMI were identified in the genoms of eight (hyper)thermophilic Archae and bacteria: *Pyrobaculum aerophilum*, *Thermoplasma acidophilum*, *Thermoplasma volcanium*, *Ferroplasma acidarmanus*, *Sulfolubus tokodaii*, *Sulfolubus solfaraticus, Aquifex aelolicus* and *Anaerocellum thermophilum* [16].

The second superfamily of PGI cupin (cPGI) was discovered in the euryarcheota species *Pyrococcus furious* (*P. furiosus*) [14, 41], *Thermococcus litoralis* [21], *Archaeoglobulus fulgidus* [15] and *Methanosarcinia mazei* [15]. This type of PGI was found in two strains of bacteria *Salmonella enterica* serovar Typhimurium and *Ensifer meliloti* [15]. The motifs $TX_3PX_3GXEX_3TXGHXHX_{6\text{-}11}EXY$ and PPX_3HX_3N were deduced as two specific motifs of the cPGI super family. Phylogenetic analysis suggested later gene transfer for the bacterial cPGIs from euryarchaeota [15]. Cupin-PGI represents a class of PGI, which are completely unrelated to the αβ sandwich fold (SIS domain) presented in the conventional PGIs [3, 7]. The polypeptide chain of cPGI from *P. furiosus* starts with a short β strand, which forms an additional strand on the cupin barrel of the adjacent monomer. The chain continues through α-helix into two β-sheets, containing three and six β-strands. The chain is completed by an α-helix and two β-strands, which pack against the three-stranded β-sheet, and lead to the C-terminus. The active site of this and other cPGIs lies in the cupin barrel adjacent to the metal-biding site [7]. The two monomers in dimer are essentially identical [35]. Table 1 presents the comparison of PGI properties from different organisms.

Table 1. Comparison of PGIs from various organisms

Organism			Temp. opt. (°C)	pH opt.	Molecular weight (kDa)		K_m (mM)			Ref.
					native	subunit	F6P	G6P	M6P	
PGI superfamily	PGI-family	*E. coli* (I)	ND	8.0	120 (α_2)	59	0.2	ND	-	32
		E. coli (II)	ND	8.0	230 (α_4)	59	0.2	ND	-	32
		Human	ND	7.5	126 (α_2)	63	0.037 ± 0.003	ND	-	24
	PGI/ PMI-family	*A. pernix*	> 98	7.6 ± 0.2	45 ± 5	36	0.44 ± 0.10 0.21 ± 0.05 (50°C)	3.5 ± 0.5	1.1 ± 0.3	16
Cupin- PGI superfamily		*P. furiosus*	90	7.0	49.3 (α_2)	23.5	0.71	1.57	-	41

ND, not determined

MECHANISMS OF THE ALDOSE-KETOSE ISOMERIZATION

cPGI from *P. furiosus* similar to conventional type of PGI showed the activity in the isomerization of F6P and G6P. A further study concerning the substrate specificity of pyrococcal cPGI indicated that this enzyme was able to isomerize non-fosforylated sugar as L-talose and D-ribulose. L-Talose was converted to L-tagatose and L-galactose with 80% and 5% conversion yield, respectively. Whereas D-ribulose was converted to D-ribose and D-arabinose with 53% and 8% conversion yield, respectively [40].

The kinetic analysis revealed that bifunctional PGI/PGM catalyzed reversible isomerization of not only G6P, but also epimeric mannose-6-phosphate (M6P) [16]. Interestingly, M6P is a classical inhibitor for PGI and cPGI [17, 41].

Catalyzed by PGIs isomerization is reaction in which a hydrogen atom is transferred between the C1 and C2 position of substrate. The second hydrogen, in the form of a proton, also moves between the O1 and O2 [35]. In the mechanism postulated for PGI, proton exchange between O1 and O2 is likely to be mediated by a water molecule without the participation of active side residue [2, 26]. The carbon-bound hydrogen can moved by one of two mechanisms, a hydride shift or a proton transfer *via* a *cis*-enediol mechanism (Fig. 1) [30, 35].

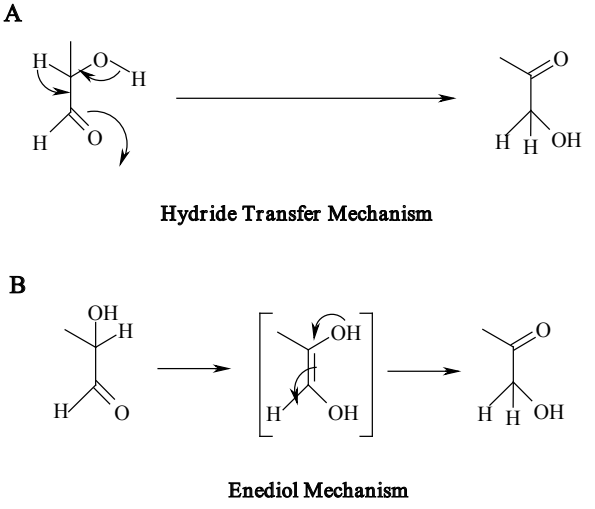

Fig. 1. The possible mechanisms for hydrogen transfer between the C1 and C2 position of substrate for phosphoglucose isomerase [after 30, 35]

Swan and his colleagues were favored a hydride transfer mechanism for cPGI from *P. furiosus* (Fig. 1A). A metal ion (Fe^{2+} *in vivo*) is rather involved in catalysis than perform a structural role. Pyrococcal PGI has a preference for straight chain substrates. This fact indicates the absence of any obvious enzymatic machinery indispensable for opening of sugar ring [35].

Berrisfold et al. [7] contrary to Swan and his co-workers postulated that the *cis*-enediol mechanism could also be employed in the *P. furiosus.* In this case, the function of metal in the *P. furiosus* PGI is to stabilize the developing charge on the intermediate.

Because of differences in opinion on mechanism of *P. furiosus* cPGI, further studies are required.

Conventional PGIs, which does not require a metal cation for activity, used acid-base mechanism with *cis*-enediol intermediate (Fig. 1B). These types of PGIs catalyze the isomerization with sugar ring opening. Lee and co-workers proposed His-388 as aa residue, which participate in the ring opening of cyclic substrates [23]. The inspection of the interaction of 5-phospho-D-arabinohydroxyamic acid (5 PAH) with rabbit PGI indicated that Glu-357 transfers a proton between C1 and C2. In the same study Arg-272 is proposed to help stabilize the *cis*-enediol(ate) intermediate [2].

POTENTIAL APPLICATIONS OF PGI AND ITS INHIBITORS

In the bacteria, EPS (compound of biofilm) provides a protective capsule against, e.g., dehydration, macrophages, antibiotics and other toxic compounds [37]. From this aspect, it is necessary to carry out a very intensive study upon enzymes (including PGI) involved in the biosynthesis EPS of pathogenic bacteria. On the other hand, bacterial EPS is widely used in many applications from food processing to pharmaceutical production and other industries [37]. An example of this type research is characterization of phosphoglucose isomerase from *Sphingomonas chungbukensis* DJ77 [36, 37].

Overexpression of AMF receptor is correlated with a poor prognosis of cancer [18]. The elevated AMF in serum or urine renders the protein tumor marker in gastrointestinal, kidney, breast, colorectal, and lung cancer. The presence of GPI in serum and urine is associated with cancer progression and indicates poor prognosis [5, 6, 11, 27].

The typical inhibitors as D-erythrose-4P, 6-phospho-D-gluconate, fructose-1-phosphate, fructose-1,6-bisphosphate and mannose-6-phosphate negatively affected various types of PGI [16, 17, 41]. Certainly, the PGI/PMM was not inhibited by mannose-6-P, which is a substrate for these type enzymes [16].

PGI are attractive targets for chemotherapeutic actions of antiparasites or antifungal drug. Firstly, parasites like *Trypanosoma brucei, Trypanosoma cruzi* and *Plasmodium falciparum* derive most their energy from glycolysis [17]. Certainly, the specific inhibition of this cycle in parasites, but not in human, is necessary. Secondly, several inhibitors were tested, which mimic the transition state intermediates in PGI and hexose phosphate domain of D-glucosamine synthase (EC 2.6.1.16) [17, 26, 43]. 5-phosho-D-arabinoate (5PA), 6-phospho-D-gluconate and 5-PAH have been reported as strong inhibitors of PGI, which mimics the transition state intermediates [17]. From these compounds, 5-PAH is the best reported inhibitor of PGI with a K_i of 2 x 10^{-7}M (pH 8.0) [2, 17]. Wojciechowski and co-workers proposed D-glucosamine synthase (L-glutamine: D-F6P amidotransferase (hexose isomerizing) as a new target for antifungal drugs [26, 43]. In fungi and bacteria, differently in mammal cells, short-time depletion of this enzyme is lethal [26, 43]. From the tested compounds 2-amino-hexitol phosphates, 2-amino-2-deoxy-D-mannitol-6-phosphate (ADMP) was a better inhibitor for *Candida albicans* D-glucosamine synthase than 2-amino-2-deoxy-D-glucitol-6-phosphate (ADPG). These two investigated compounds were not in the same extent effective against *Saccharomyces cerevisiae* PGI [26, 43].

CONCLUSIONS

Jeffery indicated that treatment that corrects only one function of "moonlighting" protein might not be sufficient to treat the disease. On the other hand, adjusting the level of one activity of the enzyme might be indispensable, because adjusting both might result in side effects [20]. From these aspects, further characterization of PGI as an example of "old proteins which were learning new tricks" [20] is necessary.

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SUMMARY

In this article, the biological functions, classification, structures, mechanisms of reaction and potential applications of the phosphoglucose isomerase and its inhibitors were discussed. The knowledge of various biological processes in which PGI is involved can be very helpful in the treatment of cancer, bacteriosis, mycosis and parasitosis.

Keywords: isomerase, biological function, classification structures, mechanism of reaction

STRESZCZENIE

W artykule przeglądowym przedstawiono biologiczne funkcje, klasyfikację, struktury, mechanizmy reakcji i potencjalne zastosowanie izomerazy fosfoglukozowej i inhibitorów tego enzymu. Wiedza o różnych biologicznych procesach, w których zaangażowana jest PGI, może być bardzo pomocna w leczeniu nowotworów, bakterioz, grzybic i parazytoz.

Słowa kluczowe: izmoeraza fosfoglukozowa, biologiczne funkcje, klasyfikacja struktury, mechanizmy relacji

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Phosphoglucose isomerase from Escherichia coli ATCC 25922 – pilot studies

Izomeraza fosfoglukozowa z *Escherichia coli* ATCC 25922 – badania pilotowe

INTRODUCTION

Phosphoglucose isomerase (D-glucose-6-phosphate aldose-ketose-isomerase; PGI; EC 5.3.1.9) is an enzyme that catalyzes reversible isomerization of glucose-6-phosphate (Robinson-Embden ester, glucose-6P) to D-fructose-6-phosphate ester (Neuberg ester, fructose-6P).

PGIs are represented by two evolutionarily distinct protein families: the PGI superfamily and the cupin superfamily. The PGI-superfamily comprises both the crenarcheal phosphogucose isomerase/ phosphomannose isomerase family (PGI/PMI family) and the PGI family, which are present in almost all bacteria including *Eschericha coli* (*E. coli*), eucarya and few euryarchaea. On the other hand, only few cupin type PGIs (cPGI) are known [8].

Phosphoglucose isomerase has sometimes been described as a "workhouse" enzyme of sugar metabolism [3]. PGI is a vital link between the Embden-Meyerhoff-Parnas, Entner-Duodoroff and pentose-phosphate pathways [14]. PGI is also involved in the gluconeogenesis. PGI has a number of other "moonlighting" functions [12].

The *E. coli* PGI mutant is able to grow glucose medium, although at reduced rate [6]. Disruption of phosphoglucose isomerase in this bacteria resulted in use the of the pentose phosphate pathway as the primary route of glucose. Furthermore, the PGI knockout *E. coli* demonstrated unexpected glyoxylate shunt activation. The Entner-Duodoroff pathway also contributed to a minor fraction of the glucose catabolism in this mutant strain [10]. In *E. coli* cells phosphoglucose isomerase is localized in two cellular compartments. Primarily the enzyme is located in the cytoplasm. About 6–10% of the PGI activity is localized in the periplasmic space. The cytoplasmic PGI participates in glucose catabolism [6]. The function of periplasmic PGI is the conversion of extracellular fructose-6P into glucose-6P, which is the only extracellular inducer for the hexose-phosphate transport system [7].

PGI is located at 91 min on the *E. coli* chromosome [1]. It does not seem to be a member of an operon or located close to other genes encoding glycolytic enzymes. The *E. coli* JM101 PGI is more

similar to the *Clarkia* (plant) sequence (87.6%) than the pig one (65.9%) [5]. The higher amino acids (aa) sequence similarity between plant plastid PGI isoenzyme and the *E. coli* PGI is consistent with the hypothesis that the nuclear gene encoding the former enzyme derived from the prokaryotic symbiont that evolved into the chloroplast [21].

The biofilm formation is recognized as an important virulence factor in many bacterial strains (e.g. *Staphylococcus aureus, Staphylococcus epidermidis* and *E. coli*). Bacterial biofilms are now known to play an important role in a range of chronic infections. Being crucial for the organism's survival, PGIs metabolizing sugars may be treated as potential targets for therapeutic control of bacterial infections.

This report describes preliminary results of the isolation, partial purification and characterization of catalytic and physicochemical properties of the phosphoglucose isomerase from *Escherichia coli* ATCC 25922. However, further research is needed to confirm and develop already performed research.

EXPERIMENTAL PROCEDURES

Bacterial strain. The reference strain *E. coli* ATCC 25922 was a kind gift from the Department of Microbiology, Medical University of Lublin. For liquid culture was used a synthetic medium, enrichment broth (Biomed, Poland) supplemented with 1% glucose. Inocula (~ 1.50 x 10^8 CFU/ml, 0.5 McF standard scales) were prepared with fresh cultures of bacteria which were streaked on CM0337 Mueller-Hinton Agar (Oxoid, England) petri plates with sterile 0.9% NaCl. Cultures were grown at 37°C and aerated with constant shaking. In our preliminary experiments the growth of *E. coli* on different carbon sources (glucose, maltose and glycerol) at final concentration 1% was measured. After inoculation the growth of bacteria were observed in nephelometer Phoenix Spec (Becton-Dickinson, USA).

Reagents and materials. All reagents were analytical quality grade and unless stated otherwise, supplied by Sigma Chemical Co., Fluka Chemie or ICN Biomedicals INC. Blue Dextran 2000 and Sephadex G-200 were purchased from Pharmacia Fine Chemicals AB, Sweden. Bio-Rad protein reagent was obtained from Bio-Rad Laboratories. DEAE-cellulose (DE-52 cellulose) was purchased from Whatman Ltd, England. N-Brom-succinimid (NBS) was obtained from Schuchardt, München, Germany. Diaflo YM-30 (cutoff, 30 kDa) ultrafiltration membranes were purchased from Amicon, Inc., USA.

Assay of PGI activity. The catalytic activity of phosphoglucose isomerase was evaluated by a coupled assay or direct assay for fructose-6P. In the coupled assay, the activity of PGI was estimated by the glucose-6P formation using dehydrogenase glucose-6P (EC 1.1.1.49) as auxiliary enzyme. The standard reaction mixture in 1 mL solution contained: 50 mM TRIS/HCl buffer, pH 7.65, 1 mM D-fructose-6P, 5 mM $MgCl_2$, 0.5 mM $NADP^+$ and 1 U of dehydrogenase glucose-6P. The reaction mixture was incubated at room temperature (RT) for 3 min and the reaction was initiated by the addition of the PGI preparations. The reaction was followed by 3 min. The activity was measured at 340 nm using spectrophotometer Genesys^{TM}6 (Thermo Electron Corporation, USA) by monitoring the NADPH formation from $NADP^+$. One unit (U) of PGI activity was defined as the amount of enzyme catalyzing 1 μmol of $NADP^+$ reduction per min under above conditions.

Phosphoglucose isomerase activity was also measured by the decreased amount of fructose-6P using colorimetric technique of Lever [13]. To our knowledge, we were the first to use p-hydroxybenzoic acid hydrazide (PAHBAH) as a reagent for the direct determination of PGI activity. The standard direct assay mixture in 0.5 mL contained: 10 mM TRIS/HCl buffer, pH 7.65, 0.15 mM D-fructose-6P, 1 mM $MgCl_2$ and various amounts of PGI. After various times of incubation at 37°C, 1.5 mL of *ex tempore* mixing liquids of 5% PAHBAH in 0.5 M HCl and 0.5 M NaOH (1: 4 v/v) were added. Next, the samples were heated at 100°C for 10 min. After cooling on ice water bath, the absorbance was measured at 410 nm using spectrophotometer Genesys™6. One unit (U) of PGI activity was defined as the amount required for the decrease of 1 μmol fructose-6P per min under above conditions. The specific activity in both methods was defined as units of enzyme activity per mg of protein. The choice of the assay to determine activity of the characterized enzyme was frequently caused by the kind of the performed experiment.

Partial purification of PGI activity. *E. coli* ATCC 25922 was grown in 1 L Ehrlemayer flasks containing 0.25 L medium for 24 hours. The bacteria cells were collected by centrifugation. Next, *E. coli* cells were washed twice in chilled 50 mM TRIS/HCl buffer, pH 7.65 and resuspended in the same chilled buffer. The suspensions of cells (2.0 mL) were frozen at -18°C until used. All purification steps were done at 4°C unless otherwise specified.

Step 1: preparation of crude extract. The suspension of cells (4 mL) was resuspended in 20 mL 50 mM TRIS/HCl buffer, pH 7.65 containing 2 mM D,L-dithiothreitol (DTT) and 5 mM $MgCl_2$. The cells were lysed by the addition of lysozyme to a final concentration 0.4 mg/mL and the viscosity of the solution was reduced by the addition of DNase I to an activity of 1 U/mL. The suspension was shaken in water bath (amplitude 6, 50 cpm Elpan, Poland,) at 37°C for 1 h. The treated cells were ruptured by sonication with apparatus Unipan UD 20 (IPPT, 180 W, 22 kHz, TECHPAN, Poland), using 3 cycles of 3 min pulses followed by a 2 min rest on ice water bath. The lysate was centrifuged at 3314 x g for 30 min at 4°C (centrifuge type 3-16K, rotor 12159, Sigma, USA) and the supernatant was collected.

Step 2: ammonium sulfate fractionation. The liquid, which was designated "crude extract", was placed in an ice bath on a magnetic stirrer and solid $(NH_4)_2SO_4$ was added, slowly to 45% saturation and the precipitate appeared was removed by a conventional centrifugation. The supernatant liquid was then brought to 70% saturation by the slow and continual addition of solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation (type 3-16K, rotor 12159, 1753 x g) dissolved in a minimal volume of 25 mM TRIS/HCl buffer pH 7.65 with 2 mM DTT (Buffer A) and dialyzed overnight against the same buffer (AS-fraction).

Step 3: DEAE-cellulose chromatography. The AS-fraction was applied to a column of DEAE cellulose (2.6 x 10 cm) that had been equilibrated with 0.2 M TRIS/HCl buffer pH 7.65 and Buffer A. The column was washed thoroughly with the same Buffer A (250 mL) and the wash, which did not contain activity of PGI, was discarded. PGI was eluted from the column with a 0.05–0.5 M linear gradient of NaCl in Buffer A (total volume of gradient 300 mL). Most active fractions were pooled and desalted immediately by ultrafiltration on the Amicon filtration apparatus (Millipore, Billarica, MA) using a YM-30 membrane and kept frozen in Buffer A.

Other methods. Protein concentration was measured with Bio-Rad protein reagent using bovine serum albumin (BSA) as standard [2] or spectrophotometrically at 280 nm. In the pool of protein concentration (mg/mL) was calculated by formula: 1.55 x A_{280nm} – 0.76 x A_{260nm}. Native polyacrylamide gel electrophoresis (PAGE) was performed on 6% gel in tubes on apparatus 175 Tube Cell (Bio-Rad, Austria). PAGE was run according to the method described by Davis [4] in TRIS/HCl continuous buffer system at pH 8.3. Gels were stained with amido black 10B in 7% acetic acid to detect protein at RT for 30 min. The destaining was done by electrophoresis (10 mA per tube) in 7% acetic acid. The solution for active PGI stainings was always freshly prepared and comprised of sources almost the same as mixture for coupled assay activity but it included 0.4 mg nitroblue tetrazolium chloride (NBT) and 0.04 mg phenazine methosulfate (PMS) per 1 mL, respectively. The native gels were incubated in the staining solution immediately after electrophoresis in the darkness at 37°C for about 30 min. The staining was stopped by rinsing the gels in deionized water. Purple bands showed the presence of activity PGI.

The PGI activity was tested over pH range 7.4–8.0 by using coupled and direct activity assays. We tested the following buffers: glycyl-glycine, pH 7.4; (N-[2-Hydroxy-ethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES), pH 7.6; morpholinopropane sulfonic acid (MOPS), pH 7.6; N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid) (TES), pH 7.6; N-tris-[hydroxymethyl]-methyl glycine (TRICINE), pH 7.6; tris-(hydroxymethyl)amino-methan (TRIS)/HCl, pH 7.65; triethanolamine (TEA)/NaOH, pH 7.7; N-tris(hydroxy-methyl)methyl-3-aminopropane sulfonic acid (TAPS), pH 7.8 and TRIS/HCl, pH 8.0.

For the determination of the effect of metal cations (5 mM) or thiols (10 mM) on PGI activity, the enzyme was dialyzed against 25 mM HEPES buffer, pH 7.6. In these experiments the catalytic activity was determined by direct assay. \

Dihydroxyacetone phosphate (DHAP), D-fructose, D-fructose-1P, D-fructose-6P, D-fructose-1,6P_2 and tagatose-6P (2 mM) were tested as possible substrates with coupled activity assay. Kinetic parameters of PGI were determined with coupled assay by varying the concentration of D-fructose-6P (0.5–15 mM).

The typical PGI inhibitors such as D-erythrose-4P, D-fructose-1P, D-fructose-1,6P_2, D-mannose-6P and 6-phospho-D-gluconate (6-phosphogluconate) (2 mM) were tested as potential inhibitors of phosphoglucose isomerase from *E. coli* ATCC 25922. The mM concentration range of phosphates sugar is usually used in the study of various types PGIs [19, 20]. To examine the effect of other inhibitors (chemical modyfing aa and the chelators, Table 2) on PGI activity, the assay was preceded by the enzyme dialysis against 25 mM HEPES buffer, pH 7.6. The tested inhibitors concentrations are usually used in the biochemical research of the enzymes. In both experiments the D-fructose-6P concentration was 1 mM and the activity was determined by a coupled assay. Inhibitors were added directly to the assay mixture. No effect of the tested inhibitors on the auxiliary enzyme was observed at the given concentration.

The molecular weight of the native phosphoglucose isomerase (isoenzyme I) was estimated by gel filtration on Sephadex G-200 (2.6 x 22 cm). To determine the void volume (V_o), the sample of Blue Dextran 2000 was used. Molecular weight standards included: apoferritin (443 kDa), alcohol

dehydrogenase (150 kDa), BSA (66 kDa) and cytochrome C (13 kDa). The calibration curve was prepared by plotting V_e/V_o values versus the logarithms of molecular weights of protein standards. The ratio of PGI V_e to the V_o was compared with corresponding ratios obtained for the standard proteins in order to determine the molecular weight of PGI by linear regression. Protein standards were assayed by measuring the absorbance at 280 nm. The elution of PGI from Sephadex G-200 column was monitored by the enzymatic activity assessment.

RESULTS

ISOLATION AND PARTIAL PURIFICATION OF THE *E. COLI* ATCC 25922 PGI

In our preliminary experiments, we determined the influence of different carbon sources (glucose, maltose and glycerol) on reference strain *E. coli* ATCC 25922 growth. The growth of characterized cells is almost equivalent to 1% glucose and 1% maltose. Little growth was found for cells cultured on 1% glycerol (data not shown). We detected a difference in enzymatic activity of PGI, although the characterized cells growth on glucose and maltose was almost identical. We estimated a two-fold increase of PGI catalytic activity for *E. coli* ATCC 25922 growing on glucose (using fructose-6P as substrate) comparing with those growing on maltose (data not shown). PGI was purified from *E. coli* ATCC 25922 cell-free extract using conventional techniques (Table 1).

Table 1. Purification of PGI from *E. coli* ATCC 25922 grown on 1% glucose

Purification step	Total activity[X] [U]	Total protein [mg]	Specific activity [U/mg]	Purification [fold]	Yield [%]
Crude extract	6.80	15.300	0.444	-	100
AS fraction	4.18	6.907	0.605	1.4	61
DEAE-cellulose (peak I)	1.13	0.999	1.131	2.5	17
DEAE-cellulose (peak II)	0.55	1.610	0.342	0.8	8

[X] – Enzymatic activity of PGI was measured by the coupled assay

After ion-exchange chromatography on DEAE-cellulose and determination of AS fraction, two peaks of PGI activity, termed I and II according to their elution sequence, were detected (Fig. 1). They were pooled separately.

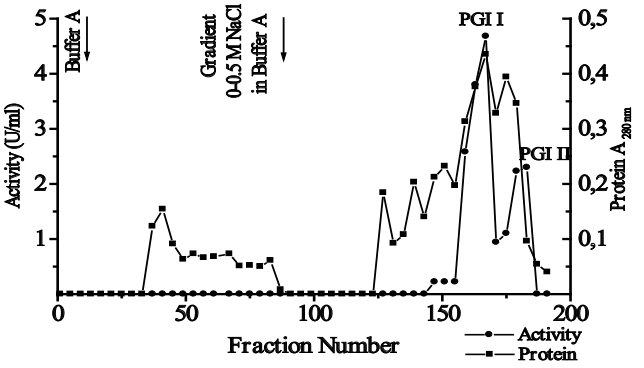

Fig. 1. Chromatography of AS fraction from *E. coli* ATCC 25922 on DEAE-cellulose column. Protein fractionation was recorded at 280 nm. The direct assay method was used to measure activity of PGI

PAGE of the PGI preparation obtained from chromatography on DEAE-celulose (peak I and peak II as one pool) showed 5 protein bands of different intensities (Fig. 2 A). Zymography of the preparation confirmed that *E. coli* ATCC 25922 has two PGI isoenzymes. The PGI isoenzymes exhibited different activities in the same method of activity detection *in situ* on the gel. A minor isoenzyme of PGI migrated more slowly to the anode than the major one and must therefore differ in size or charge (Fig. 2 B).

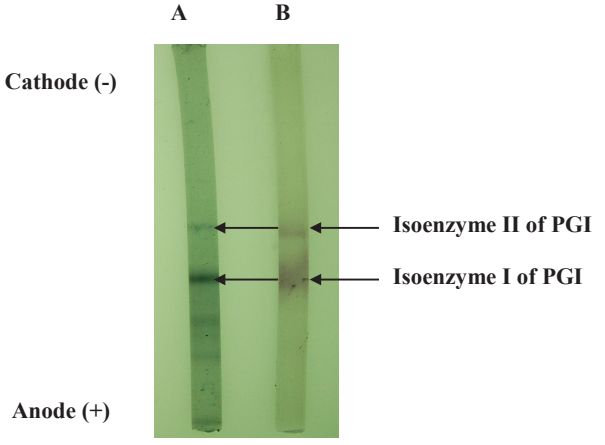

Fig. 2. PAGE analysis of pooled fractions of PGI from E. coli ATCC 25922 from DEAE-cellulose column. Lane A- staining with amido black for protein, Lane B- activity staining *in situ*

SOME PROPERTIES OF PGI (ISOENZYME I) FROM *E. COLI* ATCC 25922

The influence of buffers on enzyme activity. The experiment of activity evaluation using PAHBAH, showed enzyme activity only in TRIS/HCl buffer, pH 7.65 and glycyl-glycine buffer, pH 7.4 with 100% and 16% of relative activity, respectively. Fig. 3 ilustrates the dependence of PGI activity on buffers after determination by the coupled assay. When we determined the enzyme activity by this assay, the kind of buffer was less essential. However, the catalytic activity of PGI was higher in TRIS/HCl buffer, pH 7.65 and TRIS/HCl buffer, pH 8.0 with 100% and 98% relative activity, respectively.

The effect of metal ions on enzyme activity. The metal cations requirement for enzymatic activity was determined by the direct assay to avoid complications due to coupling enzyme. PGI from *E. coli* ATCC 25922 showed activity without addition of metal cations, but 5 mM Mg(II) stimulated activity. Then we tested the influence of Mg(II) concentration (1–15 mM) on the activity of the PGI and we observed a decrease of enzymatic activity at higher Mg(II) concentrations (data not shown). We suggest that unspecific inhibition of *E. coli* ATCC 25922 PGI resulted from the increased concentration of chloride anions. 5 mM concentration of Ca(II) and Co(II) significantly inhibited the activity of *E. coli* ATCC 25922 PGI, by 65% and 74% respectively. Other tested cations: Mn(II), Ni(II), Cd(II), Fe(II), Zn(II) and Fe(III) at this concentration completely inhibited (100% inhibition) the activity of phosphoglucose isomerase. Based on our results, we decided to use 5 mM Mg^{2+} during the direct assay of the PGI activity.

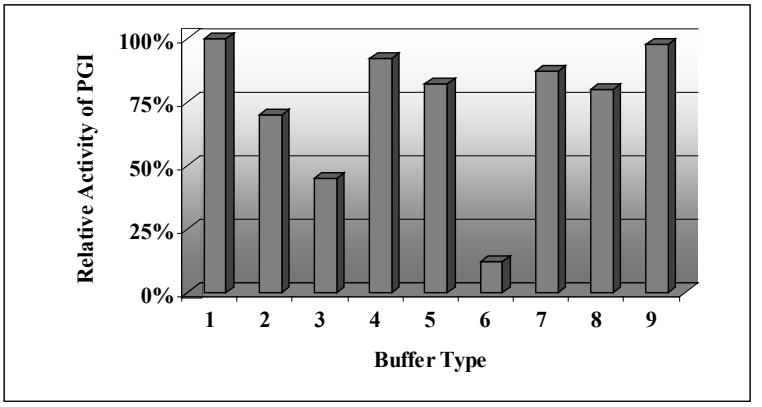

Fig. 3. Influence of PGI activity from *E. coli* ATCC 25922 on buffers.
The concentration of buffers was 50 mM. 1 – TRIS/HCl, pH 7.65; 2 – Glycyl-glycine, pH 7.4; 3 – HEPES, pH 7.6; 4 – MOPS, pH 7.6; 5 – TES, pH 7.6; 6 – TRICINE, pH 7.6; 7 – TEA/NaOH, pH 7.6; 8 – TAPS, pH 7.8; 9 – TRIS/HCl, pH 8.0

The effect of thiols on enzyme activity. D,L-dithioerythritol and D,L-dithiothreitol at constant concentrations (10 mM) activated *E. coli* ATCC 25922 PGI with 165%

and 131% relative activity compared to the control without thiols, respectively. Glutathione (reduced), D,L-cysteine and mercaptoethanol appeared to be inhibitors (data not shown). However, only mercaptoethanol completely inhibited (100%) the activity of characterized PGI at tested concentrations.

Substrate specificities and kinetic properties. Several phosphoketoses were tested as possible substrates for PGI. The activities were detected using D-fructose-6P and D-fructose-1,6P_2 with 100% and 5.5% of relative activity, respectively. The PGI activity detected using D-fructose-1,6P_2 as substrate probably resulted from impurity the agent by D-fructose-6P. The phosphoglucose isomerase from *E. coli* ATCC 25922 appeared to exhibit a high degree of D-fructose-6P substrate specificity. Other tested sugar phosphates such as DHAP, D-fructose-1P and tagatose-6P were inactive as substrates. This PGI was unable to isomerize non-phosphorylated sugar like D-fructose. The characterized PGI displayed Michaelis-Menten kinetics. The K_m value for fructose-6P and the V_{max} value were calculated from Lineweaver-Burk plot. At RT, K_m of PGI was estimated to be 1 mM for fructose-6P with the V_{max} of 0.177 μM/min (data not shown).

The effect of inhibitors on enzyme activity. The effect of various inhibitors on the PGI activity from *E. coli* ATCC 25922 was shown in Table 2. The tested compounds can be divided into three groups. The first group consists of phosphorylated compounds as D-erythrose-4P, D-fructose-1P, D-fructose-1,6P_2, D-mannose-6P and 6-phosphogluconate. The second group is composed of reagents for the aa modification such as p-chloro mercuric benzoic sodium salt (pCMB), N-ethylmaleimide (NEM), NBS, succinic anhydride and N-acetyloimidazole. Finally, the third group comprises the following chelators: EDTA and 1,10-phenanthroline. From the first group, phosphorylated agents, only two caused significant inhibition. The residual activities of 56% and 39% were monitored in the presence of D-mannose-6P and 6-phosphogluconate (at 2 mM), respectively. Especially, interesting was the lack of inhibition of PGI from *E. coli* ATCC 25922 by D-erythrose-4P. The inhibitors: pCMB (making mercaptide linkage) and NEM (alkylating compound) inhibited the enzyme activity indicated the participation of –SH group of cysteine (Cys) in enzymic catalysis. NBS at 10 mM concentration, which is a specific reagent for tryptophan (Trp) modification in proteins, did inhibit a half activity of PGI. Probably, the active site of phosphoglucose isomerase contains lysine (Lys) and tyrosine (Tyr), because this prokaryotic enzyme was inhibited in addition of succinic anhydride and N-acetyloimidazole, respectively. The fact that the tested chelators (EDTA and 1,10-phenanthroline at mM concentration) different in their structures did not significantly affected the PGI activity from *E. coli* ATCC 25922 indicated that the enzyme is not a metallprotein.

Table 2. Effect of various inhibitors on *E. coli* ATCC 25922 phosphoglucose isomerase

Inhibitor	Concentration of the inhibitor [mM]	Inhibition [%]
K	-	0
Erythrose-4P	2	0
Fructose-1P	2	5
Fructose-1,6P_2	2	20
Mannose-6P	2	44
6-phosphogluconate	2	61
pCMB	1	8
	5	45
NEM	5	73
	10	100
NBS	5	29
	10	50
Succinic anhydride	5	45
	10	92
N-acetyloimidazole	5	36
	10	38
EDTA	5	26
	10	33
1,10-phenanthroline	5	2
	10	8

M o l e c u l a r w e i g h t . Based on the gel filtration on Sephadex G-200, the active PGI I from *E. coli* ATCC 25922 had a molecular weight about 120 kDa.

DISCUSSION

In this pilot study we described the procedure of partial purification and characterized the kinetic and physicochemical properties of phosphoglucose isomerase involved in the reversible isomerization of glucose-6P to fructose-6P in *E. coli* ATCC 25922. In this research, we performed the evidence that *E. coli* ATCC 25922 cells contain two cytosolic isoenzymes of PGI. This result was

in agreement with preliminary reports of Schreyer and Böck [16] and Szynal [18] for PGIs which were isolated from *E. coli* K-10 and *E. coli* ATCC 35218 strains, respectively.

The comparison of some properties of PGI isoenzyme from *E. coli* ATCC 25922 with isoenzyme I from other *E. coli* strains like K-10 and ATCC 35218 indicates similarities. An apparent molecular weight of PGI isoenzyme I from *E. coli* K-10 [16] and *E. coli* ATCC 25922 determined by gel chromatography on Sephadex G-200 was the same 120 kDa. Although the molecular weight of PGI isoenzyme I from *E. coli* ATCC 35218 was low and its value was about 100 kDa [18]. Isoenzymes I from *E. coli* K-10 [16] and ATCC 35218 [18] strains have a homodimeric structure. We did not perform SDS-PAGE for the determination of subunit structure of PGI isoenzyme I from *E. coli* ATCC 25922, because our preparation of the enzyme was partially purified. Nevertheless, the characterized enzyme is probably homodimeric, because almost all PGIs have been previously described as homodimers of about 100–130 kDa [15].

The classical PGI from different sources were active over a broad pH range 7.5–10.0 [20]. Isoenzyme I from *E. coli* K-10 exhibited the maximum activity at pH 8.0 [16]. We determined routinely the activity of PGI from *E. coli ATCC* 25922 at pH 7.65 of TRIS/HCl buffer, but we observed a high activity at pH 8.0 of the same buffer.

The activity of PGI isoenzyme I did not depend on metal cofactors, like PGIs from other sources [14]. However, after determination of enzyme activity by direct assay, we observed that 5 mM concentration of Mg^{2+} slightly stimulated the activity of phosphoglucose isomerase isoenzyme I from *E. coli* ATCC 25922.

In our studies, a direct assay indicates the high activity of the PGI isoenzyme I from *E. coli* ATCC 25922 in the presence of D,L-dithioerythritol and D,L-dithiothreitol (10 mM). These thiols were interfered in many analytical procedures. In the coomassie protein assay, D,L-dithioerythritol is obligatory used at a lower concentration than D,L-dithiothreitol. Despite this fact, we routinely used 2 mM D,L-dithiothreitol as the protective agent of Cys –SH groups of PGI isoenzyme I from *E. coli* ATCC 25922. The present of this aa in the characterized enzyme active site is highly probable. There are two pieces of evidence for that. Firstly, the catalytic activity of PGI was significantly inhibited by non- or organic preparation of Hg^{2+} and alkylating compound NEM. Secondly, Cys are absent in the dehydrogenase glucose-6P from *Leuconostoc mesenteroides* [11], which was a coupling enzyme in the activity assay of PGI.

Phosphoglucose isomerase from *E. coli* ATCC 25922 exhibited a high specificity for D-fructose-6P substrate alike PGIs from other sources. The respective K_m value for this substrate was about 5-fold higher for the PGI from *E. coli* ATCC 25922 compared with those preliminary reported for the other *E. coli* PGIs [16, 18]. This difference is difficult to interpret. However, we evaluated the kinetic parameter by using partial purificated of enzyme (isoenzyme).

D-Erythrose-4P affected the PGI isoenzyme from *E. coli* ATCC 25922 different from other PGIs. This compound is a strong inhibitor for PGI from *Dictyostelium discoideum* [19] and for PGI/PGM from *Aeropyrum pernix* [9] with K_i values of 3.8 μM and 35±4 μM, respectively. D-erythrose-4P could hardly inhibit cupin PGI from *Pyrococcus furiosus* (K_i value of 3.9 mM) [8]. Meanwhile, this intermediate of pentose-phosphate pathway did not show (at 2 mM concentration) the inhibitory effect on PGI from *E. coli* ATCC 25922. The activity of phosphoglucose isomerase was not significantly

inhibited by other classical PGIs inhibitors such as D-fructose-1P and D-fructose-1,$6P_2$. It was only inhibited by D-mannose-6P and 6-phosphogluconate (Table 2).

The discovery of present aa like Cys, Trp, Lys and Tyr in PGI from *E. coli* ATCC 25922 is consistent with the results reported previously for PGI from *E. coli* ATCC 35218 [18]. The occurence of Lys in the PGIs active sites from other sources was postulated by Noltmann [15].

Many binding agents (chelators) of metal ions like EDTA, 1,10-phenantroline, 2,2'-bipyridyl, azide and TRIS are known. Metalloenzymes, e.g. ribitol dehydrogenase (Zn^{2+} as ion metal biding in the active site) from *Mycobacterium smegmatis* are sensitive towards these compounds [17]. PGI isoenzyme I from *E. coli* ATCC 25922 was more sensitive to EDTA than 1,10-phenanthroline at the same concentration (Table 2). Probably, this phenomenon results from a higher ability of EDTA to chelate Mg^{2+} cations existing in the assay mixture as compared with 1,10-phenanthroline. Other chelator – TRIS, did not inhibit the activity of the studied isoenzyme. In accordance with our data, the PGI isoenzyme I from *E. coli* ATCC 25922 does not have essential metal ion in the active site.

It is necessary to carry out more studies concerning isoenzymes of PGI from *E. coli* ATCC 25922. The most important is to answer the question about the physiological role of the existence of two phosphoglucose isomerase forms.

CONCLUSIONS

The existence of two different forms of PGIs (isoenzymes) in the reference strain of *E. coli* ATCC 25922 was reported. A similarity between the properties of the major phosphoglucose isomerase isoenzyme *E. coli* ATCC 25922 and isoenzymes isolated from other *E. coli* strains like K10 and ATCC 35218 was discovered. Our preliminary results indicated that PGI from reference *E. coli* ATCC 25922 strain may be included to PGI superfamily.

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SUMMARY

Phosphoglucose isomerase (EC 5.3.1.9; PGI) is an enzyme participating in the metabolism of the sugar and carrying the reversible isomerization of glucose-6-phosphate and D-fructose-6-phosphate. It plays a key role in the process of the extracellular polysaccharides (EPS) synthesis *in vivo* – creating the bacterial biofilm. The complete understanding of PGI catalysis and inhibition mechanisms may results in a discovery of new PGI inhibitors – antibacterial drugs.

In this pilot study we described the procedure of partial purification of *E. coli* ATCC 25922 PGI from cytosolic fraction. The activity of the enzymes was measured by the conversion of D-fructose-6P into D-glucose-6P. Two forms (isoenzymes I and II) of this enzyme were separated. Some properties of the dominant form (isoenzyme I) of PGI were studied. The K_m constant for D-fructose-6P was

1 mM. The enzyme had optimum activity in TRIS/HCl buffer, pH 7.65. Phosphoglucose isomerase had molecular weight 120 kDa. Our results indicated that amino acids – cysteine, tryptophan, tyrosine and lysine residues were to be presented in the active site of PGI from *E. coli* ATCC 25922. The lack of the activity inhibition of the characterized enzyme by chelators as EDTA, 1,10-phenantroline and TRIS indicated that the PGI is not a metallprotein. A large similarity between the properties of phosphoglucose isomerases isolated from *E. coli* ATCC 25922 and other *E. coli* strains such as K10 and ATCC 35218 was discovered.

Keywords: phosphoglucose isomerase; isoenzymes, PGI inhibitors, *Escherichia coli* ATCC 25922, biofilm

STRESZCZENIE

Fosfoglukoizomeraza (EC 5.3.9.1; PGI) jest enzymem uczestniczącym w metabolizmie cukrów, katalizującym odwracalną izomeryzację D-glukozo-6-fosforanu i fruktozo-6-fosforanu. Odgrywa kluczową rolę w procesie syntezy *in vivo* zewnątrzkomórkowych polisacharydów (EPS) tworzących bakteryjny biofilm. Pełne zrozumienie mechanizmów katalizy i inhibicji PGI może skutkować odkryciem nowych inhibitorów PGI – leków przeciwbakteryjnych. W pracy opisano procedurę częściowego oczyszczania PGI z frakcji cytozolowej *E. coli* ATCC 25922. Aktywność enzymu mierzono w kierunku od D-fruktozo-6P do D-glukozo-6P. Rozdzielono dwie formy (izoenzymy I i II) tego enzymu. Badano wybrane właściwości formy dominującej (izoenzymu I) PGI. Stała Michaelisa dla D-fruktozo-6P wynosiła 1 mM. Enzym miał optymalną aktywność w buforze TRIS/HCl, pH 7.65. Ciężar cząsteczkowy fosfoglukoizomerazy wynosił 120 kDa. Nasze wyniki wskazują na obecność aminokwasów: cysteiny, tryptofanu, tyrozyny i lizyny w centrum aktywnym PGI z *E. coli* ATCC 25922. Brak hamowania aktywności charakteryzowanego enzymu przez chelatory, takie jak: EDTA, 1,10-fenantrolinę i TRIS, dowodzi, że PGI nie jest metaloproteiną. Zaobserwowano duże podobieństwo właściwości pomiędzy izomerazami fosfoglukozowymi izolowanymi z *E. coli* ATCC 25922 i z innych szczepów *E. coli*, takich jak: K10 i ATCC 35218.

Słowa kluczowe: izomeraza fosfoglukozowa, izoenzymy, inhibitory PGI, *Escherichia coli* ATCC 25922, biofilm

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Islet GAD autoantibodies in patients with newly diagnosed type 2 diabetes

Autoprzeciwciała przeciw wyspowe anty GAD u chorych z nowo rozpoznaną cukrzycą typu 2

INTRODUCTION

Type 1 diabetes is an organ-specific autoimmune disease resulting from the destruction of insulin-producing pancreatic beta-cells. Islet autoimmunity is evident by the occurrence of islet-cell antibodies directed against insulin (IAA), glutamic acid decarboxylase (GADA), protein tyrosine phosphatase IA-2 (IA-2Ab) and other autoantigens [1, 5, 8].

GADA also identifies the so-called latent autoimmune diabetes in adults (LADA). In these patients, as in type 2 diabetes, disease develops after the age of 30 and has a slowly progressing autoimmune character. Based on the clinical picture, they are often misdiagnosed and treated for type 2 diabetes [1, 2, 4, 6, 9]. Several studies [1, 4, 8, 9, 11] have suggested that LADA may be found 10–20% of patients diagnosed as type 2 diabetes.

In adult-onset type 1 diabetes patients, GADA are the immune markers of higher diagnostic sensitivity [3, 5, 7, 11]. Over 80% of LADA patients develop insulin dependency within a few years after the diagnosis and have an increased risk for the occurrence of other organ-specific autoimmune diseases [1,8].

There are no generally accepted criteria for diagnosing LADA. Antibodies, especially at high titers and when are present in a combination, are the main predictors of functional injury of beta cells [2, 4, 5, 6, 13]. GAD antibody in patients with newly manifest diabetes help distinguish type 1 from type 2 diabetes, when clinical diagnosis is not unequivocal and should be offered to every diabetic patient. At present, immune-diagnosis is used too rarely in patients with diagnosed type 2 diabetes [10, 11, 13].

The aim of the study was to establish frequency and levels of GAD autoantibodies in the group of patients with newly diagnosed type 2 diabetes.

MATERIAL AND METHODS

The studied group of patients included 48 persons, 26 female and 22 male aged 36–74 (mean 54.8±10.4) years with newly diagnosed type 2 diabetes treated in our department in 2003–2005. The duration of diabetes since the diagnosis was from 1 month to one year.

Type 2 diabetes was diagnosed according to Polish Diabetes Association (2010) standards. GADA were determined by radioimmunoassay with GAD-AB (CIS reagents). The lowest detection limit of the method was the titer of 0.3.U/ml. The positive threshold for GADA was a titer higher than 1 U/ml.

The prevalence of GAD antibiodies was assessed in the entire group as well as separately for women and men subgroups. The analysis of clinical features included the patients' age and BMI. Statistical analysis was conducted using Student's test for nonparametric data. Differences of $p<0.05$ were considered statistically significant.

RESULTS

Positive GADA were found in 20 (41.7%), 12 female and 8 male patients. In these groups, only in two men (4.2% of whole studied group and 10% of group with positive GADA) the levels were high – 16.8 and 64.5 U/ml, respectively. In the other 18 subjects (37.5%), GADA levels were only slightly elevated and ranged between 1.01 and 2 U/ml (mean 1.21±0.2 U/ml). In 13 patients (27.1%), GADA titers ranged between 0.3 and 1.0 U/ml (mean 0.74±0.23). In the remaining 15 (31.2%) patients GADA were not found (titer <0.3 U/ml).

The frequency of GADA concentrations and the comparison between selected clinical parameters depending on antibody titer in the group of patients with new-onset type 2 diabetes are shown in Tab.1. In comparison with patients with no GAD antibodies, both groups of patients with GAD antibodies were characterized by a lower average age and BMI, but this difference was not statistically significant. The highest values of age and BMI were found in patients with classic type 2 diabetes, that is those in whom GAD antibodies were not found.

Table 1. Prevalence of GADA a and comparison of selected clinical parameters depending on antibody titer in the group of patients with new-onset type 2 diabetes (Mean ±SD)

	Entire group	GADA <0.3 U/ml	GADA 0.3 – 1.0 U/ml	GADA 1.01 – 2.0U/ml
Number of patients	48 (100%)	15 (31.3%)	13 (27.1%)	18 (37.5%)
Gender	F26; M22	F7; M8	F8; M5	F11; M7
Age (years)	54,8±10,4	56.5±11.5	54.3±13.7	53.1±7.3
BMI kg/ m^2	31.0±5.5	32.8±5.1	29.2±7.2	30.3±3.5

The frequency of particular GADA concentrations in subgroups of women and men is illustrated in Fig. 1. There was hardly any difference between these groups regarding the percentage of persons

without GAD antibodies (classic type 2 diabetes) or those with subthreshold GADA titers. In the subgroup of men, the percentage of patients with positive GADA titers was lower, but taking into account the fact that two patients with high GADA titers were among them, the percentage of patients with GADA titers which allowed to diagnose LADA was similar in both subgroups.

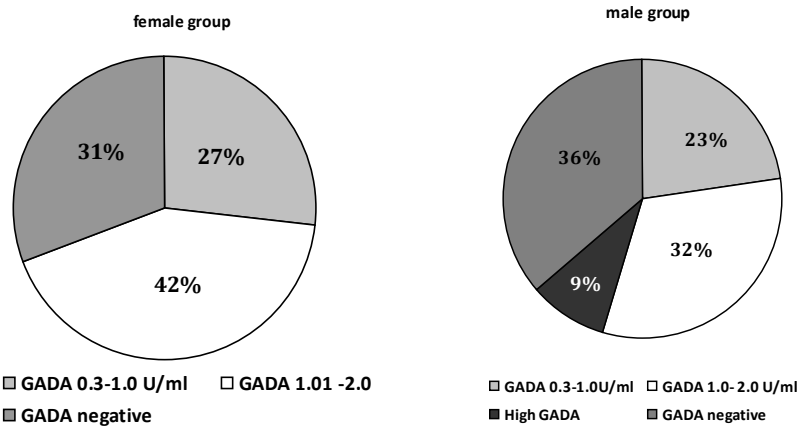

Fig. 1. A comparison of GADA frequency depending on antibody titer in male and female groups

DISCUSSION

LADA is a common and often underrecognized form of type 1 diabetes, initially often identified as type 2 [1, 2]. The prevalence of islet autoantibodies in patients with clinically diagnosed type 2 diabetes has been investigated in several studies. It was most frequently reported in 4–20% cases [1, 3, 4, 7, 8, 11, 12] and depended on the characteristics of the population studies, the kind of antibodies and used the criteria of positivity .

GAD autoantibodies are the most sensitive antigen-defined markers of autoimmune diabetes. The observation that up to 4–6% of a non-diabetic population have GAD antibodies suggests that autoimmunity toward islet cells can exist without clinical features of beta cell failure [10].

The prevalence of GAD antibodies in our group of patients with newly diagnosed diabetes was twice as high as in the studies quoted above and it mainly concerned persons with low GADA titers. A similarly high percentage of persons with low GADA titers among patients with newly diagnosed type 2 diabetes was reported by Schranz et al. [12]. The percentage of patients with high GAD antibody titers was near the lower limit of the range cited above and it was in accordance with the findings of other authors [1, 3, 7, 8].

Seeking an explanation for the high prevalence of low GADA titers among our patients we took into consideration the heterogeneity of patients with latent autoimmune diabetes in adults [10], the impact of risk factors for type 2 diabetes, such as age, obesity, and lack of physical activity, which according to Carlson et al. [15] are associated with the development of LADA. Recent studies suggest that type 2

diabetes may be a disease of the innate immune system [14]. Chronic inflammation and marked activation of acute phase response associated with aging and metabolic syndrome may be a part of autoimmune reaction against beta-cells. The occurrences described above can cause GAD antibodies in subthreshold titers to be present in patients with newly diagnosed but long-lasting diabetes which does not cause acute clinical symptoms. It should also be emphasized that according to Rosario et al. [11], approximately 20% of the patients with low GADA titers become GADA negative within 3 years since the diagnosis, which could explain less frequent diagnosis of GADA in patients with long-lasting diabetes.

Autoimmune diabetes is often classified into two groups of patients who have high or low GADA titers and are compared with each other and with those with type 2 diabetes. Patients with high GADA titers had more prominent characteristics of insulin deficiency and a profile of more severe and extended autoimmunity of low intensity. The low autoantibody levels may signify a less aggressive beta-cell autoimmunity as well as instability of the immunological system related to aging or both [3, 4, 7, 10, 12, 14].

The pathogenesis of adult-onset autoimmune diabetes has been debated recently with the possible role of autoimmunity and insulin resistance as coexistent pathogenetic factors with a variable degree of synergism, as reflected by titers of GADA [3]. Deutecom et al. [4] proposed a more continuous classification of diabetes mellitus, based on the finding that the clinical characteristics gradually changes from classic type 1 diabetes to LADA and finally to type 2 diabetes. GADA levels help in the differentiation of patients with associated insulin resistance, who usually have low titers. The differences between patients with low GADA levels and type 2 diabetics are not completely understood. Future studies should focus on determining optimal cut-off points of GAD for differentiating clinically relevant diabetes mellitus subgroups [4].

The clear difference between diabetic patients with high and low antibody titers in relation to components of the metabolic syndrome was demonstrated in several studies [4, 7, 10–12, 15]. Similarly as in other studies, in our patients higher GADA titers also corresponded to a lower mean age and BMI.

Measurement of GADA at the time of diagnosis permitted intervention during a phase when the beta cells still presented a good function. The difference between low GADA and high GADA patients becomes evident in the long term observation compatible with a slowly progressing autoimmune process which results in late beta-cell failure but always earlier than in type 2 diabetes. Another hypothesis is that these patients present a less intense autoimmune process accompanied by possible short term remission so the beta-cell destruction progress slowly [7, 10–12].

CONCLUSION

Low titer GADA were found in the majority of patients with newly diagnosed type 2 diabetes.

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SUMMARY

Autoagression against islet cells is characterized by the presence of anti-insulin antibodies and anti-glutamic acid decarboxylase antibodies (GADA), as well as other autoantigens. The marker with the highest diagnostic value in adults is GADA which helps diagnose the so-called latent autoimmune diabetes in adults (LADA). The aim of the study was to establish the prevalence and

concentrations of GADA in groups of patients with newly diagnosed type 2 diabetes. 48 (26F; 22M) patients aged from 34 to 74, mean 54.8±10.4 years took part. Diabetes was diagnosed according to the standards set by the Polish Diabetes Association. GADA was determined by radioimmunologic assay using the *GAD-AB* kit with *CIS* reagents. The level over 1U/ml was accepted as positive. The presence of GADA at the concentration >1U/ml was found in 20 (41.7%) [12F; 8M]. In this group high titers characterized only 2 men (4.2% of the whole group and 10% of patients with positive GADA) and they were 16.8 and 64.5 U/ml, respectively. In the remaining 18 patients [37.5%] (11F; 7M), GADA concentrations were only slightly elevated and they were between 1.01 and 2.0 U/ml (mean 1.21±0.2 U/ml). In 13 patients (27.1%) [8F; 5M] GADA levels were between 0.3 and 1.0 U/ml (mean 0.74±0.23). In the remaining 15 patients (31.2%) [7F; 8M] GAD antibodies were not found. In the majority of patients with newly diagnosed type 2 diabetes anti-glutamic acid decarboxylase antibodies were found in low titers.

Keywords: GAD autoantibody, newly diagnosed type 2 diabetes, latent autoimmunologic diabetes of adults (LADA)

STRESZCZENIE

Autoagresję skierowaną przeciwko komórkom wysp trzustkowych charakteryzuje obecność przeciwciał przeciwinsulinowych, przeciw dekarboksylazie kwasu glutaminowego (GADA) oraz innym autoantygenom. Markerem o najwyższej wartości diagnostycznej u osób dorosłych są GADA, pozwalające na rozpoznanie tzw. utajonej autoimmunologicznej cukrzycy dorosłych (LADA). Celem pracy było ustalenie częstości występowania i stężeń GADA w grupie chorych z nowo rozpoznaną cukrzycą typu 2. Zbadano 48 [26K; 22M] chorych w wieku od 34 do 74, średnio 54,8±10,4 lat. Cukrzycę rozpoznawano zgodnie ze standardami Polskiego Towarzystwa Diabetologicznego. GADA oznaczano metodą radioimmunologiczną z zastosowaniem odczynnika GAD-AB firmy CIS. Za dodatni uznawano poziom powyżej 1U/ml. Obecność GADA w stężeniu >1U/ml stwierdzono u 20 (41,7%) [12K; 8M]. W grupie tej wysokie miana charakteryzowały tylko 2 mężczyzn (4,2% całej grupy i 10% chorych z pozytywnym wynikiem GADA) i wynosiły odpowiednio 16,8 i 64,5 U/ml. U pozostałych 18 chorych (37,5%) [11F; 7M] stężenia GADA były tylko nieznacznie podwyższone i wahały się w granicach 1,01–2,0 U/ml (średnio 1,21±0,2 U/ml). U 13 chorych (27,1%) [8F; 5M] poziomy GADA wynosiły od 0,3 do 1,0 U/ml (średnio 0,74±0,23). U pozostałych 15 chorych (31,2%) [8M; 7K] przeciwciał anty GAD nie wykryto. U większości chorych ze świeżo rozpoznaną cukrzycą typu 2 stwierdzono obecność przeciwciał przeciwko dekarboksylazie kwasu glutaminowego w niskim mianie.

Słowa kluczowe: Autoprzeciwciała anty GAD, nowo zdiagnozowana cukrzyca typu 2, utajona, autoimmunologiczna cukrzyca dorosłych (LADA)

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Alzheimer's disease: causes, symptoms and pharmacotherapy

Choroba Alzheimera: przyczyny, objawy i farmakoterapia

INTRODUCTION

How important is memory? The study of memory can usefully investigate many aspects of pharmacological analysis. Many important issues are under discussion, including the elucidation of what memory is and which brain systems are involved in these processes. It should be noted that memories in the brain cannot be directly observed. Their presence must be inferred from clear behavioral expression. Memory, as measured by changes in an animal's behavior some time after learning, reflects many processes including acquisition, consolidation, retention, retrieval and performance. Knowledge of these processes of memory can be helpful to investigate learning and memory functions in safety pharmacology and can provide new perspectives for promising therapy in the treatment of human memory impairment like Alzheimer's disease [8].

PATHOPHYSIOLOGY OF ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a multifactorial, progressive, terminal neurodegenerative brain disorder and the most common form of dementia characterized by impaired cognitive functions. The disease was first described by doctor Alois Alzheimer, who diagnosed it in his patient – a fifty-year-old woman Auguste D. Nowadays the prevalence of Alzheimer's disease is 10.8% cases in people aged 80–90 years, although the early-onset of Alzheimer's can also occur (0.02% in people aged 30–39 years) [2]. The Alzheimer's Association estimated that in 2007 in the United States of America more than 5 million people have AD. The number of cases of AD is increasing and probably by 2050 is expected to amount approximately to 13 million persons [12].

The earliest symptoms of disease are frequently missed by the family and incorrectly thought to be age-related. First, AD leads to small deficits in cognitive functions, which become bigger during the progress of dementia. The most commonly recognized symptoms of AD in the early stages of neurodegeneration are inability, or difficulty to recollect facts, information and memory deterioration or loss (amnesia). Other symptoms of AD are difficulties in producing or comprehending spoken or written language (aphasia), impairment of the ability to carry out learned purposeful movements, such as writing, playing an instrument

(apraxia) and loss of the ability to recognize known objects or persons (agnosia). Second, in consort with neurodegeneration some behavioral disturbances and psychiatric symptoms manifest, such as personality change, mood swings, depression, misidentifications, irritability and aggression [2].

The most popular theory explaining the neuronal degeneration in AD is the amyloid hypothesis. β-amyloid is a piece from bigger protein termed amyloid precursor protein (APP). APP is very important to neuron increment, survival and repair [13]. In Alzheimer's disease APP become divided into smaller parts by enzymes β-secretase and γ-secretase through proteolysis generating β-amyloid, which form aggregates that deposit outside neurons [12]. According to amyloid hypothesis, the production and accumulation of β-amyloid peptides derived from the enzymatic processing of APP initiate the neuronal death. *β*-Amyloid plaques (also termed senile plaques) develop between neurons and cause inflammation, apoptotic cell death cascade activation, synaptic degeneration, demyelization, oxidative stress, neurotransmitter deficits and destruction of neighboring neurons [12]. In comparison to normally aging brain, in the brain of people suffering from AD a higher concentration of β-amyloid$_{42}$ is observed (it is the toxic form of amyloid containing 42 amino acids). β-amyloid peptides containing 1–42 amino acids are considered to have the biggest tendency to form into aggregates and to be more hydrophobic and neurotoxic than shorter peptides. However, the up-to-date data suggest that the most neurotoxic form of β-amyloid is its intracellular and extracellular oligomers, rather than, as it was thought, aggregates. Recent studies also suggest that the level of amyloid oligomers positively correlate with the memory impairment in patients with AD. Other studies report that β-amyloid oligomers impair the synaptic plasticity in the brain and induce synaptic declines. The trimmers are believed to be the most toxic oligomeric forms of amyloid [4].

Apart from senile plaques typical microscopic changes occurring in people suffering from AD are neurofibrillary tangles (NFT). NFT are pathological aggregates of protein which develop inside neurons. They are formed by protein termed tau (τ) after its hyperphosphorylation (protein becomes insoluble). Both amyloid plaques and neurofibrillary tangles are the histopathological hallmarks of AD [3, 12].

AD is characterized by the loss of neurons in brain. Deterioration in neuronal pathways involves neurotransmitters, especially acetylcholine – the transmitter essential to the cognitive function. Substantial degeneration of cholinergic neurons in the basal forebrain and a decrease in activity of choline acetyltransferase have been identified by scientists [10].

Knowledge about the pathophysiology of AD is pivotal to develop potential AD-modifying therapies (Fig. 1).

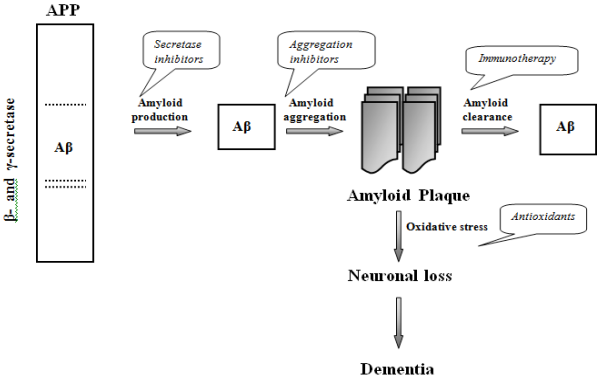

Fig. 1. Selected drug targets in pharmacotherapy of Alzheimer's disease

PHARMACOTHERAPY OF ALZHEIMER'S DISEASE

CHOLINESTERASE INHIBITORS

Cholinesterase is an enzyme which breaks down the neurotransmitter acetylcholine. Data showed that there is a relationship between the level of synaptic acetylcholine and cognitive functions improvement. Inhibition of the activity of cholinesterase leads to an increased level of acetylcholine in the brain and has positive effects on the symptoms of AD.

Cholinesterase inhibitors (AChEI) are the most popular group of drugs already registered and applied in USA and/or in Europe to treat AD. Various drugs from the group AChEI (e.g. donepezil, galanthamine, metrifonate, rivastigmine, tacrine) can cause more or less therapeutic effects measured by cognitive improvement, which depends on their level of inhibition of the enzyme cholinesterase. The major effect of AChEI is to maintain the brain cognitive functions in patients with AD at a constant level during 6 months to one year of treatment in comparison to placebo. Moreover, drugs from this group improve daily living conditions [5] and reduce some of the neuropsychiatric manifestations of AD (irritability, apathy, anxiety, paranoia) [7]. The research also suggest that AChEI and agonists of muscarinic receptors can increase the release of non-amyloidogenic and soluble derivatives of APP both *in vitro* and *in vivo* and arguably retard the formation of amyloidogenic complexes in human brain [5].

APPROACHES MODULATING SECRETASES ACTIVITY

The process of β-amyloid formation from APP in human brain is mediated by two enzymes: β-secretase and γ-secretase, while the cleavage of APP initiated by α-secretase leads to soluble products. The inhibitors of β- and γ-secretase are potential disease-modifying drugs in Alzheimer's disease. There are researches showing that β-secretase knockout mice produce less β-amyloid and that inhibitors of β-secretase injected into the hippocampus significantly reduce the production of β-amyloid *in vivo*. Reduction of β-amyloid concentration in rodent's brain was also observed after the administration of γ-secretase inhibitors. The treatment of Alzheimer's disease also includes attempts to use γ-secretase modulators, which modulates enzyme to produce less of the β-amyloid$_{42}$ and more of the shorter peptides (nontoxic forms). These drugs are shown to reduce the generation of β-amyloid by human cells [12].

ANTI-INFLAMMATORY DRUGS

Inflammation plays an important role in AD pathogenesis. The inflammatory reaction is supposed to be chronic in patients with AD. Probably, the insoluble, pathologic aggregates of amyloid lead to activation of inflammatory reaction cells (especially microglial cells and astocytes), because they are recognized as a foreign material. The data showed that long-lasting usage of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) noticeably reduces the risk of AD. It is believed that neuronal cyclooxygenase-2 (COX-2) takes part in progression of neurodegenerative processes in AD. It is

observed that transgenic mice with overexpression of COX-2 suffer from age-related cognitive and memory disorders associated with astrocytes activation and neuron's apoptosis in their brain. Moreover, the *post mortem* examinations prove the effectiveness of NSAIDs in decreasing inflammation processes in patients with AD. The mechanism of the protective activity of NSAIDs is still unknown. It is possible that except inhibiting COX activity there is another mechanism (for example, inhibition of β- or γ-secretase) explaining their effectiveness in AD. It is also found that some of the NSAIDs act directly on β-amyloid production (ibuprofen, flurbiprofen, indomethacin and sulindac induce even a 80% decrease of β-amyloid levels in transgenic animal brain). However, further research to prove the effectiveness of NSAIDs in AD is necessary [4].

IMMUNOTHERAPY

A modern and very promising approach for the AD treatment is immunotherapy including vaccination and immunization. The number of data suggesting that immunotherapy can be effective in human patients suffering from AD is rapidly growing. There are two strategies of immunotherapy. First, termed active immunization is based on usage of β-amyloid or β-amyloid fragments. The second one termed passive immunization use prepared anti-β-amyloid antibodies. The therapeutic potential of producing or delivering antibodies consists in recognizing β-amyloid by antibodies and attenuating amyloid aggregation-associated pathologies. Both active and passive immunization was assessed in mouse models of Alzheimer's disease and results are auspicious; however, passive immunization is considered to be a safer strategy than active vaccination with β-amyloid. Currently, some β-amyloid-selective monoclonal antibodies are being tested. Results of studies on the efficiency of AD-related immunotherapy show reduced brain amyloid plaques, restored neuron and synapse functioning, reduced astrocytosis and improved behaviour. However, further studies are needed to eliminate some immunotherapy-related risks like: autoimmune diseases, brain inflammation or blood brain barrier passage of antibodies [12].

NATURAL ANTIOXIDANTS

It is known that neurodegenerative diseases such as AD are associated with oxidative stress which induces neuronal apoptosis. Scientists proved that β-amyloid produces hydrogen peroxide and participates in processes which are probably related to yhe production of free radicals. Free radicals, not only peroxidize membrane lipids leading to cell destruction, but also oxidize proteins and damage nucleic acids DNA and RNA. The brain tissue is very sensitive to prooxidant-antioxidant homeostasis and high oxidative stress occurs in degeneration of brain neurons. Antioxidants such as flavonoids, polyphenols and some vitamins should have therapeutic effects on AD because they can cure the disease by protecting neurons preventing and eliminating oxidative stress [14].

F l a v o n o i d s are natural antioxidants isolated from plants. It is investigated that many of flavonoids protect rat cells and neurons from oxidative injuries like for example glutamate toxicity. There are three structural requirements of flavonoids to be effective in the protection from glutamate: unsaturated C ring, hydroxylated C3 and hydrophobicity. A representative antioxidant is Ginkgo Biloba extract. Extracts from Ginkgo Biloba leaves contain flavonoids and also ginkgolides and bilobalides.

These extracts have been reported to have brain-protecting properties by inhibiting reactive oxygen production in neurons, reducing hypoxic damage and protecting cells against apoptosis [14].

Green tea polyphenols are also reported to have potent antioxidative properties. The major constituent of Green tea salubrious polyphenols complex is epigallocatechin gallate (EGCC) which has a lot of beneficial pharmacological activities such as antimutagenic and anticarcinogenic effects. Green tea polyphenols are demonstrated to have powerful antioxidant activity against free radicals like diphenylpicrylhydrazyl radicals (DPPH), superoxide anion, hydroxyl radicals and lipid free radicals. Scientists have also found that transgenic mice overproducing β-amyloid treated EGCC have decreased β-amyloid levels and reduced amyloid plaques, which suggests that EGCC reduce amyloid generation. These findings imply the possibility of Green tea polyphenols supplementation in people suffering from AD as efficient prophylaxis [14].

There are also studies suggesting that some vitamins have important antioxidant functions and can help to protect neuron cells against oxidative stress and damage associated with neurodegenerative diseases such as AD. Very powerful antioxidants are ***vitamin C (ascorbate)*** and ***vitamin E (tocopherol)***. Decreased levels of these vitamins lead to increased oxidative stress. In one study scientists have shown that vitamin C administered intraperitoneally before testing mice in the Morris water maze test, partially attenuated cognitive deficits (amnesia) induced by scopolamine in young mice. Vitamin C also increased acetylcholinesterase activity in the medial forebrain area, which suggests that it may be connected with cholinergic signaling [6]. Results of another study verifying the role of Vitamins C and E on the cognitive functions in mice indicate a considerable improvement in the cognitive functions of aged animals but there were no significant results in young mice [1]. However, further investigation to prove the cognition-enhancing effects of vitamin C and vitamin E is needed.

CAFFEINE

Caffeine is a natural chemical compound (xanthine alkaloid) of some plants widely consumed by people in the world as coffee, tea, soft or energy drinks. Caffeine is recognized as a central nervous system stimulant which acts mainly by competitive inhibition of adenosine receptors in brain. After binding to adenosine receptors caffeine leads to the elevation of acetylcholine and serotonin levels – neurotransmitters involved in memory functioning. Moreover, caffeine intake affects an increased number of adenosine receptors in animals brain. However, for this study the most significant consequences of adenosine antagonism is the stimulation of cholinergic system, which might lead to the improvement of cognitive functions, especially concentration and memory. There are studies indicating the relation between the human cognitive performance and habitual caffeine consumption. The chronic caffeine intake was noticeably related to improved memory, especially the long-term memory compared to placebo. Moreover, low doses of long-lasting administered caffeine are considered to improve psychomotor performance and they have neuroprotective effects. These neuroprotective properties of caffeine were analyzed in some experimental models of ischemia and hypoxia and are believed to be protective in patients with AD [9].

NICOTINE

It has been reported that nicotine, a natural alkaloid found in plants from the family *Solanaceae,* is a substance which improves cognitive functions and memory by reaction with nicotinic cholinergic receptors and intensifying the cholinergic transmission. Nicotine may improve cognitive functions by direct stimulation of nicotinic cholinergic receptors or by releasing acetylcholine, glutamate, serotonin, dopamine and other neurotransmitters connected with learning and memory after interaction with presynaptic receptors [11]. Nicotine and nicotinic receptors agonists are considered to have a profitable influence on cognitive functions and memory in AD. The underlying mechanisms of neuroprotective activity of nicotine are still unclear. New findings report that nicotine has a scavenging effect on free radicals (on hydroxyl radicals and superoxide radicals even higher than vitamin C) studied by electron spin resonance techniques. These findings suggest nicotine to be a potential antioxidant. Nicotine was also found to decrease β-amyloid aggregation in unclear mechanism probably associated with regulation of metal homeostasis by nicotine [14].

There are also a lot of data which shown that there are other multiple agents which may be helpful in pharmacotherapy of AD:

MISCELLANEOUS AGENTS

Statins are inhibitors of enzyme HMG-CoA reductase and a class of drugs used to lower the cholesterol level in plasma in the treatment of dyslipidemia. Results of some research suggest that in patients using statins a considerably reduced risk of AD has been observed. The positive effect of statins in AD might be related to the decrease of β-amyloid production by increasing the γ-secretase pathway of APP processing not by their cholesterol-level-lowering properties [12].

Agonists of M1 muscarinic receptors in some research show the activity to increase the non-amyloidogenic pathway of APP processing and to decrease the levels of β-amyloid both *in vitro* and *in vivo*. M1 muscarinic agonists are probably connected with some mechanisms related to AD including hyperphoshorylation of tau protein, β-amyloid generation and cholinergic functions [12].

Antagonists of NMDA receptors are believed to be effective in AD treatment because some processes (β-amyloid production, overexpression of τ protein, brain damage from excitotoxicity and neuronal cell death) are thought to be connected with overactivation of NMDA receptors. Already approved for AD treatment, NMDA antagonist is memantine. In comparison to placebo, memantine was beneficial in patients with AD in 73% versus 45% of patients getting placebo [7,12].

CONCLUSIONS

Today there is no effective treatment of AD which can stop the disease progression. However, there are numerous trials of application of various therapies or drugs in AD with different mechanisms of action and molecular targets. Some applied drugs may help keep AD symptoms from getting worse (e.g. AChEI, natural antioxidants, anti-inflammatory drugs). The main purpose for scientists

is to find an effective drug to stop neurodegeneration or prevent the pathological changes in neurons. The most promising in this context seem to be approaches modifying the activity of secretases and immunotherapy.

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SUMMARY

AD is the most common form of dementia characterized by a noticeable decline in cognitive functions. There are several theories explaining the neuronal degeneration in AD including the β-amyloid aggregation, protein τ hyperphosphorylation, oxidative stress and inflammation. In connection with the foregoing hypothesis of AD, there are several drug targets and strategies of treating the disease. The most popular and already registered group of drugs is AChEI inhibiting the activity of enzyme cholinesterase and increasing the level of acetylcholine in brain what have positive effects on the symptoms of AD. There are also some approaches in AD to inhibit or modulate secretases activity and reduce the generation of β-amyloid by human cells or produce less of the β-amyloid$_{42}$ and more of the shorter nontoxic peptides. A very promising line for treating AD is immunotherapy including vaccination

and immunization based on the usage of β-amyloid, β-amyloid fragments or prepared anti-β-amyloid antibodies. In connection with the inflammatory hypothesis of AD new drugs reducing inflammation are searched for. It has been observed that a long-lasting therapy with NSAIDs noticeably reduces the risk of AD. It is possible that except inhibiting COX activity there is another mechanism explaining their effectiveness in AD. Adjunctive drugs in AD treatment are natural antioxidants protecting neurons, preventing and eliminating oxidative stress such as flavonoids, polyphenols, vitamin C and E or caffeine and substances such as nicotine (agonist of nicotinic cholinergic receptors). Some positive effects in AD have also been noticed after trials of using such as statins, agonists of M1 muscarinic receptors and antagonists of NMDA receptors in AD drugs. These and other trials and efforts may cause a successful disease modifying or progression-inhibiting AD treatment in the future.

Keywords: Alzheimer's disease, acetylcholine, cholinesterase inhibitors, memory and learning

STRESZCZENIE

Choroba Alzheimer'a (AD) uważana jest za najczęściej występującą chorobę otępienną. Spośród hipotez tłumaczących zanik neuronów w AD często wymienia się agregację β-amyloidu, hiperfosforylację białka τ, stres oksydacyjny oraz procesy zapalne. W oparciu o domniemane podłoże choroby opracowano szereg strategii leczenia AD, których podstawę stanowią obecnie leki z grupy inhibitorów acetylocholinoesterazy (I-AChE), które dzięki hamowaniu aktywności enzymu AChE pozwalają na zwiększenie poziomu acetylocholiny (ACh) w mózgu, co wywiera pozytywny efekt na poprawę funkcji kognitywnych. Istnieją również próby zastosowania w AD leków hamujących lub modyfikujących działanie sekretaz w celu zmniejszenia produkcji β-amyloidu, a szczególnie toksycznej formy β-amyloidu$_{42}$. Obiecującą formę leczenia AD stanowi także immunoterapia, zakładająca wprowadzenie do organizmu chorego β-amyloidu lub jego fragmentów w celu wytworzenia przeciwciał przeciwamyloidowych (immunizacja aktywna) lub też podanie gotowych przeciwciał (immunizacja pasywna). W nawiązaniu do procesów zapalnych zaobserwowanych u osób cierpiących na AD poszukuje się również nowych leków przeciwzapalnych. Stwierdzono, że długotrwałe stosowanie leków z grupy niesteroidowych leków przeciwzapalnych (NLPZ) zmniejsza ryzyko wystąpienia AD. Prawdopodobnie NLPZ poza hamowaniem aktywności cyklooksygenazy (COX) wykazują również dodatkowe działanie, tłumaczące ich efektywność w zapobieganiu AD. Wciąż badanymi lekami wspomagającymi terapię chorych na AD, z którymi wiąże się duże nadzieje, są naturalne antyoksydanty (flawonoidy, polifenole, witamina C oraz E, a także kofeina) chroniące neurony przed stresem oksydacyjnym oraz nikotyna, w stosunku do której wykazano wyraźne działanie prokognitywne. Pozytywne efekty zaobserwowano również po próbach zastosowania w AD statyn, agonistów receptora muskarynowego M1 oraz antagonistów receptora NMDA. Te oraz inne próby mogą przyczynić się do opracowania skutecznego leczenia modyfikujące przebieg AD i pozwalającego na zatrzymanie postępu choroby.

Słowa kluczowe: choroba Alzheimer'a, acetylocholina, inhibitory cholinesterazy, pamięć i uczenie się

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Antiradical and antihypertensive activity of peptides obtained from proteins pea sprouts (Pisum sativum) by enzymatic hydrolysis

Antyrodnikowa i antynadciśnieniowa aktywność peptydów otrzymanych w wyniku enzymatycznej hydrolizy białek kiełków grochu *(Pisum sativum)*

Legume protein may be useful in human food nutrition as a source of bioactive peptides with antiradical and antihypertensive properties. Numerous scientific studies have shown that sprouts are a group of plant products with important disease preventive and health protective properties. Therefore, in recent years sprouts have become increasingly significant for consumers who value a healthy lifestyle [6]. Lipids, carbohydrates and storage proteins during germination are broken down to smaller molecules and become more digestible and consequently more accessible nutrients [16]. Sprouts include a lot of bioactive compounds such as vitamins B_1 and B_2, proteins and fiber content [3].

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxpeptidase, EC 3.4.15.1) is an important enzyme involved in blood pressure regulation and in the electrolyte and fluid balance. ACE converts the inactive form of angiotensin I (decapeptide) into potent angiotensin II (octapeptide) vasoconstricting, but on the other hand, it also inactivates the antihypertensive vasodilator bradykinin [11]. As a consequence, ACE-inhibitory substances are often used in the treatment of blood pressure and hypertensive decrease. ACE inhibitor drugs, such as captopril, benazepril, and enalapril, are the first-line therapy for hypertension but they can cause serious side effects such as cough, angioedema, taste disturbances and skin rashes [12]. Therefore, scientists have carried out extensive research in the area of methods for the separation, purification and characterization of natural sources of ACE inhibitors. Many ACE inhibitory peptides are released *in vitro* from animal or plant proteins such as casein [14], fish proteins [10] and mung bean protein [7]. On the other hand, a common source of many diseases can be free radicals. These are atoms or groups of atoms with released electrons aiming at stability, which may be achieved by their coupling. They may react with proteins, lipids or DNA and consequently cause metabolism disorder. Antioxidants are chemical or natural compounds, capable of inhibiting oxidative reactions and therefore protecting human organism and food ingredients from the destructive activity of free radicals [17]. Interest in identifying foods as natural sources of antiradical compounds has increased. Several studies have proved that certain

cereal products also have antiradical activity [2]. Many of these compounds, in particular peptides, were released after gastrointestinal enzyme digestion or by *in vitro* enzymatic hydrolysis.

The aim of this work was to determine antiradical and antihypertensive activity of peptides derived from pea sprouts.

MATERIAL AND METHODS

The materials were 5-days pea sprouts (*Pisum sativum* var. Bajka). Raw pea sprouts were selected and cleaned to remove contaminants. The enzymes of pea sprouts were inactivated by heating for 15 min at 100 °C.

Preparation of protein isolate (PSPI). The pea sprouts protein isolate was prepared according to the process described by Magias with minor modifications [8].

Preparation of pea sprouts protein hydrolysate (PSPH). The 4 % (w/v) protein isolate solution was prepared and hydrolyzed with trypsin for 2 h (in optimal condition). Hydrolysis was carried out using the following hydrolysis parameters: enzyme–substrate ratio 1:20; temperature at 37 °C; pH 8.0. Hydrolysis was stopped by heat treatment at 100 °C for 10 min. Hydrolysate was clarified by centrifuging at 8000 rpm for 20 min at 4 °C to remove insoluble substrate fragments and residual enzyme. The supernatant containing the peptides was collected for further fractionation. The hydrolysate was then frozen and stored at -20 °C before further analysis.

The peptides concentration was measured by TNBS (trinitrobenzene sulfonic acid) method by Adler-Nissen [1]. The protein hydrolysate was used for purification and determination of ACE inhibitory and antiradical activity of peptides.

Fractionation of PSPH with DEAE - cellulose and Sephadx G – 15 gel filtration. The PSPH was separated by ion exchange chromatography on DEAE cellulose column (linear gradient of NaCl from 0 to 0.5 M). Fractions (2 ml each) were collected at a flow rate of 0.8 ml/h, and absorbance was measured at 220 nm to determine the elution profile of the sample. Fractions associated with each peak showing antioxidant and ACE inhibitory activity were pooled and evaporated under vacuum. The fraction exhibiting the highest antiradical and ACE inhibitory activity was purified by gel filtration using Sephadex G-15 with 0.1 mM borate buffer solution (pH 8.3) (flow rate: 0.8 ml/min). In the fractions the antiradical and ACE inhibitory activity of peptides was determined.

ABTS radical – scavenging activity assay. The scavenging activity against the ABTS radical [2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] was determined by the decolourisation assay [9]. The ABTS scavenging activity was expressed as the scavenging percent.

ACE – inhibitory activity assay. The method used for determining ACE-inhibitory activity was that described by Hollenberg with minor modifications [4]. The ACE-inhibitory activity was expressed as IC_{50} value, which was defined as the peptides concentration (mg/ml) required for half-scavenging.

RESULTS

The concentration of peptides in the PSPI was 4.2 mg/ml, after 2 h of digestion increased to 48.88 mg/ml. The results of ACE inhibitory and antiradical activity of peptides are shown in Table 1. The sprouts pea protein hydrolysate obtained by trypsin digestion was separated by ion exchange chromatography. The hydrolysate was fractionated into eight individual fractions (Fig.1). The values of ACE inhibitory and antiradical activity of peptides in separated fractions are presented in Table 2. The highest activity was determined in the first fraction (IC_{50} = 0.24 mg/ml for ACE inhibitory and 100 % free radical scavenging activity – 0.2 mg/ml peptides concentration). This fraction was further purified by gel filtration chromatography using Sephadex G-15. After separation, twenty-one individual fractions were collected (Fig.2c). In all the fractions ACE inhibitory and antiradical activity of peptides were measured (Fig.2a, b). The highest ACE inhibitory activity was noted in the twelfth fraction and antiradical in the fourth fraction (IC_{50} = 0.007 mg/ml and 28.68 %, respectively).

Tab. 1. Peptides concentration, IC_{50} value of ACE inhibitory activity (mg/ml) and antiradical activity (%) in PSPH and PSPI

Pea sprouts proteins	Peptides concentration (mg/ml)	IC_{50} value of ACE inhibitory activity (mg/ml)	Antiradical activity (%) peptides concentration 0.2mg/ml
PSPI	4.20	10.72	20.37
PSPH	48.88	3.44	98.29

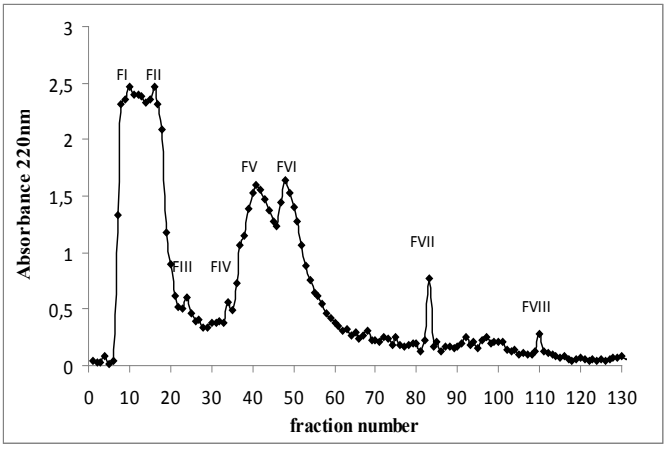

Fig. 1. Ion exchange gel chromatography profile of the trypsin hydrolysate of pea sprouts protein on DEAE-cellulose column

Tab. 2. Peptides concentration, IC_{50} value of ACE inhibitory activity (mg/ml) and antiradical activity (%) in fractions obtained after separated PSPH using DEAE cellulose

Fraction number	Peptides concentration (mg/ml)	IC_{50} value of ACE inhibitory activity (mg/ml)	Antiradical activity (%) peptides concentration 0.2mg/ml
I (7-14)	1.82	0.24	100
II (15-22)	9.40	0.85	53.98
III (23-26)	4.76	0.37	54.12
IV (33-35)	2.36	not noted	49.93
V (36-45)	1.05	not noted	51.99
VI (46-58)	2.14	0.53	51.35
VII (81-86)	1.40	0.25	50.14
VIII (109-111)	0.58	not noted	51.14

DISCUSSION

RADICAL-SCAVENGING ACTIVITY

In this experiment the obtained values of free radicals scavenging activities were 20.37 %, 98.29 % for protein isolate and hydrolysates, respectively. This result is compared with Pihlanto et al. a study by where antiradical activity increased from 5.6 % to 89.4 % for potato products after 5 h hydrolysis [9]. According to Yokomizo et al., the antioxidative activity of the hydrolysates with seven different proteases was measured. In each experiment the antiradical activity of all hydrolysates increased with hydrolysis time [17]. These results suggest that enzyme hydrolysis improves biological activity.

ACE- INHIBITORY PEPTIDE ACTIVITY

In this experiment the IC_{50} value of ACE inhibitory activity for PSPI and PSHI was 10.72 and 3.44 mg/ml, respectively. ACE inhibitory activity was found in whey and pea hydrolysates by Vermeirssen et al. and the results show that IC_{50} was 0.048 and 0.076 mg/ml, respectively [15]. According to Li et al., the alcalase mung bean protein hydrolysate showed ACE inhibitory activity with the IC_{50} value of 0.62 mg/ml [7]. However, Kuba et al. obtained IC_{50} 0.34 mg/ml for soy protein hydrolysates [5]. Many *peptides* with ACE inhibitory activity *were isolated* from fish protein hydrolysates, milk and derived products such as cheese [10, 13]. The research carried out by Srinivas and Prakash demonstrate that the IC_{50} value for α-casein protein after hydrolysis with chymotrypsin was 0.1 mg/ml [14]. These results suggest that peptides can be used as a protective substance for hypertensive patients.

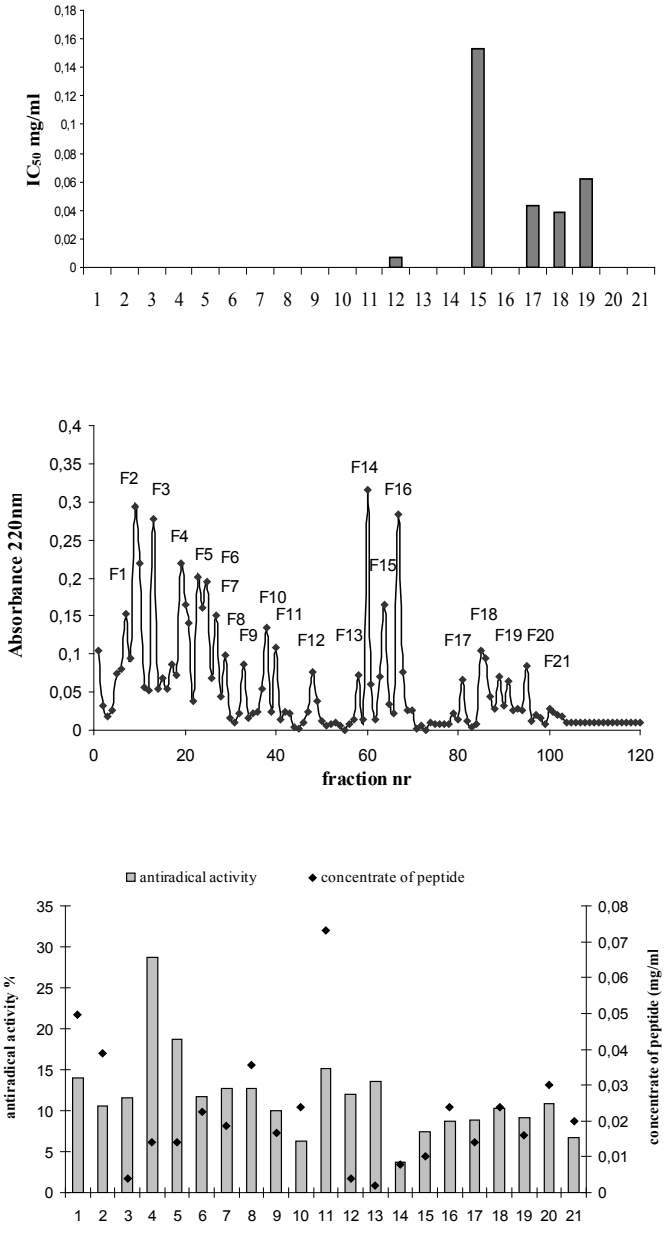

Fig. 2. Gel filtration profile of pea sprouts protein hydrolysates (c), peptides concentration and antiradical activity percent (a) and ACE inhibition activity (b) of all fractions

CONCLUSIONS

Results of this study imply that pea sprouts proteins hydrolysates may be a source of bioactive compounds with ACE-inhibitory and antioxidant activity. The bioactivities of protein hydrolysates were most likely related to peptides and/or free amino acids liberated during digestion. Therefore, pea sprouts proteins are a promising source for the production of bioactive compounds for functional foods and diet supplements that may be beneficial to human health.

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SUMMARY

Pea sprouts are a promising source for the production of bioactive compounds that can be beneficial to human health. In this study the peptides were obtained from trypsin hydrolysate from pea sprouts proteins with antiradical and antihypertensive activity. $ABTS^{+}$ was used to measure the antiradical capacities, and ACE (angiotensin I-converting enzyme) inhibitory activity of peptides was determined. The protein hydrolysate was fractionated by ion exchange chromatography on DEAE cellulose. The highest activity was noted in the fist fraction (100 % antiradical activity for 0.2 mg/ml peptides concentration and IC_{50} value of 0.24 mg/ml for ACE inhibitory activity). Further, this fraction was purified by Sephadex G-15 where the highest antiradical activity was measured in the fourth fraction (28.68 %) and ACE inhibitory activity of peptides was IC_{50} 0.007 mg/ml in the twelfth fraction. Results of this study indicate that enzymatic hydrolysates of pea sprouts protein possess potent antioxidative and antihypertensive activity.

Keywords: pea sprouts, peptide, ACE inhibitory, ABTS

STRESZCZENIE

Kiełki grochu stanowią potencjalne źródło bioaktywnych składników, które mogą pozytywnie wpływać na zdrowie człowieka. W pracy badano aktywność przeciwrodnikową i przeciwnadciśnieniową peptydów otrzymanych w wyniku trypsynowej hydrolizy białek kiełków grochu. Określono aktywność przeciwrodnikową wobec $ABTS^{+}$ oraz przeciwnadciśnieniową peptydów hamujących ACE (enzym konwertujący angiotensynę I). Hydrolizat białkowy poddano rozdziałowi przy użyciu chromatografii jonowymiennej na kolumnie DEAE-celuloza. Najwyższą aktywność przeciwrodnikową (100 % przy stężeniu peptydów 0.2 mg/ml) i inhibitującą ACE (IC_{50} 0,24 mg/ml) uzyskano w pierwszej peptydowej frakcji. Następnie frakcja ta została poddana rozdziałowi na Sephadexie G-15, gdzie największą aktywność przeciwrodnikową (28,68 %) zanotowano we frakcji czwartej, natomiast inhibitującą ACE (IC_{50} 0,007 mg/ml) w dwunastej. Na podstawie przeprowadzonych badań można stwierdzić, że w wyniku enzymatycznej hydrolizy białek kiełków grochu uwalniane są peptydy wykazujące aktywność przeciwrodnikową i przeciwnadciśnieniową.

Słowa kluczowe: kiełki grochu, peptydy, inhibitory ACE, ABTS

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Evaluation of some macroelement levels in selected dietary supplements supporting the immune system of the human organism

Ocena zawartości niektórych makroelementów w wybranych suplementach diety wspomagających system odpornościowy organizmu ludzkiego

INTRODUCTION

Dietary supplements enjoy great popularity with consumers worldwide, also in Poland. The assortment of these preparations is on a constant increase; every year many new supplements are registered. Taking preparations of this type is a comfortable way of supplementing the everyday diet with deficit substances necessary for correct functioning of the body. It is possible to apply supplements prophylactically or as supplements in therapy of different disease entities [6, 12]. Preparations taken, aimed at increasing the body's resistance, constitutean important group of supplements stimulating the immunological system [7, 11, 15]. Powdered herbalist's raw materials and dry extracts from medicinal plants are frequently included among dietary supplements; therefore, these preparations can frequently contain plenty of mineral substances [4].

Mineral elements constitute about 4% body weight of an adult man. They fulfill different functions in the body. Calcium and magnesium constitute the main building material of the bone and teeth, in which over 99% of calcium and 50%–60% of the total amount of magnesium is stored up. The remaining 1% calcium is found in tissues and extracellular liquids, in which this element fulfills other (rather than building) functions. In case of magnesium, 40%–45% of this element is stored up in cells of tissues, mainly in the muscles. Extracellular liquids contain 1% total content of magnesium [18].

The main raw plant materials used for the production of the studied dietary supplements (in the form of capsules and tablets), are: maca root and the root, bark and leaves of vilcacora and the root of Korean and American ginseng [5, 7, 11, 15].

Maca (*Lepidium meyenii* Walpers, *Lepidium peruvianum* Chacon*)* is a plant possessing nutritional and adaptogenic properties supplying the organism with natural amino acids, phytohormones, minerals and vitamins. It is strongly recommended for persons with active lifestyle, whose organism is exposed to intensive mental and physical effort. Preparations from the root find application in

states of weakness, emaciation and undernourishment of organism, during convalescence and in the Hadolescent stage, in fatigue syndromes, increasing the psychophysical activity, they are valuable immunostimulants, energizing and revitalizing the body [5].

Preparations from the root, bark or leaves of vilcacora/ cat's claw (*Uncaria tomentosa*), apart from numerous healing properties, also demonstrate strengthening action exerted on the human organism (tonicum). Thanks to the presence of pentacyclic oxoindole alkaloids which increase the activity of phagocytes, a modulating impact on immunological processes of the organism is exerted. *In vivo* and *in vitro* studies pointed in antiproliferative properties of preparations obtained from the discussed raw material in relation to the line of leukaemic cells, not-hindering the growth of the good cells [5, 15].

Ginseng (*Ginseng radix*) – called the "root of life" – is also obtained from the *Panax kind of ginseng* C.A. Meyer (proper ginseng) and *Panax quinquefolium* L. *American ginseng (*five-leaf ginseng*).*

In the 30 last years, results of over 50 clinical trials, conducted on patients and healthy volunteers, have been published. Results of those studies showed that in 13 cases (of 1572 examined persons) preparations of ginseng resulted in the improvement of physical and mental state, in 17 cases (846 patients) improvement in the physical fitness, improvement was also stated in various parameters of the metabolism in 10 more further studies. The studies on the impact of extracts from ginseng on cell metabolism, CNS, the cardiovascular system and the immunological system confirm the ability of the raw material to increase the body's resistance in the period of intensified action to factors, as free radicals, peroxides of lipids, cytotoxic and carcinogenic compounds. Preparations from this raw material demonstrate the immune system stimulating action and strengthening action. The influence of preparations from ginseng on the immunological system is connected, among others, with the presence of saponins. Saponins of ginseng have anti-oxidative and immunostimulant action, they stimulate the production of antiviral antibodies. As a result of this type of influences, an increase in the body's resistance to bacterial and viral infections and regeneration of the immunological system after experienced problematic illness occurs [7, 11].

Due to the scarcity of academic publications connected with the mineral composition of this type of preparations from non–vitamin – mineral groups and without the addition of minerals, and due to the high popularity in pharmaceutical market and frequent application exercised by members of the public, studying calcium and magnesium levels of the micronutrients important for the human body [14, 16, 17], appeared to be substantiated.

MATERIAL AND METHODS

The subject of the study were dietary supplements in the form of capsules and tablets and in liquid form (juices, their mixtures, liquid extracts from medicinal plants) supplementing the immunological system of the human body. Names and composition of these preparations are given in tables 1 and 2. 4–6 samples of each preparation were analyzed in two parallel runs. Each sample came from separate series of the production.

The 4–5 gram analytical samples for mineralization, from dietary supplements in the form of tablets, were made after prior averaging of the studied material (fragmenting tablets in the mortar). Dietary supplements in the form of gelatin capsules were weighed in one piece. In case of liquid supplements the measuring was done with pipette of 50–100 ml of liquid into quartz evaporating basins and they were evaporated to dry on a portable electric cooker (on the asbestos net). The

content of quartz melting pots (with analytical samples of tablets, capsules and liquid supplements after evaporating) was burnt initially in the flame of the burner, and then they were put to the furnace.

The samples were mineralized ”dry” in the muffle furnace at the temperature of 450ºC. The process of mineralization was hastened using water solution of the nitric acid (V) of 20% concentration (HNO_3 Suprapur, Merck). Ashes were dissolved „ hot” in 15% aqueous solution of the hydrochloric acid (HCl Suprapur, Merck). Next, mineralizates were filtered through paper filters and supplemented with deionized water to the determined volume.

Calcium and magnesium levels were determined by FAAS method (flame atomic absorption spectrometry) in M5 SOLAAR ThermoElemental, spectrometer, applying the parameters of analysis adequate for the specific element. The optimum parameters for determination of calcium [13]: wavelength – 422.7 nm; bandpass – 0.5 nm; lamp current (best sensitivity) – 75%; operating current – 5 mA; flame type – air/acetylene; flame chemistry – stoichiometric; fuel flow rate – 0.9-1.2 $l \cdot min.^{-1}$. The optimum parameters for determination of magnesium [13]: wavelength – 285.2 nm; bandpass – 0.5 nm; lamp current (best sensitivity) – 75%; operating current – 5 mA; flame type – air/acetylene; flame chemistry – stoichiometric; fuel flow rate – 0.9 – 1.2 $l \cdot min.^{-1}$.

In the same conditions, the analysis of referential material was conducted – a blend of Polish herbs „Mixed Polish Herbs” (INST-MPH-2) [3] – determining the calcium and magnesium levels in it. The declared amounts of the analyzed elements were: of calcium 1.08 ± 0.07% and of magnesium 0.292 ± 0.018%, and the determined amounts were as follows: of calcium 0.99 ± 0.03% and of magnesium 0.277 ± 0.009 %.

RESUALTS AND DISCUSSION

Liquid preparations contained the smallest amounts of calcium and magnesium: of calcium from 0.81 $\mu g \cdot ml^{-1}$ (*Ginsenol*) and 1.36 $\mu g \cdot ml^{-1}$ (*Aloe Activ*) to 250.9 $\mu g \cdot ml^{-1}$ (*Aloe vera* drinking gel) and of magnesium from 2.27 $\mu g \cdot ml^{-1}$ (*Aloe Activ*) to 155.8 $\mu g \cdot ml^{-1}$ (*Noni-vita).* In supplements in the form of capsules and tablets, the following levels were stated: of calcium from 45.92 $\mu g \cdot g^{-1}$ (*Acerola Plus)* to 25501 µg/g in *Maca 50 Plus* and 25037 $\mu g \cdot g^{-1}$ in *Maca* preparation and of magnesium : from 513.2 $\mu g \cdot g^{-1}$ in *Maca* preparation and 517.4 $\mu g \cdot g^{-1}$ in *Maca 50 Plus* to 4044 $\mu g \cdot g^{-1}$ (*Żeń-szeń* - vita-complex).

The average intake of calcium with the maximum twenty-four hour dose of the studied supplements in the form of capsules and tablets can reach: from 42.24 µg (*Acerola Plus*) to 61284 µg applying *Cat's Claw* preparation, and from liquid supplements from 4 µg (*Ginsenol*) to 12546 µg when applying gel of bitter aloes (*Aloe vera* drinking gel). Applying dietary supplements in the form of capsules or tablets, by taking the maximum twenty-four hour dose of these preparations, one can absorb on average the amount of magnesium, from 607.5 µg (*Żeń-szeń,* KRKA) to 11952 µg (*Spirulina Pacyfica),* and from liquid preparations: from 83.1 µg (*Ginsenging 400*) to 6231 µg (*Noni-vita* – juice of noni fruit).

The assimilation of calcium from the diet amounts to from 10% to 40%, depending on solubility of the chemical form and composition of the applied diet. Topical norms of calcium for the population of Poland were established on the level corresponding to the sufficient consumption (AI – Adequate Intake) for women from 1000 up to 1300 mg per day (depending on the age and the physiological state) and for men from 1000 up to 1300 mg for twenty-four hours (depending on the age) [6].

Assimilating magnesium from an average diet reaches the average of about 50%. Topical norms for magnesium for adults were stated on the level of the average demand (EAR – Estimated Average

Requirement) and of recommended consumption (RDA – Recommended Dietary Allowances). For women, depending on the age, EAR is varies from 255 to 265 mg/day, and for men from 330 to 350 mg/day, however RDA – from 310 to 320 mg/day – for women and from 400 to 420 mg/day – for men, depending on the age [6].

The main source of mineral elements for men (including calcium and magnesium) are: food and beverages, and supplementing – dietary supplements with minerals or preparations of vitamin-mineral character [6, 10, 18].

Table 1. Name, producer and composition of dietary supplements (in the form of capsules and tablets) supplementing the immunological system of the human organism

No	Preparation name	Main components
1	*Acerola Plus* (tabl.); Puritan's Pride Inc., USA	Powdered acerola fruit, powdered grains of the buckwheat, buds of the wild rose (*Rosa canina*), citrus biflavonoids, extract from the green pepper, extract from the blackcurrant, rutin, hesperidine
2	*Cat's Claw* (caps.); Now Foods, USA	Powdered cat's claw (*Uncaria tomentosa)*
3	*Maca* (caps.); A-Z Medica, Sp. z o.o., Poland	Powdered maca root (*Lepidium meyenii* Walp.)
4	*Maca 50 Plus*(caps.); A-Z Medica, Sp. z o. o., Poland	Powdered maca root (*Lepidium meyenii* Walp.), antioxidative prefix: beta-carotene, vitamins – E, C; shark's cartilage
5	*Spirulina* (tabl.); Walmark, Sp. z o. o., Czech Rep.	Sea algae (*Spirulina platensis*), Vitamins: B_1, B_2, B_6
6	*Spirulina Hawajska* (caps.); Organic by Nature Inc., USA	Sea algae (*Spirulina platensis*) 100%
7	*Spirulina Pacyfica* (tabl.); Cyanotech Corp., USA	Sea algae (*Spirulina platensis*)
8	*Vilcacora* (caps.); Andean Medicine Centre Ltd., United Kingdom	Vilcacora (*Uncaria tomentosa*)
9	*Żeń-szeń* (caps.); Herbapol Kraków, Poland	Powdered ginseng root (*Ginseng radix*), iron oxides as capsule components
10	*Żeń-szeń Ginseng*, (caps.); KRKA, Slovenia	Dry extract from ginseng root (*Panax ginseng radicis extractym siccum*), iron oxides as capsule components
11	*Żeń-szeń Ginseng* (tabl.); Naturell AB, Sweden	Standardized extract from white Korean ginseng root (*Panax ginseng* C.A. Meyer)
12	*Żeń-szeń koreański*(caps.); Walmark, Sp. z o. o., Czech Rep.	Extract from white Korean ginseng root, vitamin E (D-alpha tocopherol)
13	*Żeń-szeń, vita-complex* (caps.); Olimp Lab., Sp. z o. o., Poland	Korean ginseng (Korean *Panax ginseng extract.*), American ginseng (*Panax quinquefolium extract*}

Table 2. Name, producer and composition of liquid dietary supplements supplementing the immunological system of the human organism

No	Preparation name	Main components
1	*Aloe vera – aloe juice*; Laboratoria Natury, Alter Medica, Olfam, Sp.z o. o., Poland	Aloe juice (*Aloe vera*)
2	*Aloe vera drinking gel juice with pulp pieces* – gel; Laborat. Natury, Sp. z o.o., Bio Medica, Poland	Aloe juice with pulp pieces (*Aloe vera*)
3	*Aloes Activ – aloe juice with extract of ginseng*; Alter Medica, Poland	Aloe juice (*Aloe barbadensis*), ginseng extract (*Acanthopanax senticosus* Maxim)
4	*Aloes Young + żurawina* – aloe juice and cranberry juice with acerola fruit; Alter Medica, Poland	Aloe juice (*Aloe barbadensis* - 70%), cranberry juice (*Oxycoccus palustris* Hill syn. *Vaccinum oxycoccus* – 25%), acerola powdered fruit (*Malpighia gabra* – 5%)
5	*Bodymax Tonik*; Axellus A/S, Denamrk	Standardized extract from ginseng GGE (*Panax ginseng* C.A. Meyer), Standardized extract from Japanese gingko (*Ginkgo biloba*), vitamins: B_1, B_2, B_6, PP, pantothenic acid
6	*Ginsenging 400* – extract from ginseng with honey; MEHECO No18, Guangiming Zhong Jie, China	*Ginseng radix extractum spissum* 3:1 – 400 mg, royal jelly 300 mg in 1 ampoule –10 ml
7	*Ginsenol*; Krakowskie Zakłady Zielarskie „Herbapol”, Kraków, Poland	Ginseng root tincture 1:5 (*Ginseng radix*)
8	*Noni – noni fruit juice*; Alter Medica, Poland	Noni fruit juice (*Morinda citrifolia*)
9	Noni-vita - noni fruit juice; Laboratoria Natury, Sp. z o.o., Poland	100% Noni fruit juice (*Morinda citrifolia*)
10	*Noni Plus – juice of noni and cranberry fruits*; Alter Medica, Poland	Noni fruit juice (*Morinda citrifolia*), cranberry juice (*Oxycoccus palustris*)

Table 3. Calcium content in supplements of diet supporting the immune system of the organism, $\mu g \cdot g^{-1}$ and µg/caps.(tabl.) and intake of calcium with maximal daily dose (µg)

No	Preparation	Calcium (Ca) content ($\mu g \cdot g^{-1}$)	Calcium (Ca) content (µg/caps. (tabl.)	Intake of calcium with maximal of daily dose (µg of Ca)
		Arithmetic mean. standard deviation and content (min.- max.)		
1	*Acerola Plus* (tabl.). n *)= 4, m *) = 0.92	45.92 ± 12.94 35.92 – 65.91	42.24 ± 11.91 32.72 – 60.64	42.24 ± 11.91 32.72 – 60.64
2	*Cat's Claw* (caps.). n = 4, m = 0.60 g	17024 ± 2131 14607 - 18835	10214 ± 1279 8764 - 11301	61284 ± 7674 52584 - 67806
3	*Maca* (caps.). n = 4, m = 0.40 g	25037 ± 92 24948 - 25163	10015 ± 37 9979 - 10065	30045 ± 111 29937 - 30195
4	*Maca 50 Plus* (caps.). n = 4, m = 0.60 g	25501 ± 462 24874 - 25892	15300 ± 277 14924 - 15535	30600 ± 554 29848 - 31070
5	*Spirulina* (Walmark) (tabl.). n = 4, m = 0.65 g	436.9 ± 46.0 364.2 – 487.6	284.0 ± 29.9 236.7 – 316.9	1420 ± 149 1184 - 1585
6	*Spirulina Hawaian* (caps.). n = 4, m = 0.60 g	541.4 ± 83.5 463.5 – 617.8	324.8 ± 50.1 278.1 – 370.7	1949 ± 301 1669 - 2224
7	*Spirulina Pacyfica* (tabl.). n = 4, m = 0.50 g	283.1 ± 32.7 250.3 – 325.2	141.6 ± 16.3 125.2 – 162.6	849.6 ± 98 751.2 – 975.6
8	*Vilcacora* (caps.). n = 4, m = 0.56 g	7139 ± 890 6272 - 7966	3998 ± 50 3512 - 4461	23988 ± 300 21072 - 26766
9	*Żeń-szeń* (Herbapol Kraków) (caps.). n = 4, m = 0.30 g	1732 ± 225 1491 - 1939	519.7 ± 67.5 447.3 – 581.7	2079 ± 270 1789 - 2327
10	*Żeń-szeń Ginseng* (KRKA) (caps.). n = 4, m = 0.46 g	157.4 ± 33.9 122.1 – 190.5	72.39 ± 15.61 56.17 – 87.63	72.39 ± 15.61 56.17 – 87.63
11	*Żeń-szeń Ginseng* (Naturell) (tabl.) n = 4. m = 0.40 g	2547 ± 490 2082 - 3075	1019 ± 196 832 - 1230	2038 ± 392 1666 - 2460
12	*Żeń-szeń koreański* (Walmark) (caps.). n = 4, m = 0.50 g	1577 ± 159 1363 - 1806	788.6 ± 79.7 681.5 – 903.0	788.6 ± 79.7 681.5 – 903.0
13	*Żeń-szeń vita-complex* (Olimp) (caps.). n = 4, m = 0.78 g	3256 ± 470 2853 - 3847	2539 ± 367 2225 - 3001	2539 ± 367 2225 - 3001

n *) number of examined series of production. m *) mean mass of capsule or tablet

Table 4. The magnesium content in supplements of diet supporting immune system organism. $\mu g \cdot g^{-1}$ and µg/caps.(tabl.) and intake of magnesium with maximal of daily dose (µg)

No	Preparation	Magnesium (Mg) content ($\mu g \cdot g^{-1}$)	Magnesium (Mg) content µg/caps. (tabl.)	Intake of magnesium with maximal daily dose (µg of Mg)
		Arithmetic mean. standard deviation and content (min. –max.)		
1	*Acerola Plus* (tabl.). n *)= 4, m *)= 0.92	661.5 ± 161.5 505.0 – 842.1	608.6 ± 148.6 464.6 – 774.7	608.6 ± 148.6 464.6 – 774.7
2	*Cat's Claw* (caps.). n = 4, m = 0.60 g	1101 ± 238 924 - 1431	660.3 ± 142.6 554.4 – 858.6	3962 ± 855.6 3326 - 5152
3	*Maca* (caps.). n = 4, m = 0.40 g	513.2 ± 118.4 400.0 – 642.2	205.3 ± 47.4 160.0 – 256.9	615.9 ± 142.2 480.0 – 770.7
4	*Maca 50 Plus* (caps.). n = 4, m = 0.60 g	517.4 ± 118.6 403.7 – 628.6	310.5 ± 71.2 242.2 – 377.2	621.0 ± 142.4 484.4 – 754.4
5	*Spirulina* (Walmark) (tabl.). n = 4, m = 0.65 g	2237 ± 187 2000 - 2511	2237 ± 187 2000 - 2511	11185 ± 935 10000 - 12555
6	*Spirulina Hawaian* (caps.). n = 4, m = 0.60 g	2743 ± 503 2212 - 3341	1646 ± 302 1327 - 2005	9876 ± 1812 7962 - 12030
7	*Spirulina Pacyfica* (tabl.). n = 4. m = 0.50 g	3983 ± 285 3750 - 4399	1992 ± 143 1875 - 2200	11952 ± 858 11250 - 13200
8	*Vilcacora* (caps.). n = 4, m = 0.56 g	507.5 ± 39.4 469.1 – 561.2	284.2 ± 22.1 262.7 – 314.3	1705 ± 132.6 1576 - 1886
9	*Żeń-szeń* (Herbapol Kraków) (caps.). n = 4, m = 0.30 g	1357 ± 128 1217 - 1473	407.1 ± 38.3 365.1 – 441.9	1628 ± 153.2 1460 - 1768
10	*Żeń-szeń Ginseng* (KRKA) (caps.). n = 4, m = 0.46 g	1321 ± 151 1190 - 1536	607.5 ± 69.4 547.4 – 706.6	607.5 ± 69.4 547.4 – 706.6
11	*Żeń-szeń Ginseng* (Naturell) (tabl.). n = 4, m = 0.40 g	1590 ± 271 1189 - 1768	636.1 ± 108.4 475.6 – 707.2	1272 ± 216.8 951.2 - 1414
12	*Żeń-szeń koreański* (Walmark) (caps.). n = 4, m = 0.50 g	3748 ± 660 3142 - 4931	1874 ± 330 1571 - 2466	1874 ± 330 1571 - 2466
13	*Żeń-szeń - vita-complex* (Olimp) (caps.). n = 4, m = 0.78 g	4044 ± 582 3473 - 5004	3154 ± 454 2709 - 3903	3154 ± 454 2709 - 3903

n *) number of examined series of production. m *) mean mass of capsule or tablet

Table 5. Calcium and magnesium content in supplements of diet supporting the immune system of the human organism. $\mu g \cdot ml^{-1}$; intake of calcium and magnesium with maximal daily dose (μg)

No	Preparation	Calcium (Ca) content ($\mu g \cdot ml^{-1}$)	Intake of calcium with maximal of daily dose (μg of Ca)	Magnesium (Mg) content ($\mu g \cdot ml^{-1}$)	Intake of magnesium with maximal daily dose (μg of Mg)
		Arithmetic mean (M). standard deviation (SD) and content (min.–max.)			
1	*Aloe vera – aloe juice.* n*) = 4, v*) = 50 ml	43.10 ± 3.07 40.50 – 46.50	2155 ± 153 2055 - 2325	30.73 ± 3.96 25.15 – 33.48	1537 ± 198 1258 - 1680
2	*Aloes - aloe juice with pulp pieces* –gel. n = 4, v= 50 ml	250.9 ± 17.7 240.0 – 282.0	12546 ± 883 12000 - 14100	69.83 ± 6.19 58.79 – 73.22	3492 ± 309 2940 - 3661
3	*Aloes Activ – aloe juice with extract of ginseng.* n = 4, v = 50 ml	1.36 ± 0.41 0.98 – 1.72	68.0 ± 20.5 49.0 – 86.0	2.27 ± 0.38 1.89 – 2.80	113.3 ±19.2 94.5 – 140.0
4	*Aloes Young – aloe juice with cranberry.* n = 4, v = 60 ml	6.11 ± 1.07 5.03 – 7.46	367 ± 64.1 301.8 – 447.6	7.56 ± 0.60 6.68 – 8.04	453.5 ± 36.1 400.8 – 482.4
5	*Bodymax Tonik*. n = 4, v = 50 ml	4.71 ± 1.21 3.54 – 5.89	235.5 ± 60.6 177.0 – 294.5	8.85 ± 0.79 7.78 – 9.56	442.3 ± 39.4 389.0 – 478.0
6	*Ginsenging 400 - extract of ginseng with honey*. n = 3, v = 10 ml	6.40 ± 1.18 5.38 – 8.08	49.0 ± 33.1 53.8 – 80.8	8.31 ± 1.37 7.06 – 10.26	83.1 ± 13.7 70.6 – 102.6
7	*Ginsenol*. n = 2, v = 5 ml	0.81 ± 0.11 0.71 – 0.94	4.0 ± 0.6 3.6 – 4.7	43.22 ± 2.30 40.50 – 45.20	216.1 ± 11.49 202.5 – 226.0
8	*Noni - juice of noni fruits*. n = 5, v = 40 ml	16.00 ± 3.94 13.54 – 22.91	640.2 ± 157.5 541.6 – 910.4	105.8 ± 12.1 90.3 – 124.1	4232 ± 481.8 3612 - 4964
9	Noni-vita - juice of noni fruits. n=4, v = 40 ml	96.70 ± 9.35 84.87 – 107.2	3868 ± 374 3395 - 4388	155.8 ± 20.4 141.1 – 185.8	6231 ± 816.6 5676 - 7432
10	*Noni Plus - juice of noni and cranberry fruits*. n = 3, v = 40 ml	6.67 ± 0.90 5.94 – 7.85	266.7 ± 35.9 237.6 – 314.0	68.80 ± 3.78 63.89 – 72.65	2752 ± 151.3 2556 - 2906

n *) number of examined series of production; v*) maximal of daily dose of supplement. ml

In the studies conducted earlier on dietary supplements without the planned addition of mineral compounds it was stated that the mentioned macroelements also appeared in every kind of these preparations, only in different amounts [1, 2]. In the study [1], in which the level of calcium and magnesium in dietary supplements supplementing slimming was studied, the content of these elements ranged as follows: – for calcium from 2.69 ± 0.26 $\mu g \cdot g^{-1}$ (*Zielona herbata – Green tea* – extract) up to 39612 ± 1136 $\mu g \cdot g^{-1}$ (*Aplefit*), and for magnesium – from 44.58 ± 7.85 $\mu g \cdot g^{-1}$ (*Adipobon mono)* to 1233 ± 64 $\mu g \cdot g^{-1}$ *(Chitobon*), on average. In preparations containing antioxidants [2] calcium contents ranged in the scope of , on average, from 6.98 ± 125 $\mu g \cdot g^{-1}$ (*Aronia z luteiną*) to 2137 ± 166 $\mu g \cdot g^{-1}$ (*Vision Maxi*) and to 5299 ± 951 $\mu g \cdot g^{-1}$ in preparation of *Czosnek z pietruszką*. The preparation containing

the smallest amounts of magnesium was: *Aronia z luteiną*, on average 3.34 ± 0.50 $\mu g \cdot g^{-1}$ however, the bulk of this element was determined in *Czosnek z pietruszką* supplement: on average – 2997.8 ± 439 $\mu g \cdot g^{-1}$. Leśniewicz et al. [9] in different healing preparations studied also determined, apart from microelements, calcium content and magnesium. Amounts of calcium were in the range from 3740 $\mu g \cdot g^{-1}$ *(Alax*) to 11320 µg of calcium in 1 g of *Tonic tablets*. The content of magnesium were in the range from 1130 $\mu g \cdot g^{-1}$ (*Urogram)* to 5170 $\mu g \cdot g^{-1}$ (*Alax*). Calcium and magnesium levels were also determined in multivitamin preparations by Krejčová et al. [8] of the Czech Republic. The amounts were also in wide ranges: Ca – from 31.3 mg/tabl. to 136 mg/tabl. and Mg – from 10.5 mg/tabl. to 76.8 mg/tablet. Results of the analysis of these elements using ICP-OES after microwave digestion and ICP-OES slurry technique were compared with the amount declared by the producer.

CONCLUSIONS

Calcium and magnesium levels in the studied dietary supplements were diversified – they depended on pharmaceutical form of preparation and composition.

Indicated quantities of the studied macroelements can fill up the daily demand of the organism for calcium and magnesium in the course of the application.

It was stated that the average intake of calcium with the maximum twenty-four hour dose as for the studied capsules and tablets could be for adults from about 4.7% AI*) to 6% AI (*Cat's Claw*), and from liquid supplements – about 1% AI (*Aloe* gel), taking magnesium – from 2.9% RDA (men) to 3.8% RDA**) (women) with the maximum twenty-four hour dose of *Spirulina Pacyfic* preparation and from 1.4% RDA to 1.9% RDA from *Noni-vita*.

AI*) – (Adequate Intake) – level of sufficient consumption

RDA** – (Recommended Dietary Allowances) – recommended consumption

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SUMMARY

Dietary supplements enjoy great popularity among consumers in Poland it is possible to apply them prophylactically or as supplements in therapy of different disease entities. Preparations taken with an aim of increasing the body's resistance constitute an important group of supplements. Plant raw materials included in the studied supplements contain active substances which biologically reveal general strengthening action, restore physical fitness and intellectually stimulate the organism and improve properties of the immunological system. Powdered herbalist's raw materials composing these preparations can frequently abound in significant amounts of mineral substances. Due to the scarcity of academic publications connected with mineral composition of this type of preparations and their frequent application, examining calcium content and of magnesium (of the micronutrients important for the human body) appeared to be substantiated. The subject of the research were the supplements in the form of capsules and tablets (*Acerola Plus, Cat's Claw, Maca, Maca 50 Plus, Spirulina, Spirulina Hawajska, Spirulina Pacifica, Vilcacora, Żeń-szeń* from „Herbapol" Herbal Plant in Krakow, *Żeń-szeń – Ginseng* <KRKA>, *Żeń-szeń* <Naturell>, *Żeń-szeń koreański* <Walmark>, *Żeń-szeń* vita-complex) and fluid preparations (*Aloes–aloe juice*;, *Aloe vera drinking gel*, *Aloes–aloe juice with pulp pieces*, *Aloes Activ, Aloes Young, Bodymax Tonik, Ginsengin 400, Ginsenol, Noni, Noni-vita*, *Noni Plus*). Samples of supplements were mineralized "dry" at the temperature of 450˚C. Calcium and magnesium contents were determined in the atomic absorption spectrometer SOLAAR M 5 (Thermo Elemental).The smallest levels of calcium and magnesium were noted in the l iquid preparations: of calcium from 0.81 $\mu g \cdot ml^{-1}$ (*Ginsenol*) and 1.36 $\mu g \cdot ml^{-1}$ (*Activ Aloe*) to 250.9 $\mu g \cdot ml^{-1}$ (*Aloe Vera drinking gel*) and of magnesium from 2.27 $\mu g \cdot ml^{-1}$ (*Aloe Activ)* to 153.7 $\mu g \cdot ml^{-1}$ (*Noni-vita*). In supplements in the form of capsules and tablets, calcium content ranged from 45.92 $\mu g \cdot g^{-1}$ (*Acerola*

Plus) to over 20 000 $\mu g \cdot g^{-1}$ (*Maca, Maca 50 Plus*) and of magnesium from 513 $\mu g \cdot g^{-1}$ (*Maca 50 Plus*) and 517 $\mu g \cdot g^{-1}$ (*Maca*) to 4043 $\mu g \cdot g^{-1}$ (*Żeń-szeń* vita-complex). Calcium and magnesium contents in the studied dietary supplements were diversified depending on the pharmaceutical form of the preparation and the composition. The stated quantities of mentioned macroelements can fill up the daily demand of the organism for calcium and magnesium in the course of their application.

Keywords: dietary supplements, calcium, magnesium, flame atomic absorption spectrometer (FAAS)

STRESZCZENIE

Suplementy diety cieszą się dużą popularnością wśród konsumentów w Polsce, można je stosować profilaktycznie lub pomocniczo w terapii różnych jednostek chorobowych. Ważną grupę suplementów stanowią preparaty stosowane w celu zwiększenia odporności organizmu. Surowce roślinne zawarte w badanych suplementach zawierają substancje czynne biologicznie, wykazujące działanie ogólnie wzmacniające, przywracające sprawność fizyczną i umysłową organizmu oraz właściwości stymulujące system odpornościowy. Sproszkowane surowce zielarskie wchodzące w skład tych preparatów mogą zawierać czasami znaczne ilości substancji mineralnych. W związku z niewielką ilością publikacji naukowych związanych ze składem mineralnym tego typu preparatów i częstym ich stosowaniem przez ludzi celowe było zbadanie zawartość wapnia i magnezu (ważnych dla organizmu człowieka biopierwiastków). Przedmiotem badań były suplementy w postaci kapsułek i tabletek (*Acerola Plus, Cat's Claw, Maca, Maca 50 Plus, Spirulina, Spirulina Hawajska, Spirulina Pacifica, Vilcacora, Żeń-szeń* z Zakładu Zielarskiego „Herbapol" w Krakowie, *Żeń-szeń – Ginseng* <KRKA>, *Żeń-szeń* <Naturell>, *Żeń-szeń koreański* <Walmark>, *Żeń-szeń vita-complex*) i preparaty płynne (*Aloes–sok, Aloe vera drinking gel, Aloes–sok z miąższem, Aloes Activ, Aloes Young, Bodymax Tonik, Ginsengin 400, Ginsenol, Noni, Noni-vita, Noni Plus*). Próbki suplementów mineralizowano „na sucho" w temp. 450˚C. Zawartość wapnia i magnezu oznaczono w spektrometrze absorpcji atomowej SOLAAR M5 (Thermo Elemental). Najmniejszą ilość wapnia i magnezu zawierały preparaty płynne: wapnia od 0.81 $\mu g \cdot ml^{-1}$ (*Ginsenol*) i 1,36 $\mu g \cdot ml^{-1}$ (*Aloes Activ*) do 250,9 $\mu g \cdot ml^{-1}$ (*Aloe vera drinking gel*) i magnezu od 2,27 $\mu g \cdot ml^{-1}$ (*Aloes Activ*) do 153,7 $\mu g \cdot ml^{-1}$ (*Noni-vita*). W suplementach w postaci kapsułek i tabletek stwierdzono zawartość wapnia od 45,92 $\mu g \cdot g^{-1}$ (*Acerola Plus*) do ponad 20 000 $\mu g \cdot g^{-1}$ (*Maca, Maca 50 Plus*) i magnezu od 513 $\mu g \cdot g^{-1}$ (*Maca 50 Plus*) i 517 $\mu g \cdot g^{-1}$ (*Maca*) do 4043 $\mu g \cdot g^{-1}$ (*Żeń-szeń vita-complex*). Zawartości wapnia i magnezu w badanych suplementach diety były zróżnicowane, zależały od postaci farmaceutycznej preparatu oraz składu. Stwierdzone ilości wymienionych makroelementów mogą uzupełniać dzienne zapotrzebowanie organizmu w wapń i magnez w czasie ich stosowania.

Słowa kluczowe: suplementy diety, wapń, magnez, płomieniowa absorpcyjna spektrometria atomowa (FASA)

ANNALES
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Effect of 7-nitroindazole and N^G-nitro-L-arginine on the protective action of clobazam in the maximal electroshock-induced seizures in mice

Wpływ 7-nitroindazolu i N^G-nitro-L-argininy na ochronne działanie klobazamu w teście maksymalnego wstrząsu elektrycznego u myszy

INTRODUCTION

Clobazam (CLB) is a second-generation antiepileptic drug (AED) licensed as an adjunct therapy for patients with refractory epilepsy [25]. The drug is highly effective as adjunctive therapy for partial and generalized seizures, for intermittent therapy and for controlling non-convulsive status epilepticus [21]. Moreover, CLB is used in pediatric epileptology in treating resistant epilepsies of infancy and childhood [20].

CLB as a 1,5-benzodiazepine binds to γ subunit of the γ-aminobutyric $acid_A$ ($GABA_A$) receptors and thus, potentiates GABAergic inhibition [18]. Experimental evidence indicates that CLB exhibits anticonvulsant activity in various models of epilepsy including: the maximal electroshock (MES)-induced tonic seizures, pentylenetetrazole (PTZ)-induced clonic seizures, bicuculline- and picrotoxin-induced seizures in rodents [19, 24]. CLB is effective in suppressing motor seizures induced by PTZ in immature and developing rats [7, 22]. CLB also suppressed lateral geniculate nucleus-kindled seizures [8], olfactory bulb- and amygdala-kindled seizures in rats [6].

Nitric oxide (NO) as a gaseous molecule possesses neurotransmitter/neuromodulator properties in the brain and plays an important role in the pathophysiology of epilepsy, producing both anti- and pro-convulsant effects in various experimental models of epilepsy in rodents [2, 15, 17]. NO is produced by the oxidation of L-arginine by NO synthase (NOS – a Ca^{2+}/calmodulin-dependent enzyme), existing in three distinct isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [17]. It is generally accepted that N^G-nitro-L-arginine (NNA – a non-selective NOS inhibitor) reduces the activity of both, eNOS and nNOS, to the same extent, whereas 7-nitroindazole (7NI) is considered to be a preferential inhibitor of nNOS activity [1, 17].

Experimental evidence indicates that 7NI, the preferential nNOS inhibitor, exerted the anticonvulsant properties by elevating the threshold for maximal electroconvulsions in mice [2, 11, 15, 23]. 7NI potentiated the anticonvulsant action of phenobarbital (PB), phenytoin (PHT), valproate (VPA), oxcarbazepine (OXC), loreclezole (LCZ), pregabalin (PGB), but not that of carbamazepine (CBZ), topiramate (TPM), lamotrigine (LTG), and felbamate (FBM) in the maximal electroshock (MES)-induced seizures in mice [4, 11, 13, 15, 16].

With regards to NNA administered systemically (i.p.) at a dose of 40 mg/kg, it had no impact on the anticonvulsant effects of some various AEDs (i.e., LTG, FBM, OXC, LCZ, PGB, CBZ, PHT, and TPM) in the mouse MES model [12, 13, 16]. In contrast, NNA significantly reduced the anticonvulsant effect of PB and VPA in the MES-induced seizure test in mice [4, 5, 15]

Considering the above-mentioned fact, it was of pivotal importance to evaluate the effects of 7NI and NNA on the anticonvulsant action of CLB in the mouse MES model. Generally, the mouse MES test is thought to be an animal model of tonic-clonic seizures and, to a certain extent, of partial convulsions with or without secondary generalization in human [10]. In this model one can readily evaluate the anticonvulsant effects produced by classical and second-generation antiepileptic drugs in combination with 7NI and NNA, therefore, it was appropriate to use this test in the present study.

Moreover, the acute adverse-effect potentials of CLB in combination with 7NI and NNA were determined in the chimney test (motor performance), step-through passive avoidance task (long-term memory) and the grip-strength test (skeletal muscular strength) in mice.

MATERIAL AND METHODS

Animals and experimental conditions. All experiments were performed on male Swiss mice, kept in colony cages with free access to food and tap water, under standardized housing conditions. The animals were randomly assigned to experimental groups consisting of 8 mice each. All experimental tests were performed between 9.00 a.m. and 2.00 p.m. to minimize confounding effects of circadian rhythms. All experimental procedures described hereupon were approved by the Second Local Ethics Committee at the University of Life Sciences in Lublin (license no.: 84/2009).

Drugs. CLB (Frisium®, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany), 7NI (Sigma, St. Louis, MO, USA) and NNA (RBI, Natick, MA, USA) were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in saline and administered intraperitoneally (i.p.) in a volume of 5 ml/kg body weight: CLB, 7NI and NNA – at 30 min before the MES and all behavioral tests. The pretreatment times before testing of CLB, 7NI and NNA were based upon information about their biological activity from the literature and our previous experiments [4, 5, 11-13, 16, 17, 23].

Maximal electroshock – induced seizures. Electroconvulsions were produced by an alternating current (0.2 s stimulus duration, 50 Hz, fixed current intensity of 25 mA, maximum stimulation voltage of 500 V) delivered via ear-clip electrodes by a Rodent Shocker generator (Type 221, Hugo Sachs, Freiburg, Germany). The criterion for the occurrence of seizure activity was the tonic hindlimb extension. The protective activity of CLB administered alone or in combination with 7NI and NNA was evaluated as its median effective doses (ED_{50} in mg/kg with 95% confidence limits) against MES-induced seizures. The animals received different doses of CLB so as to obtain a variable percentage of protection against MES, allowing the construction of a dose-effect curve for CLB administered alone or in combination with 7NI and NNA, according to Litchfield and Wilcoxon

[9]. Each ED_{50} value represents the dose of CLB required to protect 50% of the animals tested against MES-induced seizures. In the present study, CLB was administered at doses ranging between 8–22 mg/kg. This experimental procedure has been described in detail in our earlier studies [11–16].

Chimney test. The effects of combination of CLB with 7NI and NNA at its ED_{50} values from the MES test on motor coordination impairment were quantified with the chimney test of Boissier et al. [3]. The time before the commencement of the chimney test (after drug administration) was identical to that for the MES test. In this test, animals had to climb backwards up the plastic tube (3 cm inner diameter, 25 cm length). Motor impairment was indicated by the inability of the animals to climb backward up the transparent tube within 60 s. Data were presented as a percentage of animals that failed to perform the chimney test. This experimental procedure has been described in detail in our earlier studies [11–14].

Grip – strength test. The effects of combinations of CLB with 7NI and NNA at its ED_{50} values from the MES test, on muscular strength (tone) in mice were quantified by the grip-strength test. The time before the commencement of the grip-strength test (after drug administration) was identical to that for the MES test. The grip-strength apparatus (BioSeb, Chaville, France) comprised a wire grid (8 × 8 cm) connected to an isometric force transducer (dynamometer). The mice were lifted by the tails so that their forepaws could grasp the grid. The mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The mean of 3 measurements for each animal was calculated and, subsequently, the mean maximal force of 8 animals per group was determined. The skeletal muscular strength in mice was expressed in Newtons (N) as means ± SE of at least 8 determinations (8 animals per group). This experimental procedure has been described in detail in our earlier study [13, 27].

Step – through passive avoidance task. Each animal was administered 7NI or NNA co-administered with CLB at doses corresponding to its ED_{50} values from the MES test on the first day before training. The time before the commencement of the training session (after drug administration) was identical to that for the MES test. Subsequently, animals were placed in an illuminated box (10 × 13 × 15 cm) connected to a larger dark box (25 × 20 × 15 cm) equipped with an electric grid floor. Entrance of animals to the dark box was punished by an adequate electric footshock (0.6 mA for 2 s). The animals that did not enter the dark compartment were excluded from subsequent experimentation. On the following day (24 h later), the pre-trained animals were placed again into the illuminated box and observed up to 180 s. Mice that avoided the dark compartment for 180 s were considered to remember the task. The time that the mice took to enter the dark box, was noted and the median latencies (retention times) with 25^{th} and 75^{th} percentiles were calculated. The step-through passive avoidance task gives information about the ability to acquire the task (learning) and to recall the task (retrieval). Therefore, it may be regarded as a measure of long-term memory [26]. This experimental procedure has been described in detail in our earlier studies [11–16].

Statistics. The ED_{50} values (in mg/kg) with their respective 95% confidence limits were calculated by log-probit analysis [9]. Subsequently, the 95% confidence limits were transformed to SE according to the method described earlier [14]. Statistical analysis of data was performed either with log-probit method for single comparison or with one-way ANOVA followed by the post-hoc Tukey-Kramer test for multiple comparisons. Qualitative variables from the chimney test were compared by use of the Fisher's exact probability test. The results obtained in the passive avoidance task were statistically evaluated using Kruskal-Wallis nonparametric ANOVA. The results from the

grip-strength test were verified with one-way ANOVA. All statistical tests were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Differences among the values were considered statistically significant if $p<0.05$.

RESULTS

EFFECT OF 7NITROINDAZOLE AND N^G-NITRO-L-ARGININE ON THE ANTICONVULSANT ACTIVITY OF CLOBAZAM AGAINST MAXIMAL ELECTROSHOCK-INDUCED SEIZURES

CLB administered i.p. 30 min before the test produced a clear-cut anticonvulsant effect and its ED_{50} value was 15.14 (12.63–18.14) mg/kg. The combination of CLB with NNA (40 mg/kg) was associated with a slight decrease in the anticonvulsant effect exerted by CLB. In such a case, the ED_{50} value for CLB increased by 5%, amounting to 15.97 (12.23–20.86) mg/kg (results not shown). In contrast, 7NI (50 mg/kg) co-administered with CLB produced a significant (by 32%) decrease in the ED_{50} value of CLB from 15.14 (12.63–18.14) mg/kg to 10.23 (8.44–12.40) mg/kg ($p<0.05$; Figure 1). In the case of the combination of CLB with 7NI (25 mg/kg), a slight (by 5%) reduction of the ED_{50} value of CLB was also observed; however, statistical analysis of data with one-way ANOVA followed by the post-hoc Tukey-Kramer test revealed that the observed reduction from 15.14 (12.63–18.14) mg/kg to 14.35 (11.96–17.21) mg/kg did not attain statistical significance (Figure 1).

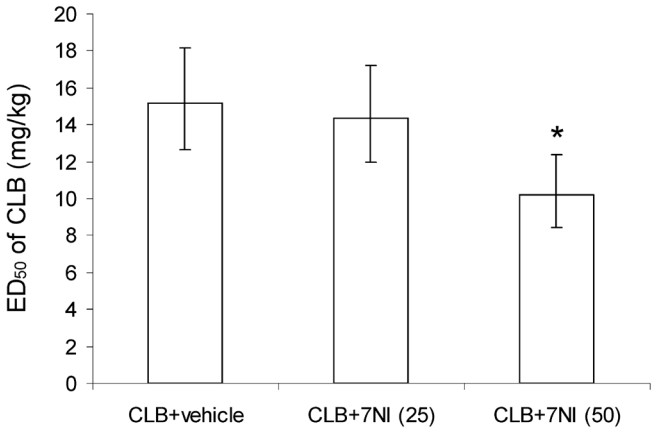

Fig. 1. Effect of 7-nitroindazole (7NI) on the protective activity of clobazam (CLB) against MES-induced seizures in mice

Columns represent median effective doses (ED_{50} in mg/kg with 95% confidence limits as the error bars) of CLB, protecting 50% of animals tested against MES-induced hindlimb extension. CLB and 7NI were administered i.p. at 30 min. prior to the MES test. Statistical analysis of data was performed with one-way ANOVA followed by the post-hoc Tukey-Kramer test for multiple comparisons. *$p<0.05$ vs. the respective control group (CLB + vehicle-treated animals)

EFFECTS OF 7NITROINDAZOLE, N^G-NITRO-L-ARGININE AND THEIR COMBINATION WITH CLOBAZAM ON MOTOR PERFORMANCE, LONG-TERM MEMORY, AND MUSCULAR STRENGTH OF ANIMALS IN THE CHIMNEY, STEP-THROUGH PASSIVE AVOIDANCE AND GRIP-STRENGTH TESTS

When CLB was administered in combination with 7NI (50 mg/kg) or NNA (40 mg/kg), at doses corresponding to its ED_{50} from the MES test, motor performance as assessed by the chimney test was unaffected (Table 1). Furthermore, none of the combinations of CLB with 7NI (50 mg/kg) or NNA (40 mg/kg) impaired long-term memory as determined in the passive avoidance test, the median retention times being 180 s (Table 1). Likewise, CLB combined with 7NI (50 mg/kg) or NNA (40 mg/kg) had no significant impact on muscular strength of animals as assessed by the grip-strength test (Table 1).

DISCUSSION

Results presented herein indicate that 7NI – the preferential nNOS inhibitor enhanced the protective action of CLB, whereas NNA – the non-selective NOS inhibitor had no impact on the anticonvulsant action of CLB in mice subjected to the MES test. Our findings are in agreement with those documenting earlier that 7NI enhanced the anticonvulsant action of some classical and second-generation AEDs in the mouse MES-induced seizure test [4, 11, 13, 15, 16]. Similarly, the lack of effect of NNA on the anticonvulsant action of CLB was consistent with previous reports showing that NNA did not affect the anticonvulsant action of classical and second-generation AEDs in the mouse MES model [12, 13, 16]. The direct comparison of effects produced by 7NI and NNA combined with CLB allowed the evaluation of effect produced by both NOS inhibitors.

Since 7NI potentiated the anticonvulsant action of CLB by reducing its ED_{50} value and NNA as the non-selective NOS inhibitor had no impact on the anticonvulsant action of CLB in the MES test in mice, one could ascertain that modulation of NO content in the brain of experimental animals by NNA had no effect on the anticonvulsant action of CLB. In contrast, 7NI could directly interact with its specific binding sites, independent on NO pathways, contributing to the enhancement of the anticonvulsant action of CLB in the MES test in mice. Recently, there has appeared a hypothesis suggesting that the effects produced by 7NI resulted from the direct effect of 7NI, which was independent on NO content in the brain [11, 15, 23].

Evaluation of acute adverse-effect profile for the combination of CLB with 7NI or NNA revealed that neither 7NI nor NNA altered motor coordination in animals challenged with the chimney test. Similarly, none of the investigated combinations of CLB with 7NI and NNA affected long-term memory in mice in the step-through passive avoidance task as well as altered skeletal muscular strength in mice subjected to the grip-strength test. These findings are also in agreement with the results from our previous studies documenting that the combinations of NNA and 7NI with classical and second-generation AEDs produced no acute adverse effects in the chimney, step-through passive avoidance and grip-strength tests in mice [11–13, 15, 16].

Table 1. Effects of 7-nitroindazole (7NI), NG-nitro-L-arginine (NNA), clobazam (CLB) and their combinations on long-term memory, skeletal muscular strength and motor performance in mice

Treatment (mg/kg)	Retention time (s)	Grip-strength (N)	Motor coordination impairment (%)
Control	180 (180; 180)	108.98 ± 4.58	0
7NI (50) + vehicle	180 (165.8; 180)	106.40 ± 4.87	12.5
CLB (10.23) + vehicle	180 (180; 180)	107.05 ± 4.77	0
CLB (10.23) + 7NI (50)	180 (122.3; 180)	105.33 ± 4.69	12.5
Control	180 (180; 180)	108.98 ± 4.58	0
NNA (40) + vehicle	180 (163.8; 180)	106.44 ± 5.00	12.5
CLB (15.97) + vehicle	180 (180; 180)	104.15 ± 4.79	0
CLB (15.97) + NNA (40)	175 (135.3; 180)	107.11 ± 4.87	25

Results are presented as: 1) median retention times (in seconds; with 25th and 75th percentiles in parentheses) from the passive avoidance task, assessing long-term memory in mice; 2) mean grip-strengths (in Newtons ± SE) from the grip-strength test, assessing muscular strength in mice; and 3) percentage of animals showing motor coordination impairment in the chimney test in mice. Each experimental group consisted of 8 animals. Statistical analysis of data from the passive avoidance task was performed with nonparametric Kruskal-Wallis ANOVA test, whereas those from the grip-strength test were analyzed with one-way ANOVA. The Fisher's exact probability test was used to analyze the results from the chimney test. All drugs were administered i.p. at times scheduled from the MES test and at doses corresponding to the ED50 values against MES-induced seizures

CONCLUSIONS

The combination of 7NI with CLB deserves more clinical attention due to its favorable effects in terms of suppression of MES-induced seizures and lack of any significant acute adverse effects in experimental animals. The combination of NNA with CLB seems to be neutral from a preclinical point of view, because NNA had no impact on the protective activity of CLB against MES-induced seizures and NNA did not exert any acute adverse effects in mice. If the results from this study could be extrapolated into clinical settings and additionally confirmed in different various experimental models of epilepsy, the combination of 7NI with CLB would occur favorable for epileptic patients as a novel treatment option in refractory epilepsy.

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Disclosure of conflicts of interest. The authors have no disclosures to declare.

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SUMMARY

The aim of this study was to determine the effect of 7-nitroindazole (7NI – a preferential neuronal nitric oxide synthase [NOS] inhibitor) and N^G-nitro-L-arginine (NNA – a non-selective NOS inhibitor) on the anticonvulsant action of clobazam (CLB – a second-generation antiepileptic drug) in the maximal electroshock (MES)-induced seizure model in mice. Electroconvulsions were produced in adult male Albino Swiss mice by means of an alternating current (50Hz, 500V, 25mA, ear-clip electrodes, 0.2s stimulus duration). The anticonvulsant action of CLB in the MES test was expressed as median effective doses (ED_{50} values) of the drug, protecting 50% of animals tested against MES-induced seizures. The acute adverse-effect potentials of CLB in combination with 7NI and NNA were evaluated in the chimney test (motor coordination), passive avoidance task (long-term memory), and grip-strength test (skeletal muscular strength) in mice. Results indicate that 7NI (50 mg/kg; i.p.) significantly enhanced the anticonvulsant action of CLB by reducing its ED_{50} value from 15.14 mg/kg to 10.23 mg/kg ($p<0.05$). Similarly, 7NI at the lower dose of 25 mg/kg also potentiated the anticonvulsant action of CLB, although the results did not attain statistical significance. In contrast, NNA (40 mg/kg; i.p.) had no impact on the anticonvulsant effect of CLB. Moreover, none of the examined combinations of CLB with 7NI and NNA affected motor coordination, long-term memory, and skeletal muscular strength in mice. Based on this preclinical study, one can conclude that 7NI significantly enhanced, whereas NNA had no effect on the anticonvulsant activity of CLB against MES-induced seizures in mice.

Keywords: 7-Nitroindazole, N^G-nitro-L-arginine, clobazam, nitric oxide, maximal electroshock seizure test

STRESZCZENIE

Celem pracy była ocena wpływu 7-nitroindazolu (7NI – preferencyjnego inhibitora neuronalnej syntazy tlenku azotu [NOS]) i N^G-nitro-L-argininy (NNA – nieselektywnego inhibitora NOS) na przeciwdrgawkowe działanie klobazamu (CLB – leku przeciwpadaczkowego drugiej generacji) w modelu maksymalnego wstrząsu elektrycznego (MES) u myszy. Drgawki elektryczne były wywoływane u dorosłych samców myszy Albino Swiss poprzez prąd zmienny (50Hz, 500V, 25mA, elektrody uszne, 0,2s czas trwania impulsu). Przeciwdrgawkowe działanie CLB w teście MES wyrażono jako mediany dawek skutecznych (wartości ED_{50}) leku, chroniące 50% zwierząt przed drgawkami wywoływanymi elektrycznie. Ostre potencjalne działania niepożądane CLB w kombinacji z 7NI i NNA oceniono w teście komina (koordynacja ruchowa), teście biernego unikania (pamięć długotrwała) i teście chwytania (siła mięśni szkieletowych) u myszy. Wyniki wskazują, że 7NI (50 mg/kg; i.p.) istotnie nasilał przeciwdrgawkowe działanie CLB poprzez zmniejszenie jego wartości ED_{50} z 15,14 mg/kg do 10,23 mg/kg ($p<0,05$). Podobnie 7NI w niższej dawce 25 mg/kg nasilał przeciwdrgawkową aktywność CLB, chociaż wyniki nie uzyskały istotności statystycznej. Przeciwnie, NNA (40 mg/kg; i.p.) nie miała żadnego wpływu na przeciwdrgawkowe działanie CLB. Ponadto żadna z badanych kombinacji CLB z 7NI i NNA nie wpływała na koordynację ruchową, pamięć długotrwałą i siłę mięśni szkieletowych u myszy. W oparciu o to badanie przedkliniczne można stwierdzić, że 7NI istotnie nasilał, podczas gdy NNA nie miała wpływu na przeciwdrgawkowe działanie CLB w teście MES u myszy.

Słowa kluczowe: 7-Nitroindazol, N^G-nitro-L-arginina, klobazam, tlenek azotu, maksymalny wstrząs elektryczny

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Effect of osthole on the protective activity of carbamazepine and phenobarbital against maximal electroshock-induced seizures in mice

Wpływ ostolu na ochronne działanie karbamazepiny i fenobarbitalu w teście maksymalnego wstrząsu elektrycznego u myszy

INTRODUCTION

Osthole [7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one] – a natural coumarin derivative is extracted from many medicinal plants, such as *Angelica pubescens, Cnidium monnieri* and *Peucedanum ostruthium* [2, 3, 5, 6, 24, 28]. Previous experimental studies have shown that osthole exerts a broad spectrum of pharmacological activities due to its anti-platelet [8], anti-tumor [35], anti-allergic [4, 22], anti-apoptotic [26], anti-oxidative [27, 30, 31], anti-proliferative [5, 7], estrogen-like [9], anti-osteoporotic [9, 10, 33], hepatoprotective [34], antidiabetic [11], and anti-inflammatory [25] effects. In addition, osthole is a potential antioxidant eliminating oxygen free radicals and inhibiting lipid peroxidation [27, 30, 31].

Accumulating evidence indicates that imperatorin [9-(3-methylbut-2-enyloxy)-7H-furo[3,2-g] chromen-7-one] – another natural coumarin derivative, possesses the anticonvulsant activity in preclinical studies by elevating the threshold for electroconvulsions [17] and enhancing the anticonvulsant action of carbamazepine, phenobarbital, phenytoin [16], and lamotrigine [21] in the mouse maximal electroshock-induced seizure model. Moreover, it has been found that both natural coumarin derivatives (imperatorin and osthole) exerted a clear-cut anticonvulsant activity against maximal electroshock-induced seizures in mice [20].

Since osthole and imperatorin exerted the antielectroshock action in mice when administered alone, and imperatorin potentiated the anticonvulsant action of some classical antiepileptic drugs, it was of importance to determine whether osthole enhances the anticonvulsant action of carbamazepine and phenobarbital in the mouse maximal electroshock-induced seizure model. It is widely accepted that the maximal electroshock seizure test is considered as an experimental model of tonic-clonic seizures and, to a certain extent, of partial seizures with or without secondary generalization [13]. Moreover, this experimental model of epilepsy is widely used for an investigation of the new drugs and for selection of the agents with antiseizure activity in vivo [13]. In this model one can readily evaluate the anticonvulsant effects produced by classical antiepileptic drugs in combination with osthole, therefore, it was appropriate to use this test in the present study.

Additionally, we investigated the combinations of osthole with classical antiepileptic drugs in relation to impairment of motor coordination, long-term memory and muscular strength by the use of the chimney test, step-through passive avoidance task and grip-strength test, respectively.

MATERIALS AND METHODS

A n I m a l s a n d e x p e r i m e n t a l . conditions. Adult male Swiss mice (weighing 22 – 26 g) that were kept in colony cages with free access to food and tap water, under standardized housing conditions (natural light-dark cycle, temperature of 23 ± 1ºC, relative humidity of 55 ± 5%), were used. After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups comprising of 8 mice. Each mouse was used only once and all tests were performed between 08.00 and 15.00 hours. Procedures involving animals and their care were conducted in accordance with current European Community and Polish legislation on animal experimentation. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. The experimental protocols and procedures described in this manuscript were approved by the Second Local Ethics Committee at the University of Life Sciences in Lublin (License no. 78/2009) and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

D r u g s . The following antiepileptic drugs were used in this study: carbamazepine (a gift from Polfa, Starogard, Poland) and phenobarbital (Polfa, Krakow, Poland). All drugs were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water and administered intraperitoneally (i.p.) as a single injection, in a volume of 5 ml/kg body wt. Fresh drug solutions were prepared on each day of experimentation and administered as follows: phenobarbital - 60 min and carbamazepine - 30 min, before electroconvulsions, motor coordination, grip-strength and long-term memory tests. The pretreatment times before testing of the antiepileptic drugs were based upon information about their biological activity from the literature and our previous experiments [14-16]. The times to the peak of maximum anticonvulsant effects for all antiepileptic drugs were used as the reference times in all behavioral tests. Osthole ([7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one]; $C_{15}H_{16}O_3$; 244.29 MW; chemical purity >97%) was extracted from roots of *Peucedanum ostruthium* (L.) Koch, which were collected from plants in September 2007, in Karpacz Gorny (Sudetes, Poland). The air-dried and powdered roots (920 g) were extracted exhaustively

(~120 h) in the Soxhlet extractor with petroleum ether. After extraction and cooling procedure osthole was crystallized and drained off. The petroleum ether extract was concentrated in a rotary vacuum evaporator. The remains were dissolved in methanol and left for final osthole precipitation (40 g of remains were dissolved in 200 ml of boiling methanol). Osthole obtained from the methanol extract was added to that from petroleum ether and recrystallized by obtaining 10 g of pure osthole. The identity of osthole was confirmed by TLC and ^{1}H-NMR analyses. Osthole was suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water and administered i.p. at 30 min before the initiation of electroconvulsions and behavioural tests.

Maximal electroconvulsions. Electroconvulsions were produced by means of an alternating current (0.2 s stimulus duration, 50 Hz, maximum stimulation voltage of 500 V) delivered via ear-clip electrodes by a Rodent Shocker generator (Type 221, Hugo Sachs Elektronik, Freiburg, Germany). The electrical system of the stimulator was self-adjustable so that changes in impedance did not result in alterations of current intensity (i.e., the system provides constant current stimulation). The criterion for the occurrence of seizure activity was the tonic hind limb extension (i.e., the hind limbs of animals outstretched 180° to the plane of the body axis). In this experiment, two experimental models of maximal electroconvulsions were used: 1) maximal electroshock seizure threshold test and 2) maximal electroshock seizure test.

Maximal electroshock seizure threshold test. To evaluate the threshold for maximal electroconvulsions, at least 4 groups of mice, consisting of 8 animals per group, were challenged with electroshocks of various intensities to yield 10 – 30%, 30 – 50%, 50 – 70%, and 70 – 90% of animals with seizures. Then, a current intensity-response relationship curve was constructed, according to a log-probit method by Litchfield and Wilcoxon [12], from which a median current strength (CS_{50} in mA) was calculated. Each CS_{50} value represents the current intensity required to induce tonic hindlimb extension in 50% of the mice challenged. Again, after administration of a single dose of osthole to 4 groups of animals, the mice were subjected to electroconvulsions (each group with a constant current intensity). The threshold for maximal electroconvulsions was recorded for 4 different doses of osthole: 50, 100, 150 and 200 mg/kg. The experimental procedure has been described in more detail in our earlier studies [14-16, 18, 20, 21].

Maximal electroshock seizure test. The protective activity of two classical antiepileptic drugs (carbamazepine and phenobarbital) was determined as their median effective doses (ED_{50} values in mg/kg) against maximal electroshock-induced seizures (fixed current intensity of 25 mA). The animals were administered with different drug doses so as to obtain a variable percentage of protection against maximal electroshock seizures, allowing the construction of a dose-response relationship curve for each antiepileptic drug administered alone, according to Litchfield and Wilcoxon [12]. Each ED_{50} value represents the dose of a drug required to protect half of the animals tested against maximal electroshock seizures. Similarly, the anticonvulsant activity of a mixture of an antiepileptic drug with osthole was evaluated and expressed as ED_{50} corresponding to the dose of an antiepileptic drug necessary to protect 50% of mice against tonic hindlimb extension in the maximal electroshock seizure test. In the present study, carbamazepine was administered at doses ranging between 4–12 mg/kg and phenobarbital at doses ranging between 10–25 mg/kg. This experimental procedure has been described in detail in our earlier studies [14-16, 18, 20, 21].

Chimney test. The chimney test of Boissier et al. [1] was used to quantify the adverse effect potential of classical antiepileptic drugs administered in combination with osthole on motor performance in mice. In this test, the animals had to climb backwards up a plastic tube (3 cm inner diameter, 25 cm length), and motor performance impairment was indicated by the inability of the mice to climb backward up the transparent tube within 60 s. The acute adverse effect potentials of classical antiepileptic drugs administered in combination with osthole were determined for antiepileptic drugs administered at doses corresponding to their ED_{50} values from the maximal electroshock seizure test when combined with osthole at a dose of 200 mg/kg. This experimental procedure has been described in detail in our earlier studies [14, 16, 18-21].

Grip – strength test. The effects of combinations of osthole with classical antiepileptic drugs at their ED_{50} values from the maximal electroshock seizure test, on skeletal muscular strength in mice were quantified by the grip-strength test of Meyer et al. [23]. The time before the commencement of the grip-strength test (after drug administration) was identical to that for the maximal electroshock seizure test. The grip-strength apparatus (BioSeb, Chaville, France) comprised a wire grid (8 x 8 cm) connected to an isometric force transducer (dynamometer). The mice were lifted by the tails so that their forepaws could grasp the grid. The mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The mean of 3 measurements for each animal was calculated and subsequently, the mean maximal force of 8 animals per group was determined. The neuromuscular strength in mice was expressed in Newtons (N) as means ± S.E. of at least 8 determinations. This experimental procedure has been described in detail in our earlier study [32].

Light – dark, step – through passive avoidance task. Each animal was administered an antiepileptic drug either singly or in combination with osthole on the first day before training. The time before the commencement of the training session (after drug administration) was identical to that for the maximal electroshock seizure test. Subsequently, animals were placed in an illuminated box (10 x 13 x 15 cm) connected to a larger dark box (25 x 20 x 15) equipped with an electric grid floor. Entrance of animals to the dark box was punished by an adequate electric footshock (0.6 mA for 2 s). The animals that did not enter the dark compartment were excluded from subsequent experimentation. On the following day (24 h later), the pre-trained animals were placed again into the illuminated box and observed up to 180 s. Mice that avoided the dark compartment for 180 s were considered to remember the task. The time that the mice took to enter the dark box, was noted and the median latencies (retention times) with 25th and 75th percentiles were calculated. The step-through passive avoidance task gives information about ability to acquire the task (learning) and to recall the task (retrieval). Therefore, it may be regarded as a measure of long-term memory [29]. This experimental procedure has been described in detail in our earlier study [19].

Statistics. Both, CS_{50} and ED_{50} values with their 95% confidence limits were calculated by computer log-probit analysis according to Litchfield and Wilcoxon [12]. Subsequently, the respective 95% confidence limits were transformed to standard errors (S.E.), as published earlier [14]. Statistical analysis of data from the electroconvulsive tests was performed with one-way analysis of variance (ANOVA) followed by the post-hoc Tukey/Kramer test for multiple comparisons. Qualitative variables from the chimney test were compared by use of the Fisher's exact probability test, whereas, the results obtained in the passive avoidance task were statistically evaluated using Kruskal-Wallis nonparametric ANOVA. The results from the grip-

strength test were verified with one-way ANOVA. All statistical tests were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Differences among values were considered statistically significant if $p<0.05$.

RESULTS

INFLUENCE OF OSTHOLE UPON THE THRESHOLD FOR ELECTROCONVULSIONS

Osthole (administered alone, i.p., 30 min prior to the test) dose-dependently raised the CS_{50} values, necessary to produce tonic hindlimb extension in 50% of animals. In this case, osthole at a dose of 200 mg/kg significantly elevated the CS_{50} value from 6.40 mA to 9.05 mA (by 41%; $P<0.01$; Table 1). In contrast, the CS_{50} values for osthole, administered at doses of 50, 100 and 150 mg/kg, did not reach statistical significance, although a slight (dose-dependent) increase in the CS_{50} values was observed in the maximal electroshock seizure threshold test in mice (Table 1).

Table 1. Effect of osthole on the threshold for maximal electroconvulsions in mice

Treatment (mg/kg)	CS_{50} (mA)	n
Vehicle	6.40 ± 0.47	16
Osthole (50)	6.51 ± 0.41	24
Osthole (100)	6.79 ± 0.37	16
Osthole (150)	7.21 ± 0.36	32
Osthole (200)	9.05 ± 0.45 **	16
F (4; 99) = 5.446; P = 0.0005		

Results are presented as median current strengths (CS_{50} in mA ± S.E.) required to produce tonic hindlimb extension in 50% of animals tested. The CS_{50} values were calculated using the log-probit method [12], followed by the method transforming 95% confidence limits to S.E. [14]. Osthole was administered i.p., at 30 min before maximal electroconvulsions. Statistical analysis of data was performed with one-way ANOVA followed by the post-hoc Tukey/Kramer test for multiple comparisons. n – number of animals at those current strengths, whose convulsant effects ranged between 16% and 84%; F – F-statistics from one-way ANOVA; P – probability value from one-way ANOVA; **p<0.01 vs. control group (vehicle-treated animals)

EFFECTS OF OSTHOLE ON THE PROTECTIVE ACTION OF CARBAMAZEPINE AND PHENOBARBITAL IN THE MOUSE MAXIMAL ELECTROSHOCK-INDUCED SEIZURE MODEL

The investigated classical antiepileptic drugs (carbamazepine and phenobarbital) administered alone exhibited a clear-cut anticonvulsant activity in the maximal electroshock seizure test in mice and their ED_{50} values are presented in Table 2. When osthole at doses of 50, 100 and 150 mg/kg was co-administered with carbamazepine, it did not significantly enhance the anticonvulsant effect of the latter drug against maximal electroshock-induced seizures (Table 2). In the case of phenobarbital,

osthole at doses of 50, 100 and 150 mg/kg did not significantly affect the antielectroshock action of phenobarbital in the mouse maximal electroshock-induced seizure model (Table 2). In all cases, the osthole-induced reduction in ED_{50} values of classical antiepileptic drugs did not attain statistical significance with one-way ANOVA (Table 2).

Table 2. Effect of osthole on the protective activity of carbamazepine and phenobarbital against maximal electroshock-induced seizures in mice

Treatment (mg/kg)	ED_{50} (mg/kg)	n
Carbamazepine + vehicle	8.87 ± 0.87	16
Carbamazepine + osthole (50)	8.18 ± 0.80	16
Carbamazepine + osthole (100)	7.65 ± 0.84	8
Carbamazepine + osthole (150)	6.89 ± 0.71	32
F (3; 68) = 1.210; P = 0.3127		
Phenobarbital + vehicle	18.17 ± 1.80	16
Phenobarbital + osthole (50)	17.72 ± 1.83	24
Phenobarbital + osthole (100)	15.31 ± 2.04	16
Phenobarbital + osthole (150)	12.35 ± 2.32	24
F (3; 76) = 1.792; P = 0.1558		

Results are presented as median effective doses (ED_{50} in mg/kg ± S.E.) of antiepileptic drugs, protecting 50% of animals tested against maximal electroshock-induced hindlimb extension. The antiepileptic drugs were administered i.p.: phenobarbital – 60 min., and carbamazepine – 30 min. prior to the maximal electroshock-induced seizure test. Osthole was administered i.p. at 30 min. before electroconvulsions. Statistical analysis of data was performed with one-way ANOVA followed by the post-hoc Tukey-Kramer test for multiple comparisons. n – total number of animals used at those doses whose anticonvulsant effects ranged between 4 and 6 probits. F – F-statistics from one-way ANOVA; P – probability value from one-way ANOVA.

EFFECTS OF OSTHOLE IN COMBINATION WITH CARBAMAZEPINE AND PHENOBARBITAL ON MOTOR PERFORMANCE, LONG-TERM MEMORY, AND MUSCULAR STRENGTH OF ANIMALS IN THE CHIMNEY, STEP-THROUGH PASSIVE AVOIDANCE AND GRIP-STRENGTH TESTS

When osthole was administered in combination with carbamazepine and phenobarbital at doses corresponding to their ED_{50}s from the maximal electroshock seizure test, motor performance as assessed by the chimney test was unaffected (Table 3). Furthermore, none of the combinations studied impaired long-term memory as determined in the passive avoidance test, the median retention times being approximately 180 s (Table 3). Likewise, osthole combined with two classical antiepileptic drugs had no significant impact on muscular strength of animals as assessed by the grip-strength test (Table 3).

Table 3. Effects of osthole and its combinations with carbamazepine and phenobarbital on long-term memory, muscular strength and motor performance in mice

Treatment (mg/kg)	Retention time (s)	Grip-strength (N)	Impairment of motor coordination (%)
Control	180 (180; 180)	104.38 ± 4.88	0
Osthole (150) + vehicle	180 (180; 180)	98.50 ± 4.27	12.5
Carbamazepine (6.9) + vehicle	180 (180; 180)	105.15 ± 4.59	0
Carbamazepine (6.9) + osthole (150)	180 (180; 180)	99.69 ± 4.47	0
Phenobarbital (12.4) + vehicle	180 (180; 180)	102.31 ± 4.68	0
Phenobarbital (12.4) + osthole (150)	180 (180; 180)	100.25 ± 4.30	0

Results are presented as: 1) median retention times (in seconds; with 25th and 75th percentiles in parentheses) from the passive avoidance task, assessing long-term memory in mice; 2) mean grip-strengths (in Newtons ± S.E.) from the grip-strength test, assessing muscular strength in mice; and 3) percentage of animals showing motor coordination impairment in the chimney test in mice. Statistical analysis of data from the passive avoidance task was performed with nonparametric Kruskal-Wallis ANOVA test, whereas those from the grip-strength test were analyzed with one-way ANOVA. The Fisher's exact probability test was used to analyze the results from the chimney test. All drugs were administered i.p. at times scheduled from the maximal electroshock seizure test and at doses corresponding to their ED50 values against maximal electroconvulsions (for more details see the legend to Table 2)

DISCUSSION

Results presented in this study indicate that osthole dose-dependently increased the threshold for electroconvulsions in mice. The natural compound administered systemically (i.p.) at a dose of 200 mg/kg significantly elevated the threshold for electroconvulsions in mice. It has been reported that osthole at doses ranging between 50 and 150 mg/kg also increased the threshold, although statistical analysis of data revealed no significance between the threshold in mice receiving osthole as compared to control animals. This finding is consistent with our previous results reporting that osthole administered i.p. exerted a clear-cut anticonvulsant action in the mouse maximal electroshock-induced seizure model [20].

Moreover, we found that osthole administered systemically (i.p.) at doses up to 150 mg/kg did not significantly affect the anticonvulsant action of two classical antiepileptic drugs (carbamazepine and phenobarbital) in the mouse maximal electroshock-induced seizure model. In contrast, imperatorin (a natural coumarin derivative) possessed a clear-cut anticonvulsant action and enhanced the anticonvulsant action of carbamazepine, phenobarbital, phenytoin and lamotrigine, but not that of valproate in the mouse maximal electroshock-induced seizure model [16, 21].

As regards the acute adverse-effect potentials of osthole in combination with classical antiepileptic drugs, one can conclude that osthole, similarly to imperatorin, did not affect long-term memory, motor performance or muscular strength in mice. Thus, one can suggest that the combined therapy of osthole with classical antiepileptic drugs is devoid of any acute side effects. Previously, we have reported that WIN 55,212-2 mesylate (WIN – a synthetic cannabinoid CB1 and CB2 receptor agonist) significantly impaired long-term memory in animals receiving WIN in combination with classical antiepileptic drugs [15]. Moreover, WIN disturbed muscular strength in mice receiving the combination of WIN with classical antiepileptic drugs [15]. Additionally, we have reported that the combination of tiagabine with valproate significantly impaired motor coordination in mice challenged with the chimney test [18]. The above-mentioned facts suggest that all behavioral tests applied in this study (chimney, passive avoidance and grip-strength tests) were sensitive enough to detect any changes in normal behavior in mice. Thus, one can ascertain that since osthole in combination with classical antiepileptic drugs is devoid of any acute adverse effects in animals, the further therapy based on these drugs might be safe and tolerable by patients. However, more advanced studies are required to establish the influence of osthole on seizure activity in various experimental models of epilepsy. It is noteworthy that osthole was tested in the present study at doses up to 200 mg/kg, whereas the ED_{50} value for this compound, as determined in the mouse maximal electroshock-induced seizure model was 253 mg/kg [20].

CONCLUSIONS

The results presented herein indicate that osthole can be used as a supplement of diet in epileptic patients. However, the coumarin derivatives exerted no significant enhancement of the anticonvulsant action of carbamazepine and phenobarbital in the mouse maximal electroshock-induced seizure model. Perhaps, the combinations of osthole with these classical antiepileptic drugs could be

advantageous in other epilepsy models, however, to confirm this hypothesis more advanced studies are required in various experimental models.

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SUMMARY

The aim of this study was to determine the effect of osthole on the anticonvulsant activity of two classical antiepileptic drugs (carbamazepine and phenobarbital) in the mouse maximal electroshock seizure model. Electroconvulsions were evoked in adult Albino Swiss mice by a current (50Hz, 500V, 0.2s stimulus duration) delivered via auricular electrodes. Acute adverse-effect profiles of the combination of osthole with carbamazepine and phenobarbital were measured in the chimney test (motor performance), passive avoidance task (long-term memory) and grip-strength test (skeletal muscular strength) in mice. Results indicate that osthole administered intraperitoneally (i.p.) at a dose of 200 mg/kg significantly elevated (by 41%; $p<0.01$) the threshold for electroconvulsions in mice. Osthole at lower doses of 50, 100 and 150 mg/kg had no significant impact on the threshold for electroconvulsions in mice. Osthole (50, 100 and 150 mg/kg, i.p.) did not significantly affect the protective action of carbamazepine and phenobarbital in the maximal electroshock-induced seizures in mice. Moreover, osthole in combination with carbamazepine and phenobarbital did not alter motor performance, long-term memory or skeletal muscular strength in experimental animals. The present study demonstrates that osthole, although elevated the threshold for electroconvulsions, had no significant effect on the anticonvulsant action of carbamazepine and phenobarbital in the mouse maximal electroshock-induced seizure model.

Keywords: Osthole, carbamazepine, phenobarbital, maximal electroshock seizure test

STRESZCZENIE

Celem pracy była ocena wpływu ostolu na przeciwdrgawkowe działanie dwóch klasycznych leków przeciwpadaczkowych (karbamazepiny i fenobarbitalu) w modelu maksymalnego wstrząsu elektrycznego u myszy. Drgawki elektryczne były wywoływane u dorosłych samców myszy Albino Swiss prądem (50Hz, 500V, 0,2s czas trwania impulsu) przez elektrody uszne. Profil ostrych działań niepożądanych kombinacji ostolu z karbamazepiną i fenobarbitalem oceniono w teście komina (koordynacja ruchowa), teście biernego unikania (pamięć długotrwała) i teście chwytania (siła

mięśni szkieletowych) u myszy. Wyniki wskazują, że ostol podany dootrzewnowo (i.p.) w dawce 200 mg/kg istotnie podnosił (o 41%; $p<0,01$) próg pobudliwości drgawkowej. Ostol w niższych dawkach 50, 100 i 150 mg/kg nie miał istotnego wpływu na próg dla drgawek elektrycznych u myszy. Ostol (50, 100 i 150 mg/kg; i.p.) nie wpływał istotnie na ochronne działanie karbamazepiny i fenobarbitalu w teście maksymalnego wstrząsu elektrycznego. Ponadto ostol w kombinacji z karbamazepiną i fenobarbitalem nie zmieniał koordynacji ruchową, pamięci długotrwałej i siły mięśni szkieletowych u badanych zwierząt. Bieżące badanie wykazało, że ostol chociaż podnosił próg pobudliwości drgawkowej, to nie miał istotnego wpływu na przeciwdrgawkowe działanie karbamazepiny i fenobarbitalu w modelu maksymalnego wstrząsu elektrycznego u myszy.

Słowa kluczowe: Ostol, karbamazepina, fenobarbital, maksymalny wstrząs elektryczny

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Experimental correction of pathological changes in salivary glands by multiprobiotic Symbiter® acidofilus under conditions of hypergastrinemia

Doświadczalna korekcja zmian patologicznych gruczołów ślinowych pod wpływem multiprobiotyku *Symbiter® acidofilus* w warunkach hipergastrynemii

INTRODUCTION

It is known that a longterm decrease of gastric secretion leads to the development of hypergastrinemia and to pathological changes in digestive organs. There is very important research of the ways to correct these undesirable consequences. Application of probiotics is one of such ways. In complex treatment of acid-dependent diseases of digestive organs probiotics are used. The usage of probiotics not only corrects infringements of digestive path microecology, but also positively influences the activity of immune and endocrine systems. Probiotic *Symbiter® Acidofilus* has polycomponent composition. Drug contains from 14 to 25 varieties of physiologically valuable microorganisms with different biological activity.

MATERIAL AND METHODS

The purpose of our research was substantiation of experimental efficiency of multiprobiotic *Symbiter® Acidofilus* for the correction of pathological changes in tissues of salivary glands tissues under conditions of hypergastrinemia. Experiments were carried out on 71 rat-males of "Wistar" line, weight 180–250g. Animals obtained omeprasole for 28 days (14 mg/kg of weight) and multiprobiotic *Symbiter® Acidofilus* (0.14 ml/kg of weight) together and separately. Development of hypergastrinemia verified by the maintenance of gastrin in blood plasma of rats (59.0±35.5 pg/ml – control group, in comparison with experimental animals – 170.7±90.7 pg/ml). In the homogenate of salivary glands we determined the activity of ornithinedecarboxylase (ODC), б-amylase, general proteinases, NO-ergic system and the maintenance of nitrites, molecules of average weight, oxidative modified proteins and ingibitors of the general proteinases.

RESULTS

Under conditions of long omeprazole introduction pathological changes in salivary glands tissues appear: intensification of free-radical oxidation, disbalance of proteolysis by decompensated type, increased activity of б-amilase, disbalance of polyamines and NO-ergic systems. We determined that the activity of NO-ergic system under conditions of correction was 1.18 times greater, than without correction, and the maintenance of nitrites – 1.02 times. Also correction of the hypergastrinemia by multiprobiotic *Symbiter® Acidofilus* led to the increase of the ODC activity 1.2 times more, б-amylase 1.08 times more and to the decrease of the maintenance of molecules of average weight 1.11 times, oxidative modified proteins 1.08 times and to the increase of the inhibitors of the general proteinases maintenance 1.06 times more.

Table 1. Metabolic changes in tissues of salivary glands under conditions of hypergastrinemia and its corrections by probiotic *Symbiter® Acidofilus*,(M ± m)

Groups of Animals / Methods	Omeprasole group	Omeprasole & Symbiter® Acidofilus group	Symbiter® Acidofilus group	Control group	
The activity of NO-synthase, мmol/g*min	5.76±0.25	6.77±0.15	3.90±0.08	3.97±0.11	P_{4-1}<0.05 P_{4-2}<0.05 P_{4-3}>0.05 P_{1-2}<0.05
The activity of ornithinedecarboxylase, nmol/g*min	244.5±10.5	293.9±8.3	269.7±9.7	269.0±8.0	P_{4-1}>0.05 P_{4-2}<0.05 P_{4-3}>0.05 P_{1-2}<0.05
The activity of general proteinases, мmol/g*min	0.383±0.018	0.391±0.008	0.332±0.006	0.327±0.01	P_{4-1}<0.05 P_{4-2}<0.05 P_{4-3}>0.05 P_{1-2}>0.05
The activity of б-amylase, mg/g*min	81.8±2.2	88.4±1.3	73.9±1.5	72.0±2.0	P_{4-1}<0.05 P_{4-2}<0.05 P_{4-3}>0.05 P_{1-2}<0.05
Maintenance of the average weight molecules, c.u.	0.321±0.024	0.290±0.012	0.228±0.009	0.243±0.016	P_{4-1}<0.05 P_{4-2}<0.05 P_{4-3}>0.05 P_{1-2}>0.05
Maintenance of the oxidative modified proteins, c.u.	0.484±0.023	0.449±0.012	0.338±0.017	0.363±0.026	P_{4-1}<0.05 P_{4-2}<0.05 P_{4-3}>0.05 P_{1-2}>0.05
Maintenance of the general proteinases ingibitors, g/kg	39.9±0.43	42.4±0.40	47.9±0.8	45.7±0.46	P_{4-1}<0.05 P_{4-2}<0.05 P_{4-3}>0.05 P_{1-2}<0.05
Maintenance of the nitrites, мmol/g	0.194±0.006	0.198±0.003	0.164±0.004	0.164±0.007	P_{4-1}<0.05 P_{4-2}<0.05 P_{4-3}>0.05 P_{1-2}>0.05

DISCUSSION

Nowdays researchers pay great attention to NO role in the development of many diseases. Therefore, research of the NO-ergic system of salivary glands under conditions of the hypergastrinemia is justified. Under conditions of long omeprazole introduction pathological changes in salivary glands tissues appear: increase in the activity of NO-synthase and the amount of nitrite-anion – a possible substrate for NO synthesis. Cell growth and differentiation require the presence of optimal concentrations of polyamines. ODC catalyses are the first and rate-controlling step in polyamine synthesis. In studies using cultures of Ehrlich ascites-tumour cells, we showed that the expression of ODC is subject to feedback regulation by the polyamines. Decrease of polyamine concentration in the cell results in a compensatory increase of ODC synthesis, whereas an increase of polyamine concentration results in suppression of ODC synthesis. These changes in ODC synthesis were attributed to changes in the efficiency of ODC mRNA translation, because the steady-state amount of ODC mRNA remained constant. Under conditions of long omeprazole introduction pathological changes in salivary glands tissues appear: disbalance of polyamines and NO-ergic systems. The use of multiprobiotic *Symbiter® Acidofilus* normalises the balance of the polyamines and NO-ergic systems.

Most reactive oxygen species (ROS) in living organisms are produced as byproducts of many processes. Being highly active, ROS interact with virtually all cellular components particularly modifying their properties. Functional consequences of protein modification by ROS depend on the nature of ROS and protein as well as particular conditions of their interaction. The process in which a protein is broken down, partially into peptides, or, completely, into amino acids, by proteolytic enzymes, is present in bacteria and in plants but most abundant in animals. Food proteins are attacked in the stomach by pepsin and in the small intestine mainly by trypsin and chymotrypsin from the pancreas. Proteolytic enzymes are secreted as zymogens, which are themselves converted by proteolysis to their active forms. Many other zymogens or precursors undergo proteolysis to form active enzymes or proteins (e.g. fibrinogen to fibrin). In cells, proteolytic degradation of old proteins is part of cellular homeostasis. Under conditions of long omeprazole introduction pathological changes in salivary glands tissues appear: intensification of free-radical oxidation, disbalance of proteolysis by decompensated type and increased activity of б-amilase. Experimental correction by multiprobiotic *Symbiter® Acidophilus* promotes normalisation of pathological changes in salivary glands of rats during long introduction proton pomp inhibitor because of free-radical oxidation and proteolytic processes are depressed.

CONCLUSIONS

Under conditions of long hypergastrinemia pathological changes in salivary glands tissues appear: intensification of free-radical oxidation, disbalance of proteolysis by decompensated type, increased activity of б-amilaze, disbalance of polyamines and NO-ergic systems. Correction of omeprazole-inducted hypergastrinemia using multiprobiotic *Symbiter® Acidofilus* normalises synthesis of regulatory polyamines, NO, б-amylase, proteolysis and reduces of the free-radical processes.

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SUMMARY

Under conditions of long omeprazole introduction pathological changes in salivary glands tissues appear: intensification of free-radical oxidation, disbalance of proteolysis by decompensated type, increased activity of б-amilase, disbalance of polyamines and NO-ergic systems. Experimental correction by multiprobiotic *Symbiter® Acidophilus* promotes normalisation of pathological changes in salivary glands of rats during long introduction of proton pomp inhibitor because free-radical oxidation and proteolytic processes are depressed, normalises balance of the polyamines and NO-ergic systems.

Keywords: salivary glands, omeprazole, hypergastrinemia, polyamines, NO-ergic systems, *Symbiter® Acidophilus*

STRESZCZENIE

W warunkach długotrwałego stosowania omeprazolu dochodzi do rozwoju zmian patologicznych w obrębie gruczołów ślinowych: intensyfikacji utleniania wolnych rodników, zaburzeń równowagi proteolizy typy dekompensacyjnego, wzrostu aktywności 6-amylazy, zaburzenia równowagi systemów poliamin I NO-ergicznego. Doświadczalna korekcja z zastosowaniem *Symbiter® Acidophilus* promuje normalizację zmian patologicznych w gruczołach ślinowych szczurów podczas długotrwałego stosowania inhibitora pompy protonowej, ponieważ utlenianie wolnych rodników i procesy proteolityczne są hamowane i normalizowana jest równowaga systemów poliamid i NO-ergicznego.

Słowa kluczowe: gruczoły ślinowe, omeprazol, hipergastrynemia, poliamidy, system NO-ergiczny, *Symbiter® Acidophilus*

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Synthesis of novel fused thiopyrano[2,3-d]thiazole derivatives as potential anticancer agents

Synteza nowych pochodnych tiopirano[2,3-d]tiazolu jako potencjalnych leków przeciwnowotworowych

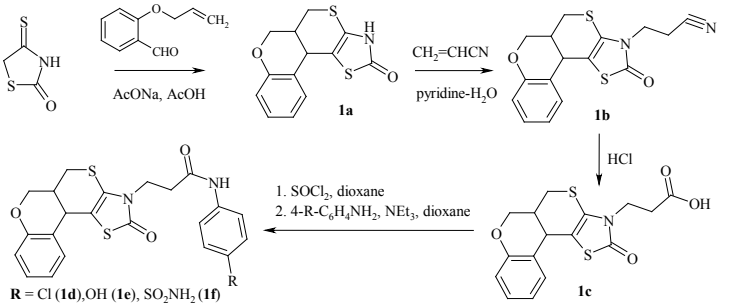
INTRODUCTION

Biological studies of thiopyrano[2,3-*d*]thiazole derivatives, which mimic some biophore fragments of 5-ylidene-4-thiazolidinones [2, 4, 5, 7], allowed us to confirm our previous hypothesis about the development of pharmacological activity of the mentioned heterocyclic systems, and this served as a basis for the synthesis of compounds rows with high biological potential [1, 8]. The fact of presented biological activity gives us reasons for proceeding in synthesis of new fused heterocycles based on 4-thiazolidinone derivatives, exactly chromeno[4',3':4,5]thiopyrano[2,3-*d*] thiazole derivatives as potential anticancer agents.

MATERIAL AND METHODS

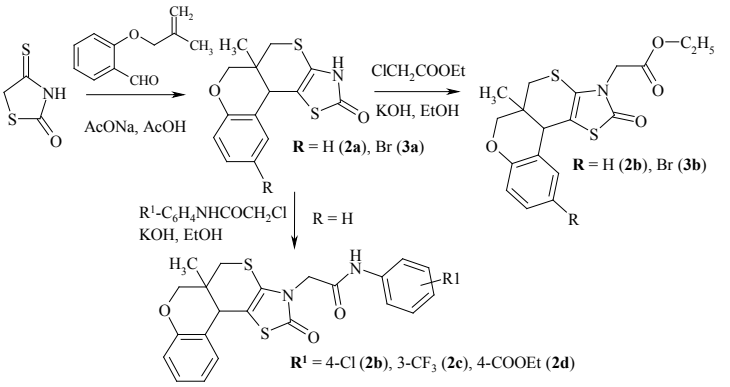
Starting 3-unsubstituted chromeno[4',3':4,5]thiopyrano[2,3-*d*]thiazole derivatives (**1a**, **2a, 3a**) were synthesized using 4-thioxo-2-thiazolidinone and allyloxo- or (2-methylallyloxy)-benzaldehydes according to domino-Knoevenagel-*hetero*-Diels-Alder reaction [6, 9]. For introducing carboxylic group functionality into 3-(chromeno[4',3':4,5]thiopyrano[2,3-*d*]thiazole)-propionitrile (**1b**) cyanoethylation reaction of compound **1a** and acrylonitrile was carried out. This derivative was transformed into respective carboxylic acid **1c** by treatment of hydrochloric and acetic acids mixture. Compounds (**1d-1f**) were synthesized via forming of corresponding acid chloride which was used in the acylation reactions of primary amines (Scheme 1).

Scheme 1



Following alkylation reaction of fused heterocycles (**2a, 3a**) with ethylchloroacetate, and various chloroacetamides, via intermediate *N*-potassium salts, novel 3-substituted chromeno[4',3':4,5] thiopyrano[2,3-*d*]thiazole derivatives (**2b-2d, 3b**) were synthesized (Scheme 2).

Scheme 2



The structures of all newly synthesized compounds were confirmed by elemental analyses, ^{1}H and ^{13}C NMR spectroscopy.

Anticancer activity evaluation of synthesized compounds was carried out in the National Cancer Institute (NCI, Bethesda, Maryland, USA) [3, 10]. Results were observed on 60 tumor cell lines representing all forms of cancer (such as, non-small cell lung cancer, colon cancer, breast cancer, ovarian cancer, leukemia, renal cancer, melanoma, prostate cancer) at single concentration of 10^{-5} M.

RESULTS AND DISCUSSION

Among 10 tested compounds six active or moderate active ones (**1a**, **1b**, **1d**, **2b**, **2d**, **3b**) were found and for two others (**1e, 1f**) primary anticancer assays are in progress.

Table 1. Prescreening results of antitumor activity for synthesized compounds

Comp.	Mean growth inhibition percent/ activity range ,%	The most sensitive lines (growth, %)
2a	96.05 / 30.63 ÷147.33	**RC:** TK-10 (30.63). UO-31 (30.88)
3b	49.23 / 7.34 ÷ 101.18	***L***: CCRF-CEM (7.34). SR (10.12). K-562 (10.55); ***BC***: MDA-MB-435 (7.52); ***CC***: HT-29 (9.91)
2b	78.77 / 46.95 ÷ 116.20	***L***: RPMI-8226 (46.95); ***BC***: T-47D (52.40)
2d	87.03 / 0.43 ÷ 156.66	***L***: CCRF-CEM (0.43). RPMI-8226 (35.25); ***NsCLC:*** HOP-92 (46.11); ***BC***: T-47D (49.33)
1b	101.04 / 8.59 ÷ 304.74	***BC***: HS 578T (8.59); ***NsCLC:*** EKVX (39.22); ***CNSC:*** SF-268 (58.00)
1d	91.69 / 60.09 ÷ 126.78	***CNSC:*** SNB-75 (60.09)

NsCLC – non-small sell lung cancer; *BC* – breast cancer, *OC* – ovarian cancer, *L* – leukemia, *RC* –renal cancer, *M* – melanoma, *CNSC* – CNS cancer

It is worth to mention that the highest growth inhibition was observed for compounds **1b**, **2d** and **3b**. Compound **1b** is rather active towards breast cancer cell line HS 578T: GI=8.59%; compound **2d** is active towards leukemia cell line CCRF-CEM: GI=0.43%; and compound **3b** shows anticancer activity relatively leukemia cell lines: CCRF-CEM: GI=7.34%, SR GI=10.12%, K-562 GI=10.55%; breast cancer cell line: MDA-MB-435: GI=7.52%; and colon cancer: HT-29: GI=9.91%. Compound **3b** was revealed to be the most active antitumor agent. The structure of this substance differs from the other tested compounds by the presence of bromine atom in the core heterocycle and by the presence of aliphatic ester substituent in the position 3. It allows us to consider the character of substituent in the 3 position of basic condensed system as the crucial factor in revealing anticancer activity by chromeno[4',3':4,5]thiopyrano[2,3-*d*]thiazole derivatives.

CONCLUSIONS

Preparative synthesis method of novel 3-substituted (5aRS,11bSR)-3,5a,6,11b-tetrahydro-2*H*,5*H*-chromeno[4´,3´:4,5]thiopyrano[2,3-*d*][1,3]thiazol-2-one and 5a-methyl–(5aRS,11bSR)-3,5a,6,11b-tetrahydro-2*H*,5*H*-chromeno[4´,3´:4,5]thiopyrano[2,3-*d*][1,3]thiazol-2-one derivatives has been worked out. Anticancer activity studies of synthesized substances revealed that the highest antitumor activity was shown by compounds **1b**, **2d** and **3b**. These compounds possess a distinctive pattern of selective action against breast cancer, colon cancer and leukemia cell lines. Results of this study prompt us to in-depth anticancer studies of fused thiazole derivatives as possible "drug-like" molecules.

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SUMMARY

The paper presents a synthetic strategy for chromeno[4',3':4,5]thiopyrano[2,3-*d*]thiazole derivatives search and a study of their probable anticancer activity. To verify the chemical structures of synthesized substances, we performed ^{1}H and ^{13}C NMR analysis on the chemical samples. Anticancer cytotoxicity of these heterocyclic compounds was studied according to NCI protocol. Among others, compounds **1b**, **2d** and **3b** showed the highest antitumor activity in the 60 cell line assay. These compounds possess a distinctive pattern of selective action against breast cancer, colon cancer and leukemia cell lines.

Keywords: synthesis, novel anticancer agent, thiopyrano[2,3-d]thiazole derivatives

STRESZCZENIE

W pracy przedstawiono strategię syntezy pochodnych chromeno[4',3':4,5]tiopirano[2,3-*d*] thiazolu i wyniki badań nad ich prawdopodobnym działaniem przeciwnowotworowym. W celu zweryfikowania struktury chemicznej zsyntetyzowanych substancji przeprowadzono analizę z zastosowaniem ^{1}H i ^{13}C NMR. Cytotoksyczność przeciwnowotworowa tych substancji

heterocyklicznych była badana zgodnie z protokołem NCI. Spośród wszystkich substancji 1b, 2d i 3b wykazały najwyższą aktywność przeciwnowotworową w 60 oznaczeniach linii komórkowych. Te substancje wykazywały wyraźne działanie na linie komórkowe komórek raka piersi, jelita grubego i białaczki.

Słowa kluczowe: synteza, nowe środki przeciwnowotworowe, pochodne tiopirano[2,3-d]tiazolu

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Investigation of fibronectin expression by white blood cells in diffuse liver disease of viral etiology

Badania nad ekspresją fibronektyny przez krwinki białe w rozlanych chorobach miąższu wątroby o etiologii wirusowej

INTRODUCTION

Problems of liver chronic damages are major and complex in modern medicine. Chronic hepatitis B and C are serious diseases that often lead to cirrhosis due to significant disruptions in the liver. An accompanying parameter of their clinical course is endotoxicosis in inhibition of which a multifunctional glycoprotein fibronectin (FN) is directly involved as an opsonin [3].

FN is presented in plasma, extracellular matrix, and basement membranes of the cells of many tissues, such as hepatocytes, fibroblasts of connective tissue, etc. It can be associated with membranes of different blood cells. Investigation of surface-associated FN has been intensified in connection with FN participation in non-specific host defense and regulation of T-lymphocyte proliferation.

However, little is known about FN distribution inside the cells. Studies of FN synthesis activation inside the cells involved directly in immune responses and connections of this process with different pathologies can be of great use.

The objectives of this paper were to investigate surface-associated and intracellular FN expression by white blood cells in diffuse liver disease of viral etiology.

MATERIAL AND METHODS

Analysis of FN level was carried out in blood plasma of healthy donors (n=7), patients with chronic hepatitis B or/and C in acute phase (n=7) and cirrhosis in active phase (n=7) by immunodot using polyclonal antibodies to plasma fibronectin (received in our laboratory). Expression of FN was examined by flow cytometry using monoclonal antibodies to fibronectin (AbD Serotec, UK).

RESULTS

The FN level in plasma of patients with chronic hepatitis was not changed (368.81 mkg/ml); it was reliably decreased in plasma of patients with liver cirrhosis (253.2 mkg/ml, $p>0.05$). Analysis of FN expression by blood cells showed that the most significant changes occurred in lymphocytes. In chronic hepatitis the amount of lymphocytes with surface-associated and intracellular FN increased by 36% and 55%, respectively, in comparison with normal blood cells. In cirrhosis of liver the amount of lymphocytes expressing intracellular FN increased by 59% (Table 1).

Table 1. Fibronectin expression by white blood cells in liver diseases

	Chronic hepatitis B or/and C		Cirrhosis of liver		Control	
Cells	On the surface, %	Inside the cell, %	On the surface %	Inside the cell, %	On the surface %	Inside the cell, %
Lymphocyte	136.04±7.77*	154.50±25.19*	114.67±21.53	158.99±25.8**	100±5.97	100±3.56
Monocyte	100.98±0.91	95.54±10.31	100.2±0.68	103.37±0.3	100±0.50	100±2.09
Granulocyte	100.94±0.11	104.44±0.09	100.32±0.76	103.57±0.09	100±1.00	100±3.87

* level of reliability during $p>0.05$; ** $p>0.01$

DISCUSSION

Fibronectin can be associated with membranes of T-lymphocytes, polymorphonuclear neutrophils and monocytes [4, 5]. Our experiments showed an increasing level of fibronectin inside the fraction of lymphocytes in chronic hepatitis and liver cirrhosis.

It has been revealed that fibronectin may be present inside T-lymphocytes [2]. According to Blum et al. endogenous FN interacts through its ED-A and ED-B domains with ganglioside sites on the cell membrane. Functions of this endogenous fibronectin are still unclear [1].

In liver diseases increased expression of FN is a result of increasing lymphocyte populations with intracellular and surface-associated FN which can be synthesized by these cells or captured by cell receptors from blood stream. This can be connected with the functional roles of FN in the diseases.

Further investigation of fibronectin expression by white blood cells can be useful for studying molecular mechanisms of immune processes and their dysfunctions in liver diseases and for searching molecular targets of new medications.

CONCLUSIONS

The level of plasma fibronectin was not changed in chronic hepatitis and it was decreased in liver cirrhosis.

Fibronectin expression by lymphocytes is increased considerably both in chronic hepatitis and liver cirrhosis.

Investigation of fibronectin expression by white blood cells can be useful for studying molecular mechanisms of immune processes and searching for new medicines.

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SUMMARY

We investigated plasma and cell fibronectin level in 14 patients with chronic liver diseases. The level of plasma fibronectin was not changed in chronic hepatitis and it was decreased in liver cirrhosis. In chronic hepatitis the number of lymphocytes expressed fibronectin on the surface and inside the cells was increased; in cirrhosis only the lymphocyte amount with intracellular FN was increased. Investigation of the expression of fibronectin by blood cells can be useful for studying molecular mechanisms of immune processes and searching for new medicines.

Keywords: fibronectin, chronic hepatitis, expression, white blood cells, cirrhosis

STRESZCZENIE

Oznaczono stężenia osoczowe i komórkowe fibronektyny u 14 pacjentów z przewlekłymi schorzeniami wątroby. Stężenia osoczowe fibronektyny nie ulegały zmianie w przewlekłym zapaleniu wątroby, były natomiast obniżone u pacjentów z marskością wątroby. W przewlekłym zapaleniu wątroby liczba limfocytów z ekspresją fibronektyny na powierzchni i wewnątrz komórki była zwiększona; w marskości wątroby podwyższona była jedynie liczba limfocytów z wewnątrzkomórkową ekspresją FN. Badanie ekspresji fibronektyny na komórkach krwi może być pomocne w badaniach nad molekularnymi mechanizmami procesów immunologicznych oraz w poszukiwaniach nowych leków.

Słowa kluczowe: fibronektyna, przewlekłe zapalenie wątroby, ekspresja, krwinki białe, marskość

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Synthesis of new 4-azolidinones with 3,5-diaryl-4,5-dihydropyrazole moiety and evaluation of their antitumor activity in vitro

Synteza nowych 4-azolidynonów z cząsteczką 3,5-diaryl-4,5-dihydropirazolu i ocena ich aktywności przeciwnowotworowej *in vitro*

INTRODUCTION

A systematic study of 4-thiazolidones derivatives with pyrazoline fragment in molecules allowed us to identify a number of high-active compounds as potential antitumor agents [7]. Generally, combination of thiazolidine template with diazole heterocycles is a perspective approach to drug-like molecules design, considering an antitumor potential of 4-thiazolidinone derivatives. The mechanisms of antitumor activity of 4-thiazolidinones can be associated with their affinity to anticancer biotargets such as JNK-stimulating phosphatase-1 (JSP-1) [4], tumor necrosis factor TNFα [3], anti-apoptotic biocomplex Bcl-X_L-BH3 [6], integrin $\alpha_v\beta_3$ receptor [5], etc. Besides, affinity of diazole derivatives was determined to the number of known biotargets. Among pyrazoles or pyrazolines inhibitors of cyclin-dependent kinase [10], heat shock proteins [2], vascular endothelium growth factors [1] and P-glycoprotein [9] were identified. The aim of our research was the synthesis of novel 4-azolidinones with 3,5-diaryl-4,5-dihydropyrazole moieties in positions 3 and 5 of core scaffold and evaluation of their antitumor activity *in vitro*.

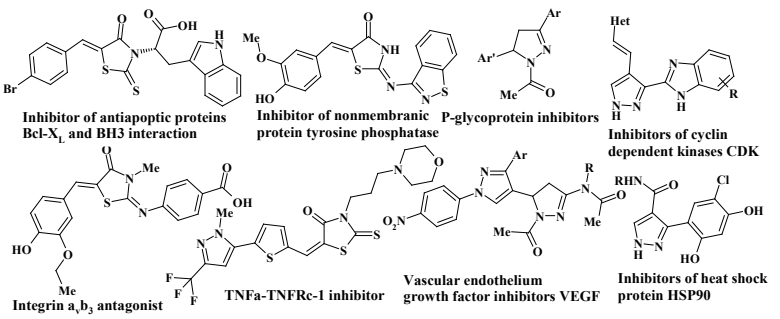


Fig. 1. 4-Thiazolidinones and diazoles as potential antitumor agents (world experience)

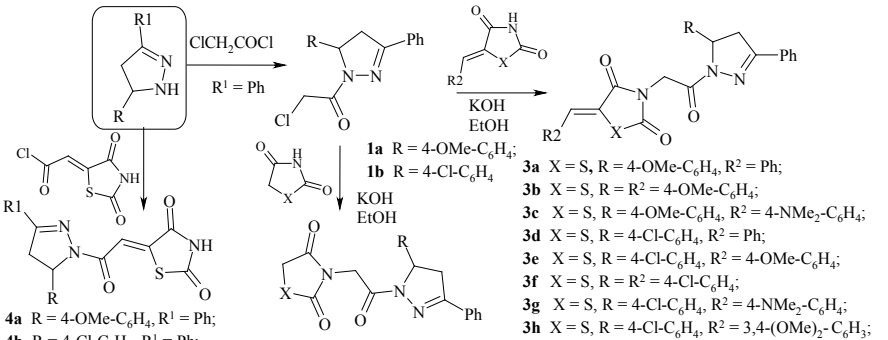
MATERIAL AND METHODS

Starting 3,5-diaryl-4,5-dihydropyrazoles [12], 5-arylidene-2,4-azolidinones potassium salts [9], (2,4-dioxothiazolidine-5-ylidene)-acetyl chloride [15] were obtained according to the methods described previously. For synthesis of new 4-thiazolidinone derivatives, the reactions of acylation and alkylation were used. The structure of the synthesized compounds was confirmed by ^{1}H NMR spectra.

RESULTS AND DISCUSSION

3,5-Diaryl-4,5-dihydro-1*H*-pyrazoles easily react with chloroacetyl chloride yielding 2-chloro-1-(3,5-diaryl-4,5-dihydropyrazol-1-yl)-ethanones 1a-1b. Compounds 1 were tested as alkylating agents in the reactions with 2,4-azolidinones and their 5-arylidenederivatives potassium salts in refluxing ethanol or mixture DMF-ethanol. Thus, the corresponding compounds 2 and 3 were obtained. Following the reaction of 3,5-diaryl-4,5-dihydro-1*H*-pyrazoles and (2,4-dioxothiazolidine-5-ylidene)-acetyl chloride the group of 5-[2-(3,5-diaryl-4,5-dihydropyrazol-1-yl)-2-oxoethylidene]-thiazolidine-2,4-diones 4 was synthesized (Scheme).

Scheme



Structures of the synthesized compounds were confirmed by ^{1}H NMR spectra. Protons CH_2-CH of pyrazoline fragment in the ^{1}H NMR spectra of synthesized compounds showed characteristic patterns of an AMX system. The chemical shifts of the protons H_A, H_M, and H_X were assigned to about δ~3.26-3.45, δ~3.96-4.17, and δ~5.68-6.13, respectively, with corresponding coupling constants of J_{AM} = 17.9-18.6, J_{AX} = 10.4-11.6, and J_{MX} = 2.9-4.5 Hz. The chemical shifts of the protons of the methylene group (CH_2CO) 2a-2c, 3a-3j were assigned at δ~4.45-4.86 and δ~4.56-4.94 and the protons of methylene group of azolidine cycle in compounds 2a-2c showed up as singlet at δ~3.96-4.28. The chemical shift for the methylidene group of 5-arylidenederivatives 3 is insignificantly displaced in weak magnetic

field, δ~6.90 (hydantoin derivatives 3i-3j) and δ~7.90 (2,4-thiazolidindione derivatives 3a-3h), and clearly indicated that only *Z*-isomers were obtained [13].

Primary anticancer assay of the synthesized compounds (2a, 2b, 2i, 4a, 4d, 4e) was performed at approximately sixty human tumor cell lines panel derived from nine neoplastic diseases, in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda [8, 14].

The tested compounds (2a, 2b, 2i, 4a) displayed moderate antitumor activity with average values GP 51.05-97.11% (Table). Selectivity pattern analysis of cell lines of disease origin can definitely affirm selective action of compound 2a on Non-Small Lung Cancer cell line NCI-H522 (GP= -68.70%), Ovarian Cancer – IGROV1 (GP= -51.27%), Renal Cancer – CAKI-1 (GP= -50.64%), compound 2i – on CNS cancer line SF-295 (GP= -4.03%), compound 4a – on Leukemia sell line SR (GP = 39.09%). Finally, compounds 4d and 4e possessed considerable activity (mean growth for 4d – 60.11% and 4e – 51.05%) and were selected for advanced assay against a panel of approximately sixty tumor cell lines at 10-fold dilutions of five concentrations (100μM, 10μM, 1μM, 0.1μM and 0.01μM) [8, 14].

Table 1. Anticancer screening data at concentration 10^{-5}M

Comp	60 cell lines assay in 1 dose 10^{-5} M conc				Active (selected for 5-dose 60 cell lines assay)
	mean growth %	range of growth %	the most sensitive cell lines	growth % of the most sensitive cell line	
2a	84.37	-68.70 to 123.93	NCI-H522 (lung cancer) IGROV1 (ovarian cancer) CAKI-1 (renal cancer)	-68.70 -51.27 -50.64	Inactive
2b	97.11	64.96 to 119.33	SF-295 (CNS cancer)	64.96	Inactive
2i	86.17	-4.03 to 121.25	SF-295 (CNS cancer)	-4.03	Inactive
4a	96.64	39.09 to 147.38	SR (leukemia)	39.09	Inactive
4d	60.11	-27.33 to 160.47	HL-60(TB) (leukemia) SF-295 (CNS cancer)	-27.33 -13.37	Active
4e	51.05	-5.27 to 109.26	RPMI-8226 (leukemia) SF-295 (CNS cancer)	2.48 -5.27	Active

The tested compounds (4d, 4e) showed a broad spectrum of growth inhibition activity against human tumor cells, as well as some distinctive patterns of selectivity on leukemia (Fig. 2). Compound 4d was found to be a highly active growth inhibitor of the leukemia cell lines RPMI-8226 ($logGI_{50}$ = -5.67), CCRF-CEM ($logGI_{50}$ = -5.55) and SR ($logGI_{50}$ = -5.55). Compound 4e showed selectivity on leukemia cell line MOLT-4 ($logGI_{50}$ = -5.79) and non-small cell lung cancer cell line HOP-92 ($logGI_{50}$ = -5.93). Generally, pyrazoline substituted derivative 4e demonstrated the most marked

effect among all synthesized compounds and possessed significant activity with the mean $logGI_{50}$ value -5.44.

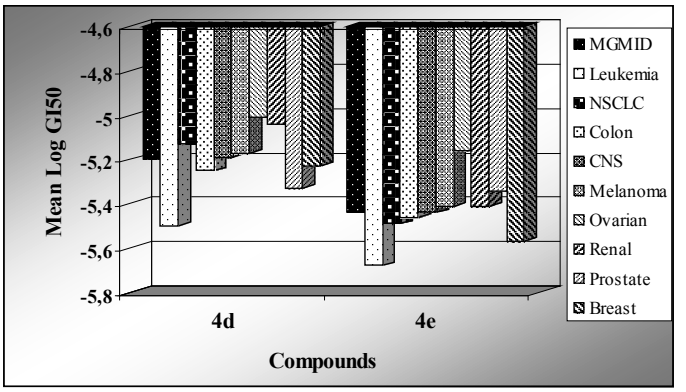


Fig. 2. Anticancer selectivity pattern of the most active compounds

CONCLUSIONS

A group of novel 4-azolidinone derivatives with pyrazoline moieties was synthesized using reactions of acylation and alkylation. Antitumor activity screening of the synthesized compounds showed their moderate activity with high selectivity to individual lung, renal, ovarian and CNS cancer cell lines. In conclusion, these preliminary results allowed to identify the most active compound 4e as a prospective antitumor agent (average $logGI_{50}$ and logTGI values -5.44 and -4.52, respectively) with selective influence on leukemia.

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SUMMARY

In the present paper, a synthetic approach for design of new pyrazoline substituted 4-azolidones were described. Six of the synthesized compounds were tested according to NCI protocol and two of them (**4d**, **4e**) displayed antitumor activity on leukemia, melanoma, lung, colon, CNS, ovarian, renal, prostate and breast cancers cell lines.

Keywords: 4-azolidinones, 3,5-diaryl-4,5-dihydropyrazoles, acylation, alkylation, ^{1}H NMR spectra, antitumor activity

STRESZCZENIE

W pracy opisano proces syntezy nowego 4-azolidonu z podstawnikiem pirazolinowym. Zgodnie z protokołem NCI przebadano sześć uzyskanych substancji, przy czym dwie z nich (4d, 4e) wykazały aktywność przeciwnowotworową w stosunku do linii komórkowych białaczki, czerniaka, raka płuc, okrężnicy, OUN, jajników, nerek, prostaty i piersi.

Słowa kluczowe: 4-azolidynony, 3,5-diaryl-4,5-dihydropirazole, acylacja, alkilacja, spektra ^{1}H NMR, aktywność przeciwnowotworowa

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Blockade of the endoplasmic reticulum stress sensor inositol requiring enzyme-1 changes the expression of cyclin and growth arrest-specific genes in glioma cells

Blokada enzymu-1 zależnego od inozytolu sensora stresu retikulum endoplazmatycznego zmienia ekspresję cyklin genów hamujących wzrost (GAS) w komórkach glejaka

INTRODUCTION

Astrocytes represent the most abundant cell type in the mammalian brain and play an important role in the maintenance and regeneration of neuronal functions. Ischemia have been shown to induce a set of complex intracellular signaling events known as the Unfolded Protein Response (UPR), which is mediated by inositol requiring enzyme-1 (IRE-1), to adapt for cell survival or, alternatively, to enter cell death programs through endoplasmic reticulum-associated machineries [2-5]. As such, it participates in the early cellular response to the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum, occurring under both physiological and pathological situations. Two distinct catalytic domains of the dual enzyme IRE1, a serine/threonine kinase and an endoribonuclease contribute to IRE-1 signaling. IRE-1-associated kinase activity autophosphorylates and dimerizes IRE-1, leading to the activation of its ribonuclease domain, to the degradation of a specific subset of mRNA and to the initiation of the pre-XBP1 (X-box binding protein 1) mRNA splicing. Mature XBP1 mRNA encodes a transcription factor that stimulates the expression of UPR-specific genes [7, 10, 11, 14, 15]. Recently, single mutations in *IRE1α* gene were detected in human cancers and IRE1 was proposed as a major contributor to tumor (including glioblastoma) progression among protein kinases. IRE1 transduction pathway is linked to the neovascularization process, tumor growth and cellular death processes because a complete blockade of IRE1 activity had anti-tumor effects [1, 6, 8, 9, 12].

We studied effect of IRE-1-deficiency on the expression of different cyclin (A2, D3, E2 and G2) and cyclin-dependent kinase (CDK4 and CDK5) genes as well as growth arrest-specific genes GAS1 and GAS6 in glioma cell line U87 and modified glioma cells without IRE-1 kinase and ribonuclease activities.

MATERIAL AND METHODS

IRE-1 wild-type and knockout glioma cell line U87 were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. RNA was extracted using Trizol reagent (Invitrogen, USA) [13]. Expression of cyclin A2, D3, E2, G2, CDK4 and CDK5 as well as growth arrest-specific genes GAS1 and GAS6 in glioma cell line U87 and its subline with IRE-1-deficiency was measured by quantitative polymerase chain reaction using „Stratagene Mx 3000P cycler" (USA) and SYBRGreen Mix (AB gene, Great Britain). QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis. Polymerase chain reaction was performed in triplicate. The analysis of quantitative PCR was performed using a special computer program "Differential expression calculator" and statistical analysis – in Excel program.

RESULTS

As shown in Fig. 1 and 2, cyclin A2, D3 and G2 mRNA expression significantly increased (+39, +74 and +70 %, correspondingly) in IRE-1-deficient glioma cells as compared to control cell line. Both cyclin A2 and D3 mRNA expression in wild-type and IRE-1-deficient glioma cells is suppressed in hypoxic conditions. Much stronger (3 fold) induction of mRNA expression in IRE-1-deficient cells was shown for cyclin E2 (Fig. 2). Hypoxia also suppressed the expression of cyclin E2 mRNA both in wild-type and IRE-1-deficient glioma cells however, the expression of cyclin G2 mRNA was significantly induced (4 fold) under hypoxia in wild-type glioma cells and slightly less (3 fold) in cells with IRE-1-deficiency as compared to control cells (Fig. 2). Expression of both cyclin-dependent kinases CDK4 and CDK5 in IRE-1-deficient glioma cells did not change significantly as compared to control cells, but hypoxia is reduced expression of both kinases both in wild-type and IRE-1-deficient glioma cells (Fig. 3). We also showed that expression of both growth arrest-specific genes (GAS1 and GAS6) is hypoxia responsive and strongly enhanced in glioma cells without IRE-1 kinase and ribonuclease activities: in 7.3 and 6.7 fold, correspondingly (Fig. 4).

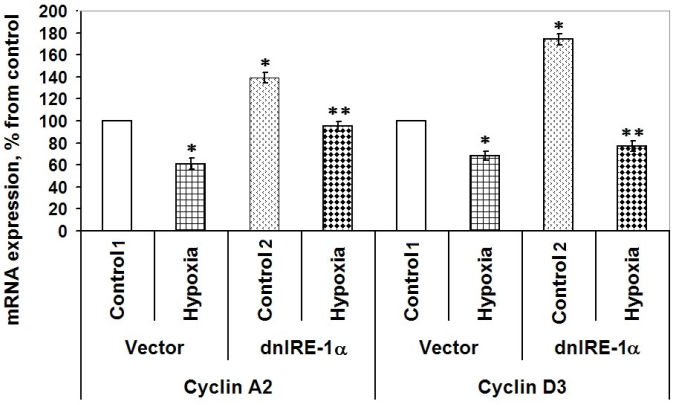

Fig.1. Expression of cyclin A2 and D3 mRNA in glioma cell line U87 and its subline with IRE-1-deficiency measured by quantitative polymerase chain reaction. Values of cyclin A2 and D3 mRNA expressions were normalized to β-actin mRNA expression. In Fig. 1-4: * - $P < 0.05$ as compared to control 1; ** - $P < 0.05$ as compared to control 2; $\boldsymbol{n} = 3$

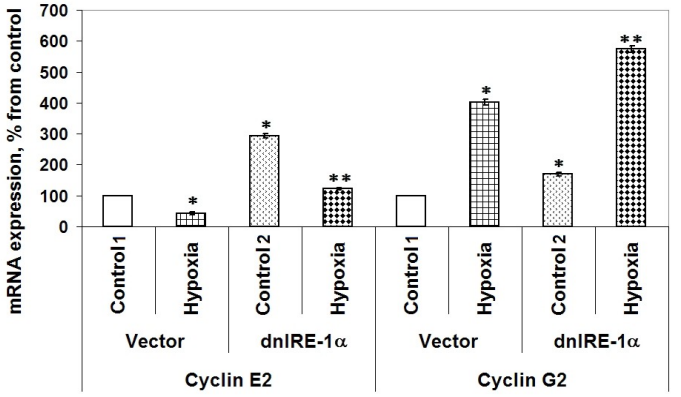

Fig. 2. Expression of cyclin E2 and G2 in glioma cell line U87 and its subline with IRE-1-deficiency measured by quantitative polymerase chain reaction. Values of cyclin E2 and G2 mRNA expressions were normalized to β-actin mRNA expression

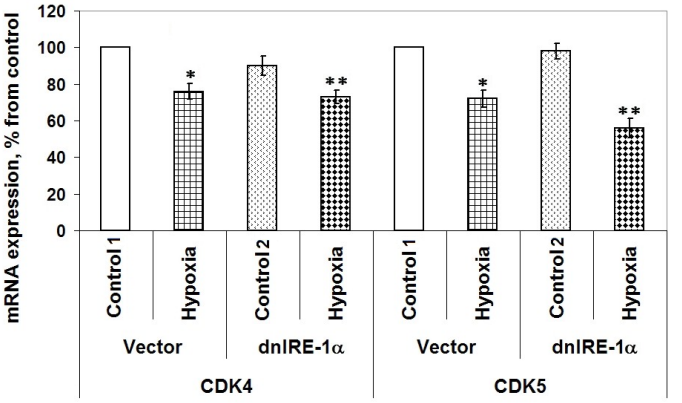

Fig. 3. Expression of cyclin-dependent kinase CDK4 and CDK5 in glioma cell line U87 and its subline with IRE-1-deficiency measured by quantitative polymerase chain reaction. Values of CDK4 and CDK5 mRNA expressions were normalized to β-actin mRNA expression

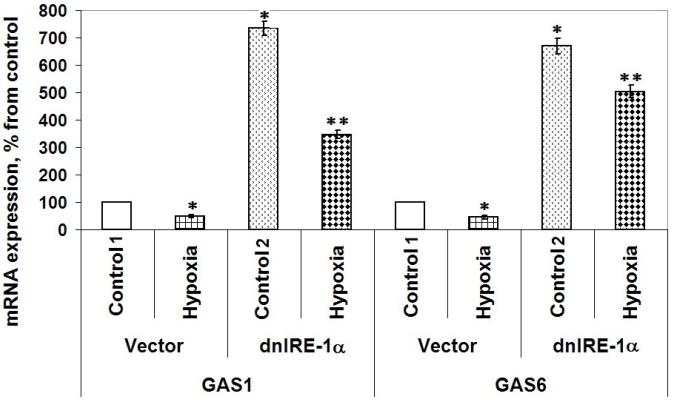

Fig. 4. Expression of growth arrest-specific genes GAS1 and GAS6 in glioma cell line U87 and its subline with IRE-1-deficiency measured by quantitative polymerase chain reaction. Values of cyclin GAS1 and GAS6 mRNA expressions were normalized to β-actin mRNA expression

DISCUSSION

Cyclin A2, D3, G2 and E2 as well as cyclin-dependent kinases CDK4 and CDK5 play a significant role in the cell cycle control and tumor growth [10, 15]. We observed induction of cyclin genes expression in IRE-1-deficient glioma cells which have suppressed cell proliferation and angiogenesis [6, 14]. Recently, IRE1 was proposed as a major contributor to tumor (including glioblastoma) progression among protein kinases because IRE1 transduction pathway is linked to the neovascularization process, tumor growth and cellular death processes [1, 10, 11, 15]. However, single mutations in *IRE1*α gene were detected in many human cancers. We also showed that a complete blockade of IRE1 activity significantly increased the expression of growth arrest-specific genes which correlate with anti-tumor effects of this IRE1 blockade.

CONCLUSIONS

Results of these investigations clearly demonstrated that the expression of different cyclin as well as growth arrest-specific genes in glioma cells is regulated by hypoxia and significantly depends from IRE-1 kinase and ribonuclease activities. Thus, cyclin A2, D3, E2 and G2 as well as GAS1 and GAS6 genes possibly participate in cell adaptive response to endoplasmic reticulum stress associated with accumulation of misfolded proteins in the endoplasmic reticulum.

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SUMMARY

We studied effect of inositol requiring enzyme-1 (IRE-1) deficiency on the expression of different cyclin and cyclin-dependent kinase (CDK) genes as well as growth arrest-specific (GAS) genes in glioma cells and modified glioma cells without IRE-1 kinase and ribonuclease activities. Cyclin A2, D3, E2 and G2 mRNA expression was significantly increased in IRE-1-deficient glioma cells as compared to control cell line. Blockade of IRE-1 activities significantly induced the expression of growth arrest-specific genes GAS1 and GAS6. Results of these investigations clearly demonstrated that the expression of growth arrest-specific genes and different cyclins in glioma cells significantly depends on IRE-1 kinase and ribonuclease activities.

Keywords: inositol requiring enzyme-1, gene expression, cyclin A2, D3, E2 and G2, cyclin-dependent kinase CDK4, growth arrest-specific genes GAS1 and GAS6, glioma cells

STRESZCZENIE

Zbadano wpływ niedoboru enzymu-1 zależnego od inozytolu (IRE-1) na ekspresję cyklin i genów kinazy zależnej od cyklin (CDK) jak też genów hamujących wzrost (GAS) w komórkach glejaka i modyfikowanych komórkach glejaka bez aktywności kinazy IRE-1 i rybonukleazy. Ekspresja cyklin A2, D3, E2 i G2 mRNA była istotnie wyższa w komórkach z niedoborem IRE-1 w porównaniu z kontrolną linią komórek. Zahamowanie aktywności IRE-1 w sposób istotny indukowało ekspresję genów hamujących wzrost GAS1 i GAS 6. Wyniki badań wskazują, iż ekspresja genów GAS oraz cyklin w komórkach glejaka zależy w głównej mierze od aktywności kinazy IRE-1 i rybonukleazy.

Słowa kluczowe: enzym-1 zależny od inozytolu, ekspresja genu, cykliny A2, D3, E2 i G2, kinaza cyklino-zależna CDK4, geny hamujące wzrost GAS1 i GAS6, komórki glejaka

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Ca^{2+}-transporting ATP-hydrolyzing systems activity in lymphocytes of peripheral blood from men with erectile dysfunction

Aktywność systemów hydrolizujących ATP zależnych od Ca^{2+} w limfocytach krwi obwodowej mężczyzn z zaburzeniami erekcji

INTRODUCTION

The most widely spread male sexual disorder is erectile dysfunction (ED), which is a stable disability to achieve and keep erection [8, 10]. 40–52% of male from age group 40 plus have different forms of this disease. It is proved that 63–69% have ED of psychogenic origin, 9–20% of natural origin and 6-11% have a combined form [1, 15].

The main risk factors are depression and psychological traumas, diabetes mellitus, alcohol misuse, hypertension, atherosclerosis, hyperlipidhemia [7, 11, 14, 15]. Organism adopts to stress factors (one of which is erectile dysfunction) with the help of nervous, endocrine and immune systems [4, 12, 13].

In recent years we have noticed a increasing interest in lymphocytes of peripheral blood caused by their role in processes influencing the level of homeostasis of the whole organism. Lymphocytes are involved in pathological process not only in the conditions of blood diseases; they suffer serious structural and functional changes in the conditions of different genesis diseases [3, 4].

An important index of the functional condition of cells, especially lymphocytes, is ionic homeostasis, especially homeostasis Ca^{2+}, which is supported by Ca^{2+}-transportation systems. Ca^{2+} is an intracellular messenger and it regulates almost all cell functions, especially contraction-relaxation of muscles [2, 14]. Functions of Ca^{2+}, Mg^{2+}-ATPase in plasmatic membrane and endoplasmatic reticulum which provides the level of intracellular calcium homeostasis are under a modulating influence of both external and internal cellular factors. We assume that lymphocytes can be an appropriate model for studying biochemical indexes in the development of erectile dysfunction.

The aim of the research was to study the activity of Ca^{2+}-dependent ATP-hydrolases on the example of lymphocytes as the model of peripheral blood of males of different age groups with erectile function disorders. Both general Ca^{2+},Mg^{2+}-ATPases activity and the activity of this ferment in plasmatic membrane and endoplasmatic reticulum were studied with the use of appropriate blockers of ATPases.

MATERIAL AND METHODS

The objects of the research were lymphocytes of peripheral blood of clinically healthy men (group 1, control), men with psychogenic mono syndrome erectile dysfunction in the age group 18–29 years (group 2) and men of age group 50–63 years with a mixed form of erectile dysfunction influenced by dyslipidhemia, hypertension, abdominal obesity and partial androgenic deficiency (group 3). Mononuclear lymphocytes of peripheral blood were extracted from heparinized freshly taken blood in the gradient concentration of philol-urographine [3]. The number of cells was counted in Horyayev camera using 0.1% tripane blue as a pigment. Viability of lymphocytes in all tests results was not lower then 95%. To discover latent ATPase activities, a 0.2% solution of saponine was added to, lymphocyte suspension [14].

General Na^+,K^+,Mg^{2+}-ATPases activity was tested in incubation environment (mM): 30 NaCl, 120 KCl, 5 $MgCl_2$, 3 ATP, 3 Hepes-Tris buffer (pH 7,4), 0.2 % saponine solution, 0.5 mg proteine/ml. Testing of Ca^{2+},Mg^{2+}-ATPase activity was conducted in the presence of 10 μM $CaCI_2$, a Ca^{2+}-independent - 1 mM EGTA [9]. For testing Ca^{2+},Mg^{2+}-ATPase activity of plasmatic membrane and endoplasmatic reticulum, a number of non specific and specific blocker of ATPases were used; they were eosine Y (1 mM), tipsigargine (0.1 μM), oubaine (1 mM), sodoum azide (1 mM) [14]. Incubating time – 5 min, temperature 37^0 C. The statistical analysis of the obtained data was made using Student criteria. Statistic and kinetic calculations were made on IBM PC.

RESULTS AND DISCUSSION

Cells Ca^{2+},Mg^{2+}-ATPase activity is one of the indexes which reflect their functional condition [2, 14]. It is known that a cell has two Ca^{2+}-depending ATPases: Ca^{2+},Mg^{2+}-ATPase of plasmatic membrane and Ca^{2+},Mg^{2+}-ATPase of endoplasmatic reticulum [14].

General Ca^{2+},Mg^{2+}-ATPase activity of saponine permiabilized lymphocytes of peripheral blood of clinically healthy men of age group 20–29 years shows 10.7±0.8 μM P_i/h · protein mg (n=48), men of group 2 – 10.5±0.8 (n=27), group 3 - 8.1±0.7 μM P_i/h · protein mg (n=14). It's discovered that diabetes mellitus of 1st and 2nd type the Ca^{2+},Mg^{2+}-ATPase activity is reduced [9]. The level of saturated acid increases and the level of polyunsaturated acid decreases which results in a decreasing level of membrane-joined ATPase activity. Active LP processes in pathological condition results in changes in membrane-joined ferments activity level, especially of Na^+,K^+-ATPase.

Using specific and not specific blockers of different ATPases, we identified the contribution of each of them to the keeping of Ca^{2+}-homeostasis in the cell (Fig. 1).

The component of general ATPase activity which was inhibited by specific blocker Ca^{2+},Mg^{2+}-ATPase of endoplasmatic reticulum 0.1 μM tapsyharine on the background of action Na^+,K^+-ATPase blocker oubaine (1 mM) and H^+-ATPase mitochondrial blocker sodium azide (1 mM) in control point was equal to 2.6±0.3 μM P_i/hour · protein mg. The component which was additionally inhibited by 1 mM eosyne Y was equal to 2.8±0.3 μM P_i/h · protein mg. This is the activity of Ca^{2+},Mg^{2+}-ATPase lymphocytes plasmatic membrane.

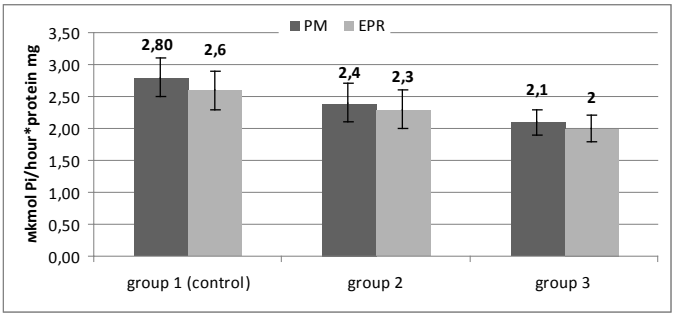

Fig. 1. Ca^{2+},Mg^{2+}-ATPases activities of plasmatic membranes and endoplasmatic lymphocytes reticulum of peripheral blood

The second group of men showed the activity of ferment in PM – 2.55±0.22 μM P_i/h · protein mg, and in EPR – 2.32±0.22 μM P_i/h · protein mg. Group 3 showed the activity of PM Ca^{2+},Mg^{2+}-ATPase equal 2.13±0.2 μM P_i/h · protein mg and the activity of EPR Ca^{2+},Mg^{2+}-ATPase equal – 2.06 ±0.2 μM P_i/h · protein mg. We can see that the activity of Ca^{2+},Mg^{2+}-ATPase both PM and EPR decrease in conditions of erectile dysfunction but a more significant decrease takes place as the age of the patients grow. So in conditions of erectile dysfunction development, the activity of ferment decreases. It results in slowing down of outflow Ca^{2+} from cytosole and this can be evidence of the fact that the concentration Ca^{2+} in the cell increases. Accumulating of Ca^{2+} in the cells and a decrease of ATP level results in a break down of ionic pumps and overload of cytosole with calcium. On the other hand, increasing Ca^{2+} concentration results in activation of Ca^{2+}-dependant phospholipase A_2 which stimulates accumulation of lipophractions and free fatty acids in membranes [2]. These data confirm others which show that high accumulation of calcium ions in lymphocytes in conditions of diabetes mellitus and hypertension is caused by decreasing Ca^{2+},Mg^{2+}-ATPase activity in the plasmatic membrane and by modulation of Na^+/Ca^{2+}-exchange. Increasing of ionized calcium concentration in cytoplasm in different pathologic conditions is widely spread. These data are confirmed by those received on platelets [5, 6]. For example, in condition of hypertension to avoid excessive flow of Ca^{2+} to cells blockers of Ca^{2+}-channels are widely used (diltiasem, nifedipine, nicardipine etc) [2].

Disorder in the functioning of the complicated system of Ca^{2+}-connecting and Ca^{2+}-transporting mechanisms logically causes disorders in calcium homeostasis, regulation function Ca^{2+} and numerous pathological changes and metabolicic shifts which are harmful to the cells.

CONCLUSIONS

Erectile dysfunction of combined genesis in the presence of dislipidemia is accompanied by both decreasing of general Ca^{2+},Mg^{2+}-ATPase activity, and Ca^{2+},Mg^{2+}-ATPase activity of plasmatic membrane and endoplasmatic reticulum of lymphocytes, which indicates the increase of ionized calcium concentration in cells and disorders in regulation mechanisms of the cells.

With aging of the patients with sexual function, disorders decreasing of Ca^{2+},Mg^{2+}-ATPases activity grow.

Lymphocytes of peripheral blood can become a convenient and appropriate model for studying erectile dysfunction mechanisms.

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SUMMARY

Ca^{2+},Mg^{2+}-ATPases properties in lymphocytes of peripheral blood from men with erectile dysfunction were studied. It was shown that with age total Ca^{2+},Mg^{2+}-ATPase activity and its activity in plasmatic membrane and endoplasmatic reticulum decreases, which leads to Ca^{2+} concentration growth in cytosole.

Keywords: Ca^{2+},Mg^{2+}-ATPase, lymphocyte, erectile dysfunction

STRESZCZENIE

Zbadano właściwości Ca^{2+},Mg^{2+}-ATPazy limfocytów krwi obwodowej u mężczyzn z zaburzeniami erekcji. Wykazano, iż wraz z wiekiem całkowita, jak też związana z błonami plazmatycznymi i retikulum endoplazmatycznym aktywność Ca^{2+},Mg^{2+}-ATPazy obniża się, co prowadzi do wzrostu stężenia Ca^{2+} w cytozolu.

Słowa kluczowe: Ca^{2+},Mg^{2+}-ATPaza, limfocyty, zaburzenia erekcji

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Effect of maleimide derivative on oxidative stress and glutathione antioxidant system in 1,2-dimethylhydrazine induced colon carcinogenesis in rat

Wpływ pochodnych maleimidu na stres oksydacyjny i glutationowy system antyoksydacyjny w karcinogenezie okrężnicy indukowanej 1,2-dimetylohydrazyną

INTRODUCTION

Protein kinases are the most exploited targets in modern drug discovery due to key the roles of these enzymes in human diseases, including cancer [5]. Novel protein kinases inhibitor maleimide derivative 1-(4-Cl-benzyl)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrol-2.5-dione (MI-1) has been synthesized at the Taras Shevchenko National University of Kyiv. MI-1 exhibits cytostatic effects on interferon resistant cell line, transformed cell lines HEK293, *MCF-7;* the most significant influence has been determined on SW620 (human caucasian colon adenocarcinoma) [4]. The 1,2-dimethylhydrazine (DMH)-induced colon cancer is morphologically similar to human colon cancer [8].

The aim was to investigate the levels of protein carbonyl groups and thiobarbituric acid reactive substances (TBARS) and glutathione antioxidant system after treatment with MI-1 in case of DMH-induced colon cancer in rats and c-kit expression that is often mutated in colorectal carcinoma.

MATERIAL AND METHODS

MI-1 as a fat-soluble substance was dissolved in sunflower oil and injected by intragastric way in doses 0.027, and 2.7 mg/kg b.w. everyday for 20 weeks. Colon carcinogenesis was induced by weekly subcutaneous injections of DMH dissolved in physiological solution (20 mg/kg body weight) for 20 weeks. Rats were divided into 8 groups, 3 of them are controls. Group 1 (control) was administered physiological solution, group 2 – DMH, group 3 (control) – sunflower oil, group 4 – MI-1, 0.027 mg/

kg, group 5 – MI-1 2.7 mg/kg, group 6 – physiological solution and sunflower oil, group 7 – DMH and MI-1, 0.027 mg/kg, group 8 – DMH and MI-1, 2.7 mg/kg.

The contents of thiobarbituric acid reactive substances (TBARS) [9], protein carbonyl groups [7], reduced glutathione (GSH), and glutathione peroxidase (GSHPx), glutathione-S-transferase (GST) activities [9] were determined in intestinal mucosa homogenate. The protein level of *c-kit tyrosine kinase (CD117, 145 kDa)* was identified using *Western blotting*.

RESULTS

The content of protein carbonyl groups and TBARS (Fig. 1) in the intestinal mucosa homogenate was significantly increased (nearly twice) than control sample. MI-1 does not change the level of protein carbonyl groups in dose 0.027 mg/kg, while in dose 2.7 mg/kg this parameter is diminished by 30%. But TBARS concentration is decreased by 29% in both doses. Both MI-1 and its combined administration with DMH did not cause significant changes of these parameters.

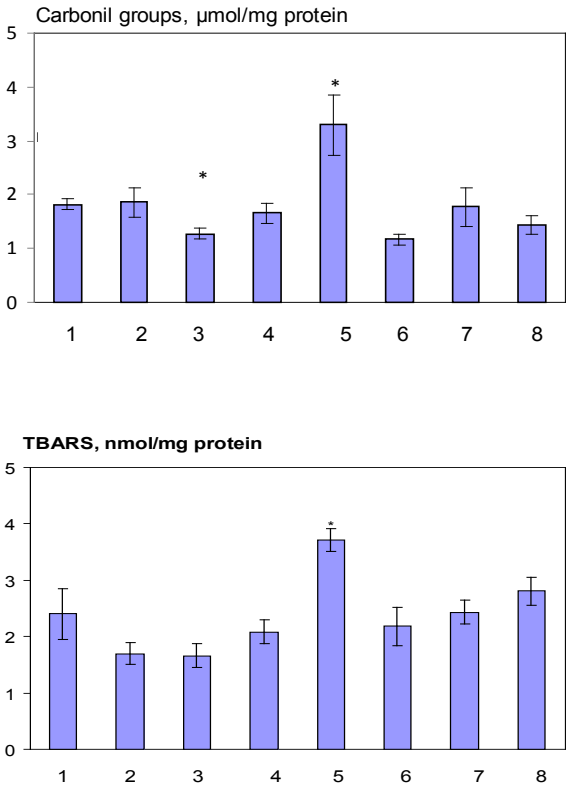

Fig. 1. Content of carbonil groups and TBARS in intestinal mucosa of rats: 1 – control, sunflower oil; 2 – MI-1, 0.027 mg/kg; 3 – MI-1, 2.7 mg/kg; 4 – control, physiological solution; 5 – DMH; 6 – control, physiological solution + sunflower oil; 7 – DMH + MI-1, 0.027 mg/kg; 8 - DMH + MI-1, 2.7 mg/kg

In DMH treated rats the levels of GSH-dependent parameters were elevated in comparison to control rats. DMH significantly increases the level of GSH by 90%, GST activity by 62% and insignificantly GSHPx activity by 26% (Tab. 1). MI-1 reveals a tendency to decrease GSHPx activity by 27% in both doses and insignificantly decreases GST activity and the level of GSH. The co-administration of DMH and MI-1 did not cause significant changes of the explored parameters.

Table 1. The level of reduced glutathione and glutathione peroxidase and glutathione-S-transferase activities in intestinal mucosa of rats

Groups	GSH (nmol/ mg protein · min)	GSHPx (nmol/ mg protein · min)	GST (μmol/ mg protein · min)
Control (oil)	1.03 ± 0.13	4.14 ± 0.09	0.16 ± 0.01
MI-1 0.027 mg/kg	0.89 ± 0.03	3.20 ± 0.25	0.14 ± 0.03
MI-1 2.7 mg/kg	0.97 ± 0.14	3.01 ±0.31*	0.13 ± 0.02
Control (phys. sol.)	0.83 ± 0.09	3.88 ±0.40	0.08 ± 0.01
DMH	1.53 ± 0.30*	4.89 ± 0.49	0.13 ± 0.02
Control (phys.sol.+ oil)	1.04 ± 0.01	3.93 ± 0.49	0.20 ± 0.03
DMH+MI-1 0.027 mg/kg	1.16 ± 0.16	4.44 ± 0.49	0.22 ± 0.03
DMH+MI-1 2.7 mg/kg	1.30 ± 0.11	4.87 ± 0.70	0.15 ± 0.02

Values are Mean ± SD derived from 8 rats. * – $p < 0.05$ compared with control group

The c-kit antibody detected one major band between 130 and 170 kDa which corresponds to Mr 145 kDa (Fig. 2). This band represents the c-kit tyrosine kinase receptor. The protein level of the c-kit was detected only on two tracks corresponding to DMH-treated group and its combined administration with MI-1 in dose 0.027 mg/kg.

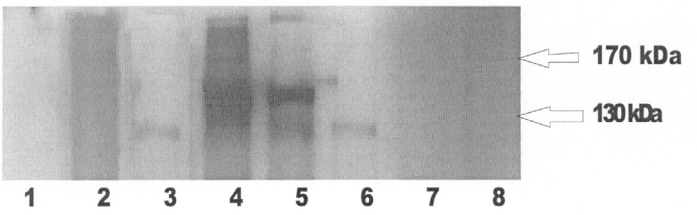

Fig. 2. The protein level of c-kit tyrosine kinase (Mr 145 kDa) in rats; 1 – control, sunflower oil; 2 – control, physiological solution; 3 – control, physiological solution + sunflower oil; 4 – DMH; 5 – DMH + MI-1, 0.027 mg/kg; 6 – MI-1, 0.027 mg/kg; 7 – DMH + MI-1, 2.7 mg/kg; 8 – MI-1, 2.7 mg/kg

DISCUSSION

The increase of the level of TBARS and protein carbonyl groups in DMH-treated rats has been shown. Theses results correlate with our previous finding [6] and increased plasma and tissue malone

dialdehide (MDA) content in colorectal cancer patients [2]. It has been claimed that MDA acts as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes. However, MI-1 administration to DMH treated animals showed a reduction in the levels of TBARS. It was attributed to MI-1 antiperoxidative property.

GSH and GSH-dependent enzymes are important in neoplastis diseases and they play a crucial role in the defence against reactive oxygen species, detoxification of xenobiotics and carcinogens [3]. We observed enhanced levels of GSH and GST, GSHPx activities in intestinal mucosa of DMH-treated rats. This may be due to the increased cell proliferation involved in the pathogenesis of DMH-induced colon cancer. It was previously demonstrated that GSH, GSHPx, GST and GSH-reductase was expressed in greater amounts in neoplastic cells, conferring a selective growth advantage [8]. Other studies showed similar changes in GSH-dependent enzymes in cases of colorectal cancer, while GSH level increased or decreased depending on studies [10]. MI-1 caused a tendency to decrease GSHPx activity; however, the level of GSH, as substrate for GSHPx, did not change. On the administration of MI-1 to DMH-treated rats, the GSH levels and GSHPx, GST activities were decreased to control values. It suggests that MI-1 reveals the ability to inhibit the progression of cancer.

The expression of the c-kit proto-oncogene has been reported in a number of cancer cell lines, and human colorectal tumors also express this proto-oncogene [1]. It is known that the activation of the c-Kit/SCF pathway has been detected in several human colon cancer cell lines. We revealed the level of c-kit only in DMH-treated group and its combined administration with MI-1 in dose 0.027 mg/kg, whereas MI-1 in dose 2.7 mg/kg blocks c-kit expression in DMH-induced colon carcinogenesis in rat and might be a potential chemotherapeutic agent for the treatment of colon cancer.

CONCLUSIONS

Novel maleimide derivative MI-1 did not significantly disturb peroxidation/antioxidation system of intestinal mucosa, and caused recovery of the contents of TBARS, protein carbonyl groups, GSH, and GSHPx, GST activities in DMH-treated rats. MI-1 blocks expression of c-kit proto-oncogene in DMH-induced colon carcinogenesis in rat. Therefore, MI-1 might be a potential chemotherapeutic agent for the treatment of colon cancer.

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SUMMARY

The levels of protein carbonyl groups, thiobarbituric acid reactive substances (TBARS), reduced glutathione, and glutathione-dependent enzymes, and c-kit expression in intestinal mucosa of rats after treatment with maleimide derivative 1-(4-Cl-benzyl)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrol-2.5-dione (MI-1) in doses 0.027 and 2.7 mg/kg have been studied. Colon cancer was induced by 1,2-dimethylhydrazine (DMH). The increase of the level of TBARS and protein carbonyl groups and glutathione antioxidant system in DMH-treated rats was shown. MI-1 restores changes of the studied parameters and blocks c-kit expression in intestinal mucosa of DMH-treated rats.

Keywords: maleimide derivative, 1,2-dimethylhydrasine, colon cancer, oxidative stress

STRESZCZENIE

Określono poziomy grup karbonylowych białek, produktów peroksydacji reagujących z kwasem tiobarbiturowym (TBARS), glutationu zredukowanego, enzymów zależnych od glutationu, i ekspresję c-kit w śluzówce jelit szczurów po podaniu pochodnej maleimidu 1-(4-Cl-benzyl)-3-Cl-4-(CF3-phenylamino)-1H-pyrrol-2.5-dionu (MI-1) w dawkach 0,027 and 2,7 mg/kg m.c. Raka okrężnicy indukowano przy użyciu 1,2-dimetylohydrazyny (DMH). Wykazano wzrost poziomów TBARS i grup karbonylowych białek oraz glutationowego systemu antyoksydacyjnego u szczurów, którym podawano DMH. Zastosowanie MI-1 powodowało cofnięcie się opisanych zmian w oznaczanych parametrach i blokowało ekspresję c-kit w śluzówce jelit szczurów.

Słowa kluczowe: pochodna maleimidu, 1,2-dimetylohydrazyna, rak okrężnicy, stres oksydacyjny

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Dual acting COX/LOX nonsteroidal anti-inflammatory drugs versus traditional COX-2 inhibitors

Podwójne działanie COX/LOX niesteroidowych leków przeciwzapalnych w odniesieniu do tradycyjnych inhibitorów COX-2

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs), inhibiting synthesis of prostaglandins by cyclooxygenase (COX), are some of the most commonly prescribed medications in the treatment of inflammatory states. Two COX isoforms, COX-1 and COX-2 have been identified. It has been suggested that constitutive COX-1 is involved in homoeostatic processes, whereas COX-2 is the isoform that plays a major part in the inflammatory process and the pain associated with it. On the basis of this assumption, selective COX-2 inhibitors were developed; these were intended to have the anti-inflammatory properties of classical NSAIDs but without affecting the integrity of the gastric mucosa. Nevertheless, there is accumulating evidence that COX-1 and COX-2 have overlapping actions and that both isoforms are involved in homoeostasis processes, just as both are modulators of inflammatory reactions [7]. Lipooxygenase (LOX) pathway also plays an important role in inflammation. Leucotriens and lipoxins produced via LOX activity play a role in the damage of gastric mucosa [6]. Compounds that combine COX/LOX inhibition are potential new drugs to treat inflammation. Dual inhibitors, by acting on the two major arachidonic acid metabolic pathways – cyclooxygenase (COX) and lipooxygenase (LOX) – possess a wide range of anti-inflammatory activity. Besides that, dual inhibitors appear to be almost exempt from gastric and cardiovascular toxicity, which is the most troublesome side effect of COX inhibitors [2]. Therefore, the purpose of the research was to compare the action of COX-2 selective inhibitor celecoxib and thiazolidin derivatives possessing dual COX/LOX inhibition on processes of lipoperoxidation and activity of the antioxidant protection system in heart tissue and gastric mucosa.

MATERIAL AND METHODS

The structure of this study and animal experimental procedures were approved by the Ethical Committee of Lviv National Medical University. 40 male albino rats weighing 200–250 g were used.

Animals were divided into 4 groups: 1 – intact animals were used as controls; 2 – COX-2 inhibitor celecoxib was introduced per os for 14 days (10 mg/kg); 3 – {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-acetic acid was introduced per os for 14 days (10 mg/kg); 4 – {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-benzene-sulfonamide was introduced per os for 14 days (10 mg/kg). Under general anesthesia, rats were sacrificed by decapitation and stomach and heart were excised, and opened longitudinally. For histological investigation, additional samples were fixed in 10% formalin, then embedded in paraffin and sections were prepared and stained with hematoxylin-eosin. After that, tissue samples were homogenized in saline (or phosphate buffer pH 6.0) 1:4, centrifuged at 5000 rpm and supernatant was used for determination of biochemical parameters. Lipid peroxidation level was expressed as MDA concentration in homogenates of heart tissue and gastric mucosa. It was measured according to the procedure of Timirbulatow et al. [8]. The content of nitrogen oxide in homogenate was determined by methods of Green, David, 1982 [3]. Activity of enzymes of the antioxidant protection system was evaluated on the basis of determination of SOD [1] and catalase activity by the method of Korolyuk [5].

RESULTS

Under a long-term blockage of COX-2, changes of gastric mucosa morphology in rats were as follows: impaired mucous barrier of gastric mucosa, desquamation of cells, edema. Under the effect of celecoxib, width of gastric mucosa increased by 7% and was 217.96 ± 6.73 μm, the area of transverse section of the nuclei of endocrine cells was 1.82 ± 0.11 μm^2, the area of transverse section of the nuclei of parietal cells was 2.73 ± 0.21μm^2 having increased by 31% (Fig.1). Morphological changes due to injection of {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-acetic acid that are evidence of a higher degree of preserved integrity of the mucous barrier components, decreased the number of ulcerative lesions, increased the density of epithelial cells of the surface of mucous membrane, reduced the degree of edema. Due to injection of {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-benzene-sulfonamide, the protective effect upon the status of mucous membrane was less manifested whereas in the area of the base of the gastric glands and submucous region morphological changes did not differ from the state of the norm (Fig. 1).

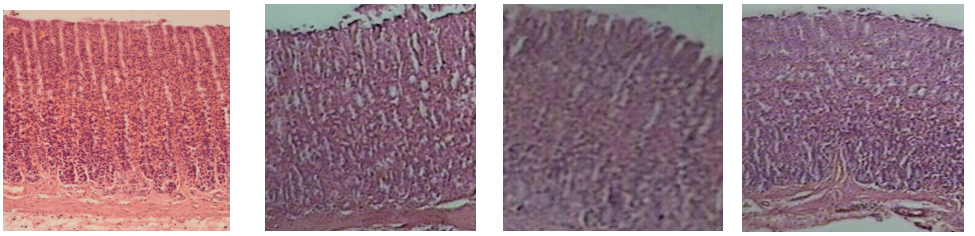

Fig. 1. Histological changes of the gastric mucosa caused by the action of: 2 – celecoxib; 3 –2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-py-rrolidin-1-yl}-acetic acid; 4 – {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-benzene-sulfonamide in comparison with – 1 – intact animals gastric mucosa. Magnification of 1:56

COX-2 inhibition by celecoxib caused the increase of MDA content in heart tissue by 37%, indicating activation of lipoperoxidation. After inhibition, both COX and LOX by {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-acetic acid and {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-benzene-sulfonamide MDA content in heart tissue was also increased, but less than after celecoxib action (by 28% and 30%, subsequently). MDA concentration almost was not changed in gastric mucosa after action of these 3 types of inhibitors (Fig. 2).

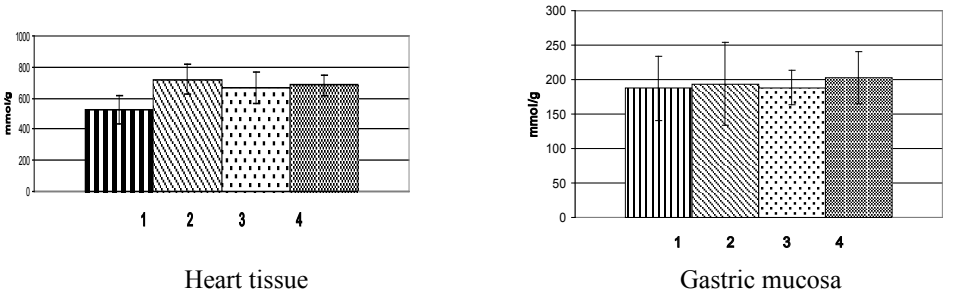

Fig. 2. MDA concentration in heart tissue and gastric mucosa: 1 – control group; 2 – celecixib; 3 – 2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-py-rrolidin-1-yl}-acetic acid; 4 – {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-benzene-sulfonamide

NO concentration was 21% higher than normal in heart tissue after COX-2 blockage, whereas agent 2 caused increase of NO concentration only by 7%. NO content almost was not changed in gastric mucosa after celecoxib action. COX/LOX dual inhibition led to a considerable rise in NO concentration in gastric mucosa (by 40% and 22%) (Fig. 3).

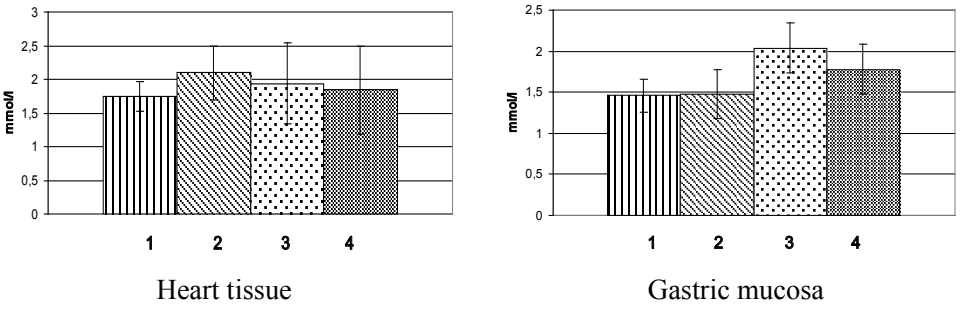

Fig. 3. NO content in heart tissue and gastric mucosa: 1 – control group; 2 – celecixib; 3 – 2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-py-rrolidin-1-yl}-acetic acid; 4 – {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-benzene-sulfonamide

Inhibition of COX-2 as well as COX/LOX dual inhibition led to the increase activity of the antioxidant protection system enzymes (catalase, SOD) in both investigated tissues. Celecoxib possesses antioxidant properties itself and increases the activity of SOD (fig. 4). Celecoxib application caused increased catalase activity in heart tissue by 28%, in gastric mucosa by 26%, {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-acetic acid increased catalase activity in heart tissue by 24%, in gastric mucosa by 29%, {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-benzene-sulfonamide enhanced catalase activity in heart tissue by 20%, in gastric mucosa 23%.

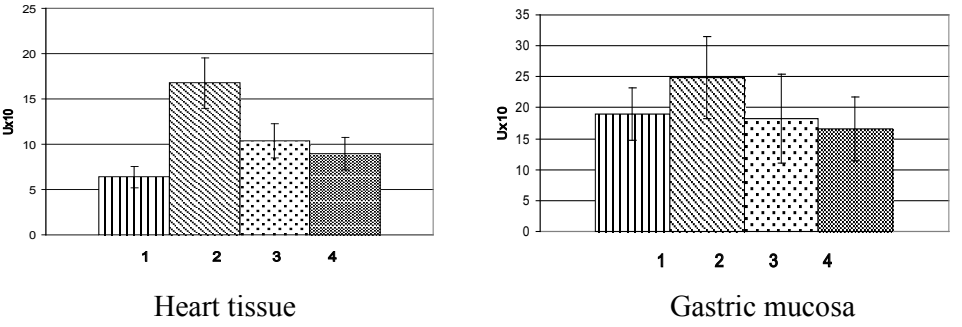

Fig. 4. Activity of SOD in heart tissue and gastric mucosa: 1 – control group; 2 – celecixib; 3 – 2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-py-rrolidin-1-yl}-acetic acid; 4 – {2,5-Dioxo-3-[4-oxo-5-(3-phenyl-allylidene)-2-thioxo-thiazolidin-3-yl]-pyrrolidin-1-yl}-benzene-sulfonamide

DISCUSSION

Recent investigations have established that COX-2 selective inhibitor celecoxib in addition to its direct anti-inflamatory action can also act through COX-independent mechanisms. Currently, celecoxib derivatives have been developed to inhibit PKB/AKT or to disrupt the mitochondrial membrane potential and have anticarcinogenic activity without inhibiting COX [4]. This indirect action of celecoxib could be a reason why we have obtained morphological changes in gastric mucosa after prolonged application of this inhibitor.

Celecoxib did not cause any significant metabolic changes in gastric mucosa as well as thiazolidin-derivatives, possessing dual COX/LOX inhibition. It was shown before [6] that COX-2 selective inhibitors do not own gastrotoxicity. At the same time, their use is associated with the increase of cardiovascular risk by creating an imbalance between thromboxane A_2 and prostaglandin I_2, leading to vasoconstriction and thrombosis. In our investigations it was established that under prolonged inhibition of COX-2 lipoperoxidation processes in heart tissue where intensified, which can be the result of its thrombotic action.

CONCLUSIONS

Long-term blockage of COX-2 caused the impairement of mucous barrier of gastric mucosa, desquamation of cells, edema. Morphological changes due to injection of dual COX/LOX inhibitors which are evidence of a higher degree of preserved integrity of the mucous barrier components, decreased the number of ulcerative lesions, increased the density of epithelial cells of the surface of mucous membrane, reduced the degree of edema.

COX-2 inhibition by celecoxib led to intensification of lipoperoxidation processes in heart tissue. Activity of enzymes of the antioxidant protection system was increased under these conditions.

Changes in NO content and activity of lipoperoxydation processes after prolonged dual COX/LOX inhibition were less marked in both tissues, comparing with the action of celecoxib.

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SUMMARY

Dual COX/LOX inhibitors, by acting on the two major arachidonic acid metabolic pathways – cyclooxygenase and lipooxygenase, possess a wide range of anti-inflammatory activity. Besides that, dual inhibitors appear to be almost exempt from gastric and cardiovascular toxicity. COX-2 prolonged inhibition by celecoxib led to intensification of lipoperoxidation processes in heart tissue. Activity of enzymes of the antioxidant protection system was increased under the action of celecoxib as well as under the action of thiazolidin-derivatives, possessing dual COX/LOX ingibitory activity. Changes after prolonged dual COX/LOX inhibition were less marked in both tissues, comparing with the action of celecoxib.

Keywords: NSAIDs, cyclooxygenase, lipooxygenase, lipoperoxydation, nitric oxide

STRESZCZENIE

Podwójne inhibitory COX/LOX, poprzez działanie na dwa główne szlaki metaboliczne kwasu arachidonowego – cyklooksygenazę i lipooksygenazę, posiadają szerokie spektrum aktywności przeciwzapalnej. Ponadto podwójne inhibitory wydają się prawie całkowicie pozbawione działań toksycznych na żołądek i układ sercowo-naczyniowy. Długotrwałe hamowanie COX-2 przez celekoksyb prowadzi do intensyfikacji procesów lipoperoksydacji w tkance serca. Aktywność enzymów ochronnego systemu antyoksydacyjnego była zwiększona pod działaniem celekoksybu, podobnie jak w efekcie działania pochodnych tiazolidynowych, posiadających podwójną aktywność hamującą COX/LOX. Zmiany pojawiające się po długotrwałym stosowaniu inhibitorów COX/LOX w obydwu narządach były jednak w mniejszym stopniu zaznaczone niż po stosowaniu celekoksybu.

Słowa kluczowe: NSAID, cyklooksygenaza, lipooksygenaza, lipoperoksydacja, tlenek azotu

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Non-invasive markers of hepatic encephalopathy under chronic hepatitis C and 2-oxoglutarate treatment

Nieinwazyjne markery encefalopatii wątrobowej w przebiegu wirusowego zapalenia wątroby typu C i po podaniu 2-oksoglutaranu

INTRODUCTION

Chronic hepatitis causes progressive brain damage (hepatic encephalopathy, HE). A key role for ammonia in the pathogenesis of both HE and brain edema is now firmly supported by clinical and experimental data. The astrocytes play the key role in HE pathogenesis. The metabolism of ammonia to glutamine providion by astrocytes down regulates the osmotic disturbance and mitochondrial dysfunction with oxidative stress in the brain [4].

The main goal of our work was investigating the level of non-invasive markers of hepatic fibrosis and astroglia damage under chronic hepatitis C (CHC) and 2-oxoglutarate treatment. The non-invasive markers of chronic hepatitis C (CHC) – total protein, urea and hyaluronic acid in the blood serum are well known [2]. However, blood serum markers for astrocytes disturbance are less studied.

MATERIAL AND METHODS

The experimental CHC was developed according to patent № u2006004614 [7]. 32 Wister rats were used for the experiment according to European ethical rules. In short, carbon tetrachloride (CCL4) at a dose of 0.25 ml 50% solution in the refined oil was administered under the skin of back rat paws four times every 5 days, and after that – the complete Freund's adjuvant 0.5 ml, containing 0.5 mg of BCG and 5 mg of liver protein homogenate, was injected in the root of the rat tail; after 8 days the azathioprine was administered at a dose of 50 mg/kg, next 7 days after – complete Freund's adjuvant with liver homogenate 0.25 ml, then 5 days after – azathioprine, 4 days after – Freund's adjuvant with liver homogenates 0.25 ml. 2-oxoglutarate was given to the rats in drinking water (0.228%) during 2 weeks after CHC development.

The level of S-100b protein was measured with ELISA using monospecific polyclonal antiserum against S100b (Sigma, USA) and highly purified S100b (Sigma) as a standard. Optical density was measured with the help of Anthos-2010 absorbance reader (Anthos Labtec Instruments, Austria). The concentration of total protein was measured using the Bradford protein assay [3]. The level of urea in the blood serum was measured with Urea kit (Reagent, Ukraine), the concentration of hyaluronic acid – according to Gold methods [5]. Statistical analysis was performed using Statistica software (version 5, StatSoft, Tulsa, OK, USA). Values are shown as mean ± standard error of the mean (SEM).

RESULTS

The development of CHC induced decreased level of the total serum protein (49.9±4.0 mg/ml compared to the control 62.4±2.2 mg/ml) and urea in the blood serum (5.62±0.22 mmol/l compared to the control 7.26±0.13 mmol/l). The level of hyaluronic acid in the blood serum was increased to 2.74±0.11 μg/ml compared to the 2.1±0.07 μg/ml under CHC condition (Fig. 1). The development of CHC leads to elevation of the calcium-binding protein S-100b level (specific for astrocytes) in all studied brain regions (data not presented). The immunohistochemical data show that the number of astrocytes in the cerebellum changed in the Alzheimer type II cells was elevated. It was coincident with increased level of the S-100b in the blood serum. The treatment with 2-oxoglutarate 0.228% in drinking water 2 weeks after CHC development prevents increasing uptake of that protein to the blood and decreasing total serum protein, while the increased level of hyaluronic acid and urea were still saved in the blood.

DISCUSSION

Strong elevation (more than to four times) of S-100b concentration in the blood indicated decreased properties of blood brain barer (BBB) during chronic hepatitis C development. Brain accumulation of calcium occurs in a number of pathological conditions. Biochemical data indicated the elevation of S-100b level in cytosolic fraction of different brain regions of rats with CHC which reflects astrocyte reaction to the liver toxicity. Hepatic encephalopathy (HE) is a common reversible neuropsychiatric syndrome associated with chronic and acute liver dysfunction and significant morbidity and mortality. Although a clear pathogenesis is yet to be determined, elevated ammonia in the serum and the central nervous system are the mainstay for pathogenesis and treatment. An increased ammonia level raises the amount of glutamine within astrocytes, causing an osmotic imbalance resulting in cell swelling and ultimately brain oedema [4]. Early studies noted a significant inhibition of brain mitochondrial ketoglutarate dehydrogenase at pathological concentrations of ammonia (0.2–2 mM), possible calcium accumulation have been proposed as a deleterious effect on KGDH activity [6]. Moreover, minimal hepatic encephalopathy (MHE) is a neuro-cognitive dysfunction which occurs under hepatic disorders characterized by a specific, complex cognitive dysfunction which is independent of sleep dysfunction or problems with overall intelligence [1]. Minimal hepatic encephalopathy can have a far-reaching impact on the quality of life, and progression to overt hepatic encephalopathy. The non-invasive testing for MHE and a subsequent therapy is very important to consider this under different hepatic disorders. The level of increased concentration of S-100b in the

blood serum can help to indicate the level of risk to hepatic encephalopathy development. Effective treatment options for hepatic encephalopathy are still limited. Based on the principle that ammonia contributes to the pathogenesis of hepatic encephalopathy, current therapeutic approaches are directed at enhancing its elimination. Management includes prompt treatment of precipitating factors (infection, gastrointestinal bleeding, electrolyte disturbances, hepatocellular carcinoma, dehydration, hypotension, and the use of benzodiazepines, psychoactive drugs, and/or alcohol). Newer therapies being investigated in humans with clinical promise include 2-oxoglutarate. Since ammonia freely crosses the blood-brain barrier and astrocytes are responsible for maintaining the BBB, the presence of extra 2-oxoglutarate in the blood could produce a rapid glutamine synthesis. It could, therefore, prevent the entry of high amounts of ammonia from circulation to attenuate neurotoxicity. Our data indicate that the treatment with 2-oxoglutarate in a dose 0.228% in the drinking water during 10 days after CHC development down regulates the level of S-100b in the blood.

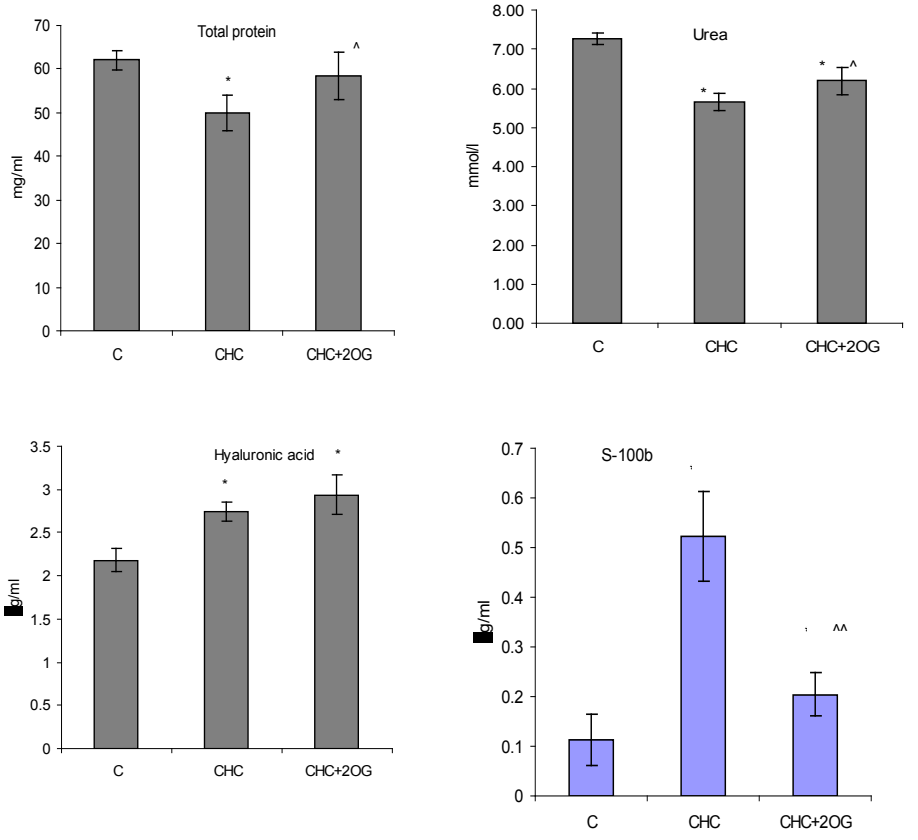

Fig. 1. The level of the non-invasive markers of liver fibrosis and astrocyte damage in the blood serum; C – control; CHC – chronic hepatitis C; CHC+2OG – treatment with 2-oxoglutarate, 0.228% in drinking water during 2 weeks after CHC development; n=6; * $p<0.001$ (compared to the control group); ^ $p<0,1$; ^^ $p<0.001$ (compared to the CHC group)

CONCLUSIONS

The obtained data allow us to suggest that the S-100b level in the blood serum may be used as a marker of astrocytes damage induced by liver toxicities under chronic hepatitis C. The treatment with 2-oxoglutarate can prevent serious brain damage.

This study was supported by Essentys AB (Ideon beta, Lund, Sweden).

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SUMMARY

The level of the non-invasive markers of hepatic fibrosis and astroglia damage under chronic hepatitis C (CHC) and 2-oxoglutarate treatment was investigated. The obtained data allow for the suggestion that S-100b level in the blood serum may be used as a marker of hepatic encephalopathy under CHC, the treatment with 2-oxoglutarate can prevent serious brain damage caused by ammonia effect.

Keywords: chronic hepatitis C, encephalopathy, S-100b, 2-oxoglutarate

STRESZCZENIE

Określono poziomy nieinwazyjnych markerów zwłóknienia wątroby i zniszczenia astrogleju w efekcie przewlekłego zapalenia wątroby typu C i podawania 2-oksoglutaranu. Uzyskane dane sugerują, że poziomy S-100b w surowicy krwi mogą być stosowane jako marker encefalopatii wątrobowej w przebiegu WZW typu C, zaś podanie 2-oksoglutaranu może zabezpieczyć przed poważnymi uszkodzeniami mózgu spowodowanymi działaniem amoniaku.

Słowa kluczowe: przewlekłe zapalenie wątroby typu C, encefalopatia, S-100b, 2-oksoglutaran

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The comparative effect of rabeprazole vs. omeprazole on gastric acid and mucoid-electrolite secretion in patients with peptic ulcer disease

Badania porównawcze wpływu rabeprazolu vs omeprazolu na wydzielanie kwasu żołądkowego i śluzowo-elektrolitowe u pacjentów z chorobą wrzodową

INTRODUCTION

Proton pump inhibitors (PPI) are widely used in the treatment of acid-related diseases. Admission of PPI results in a rapid removal pain syndrome and healing of erosive and ulcerous defects [1, 6].

Despite the fact that the pharmacological activity of PPI of first and second generations is identical, there is a big difference between omeprazole and rabeprazole [4]; including different time of activation due to pH of gastric juice, time interval of achieving desired pH for *H. pylori* eradication, antihelicobacter activity of prescribed drugs, bioavailability then taking additional dose, "first dose" effect [2, 3, 7]. These differences in omeprazole and rabeprazole are shown by different clinical presentations, "night rises of acidity" when taking omeprazole comparing to rabeprazole [10].

In the mechanisms of action, rabeprazole is more effective than omeprazole, lansoprazole or pantoprazole in acid suppression, increasing intragastric pH and maintaining pH>4 [5, 9]. The favourable phrarmacodynamic profile for rabeprazole has been shown to result in high eradication rates for *H. pylory* than other drugs of this group [8]. However, little it is still known about the mucoid-protecting action of rabeprazole vs. omeprazole.

The aim of the study was to evaluate the effects of rabeprazole vs. omeprazole on gastric acidity and mucoid-electrolyte secretion in patients with peptic ulcer disease after two weeks' treatment with these PPI.

MATERIAL AND METHODS

The examined group consisted of 42 patients. 6 (15%) of them had stomach peptic ulcer, 19 (47%) had duodenal ulcer and 17 (38%) had combined stomach and duodenal ulcers. The mean age was 34.0 ± 3.4 years. The mean time of illness was 5.0 ± 1.1 years. Patients were divided in 2

adequate groups by age and course of disease: first group – 27 patients, which took the following combination for 14 days: omeprazole – 20 mg/daily, amoxiciline – 1000 mg/daily, clarithromycine – 500 mg/ twice daily; second group – 15 patients, which took the following combination for 14 days: rabeprazole – 20 mg/daily, amoxiciline – 1000 mg/daily, clarithromycine – 500 mg/twice daily. All patients were confirmed positive *H. pylori* by 14 C urea breath test and urease test. The healing of ulcers was controlled by fibrogastroscopy after 2 weeks of treatment. The severity of ulcer pain was investigated during the treatment.

The volume of gastric aspirate, hydrogen ion concentration, pepsin concentration were measured and compared before and after treatment with rabeprazole or omeprazole. N-acetylneuraminic acids (NANA) and Na^+-ions concentrations were studied in gastric juice and insoluble mucus.

RESULTS

After 2 weeks' treatment with omeprazole and antibiotics, ulcers healed in 70% of patients, although all patients kept complaints about active antral gastritis.

Prescription of omeprazole led to the lowering of volumetric secretion by 54%, pepsin concentration by 13%, general acidity by 4%. During this treatment we did not observe significant changes of NANA concentration in gastric juice and insoluble mucus. In the majority of cases Na+-ions concentration was higher in gastric juice comparing to insoluble mucus.

After 2 weeks' treatment with rabeprazole healing of ulcerous defects controlled by gastroscopy was achieved in 100% cases, still with active antral gastritis and duodenitis.

Administration of rabeprazole led to lowering of volumetric secretion by 50%, general acidity by 72% and pepsin concentration by 82%. With the significant lowering of gastric acidity, the level of mucus-electrolyte secretion risen.

Taking into account the overall stability of NANA concentration and Na+-ions in insoluble mucus, the volume of those components in gastric juice almost doubled. After 2 weeks of treatment with rabeprazole, Na+-ions concentration was higher in gastric juice comparing to insoluble mucus, which correlates with increase in occurance of duodeno-gastric reflux.

DISCUSSION

During the treatment with omeprazole in some part of the patients with peptic ulcer there were the remaining symptoms of active antral gastritis and duodenitis. Omeprazole decreases gastric acidity by lowering volumetric secretion, at the same time without affecting general acidity, pepsin and NANA concentrations, which can be the reason for recurrent ulcers. Comparing to omeprazole, administration of rabeprazole leads to faster pain relief and better ulcer healing results, which can be explained by better lowering of volumetric secretion, practically full block of acid secretion and pepsin release. Another advantage of rabeprazole is its availability to increase mucus and electrolyte secretion. Rabeprazole is considered to be a more effective drug in peptic ulcers healing comparing to omeprazole.

CONCLUSIONS

Rabeprazole demonstrated a greater inhibition of acid secretion and peptic release than omeprazole. Another advantage of rabeprazole is its availability to increase mucoid secretion in comparison with omeprazole. At the same time, administration of rabeprazole or omeprazole increases the frequency of duodeno-gastric reflux.

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SUMMARY

Rabeprazole provides superior acid suppression and inhibits pepsin release in comparison to omeprazole. Better ulcer healing results after management by rabeprazole are caused by its gastric mucosa protecting effect in compare with omeprazole.

Keywords: peptic ulcer, rabeprazole, omeprazole

STRESZCZENIE

Rabeprazol zapewnia lepszą supresję kwasu i hamowanie uwalniania pepsyny niż omeprazol. Lepsze wyniki leczenia wrzodów po zastosowaniu rabeprazolu w porównaniu z omeprazolem spowodowane są jego wpływem ochronnym na śluzówkę żołądka.

Słowa kluczowe: wrzód żołądka, rabeprazol, omeprazol

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Effect of pro-inflammatory cytokine-mediated mechanism on quality of gastrointestinal restitutio ad integrum

Wpływ mechanizmu prozapalnego mediowanego cytokinami na jakość restytucji żołądkowo-jelitowej *ad integrum*

INTRODUCTION

The existing treatment of acid-related diseases is mostly based on potent acid suppression. Recent studies have revealed that the widespread use of prolonged acid suppression is associated with dismotility in the upper gastrointestinal (GI) tract, hypergastrinemia, dysbiosis [1]. Chronic gastroesophageal reflux disease (GERD) bears the risk of Barrett's esophagus that is a premalignant stage of esophageal adenocarcinoma. WHO warns that the risk of Barrett's esophagus increases by 30 to 50% per decade of life in patients with GERD. Inflammatory reaction is central to the majority of manifestations of GERD and a starting point for abnormal *restitutio ad integrum*. *Restitutio ad integrum* is a total recovery performed by natural healing systems of an organism and the key characteristic of the GI epithelial barrier [6, 11]. Melatonin (MT) may take part in protecting the esophagus from tissue damage [2, 7]. It is well known that melatonin possesses GI resistance to luminal damaging agents due to the free radical scavenging cascade, vasodilatatory and cytoprotective effects [4, 5, 9]. The main role in the *restitutio ad integrum* is played by intercellular and apical glycoconjugates. They represent a signaling system of cell functioning. The aim of our study was to examine if inflammatory signaling contributed to the esophageal phenotype of cytoprotection and healing activity during MT pretreatment.

MATERIAL AND METHODS

Male Wistar rats which were used in our studies were divided into five groups. One was the intact control group. The other rats were inducted erosive esophageal acidic lesions by perfusion solution of 0.25 N HCl with pepsin and non-erosive esophageal mucosa (EM) lesions by water-immersion restraint *stress* (WRS) by Takagi, 1964 without and with melatonin (20 mg/kg/ip) treatment. The

healing process of EM was monitored after 24h of injury. To estimate the damage, inflammation and hyperplasia of the EM and lectin histochemistry examination, segments of the lower third of esophagus were extracted from the animals body. A score system was used for estimation of the degree of epithelial changes. The experiment included revealing of both the macroscopic morphological changes and histological changes. To access the glycoconjugates of EM lectin sets were used. The lectin set included using peanut agglutinin (PNA, specific to BDGal→DGalNAcDGal), Sambucus nigra agglutinin (SNA, specific to Neu5 Ac(2-6)Gal), Helix pomatia agglutinin (HPA, specific to DGalaNAc), wheat germ agglutinin (WGA, specific to DGlcNeuNAc) conjugated to peroxidase (purchased from Lectinotest Lab, Ukraine). Images of histological slices were investigated using a digital video camera connected to a microscope (MBI-15-2, LOMO, Russia) and were processed using the AVerMedia FZC Capture image analysis program (AVerMedia Technologies, Inc., USA). To study the functioning of the proinflammatory signaling mechanism in the serum; IL-1beta, TNFalpha cytokines were examined by enzyme-linked immuno sorbent assay (ELISA) by kits "Diaclone" (France). For a comparison of data, we used a paired Newman-Keuls's test with the level of significance at $P< 0.05$.

RESULTS

In the groups of rats with the MT pre-treatment less damage to EM, hyperplasia of basal membrane and akantosis was revealed. There was a variety of expressions and epithelial patterns of lectins (PNA, SNA, WGA) in different groups of rats. Generally, the lectin expression decreases after acidic perfusion in different epithelial layers, but it increases in rats with the MT pretreatment by modification of glycoprotein pattern in EM. These changes are accompanied by increased activity of mucus-producing cells. Lectin expression in NEE inducted by stress is different compared to the expression caused by acidic injury. PNA expression is weaker, but SNA expression is stronger in different epithelial layers. With melatonin pretreatment the most intense PNA expression was found in stratum spinosum and basal cells. The strongest SNA expression was found in the basal layer. WGA expression shows no visible differences. Moreover, changes in carbohydrate moieties of glycoconjugates revealed MT-induced activation of cytoprotection mechanism that caused marked thinning of esophageal epithelium and a decrease of papillary due to faster luminal cell migration and more rapid epithelial regeneration after injury. In the control group, IL-1beta content was 24.16±0.30 pg/ml, TNFalpha – 1.05±0.11 pg/ml. Induction of acid-peptic esophageal lesions led to increased IL-1beta to 55.35 ± 7.88 pg/ml, TNFalpha – 5.56 ± 0.55 pg/ml ($p <0.05$). Melatonin treatment reduced IL-1beta to 46.4% and TNFalpha – 16.2% in comparison to the previous group ($p<0.05$). In WRS-induced EM lesions IL-1beta increased to 38.7%, TNFalpha – 65.6% relatively to control ($p <0.05$). Melatonin caused a decrease of IL-1beta to 21.8%, TNFalpha – 35.1% compared to the control. Acidic perfusion induced topical destructive EM lesions and HSI was 2.5-folds time more than with melatonin treatment; WRS-induced non-erosive esophagitis was with constantly increased HSI in 150% in comparison to melatonin treated rats showing the same effect as in the model of acidic esophagitis. These changes show an obvious anti-inflammatory effect of melatonin.

DISCUSSION

MT, potent antioxidant, vasodilatatory agent is intensively studied in recent investigations, especially in experimental and clinical gastroenterology. Prolonged inflammation is the key factor in abnormal *restitutio ad integrum* which caused metaplasia in foregut epithelial barrier [3, 8]. The presented *in vivo* studies demonstrated in two experimental models of esophageal injury that acidic-pepsin erosive esophagitis and stress-induced non-erosive esophagitis that MT strongly exhibits anti-inflammatory activity via decreased synthesis of the IL-1beta TNFalpha. The exposed phenomenon regarding changes of the esophageal barrier induced during MT treatment healing indicate modification of the synthesis of DGlcNeuNAc, Neu5 Ac(2-6)Gal, DGalaNAc specific glycoconjuges associated with signs of hyperkeratosis, hyperproliferation and local microcirculatory lesions. MT exerts an anti-inflammatory effect on the EM, particularly on inhibition leukocytes infiltration, which stimulate the quality of *restitutio ad integrum.* Also, important changes in the EM expression of mucin and in their glycosylation state were shown in this stress-induced studies of non-erosive esophagitis because they are closely associated with the development silent signs of precancer-related processes such as Barrett esophagus. In this study we established the phenotype of esophageal epithelial barrier profile of glycoconjugates during the healing process is a result of involvement of glycosylation in the essential biological process of inflammation [10] and modification of glycoconjugates are novel valid biomarkers of it. Of course, these investigations did not elucidate the full mechanism of *restitutio ad integrum*. In fact, future studies with molecular tools are necessary for identification of the signaling pathways of aberrant glycosylation. Moreover, the glycoconjugates profile with pre-malignant esophageal tissue (during Barrett esophagus) shoud be investigated.

In summary, the results of the present study indicate that the quality of *restitutio ad integrum* of erosive and non-erosive esophagitis is related to pro-inflammatory cytokine-mediated mechanism, which is the basis for the formation phenotype of EM protective responses. MT, perspective and potent anti-inflammatory agent, would be beneficial to antiulceric and healing action.

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SUMMARY

We studied the morphofunctional changes in esophageal mucosa after the experimental injury by acid-pepsin perfusion and stress inducted injury with and without melatonin treatment. Melatonin-treatment enhanced epithelial proliferation and accelerated the healing activity of the esophageus tissue. Melatonin, a perspective and potent anti-inflammatory agent, can be used with antiulceric and healing scope.

Keywords: esophageal mucosa, melatonin, ulcer

STRESZCZENIE

Oceniono zmiany morfotyczno-funkcjonalne w śluzówce przełyku po jej eksperymentalnym uszkodzeniu pod wpływem perfuzji kwasowo-pepsynowej i uszkodzeniu indukowanym stresem bez leczenia i po leczeniu melatoniną. Podawanie melatoniny zwiększało proliferację nabłonka i przyspieszało proces zdrowienia tkanki przełyku. Melatonina, potencjalny środek przeciwzapalny, może być stosowana w celu zapobiegania rozwojowi wrzodów jak też w celu ich leczenia.

Słowa kluczowe: śluzówka przełyku, melatonina, wrzód

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Effects of Bergenia crassifolia lyophilized water extract on carbon tetrachloride-induced chronic liver injury in rats

Wpływ liofilizowanego wodnego ekstraktu *Bergenia crassifolia* na przewlekłe uszkodzenie wątroby indukowane czterochlorkiem węgla

INTRODUCTION

Bergenia crassifolia is used in clinical practice as astringent, anti-inflammatory, antimicrobial, haemostatic agent. Decoction and fluid extract made of the rhizome of *Bergenia* are recommended for the treatment of colitis, enterocolitis, stomatitis, gingivitis, uterine bleeding. Drug dosage forms made of leaves of this plant were shown to produce antibacterial, antioxidant, cerebroprotective, choleretic, diuretic effects.

Bergenia crassifolia contains a complex of biologically active substances like flavonoids, coumarins, phenolglycosides, polysacchirides, vitamins, organic acids, aminoacids et. al [6]. One of the most important active substance of *Bergenia crassifolia* is bergenin. Recently it has been shown to have hepatoprotective activity and flavonoids have been shown to possess antioxidant properties [3, 7].

The aim of this study was to investigate the ability of *Bergenia crassifolia* lyophilized water extract (BCLWE) to prevent chronic CCl_4 -induced liver injury.

MATERIAL AND METHODS

BCLWE was prepared by original technology as described previously [2]. Adult male rats weighing 220–280 g were used. Animals were maintained on a standard diet, given water *ad libitum*, and housed in a temperature- and humidity-controlled room, under a constant 12-h light/dark cycle.

The Local Ethics Committee approved the study. The procedures involving the animals and their care conformed to the institutional guidelines and were in compliance with national and international laws and guidelines for the Use of Animals in Biomedical Research.

Animals were randomly divided into three experimental groups: 1st – control group; 2nd – animals receiving CCl_4; 3rd – animals receiving CCl_4 and BCLWE. Each group consisted of 10 animals.

Chronic liver injury was induced by repeated intragastric administration of 20% CCl_4 solution in olive oil (2 ml/kg b.wt.) two times a week for 30 days. BCLWE (80 mg/kg b.wt.) was administered daily in the form of suspension intragastrically throughout the experimental period. The control group received an equivalent amount of water intragastrically every day throughout the experimental period.

Three days after the last CCl_4 administration, the rats, were sacrificed under ether anesthesia; the liver was removed for examination and blood samples were taken. Serum albumin, bilirubin, aspartate transaminase, alanine transaminase, alkaline phosphatase, gamma-glutamyltranspeptidase activities, cholesterol were determined using biochemical analyzer BTS-370 (Spain).

For histology, the right lobe of each liver was fixed in 10% buffered formalin for 24 h and embedded in paraffin. For each staining, to prevent any bias because of the sampling error, the quantification was performed by the analysis of at least 10 different regions and examined by the same experienced pathologist who was blinded to laboratory data. The sections were stained with haematoxylin and eosin, chromotrope aniline blue. Haematoxylin and eosin slides were used for morphologic evaluation, assessment of steatosis, infiltration and hepatocellular ballooning. Collagen fibers were demonstrated by chromotrope aniline blue. Following the previously described methods, the hepatocyte injury (alteration) was semi-quantitatively assessed using a scoring system of 0 = microvesicular steatosis in the perivenular region (no injury); 1 = microvesicular steatosis in the perivenular region and single swollen hepatocytes (mild injury); 2 = microvesicular steatosis in the perivenular region and mid-zone, periseptal hepatocyte balooning (moderate injury); and 3 = diffuse microvesicular steatosis, periseptal hepatocyte balooning, apoptosis and coagulation (severe injury).

Fibrosis was also assessed using semiquantitave scoring system: 0 = normal portal tracts (no fibrosis); 1 = sclerosis of portal tracts and single short connective tissue septa (mild fibrosis); 2 = multiple long connective tissue septa (porto-central) (moderate fibrosis) 3 = cirrhosis (nodularity) (severe fibrosis).

The data are expressed as means ± SD. One-way analysis of variance, followed by Newman-Keuls test, were performed for multiple comparison among the groups. Values of $P< 0.05$ were considered statistically significant.

RESULTS

The serum biochemical and histological changes in three groups of rats are summarized in Table 1.

Table 1. Biochemical and histological variables in rats

	Group 1 Control	Group 2 CCl_4	Group 3 CCl_4 + BCLWE
Albumin	23.81±1.47	23.51±1.31	23.85±1.38
Bilirubin	2.93±0.55	3.07±0.46	2.74±0.44
Cholesterol	2.09±0.24	2.01±0.41	2.19±0.25
AST U/L	173.01±19.12	389.61±120.22*	275.76±42.01**
ALT	160.07±24.54	329.67±94.87*	224.43±41.22**
GGTP	40.53±14.59	43.49±8.95	46.16±11.39
ALP	279.91±38.19	770.38±104.38*	743.16±108.67*
Alteration (n(%))		*	**
0	8 (80)	-	-
1	2 (20)	2 (20)	8 (80)
2	-	4 (40)	2 (20)
3	-	4 (40)	-
Fibrosis (n(%))		*	
0	9 (90)	-	-
1	1 (10)	-	1 (10)
2	-	8 (80)	9 (90)
3	-	2 (20)	-

*p< 0.05 vs control group, **p< 0.05 vs CCl_4 group

Chronic administration of CCl_4 led to the increase of serum activity of AST, ALT, ALP, while serum albumin, bilirubin, cholesterol and GGTP levels were unchanged. Most rats showed a high degree of alteration and fibrosis: diffuse or focal microvesicular steatosis, multiple foci of necrosis and apoptosis; porto-portal and porto-central septa with nodule formation – cirrhotic transformation. Morphological findings were statistically significant for alteration and for fibrosis.

Compared with the CCl_4 group, in the CCl_4 + BCLWE group serum enzymatic activity had a tendency to improve. Serum AST and ALT levels were lower in the CCl_4 + BCLWE group, while serum ALP level was about the same in both groups. There were no significant differences in serum albumin, bilirubin, cholesterol and GGTP levels among three groups of rats. In rats receiving CCl_4 + BCLWE degrees of parenchymal injury and fibrosis are lower. The histological examination in most animals shows microvesicular steatosis in the perivenular region and mid-zone; signs of cellular death are not prominent. Fibrosis is manifested by enlarged portal tracts, several short and porto-portal connective tissue septa associated with indistinct nodularity. However, these differences are not statistically significant.

DISCUSSION

In the present study administration of BCLWE partially prevented elevation of serum ALT and AST, it attenuated parenchymal injury and fibrosis in rat liver induced by chronic administration of CCl_4. Hepatoprotective effect was confirmed by a statistically significant decrease in cell injury score – signs of necrosis and apoptosis were less prominent in animals treated with BCLWE. Antifibrotic effect was not so distinct. The signs of fibrosis were of moderate degree, but these differences were not statistically significant. These results coincide with that of M.W. Chung [3] who established that bergenin and acetyl bergenin ameliorated the liver damage induced by bile duct ligation in rats. In this study, bergenin and acetyl bergenin prevented elevation of serum AST and ALT, reduced accumulation of hydroxyproline, decreased the damaged area. The mechanisms underlying these effects are not known. Based on the results of Lim et al. [5], hepatoprotective effects of bergenin and acetyl bergenin are related to glutathione-mediated detoxification and their free radical suppressing activity. Antifibrotic activity of BCLWE may be attributed to bergenin-induced stimulation of prostaglandins E1 and E2 synthesis which inhibit the growth of hepatic stellate cells that are essential for the development of liver fibrosis [1].

CONCLUSIONS

BCLWE exerts a hepatoprotective effect against chronic CCl_4-induced lesions of liver tissue.

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SUMMARY

BCLWE exerts a hepatoprotective effect against chronic CCl_4-induced lesions of liver tissue in rats, which is confirmed by prevention of serum ALT and AST activity elevation and progression of liver cell injury and fibrosis.

Keywords: Bergenia crassifolia, hepatic fibrosis, carbon tetrachloride

STRESZCZENIE

Liofilizowany ekstrakt wodny *Bergenia crassifolia* wykazuje działanie hepatoprotekcyjne na tkankę wątroby z przewlekłym uszkodzeniem spowodowanym CCl_4, co potwierdzono brakiem wzrostu ALT i AST w surowicy oraz postępu uszkodzenia komórek i włóknienia wątroby.

Słowa kluczowe: Bergenia crassifolia, zwłóknienie wątroby, czterochlorek węgla

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Influence of the mixed-metal Cu/Fe complex $[Cu(dmen)_2][Fe(CN)_5(NO)]$ (dmen=N,N-dimethylethylenediamine) on serum iron and copper levels in experimental anemia of rats

Wpływ mieszanego kompleksu metali Cu/Fe $[Cu(dmen)_2][Fe(CN)_5(NO)]$ (dmen=N,N-dimetyletylenediamina) na poziomy żelaza i miedzi w surowicy krwi w doświadczalnej niedokrwistości u szczurów

INTRODUCTION

It is well known that iron and copper play an important role in red blood cells differentiation. Iron is part of hemoglobin and is necessary for hemoglobin synthesis in red blood cells precursors [8]. Hemoglobin transports oxygen from lungs to cells of various tissues. Copper is part of the proteins that transport and store iron [2, 3, 12, 13, 14]. That is why iron and copper deficiency in the organism causes anemia [4, 7]. The level of these ions can be increased using various medicines, including the novel mixed-metal complex $[Cu(dmen)_2][Fe(CN)_5(NO)]$ (dmen = N,N-dimethylethylenediamine) (code KL447) that has been synthesized at the Taras Shevchenko National University of Kyiv. The complex contains both iron and copper ions. The aim of our study was to investigate the effect of this complex on the dynamics of serum iron and copper in experimental anemia of rats.

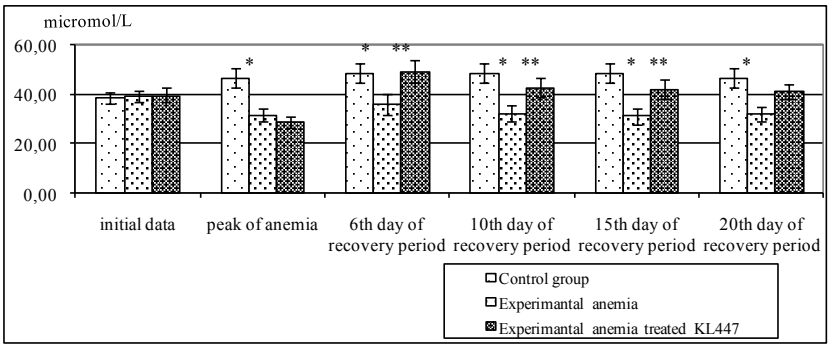
MATERIAL AND METHODS

Experiments were carried out on pubertal age outbred female rats (weight 170–200 g). The rats were divided into three groups: kontrol, experimental anemia and experimental anemia treated with KL447 (eight animals per group). The anemia was caused by the blood loss (1 ml/100 g body weight) 5 times for 10 days. KL447 was dissolved in distilled water and administered *per os* in a daily dose of 25 mg/kg body weight (3 mg/kg Fe(II) and 3.5 mg/kg Cu(II)) since the next day after the last blood

loss during 20 days. The blood was collected from the tail vessels. Serum iron and copper levels were determined using Pliva Lachema's kits and a SUMAL spectrophotometer at the peak of anemia (the next day after the last blood loss), and on the 6th, 10th, 15th and 20th days of the recovery period. Statistical analyses were performed in Excel. The experimental data were normally distributed. Difference between the respective values was statistically significant at $p \leq 0.05$ using Student's t-test.

RESULTS

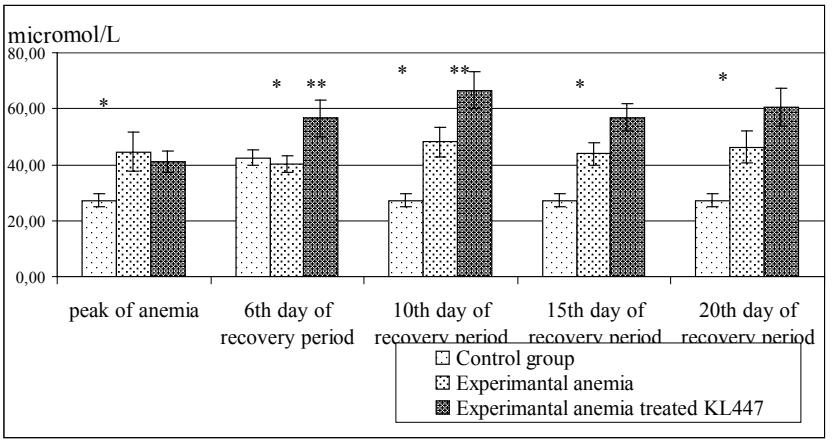
There was no significant difference in serum iron levels between the groups before the experiment (Fig.1). The serum iron levels in the control group revealed a tendency to increase at the peak of anemia in the experimental groups and increased on the 6th day of the recovery period in the experimental groups vs data before the experiment. These changes related to the release of iron ions from storage to be included in erythrocytes recovery (after the blood loss for examination of this parameter). That is why we reduced the blood loss in the control group and compared the last data of iron serum levels in this group with the data of the experimental (anemic) groups on the 10th, 15th and 20th days of recovery period.



* significant difference compared with the control group at $p \leq 0.05$, ** significant difference compared with the group of experimental anemia at $p \leq 0.05$

Fig.1. Influence of mixed-metal complex KL447 on serum iron levels of rats

Serum copper levels were increased in the control group (after the blood loss for examination of this parameter) as well as iron on the 6th day of the recovery period in the experimental groups vs the data at the peak of anemia in the experimental groups (Fig. 2). That is why serum copper levels in the experimental groups were compared with this parameter in the control group at the peak of anemia.



* significant difference compared with the control group at $p \leq 0.05$, ** significant difference compared with the group of experimental anemia at $p \leq 0.05$

Fig. 2. Influence of mixed-metal complex KL447 on serum copper levels of rats

At the peak of anemia, serum iron levels (Fig.1) decreased (31.67±2.47; 28.71±2.55 μmol/l) and the copper level (fig. 2; 44.6±6.97; 41.13±3.75 μmol/l) increased in two anemic groups vs the control group (46.73±3.89; 27.29±2.43 μmol/l, respectively). The serum iron levels were decreased and the copper level was increased during 20 days of the recovery period in the non-treated anemic group vs the control group. KL447 increased serum iron (on the 6th, 10th and 15th days) and copper (on the 6th and 10th days) levels vs the anemic group. The tendency of iron to increase on the 20th and copper on the 15th days of the recovery period retained in the anemic group treated with KL447.

DISCUSSION

Chronic blood loss causes iron defficiency and anemia [5, 9]. In our investigation the blood loss (approximately 10% of the circulating blood volume for five times) in rats results in reduced hemoglobin and serum iron levels. But the serum copper level change in an opposite direction – it increases. These data indicate the role of copper in iron trasport. It is known that copper is part of duodenal enterocytes protein haphaestin that take part in iron absorption [12]. Plasma protein ceruloplasmin (containing copper) plays a role in the mobilization and oxidation of iron from tissue stores with subsequent incorporation into transferrin [3]. Mitochondrial copper-containing proteins frataxin and cytochrome-c-oxidase serve heme synthesis in the erythroblasts [2, 13]. Cu,Zn-Superoxide dismutase catalyses the dismutation of superoxide anions in the cytoplasm and it protects cells against free radicals [14]. That is why the increase of copper serum levels in experimental anemia in the our study can be related to the active synthesis and inclusion of copper in the proteins that participate in the transport of iron. Increase of serum iron levels after treatment with KL447 was shown. KL447 and nitroprusside ($Na_2[Fe(CN)_5NO]\cdot 2H_2O$) have the same anion. Our data correlate with the results of the study nitroprusside influence on serum iron levels in rabbits obtained by

Engeser et al. [1]. Nitroprusside can release iron [11]. It increases heme oxygenase-1 expression and promotes iron regulatory proteins degradation in mouse monocyte macrophage cell line via an increase in intracellular iron [6, 15]. Serum iron levels increase with induction of hepatic heme oxygenase-1 [10]. Moreover, nitroprusside elevates the iron content of the liver more effectively (60%) in comparison with $FeSO_4$ (30%) [1].

CONCLUSIONS

These results indicate that the mixed-metal Cu/Fe complex KL447 causes recovery of the serum iron levels and increases the serum copper levels in the experimental anemia of rats and it could be a potential substance for stimulation of erythropoiesis.

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SUMMARY

The aim of the study was to investigate the effect of novel mixed-metal complex $[Cu(dmen)_2][Fe(CN)_5(NO)]$ (dmen=N,N-dimethylethylenediamine) (code KL447) on dynamics of serum iron and copper in the experimental anemia of rats. At the peak of anemia the serum iron levels were shown to decrease and the copper level – to increase and these retained during 20 days of the recovery period in the anemic group. KL447 increases serum iron and copper levels. These results indicate that the mixed-metal Cu/Fe complex causes the recovery of the serum iron levels and increases the serum copper levels in the experimental anemia of rats and it could be a potential substance for stimulation of erythropoiesis.

Keywords: mixed-metal Cu/Fe complex, serum iron, serum copper

STRESZCZENIE

Celem badań była ocena wpływu nowego kompleksu metali $[Cu(dmen)_2][Fe(CN)_5(NO)]$ (dmen=N,N-dimetylethlenediamina) (kod KL447) na dynamikę żelaza i miedzi w surowicy krwi w przebiegu doświadczalnej niedokrwistości u szczurów. W okresie maksymalnego nasilenia anemii stężenie żelaza w surowicy było obniżone, zaś miedzi – podwyższone i stan ten utrzymywał się przez kolejne 20 dni okresu zdrowienia. KL447 zwiększał poziomy żelaza i miedzi w surowicy. Wyniki przeprowadzonych badań wskazują, iż kompleks KL447, przyczyniając się do wzrostu poziomów Fe i Cu w surowicy krwi, może być potencjalną substancją wykorzystywaną do stymulacji erytropoezy.

Słowa kluczowe: mieszany kompleks metali Fe/Cu, miedź, żelazo, surowica

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Hypoglycemic and antioxidant action of melatonin in alloxan diabetic rats

Działanie hipoglikemiczne i antyoksydacyjne melatoniny u szczurów z cukrzycą alloksanową

INTRODUCTION

Melatonin is a circulating hormone that is primarily released from the pineal gland. It is best known as a regulator of seasonal and circadian rhythms; its levels are high during the night and low during the day. Oral melatonin supplementation may be useful for known sedative and antioxidant properties. Interestingly, insulin levels also exhibit a nocturnal drop, which has previously been suggested to be controlled, at least in part, by melatonin. Increased oxidative stress and impaired anti-oxidant defense have been suggested as contributory factors for initiation and progression of complications in diabetes mellitus. Alloxan is a classical diabetogen which is used to achieve beta-cell destruction and type 1 diabetes due to its selective cytotoxic effect on pancreatic beta-cells. The present study was to determine the protective effects of melatonin in alloxan-induced diabetic rats, and to analyze the parameters related to diabetes and oxidative stress.

The object of this experimental research was to ascertain the influence of exogenous melatonin on the level of basal glycemia (BG), glycosylated hemoglobin (Hba1c), total antioxidant activity (TAOA), malonic dialdehyde (MDA) and glutathione (G-SH) in rats under conditions of alloxan diabetes.

MATERIAL AND METHODS

The experiments were carried out on 67 sexually mature male albino, not thoroughbred rats with the body mass – 0.18–0.20 kg. The animals were kept in a vivarium under conditions of natural lighting at a constant temperature, air humidity and free access to water and food. Alloxan diabetes was evoked via injecting the rats with a 5% solution of alloxan monohydrate intraperitoneally in a dose of 170 mg/kg following a 24 hour period of fasting. The melatonin preparation was used in the research (the manufacturer – "Sigma", USA). The animals were divided into three subgroups: 1) intact rats (control group); 2) alloxan diabetic rats; 3) alloxan diabetic animals which were introduced the melatonin preparation intraperitoneally in a dose of 10 mg/kg at 8 a. m. daily during 6 weeks

starting with a 5-th 24 hour period after the injection of alloxan. Blood was taken from the tail vein to evaluate the basal glycemia (BG) level with the use of One Touch Ultra (LifeScan, USA). On the third (critical) day the death of a part (50%) of the alloxan diabetic animals was observed. Determinations of the content of Hba1c were conducted by a biochemical analyzer D 10 ("Bio-Rad Laboratoria Inc.", France). TAOA, MDA and G-SH were determined with standard methods.

RESULTS

In accordance with the findings, an introduction of alloxan monohydrate caused a sharp rise of the level of basal glycemia (BG) on an empty stomach in a part of the rats (by 111% in comparison with the indices of control group of animals); such animals formed a group of rats with overt diabetes (BG ≥ 8.0 mmol/l). The level of BG did not reliably differ in the rest of the alloxan diabetic animals from the indices of the intact rats (BG ≤ 6.9 mmol/l); such animals were placed into a group of alloxan diabetic rats with occult diabetes. The introduction of melatonin during 6 weeks was conductive to normalization of the level of BG in the group of animals with overt diabetes and a decrease of the BG level (by 37% in comparison with intact rats) in the animals with occult diabetes, indicating a hypoglycemic action of the melatonin preparation.

The content of HbA1c in the blood of the rats with overt and occult diabetes increased by 221 and 122%, respectively, as compared with the indices of the control group of animals. The introduction of melatonin over a period of 6 weeks contributed to a decrease of the blood HbA1c content of the rats with overt diabetes by 45%, as compared with the values of untreated animals. On the other hand the HbA1c content in the group of animals with occult DM did not considerably differ from the values of intact control.

Table 1. Changes of the state of antioxidative defence on the background of alloxan diabetes in rats (M±m, n=6)

Indexes / Groups	BG on 4-th day, mmol/l	After 6 weeks of alloxan introduction				
		BG, mmol/l	HbA1c,%	TAOA,%	MDA, mkmol/g tissue	G-SH, mkmol/g tissue
1.Control group	5.5±0.49	5.5±0.40	4.7±0.77	83.2±2.30	30.0±1.58	7.2±0.41
2.Overt diabetes	11.5±2.15[a]	13.8±2.70[a]	15.1±1.13[a]	63.2±1.45[a]	48.1±1.21[b]	4.3±0.27[a]
3.Overt diabetes + insulin	12.0±2.49[a]	5.3±0.45[b,d]	7.8±1.58[b]	76.5±2.21[b]	27.3±1.56[b]	6.7±0.69[b]
4.Overt diabetes + melatonin	11.6±1.80[a]	5.8±1.41[b,d]	8.3±1.08[a,b]	72.7±2.24[a,b]	38.2±1.32[a,b]	6.8±0.38[b]
5.Occult diabetes	5.3±0.25[b]	6.1±0.39[b]	10.2±0.51[ab]	70.1±1.94[a,b]	40.4±1.23[a,b]	8.6±0.50[a,b]
6.Occult diabetes + melatonin	5.1±0.36[b]	3.7±0.36 [a,b,c,d]	6.2±0.28[b,c]	80.6±2.71[b,c]	28.7±1.15[b,c]	7.1±0.31[b,c]

The blood plasma total antioxidant activity (TAOA) in the animals with overt and occult DM increased by 24 and 17%, respectively, reliably lower than the values of the intact animals and that is, evidently, associated with a depletion of the system of the body's antioxidant defense in alloxan diabetic rats. The introduction of the melatonin preparation resulted in normalization of this particular index in the group of animals with occult DM and its reliable elevation by 15 % in the group in the group of animals with overt DM, as compared with the control indices.

The level of malonic dialdehyde (MDA) reliably increased in the groups of animals with overt and occult DM by 60 and 30%, respectively, compared with the indices of intact animals and that is indicative of intensification of free radical lipid peroxidation in the case of DM. The introduction of exogenous melatonin resulted in normalization of this particular index in the animals with occult diabetes DM and a considerable decrease of the MDA level in the group of animals with overt DM by 20%, as compared with the indices of untreated rats. A reliable decrease of the content of G-SH by 40% occurred in the liver of the rats with overt DM, whereas a reliable increase of this particular index by 20% was observed in the animals with occult DM in comparison with the indices of intact animals and that conforms to bibliographical findings. An increase of the G-SH content in the rats with occult DM is likely to take place at the expense of its intensified regeneration from its oxidative form. The administration of the melatonin preparation induced normalization of the index in question in the alloxan diabetic animals of both groups.

DISCUSSION

The introduction of melatonin during 6 weeks was conductive to normalization of the level of BG in the group of animals with overt diabetes and a decrease of the BG level in the animals with occult diabetes, indicating a hypoglycemic action of the melatonin preparation. The introduction of melatonin contributed to a decrease of the blood HbA1c content of the rats with overt diabetes, as compared with the values of untreated animals; while the HbA1c content in the group of animals with occult DM did not considerably differ from the values of intact control. The introduction of the melatonin preparation resulted in normalization of TAOA index in the group of animals with occult DM and its reliable elevation in the group of animals with overt DM, as compared with the control indices. The introduction of melatonin resulted in normalization of MDA level index in the animals with occult diabetes DM and a considerable decrease of the MDA level in the group of animals with overt DM, as compared with the indices of untreated rats. An increase of the G-SH content in the rats with occult DM is likely to take place at the expense of its intensified regeneration from its oxidative form. The administration of the melatonin preparation induced normalization of the index of G-SH content in question in the alloxan diabetic animals of both groups.

CONCLUSIONS

The introduction of melatonin during six weeks to alloxan diabetic rats is conducive to a decrease in them of the level of BG and HbA1c, as well as stabilization of the indices of the body's antioxidant defense disturbed under the conditions of an absolute deficit of insulin.

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SUMMARY

This study investigated the possible protective effects of melatonin as an antioxidant against alloxan-induced diabetic liver injury in rats. The introduction of melatonin to alloxan diabetic rats is conducive to a decrease in them of the level of basal glycemia BG and HbA1c, as well as stabilization of the indices of the body's antioxidant defense disturbed, namely total antioxidant activity (TAOA) of blood plasma, malonic dialdehyde (MDA) and glutathione (G-SH) in rats liver.

Keywords: melatonin, alloxan diabetes, carbohydrate metabolism, antioxidant protection, liver, rats.

STRESZCZENIE

W pracy ocenie poddano prawdopodobny antyoksydacyjny efekt ochronny melatoniny w przebiegu uszkodzenia wątroby szczurów z cukrzycą alloksanową. Podanie melatoniny u szczurów z cukrzycą prowadziło do spadku wartości glikemii podstawowej i HbA1c, jak również stabilizacji wskaźników systemu antyoksydacyjnego organizmu: całkowitej aktywności antyoksydacyjnej (TAOA) osocza krwi, dialdehydu malonowego (MDA) oraz glutationu (G-SH) w wątrobie szczurów.

Słowa kluczowe: melatonina, cukrzyca alloksanowa, metabolizm węglowodanów, ochrona antyoksydacyjna, wątroba, szczury

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Synthesis and antitumor activity evaluation of new 2-(4-alkoxyphenylamino)thiazol-4(5H)-ones derivatives

Synteza i ocena aktywności przeciwnowotworowej nowej pochodnej 2-(4-alkoksyfenylamino) tiazol-4(5H)-onu

INTRODUCTION

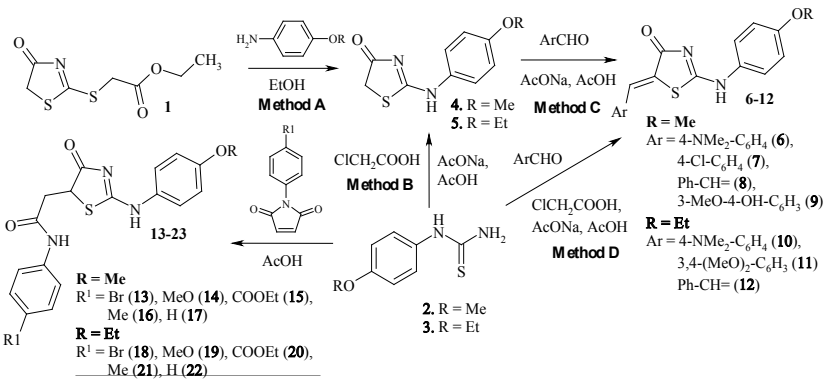
Synthetic and screening research was carried out in the field of 4-thiazolidone derivatives synthesis. Our cooperation with the U.S. National Cancer Institute (NCI) have allowed identification of significant antitumor potential of 2-(oxyphenylamino)thiazol-4(5*H*)-ones. In this group 3 lead compounds were selected, namely (5-[2-chloro-3-(4-nitrophenyl)-2-propenylidene]-2-(3-hydroxyphenylamino) thiazol-4(5*H*)-one, 5-(4-chlorophenylmethyli-dene)- and 5-(4-fluorophenylmethylidene)-2-(4-hydroxyphenylamino)thiazol-4(5*H*)-ones [5, 6]. Currently, these compounds are under in-depth study according to NCI Biological Evaluation Committee decision. The influence of the hydroxyl group replacement by the alkoxygroup in phenyl ring on antitumor activity was studied as continuation of this research.

MATERIAL AND METHODS

A synthetic approach to 2-substituted thiazolidones was based on ethoxycarbonylmethylthio moiety usage as leaving group (Scheme). Reaction of 2-carbethoxymethylthio-2-thiazol-4(5*H*)-one (1) with 4-methoxy- and 4-ethoxyanilines yielded the target 2-(4-alkoxyphenylamino)thiazol-4(5*H*)-ones (4, 5, method A). Synthesized compounds are methylene active heterocycles. On the other hand, it was previously shown that the presence and nature of moiety in position 5 of thiazolidinones played the key role in realization and character of pharmacological effects [3–7, 11]. The abovementioned became a background for the synthesis of new 5-arylidenederivatives (6-12), using standard Knoevenagel reaction procedure (method C, medium – acetic acid, catalyst – fused sodium acetate). 5-Arylidenederivatives were prepared alternatively by one-pot methodology involving reaction of arylthioureas [10] with chloroacetic acid and appropriate aromatic aldehydes in the presence of fused

sodium acetate in refluxing acetic acid (method D). It should be noted that methods A, B and C, D practically do not differ in outputs and purity of products that can alternatively be used for the synthesis of target compounds [5, 6, 8]. The condensation of appropriate arylthioureas (2, 3) with arylmaleimides gave a series of 2-[2-(4-meth(eth)oxyphenylamino)-4-oxo-4,5-dihydrothiazol-5-yl]-*N*-arylacetamides (13-22).

Scheme



The presence of amino-imino tautomerism was confimed by ^{1}H NMR spectroscopy; structures of all newly synthesized compounds were confirmed by elemental analyses, ^{1}H NMR and mass spectroscopy.

The presented row of heterocyclic compounds was studied for anticancer activity. First, the screening was carried out on 3 cancer cell lines (NCI-H460, MCF-7 and Sf-268) or on 60 cell lines covering almost the entire range of human cancers (lung, breast, ovaries, large intestine, kidney, prostate and CNS cancer lines, as well as leukemia and melanoma). Cell growth was compared with control in both cases and was determined by the percentage of growth.

Comprehensive *in vitro* screening consisted in a study of its antitumor effect in five concentrations at 10-fold dilution (100μM, 10μM, 1μM, 0.1μM and 0.01μM) for 57 lines of human cancer cells, which are the same to stage prescreening. Based on the cytotoxicity assays, three antitumor activity dose–response parameters were calculated for each experimental agent against each cell line: GI_{50} – molar concentration of the compound that inhibits 50% net cell growth; TGI – molar concentration of the compound leading to total inhibition; and LC_{50} – molar concentration of the compound leading to 50% net cell death. If the logarithmic value of the investigated parameters ($lgGI_{50}$, lgTGI and $lgLC_{50}$) are smaller than -4.00, compounds are considered to be active [1, 2, 9].

RESULTS AND DISCUSSION

Results of the prescreening of compounds 5, 9, 12, 19 and 20 have not shown the desired effect (Table 1), whereby some lines even stimulated cell growth. However, it is worth noting that 2-(4-metoxyphenylamino) thiazole-4(5*H*)-one (5) showed high selectivity suppression of mitotic activity line ovarian cancer IGROV1, and its 5-(3-methoxy-4-oxybenzylidene) derivative 9 – line breast cancer MBA-MB-435. As seen from the

data in Table 1, compound 15 showed high levels of cytotoxicity and was selected for in-depth *in vitro* studies. Interestingly, the substitution at position 4 ethoxygroup in aminophenol residue (compound 20) on metoxygroup (compound 15) leads to significant increase in cytotoxic effect.

Table 1. Cytotoxicity of synthesized compounds

Comp.	Mitotic activity (3 lines, 10^{-4} M), %	Average mitotic activity of 60 lines / range mitotic activity (10^{-5} M), %	The most sensitive cell line (mitotic activity,%)
5	–	100.90 / 17.89 ÷ 156.00	Ovarian Cancer: *IGROV1* (17.89%) Breast Cancer: *BT-549* (56.81%)
9	–	78.85 / 10.04 ÷ 112.63	NSC Lung Cancer: *HOP-32* (32.95%) Colon Cancer: *HCT-15* (51.82%) Leukemia: *K-562* (50.21%) Breast Cancer: *MBA-MB-435* (10.04%). MBA-MB-468 (45.93%)
12	MCF7 – 120% NCI-H460 – 112% SF-268 – 87%	–	–
15	–	59.84 / -41.66 ÷ 115.04	Renal Cancer: *UO-31* (-41.66%) Colon Cancer: *KM12* (1.50%). *HCC-2998* (-13.44%) Ovarian Cancer: *OVCAR-3* (14.31%) Breast Cancer: *MDA-MB-231/ATCC* (3.44%)
19	MCF7 – 103% NCI-H460 – 112% SF-268 – 78%	–	–
20	MCF7 – 96% NCI-H460 – 156% SF-268 – 112%	–	–

In-depth investigation of compound 15 confirmed its high antitumor potential. In general, it is worth to mention the substantial level of effective growth inhibition (Table 2) practically of all tumor cell lines (percentage of "active line" was 84.2% with an average of $lgGI_{50}$ = -5.11). With regard to individual cell lines $lgGI_{50}$ the highest value was observed for MOLT-4 (leukemia).

In evaluating the antitumor profile lead compound in various types of cancer the relative selectivity of CNS cancer line should be noted. A very interesting picture was observed for cell line U251 (CNS cancer), an effective level of inhibition, cytotoxic and cytostatic effects were approximately at the same level ($lgGI_{50}$ = -5.74, lgTGI = -5.74, $lgLC_{50}$ = 5.14). Thus, the level of antitumor profile of compound 15 may be considered as potential "lead structure", which is characterized by high effective growth inhibition of all tested cell lines (range $lgGI_{50}$ within -4.00 ÷ -5.91) and significant cytostatic (TGI range within -4.00 ÷ -5.44) activity.

Table 2. Results of in-depth *in vitro* screening compounds 15 in concentration gradient 10^{-4}-10^{-8} M

Parameter	Number of „active" lines / %	Average activity / range	The most sensitive cell line (value of the parameter effect)
$lgGI_{50}$	48 / 84.2%	-5.11 / -5.91 ÷ -4.00	*$lgGI_{50}$ < -5.50* Leukemia: *MOLT-4* (-5.91) NSC Lung Cancer: *HOP-62* (-5.54). *HOP-92* (-5.66). *NCI-H322M* (-5.52) Colon Cancer: *HCT-116* (-5.58) CNS Cancer: *SF-539* (-5.63). *SNB-75* (-5.73). *U251* (-5.74) Melanoma: *MALME-3M* (-5.85). *SK-MEL-28* (-5.57) Renal Cancer: *CAKI-1* (-5.55). *TK-10* (-5.68) Breast Cancer: *MBA-MB-231/ATCC* (-5.64)
lgTGI	26 / 45.6%	-4.36 / -5.44 ÷ -4.00	*lgTGI ≤ -5.00* NSC Lung Cancer: *HOP-62* (-5.00). *HOP-92* (-5.11) CNS Cancer: *SF-539* (-5.09). *SNB-75* (-5.16). *U251* (-5.44) Melanoma: *MALME-3M* (-5.42). *SK-MEL-28* (-5.15) Renal Cancer: *TK-10* (-5.15) Breast Cancer: *MBA-MB-231/ATCC* (-5.32)
$lgLC_{50}$	14 / 24.6%	-4.11 / -5.14 ÷ -4.00	*$lgGI_{50}$ < -4.50* CNS Cancer: *SF-539* (-4.53). *U251* (-5.14) Melanoma: *MALME-3M* (-4.79) Renal Cancer: *TK-10* (-4.58) Breast Cancer: *MBA-MB-231/ATCC* (-4.99)

CONCLUSSIONS

Results of this study prompt us to in-depth anticancer studies of 2-arylaminothiazol-4(5*H*)-one derivatives as possible "drug-like" molecules. 4-Ethoxycarbonylphenylamide of 5-carboxymethyl-2-(4-methoxyphenylamino)thiazol-4(5*H*)-one (15) was selected as a lead-compound with high antitumor activity and selective action against CNS-cancer and melanoma.

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SUMMARY

5-Substituted 2-(4-alkoxyphenylamino)thiazol-4(5*H*)-ones were synthesized. Anticancer activity of synthesized compounds toward 60 human tumor cell lines panel in National Cancer Institute was evaluated. 4-Ethoxycarbonylphenylamide of 5-carboxymethyl-2-(4-methoxyphenylamino)thiazol-4(5*H*)-one was selected as lead-compound with high antitumor activity.

Keywords: 2-arylaminothiazol-4(5*H*)-ones, [2+3]-cyclocondensation, Knoevenagel reaction, antitumor activity

STRESZCZENIE

Zsyntetyzowano 5-podstawioną pochodną 2-(4-alkoxyphenylamino)thiazol-4(5*H*)-onu. Aktywność przeciwnowotworową zsyntetyzowanego składnika w kierunku panelu 60 nowotworowych linii komórkowych zbadano w Narodowym Instytucie Raka. Jako główny składnik z najwyższą aktywnością przeciwnowotworową wskazano 4-etoksykarbonylfenylamide 5-karboksymetyl-2-(4-metoksyfenylamino) tiazol-4(5*H*)-on.

Słowa kluczowe: 2-arylaminotiazol-4(5*H*)-ony, [2+3]-cyklokondensacja, reakcja Knoevenagela, aktywność przeciwnowotworowa

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Serological markers of cognitive deficit development in the acute period of traumatic brain injury

Markery serologiczne rozwoju deficytu poznawczego w ostrym okresie pourazowego uszkodzenia mózgu

INTRODUCTION

Traumatic brain injury is one of the most important causes of invalidisation all over the world. Cognitive deficit appears in 70 % of patients who survived craniocerebral trauma. Success in the treatment of posttraumatic dementia mainly depends on the early diagnostics and appropriate pathogenic therapy. Diagnostic criteria of early posttraumatic cognitive deficit are still unclear. There are a number of neuropsychological tests which nowadays are used for diagnostics of cognitive decline in different patients but most of them need additional application of objective criteria for the verification of early cognitive deficit [1]. A number of serological markers such as protein S-100, Tau-protein and others failed the test for the applicability as clinical diagnostic markers of the development of posttraumatic dementia [1].

Long-term oxidative and nitrosative stresses are believed to be one of the major contributing factors in progression of neuronal degeneration and decline of cognitive function in posttraumatic period. Compensative activation of antioxidant defense system is one of the most important mechanisms of adequate systemic regeneration after the trauma. Experimental data showed retardation in mental development in animals with impaired function of superoxide dismutase (SOD), catalase, glutathione-transferase [1]. Nitric oxide is implicated in the processes of memory and learning both as neuromediator and endocrine regulator of neurophysiological processes. Under the condition of oxidative stress, development excessive production of NO results in the formation of toxic peroxinitrite anion and nitrosative stress development.

The aim of our work was to investigate the state of cognitive functions in patients in acute period of mild and moderate traumatic brain injury according to the standard tests of neuropsychological diagnostics and to evaluate the sensitivity of assay of lipid peroxidation activity, SOD and catalase activities and NO-metabolites concentration as serological tests for the routine diagnostics of early posttraumatic cognitive deficit.

MATERIAL AND METHODS

We examined 27 patients in the acute period of mild and moderate craniocerebral trauma. The state of cognitive functions was examined with the use of standard test – FAB (frontal assessment battery) [2] and MMSE (mini-mental state examination) [3]. Parameters of prooxidant-antioxidant metabolic status and nitric oxide metabolites were estimated in erythrocyte hemolysate and blood plasma. Concentration of NO_2^- was measured with the method [5], activity of SOD by [4], activity of catalase by [7] in erythrocyte hemolysate, concentration of MDA in blood serum by [8].

RESULTS AND DISCUSSION

It was revealed that 83% of the examined patients have posttraumatic cognitive deficit. The correlation analysis showed that in the scoring between the scales of FAB and MMSE) and MDA concentrations there is a negative correlation average force ($r = -0.37$ scale for MMSE, $p < 0.05$, and $r = -0.48$ scale for FAB, $p < 0.05$). The same strength and directivity correlation was typical of the activity of catalase ($r = -0.31$, $p < 0.05$, and $r = -0.33$, $p < 0.05$, for the scale and MMSE FAB, respectively). A strong positive correlation was determined between SOD activity and the amount of points by the FAB in both clinical groups of patients. Regression analysis of changes in SOD activity according to the degree of the loss of cognitive functions by MMSE scale was the same for directivity (positive), but the average force. Superoxide dismutase is one of the most effective metabolic mechanisms for the inactivation of reactive oxygen species. Elevated SOD activity can be therapeutically useful by protecting against oxidative stress-induced neurotoxicity. It was found that mice with genetic overexpression of SOD do not show the aging-induced decline in learning and memory that control, wild type mice show [1].

A strong negative correlation was revealed between the growth of total NO_2^- concentrations in serum of patients and total score on the scale of FAB is a ($r = -0.84$). A significant negative correlation is also observed when comparing the total score for the MMSE scale and the content of serum nitric oxide metabolites, but the strength of the relationship between these parameters is much lower ($r = -0.30$).

CONCLUSIONS

The combination of neuropsychological testing with scale FAB and laboratory determination of NO_2^- and SOD activity can be a useful diagnostic tool for posttraumatic patients who require extra attention from neuropsychologists for more adequate treatment in the acute period of craniocerebral trauma. The choice of the mentioned markers is pathogenically substantiated and can be used in routine clinical practice for precise diagnostics of posttraumatic cognitive deficit.

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SUMMARY

The aim of our work was to investigate the state of cognitive functions in patients in acute period of mild and moderate traumatic brain injury according to the standard tests of neuropsychological diagnostics and to evaluate the sensitivity of assay of lipid peroxidation activity, SOD and catalase activities and NO-metabolites concentration as serological tests for the routine diagnostics of early posttraumatic cognitive deficit. The combination of neuropsychological testing with scale FAB and laboratory determination of NO_2^- and SOD activity can be a useful diagnostic tool for posttraumatic patients who require extra attention from neuropsychologists for more adequate treatment in the acute period of craniocerebral trauma. The choice of the mentioned markers is pathogenically substantiated and can be used in routine clinical practice for the precise diagnostics of posttraumatic cognitive deficit.

Keywords: traumatic brain injury, cognitive deficit, nitric oxide, superoxide dismutase

STRESZCZENIE

Celem badań było określenie stanu funkcji poznawczych u pacjentów w ostrym okresie niewielkiego i umiarkowanego pourazowego uszkodzenia mózgu w odniesieniu do standardowych testów stosowanych w diagnostyce neuropsychologicznej i dla określenia czułości oznaczeń aktywności procesów peroksydacji lipidów, aktywności SOD i katalazy oraz stężenia metabolitów NO jako testów serologicznych rutynowej diagnostyki wczesnego pourazowego deficytu poznawczego. Kombinacja testów neuropsychologicznych ze skalą FAB i oznaczeniami laboratoryjnymi stężenia NO_2^- i aktywności SOD mogą być pomocnym narzędziem diagnostycznym u pacjentów pourazowych, którzy wymagają wyjątkowej uwagi neuropsychologów w kierunku wdrożenia bardziej adekwatnego leczenia w ostrym okresie urazu czaszkowo-mózgowego. Wybór wspomnianych markerów jest patogenicznie uzasadniony i mogą być one stosowane w rutynowej praktyce klinicznej w celu precyzyjnej diagnostyki pourazowego deficytu poznawczego.

Słowa kluczowe: pourazowe uszkodzenie mózgu, deficyt poznawczy, tlenek azotu, dysmutaza ponadtlenkowa

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The influence of the short peptide of arginyl-alfa-aspartyl-lysyl-valyl-tyrosyl-arginine on the activity of NO-synthase system and processes of lipoperoxidation in experimental gastric lesions in rats

Wpływ krótkiego peptydu arginyl-alfa-aspartyl-lizyl-walyl-tyrozyl-argininy na aktywność systemu syntazy NO i procesy lipoperoksydacji w doświadczalnym uszkodzeniu żołądka u szczurów

INTRODUCTION

It is known that short-chained peptides participate in the regulation of a variety of physiological functions in humans, particularly in cytoprotection of gastric mucosa (GM). The gastroprotective effects were evaluated in such short-chained peptide substances as glyprolins and pentadecacapeptide BDC 157 [5, 10, 13]. The positive influence of the hexapeptide arginyl-alfa-aspartyl-lysyl-valyl-tyrosyl-arginine ("Imunofan") was also reported on the course of the peptic ulcer but the mechanisms of its cytoprotective influence on GM is not clear [9]. That is why the aim of the present investigation was to study the role of Imunofan in the processes of lipoperoxidation and the status of L-arginine/NOS/NO system in experimental gastric lesions (GL) in rats.

MATERIAL AND METHODS

Investigations were conducted on 122 rats. The structure of this study and animal experimental procedures were approved by the Ethical Committee of Lviv National Medical University. Stress GL was induced by intraperitoneal injection of epinephrine (2 mg/kg) [2]. We conducted 4 series of the experiments: 1) the study of epinephrine action on GM; 2) the study of Imunofan action (1 μg/100g) on GM on the background of stress; 3) the study of the combined action of Imunofan (1 μg/100g) and L-arginine on the background of stress; 4) the study of the combined action of Imunofan (1 μg/100g) and selective iNOS blocker aminoguanidine (20 mg/kg) on GM on the background of stress.

With the purpose of investigating the NO-synthase system status in GM homogenates, the following indices were determined: activity of NO-synthase [11] and the content of NO [4] in GM and the level of L-arginine in plasma [1]. Processes of lipoperoxidation were analized by the contents of tiobarbituric acid products (TBAP) [12], antioxidant system status – by superoxidedismuthase

activity (SOD) [3] and catalase [7]. The area and severity of GL were investigated using the method of planiometry and 12-grade scale. The results were processed using the method of variation statistics.

RESULTS AND DISCUSSION

The rats introduced to epinephrine developed severe GL in the form of erosions, ulcers and hemorhages. The mean damaged area made up 38.5±5 mm^2 and 10.9±2 grades. In rats who were administered Imunofan under conditions of stress the damaged area of GL was 42% smaller and made up 22±4 mm^2, the severity of GL decreased by 39.44% and made up 6.6±2.8 grades. The group of animals introduced to the combined action of Imunofan and L-arginine on the background of stress GM remained almost intact and the damaged area made up 2.75±0.5 mm^2 and 2.5±0.4 grades respectively. Under conditions of NOS blockage by amine guanidine and Imunofan administration the damaged area of GM made up 6.6±1.7 mm^2 and 3.6±1.2 grades. Thus, the combined administration of Imunofan and L-arginine induced significant gastroprotective effect.

In healthy conditions, the activity of eNOS in GM is dominating and iNOS activity is low [6]. In our experiments, the proportion between eNOS та iNOS in intact animals made up 3.8 and the level of NO in GM made up 16.1±2.55 μmol/l, the concentration of L-arginine in plasma was 39.2±2.09 μmol/ml.

In stress, the NOS activity was significantly increased: total NOS activity increased by 133% from 0.876 ± 0.42 to 2.65 ± 0.827 nmol/min·g ($p<0.01$), eNOS activity did not change significantly and iNOS activity was 6 times higher ($p<0.001$). The content of NO increased from 16.1±2.55 to 24.1±2.6 μmol/l (by 50 %, ($p<0.05$), and the concetration of L-arginine in plasma decreased by 37% ($p<0.05$). The proportion between eNOS and iNOS made up 0.68, and between NO in GM and L-arginine in plasma – 1.22, respectively.

In stress-induced GL, the activity of lipoperoxidation processed also increased – the content of the products of TBAP increased from 224.5±24.4 to 312.4±10.7 μmol/g ($p<0.05$); SOD activity increased by 57%, ($p<0.05$); catalase activity did not change significantly. Thus, in experimental epinephrine-induced GL, massive structural and hemorrhagic damages of the GM were supervised, accompanied by the increase of iNOS activity and content of NO in GM. The level of L-arginine in plasma decreased appropriately. The activity of lipoperoxidation and SOD activity increased.

Imunofan administration in dose of 1 μg/100 g of body weight under the influence of epinephrine induced significant decrease of the total NOS activity by 56% ($p<0.05$), eNOS activity decreased by 41%, and iNOS – by 62%. The content of NO decreased by ($p<0.05$) in comparison to animals who were introduced to epinephrine. The tendency to a decrease of the activity of lopoperoxidation processes was also noted, meanwhile SOD activity decreased by 54%. Thus, Imunofan administration induces the decrease of the damaged area, NOS activity and the content of NO while L-arginine concentration increased.

Imunofan action on the background of iNOS blockage by amine guanidine induced tendency to enhance the inhibition of iNOS activity and the content of NO in GM decreased; respectively. The content of TBAP diminished by 11%, SOD activity had a tendency to decrease in comparison to Imunofan effect. The concentration of L-arginine in blood did not change significantly. Thus, iNOS

blockage under the influence of Imunofan enhanced iNOS inhibition, lipoperoxidation processes, a decrease of NO and SOD activity. Meanwhile L-arginine concentration in plasma increased.

A combined action of L-arginine and Imunofan induced a significant decrease of the activity of total NOS and a decrease of iNOS and the proportion between the activity of total NOS and iNOS made up about 1. NO content did not change significantly and L-arginine concentration was higher, TBA products decreased by 15%, meanwhile SOD and catalase activity did not change significantly in comparison to monotherapy with Imunofan.

Analyzing the obtained data, we conclude that the ulcerogenic effect of epinephrine induces the development of oxidative stress, activation of proinflammatory enzymes - iNOS and COX-2, increase of GM infiltration by polymorphonuclear leukocytes, increase of proinflammatory cytokins production – IL-1β, IL-6 and TNF-α in GM [14]. In oxidative stress, the generation of active forms of oxygen and fatty acid radicals is significantly increased, leading to the damage of the blood vessels membranes, secretory cells and epiteliocytes, which induces the formation of GL [9].

Epinephrine enduces GL formation with a typical increase of TBAP, activation of iNOS, increase of NO and peroxinitrite production. Increased NO-synthase activation leads to the decrease of L-arginine level in blood, which indicates the activation of L-arginine transportation from blood into the cells of the damaged area and its utilization by iNOS. Imunofan inhibits NO-synthases activity in GM [4, 5]. It was proved that Imunofan shows a significant immunomodulatory effect, stimulating differentiation and proliferation of T-lymphocytes, increasing antibodies production, phagocytic activity of macrophages and polymorphonuclear leukocytes and it inhibits the processes of lipoperoxidation [9].

In our experiments Imunofan induced the cytoprotective processes, which was determined on the basis of the area and severity of GL, accompanied by the decreased activity of eNOS and iNOS, NO, L-arginine and TBAP. The obtained indices confirm the cytoprotective action of Imunofan. This effect of Imunofan may be explained by its degradation into di- and tripeptides, containing arginine, which may act as NO-synthase blockers, and its stimulating influence on polymorphonuclear leukocytes.

CONCLUSIONS

Administration of Imunofan in stress decreases the severity of GL, the activity of eNOS, iNOS, NO, SOD in GM and it increases the level of L-arginine in plasma. As the most significant gastroprotection was evaluated under the conditions of the combined action of Imunofan and L-arginine, we suggest this combination to be recommended for the treatment of inflammatory diseases of the stomach.

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SUMMARY

In the experiments on rats, under conditions of modeled ulcer of the stomach, the role of arginyl-alfa-aspartyl-lysyl-valyl-tyrosyl-arginine – Imunofan was investigated on the processes of lipoperoxidation and the status of the system of L-arginine/NOS/NO. It was shown that Imunofan action at the background of ulcerative lesions of the stomach caused a steep decline of the activity of NO-synthases, decrease of NO content in GM and increase of L-arginine concentration in the plasma of blood. The action of Imunofan at the background of blockage of iNOS with amine guanidine induced the inhibition of iNOS activity and a decrease of NO content in GM, whereas the concentration of L-arginine in the plasma of blood increased. Under conditions of combined action of Imunofan with L-arginine, the activity of NO-synthases reduced, the content of nitrogen oxide slightly increased, and the concentration of L-arginine in the plasma of blood was determined higher than in the control animals. The action of Imunofan also caused a decrease of the processes of lipoperoxidation and SOD activity.

Keywords: arginyl-alfa-aspartyl-lysyl-valyl-tyrosyl-arginine, gastroprotection, lipoperoxidation, NO-synthase system.

STRESZCZENIE

W badaniu na szczurach w warunkach modelowanych wrzodów żołądka badano wpływ arginyl-alfa-aspartyl-lizyl-walyl-tyrozyl-argininy – Imunofanu na procesy lipoperoksydacji i stan systemu L-arginina/NOS/NO. Wykazano, że Imunofan w przebiegu wrzodziejącego uszkodzenia żołądka powodował stopniowy spadek aktywności syntaz NO, spadek zawartości NO w śluzówce żołądka i wzrost stężenia L-argininy w osoczu krwi. Imunofan w przypadku blokady iNOS za pomocą guanidyny indukował inhibicję aktywności iNOS i spadek zawartości NO w śluzówce żołądka, podczas gdy stężenie L-argininy w osoczu krwi było podwyższone. W warunkach jednoczesnego oddziaływania Imunofanu i L-argininy aktywność syntaz NO była zmniejszona, zawartość tlenku azotu lekko podwyższona, a stężenie L-argininy w osoczu krwi wyższe niż u zwierząt kontrolnych. Imunofan powodował także spadek aktywności procesów lipoperoksydacji i SOD.

Słowa kluczowe: arginyl-alfa-aspartyl-lizyl-walyl-tyrozyl-argininy, gastroprotekcja, lipoperoksydacja, układ syntazy NO

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   c) results (text, tables or other methods presenting the results),
   d) discussion of the results, which is a critical comparison of the studies with the data from the literature, conclusions - brief generalizations, and possibly determination of further directions of studies

e) references - prepared on a separate page, and marked with numbers in the alphabetical order. Each item should include the following: the author's/authors' surname(s), first letter of the first names, title of the paper (article), abbreviated name of the periodical according to the Cumulated Index Medicus, volume, first page, year. In case of a quoted book, the title should be followed by the publisher, place of publication (the first one - in case of a few),year. Page(s) can be indicated. In case of three or more authors, only the first one should be named followed by „et al."
Some sample reference styles are listed below:
Articles in journals
*a. Standard journal article (one or tree authors):*1. Salling MC, Faccidomo S, Hodge CW: Nonselective suppression of operant ethanol and sucrose self-administration by the mGluR7 positive allosteric modulator AMN082. Pharmacol. Biochem. Behav., 91, 14, 2008.
b. More than three authors:
1. Carrilho C et al.: Distribution of HPV infection and tumour markers in cervical intraepithelial neoplasia from cone biopsies of Mozambican women J. Clin. Pathol., 58, 61, 2005.
Book:
1. Besner D, Humphreys GW, editors (1991). *Basic processes in reading: visual word recognition*. Hillsdale, NJ: Erlbaum.
Chapter in a book:
1. Sanger DJ (1986). Drug taking as adjunctive behaviour. In: *Behavioural analysis of drug dependence*. Goldberg R, Stolerman IP (editors). New York: Academic Press; p. 123.

f) summaries and key words (in Polish and English together with the title of the paper, a maximum of 6 keywords),

g) legends of figures marked with Arabic numbers and in the sequence they appear in the text.

h) in the text, literature is quoted providing the numbers from the list, and not the name and year of publication.

i) abbreviations and symbols. Use only standard abbreviations. Avoid abbreviations in the title and abstract. The full term for which an abbreviation stands should precede its first use in the text.

11. The original or review paper may include 15 at the most, and in case of short reports or introductory reports - 5 references.

12. The paper should use basic, conventional units of measure or the international system of units (SI).

13. The manuscript should be submitted to the editorial office in two versions: an electronic (compact disc – CD-R) and a printed (original and one copy). The author is responsible for the consistence of the electronic version with the printed one. The electronic version should be prepared in MS Word 97-2003 or 2007 (*.doc; *.docx). The text should be written in Times New Roman (CE) fonts, 12 points, with double spacing and the margins of 2.5 cm. The printed copy should be prepared on a white A-4 paper, one side. The paragraphs are to be clearly marked with distinct indentations.

14. Preparation of the figures and tables. Each table/figure should be typed/printed on a separate sheet. Tables should not be submitted as photographs. Each table should be assigned with Arabic numeral, e.g. (Tab. 3) and a brief title. Figures of good quality should be submitted in form of a computer copy. Tables and figures (in an electronic form) should be prepared in MS Word and submitted in *.jpg (jpeg) or *.tif (tiff) formats respectively. TIFF and EPS files, with fonts embedded, are preferred. If scanned, line art should be at a resolution of 800 dpi, and halftones and color at 300 dpi. All color values should be CMYK. Figures should be marked in pencil on the back with Arabic numbers, the top and the bottom, and with the surname of the first author.

References to figures and tables should be listed in order of appearance in the text and should be in Arabic numerals in parentheses, e.g. (Fig. 2). No abbreviations should be used in tables. Unintelligible prints with manual corrections and those not satisfying technical requirements will be sent back to the authors.

15. Papers submitted to the editorial office are first subjected to language correction. Next, the authors receive their papers with the the so-called author's correction. Slight changes are possible only at the stage of the editorial process. If the corrected paper has more than 5 stylistic and/or grammatical corrections on one page, the author is obliged to complete corrections and send the improved material to the editorial office in the form of a new printed copy and a new electronic version. Corrected papers must be returned within the fixed time. In case the author does not send the corrected paper back on time, it means their silent consent for the exclusive correction by the editors (with a small number of errors); in case of a large number of errors and inaccuracies - it means their consent to have the paper rejected.

16. Authors do not receive royalties for the published papers.

17. Hard copies of the manuscript are to be submitted to the address of the editorial office:
Prof. dr hab. Jolanta Kotlińska, Department of Pharmacology and Pharmacodynamics, Medical University, Staszica 4, 20-081 Lublin, Poland.