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REVIEW / PRACA POGLĄDOWA

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GASTROINTESTINAL ENDOCRINE CELLS AND THEIR TRANSFORMATION INTO NEUROENDOCRINE TUMOURS

KOMÓRKI ENDOKRYNOWE CEWY POKARMOWEJ ORAZ ICH TRANSFORMACJE W GUZY NEUROENDOKRYNNE

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Summary

APUD endocrine cells are characterized by synthesis and secretion of active amines and peptide hormones which are capable to regulate functions of gastrointestinal tract. All tumours derived from the dispersed neuroendocrine system APUD cells were classified as GEP NETs (gastroenteropancreatic neuroendocrine tumours). These tumours are rare , they are able to synthesize, store and release biogenic amines and peptide hormones. This article presents morphology, functions, origin and means of identification of endocrine cells. Neuroendocrine tumours increase slowly, but they have a high malignant potential.

Streszczenie

Wspólną cechą komórek endokrynnych APUD jest synteza i wydzielanie amin oraz hormonów peptydowych regulujących funkcjonowanie układu pokarmowego. Z układu rozproszonych komórek APUD wywodzą się guzy neuroendokrynne przewodu pokarmowego i trzustki (GEP-NET), które są rzadkimi nowotworami. W pracy scharakteryzowano najważniejsze typy komórek endokrynnych, przedstawiono hipotezy ich pochodzenia oraz identyfikacji. Opisano również grupy nowotworów wywodzące się z komórek neuroendokrynnych, które charakteryzują się powolnym wzrostem i dużym stopniem złośliwości.

Key words: APUD endocrine cells, characterization of APUD cells, neuroendocrine tumours of GEP-NETs
Słowa kluczowe: komórki endokrynne APUD, charakterystyka APUD, guzy neuroendokrynne typu GEP-NET

Endocrine cells are present in epithelium of gastrointestinal tract, respiratory system, pancreas, adrenal and thyroid. They are characterized by hypochromia of cytoplasm, most of them have the ability to reduce chromium and silver salts. There is evidence that chromogranin, which is present in all endocrine cell-secreting granules, can be a good marker for endocrine cells [1]. These cells have also the ability to take up amine precursors and decarboxylate them. Therefore the term APUD stands for Amine Precursor Uptake and Decarboxylation [2].

According to Pearse and Takor-Takor there are 40 types of APUD cells of different origin and capacity of amin precursors accumulation and their decarboxylation.

ORIGIN OF APUD CELLS

There are at least two hypotheses on the origin of APUD cells. One of them suggests that gastrointestinal endocrine cells origin from endodermal cells. According to this hypothesis all cells derive from stem cells and during this process endocrine cells differentiate [3]. Second hypothesis, proposed by Pearse, suggests that gastrointestinal endocrine cells originate from ectoderm of neural crest [4].Chromogranin is a marker for endocrine cells. This protein was first noticed in intestine of 8-week old human fetus. In the 13th week of fetal development there are 13 types of endocrine cells of different morphology. It is believed that in the second trimester of pregnancy the number of APUD cells in gastrointestinal tract is similar to that in adolescence .

APPEARANCE, STRUCTURE AND FUNCTIONS OF APUD CELLS

APUD cells appear singly and are spread among epidermal cells of gastrointestinal tract, respiratory system and epidermal cells of some glands. Endocrine cells of stomach mucosa can be found in neck and fundic glands. They are never present in epidermal layer of gastric pits' mucosa [5].

APUD cells have a typical structure. In most cases they present pyramidal shape with the base on basilemma of epithelium. They are small, with 100-200 μm in diameter. Bipolarity is their characteristic feature. They contain big, spherical nucleus above which there is Golgi apparatus – a centre of hormone synthesis and secretory vesicle formation. These vesicles are cumulated at secretory end of cell [1,6]. Secretory vesicles which contain polypeptide reach the size of 100-200 nm. There is also a great number of rybosomes present in APUD cells, which indicates high protein synthesis and the high density of mitochondrions means high intensity of energetic processes [7].

There are two types of cells – opened and closed type – depending on the presence or absence of cytoplasmatic inset which binds the cell with gastrointestinal tract. Opened type cells also have singular, irregularly placed microvilli in their narrow top part. Endocrine cells of the stomach corpus belong to the closed type without contact with gland lumen [1]. What differentiates APUD cells from other cells of endocrine secretion is the fact that they never form groups [7].

By secreting biologically active substances (amines and peptides) endocrine cells regulate functions of gastrointestinal tract: its motor activity, digestive and secretive functions. Some of amines (P substance, somatostatin, VIP, bombesin, neurotensin, motilin and CCK peptide) have mediating function in the endings of some nerve fibers of gastrointestinal tract [1]. Cells which do not contain bioamines can, after stimulation, pick up specific aminoacids as amines' precursors, and transform them into amines in the presence of aminoacid decarboxylase [8].

For endocrine cells present in gastrointestinal tract and for their tumors Japanese authors proposed the term GEP EC – gastroenteropancreatic endocrine cells. Endocrine cells are usually named with capital letters which stand for the hormone which they secrete, e.g. S cells – secretin, G cells – gastrin, etc.G, S, I, K and EC cells are usually present in duodenum and in jejunum, D, H and ECL cells – in small intensine, N cells mainly in jejunum and ileum [9].

The classification of APUD cells is based on their secretory vesicles' placement [10].Consequently , there are two groups of endocrine cells present in epithelium of stomach mucosa. First group contains cells of the corpus and fundus of stomach, second group contains cells from pylorus region. ECL, D, Ecn, X/A, D1 and P cells belong to the first group. In the mucosa of stomach fundus there are only ECL cells. But in the pylorus region there are G,D, Ecn cells [10].

ECL, G and D cells are the numerous APUD cells of stomach mucosa as they make up to 75% of all endocrine cells of cardiac and stomach corpus mucosa. In cardiac mucosa 50% of cells are the G cells, 30% - ECn cells and 15% - D cells [10].

Functioning of endocrine cells is dependent on binding receptors with appropriate ligand, which can be a hormone or a nutrient. In this way APUD cells receive impulse for secretion of vesicles' content into tissue fluid from connective tissue [6]. Also luminal has the influence on secretion of gastrointestinal hormones, produced by endocrine G and D cells [13,14].

There are three hypotheses of amine and peptide secretion by APUD cells.

First of them suggests that secretory vesicles' content can be excrete due to emiocytosis (reversed pinocytosis) – whole vesicle moves towards cell's surface, its membrane fuses with cellular membrane and the vesicle's content is released outside. The second hypothesis says that due to vesicles destruction their content is dissolved in cytoplasm and then is moved outside the cell by diffusion. There is also a third hypothesis suggesting that cell reacts in different ways depending on the type of stimulation.

Different organs' functioning is controlled by hormones (amines and peptides) which are secreted by endocrine cells. They influence the secretion and repression of saline acid and pepsin in stomach as well as secretion of pancreatic hormones and initiation of gallbladder wall contractions. These hormones regulate peristalsis and expand blood vessels [16].

CHARACTERISTICS OF APUD CELLS

1. G cells

They are present mainly in pylorus region of the stomach, especially in the central part of gastric mucosa and in the neck region of fundus and corpus of stomach [17]. G cells are pyramid-shaped or round, with a clear narrowing of the peak. Their secretory granules containing gastrin are in basal and apical part of cytoplasm of the cell and reach a diameter of 150-300 nm.

Gastrin is a heterogenic hormone, occurring in several forms, which in terms of functions do not differ. Release occurs with the involvement of gastrin-releasing peptide (GRP). Gastrin is secreted mostly in the stomach, which is probably due to both the GRP and the indirect effect of other various substances on the G cells, for example, compounds resulting from the digestion of proteins, as well as coffee, wine, beer and calcium ions. In addition, some influence on the release of gastrin from G cells results from volume enhancement of the stomach, which activates intraparietal cholinergic reflexes stimulating G cells.

During the intestinal phase gastrin release is weak and takes place both under the influence of substances resulting from digestion of proteins and also with the GRP released locally by duodenum neurons. Moreover, the release of gastrin is negatively affected by acidic environment (pH<3). At pH = 1 complete inhibition of the functioning of G cells is observed. Influence of acidic environment on the release of gastrin is called cardiac autoregulation and is associated with the release of somatostatin from D cells and its paraendocrine effect, which inhibits G cells [18].

Gastrin has a wide range of biological activity as it is associated with gastric secretion of H^+ ions and the induction of proliferation of mucosal cells of the stomach, duodenum and colon. Gastrin stimulates the secretion of H^+ ions from the parietal cells mainly through the stimulation of ECL cells, and through histamine release, which activates appropriate receptors of the parietal cells. Large doses of gastrin increase motor activity of the gastrointestinal tract, stomach, small intestine, colon and gall bladder in particular.

Gastrin also affects the contractions of the lower muscle of esophageal sphincter, preventing gastroesophageal reflux and inhibits the contraction of ileocecal sphincter and hepato-pancreatic bulb. Enzymatic inactivation of gastrin occurs in the capillaries of kidney and small intestine.

2. D cells

These cells are present in the whole gastrointestinal tract and pancreas and produce somatostatin. Especially large number occurs in duodenum. In the gastric pylorus and corpus the closed type of D cells occure. They have fusiform or pyramidal shape. They have long cytoplasmic processes and paraendocrine effect on neighboring cells. Their secretory granules reach the diameter of 300-400 nm.

About 20% of D cells have long aksons, whose function is to keep contact with G cells and parietal cells of the stomach. Furthermore, in the pyloric region of gastric part D cells have specialized structures by means of which they contact with stomach lumen.

D cells are located in close proximity to other endocrine cells of the stomach, intestines and pancreas. Somatostatin effect is "paracrine" which means that it inhibits the release of gastrointestinal hormones. Furthermore somatostatin inhibits gastrin, CCK and insulin secretion [9].

3. Ecn cells (enterochromaffin cells)

These cells are located in the epithelium of the gastric glands, they are cells of closed type and produce serotonin. Like D cells, they interact with neighbouring cells with long cytoplasmic processes. They are characterized by argyrophylia and react with monoclonal sera containing antibodies against serotonin [10].

4. EC1 cells

These cells appear only in the corpus of stomach and produce P substance. However, there is no evidence that this substance infiltrates into the blood. The secretory granules reach the diameter of 200-300 nm. Substance P increases motor activity of small intestine [20].

5. EC2 and Mo cells

These cells are present in duodenum mucosa. Their secretory granules reach the diameter of 200-300 nm and in alkaline environment release motilin, which increases motor activity of stomach and intestine and inhibits stomach evacuation [9].

6. S cells

S cells, like the ones mentioned above, can be found in the mucosa of the duodenum and upper jejunum. These cells produce secretin. The secretory granules reach the diameter of 180-220 nm. Secretin release occurs under the influence of H^+ ions and under the influence of peptides, amino acids and fatty acids as well as during the digestion of proteins and fats.

Secretin stimulates the pancreas, bile ducts and duodenal glands to HCO_3^- secretion, enhances the secretion of pancreatic enzymes, inhibits gastric motility and initiates the secretion of pepsin, reinforces trophic effect of CCK on the pancreas, and is beneficial for blood flow and metabolism of the pancreas. In addition, it neutralizes acidic environment of the stomach and duodenum [9].

7. N cells

These cells are located in jejunum and ileum mucosa. Under the influence of compounds formed during the digestion of fats and proteins, they release neurotensin. Neurotensin increases the secretion of pancreatic juice and intestinal and bowel motility, increases blood flow and inhibits gastric acid secretion [9].

8. K cells

They are located in duodenum and ileum mucosa. They produce GIP – gastric inhibiting peptide, which is released after the stimulation of fats, glucose and amino acids [21].

GIP inhibits the secretion of hydrochloric acid and pepsin, decreases gastric motility, stimulates intestinal secretion and increases insulin release [9].

9. I cells

They are present in duodenum and ileum and produce cholecystokinin peptide (CCK). The secretory granules reach 250-300 nm in diameter. CCK stimulates the secretion of stomach enzymes, influences the contractions of the gallbladder sphincter with parallel relaxation of hepato-pancreatic bulb, causes contractions of pyloric sphincter and growth of the small intestine motility. CCK also regulates blood flow increase, the intensity of pancreatic metabolism and the release of glucagon [9].

10. D1 cells

These cells are located mainly in duodenum and ileum and secrete vasoactive intestinal peptide (VIP), which is present in stomach, intestine and portal circulation area. VIP influences the process of cell respiration, inhibits the secretion of hydrochloric acid and pepsin in stomach, stimulates lipolysis processes and hepatic glycogenesis [9].

11. ECL cells

They represent the largest group of endocrine cells present in the mucosa of the fundus of stomach. They have the ability to synthesize and secrete histamine.

These cells constitute 30-44% of all endocrine cells of corpus and gastric fundus mucosa, they interact with the main cells of the glands of gastric corpus and fundus. Identification of ECL cells is possible with monoclonal antibodies against histamine and/or histamine decarboxylase [12].

ENDOCRINE CELLS IDENTIFICATION

APUD cells do not stain with traditional methods. They can be visualized by impregnation with silver salts, and therefore they were originally called argentaffin or argyrophilic cells. APUD cells have the ability to spontaneously reduce silver salts, also after addition of reducing substances, such as hydroquinone. Endocrine cells stain yellow after chromium salts treatment and that is why they are called “yellow cells” as well as chromatophilic. In the past they were called chromaffin cells, as they form brown deposits with sodium dichromate. More accurate identification of endocrine cell types is possible with the use of electron microscope or appropriate immunohistochemical markers. Markers which allow to identify specific peptides produced by these cells are particular

importance [22]. Neuron-specific enolase (NSE), synaptophysin and chromogranin are used for the diagnosis of stomach endocrine cells [16].

For detailed identification of endocrine cells monoclonal antibodies (e.g. against gastrin, somatostatin, serotonin, etc.) are used. This method is widely used in quantitative evaluation in pathomorphology [16].

TUMOURS DERIVED FROM APUD CELLS

Endocrine cells located in the gastrointestinal tract can occasionally go under hiperplasia or tumour transformation. Tumors developing from the APUD neuroendocrine cells represent rare, heterogeneous tumor group and have different degree of malignancy. They affect both men and women with similar frequency. They can be hormonally active or passive.

Tumours which origin from APUD cells are called *apudoma* [7]. This term refers to such tumours as: cancers, adenomas, carcinoids and all simple hypertrophies [23]. These are hormone active tumours, which secrete hormones produced by APUD cells (serotonin, corticotrophin, histamine, dopamine, P substance, prostaglandins, callikrein, motilin) [24]. *Apudoma*-type tumours can secrete one or more hormones. The same hormone can be produced by several tumours present in different parts of one patient's body. Identification of *apudoma* tumours is based on histological and immunocytochemical investigation [7].

"Carcinoid syndrome" was described as a disease at the beginning of the XXth century. In 1980 WHO proposed "carcinoid" term for all neuroendocrine tumours which origin from the diffuse neuroendocrine system. In the year 2000 a new classification of GEP-NETs (gastroenteropancreatic neuroendocrine tumours) was accepted [25].

Carcinoids are rare tumours, considered as neuroendocrine tumours, which originate from the diffuse neuroendocrine system cells (APUD) [26]. Synaptophysin, neuron-specific enolase and chromogranin A are considered to be specific markers for neuroendocrine carcinoids [24]. These tumours are characterized by slow growth and high malignancy. Histologically they present positive reaction to silver salts staining and are usually present in small intestine. They represent 1% of all primary tumours of gastrointestinal tract and can be found from the end of esophagus up to anus. Taking into consideration the frequency, 39% of carcinoids can be present in small intestine, 26% in vermiform appendix, 15% in anus, 5-7% in colon, 2-4% in stomach, 2-3% in pancreas and 1% in liver. Approximately 10-20% of gastrointestinal carcinoids can be connected with other tumours, e.g. typical intestine tumors (carcinomas and adenocarcinomas) [24].

Small intestine carcinoids

Taking into account their ontogenic origin (specific parts of gut) they were divided into three groups [23, 24, 26]:

1) of foregut origin – lungs, bronchi, stomach and duodenum carcinoids; they secrete serotonin and reduce silver salts after addition of reducing substances (argentaffin-negative, argyrophilic-positive),
2) of midgut origin – small intestine and appendix carcinoids; they secrete serotonin and reduce silver salts both simultaneously and after addition of reducing substances (argentaffin- and argyrophilic-positive),
3) of hindgut origin – distal part of colon and anus; do not produce specific substances, reduce silver salts neither simultaneously nor after addition of reducing substances (argentaffin- and argyrophilic-negative).

The most common hormone-active intestine tumour is orthoendocrine carcinoid which originates from EC cells. About 1/3 of these tumors are benign and locate in appendix, further 1/3 can be found in jejunum and ileum and are mildly malignant.

Malignant tumours of small intestine origin secrete serotonin (5-HT) and callicrein. Some of these tumours are able to synthesize prostaglandin E, P substance, motilin and calcitonin. Serotonin and bradykinin are pharmacologically active substances, they get directly through portal vein and initiate carcinoid syndrome. The symptoms can be acute or chronic [23].

The diagnosis can be made according to clinical symptoms and high calcitonin level in blood. In half of the patients high level of pancreatic peptide in plasma is observed.

The tumour's size at the time of diagnosis is very important. There is 50% increased risk of metastasis into lymph nodes and other organs (liver, lungs, bones, skin, brain and even heart) when tumor reaches a diameter of 2 cm or more. Therapy is based on somatostatin analogues, surgical excision or cytoreductive treatment [24, 27].

Hormonally active duodenum tumors

They differentiate from neuroectodermal cells of the primordium intestine and represent approximately 1-5% of intestine carcinoids [27].

In most of the cases these tumours develop from G cells – they are called *gastrinoma*, which can be present subsequently with general changes in connective tissue, they can be associated with Recklinghausen's disease and adrenal chromaffin tumours. Among all gastrinomas duodenal tumours represent 43%.

Second group of neuroendocrine duodenal tumours are tumors with high level of somatostatin (*somatostatinoma*). They are more aggressive then the first group, mostly malignant and in nearly all cases they develop in the area of Vater papilla.

Third group are gangliocytic paragangliomas, which synthesize mainly somatostatin and pancreatic peptide (PP). They are usually benign [27].

Duodenal carcinoids, histopathologically, have typical features of neuroendocrine tumours. They are argyrophilic and contain typical markers, such as chromogranin A, NSE (neuron-specific enolase) and synaptophysin. Immunohistochemistry, apart from gastrin and serotonin, detects: catecholamines, secretine, ADH (anti diuretic hormone), ACTH (adrenocorticotropic hormone), MSH (melanocyte stimulating hormone), insuline, glucagone, VIP (vasoactive intestinal polypeptide) and calcitonin. Cells containing gastrin and somatostatin are of the greatest number [27].

Gastric carcinoids

They represent 11-40% of all carcinoids of gastrointestinal tract. They differentiate from ECL cells in most of the cases and have receptors for somatostatin, CCK-B and gastrin. They are gastrin-sensitive, and this stimulates them for histamine release. Histamine stimulates parietal cells. Development of stem cells, which ECL cells origin from, is influenced by gastrin. ECL cells hyperplasia is continuous process and leads to hypergastrinaemia [26].

According to their histopathological features and clinical symptoms, gastric carcinoids were classified into three types, different in biological functioning and prognosis:

Type 1 – is related to CAG (chronic atrophic gastritis), hypergastrinaemia and achlorhydria. It is responsible for 75% of gastric carcinoids. Tumors occur in mucosa of the gastric corpus, in 57% they are multiple tumours. G and ECL cells hyperplasia is common. The frequency of metastasis is low [26] .

Type 2 – occurs with Zollinger-Ellison Syndrome (ZES, recurrent duodenal ulcers, pancreatic adenomas and increase in gastric acidity) and MEN 1 (multiple endocrine neoplasia type 1). This type is related to hypergastrinaemia and hyperchlorhydria (there is not atrophy of gastric corpus mucosa, strongly developed glandular layer with great number of parietal cells occurs). This type represent 5-10% of gastric carcinoids. Hyperplasia affects G cells first, then ECL cells of the corpus and fundus of stomach. This is caused by *MEN 1* gene mutation. *MEN 1* is located on chromosome 11 and encodes menin [26].

Type 3 – these are tumours which develop under stomach mucosa and represent about 15-25% of gastric carcinoids. They consist of ECL cells and small number of X and EC cells. Tumours are usually single and grow submucosal on unchanged gastric mucosa. They often form metastases to nearby lymph nodes. The disease has poor prognosis factors with acute character. Chromogranin level is higher than in two other types [26].

GEP-NET type of neuroendocrine tumours of gastrointestinal tract are important from the diagnostic point of view [28]. They can differ in localization and size. In 2004 the European Neuroendocrine Tumour Society published principles for diagnosis and treatment of gastrointestinal neuroendocrine tumours [29]. Clinicians and researchers agreed as to guidelines on treatment of patients with gastrointestinal neuroendocrine tumours [30]. Therapeutic procedures in GEP-NET type neuroendocrine tumours were described in details in B. Kos-Kudła and A. Zemczak papers [31]. Basic steps during diagnosis of neuroendocrine tumours are hormone activity evaluation (during histopathological analysis) and diagnostic imaging [32].

Use of molecular methods and understanding of gene-phenotype correlation which leads to *RET* oncogene mutation in the early stage of *MEN 1* and *MEN 2* (which is one of the most common neuroendocrine gastrointestinal tumours) can improve efficient treatment [33]. In recent years, several innovative discoveries were made in the field of genetics and the diagnosis and therapy of endocrine

pancreatic tumours, which will lead to better diagnosis and treatment of patients with this tumour [34].

The detection of tumours increases along with the distribution of modern diagnostic methods [31]. Diagnostic tools are biochemical analysis, ultrasound, pathomorphological methods, CT (computed tomography), magnetic nuclear resonance and radioisotope methods with labelled somatostatin derivatives. Somatostatin receptors scintigraphy is believed to be the most effective diagnostic method and its sensitivity in diagnosis of primary changes is higher than the imaging techniques. Scintigraphy also allows to detect metastases and preliminarily assesses tumour malignancy [31].

REFERENCES

1. Ostrowski K.: Histologia. Wydawnictwo Lekarskie PZWL, Warszawa 1995; 623-625
2. Pearse A.G.E., Takor-Takor T.: Embryology of the diffuse neuroendocrine system and its relationship to the common peptides . Fed. Proc.,1979; 2288-2294
3. Bożiłow W.: Histologia szczegółowa, Wydawnictwo Akademii Medycznej im. Ludwika Rydygiera, Bydgoszcz 2000;155-157
4. Pearse A.G.E.,Polak J.M.: Neural crest origin of the endocrine polypeptide (Apud) cells of the GI and pancreas. Gut, 1971; 12: 783-787
5. Kozłowski W., Jochymski C., Dąbek A., i inni.: Badania histochemiczne i immunohistoenzymatyczne komórek endokrynowych (ECL, EC,G i D) błony śluzowej żołądka u dzieci z *Helicobacter pylori* . Przegląd Pediatryczny, 1993; XXIII, 3: 249-254
6. Sawicki W.: Histologia, Wydawnictwo Lekarskie PZWL 2003; 404-406
7. Brongel L.: Komórki z grupy APUD i nowotwory z nich się wywodzące. Polski Przegląd Chirurgiczny, 1980; 52(5): 441-450
8. 8. Boyd C.A.R.: Amine uptake and peptide hormone secretion: APUD cells in a new landscape. Joural of Physiology, 2001; 531.3: 581-582
9. Konturek S.: Fizjologia człowieka t.V – Układ trawienny i wewnątrzwydzielniczy, Wydawnictwo Uniwersytetu Jagiellońskiego, Kraków 2000; 1-338
10. Bordi C., D´Adda T., Azzoni C., Ferraro G.: Classification of gastric endocrine cells at light and microscopical levels. Microsc. Res. Tech. 2000; 48: 258-271
11. Fenoglio-Preiser C.H.: Gastrointestinal Pathology. Ed. Lippincott-Raven. Philadelphia, New York, 1999
12. Kozłowski W., Jochymski C., Dąbek A.,i inni: Aktualny stan badań patomorfologicznych komórek endokrynowych błony śluzowej żołądka. Przegl. Wojsk.Med. 2001; 43 (3): 199- 204.
13. Mei-Rong He, Yu-Gang Song, Fa-Chao Zhi: Gastrointestinal hormone abnormalities and G and D cells in functional dyspepsia patients with gastric dysmotility. Gastroenterol. 2005 January 21; 11(3); 443-446
14. Muth E.R., Koch K.L., Stern R.M .: Significance of autonomic nervous system activity in functional dyspepsia. Dig Dis Sci 2000; 45: 854-863
15. Greenspan F. S., Gardner D. G.: Endokrynologia ogólna i kliniczna.Wyd. Czelej Sp. z o.o. Lublin, 2004: 1-995
16. Park D.I., Rhee P.L., Kim Y.H., et al.: Role of autonomic dysfunction in patients with functional dyspepsia. Dig Liver Dis 2001; 33: 464-471
17. Mirecka J.: Komórki dokrewne cewy pokarmowej II. Rozmieszczenie oraz podstawy identyfikacji ultrastrukturalnej. Przeg Lek 1977; 4: 419-422
18. Kozłowski W., Loba J., Klimczok J.: Badania immunohistochemiczne komórek G i D błony śluzowej żołądka u chorych z cukrzycą typu I. Biul WAM 1994, t XXXVII (1/4)
19. Polak J.M., Pearse A.G.E., Grimelius, Bloom S.R., Arimura A.: Growth-hormone release- inhibiting hormone in gastrointestinal and pancreatic D cells. Lancet 1975; 1:1220
20. Kobayashi S., Fujita T., Sasagawa T.: The endocrine cells of humane duodenal mucosa. An electron microscope study. Arch. Histol. Jap. 1970; 31: 477-481
21. Ganong W. F., Fizjologia Wyd Lek PZWL 2007: 1-820
22. Stevens A., Lowe J.: Histologia człowieka Wyd Lek PZWL, 2000: 1-408
23. Welbourn R., B.: Hormonalnie czynne nowotwory przewodu pokarmowego. Hexagon; 1983; 2: 8-17
24. Życińska K., Wardyn A.K, Olędzka-Oręziak M.: Zespół rakowiaka – nowe możliwości diagnostyczne i terapeutyczne. Terapia 2000; 8,12: 52-55
25. Nasierowska –Guttmajer A., Malinowska M.: Guzy neuroendokrynne układu pokarmowego (GEP-NET) – dyskusja wokół nazewnictwa i klasyfikacji. Przegl Gastroenterol 2006; 1,1: 16-21
26. Stachura T., Strzałka M., Bolt L.: Rakowiaki typu 1 i rozrosty z komórek ECL błony śluzowej żołądka. Przeg Lek 2003; 60, 12: 782-788
27. Wojtyczka A, Wojtyczka U.: Guzy neuroendokrynne górnego odcinka przewodu pokarmowego II. Dwunastnica, Pol Przeg Chir 1997; 69,8: 872-879
28. Krassowski J.: Insulinoma: najczęstsze błędy diagnostyczne. Endokrynologia Pol. 2003; 5(54): 700-701
29. Plockinger G. I inni: Guidelines for the Diagnosis and Treatment of Neuroendocrine Gastrointestinal Tumours. Neuro-endocrinology 2004; 80: 394-424

30. Rindi G. i inni: Consensus Guidelines for the Management of Patients with Digestive Neuroendocrine Tumours: Why Such Guidelines and How We Went about It. Neuroendocrinology 2006; 84: 155-157
31. Kos-Kudła B., Zemczak A.: Współczesne metody rozpoznawania i leczenia guzów neuroendokrynnych układu pokarmowego. Endokrynologia Pol. 2006; 2(57): 174-186
32. Kos-Kudła B. i inni.: Zalecenia diagnostyczno-lecznicze w guzach neuroendokrynnych układu pokarmowego. Endokrynologia Pol. 2008, 59: 41-45
33. Carling T.: Multiple endocrine neoplasia syndrome: genetic basis for clinical management. Current Opinion of Oncology. 2005; 17:7-12
34. Kate V. Viola: Current advances in the diagnosis and treatment of pancreatic tumors.Current Opinion of Oncology. 2005; 17: 7-12

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REVIEW / PRACA POGLĄDOWA

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THE ROLE OF VEGF IN TYPE 2 DIABETES

ROLA VEGF W CUKRZYCY TYPU 2

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S u m m a r y

On the threshold of the XXIst century diabetes has become an important problem and a serious threat not only to our health but also to our life. Diabetes constitutes one of the major independent cardiovascular risk factors. People diagnosed with diabetes bear a higher risk of coronary disease. The consequence of long-standing hyperglycemia is development of micro – and macroangiopathy. Intensified glycation and oxidation processes damage the blood vessel wall resulting in disturbed synthesis of nitric oxide (NO). Endogenous NO is produced through the conversion of 1-arginine to 1-cytruline by the nitric oxide synthase (NOS). Accumulation of advanced glycation end products (AGEs) is an important biochemical abnormality, associated with diabetes, which disturbs a balance between NOS isoforms. Both, increased NO production and decreased NO availability may cause tissue hypoxia.

Hyperglycemia, oxidative stress, hypoxia, endothelial dysfunction associated with diabetes intensify the expression of vascular endothelial growth factor gene (VEGF). VEGF is the most important cytokine that is responsible for diabetic microvascular and macrovascular complications. It is a molecule with angiogenic properties. VEGF has an influence on endothelial cells by increasing vessel permeability. Besides, it regulates endothelial cell proliferation. VEGF plays an important role in the pathogenesis of diabetic retinopathy and nephropathy. On the other hand, VEGF is an indispensable factor in wound healing, placenta formation and neovascularization. It also performs a neuroprotective function in the nervous system and is an essential cytokine for development of nerve cells.

Recent studies on VEGF show that it could be a target for gene therapy applied in the treatment of cancer, neurodegenerative diseases and diabetes complications.

S t r e s z c z e n i e

U progu XXI wieku cukrzyca stała się ważnym problemem endokrynologicznym i stanowi poważne zagrożenie nie tylko dla zdrowia, ale przede wszystkim dla życia człowieka. U chorych na cukrzycę wzrasta ryzyko wystąpienia incydentów wieńcowych, a konsekwencją długo trwającej hiperglikemii jest rozwój mikro- i makroangiopatii. Nasilone procesy glikacji i oksydacji uszkadzają ścianę naczyń krwionośnych, skutkiem czego zaburzona jest synteza tlenku azotu (NO). Tlenek azotu powstaje w reakcji katalizowanej przez syntazę tlenku azotu (NOS). Powstanie końcowych produktów glikacji zaburza równowagę pomiędzy izoformami NOS. Zmniejszenie ilości i biodostępności NO powoduje niedotlenienie tkanek.

Hiperglikemia, stres oksydacyjny, hipoksja, zaburzenie funkcji śródbłonka towarzyszące cukrzycy prowadzą do nasilenia ekspresji genu dla naczyniowo-śródbłonkowego czynnika wzrostu (VEGF). VEGF jest najważniejszą cytokiną odpowiedzialną za powstawanie naczyniowych powikłań cukrzycy, jest czynnikiem angiogennym. VEGF wpływa przede wszystkim na komórki śródbłonka naczyniowego poprzez zwiększenie przepuszczalności naczyń, indukuje proliferację komórek. Odgrywa zatem kluczową rolę w patogenezie retinopatii i nefropatii

cukrzycowej. Z drugiej strony VEGF jest niezbędnym czynnikiem w procesie gojenia ran, powstawania łożyska, tworzenia naczyń w okresie życia płodowego. Pełni funkcje neuroprotekcyjną w układzie nerwowym oraz jest niezbędną cytokiną do prawidłowego rozwoju komórek nerwowych.

Ostatnie lata badań nad VEGF wskazują, iż może być on celem terapii genowej stosowanej w leczeniu nowotworów, chorób neurodegeneracyjnych oraz narządowych powikłań cukrzycy.

Key words: diabetes, endothelial cells, angiogenesis, VEGF
Słowa kluczowe: cukrzyca, śródbłonek naczyniowy, angiogeneza, VEGF

INTRODUCTION

Diabetes is a widespread and an increasing health problem in the XXIst century [1]. In 2003 diabetes was diagnosed in approximately 194 millions of subjects worldwide. It is prognosed to increase up to 333 millions over the next 20 years [2]. About 25% of the Western population demonstrate some features of the insulin resistance syndromes which are strongly associated with an increased risk of type 2 diabetes (T2DM) [3].

Diabetes is associated with increased mortality because of its complications: microvascular (retinopathy, nephropathy, neuropathy) and macrovascular (atherosclerosis, coronary heart disease, stroke) [2, 4, 5]. In patients with diabetic vascular disease, apoptosis, migration and proliferation of endothelial cells contribute to uncontrolled expansion and damage of the vascular system [2, 4].

Endothelium is the most internal layer of blood vessels. Moreover, it is the biggest organ in our body. It acts as a multiple function organ: takes part in coagulation and fibrinolysis, regulates blood pressure through maintaining the vessel wall tone with the use of vasoconstrictor and vasodilators [6]. Due to its location, the endothelium actively conveys biological molecules into the surrounding tissues. Furthermore, it is a source of many factors that assure hemostasis and regulate cell proliferation, especially vascular endothelial growth factor (VEGF) [7, 8]. Hyperglycemia, hypoxia, oxidative stress or inflammation are factors that stimulate endothelial function [6].

Hyperglycemia is an important cause when it comes to promotion of endothelial dysfunction. The glucose molecule due to its unreactivity makes a great cellular fuel. Nevertheless, its transformation into advanced glycation end products (AGEs) could be harmful for tissues. AGEs are produced in reaction that does not require enzymatic catalysis [3, 9]. Subsequently there is also an interaction of AGEs with specific RAGE - receptors which are located on the surface of various tissues particularly on endothelial cells [7]. AGE/RAGE-complex induces oxidative reactions in which reactive oxygen species (ROS) are generated. AGE/RAGE complex also activates nuclear factor κB (NF-κB) that regulates expression of a variety of cytokines and genes [2, 3, 7]. As a consequence of AGEs accumulation, blood vessels become non-elastic and stiffened, which, in turn, contributes to increase of blood pressure in diabetic patients.

It is known that hyperglycemia is responsible for a decreasing bioavailability of NO and its production by endothelial nitric oxide synthase (NOS) [10]. NO is the most important factor that is released by endothelial cells. Moreover, NO relaxes blood vessels and increases blood flow [11]. It is formed in enzymatic reaction catalyzed by nitric oxide synthase [12]. We can distinguish three isoforms of NOS in tissues: endothelial – eNOS, neuronal – nNOS and inducible – iNOS. Two isoforms: nNOS and eNOS are produced constantly in picomolar levels – they are termed "constitutive" whereas iNOS is produced in response to several cytokines and immune factors. A crucial role in regulation of iNOS expression plays NF-κB [13].

Endothelial dysfunction is defined as an imbalance between vasodilating and vasoconstricting factors. Reduction in nitric oxide bioavailability and its production by eNOS lead to an increased ROS [11]. It is suggested that oxidative stress and endothelial dysfunction occur at an early stage of diabetes. Oxidative stress is defined as a change in the anti- and pro-oxidant balance. This pathological condition causes damage to molecules and cell's dysfunction. When ROS are generated at low concentration they can function as signaling molecules. However, at higher concentration ROS can cause cellular injury. The main mechanism which leads to ROS generation by NADPH in diabetes is protein kinase C (PKC) activation. Moreover, hyperglycemia is responsible for PKC activation [1, 5]. In pathological conditions with endothelial dysfunction nitric oxide is produced by iNOS at a very high concentration (even 1000-fold

higher than nNOS and eNOS) and it reacts with superoxide in the vessel wall. In this way, NO limits its availability [5, 12]. Thus in diabetes we can observe some NO paradoxes. Although it is present at a high level – it cannot fulfill its biological function and becomes harmful to surrounding cells. Decreased NO level, which is generated by eNOS, contributes to micro- and macroangiopathy progress. On the other hand, NO produced by iNOS in a huge quantity deepens endothelial dysfunction and causes hypoxia [7]. Hypoxia activates gene transcription of erythropoietin, VEGF and iNOS. Cellular response to hypoxia induces a mechanism that will enable cells to survive in new conditions. These mechanisms are: angiogenesis, glycolysis, erythropoiesis [14].

THE ROLE OF VEGF IN DIABETES

Senger et al. were first to describe the vascular endothelial growth factor. It was in 1983. At that time, VEGF was classified as a protein which increased vascular permeability and it was called vascular permeability factor (VPF). It was isolated from a guinea-pig hepatocarcinoma cell line. Several studies were conducted and in 1989 an endothelial growth factor, which was named VEGF, was identified [8]. VEGF is one of the most important secreted glycoprotein. In a native medium it appears as a basic homodimer binding heparin. Vascular endothelial growth factor, a potent and specific mitogen for endothelial cells in vessels, is produced not only by endothelial cells but also by fibroblasts, smooth muscle cells and macrophages [8, 15].

It is known that VEGF family is comprised of seven growth factors: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PIGF (placental growth factor). Each factor possesses different biological and physical properties [8]. VEGF-A, VEGF-B, VEGF-D, VEGF-E and PIGF are responsible for angiogenesis whereas, VEGF-C takes part in lymphatic vessels development. VEGF-A,-B, -C are necessary for cell progress [4].

VEGF-A is the most well-studied "element" of VEGF family. The gene, which is responsible for vascular endothelial growth factor, is located on chromosome 6 (6p21.3). It consists of eight exons and it is hypoxia-inducible gene [16]. Furthermore, VEGF expression is regulated by many factors: UV, nitric oxide, cobalt ion, ROS, mechanical injury and other growth factors and cytokines [7, 14, 17]. Moreover, in diabetes VEGF expression is induced by AGEs. Binding AGEs with RAGE leads to NF-κB activation [18, 19]. VEGF gene is highly polymorphic which influences vascular complications development in diabetic patients [20]. Twenty five different polymorphisms of VEGF gene are known. They are organized into haplotypes. In addition, 4 haplotypes have been identified in a promotor region [16]. As a consequence of alternative splicing of VEGF gene, various isoforms are produced: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$ [4, 15]. $VEGF_{145}$ is the main isoform that is expressed by ovarian and breast cancer tissue. $VEGF_{148}$ is detected in human glomeruli and breast cancer cells [21]. The $VEGF_{165}$ plays a key role in pathological angiogenesis [4] .

Members of VEGF family are the multifunctional cytokines that influence vascular endothelium and initiate a cascade of signaling events. VEGF acts by its special receptors that include tyrosine kinase receptors. VEGF is selective for vascular endothelium because of its major receptors that are mainly expressed by endothelium [8, 15, 22]. There are three transmembranous tyrosine kinase receptors for VEGF: VEGF-R1 (Flt-1), VEGF-R2 (KDR/Flk-1), VEGF-R3. Endothelial cells express also neuropilin (neuropilin-1 and neuropilin-2) which selectively bind with the 165 amino acid form of VEGF. Binding of VEGF with receptors induces autophosphorylation and signal transduction [21]. VEGF-R1 has a great affinity to VEGF-B and is a key regulator of vascular development and plays a crucial role in blood vessel formation. It is located on the surface of endothelial cells, macrophages and monocytes. VEGF-R2 also binds VEGF but this linkage is weaker. Nevertheless, VEGF-R2 is necessary for endothelial cell proliferation and differentiation. It is expressed on endothelial cells, megakaryocytes and thrombocytes. This receptor is present on the cells during embryonal life because it stimulates angiogenesis and vasculogenesis. VEGF-R3 is responsible for lymphatic vessels formation. It is observed on vascular endothelium in the human embryo. However, its expression in lymphatic vessels is also observed in adults [8, 15]. Neuropilins represent another kind of receptors. They function as co-receptors. Neuropilin-1 is located on the endothelial cells and plays an important role in heart vascularization [15]. Besides, it consolidates a signal transduction by VEGF-R2 [15, 22].

Vascular endothelial growth factor plays a crucial role in physiological angiogenesis (development of new blood vessels from existing vascular structures), tissue regeneration and wound healing. It is essential for endometrium, placenta and corpus luteum formation, thus VEGF is a very important growth factor for normal embryonic development. VEGF expression is increased in pathological events such as tumor development, angiogenesis, psoriasis or retinopathy and nephropathy [14]. Its expression is also observed in autoimmune diseases (rheumatoid arthritis) and neurodegenerative diseases (Alzheimer's Disease) [8].

Angiogenesis is the most important process during wound healing and organ regeneration. The first step in angiogenesis is to increase vessels permeability. VEGF mediates in this process through vascular fenestration and tight junction modulation. In this way, water and other molecules abandon blood vessels [4]. Vasculogenesis is another process which leads to vessel formation – it forms new blood vessels from endothelial progenitor cells (EPCs). VEGF-R2 is expressed on EPCs thus VEGF plays a crucial role in vasculogenesis [4]. Neurogenesis is also regulated by VEGF which has a neuroprotective function. Besides, it takes part in normal astrocytes, glial and Schwann cell development [4, 16]. As far as vessels are considered, VEGF can be characterized as a factor responsible for cell proliferation, increasing blood vessels permeability and cell's phenotype modulation [17]. In addition, vascular endothelial growth factor is a multifunctional cytokine that plays very important role in pathogenesis of vascular complication in diabetes [20]. Evidence suggests that VEGF may play a pivotal role in microvascular complications of diabetes. However, the underlying molecular mechanism by which VEGF leads to diabetic microangiopathy has only recently begun to be unraveled.

In diabetes growth factors' expression, especially this of VEGF, is observed in many tissues. This is the answer for hypoxia and hyperglycemic condition that leads to organ complications [21]. Hypoxia, increased blood vessel permeability and neovascularization are the characteristics of diabetic retinopathy [20]. VEGF is produced by various types of vision cells: retinal capillary, pericytes, glial cells, endothelial cells. According to some authors there is a correlation between VEGF gene polymorphism and retinopathy development in diabetic patients [20]. The mechanism through which VEGF induces vessel formation is multifactorial. Both hypoxia and AGEs increase VEGF expression. Higher VEGF level leads to intensive VEGF receptors' expression which, in turn, contributes to pathological angiogenesis. Apart from mitogenic effects, VEGF favours the proteolysis of membrane elements and vascular fenestration. This leads to increase of vessel permeability, which is the first step in the angiogenesis [4].

VEGF may also take part in pathogenesis of diabetic nephropathy. It is known that VEGF functions through its specific receptors. In vivo renal cells show a strong expression of two types of VEGF receptor: VEGF-R1 and neuropilin-1. However, in vitro VEGF-R2 expression has been observed. It is suggested that signal transduction through VEGF-R2 can influence nitric oxide bioavailability. There are hypotheses that after binding $VEGF_{165}$ with VEGF-R2, iNOS is activated [4, 18]. Hyperglycemia due to AGEs accumulation intensifies not only VEGF but also VEGF-R2 expression [18].

There is a positive correlation between VEGF level and the risk of coronary heart disease in diabetic patients. Increased VEGF level during myocardial hypoxia leads to EPCs mobilization from bone marrow endothelial progenitor cells. EPCs contribute to proliferation and blood vessel formation. Nevertheless, hypertension and hypercholesterolemia, which are associated with diabetes, decrease EPCs number resulting in ineffective angiogenesis [4].

Mutual relation between VEGF and micro- and macroangiopathy is not a simple dependency, likewise diabetes is not an isolated disease. Mechanisms, which induce VEGF synthesis, are multifactorial and are consequence of processes that take place in patient with diabetes.

Worse wound healing observed in diabetic patients is a consequence of inflammation which accompanies diabetes. This leads to macrophage dysfunction and decreases VEGF concentration. Reduced VEGF secretion in an injury area results in reduced EPCs, their dysfunction and defective angiogenesis [4].

THE OTHER FACE OF VEGF – PROSPECTS

Several studies have been conducted to use VEGF in gene therapy [23]. Thanks to gene manipulation with the use of molecular biology methods a new way of treatment may be introduced for those with complications related to abnormal VEGF expression. The basis of gene therapy is the administration of exogenous DNA which will influence genes responsible for pathological processes [23]. In diabetic patients who have undergone gene therapy with VEGF the number of feet amputations was diminished [4, 23]. Moreover, improvement in wound healing has been

reported in diabetic mice which were given intradermal injection with adenoviruses encoding VEGF-C [4].

In the very recent study intramuscular gene transfer using plasmid vascular endothelial growth factor (VEGF) was used in patients to treat diabetic polyneuropathy [24]. It was found that plasmid VEGF gene transfer improved diabetic neuropathic symptoms, meeting primary end-point criteria for efficacy. The results, however, are not conclusive but may justify further clinical study.

REFERENCES

1. Hadi H.A.R., Hadi J.A.I. Endothelial dysfunction In diabetes mellitus. Vasc. H. and R. Manag. 2007;3:853-876
2. Wright J.R.E., Scism-Bacon J.L., Glass L.C. Oxidative stress in type 2 diabetes: the role of fasting and posprandial glycemia. Int. J. Clin. Pract. 2006;60: 308-314
3. Kohler H.P. Insulin resistance syndrome: interaction with coagulation and fibrinolysis. Swiss. Med. Wkly. 2002;132:241-252
4. Wirostko B., Wong T.Y., Simo R. Vascular endothelial growth factor and diabetic complications. Prog. In Ret. And Eye Res. 2008;27:608-621
5. Su Y., Liu X.M., Sun Y.M. et al. The relationship between endothelial dysfunction and oxidative strss in diabetes and prediabetes. Int. J. Clin. Pract. 2008;62:877-882
6. Hartge M.M., Unger T., Kintscher U. The endothelium and vascular inflammation in diabetes. Diabetes. Vasc. Dis. Res. 2007;4:84-88
7. Bakker W., Eringa E.C., Spikema P., et al. Endothelial dysfunction and diabetes: roles of hyperglycemia, impaired insulin signaling and obesity. Cell. Tissue. Res. 2009;335:165-189
8. Carvalho J.F., Blank M., Shoenfeld Y. Vascular endothelial growth factor (VEGF) in autoimmune diseases. J. Clin. Immun. 2007;3:246-254
9. Giuliano D., Ceriello A., Esposito K. Glucose metabolism and hyperglycemia. Am. J. Clin. Nutr. 2008;87:217-221
10. Bagg W., Ferri C., Cesideri G., et al. The influences of obesity and glycemic control on endothelial activation in patients with type2 diabetes. J. Clin. Endocrinol. Metab. 2001;86:5491-5497
11. Hamilton S.J., Chew G.T., Watts G.F. Therapeutic regulation of endothelial dysfunction In type 2 diabetes mellitus. Diabetes. Vasc. Dis. Res. 2007;4:89-102
12. Mackiewicz U., Mączewki M., Beręsewicz A. Różne twarze tlenku azotu. Kardiol. Pol. 2002;57:IV-16
13. Oszajca K., Szemraj J., Barkowiak J. The influence of nitric oxide on the regulation of plasminogen activator inhibitor type 1 and tissue-type plasminogen activator expression. Post. Biochem. 2005;51:407-413
14. Kimura H., Weisz A., Ogurat T., et al. Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. The J.B. Chem.2001;3:2293-2298
15. Barańska P., Jerczyńska H., Pawłowska Z. Vascular endothelial growth factor-structure and functions. Post. Biochem. 2005;51:12-21
16. Del Bo R., Ghezzi S., Scarpini E., et al. VEGF genetic variability is associated with increased risk of developing Alzheimer"s disease. 2009;article in press
17. Castilla M.A., Caramelo C., Gazapo R.M., et al. Role of vascular endothelial growth factor (VEGF) in endothelial cell protection against cytoxic agents. L. Scien. 2000;67:1003-1013
18. Pala L., Cresci B., Manuelli C. et al. Vascular endotheliai growth factor receptor-2 and low affinity VEGF binding sites on human glomerular endothelial cells: Biological effects and advanced glycosylation end products modulation. Microvasc. Res. 2005;70:179-188
19. Lim H.S., Blann A.D., Chong A.Y., et al. Plasma vascular endothelial growth factor, angiopoietin-1 and angiopoietin-2 in diabetes. Diab. Care. 2004;27:2918-2924
20. Buraczyńska M., Książek P., Baranowicz-Gaszczyk I., et al. Association of the VEGF gene polymorphism with diabetic retinopathy in type 2 diabetes patients. Nephrol. Dial. Transplant. 2007;22:827-832
21. Zygalski E., Kaklamanis L., Nikolaou N.I. et al. Expresion profile of Total VEGF, VEGF splice variants and VEGF receptors In the myocardium and arterial vasculature of diabetic and non-diabetic patients with coronary artery disease. Clin. Biochem. 2008;41:82-87
22. Sengupta S., Gherardi E., Sellers L.A., et al. Hepatocyte growth factor/scatter factor can induce angiogenesis independently of vascular endothelial growth factor. Arterioscler. Thromb. Vasc. Biol. 2003;23:69-75
23. Dulak J., Józkowicz A., Guevara I., et al. Gene transfer of vascular endothelial growth factor and endothelial nitric oxide synthase – implications for gene therapy in cardiovascular diseases. Pol. J. Pharmacol. 1991;51:233-241
24. Ropper AH., Gorson KC., Gooch CL. et al. Vascular endothelial growth factor gene transfer for diabetic polyneuropathy: a randomized, double-blinded trial. Ann Neurol 2009;65:386-93

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ORIGINAL ARTICLE / PRACA ORYGINALNA

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TRAITS OF BODY MUSCULATURE IN STUDENTS OF MEDICINE AND PHYSICAL EDUCATION

CECHY UMIĘŚNIENIA CIAŁA U STUDENTÓW STUDIÓW MEDYCZNYCH ORAZ WYCHOWANIA FIZYCZNEGO

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Summary

Introduction. Active body mass is a body component which may play the role of change-sensitive sensor since it is able to react quickly to different exogenous factors. The primary aim of this paper was to examine the relations of the so-called traits of body musculature between the female and male groups of subjects.

Material and methods. The research was carried out in a group of 695 students divided into four groups – two male groups and two female groups. The somatotype of students was specified according to the method proposed by Sheldon and the body was tested by segmental bioelectrical impedance analysis (SBIA).

Results. The female and male students of Physical Education obtained significantly higher values in the so-called body musculature traits, compared to their peers from Collegium Medicum. Between the examined traits within each of the groups there was a high correlative dependence.

Conclusions. Intensive physical activity contribute to the increase of protein body mass, total content of fluid and lean body mass (LBM).

Streszczenie

Wstęp. Aktywna masa ciała jest to komponent ciała, który może pełnić rolę czułego sensora zmian, gdyż jest w stanie szybko reagować na działanie różnych egzogennych czynników. Podstawowym celem niniejszej pracy było zbadanie relacji tzw. cech umięśnienia ciała pomiędzy badanymi żeńskimi i męskimi grupami studentów.

Materiał i metody. Badania prowadzone były na grupie 695 studentów podzielonych na cztery podgrupy – dwie męskie i dwie żeńskie. Somatotyp badanych studentów określono wg typologii Sheldona oraz zbadano skład ciała metodą SBIA.

Wyniki. Studentki i studenci wychowania fizycznego uzyskali istotnie wyższe wartości w tzw. cechach umięśnienia ciała w stosunku do swych rówieśników z Collegium Medicum. Pomiędzy badanymi cechami wewnątrz każdej z badanych grup zanotowano wysoką korelacyjną zależność.

Wnioski. Intensywna aktywność fizyczna przyczynia się do zwiększenia proteinowej masy ciała, ogólnej zawartości płynów oraz szczupłej masy ciała (LBM).

Key words: body musculature, SBIA, medical and physical education students
Słowa kluczowe: umięśnienie ciała, SBIA, studenci studiów medycznych i wychowania fizycznego

INTRODUCTION

Active body mass is a specific trait of a living human due to its structure, distribution and functions. It is a body component which may play the role of change-sensitive sensor since it is able to react quickly to different exogenous factors. Active body mass, including skeletal mass, represents – as everybody knows – the biggest homogeneous (in the morphofunctional sense) body tissue and, together with fat body mass, considerably affects the human somatotype. At the same time it is an unusually plastic structure (in particular skeletal muscles) that quickly adapts to the exogenous factors of the environment – physical exercise, and eco-sensitive to other lifestyle factors, in particular eating habits [1, 2 et al.]. A beneficial effect of physical activity on our organism is confirmed by a lot of scientific research. Many beneficial changes that result from regular exercises can be noticed easily. They most often include a straight lean figure, better muscular structure of the body, a brisk and springy stride, and no symptoms of tiredness after physical work. Beneficial changes occur both at the macro- and microscopic level [3, 4].

According to the traditional two-component model proposed by Siri [5], the human body consists of fat mass and fat-free mass. On the basis of similar principles Forbes [6] divides the body mass into two components: metabolically active and metabolically inactive. The first one, i.e. the fat-free metabolically active component, consists in 73.8% (according to other authors 72.3% [7, 8]) of: water, 19.4% – protein and 6.8% – mineral elements [9], and considerably correlates with muscle mass [10, 11, 12, 13]. The research regarding sportsmen showed that intensive weight training leads to increased reserve of organic water in the body [14], and water, in turn, correlates with hypertrophy of muscles [15]. Based on the degree of body hydration, Pace and Rathbun [7] proposed a formula for the assessment of so-called lean body mass (LBM) which was used in our research to assess total and segmental lean body mass (LBM, sLBM) and was used as one of the body musculature indices.

Other means used to measure body musculature was the value of mesomorphic component of somatotype according to Heath-Carter's method [16] used by other researchers [15, 17] for the same purpose, and arm circumference having subtracted subcutaneous fat [18, 19], as well as protein body mass [20].

The segmental bioelectrical impedance method, applied in this research, was used in a number of other researches assessing the segmental distribution of body fluids [21, 22], regional muscle mass [18] and lean body mass [23, 24].

The primary aim of this paper was to examine the relations of the so-called traits of body musculature between the female and male groups of subjects and their correlative interdependence within each of the groups.

MATERIAL AND METHODS

The research was carried out in a group of 695 students divided into four groups – two male groups and two female groups. The male group (192 people) aged 22.69 ± 1.78 was made up of Collegium Medicum students (98 people) and Physical Education students (94 people). The female group included 503 students aged 22.53 ± 1.9 8 (385 from Collegium Medicum and 118 physical education students).

The research was conducted over a few years in the Chair and Department of Anthropology at the Nicolaus Copernicus University Collegium Medicum in Bydgoszcz. The students underwent anthropometric measurements with Martin's technique which permitted to describe their somatotype according to Sheldon's typology with the Heath-Carter's modification [16], and their BMI was calculated. The body composition was investigated with segmental bioelectrical impedance analysis (SBIA) using the Biospace Co. Ltd. *In Body 3.0* apparatus. The principle of the SBIA method is measurement of electrical resistance in the body, which mainly depends on tissue hydration and electrolyte concentration [25, 26].

The assessment of lean body mass (LBM) and segmental lean mass of limbs and trunk (sLBM) was made on the basis of total and segmental content of organic water in the body [6, 7]:

LBM (%) = % water / 0,732

and

sLBM (%) = % segmental water / 0,732

Basic statistical characteristics (arithmetical mean and standard deviation) were calculated. The differences between the groups of students were

evaluated with a t-Student test and the correlation analysis was applied.

RESULTS

The results of the research are presented in Tables I to IV.

Table I. *Statistical characteristics of traits of female students of Collegium Medicum (n_1=385) and female physical education students (n_2=118)*

Tabela I. *Charakterystyki statystyczne cech studentek Collegium Medicum (n_1=385) i studentek Wychowania Fizycznego (n_2=118)*

Cecha (Feature)	Studentki (female students)			Test t Studenta (T-Student test)
	Całość (Total) N=503	Collegium Medicum (Collegium Medicum) n_1=385	Wych. Fizyczne (physical education) n_2 =118	
	M ± SD	M ± SD	M ± SD	
Woda całkowita (Total Body Water) [l]	31,98 ± 3,99	31,46 ± 3,75	33,69 ± 4,27	**p ≤ 0.001**
Masa proteinowa (Protein Mass) [kg]	11,65 ± 1,45	11,46 ± 1,37	12,26 ± 1,56	**p ≤ 0.001**
Szczupła masa ciała [kg] LBM: (Lean Body Mass)	46,19 ± 5,74	45,44 ± 5,41	48,63 ± 6,12	**p ≤ 0.001**
Segmentalna (Segmental): $sLBM_{pkg}$	2,27 ± 0,40	2,21 ± 0,37	2,48 ± 0,41	**p ≤ 0.001**
$sLBM_{lkg}$	2,23 ± 0,40	2,17 ± 0,37	2,43 ± 0,42	**p ≤ 0.001**
$sLBM_t$	19,95 ± 2,43	19,59 ± 2,28	21,12 ± 2,53	**p ≤ 0.001**
$sLBM_{pkd}$	7,18 ± 1,02	7,09 ± 0,98	7,50 ± 1,09	**p ≤ 0.001**
$sLBM_{lkd}$	7,18 ± 1,01	7,08 ± 0,97	7,49 ± 1,09	**p ≤ 0.001**
Segmentalna dystrybucja płynów(W) [l]: sW_{pkg}	1,66 ± 0,29	1,62 ± 0,27	1,81 ± 0,30	**p ≤ 0.001**
(Segmental Fluid Distribution) sW_{lkg}	1,63 ± 0,29	1,59 ± 0,27	1,78 ± 0,30	**p ≤ 0.001**
sW_t	14,61 ± 1,92	14,33 ± 1,67	15,54 ± 2,33	**p ≤ 0.001**
sW_{pkd}	5,26 ± 0,75	5,19 ± 0,72	5,49 ± 0,80	**p ≤ 0.001**
sW_{lkd}	5,26 ± 0,71	5,19 ± 0,71	5,49 ± 0,80	**p ≤ 0.001**
Obwód mięśniowy ramienia [cm] (Muscular circumference of arm)	21,25 ± 1,58	21,04 ± 1,57	21,93 ± 1,45	**p ≤ 0.001**
Wysokość ciała [cm] (Height)	166,6 ± 6,47	166,1 ±6,12	168,1 ± 7,16	**p ≤ 0.01**
Masa ciała [kg] (Body mass)	59,6 ± 9,13	59,2 ± 9,39	60,7 ± 8,13	-
Wskaźnik BMI [kg/m^2] (Body Mass Index)	21,44 ±2,83	21,45 ± 3,04	21,41 ± 2,83	-
Mezomorfia – II (Mesomorphy)	2,41 ± 1,46	2,30 ± 1,49	2,83 ± 1,25	**p ≤ 0.01**

LBM_{pkg} - prawa kończyna górna (right upper limb)-
LBM_{lkg} – lewa kończyna górna (left upper limb)
LBM_t – tułów (trunk)
LBM_{pkd} – prawa kończyna dolna (right lower limb)
LBM_{lkd} – lewa kończyna dolna (left lower limb)

Between the groups of female students occurred a significant difference in body height. The average female students of Physical Education, with similar body mass and BMI, were two centimetres taller than their peers from Collegium Medicum (Table I). The two groups of male students were more homogeneous in respect of basic traits and did not differ significantly from each other (Table II). In respect of body composition, however, the groups, both female and male, were considerably different. The female and male students of Physical Education obtained significantly higher values in the so-called body musculature traits, compared to their peers from Collegium Medicum. Only the value of lean body mass concerning the left lower limb ($sLBM_{lkd}$) and fluid distribution in this area (sW_{lkd}) in the students of both groups were similar.

Tabela II. *Charakterystyki statystyczne cech studentów Collegium Medicum (n_1=98) i studentów wychowania fizycznego (n_2=94)*

Table II. *Statistical characteristics of traits of male students of Collegium Medicum (n_1=98) and male physical education students (n_2=94)*

Cecha (Feature)	Studenci (male students)			Test t Studenta (t-Student test)
	Całość (Total) N=192	Collegium Medicum (Collegium Medicum) n_1=98	Wych. Fizyczne (physical education) n_2=94	
	M ± SD	M ± SD	M ± SD	
Woda całkowita (Total Body Water) [l]	46,46 ± 5,25	45,58 ± 5,13	47,37 ± 5,23	**p ≤ 0.05**
Masa proteinowa (Protein Mass) [kg]	16,92 ± 1,92	16,61 ± 1,87	17,24 ± 1,91	**p ≤ 0.05**
Szczupła masa ciała [kg]	66,85 ± 7,50	65,63 ± 7,34	68,12 ± 7,49	**p ≤ 0.05**
LBM: (Lean Body Mass) Segmentalna				
(Segmental): $sLBM_{pkg}$	3,95 ± 0,58	3,79 ± 0,54	4,11 ± 0,58	**p ≤ 0.001**
$sLBM_{lkg}$	3,90 ± 0,57	3,75 ± 0,54	4,05 ± 0,56	**p ≤ 0.001**
$sLBM_t$	29,71 ± 3,26	28,91 ± 3,11	30,55 ± 3,22	**p ≤ 0.001**
$sLBM_{pkd}$	10,26 ± 1,15	10,10 ± 1,11	10,43 ± 1,18	**p ≤ 0.05**
$sLBM_{lkd}$	10,23 ± 1,15	10,08 ± 1,12	10,38 ± 1,17	-
Segmentalna dystrybucja płynów(W) [l]: sW_{pkg}	2,89 ± 0,42	2,78 ± 0,40	3,01 ± 0,42	**p ≤ 0.001**
(Segmental Fluid sW_{lkg}	2,85 ± 0,42	2,74 ± 0,40	2,96 ± 0,42	**p ≤ 0.001**
Distribution) sW_t	21,79 ± 2,49	21,27 ± 2,49	22,33 ± 2,39	**p ≤ 0.01**
sW_{pkd}	7,51 ± 0,84	7,39 ± 0,82	7,64 ± 0,85	**p ≤ 0.05**
sW_{lkd}	7,49 ± 0,84	7,38 ± 0,82	7,60 ± 0,85	-
Obwód mięśniowy ramienia [cm] (Muscular circumference of arm)	26,20 ± 2,15	25,68 ± 2,24	26,74 ± 1,93	**p ≤ 0.001**
Wysokość ciała [cm] (Height)	181,0 ± 6,26	180,7 ± 6,20	181,3 ± 6,34	-
Masa ciała [kg] (Body mass)	76,8 ± 9,43	77,1 ± 10,15	76,6 ± 8,65	-
Wskaźnik BMI [kg/m^2] (Body Mass Index)	23,44 ± 2,55	23,59 ± 2,99	23,28 ± 2,55	-
Mezomorfia – II (Mesomorphy)	3,86 ± 1,23	3,64 ± 1,36	4,09 ± 1,04	**p ≤ 0.05**

LBM_{pkg} - prawa kończyna górna (right upper limb)
LBM_{lkg} – lewa kończyna górna (left upper limb)
LBM_t – tułów (trunk)
LBM_{pkd} – prawa kończyna dolna (right lower limb)
LBM_{lkd} – lewa kończyna dolna (left lower limb)

Between the examined traits within each of the groups there was a high correlative dependence (Tables III and IV). The only non-significant correlation was between mesomorphic component and segmental lean body mass of the lower limb. The lack of interdependence between these traits is difficult to explain.

Tabela III. *Współczynniki korelacji cech umięśnienia ciała u studentek Collegium Medicum i Wychowania Fizycznego*
Table III. *Correlation coefficient of body musculature features in female students of Collegium Medicum and female physical education students*

a. Studentki Collegium Medicum (Female students of Collegium Medicum)

	m.ciała	proteiny	LBM	II	AMC	m.PR	m.LR	m.T	m.PN
proteiny	0.859 **								
LBM	0.855 **	0.994 **							
II	0.278 **	0.143 **	0.137 **						
AMC	0.851 **	0.811 **	0.811 **	0.408 **					
Mięśnie PR	0.818 **	0.967 **	0.964 **	0.207 **	0.885 **				
LR	0.827 **	0.966 **	0.963 **	0.209 **	0.891 **	0.987 **			
T	0.837 **	0.982 **	0.978 **	0.159 **	0.849 **	0.985 **	0.985 **		
PN	0.751 **	0.938 **	0.932 **	0.001	0.586 **	0.843 **	0.840 **	0.883 **	
LN	0.747 **	0.933 **	0.928 **	0.007	0.578 **	0.837 **	0.834 **	0.878 **	0.996 **

b. Studentki Wychowania Fizycznego (Female physical education students)

	m.ciała	proteiny	LBM	II	AMC	m.PR	m.LR	m.T	m.PN
proteiny	0.928 **								
LBM	0.927 **	0.999 **							
II	0.293 **	0.176	0.176						
AMC	0.802 **	0.798 **	0.799 **	0.496 **					
mięśnie PR	0.305 **	0.350 **	0.353 **	0.122	0.311 **				
LR	0.303 **	0.349 **	0.353 **	0.096	0.304 **	0.982 **			
T	0.328 **	0.372 **	0.375 **	0.088	0.302 **	0.987 **	0.987 **		
PN	0.300 **	0.370 **	0.373 **	-0.019	0.219 *	0.852 **	0.852 **	0.897 **	
LN	0.299 **	0.372 **	0.375 **	-0.020	0.219 *	0.852 **	0.855 **	0.900 **	0.996 **

Tabela IV. *Współczynniki korelacji cech umięśnienia ciała u studentów Collegium Medicum i Wychowania Fizycznego*
Table IV. *Correlation coefficient of body musculature features in male students of Collegium Medicum and male physical education students*

a. Studenci Collegium Medicum (male students of Collegium Medicum)

	m.ciała	proteiny	LBM	II	AMC	m.PR	m.LR	m.T	m.PN
proteiny	0.883 **								
LBM	0.882 **	0.999 **							
II	0.385 **	0.351 **	0.352 **						
AMC	0.799 **	0.805 **	0.805 **	0.606 **					
mięśnie PR	0.794 **	0.964 **	0.965 **	0.386 **	0.834 **				
LR	0.792 **	0.963 **	0.963 **	0.399 **	0.836 **	0.985 **			
T	0.806 **	0.976 **	0.976 **	0.362 **	0.818 **	0.993 **	0.993 **		
PN	0.594 **	0.833 **	0.835 **	0.056	0.404 **	0.777 **	0.773 **	0.805 **	
LN	0.592 **	0.834 **	0.836 **	0.058	0.408 **	0.778 **	0.776 **	0.806 **	0.995 **

b. Studenci Wychowania Fizycznego (male physical education students)

	m.ciała	proteiny	LBM	II	AMC	m.PR	m.LR	m.T	m.PN
proteiny	0.933 **								
LBM	0.933 **	1.000 **							
II	0.366 **	0.251 *	0.250 *						
AMC	0.763 **	0.793 **	0.792 **	0.577 **					
Mięśnie PR	0.708 **	0.832 **	0.831 **	0.241 *	0.774 **				
LR	0.732 **	0.850 **	0.850 **	0.282 **	0.821 **	0.964 **			
T	0.762 **	0.879 **	0.879 **	0.249 *	0.800 **	0.965 **	0.991 **		
PN	0.718 **	0.842 **	0.844 **	0.017	0.478 **	0.787 **	0.784 **	0.825 **	
LN	0.716 **	0.844 **	0.846 **	0.019	0.481 **	0.796 **	0.793 **	0.833 **	0.995 **

DISCUSSION

Our research showed statistically significant differences in the so-called traits of body musculature within the female and male groups of physiotherapy students and physical education students. A lot of other research also demonstrated distinct hypertrophy of skeletal muscles in persons practicing weight or speed sports disciplines in comparison with control groups [3, 4, 27, 28, 29], as well as in persons undergoing intensive electrostimulation of muscles [4, 30], and even in sportsmen practicing endurance sports disciplines [31]. A lot of research was devoted to differences in body composition including muscle mass, lean body mass and limb circumference, in groups of physically active and inactive people [18, 25, 26 et al.].

Numerous researches confirm that expansive physical activity leads to increased strength and muscle mass [3, 4, 29, 32 et al.] and, consequently, lean body mass index LBM [28, 33, 34], which is logic and

predictable. Kitagawa and Miyashita [33] note a high correlation between strength and muscle mass on the one hand, and lean body mass (LBM) on the other hand. According to Spenst et al. [35], high values of LBM can be found among bodybuilders, weightlifters and sprinters, in other words – in typical weight sports disciplines, whereas they are relatively lower in swimmers, long-distance runners and non-trained persons.

Similarly to our study, Knechtle et al. [23] note a statistically significant increase in protein body mass, total content of body fluids and LBM in trained persons, whereas Modlesky et al. [15] find a close relationship between mesomorphy and body musculature, and between the degree of muscle hypertrophy and LBM index. On the other hand, many researches show a high correlation between muscle mass (and LBM) and protein body mass [20, 36, 37, 38, 39 et al.], which was found in our research as well. The research by Grund et al. [18] indicates a connection between intensive training and increased muscle mass, LBM, total content of organic water and arm circumference, which was also found in our research.

The results, following a correlation analysis, showed a high interdependence of almost all body musculature traits in all four groups of students, which was highly predictable.

Conclusions: Intensive physical activity contribute to the increase of protein body mass, total content of fluid and lean body mass (LBM).

REFERENCES

1. Cheek D.B., Schultz R.B., Parra A., Reba R.C.: Overgrowth of lean and adipose tissue in adolescent obesity. Pediatr.Res.1970; 4: 268-271.
2. Forbes G.B.: Lean body mass and fat in obese children. Pediatrics 1964; 34: 308-311.
3. Čabrić M.: Morphometric analyses of the muscles of weight-lifters. Med. Sport. 2002; 18, 9: 397-402.
4. Čabrić M.: Morfometryczna analiza zmian struktury mięśni szkieletowych pod wpływem hiperfunkcji.(Monografia),Wyd. A.M, Bydgoszcz 2003.
5. Sir W.E.: Body composition from fluid space and density: analysis and methods. In: Techniques for Measuring Body Composition. (Ed. Brozek J. Hanschel A.). Natl. Acad. Sc 1961; 223-244.
6. Forbes G.B.: Human Body Composition and Activity. Springer-Verlag, London 1987
7. Pace N., Rathbun E.N.: Studies on Body Composition. III. The Body Water and Chemically Combined Nitrogen Content in Relation to Fat Content. J. Biol. Chem.1945; 158: 685-691.
8. Wieliński D.: Komponenty ciała człowieka w aspekcie tradycyjnych i najnowszych metod badawczych. Monografie, nr 338, Poznań 2000.
9. Brozek J., Grande F., Anderson J.T., Keys A.: Densitometry analysis of body composition: revision of some quantitative assumptions. Ann. N. York Acad. Sc. 1963; 110: 113-140.
10. Levine J.A., Abboud L., Barry M., Reed J.E., Sheedy P.F., Jensen M.D.: Measuring leg muscle and fat mass in humans: comparison of CT and dual-energy X-ray absoptiometry. J. App. Phys. 2000; 88: 452-456.
11. Visser M., Fuerst T., Lang T., Salamons L., Harris T.B.: Validity of fan-beam dual-energy X-ray absorptiometry for measuring fat-free mass and leg muscle mass. J. App. Phys. 1999; 87: 1513-1520.
12. Wang W., Wang Z., Faith M.S., Kotler D., Shih R., Heymsfield S.B.: Regional skeletal muscle measurement: evaluation of new dual-energy X-ray absorptiometry. J. App. Phys 1999; 87: 1163-1171.
13. Wang Z.M., Visser M., Ma R., Baumgartner R. N. , Kotler D., Gallagher D., Heymsfield S.B.: Skeletal muscle mass: evaluation of neutron activation and dual-energy X-ray absorptiometry methods. J. App. Phys. 1996; 80: 824-831.
14. Campbell W.C., Crim M.C., Young V.R., Evans W. J.: Increased energy requirements and changes in body composition with resistance training in older adults. A.J. Clin. Nutr. 1994; 60: 167-175.
15. Modlesky C.M., Cureton K.J., Lewis R.D., Prior B.M., Sloniger M.A., Rowe D.A.: Density of the fat-free mass and estimate of body composition in male weight trainers. J. App. Phys. 1996; 80: 2085-2096.
16. Carter J.E.L., Heath B.H.: Somatotyping - Development and Applications. Cambridge University Press, Cambridge 1990.
17. Prior B.M., Modlesky C.M., Evans E.M., Sloniger M.A., Saunders M.J., Lewis R.D., Cureton K.J.: Muscularity and the density of the fat-free mass in athletes. J. App. Phys 2001; 90: 1523-1531.
18. Grund A., Krause H., Kraus M., Siewers M., Rieckert H., Möller M.J.: Association between different attributes of physical activity and fat mass in untrained, endurance- and resistance-trained men. E. J. App. Phys. 2001; 84: 310-320.
19. Heymsfield S.B., McManus C., Smith J.: Anthropometric measurement of muscle mass: revised equations for calculating bone-free arm muscle area. Am .J. Clin. Nutr. 1982; 36: 680-690.
20. Proctor D.N., O´Brien P.C., Atkinson E.J., Nair K.S.: Comparison of techniques to estimate total body skeletal muscle mass in people of different age groups. Am. J. Phys 1999; 277: (Endocrinol.Metab.40),E489-E495.
21. Scheltinga M.R., Jacobs D.O., Kimbrough T.D., Wilmore D.W.: Identifying body fluid distribution by measuring electrical impedance. J. of Trauma-Injury Infection & Critical Care 1992; 33 (5): 665-670.

22. Wotton M.J., Thomas B.J., Cornish B.H., Ward L.C.: Comparison of whole body and segmental bioimpedance methodology for estimating total body water. Ann. N. York Acad. Sc. 2000; 904: 181-186.
23. Knechtle B., Salas-Fraire O., Andonie J.L., Kohler G.: Effect of a multistage ultra-endurance triathlon on body composition: World Challenge Deca Iron Triathlon 2006. J. Sports Med. 2007; 42: 121-125.
24. A.D., Andreoti A.: Segmental bioelectrical impedance analysis. (Review), Lorenzo Current Opinion in Clinical. Nutrition & Metabolic Care 2003; 6 (5): 551-555.
25. Čabrić M., Krakowiak H., Janczak R.: Badania zależności między typem budowy ciała a składem ciała u młodych kobiet. Postępy Rehabilitacji 2002; 2, 1: 1-5.
26. Čabrić M., Krakowiak H., Sokołowska E., Janczak R.: Badania zależności między wybranymi cechami morfologicznymi a sprawnością fizyczną u kobiet w różnym wieku. Scripta Periodica, III 2000; 2, suppl.1/2: 541-546.
27. Čabrić M.: Quantitative Analyses of Skeletal Muscles of Hanball Players. Scripta Periodica III 2000; 3: 187-192.
28. Janiak J., Krawczyk B.: Relationships between force and total or lean body mass in highly experienced combat athletes. Biol. Sport 1995; 12, 8: 107-111.
29. Maughan R.J., Watson J., Weir J.: Strength and cross-sectional area oh human skeletal muscle. J. App. Phys. 1983; 338: 37-49.
30. Čabrić M., Appell H-J., Rešić A.: Fine structural changes in electrostimulated human skeletal muscle. E. J. App. Phys.1988; 57: 1-5.
31. Raschka C., Plath M., Cerull R.: The body muscle compartment and its relationship to food absorption and blood chemistry during an extreme endurance performance. Z. Ernährungswiss.1991; 30: 276-288.
32. Brown C.H., Wilmore J.H.: The effect of maximal resistance training on the strength and body composition of women athletes. Med. Sc. Sports, 1974; 6: 174-177.
33. Kitagawa K., Miyashita M.: Muscle strengths in relation to fat storage rate in young men. E. J. App. Phys. 1978; 38: 189-196.
34. Kuta I., Parizkova J., Dycka J.: Muscle strength and lean body mass in old men of different physical activity. J. App. Phys. 1970; 29: 168-171.
35. Spens L., Martin A., Drinkwater D.: Muscle mass of competitive male athletes. J. Sport Science 1993; 11: 3-8.
36. Nelson M., Fitarone M.A., Layne J.E., Trice I., Economos C.D., Fielding M.A., Pierson R.N., Evans W.J.: Analysis of body-composition techniques and models for detecting change in soft tissue with strength training. Am. J. Clin. Nutr.1996; 63: 678-686
37. Proctor D.N., Balagopal P., Nair K.S.: Age-related sarcopenia in humans is associated with reduced synthetic rates of specific muscle proteins. J. Nutr.1998; 128, suppl. 2: 351S-355S.
38. Rogers M.A., Evans W.J.: Changes in skeletal muscle with aging: effects of exercise training.[In:] Exercise and Sport Sciences Review Edited by J. O. Holloszy. Williams & Wilkins, Baltimore 1993; 21: 65-102.
39. Welle S., Thornton C., Totterman S., Forbes G.: Utility of creatinine exertion in body-composition studies of healthy men and women older than 60 years. Am. J. Clin. Nutr. 1996; 63: 151-156.

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ORIGINAL ARTICLE / PRACA ORYGINALNA

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EVALUATION OF THE EFFECT OF MILD HYPERTHERMIA ON MORPHOLOGY IN CHO AA8 CELL LINE

OCENA WPŁYWU ŁAGODNEJ HIPERTERMII NA MORFOLOGIĘ KOMÓREK LINII CHO AA8

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Summary

Introduction. Hyperthermia is one of many factors that can improve cancer therapy in the future. Many studies proved that hyperthermia may cause an increased blood flow, which results in improved drug delivery, but also better oxygenation. The aim of this investigation was to determine the effect of mild hyperthermia on morphology and hyperthermia-induced cell death in CHO AA8 cell line.

Material and methods. The material for the study was the Chinese Hamster Ovary AA8 cell line. After 48 hrs cell culture, the cells were exposed to 41°C for 2 hrs and then left to recover in 37°C for 24 hrs. Control cells were incubated under analogous conditions without exposition to hyperthermia. The effect of mild hyperthermia was evaluated by light and electron microscopy. The hyperthermic induction of apoptosis and necrosis was measured by using FACScan flow cytometer.

Results. The exposition of CHO AA8 cells to 41°C for 2 hrs resulted in swollen cells with fragmentized nuclei that resembled micronuclei, increased number and survival of cells. The results at the ultrastructural level showed changes in the shape and structure of mitochondria and the cell nucleus as well as disturbances in endoplasmic reticulum. Moreover, lysosome- and vacuole-like structures were observed, also with enclosed organelles. Flow cytometry analysis revealed a decrease in the percentage of both apoptotic and necrotic cells.

Conclusions. The results of this study allow us to suppose that exposure of CHO AA8 cells to 41°C for 2 hrs affects the morphology and cell survival. We also observed cells with a mitotic catastrophe phenotype (giant multinucleated cells) presumably not associated with apoptosis. Furthermore, a comparison of the light microscopy and flow cytometry results confirms an important role of p53 in hyperthermia-induced cell death and suggests the increased expression of heat shock proteins (HSPs).

Streszczenie

Wstęp: Hipertermia jest jednym z wielu czynników mogących mieć w przyszłości szerokie zastosowanie w terapii przeciwnowotworowej. Liczne badania dowiodły, że podwyższona temperatura może powodować zwiększony przepływ krwi, a co za tym idzie, bardziej efektywną absorpcję leków oraz zwiększoną oksygenację. Celem niniejszych badań była ocena wpływu łagodnej hipertermii na morfologię oraz rodzaj indukowanej śmierci fibroblastów CHO AA8.

Materiał i metody: Materiał do badań stanowiła linia komórkowa CHO AA8. Po 48 h hodowli komórki poddano działaniu 41°C przez 2 h, następnie hodowlę kontynuowano przez kolejne 24 h w warunkach standardowych. Kontrolą były komórki utrzymywane w tych

samych warunkach, bez ekspozycji na podwyższoną temperaturę. Efekt działania łagodnej hipertermii oceniany był przy użyciu mikroskopii świetlnej oraz elektronowej. Hipertermiczną indukcję apoptozy i nekrozy oceniano metodą cytometrii przepływowej.

W y n i k i : Ekspozycja komórek na działanie temperatury 41°C przez 2 h wpłynęła na pojawienie się komórek obrzmiałych z pofragmentowanym jądrem, jak również wzrost ich liczebności i przeżywalności. Badania na poziomie mikroskopu elektronowego pokazały, że hipertermia wpływa na kształt i strukturę mitochondriów oraz jądra komórkowego, zaburzenia w obrębie siateczki śródplazmatycznej, a także obecność struktur lizosomo- oraz wakuolo-podobnych, również z zawartymi wewnątrz organellami. Cytofotometryczna ocena komórek wykazała spadek liczby komórek apoptotycznych i nekrotycznych w odniesieniu do przeprowadzonej kontroli.

W n i o s k i : Uzyskane wyniki pozwoliły stwierdzić, że zastosowany profil temperaturowy wpływa zarówno na ich kształt, jak i przeżywalność fibroblastów linii CHO AA8. W wyniku działania temperatury obserwowano także komórki o fenotypie katastrofy mitotycznej (duże komórki z rozległą mikronukleacją), prawdopodobnie niezwiązanej z procesem apoptozy. Ponadto, porównanie wyników uzyskanych za pomocą mikroskopu świetlnego oraz cytometru przepływowego potwierdzają istotność białka p53 w hipertermicznej śmierci komórki, jak również mogą sugerować wzrost poziomu ekspresji białek szoku cieplnego.

Key words: hyperthermia, CHO AA8, mitotic catastrophe, apoptosis

Słowa kluczowe: hipertermia, CHO AA8, katastrofa mitotyczna, apoptoza

INTRODUCTION

Hyperthermia is one of well known physical modalities which are used in combination with radiation or chemicals such as cytostatics in the cancer therapy [1-2]. However, the clinical importance of hyperthermia is still an object of intensive studies and discussions. Depending on the hyperthermia efficiency in destroying normal tissue, the temperature used in cancer therapy is limited to 40-46°C. Besides efficiency in cell killing, it was shown that hyperthermia interacts synergistically with ionizing radiation but also with many cytostatic drugs [3]. Processes such as regulation of gene expression or interaction between cell and external environment are exposed to hyperthermia effect or other stress factors [4-5]. Changes observed in the cell physiology after hyperthermia treatment, called the "heat-shock response" are activated by the series of alterations and result in the impact on cell survival [6]. It is believed that thermal response can be caused by denaturation and disrupted protein folding. The heat-shock response also could be activated by oxidative stress and metabolic alterations [7].

Studies on the function of heat shock proteins showed that these proteins are molecular chaperones activated by denaturation. Heat shock proteins prevent protein aggregation and loss of its biological functions [7-8]. Moreover, heat shock proteins are involved in the control of cell signaling and cell cycle, and also in modulation of the immunological response [9-10].

Meanwhile, P53 tumor suppressor plays an important role in maintaining genomic stability during a cell cycle, apoptosis, and DNA repair [11-13]. Recent studies proved that genetic alterations in transformed cells cause their different susceptibility to external stress, and the loss of p53 function affects the cellular response to anticancer compounds [14-16].

To determine the effect of mild hyperthermia on morphology and hyperthermia-induced cell death we used CHO AA8 cell line with single missense mutation in p53 gene, which limits the possibility of cell cycle arrest in G1 phase, without affecting G2/M transition. It can be useful for study of the p53-independent apoptotic pathways and from this point of view, studies on CHO AA8 cells seem justified.

MATERIAL AND METHODS

Cell culture. The study material was the Chinese hamster cell line AA8, kindly provided by Prof. M. Zdzienicka (Department of Molecular Cell Genetics, Nicolaus Copernicus University Collegium Medicum in Bydgoszcz, Poland). The CHO AA8 cells were grown at 37ºC in a 5% CO_2 atmosphere in minimum essential medium eagle supplemented (Sigma-Aldrich) with 10% (v/v) fetal bovine serum (FBS; Gibco) and mixture of penicillin and streptomycin (Sigma-Aldrich). Before starting the culture, the density of cells was 2×10^4 cells/ml.

Heat treatment and cell survival. After 48 hour cell culture, the CHO AA8 cells were heat-shocked at 41ºC for 2 hours. Immediately after the heat treatment, the cells were incubated at 37ºC in a conventional 5% CO_2 incubator. Control cells were cultured identically without the exposition to a heat-shock. Cell viability was assessed by the trypan blue dye exclusion method.

Mayer's hematoxylin staining. Cells grown on coverslips were fixed in 4% paraformaldehyde for 20 min at room temperature and afterwards rinsed with PBS (3×5 min). Then the cells were embedded in 0.1M glycine solution (5 min, RT). Following a double rinsing with PBS, the cells were stained with Mayer's hematoxylin (5 min, RT). After that, the cells were rinsed under running tap water for 20 min, dehydrated through a series of alcohols and xylens and mounted in xylene-based mounting medium. The fixed preparations were examined using Eclipse E800 microscope (Nikon) with NIS-Elements 3.30 image analysis system and CCD camera (DS-5Mc-U1; Nikon).

Transmission electron microscopy. For ultrastructural analysis, the cells were fixed for 30 min at room temperature with 3.6% glutaraldehyde and then moved to 0.1M cacodylate buffer (pH 7.4). Following postfixing with 2% osmium tetraoxide in cacodylate buffer for 1.5 h, the studied material was dehydrated through graded series of alcohols and embedded in Epon 812. Parts of the material selected from semi-thin sections were cut into ultra-thin sections by using Richet Om U3 ultramicrotome and then stained with uranyl acetate and lead citrate. The prepared material was examined using JEM 100 CX electron microscope (JEOL).

Flow cytometry. Fibroblasts surface exposure of phosphatidylserine was determined with annexin V-fluorescein isothiocyanate (FITC) binding and flow cytometry. 7-AAD was used for nuclei counterstaining. Staining with annexin V-FITC and 7-AAD was conducted according to the manufacturer's instructions (BD Biosciences). Briefly, exposed to a heat-shock and untreated cells were trypsinized and centrifuged (300g for 5 min). Following supernatant removal, 195 µl of binding buffer and 5 µl of annexin V-FITC were added. The cells were incubated for 15 min at room temperature, in dark. After centrifugation (300g for 5 min) and removal of supernatant, 190 µl of binding buffer and 10 µl of 7-AAD were added to the cell pellet. The fluorescence of 20,000 events per sample was analyzed using FACScan flow cytometer (Becton Dickinson) and FlowJo software (Tree Star) on the assumption that viable cells are both Annexin V-FITC- and 7-AAD-negative cells; cells that are in early apoptosis are Annexin V-FITC-positive and 7-AAD-negative; cells that are in late apoptosis are both Anexin V-FITC- and 7-AAD-positive; whereas necrotic cells are Anexin V-FITC-negative and 7-AAD-positive (BD Biosciences).

Statistical analysis. The nonparametric Mann-Whitney U test was used to assess quantitative experiments. Results were considered at $p<0.05$. The GraphPad Prism 5.0 (GraphPad Software) was used for the statistical analyses.

RESULTS

Light microscopy studies

The trypan blue staining showed an increase in the mean percentage of trypan negative CHO AA8 cells after 2h hyperthermia treatment at 41°C. Statistical analysis showed a statistically significant differences ($p<0.05$) in the average percentage of surviving heat-shocked CHO AA8 cells in comparison to control (91,92% viable cells after hyperthermia treatment and 84,83% in control) (Table I). Moreover, counting of the total cell number also revealed a statistically significant differences ($p<0.05$) between cells exposed to hyperthermia and control group. Increase of the cell number after heat-shock in proportion to control (199×10^4/ml and $155{,}33\times10^4$/ml, respectively) was observed (Table II). On the basis of Mayer's hematoxylin staining we observed hyperthermia-induced changes in cell shape and size of CHO AA8 fibroblasts. The cells cultured in standard conditions showed spindle-like morphology, characteristic for fibroblasts, and only a small population of cells was morphologically changed (Figure 1 A-C).

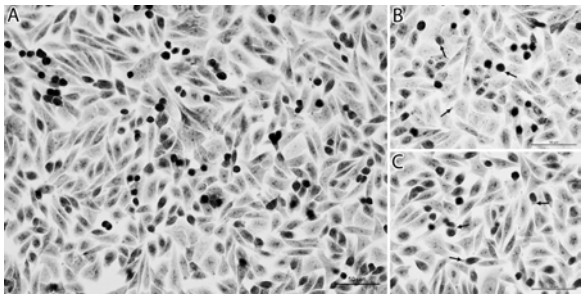

Fig. 1. A-C. *Control. The CHO AA8 cells without exposition to hyperthermia, stained with Mayer's hematoxylin. Characteristic for CHO AA8 fibroblasts the spindle-like morphology was observed, only a small population of cells was morphologically changed. Black arrows indicate shrunken cells with surface blebbing positively stained with Mayer's hematoxylin.*

Table I. *The effect of mild hyperthermia on cell viability of CHO AA8 cells. Data in the table presents the mean percentage of viable cells, standard deviation and median determined by the trypan blue exclusion test*

	Mean [%]	Standard deviation	Median
Control	84.83	5.15	86.5
Hyperthermia (41°C/2h)	91.92	2.94	92.5

Table II. *The effect of mild hyperthermia on viable cell number of CHO AA8 cells. Data in the table presents the mean number of viable cells, standard deviation and median determined by the trypan blue exclusion test*

	Mean	Standard deviation	Median
Control	155.33×10^4/ml	9.76×10^4/ml	153×10^4/ml
Hyperthermia (41°C/2h)	199×10^4/ml	15.91×10^4/ml	201×10^4/ml

Among these morphologically changed, ***shrunken cells*** with surface ***blebbing*** positively stained with Mayer's hematoxylin were observed. After hyperthermia treatment we observed mostly swollen cells with fragmentized nuclei that resembled micronuclei (Figure 2 A-E). There were also observed giant cells with one big nucleus (Figure 2 A, D). The heat-shocked cells revealed also an increase in chromatin condensation and the number of nucleoli, in comparison to control (Figure 2 A-E). Not many cells were reduced in volume and revealed extensive membrane blebbing (Figure 2 A-E).

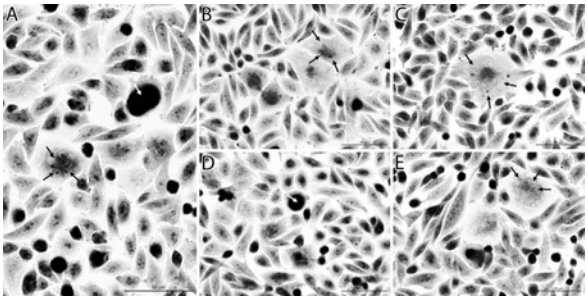

Fig. 2. A-E. *The CHO AA8 cells after 2h hyperthermia treatment at 41°C, stained with Mayer's hematoxylin. After hyperthermia treatment swollen cells with fragmentized nuclei that resembled micronuclei were observed (black arrows). There were also observed giant cells with one big nucleus (white arrows).*

Electron microscopy studies

To confirm the results observed at the light microscopy level, our studies were complemented with ultrastructure analysis. At the electron microscopy level, kidney-shaped nuclei were observed in the majority of control cells (Figure 3 A, B). After 2 hrs of treatment with hyperthermia, the nuclei changed their shape and became irregular with numerous indentations and inclusion bodies. Increase in the number of nucleoli, chromatin condensation and marginalization near to the nucleus membrane were also observed. Moreover, changes in the area of cytoplasm were observed. After heat-shock we observed high cytoplasmic vacuolization, also with contained inside organelles or cytoplasmic components (Figure 3 C-F). Mitochondria became swollen or/and flattened, but also the loss of mitochondrial cristae was observed (Figure 3 E). Our studies at the electron microscopy level also showed changes in the structure of other cellular organelles. The canals of endoplasmic reticulum were dilated (Figure 3 E). The lysosome-like structures were observed (Figure 3 F).

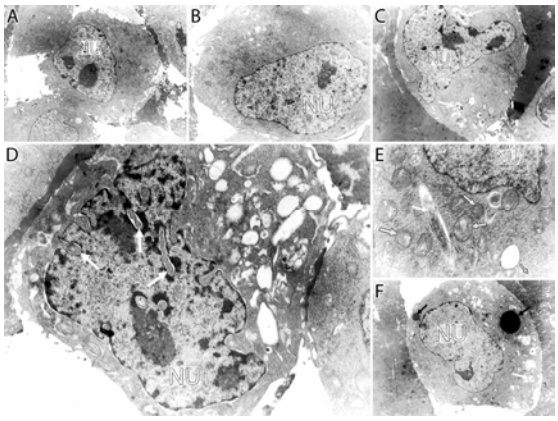

Fig. 3. *The ultrastructure of CHO AA8 cells. Control: A-B; 41°C/2h: C-F. In the majority of control cells, nuclei were kidney-shaped (NU; Figure 3 A, B) After hyperthermia treatment nuclei became irregular with numerous indentations and inclusion bodies (white arrows; Figure 3 D). Moreover, there was a high cytoplasmic vacuolization observed, also with organelles contained inside or cytoplasmic components (white arrows; Figure 3 C-F). Mitochondria became swollen or/and flattened, but also the loss of mitochondrial cristae was observed (grey arrows, Figure 3 E). The canals of endoplasmic reticulum were dilated (transparent arrow; Figure 3 E). The lysosome-like structures were observed (black arrows; Figure 3 F). Magnification: A ×1900; B ×2900; C ×3800; D ×15900; E ×7900; F ×2600.*

Flow cytometry

Cell death was measured by double staining with Annexin V-FITC and 7-AAD. The flow cytometry analysis showed a decrease in the number of cells with externalized phosphatidylserine after mild hyperthermia treatment. Under the influence of 41°C/2h a decrease in the percentage of early (Annexin V-FITC$^+$/7-AAD$^-$; from 0.66% observed in control cells to 0.30% in cells after hyperthermia treatment) and late (Annexin V-FITC$^+$/7-AAD$^+$; from 3.16% observed in control cells to 1.32% in cells after hyperthermia treatment) apoptotic cells was observed. The temperature profile used caused a decrease in the number of necrotic cells as well (Annexin V-FITC$^-$/7-AAD$^+$; from 3.16% observed in control cells to 1.32% after hyperthermia treatment). Moreover, the Annexin V-FITC/7-AAD assay showed an increase in the percentage of living cells (Annexin V-FITC$^-$/7-AAD$^-$; from 12.38% observed in control cells to 3.02% after hyperthermia treatment) (Figure 4).

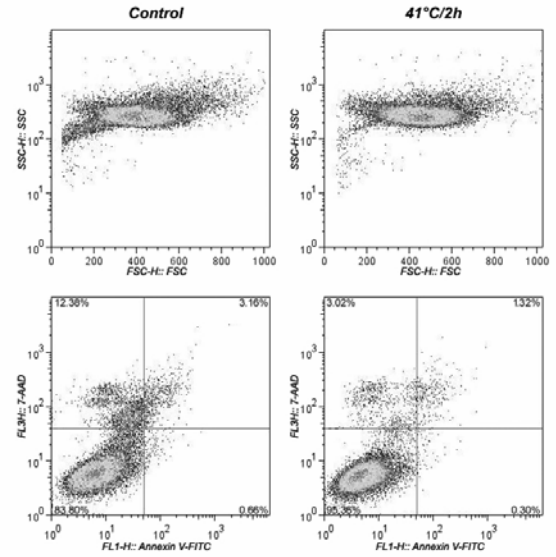

Fig. 4. *The effect of hyperthermia on cell death of CHO AA8 cells as detected by flow cytometry. Data are expressed as: percent of Annexin V-FITC- and 7-AAD-negative cells (live cells; lower left quadrants); percent of Anexin V-FITC-positive and 7-AAD-negative cells (early apoptosis; lower right quadrants); percent of Anexin V-FITC- and 7-AAD-positive cells (late apoptosis; upper right quadrants); percent of Anexin V-FITC-negative and 7-AAD-positive cells (necrosis, upper left quadrants).*

DISCUSSION

During the last few decades, the use of increased temperature in cancer therapy has been intensively investigated [1, 2, 3]. Hyperthermia causes many cellular-level changes affecting almost all structural and functional cellular systems, e.g. chromatin organization, regulation of gene expression, cell membranes, ion homeostasis, but also synthesis of nucleic acids [6, 17-18].

The material to present investigation was the Chinese hamster ovary (CHO) cell line AA8. These cells are heterozygous at the aprt locus, but also reveal the resistance to the low concentration of 8-azaadenine (8AA) [19]. The CHO AA8 cell line has also mutation at codon 211 in the exon 6 of p53 sequence resulting in the change from Thr (ACA) to Lys (AAA). The single missense mutation in p53 gene of CHO AA8 cell line limits the possibility of cell cycle arrest in G1 phase, without affecting G2 cell cycle checkpoint [20-21].

The lack of p53 gene expression can result in both inhibition of the apoptosis process and activation its alternative pathways. The action of p53 as a tumor suppressor involves inhibition of cell proliferation through cell cycle arrest and regulation of DNA repair pathway(s). Masunaga *et al*. (2003) showed higher expression of the proapoptotic protein Bax during hyperthermia-induced apoptosis, which suggests the participation of this protein in hyperthermic cell death [22]. In the present study the decrease in the percentage of apoptotic cells was observed. These results suggest that p53 function is crucial for protecting cells from mild hyperthermia-induced cell death.

Cellular response to heat-shock may also be a consequence of adaptive changes, which increase the ability of cells to survive in lethal conditions. Recently, more and more papers suggest that heat shock proteins, i.e. Hsp27, Hsp70 and Hsp90 inhibit and reduce apoptosis signals in different model systems [23-25]. Saleh *et al*. (2000) demonstrated the antiapoptotic effect of Hsp70 throughout its direct association with ***caspase***-recruitment domain (CARD) of apoptotic protease activating factor 1 (Apaf-1) and thus inhibition of apoptosome activity. Interaction between Hsp70 and Apaf-1 protects against oligomerization of Apaf-1 and its association with procaspase-9 [23]. It was also indicated by Pandey *et al*. (2000) that Hsp90 prevent cytochrome c-dependent oligomerization of Apaf-1 [26]. Moreover, Bruey *et al*. (2000) showed

that Hsp27 proteins, released from mitochondria, may also play a role in negative regulation of apoptosis through association with cytochrome c [24].

The results presented hereby show that exposition of Chinese hamster ovary cells AA8 to 41°C changes their morphology at the light and electron microscopy level. Numerous morphological changes (e.g. irregular nuclei with numerous indentations and inclusion bodies, chromatin condensation and marginalization, lysosome- and vacuole-like structures, dilated endoplasmic reticulum) observed in the heat-shocked cells demonstrate the response of cells to stress conditions. As the result of heat shock many changes occur in mitochondria. Often, they become swollen or/and flattened, but also alterations in mitochondrial cristae can be observed. Moreover, it has been shown that after hyperthermia treatment the perimitochondrial space dilation and a total mitochondrial damage can occur [27-29]. Alterations in mitochondria structure play an important role in decrease of pH, disruptions of mitochondrial membrane potential, and the loss of mitochondrial complex integrity [28]. Arancia *et al*. (1989) showed, that mitochondria exposed to lower temperatures contained dense mitochondrial matrix and the part of cristae in those mitochondria was diluted or bleb-shaped. It was also pointed that higher temperatures (43°C and 45°C) cause decrease in mitochondrial matrix density, irregular distribution of cristae, but also accumulation of mitochondria in perinuclear space [30]. Cells exposed to 45°C revealed positive correlation between alterations in mitochondria structure and cell death, which were not seen in cells exposed to 43°C [28-29]. There are also significant effects of hyperthermia on the mitochondrial membrane potential resulting in the change in redox status, and as a consequence increased sensitivity to hyperthermia [31-34]. Roti Roti (2008) suggests that depolarization of the mitochondrial membrane results in outburst of reactive oxygen species, which alter protein stability rendering proteins sensitive to increased temperature [35].

In the present study there were also observed cells which resemble the mitotic catastrophe phenotype. However, flow cytometry analysis showed decreased number of cells with phosphatidylserine in the outer membrane leaflet. Nakahata *et al*. (2002) showed the decrease in stability of centrosomes in heat-shocked at 43°C for 2 hrs human tumor cells. It was also pointed that abnormal centrosomes result in non-apoptotic mitotic catastrophe [36]. However, Grzanka *et al*. (2008) showed the increase of cells with mitotic catastrophe phenotype, but also cells with several features of apoptosis after hyperthermia treatment of CHO AA8 cells (44.5°C for 30 min). Hence, these findings may suggest that one of the modes of heat-shocked CHO AA8 cell death is mitotic catastrophe, which probably ends in apoptosis [37].

The results obtained in this study indicate that the mode of hyperthermic cell death depends on the temperature profile used as well as the time of exposure. Furthermore, the exposition of CHO AA8 cells to 41°C for 2 hrs causes *p53-independent* death by *mitotic catastrophe.* However, to confirm this, further studies using different cell lines and additional research methods are required.

CONCLUSIONS

1. The results allow us to conclude that beside the influence of 41°C/2h treatment on cell shape and size of CHO AA8 fibroblasts, the temperature profile used increased the cell survival and the total number of cells.
2. The observations at the electron microscopy level revealed numerous alterations in the area of cell nucleus, but also in remaining cellular organelles. These results let us suppose that temperature profile used causes permanent cellular changes.
3. Moreover, as a result of 41°C/2h treatment, the cells with mitotic catastrophe phenotype and decreased phosphatidylserine in the outer membrane leaflet appeared. This allows to suggest that cell death observed by us results in non-apoptotic mitotic catastrophe.
4. Decrease in percentage of apoptotic and necrotic cells, concurrence with increase of cell survival and number of heat-shocked CHO AA8 cells may suggest the significance of p53 in hyperthermia-induced cell death, but also an increased expression of heat shock proteins.
5. The changes within the cells, but also the mode of stress-induced cell death depend on the cell type and the temperature profile used.

REFERENCES

1. Juffermans J.H., Hanssens P.E., van Putten W.L., *et al.* Reirradiation and hyperthermia in rectal carcinoma: a retrospective study on palliative effect. *Cancer*. 2003; 98:1759-66.

2. Albregts M., Hulshof M.C., Zum Vörde Sive Vörding P.J., *et al.* A feasibility study in oesophageal carcinoma using deep loco-regional hyperthermia combined with concurrent chemotherapy followed by surgery. *Int J Hyperthermia*. 2004; 20:647-59.
3. González Gonzáles D., van Dijk J.D.P., Blank L.E.C.M. Radiotherapy and hyperthermia. *Eur J Cancer*. 1995; 31A:1351-55.
4. Lindquist S. The heat-shock response. *Annu Rev Biochem*. 1986; 55:1151-91.
5. Nover L. HSFs and HSPs--a stressful program on transcription factors and chaperones. Stress Proteins and the Heat Shock Response, sponsored by Cold Spring Harbor Laboratory, Cold Spring Harbor, NY USA, April 29-May 2, 1991. *New Biol*. 1991; 3:855-9.
6. Coss R.A., Linnemans W.A. The effects of hyperthermia on the cytoskeleton: a review. *Int J Hyperthermia*. 1996; 12:173-96
7. Noble E.G., Milne K.J., Melling C.W. Heat shock proteins and exercise: a primer. *Appl Physiol Nutr Metab*. 2008; 33:1050-65.
8. Park H.G., Han S.I., Oh S.Y., *et al*. Cellular responses to mild heat stress. *Cell Mol Life Sci*. 2005; 62:10-23.
9. Calderwood S.K., Mambula S.S., Gray P.J. Jr, *et al*. Extracellular heat shock proteins in cell signaling. *FEBS Lett*. 2007; 581:3689-94.
10. Calderwood S.K., Mambula S.S. Gray P.J. Jr. Extracellular heat shock proteins in cell signaling and immunity. *Ann N Y Acad Sci*. 2007; 1113:28-39.
11. Fei P., El-Deiry W.S. P53 and radiation responses. *Oncogene*. 2003; 22:5774-5783.
12. Caelles C., Helmberg A., Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature*. 1994; 370:220-223.
13. Geske F.J., Nelson A.C., Lieberman R., *et al*. DNA repair is activated in early stages of p53-induced apoptosis. *Cell Death Differ*. 2000; 7:393-401.
14. Harris C.C. Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. *J Natl Cancer Inst*. 1996; 88:1442-55.
15. Rosen E.M., Fan S., Rockwell S., et al. The molecular and cellular basis of radiosensitivity: implications for understanding how normal tissues and tumors respond to therapeutic radiation. *Cancer Invest*. 1999; 17:56-72.
16. Hoeijmakers J.H. Genome maintenance mechanisms for preventing cancer. *Nature*. 2001; 411:366-74.
17. Streffer C. Metabolic changes during and after hyperthermia. *Int J Hyperthermia*. 1985; 1:305-19.
18. Burdon R.H. Heat shock and the heat shock proteins. *Biochem J*. 1986; 240:313-24.
19. Thompson L.H., Fong S., Brookman K. Validation of conditions for efficient detection of HPRT and APRT mutations in suspension-cultured Chinese hamster ovary cells. *Mutat Res*. 1980; 74:21-36.
20. Hu T., Miller C.M., Ridder G.M., *et al*. Characterization of p53 in Chinese hamster cell lines CHO-K1, CHO-WBL, and CHL: implications for genotoxicity testing. *Mutat Res*. 1999; 426:51-62.
21. Tzang B.S., Lai Y.C., Hsu M., *et al*. Function and sequence analyses of tumor suppressor gene p53 of CHO.K1 cells. *DNA Cell Biol*. 1999; 18:315-21.
22. Masunaga S., Ono K., Takahashi A., *et al*. Usefulness of combined treatment with mild temperature hyperthermia and/or tirapazamine in the treatment of solid tumors: its independence of p53 status. *Cancer Sci*. 2003; 94:125-33.
23. Saleh A., Srinivasula S.M., Balkir L., *et al*. Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat Cell Biol*. 2000; 2:476-83.
24. Bruey J.M., Ducasse C., Bonniaud P., *et al*. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat Cell Biol*. 2000; 2:645-52.
25. Lanneau D., de Thonel A., Maurel S., *et al*. Apoptosis versus cell differentiation: role of heat shock proteins HSP90, HSP70 and HSP27. *Prion*. 2007; 1:53-60.
26. Pandey P., Saleh A., Nakazawa A., et al. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *EMBO J*. 2000; 19:4310-22.
27. Welch W.J., Suhan J.P. Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. *J Cell Biol*. 1985; 101:1198-211.
28. Cole A., Armour E.P. Ultrastructural study of mitochondrial damage in CHO cells exposed to hyperthermia. *Radiat Res*. 1988; 115:421-35.
29. Wheatley D.N., Kerr C., Gregory D.W. Heat-induced damage to HeLa-S3 cells: correlation of viability, permeability, osmosensitivity, phase-contrast light-, scanning electron- and transmission electron-microscopical findings. *Int J Hyperthermia*. 1989; 5:145-62.
30. Arancia G., Crateri Trovalusci P., Mariutti G., *et al*. Ultrastructural changes induced by hyperthermia in Chinese hamster V79 fibroblasts. *Int J Hyperthermia*. 1989; 5:341-50.
31. Senisterra G.A., Huntley S.A., Escaravage M., *et al*. Destabilization of the Ca2+-ATPase of sarcoplasmic reticulum by thiol-specific, heat shock inducers results in thermal denaturation at 37 degrees C. *Biochemistry*. 1997; 36:11002-11.
32. Dressler C., Beuthan J., Mueller G., *et al*. Fluorescence imaging of heat-stress induced mitochondrial long-term depolarization in breast cancer cells. *J Fluoresc*. 2006; 16:689-95.
33. Nijhuis E.H., Poot A.A., Feijen J., *et al*. Induction of apoptosis by heat and gamma-radiation in a human lymphoid cell line; role of mitochondrial changes and caspase activation. *Int J Hyperthermia*. 2006; 22,:687-98.
34. Yu D.Y., Matsuya Y., Zhao Q.L., *et al*. Enhancement of hyperthermia-induced apoptosis by a new

synthesized class of furan-fused tetracyclic compounds. *Apoptosis*. 2007; 12:1523-32.
35. Roti Roti J.L. Cellular responses to hyperthermia (40-46 degrees C): cell killing and molecular events. *Int J Hyperthermia*. 2008; 24:3-15
36. Nakahata K., Miyakoda M., Suzuki K., *et al*. Heat shock induces centrosomal dysfunction, and causes non-apoptotic mitotic catastrophe in human tumour cells. *Int J Hyperthermia*. 2002; 18:332-43.
37. Grzanka D., Stepien A., Grzanka A., *et al*. Hyperthermia-induced reorganization of microtubules and microfilaments and cell killing in CHO AA8 cell line. *Neoplasma*. 2008; 55:409-15.

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ORIGINAL ARTICLE / PRACA ORYGINALNA

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SIRNA MEDIATED EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) GENE INHIBITION IN THE MODEL OF NON-SMALL CELL LUNG CANCER: EGFR BLOCKADE PRODUCES CHANGES POTENTIALLY INDUCING ANTI-TUMOR IMMUNITY

HAMOWANIE GENU RECEPTORA NASKÓRKOWEGO CZYNNIKA WZROSTU (EPIDERMAL GROWTH FACTOR, EGFR) PRZY UŻYCIU SIRNA W MODELU NIEDROBNOKOMÓRKOWEGO RAKA PŁUCA: BLOKOWANIE EGFR POWODUJE ZMIANY POTENCJALNIE INDUKUJĄCE PRZECIWNOWOTWOROWĄ ODPOWIEDŹ UKŁADU IMMUNOLOGICZNEGO

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Summary

Background. Development of new research methods, including molecular biology, results in new perspectives of lung cancer treatment and prognosis. Up to date small interfering RNA (siRNA) molecules have been used in several studies to silence oncogenes in the respiratory system. One of them, epidermal growth factor (EGF) receptor gene plays an important role in lung carcinogenesis, since EGFR initiates signal transduction for cancer cell cycle progress and survival.

Methods. A549 line cells, model of non-small cell lung cancer (NSCLC), were transfected with specific anti-EGFR siRNA sequence. Transfection efficiency was estimated by RT-PCR. Apoptosis was detected by Annexin V staining (early apoptosis) and cell cycle analyses with PI stained, permeabilized cells (late apoptosis). Expression (incl. calculation of MFI) of CD 80, CD86, CD83, IGFR, EGFR was analyzed before and after transfection.

Results. (Median of 5 experiments): Silencing of EGFR expression in A549 cells caused increase in both late (13.7% vs 8.6%) as well as early apoptosis (21.6% vs 12.8%) rate. A549 cells transfected with siRNA anti-EGFR showed significant ($p<0.05$) increase in CD86 (2.5 vs 16%) expression. Increase in CD80 and CD83 expression in transfected cells was not significant, MFI was significantly lower in A549 cells transfected with specific siRNA, anti-EGFR as compared to cells transfected with nonspecific control siRNA.

Conclusions. siRNA-mediated EGFR gene inhibition in A549 cells resulted in some functional and phenotypic changes potentially responsible for stimulation of anti-tumor immune response. EGFR seems to be a promising target of immune gene therapy in NSCLC.

S t r e s z c z e n i e

W p r o w a d z e n i e. Rozwój nowych technik badawczych, zwłaszcza molekularnych, daje nadzieję na poprawę skuteczności leczenia i lepsze rokowanie w raku płuca. Dotychczas małe interferencyjne cząsteczki RNA (siRNA) były wykorzystywane w wielu badaniach do wyciszania onkogenów układu oddechowego. Jeden z nich, EGFR odgrywa ważną rolę w karcinogenezie, ponieważ inicjuje sygnał transdukcji dla progresji cyklu podziałowego i przetrwania komórek raka.

M e t o d y. Komórki linii A549 stanowiącej model niedrobnokomórkowego raka płuca transfekowano specyficznymi sekwencjami siRNA anty-EGFR. Wydajność transfekcji oszacowano metodą RT-PCR. Apoptozę zbadano barwieniem aneksyną V (wczesna apoptoza) i wykonano analizę cyklu komórkowego przy pomocy barwienia PI permeabilizowanych komórek (późna apoptoza). Ekspresja cząsteczek CD 80, CD86, CD83, IGFR, EGFR była analizowana (włączając pomiar MFI) przed i po transfekcji.

W y n i k i (Mediana z 5 doświadczeń): Wyciszenie ekspresji genu EGFR prowadziło do wzrostu odsetka komórek zarówno w późnej (13,7% do 8,6%) jak i we wczesnej apoptozie (21,6% do 12,8%), co wykazano barwieniem z aneksyną V. Komórki A549 transfekowane siRNA anty-EGFR wykazują znamienny ($p<0.05$) wzrost ekspresji CD86 (16% do 2,5%). Wzrost ekspresji CD80 i CD83 w komórkach transfekowanych nie był znamienny. MFI była znacząco niska w komórkach A549 transfekowanych swoistymi cząsteczkami siRNA anty-EGFR w porównamiu do komórkek transfekowanych nieswoistymi sekwencjami kontrolnymi.

W n i o s k i. Hamowanie ekspresji genu EGFR w komórkach A549 spowodowało szereg zmian funkcjonalnych i fenotypowych potencjalnie odpowiedzialnych za pobudzenie odpowiedzi przeciwnowotworowej. EGFR wydaje się obiecującym celem immuno- i genoterapii niedrobnokomórkowego raka płuca.

Key words: small interfering RNA, RNA interference, epidermal growth factor receptor, non-small cell lung cancer

Słowa kluczowe: małe interferencyjne cząsteczki RNA, interferencja RNA, receptor epidermalnego czynnika wzrostu, niedrobnokomórkowy rak płuca

INTRODUCTION

Lung cancer is the most common cause of cancer death in the worldwide [1]. It is generally subdivided into two main types: small cell lung cancer (SCLC) and non-small cell (NSCLC) one [1]. The non-small cell type is much more common, accounting for 80 percent of all lung cancer patients [1]. Lung cancer is usually extremely difficult to treat, so researchers continue to look for new treatment opportunities [1].

Genetic engineering brings new hope due to its ability to modify cell genome, including functional regulation of genes responsible for tumor transformation and progress [2.3]. Since the phenomenon of RNA interference was discovered, the new tool, small interfering RNA (siRNA) has been widely used for gene silencing in mammalian cells [4].

RNA interference (RNAi) is a process in which double-stranded RNA triggers the degradation of a homologous messenger RNA [5.3]. This degradation is mediated by oligonucleotide (about 20 bases) siRNA molecules [2]. In mammalian cells, RNAi is initiated by the enzyme called Dicer, which is a member of dsRNA-specific endonuclease III family. Dicer cuts longer dsRNA strands into siRNA duplexes, 21-23 nucleotides long. Then siRNA is incorporated into a protein complex RISC (RNA-Induced Silencing Complex) recognizing complemenatary mRNA on the single-stranded siRNA template and finally cleaving target RNA [5.6]. It was demonstrated that synthetic 21-nucleotide dsRNA introduced into mammalian cells could efficiently silence gene expression [5].

In this study, we used synthetic, ready to-use siRNA targeting EGFR to silence EGFR gene expression in NSCLC cell line, A549.

EGFR is a member of the epidermal growth factor receptor family and encodes cell superficial protein with tyrosine kinase activity [7.8]. Its overexpression is a frequent event observed in NSCLC but no correlation with poor prognosis and shorter survival time was found [9]. Activation of EGF receptor initiates signal transduction in lung cancer cells [10]. The downstream effectors of EGFR involve inter alia PI3-K, RAS, RAF and mitogen-activated protein kinase (MAPK) and series of transcription factors [10]. They finally determine the activation of several nuclear proteins driving the cell growth and proliferation, especially cycle progress from G1 to S phase [11]. Characteristic genetic changes causing direct EGFR over-expression in NSCLC cells concern both increase of receptor numbers, and, due to its gene amplification, activating mutations [12]. In general, constitutively active, superficially overexpressed EGFR molecules send continuous false mitogenic signal to the nucleus, provoking unlimited tumor growth [11]. The blockade

of EGFR gene potentially allows to inhibit the proliferation of cancer cells and makes them more sensitive to other therapeutic tools [13]. Therefore, the blocking effect of the receptor should lead to the reduction of transforming activity in vitro and prevent tumor initiation in experimental models in vivo, as well as increase tumor cell sensitivity to agents that can trigger apoptosis.

In the current experiment, we investigated the possibility whether RNAi could silence EGFR gene in a model line of human NSCLC, A549.

MATERIALS AND METHODS

1. A549 cell line culture.

A549 is a well-characterized human lung adenocarcinoma cell line, (*American Type Culture Collection,* Cat. No. CCL 185). Cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM, *Gibco, BRL*) supplemented with 10% fetal bovine serum (FBS, *Gibco, BRL*) in a well humidified atmosphere of 5% CO_2 at 37°C. It is a good model for NSCLC researches, *inter alia* the cells express molecules of EGFR family.

2. SiRNAs preparation.

The siRNAs duplexes, corresponding to EGF receptor gene, have been designed using the HiPerformance Design Algorithm (*Novartis AG*), integrated with a stringent inhouse homology analysis tool. They were chemically synthesized by *Qiagen*:

1st. siRNA-EGFR name: HS_EGFR_10_HP Validated siRNA (cat. No. SI02660140) contained: sense (Batch No. 226417); antisense (Batch No. 226418).

2nd. siRNA-EGFR name: HS_EGFR_11_HP Validated siRNA (cat. No. SI02660147) contained: sense (Batch No. 226419); antisense (Batch No. 226420).

Additionally, control unrelated nonspecific dsRNA sequence was prepared:

siRNA-unrelated named negative control (i.e. non silencing) siRNA, (cat. No. 1022079) contained: sense 5'-UUCUCCGAACGUGUCACGUdTdT (Batch No. 148483); antisense 5'-ACGUGACACGUUCGGAGAAdTdT (Batch No. 148636).

The positive siRNA control was the sequence known to provide high knockdown of MAPK1 as a target gene (Mm/Hs_MAPK1 Control siRNA, *Qiagen*, cat. no. 1022564).

3. Transfection with the use of specific small interfering RNA (siRNA) directed against transcripts of anti-EGF receptor (EGFR) and nonsilencing control siRNA.

We used transfection protocol by *Qiagen* according to the firm reference (*HiPerFect Transfection Reagent Handbook, 3rd edition, II 2007*). In brief, the day before transfection, the cells were seeded (16 x 10^4 cells per well) in a 12-well plate in culture medium (DMEM, serum and antibiotics). The cells were incubated under normal growth conditions (37°C and 5% CO2). On the day of transfection, two different complexes (mixes for 12 wells) were prepared: 1) Complex containing 36µl sequence 2µM siRNA (negative control siRNA), 1164µl DMEM and 72µl HiPerFect Transfection Reagent; 2) Complex containing 18 µl 1st sequence 2µM siRNA-EGFR (Cat. No. SIO2660140) and 18µl 2nd sequence 2µM siRNA-EGFR (Cat. No. SIO2660147), 1164µl DMEM, 72µl HiPerFect. The samples were incubated for 5-10 min at room temperature to enable the formation of transfection complexes. Then, the complexes were drop-wise added onto the cells (106 µl) cultured under their normal growth conditions. The plates were gently swirled to ensure uniform distribution of the transfection complexes. The cells were monitored for gene silencing effect after 24 hrs. They were harvested by trypsinization 24 hrs after transfection and analyzed with the methods described below.

4. RT-PCR

Total RNA from each experiment group (non-transfected, transfected, siRNA-EGFR and control siRNA) was extracted with High Pure RNA Isolation Kit (*Roche Diagnostics GmbH*). Quality and integrity of the RNA were checked by 1,5% agarose gel electrophoresis. RNA was reversely transcribed to cDNA with *Reverse Transcription System* reagents *(Promega Corporation)*. The appropriate primers for EGFR were prepared. 1st pair: forward 5'ATAGTCGCCCAAAGTTCCTGAGT3' and reverse: 5'TTTGGCTTGGCTTCCTTGGGA AAG3' (685bp, *DNA Gdańsk II*). Thermal cycler parameters for EGFR included one cycle at 94°C for 2 min, and 35 cycles involving denaturation at 94°C for 30s, annealing at 60°C for 30s and extension at 72°C for

40s, followed by a final extension at 72°C for 10 min. The PCR products were analyzed on 1.5% agarose gels.

5. A549 phenotyping. Flow cytometry.

Cells were centrifuged (300×g, 6 min), the pellet was resuspended in PBS. Each sample containing 5 x 10^4 cells was incubated with saturating amounts of monoclonal antibodies (MoAbs, *BD Pharmingen*^{TM}) for 30 min in the dark. MoAbs were directed against human superficial CD80 (FITC), CD83 (PE Cys5), CD86 (PE), EGFR (PE), CD221 (PE) antigens (respective fluorochromes in brackets). Negative isotype control was used in each sample series. After incubation, cells were washed in PBS containing 0,1% sodium azide (300×g, 8 min) and resuspended in 250µl of PBS with 1% formaldehyde. Cells were acquired immediately in flow cytometer (FACScan, *Becton-Dickinson Immunocytometry Systems*).

6. Early apoptosis detection with Annexin V-FITC/PI staining

Detection of early apoptosis in A549 cells was performed according to the instruction of manufacturer (Annexin V-FITC Apoptosis Detection Kit, *BD Pharmingen*^{TM}). All the following steps were performed on ice. In brief, cells (1x10^5 /ml) were washed, resuspended in 96 µl of icecold diluted binding buffer and incubated with 1 µl Annexin V-FITC and 2.5 µl propidium iodide for 10min in the dark. The cell samples were then diluted with 250 µl of binding buffer and measured immediately by flow cytometry. Negative control was carried out for each sample (incubation with binding buffer alone). For positive control, the cells were preincubated on ice with 3% formaldehyde in binding buffer for 30 min.

7. Late apoptosis detection with cell permeabilization and PI staining

The 1x10^5 cells were incubated with 0,5ml of 0,3% saponin (*Sigma*) for 20min. in the dark at 4^{o}C, washed with 1ml of 0,1% saponin and centrifuged (400×g, 5 min). Cell pellet was resuspended in solution containing 10µl of propidium iodide (PI, 500µg/ml), 50 µl of RNAse (1mg/ml) and 440 µl of PBS. The final incubation was carried out for 20 min in the dark, at 4^{o}C. The cells were resuspended in 1ml volume (adjusted with PBS) and measured by flow cytometry [14].

8. Statistical analysis

Statistica 7 software was used to perform statistical analysis. Wilcoxon test was used to compare matched pairs. The experiment was repeated five times. The results were presented as median ± SEM. Differences were considered to be significant at P≤0.05.

The study was approved by the Bioethics Committee at the Nicolaus Copernicus University in Toruń (registration number KB 690/2007).

RESULTS

Inhibition of EGFR gene expression in A549 cells transfected with siRNA anti-EGFR was confirmed by the RT-PCR technique.

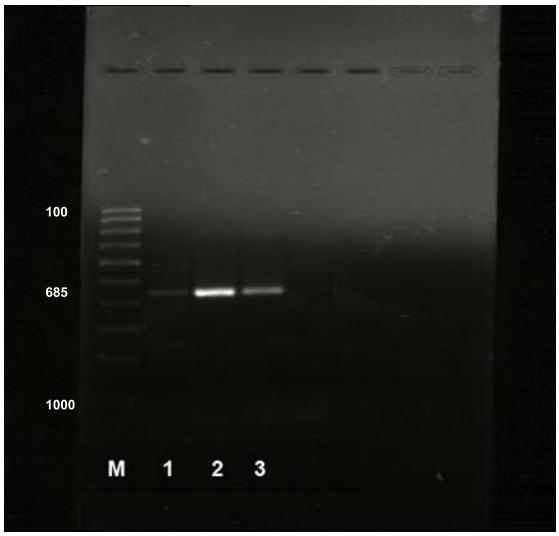

Fig. 1. *Agarose gel electrophoretic analysis of cDNA expression by RT-PCR with specific primer pairs for EGFR gene. A549 cells. M – marker; 1 – transfected with siRNA anti-EGFR; 2 – nontransfected; 3 – transfected with control siRNA*

Ryc. 1. *Elektroforetyczna analiza ekspresji cDNA na żelu agarozowym, RT-PCR ze specyficznymi parami starterów dla genu EGFR. Komórki A549. M – marker; 1 – transfekowane za pomocą siRNA anty-EGFR; 2 – nietransfekowane; 3 – transfekowane za pomocą kontrolnego siRNA*

As shown in Figure 1, specific siRNA sequences, targeting EGFR significantly down-regulated gene expression in A549 cells.

Table I presents the results of the cell cycle analysis. The increase in percentage of Sub-G1 phase cells as well as proliferation decline were found after specific transfection, as compared to control transfected cells.

Table I. *Results of A549 cell cycle (NTR, nontransfected; TR, transfected with siRNA). Results are presented as the median of 5 measurements (percentage of all cells); * p <0.05 as compared to siRNA negative control sequence transfected cells*

Tabela I. *Wyniki analizy cyklu komórkowego linii A549 (NTR, nietransfekowane; TR, transfekowane za pomocą siRNA). Wyniki przedstawiono jako medianę z 5 pomiarów (odsetek wszystkich komórek); * p <0.05 w porównaniu komórkami transfekowanymi negatywną sekwencją kontrolną siRNA*

	NTR	TR siRNA control	TR siRNA anti-EGFR
Sub-G1 (late apoptosis)	**0,9**	**1,2**	**6,8 ***
G0/G1	**73,1**	**74,3**	**67,8**
G2/M	**12,5**	**9,5**	**6,5 ***
S	**13,3**	**14,9**	**10,2**
S/G2/M	**25,8**	**24,4**	**16,7 ***

Table II shows the results of transfected and nontransfected cells in Annexin V/FITC with PI staining. Significant increase of both early (Anenexin V+/PI-) and late (Anenexin V+/PI+) apoptotic cell percentage was found in siRNA anti-EGFR transfected cells.

Table II. *Results of early and late apoptosis of A549 cells (percentage of nontransfected – NTR; transfected with siRNA – TR: control sequence, anti-EGFR,). Results are presented as the median of 5 measurements; * p <0.05 as compared to siRNA control sequence transfected cells*

Tabela II. *Wyniki badania wczesnej i późnej apoptozy komórek A549 (odsetek komórek nietransfekowanych – NTR, transfekowanych – TR siRNA kontrolnym, siRNA anty-EGFR). Wyniki przedstawiono jako medianę z 5 pomiarów; *p <0.05 w porównaniu z transfekcją komórek sekwencją kontrolną siRNA*

	NTR	**TR siRNA control**	**TR siRNA anti-EGFR**
1. Annexin V+/PI- (early apoptosis)	6,7	12,8	21,6 *
2. Annexin V+/PI+ (late apoptosis)	3,1	8,6	13,7 *
Apoptosis (1+2)	8,7	20,7	40,6 *

A549 superficial EGFR expression was high, almost 100% of cells were positive with anti-EGFR MoAb, after transfection with specific siRNA. However, in the latter, the mean fluorescence intensity (MFI) of positive cells was calculated and the growth of EGFR MFI (in each series compared to the isotype control sample) was significantly lower in siRNA anti-EGFR transfected cells than in control siRNA transfected as well as in nontransfected cells (Fig. 2.).

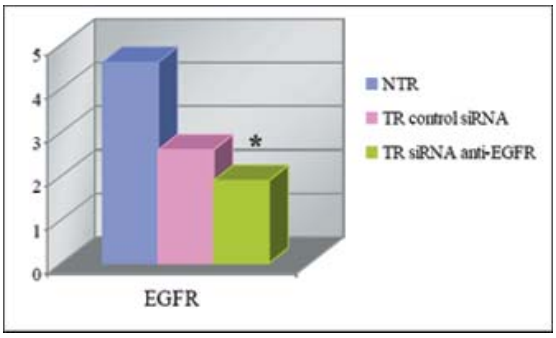

Fig. 2. *Results of Mean Fluorescence Intensity (MFI) EGFR positive cells. The increase of MFI (as compared to the respective isotype control stained sample) is shown, results are presented as the median of 5 measurements; * p <0.05 as compared to siRNA control sequence transfected cells*

Ryc. 2. *Wyniki średniej intensywności fluorescencji (Mean Fluorescence Intensity, MFI) komórek dodatnich z EGFR (w porównaniu z odpowiednią próbką serii barwioną kontrolą izotopową). Wyniki przedstawiono jako medianę z 5 pomiarów; * p <0,05 w porównaniu z komórkami transferowanymi sekwencją kontrolną siRNA*

Other results of A549 cell typing are presented in the Table III. SiRNA anti-EGFR cells expressed higher levels of CD86 and CD221 (receptor for IGF, insulin-like growth factor), statistically significant. Increase in CD80 and CD83 superficial molecules expression was statistically non-significant.

Table III. *Results of Wilcoxon test applied for A549 cells transfected with siRNA anti-EGFR in comparison to negative control transfected cells. Results are presented as the p-level of 5 measurements; * p <0.05 was statistically significant*

Tabela III. *Wyniki testu Wilcoxona dla komórek A549 transfekowanych siRNA anty-EGFR w porównaniu z komórkami transferowanymi negatywną sekwencją kontrolną. Wyniki przedstawiono jako poziom istotności (p-level) z 5 pomiarów; * p <0.05 uznano za statystycznie znamienne*

	p-level TR siRNA anti-EGFR
CD83	**ns**
CD80	**0.06**
CD86	**0.02 ***
CD221	**0.03 ***

Since the phenotype results (especially those concerning co-stimulatory molecules – CD80, 83 and 86) were of special importance for the study results interpretation, we summarized them in the Figure 3.

The attention should be paid to the significant ($p<0.05$) increase in the percentage of CD86 positive cells after specific siRNA-mediated EGFR blockade (16 vs 2,5% in negative control transfected cells) .

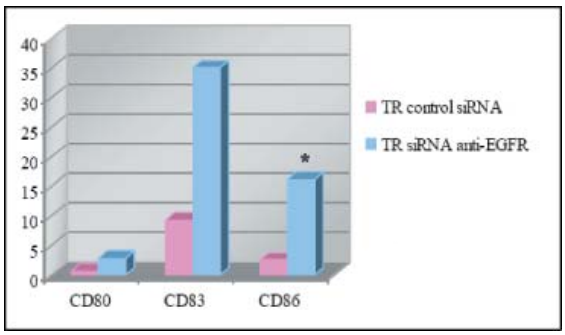

Fig. 3. *Results of phenotyping of superficial costimulatory molecules on A549 cells. TR, transfected cells. Results are presented as the median of 5 measurements (percentage of all cells); * p <0.05 as compared to siRNA control sequence transfection*

Ryc. 3. *Wyniki typowania cząsteczek kostymulujących na komórkach A549. TR, komórki transfekowane. Wyniki przedstawiono jako medianę z 5 pomiarów (odsetek wszystkich komórek), *p <0.05 w porównaniu z transfekcją sekwencją kontrolną*

DISCUSSION

The methods used in the current study are relevant to the present trends: many experiments use RNAi mechanism to silence oncogenes. The growth factors and their receptors are the special point of interest, as they drive cell division, development and invasion [15]. Many researchers highlight the role of EGF receptors family in lung carcinogenesis. It was experimentally proven that EGFR over-expression in lung cancer is a prognostic factor for survival as well as treatment efficiency [16.9]. A lot of modern therapy modalities base exactly on EGF and its receptors system inhibition; monoclonal antibodies, reversible as well as nonreversible small molecules protein kinase inhibitors were in use with EGFRs as targets [17].

Additionally, it has been recently observed that both triple-helix and siRNA mediated IGF receptor inhibition caused increased apoptosis and low proliferation rate of glioma and NSCLC cells [14]. Interestingly, IGFR-inhibition characteristically modified the tumor cell phenotype. The changes involved the distinct rise of CD80 and CD86 expression, both molecules engaged in efficient antigen presentation to T lymphocytes, and were relevant to enhanced tumor susceptibility to the immune system response [18]. We hypothesized then that parallel changes are to be observed in NSCLC cells after blockade of another key growth factor receptor.

In this area, the phenomenon of RNAi seems to be especially promising. RNAi usually inhibits target gene expression in about 75% of its initial level [19. 20]. Subsequently specific siRNA sequences were used in the study. Our experiment was successful, the efficiency of EGFR silencing was proven with two independent methods (MFI of EGFR molecule flow cytometric assessment and RT-PCR). Since siRNA molecules can damage transfected cells nonspecifically, the respective control system with non-silencing siRNA sequences was applied in order to get reliable conclusions.

Our results concerning EGFR expression, apoptosis and cell cycle were similar to the data obtained by other authors [21]. *Zhang et al* transfected A549 cells with specific siRNA anti-EGFR and found decline in percentage of EGFR positive cells, decreased mRNA transcription (about 30% of baseline) and significant inhibition of cancer grow *in vivo* [22]. *Li Bai et al* tested the impact of shRNA-mediated anti-EGFR specific transfection of NSCLC cells and found significant decrease of EGF receptor expression and apoptosis induction. Additionally, transfected lung cancer cells were more sensitive to chemotherapy [23].

These observations are completed herein with very interesting finding that EGFR gene siRNA-silenced lung cancer cells expressed significantly higher percentage of CD86 co-stimulatory molecule. However, the parallel CD80 over-expression on A549 cell was less characteristic, without statistical significance. It should be also considered that CD86 seems to be less important in anti-tumor defense, since it drives the immune system towards Th2, i.e. humoral response rather, than Th1, i.e. the cellular one [24]. Thus, our results are promising but their actual meaning needs to be proven and clarified by further investigation. Up to date, tumor cell immunogenicity seems to be more pronounced after IGF system silencing [25].

Another original result of the current study shows that surface CD83 antigen, the marker of mature APC including dendritic cells, was detected on A549 cells. The CD83 may be shed from tumor cell surface in order to disintegrate the local immune system [26].

There are also reports that CD83 positive APC cells induce immunotolerance to tumor [27]. On the other hand, the CD83 over-expression on A549 EGFR gene-silenced cells, may reflect the tumor disability to shed the molecule.

In the study EGFR inhibition did not influence significantly another growth factor receptor (IGFR-I, receptor for interleukin growth factor type I, CD221) expression.

Generally, some of our results indicate the actual potential of siRNA-mediated EGFR inhibition in immune gene therapy of non-small cell lung cancer. Additional tests and novel experimental models should be yet carried out to confirm this therapeutic opportunity as a realistic promising approach.

CONCLUSIONS

In the current study, EGFR gene expression was inhibited successfully in non-small cell lung cancer cell line A549 with the use of specific siRNA sequences.

EGFR gene silencing carried out in A549 cells resulted in increased apoptosis rate and in decline of cell proliferation.

Increase of B7 co-stimulatory molecules expression on siRNA anti-EGFR transfected cells was significant only for CD86 marker. However, all in all, phenotype changes in transfected cells may reflect enhanced tumor susceptibility to the immune system and make EGFR gene a promising target for future immune gene therapy trials in NSCLC patients.

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REFERENCES

1. Molina J.R., Yang P., Cassivi S.D. et al: Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc. 2008; 83 (5): 584-94.
2. Czajka-Uhryn M., Bednarek I.: Interferencja RNA — nowe narzędzie molekularne w modulacji zjawiska oporności wielolekowej. Ann. Acad. Med. Siles. 2005; 59 (3).
3. Takeshita F., Ochiya T.: Therapeutic potential of RNA interference against cancer. Japanese Cancer Association 2006; 97 (8): 689– 696.
4. Leung R.K, Whittaker P.A.: RNA interference: from gene silencing to gene-specific therapeutics. Pharmacol Ther. 2005; 107 (2): 222-39.
5. Chiu Y., Rana T.M.: SiRNA function in RNAi: A chemical modification analysis. RNA 2003; 9: 1034–1048.
6. Majorek M., Guzenda P., Lamparska-Przybysz M.: Krótkie interferujące RNA w onkologii. Współczesna Onkologia 2006; 10; 8: 367–372
7. Bielawski K.: Budowa i funkcje receptorów ErbB (HER). Współczesna Onkologia 1999; 6: 241–243.
8. Mendelsohn J., Baselga J.: Epidermal growth factor receptor targeting in cancer. Semin Oncol. 2006; 33(4): 369-385.
9. Nicholson R.I, Gee J.M.W., Harper M.E.: Egfr and cancer prognosis. Europ. J. Cancer 2001; 37 (9s).
10. Ullrich A., Schlessinger J.: Signal transduction by receptors with tyrosine kinase activity. Cell, 1990; 61: 203-212.
11. Błasiak J., Majsterek I.: Kinazy Tyrozynowe. Nowy cel terapii przeciwnowotworowej. Postępy Biochemii 2005; 51 (3): 251-260.
12. Franklin W.A., Veve R.: Epidermal growth factor receptor family in lung cancer and premalignancy. Semin Oncol. 2002; 29 (s4): 3-14.
13. Sordella R., Bell D.W., Haber D.A. et al: Gefitinib-Sensitizing EGFR Mutations in Lung Cancer Activate Anti-Apoptotic Pathways. Science. 2004; 305 (5687): 1163 – 1167.
14. Kopiński P., Sładek K., Szczeklik J. et al.: Expression of insulin-like growth factor-I (IGF-I) in alveolar macrophapeg and lymphocytes obtained by bronchoalveolar lavage (BAL) in interstitial lung diseases (ILD). Assessment of IGF-I as a potential local mitogen and antiapoptotic cytokine. Folia Histochem. Cytobiol. 2006; 44 (4): 249-258.
15. Rajkumar T.: Growth factors and growth factor receptors in cancer. Current Science, 2001; 81(5): 535-541
16. Fang K., Chen M.H.: Transfection of anti-sense complementary DNA of human epidermal-growth-factor receptor attenuates the proliferation of human non-small-cell-lung-cancer cells. Int J Cancer. 1999 5; 81 (3): 471-8.
17. Bates S.E., Fojo T.: Epidermal Growth Factor Receptor Inhibitors: AMoving Target? Clin. Cancer Res. 2005; 11 (20): 7203-7205.
18. Szpechciński A., Trzos R., Jarocki P. et al: Presence of MHC-I in rat glioma cells expressing antisense IGF-I-Receptor RNA. Ann. Acad. Med. Bialos. 2004; 49 (1s): 98-104.
19. Tech Review: Off-Target Effects: Disturbing the Silence of RNA interference (RNAi) Literature Code: 00082-06-E-02-U
20. Semizarov D., Frost L., Sarthy A.: Specificity of short interfering RNA determined through gene expression signatures. Proc Natl Acad Sci U S A. 2003; 100 (11): 6289-91

21. Faltus T., Yuan J., Zimmer B. et al: Silencing of the HER2/neu Gene by siRNA Inhibits Proliferation and Induces Apoptosis in HER2/neu-Overexpressing Breast Cancer Cells. Neoplasia 2004; 6 (6): 786–795
22. Zhang M.: Silencing the epiderma growth factor receptor gene with RNAi May be developer as a potential therapy for non small cell lung cancer. Genet. Vaccines Ther. 2005; 30: 3-5.
23. Bai L., Zhu R.: Potential role of short hairpin RNA targeting epidermal growth factor receptor in growth and sensitivity to drugs of human lung adenocarcinoma Wells. Biochemical Pharmacology 2006; 71 (8): 1265-1271.
24. Martin B.K., Frelinger J.G., Ting J.P.: Combination Gene Therapy with CD86 and the MHC Class II Transactivator in the Control of Lung Tumor Growth. The Journal of Immunology. 1999; 162: 6663-6670.
25. Dong A.Q.: Down-regulation of IGF-IR using small, interfering, hairpin RNA (siRNA) inhibits growth of human lung cancer line A549 in vitro and nude mice. Cell Biol. Int. 2007; 31 (5): 500-507.
26. Baleeiro R.B., Barbuto J.A.: Local secretion/shedding of tumor-derived CD83 molecules as a novel tumor escape mechanism. Mol Immunol 2008; 45 (12): 3502-3504.
27. Markowicz S.: Komórki dendrytyczne – od poznania funkcji w układzie odpornościowym do zastosowania w klinice chorób nowotworowych. Centrum Onkologii – Instytut im. Marii Skłodowskiej – Curie. Wa-wa 2007.

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THE SACROILIAC JOINT AS A FACTOR IN THE FORMATION OF LUMBO-SACRAL PAIN

STAW KRZYŻOWO-BIODROWY CZYNNIKIEM ODPOWIEDZIALNYM ZA POWSTANIE BÓLÓW OKOLICY LĘDŹWIOWO-KRZYŻOWEJ

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S u m m a r y

T h e a i m o f t h e s t u d y is to show the correlation between the low back pain syndrome and the limited mobility in the sacroiliac joint and an assessment of the factors aggravating the symptoms of this pain syndrome.

M a t e r i a l s a n d m e t h o d s. 15 patients, aged 20-70, of both sexes, took part in the study. The study was based on an analysis of the patients' body posture, patients' gait, medical history regarding their locomotor system and other diseases if the patient suffered from pain in the lumbosacral area. Apart from that, the mobility of the spine's joints and the sacroiliac joints was assessed using physical tests: repeated movements tests for pain, Laseque's test, Patrick's test, standing flexion test and active movements test. The results were statistically analyzed.

R e s u l t s. In the study group it was discovered that the frequency of sacroiliac joint dysfunctions statistically increases in patients older than 45 but there is no correlation between sex and the dysfunctions. Pain in the LS area was present in all patients, pain in the buttocks area in 8 (47%), pain radiating to the lower limbs in 7 (41%) patients. Pain of intensity higher than 6 points according to the *Visual Analogue Scale* was observed in 11 (65%) patients, a necessity to assume an unnatural body position in order to relieve the pain was noted in 5 (29%) patients. No correlation was shown between body mass and sacroiliac joint dysfunctions but a correlation between the dysfunctions and abnormal lordosis as well as increased abdominal press (during coughing, sneezing and tenesmus) was found. On the basis of the repeated movements functionality tests, pain at the end of the movement was found in 3 patients (21%) and 4 (26%) felt no change. Laseque's test was positive in 12 (80%) patients and Patrick's test was positive in 11 (75%) patients. The standing flexion test proved to be the differentiating test and it showed a centralization of the symptoms during the overstraightening movement in 31% of the patients and 29% felt pain at the end of the movement. Functionality test of sacroiliac ligaments was positive in 11 patients and in the case of the remaining patients (27%) it was negative and there was functional shortening and an overload of the sacroiliac ligaments.

C o n c l u s i o n. The conducted study showed that functional changes in the area of the sacroiliac joint affect the etiology of low back pain. Intensity of the changes depends on the age of the patient, existing lordosis and patient's physical activity.

S t r e s z c z e n i e

Celem badań była ocena korelacji pomiędzy ograniczeniem ruchomości w stawie krzyżowo-biodrowym a zespołem bólowym dolnego odcinka kręgosłupa oraz ocena czynników nasilających dolegliwości w ww. zespole. Badaniem objęto 15 chorych obojga płci, w wieku 20-70 lat. Badania polegały na analizie chodu, postawy ciała, wywiadu dotyczącego układu ruchu oraz przebytych chorób u pacjentów z bólami okolicy krzyżowo-lędźwiowej. Ponadto, oceniono ruchomość w stawach kręgosłupa oraz w stawach krzyżowo-biodrowych za pomocą testów fizykalnych: testu powtarzanymi ruchami na wywołanie dolegliwości bólowych, testu Laseque'a, Patricka, wyprzedzania oraz czynnego wykonywania ruchów.

Key words: sacroiliac joint, dysfunctions, blockage, functional tests
Słowa kluczowe: staw krzyżowo-biodrowy, dysfunkcja, zablokowanie, testy funkcjonalne

INTRODUCTION

Low back pain is a functional disorder of the spine which is caused by limited mobility of the lumbosacral spine or a blockage of the sacroiliac joint [3]. It is being observed in younger and younger patients [2]. Its occurrence is mostly linked with a limitation of daily activity and passive way of spending free time [2].

The pathological mechanism of the formation of low back pain is multidirectional: it may be caused by an organic disease, functional disorders in the spinal or tissue area or of the spinal structures, but also nociceptive intervertebral joint capsules, interspinal and longitudinal ligaments, dura mater, vertebral body, spinal nerve root sheaths, connective tissue of the nerves, local muscles, blood vessels of the spinal canal, exterior spheres of the intervertebral discs and the sacroiliac joint [1].

For many years it was thought that, due to the characteristic of the joint (a synchondrosis), no movement in this joint is possible. [7].

Studies have shown that during the bending of the lumbar spine a swinging motion of the sacrum takes place – it is called nutation (bending forwards) and counternutation (bending backwards). The movements of this joint can also be observed during breathing or walking. This means that every dysfunction of the sacroiliac joints causes a whole cascade of improper sacrum placements, starting from the improper placement of lumbar vertebrae, vertebrae arches, the formation of lumbar scoliosis, tilting of the pubic symphysis and even spondylolisthesis [7].

In anamnesis low back pain is characterized as: one sided, blunt pain placed above the buttocks and able to radiate along the groin, thigh and calf [4].

AIM OF THE STUDY

The aim of the study is to show the correlation between low back pain and the blockage of the sacroiliac joint and an assessment of the factors aggravating the symptoms of this pain syndrome.

MATERIALS AND METHODS

15 patients, aged 20-70, of both sexes took part in the study. The study was based on an analysis of the patients' body posture, patients' gait, medical history regarding their locomotor system and other diseases if the patient suffered from pain in the lumbosacral area. Apart from that, the mobility of the spine's joints and the sacroiliac joint was assessed using physical tests: repeated movements tests for pain, Laseque's test, Patrick's test, standing flexion test and active movements test. The results were statistically analyzed.

THE RESULTS OF THE INVESTIGATIONS

Among the 30 patients who were under the care of the REH-MED (rheumatology and rehabilitation) clinic there were men and women aged 20 – 70 years. A conducted survey showed a positive correlation between the presence of the dysfunction of sacroiliac joint and being over 45 years of age. As many as 12 (80%) patients from the whole group complained about pain (Fig. 1). However there was no correlation between sex and the dysfunction.

Pain in the LS area was present in all 15 (100%) patients, pain in the buttocks area in 8 (47%), pain radiating to the lower limbs in 7 (41%) (fig.4).

While assessing the intensity of the pain by the Visual Analogue Scale (range 0-10 points), pain in the lower back (above 6 points) was observed in 11 (65%) patients (fig 2).

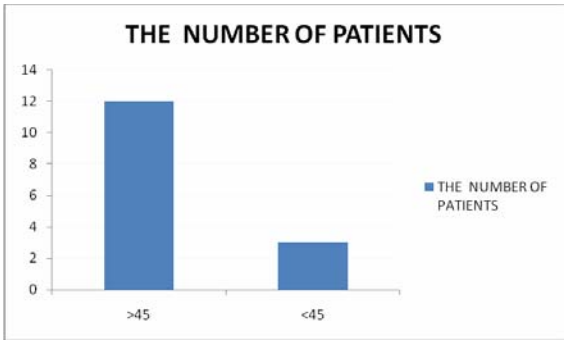

Fig. 1. *Analysis of the sacroiliac joint pain in relation to the age of the patients*

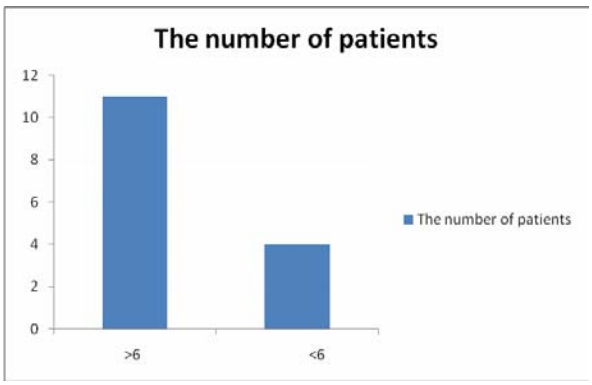

Fig.2. *VAS pain scale in the patients who took part in the study*

A necessity to assume a forced body position in order to relieve the pain was noted in 10 (71%) patients and the remaining 5 (29%) observed that pain was lessened in a static position and aggravated during movement (fig. 3) 10 patients (67%) observed an aggravation of the pain during standing and walking, 2 (13%) when bending and 3 (20%) when sitting (fig. 5). The study showed no correlation between body mass and sacroiliac joint dysfunctions, even though only 5 (33 %) patients were of proper weight (fig. 6).

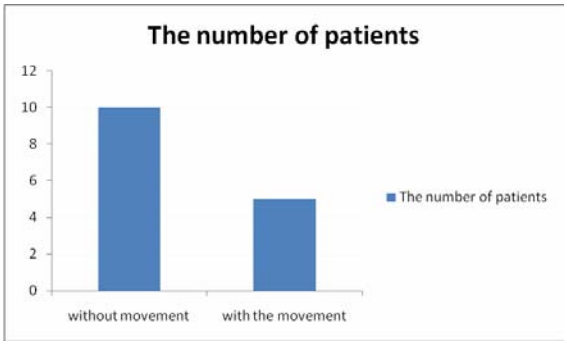

Fig. 3. *Patient's sensations depending on the activity performed*

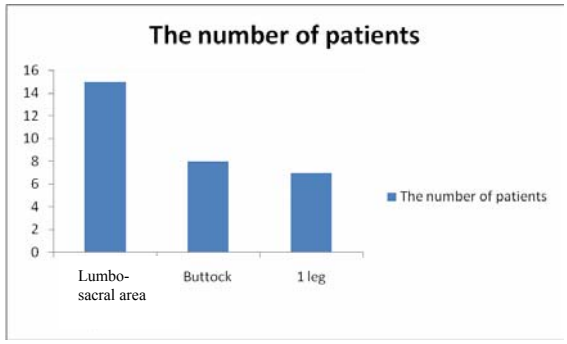

Fig. 4. *Localization of the pain in the patients who took part in the study*

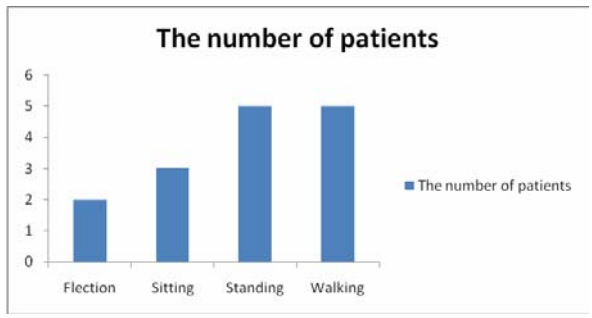

Fig. 5. *Aggravation of the pain in the study group*

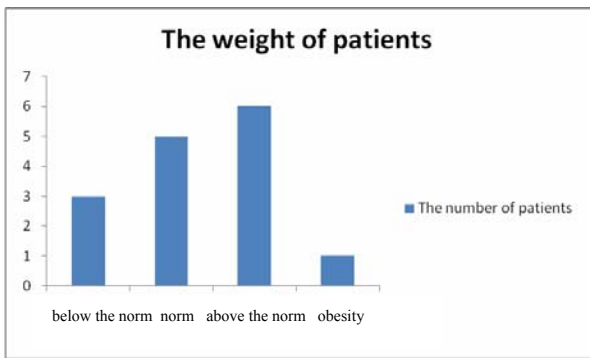

Fig. 6. *Body mass in relation to the dysfuntions of the sacroiliac joints in the study group*

A significant correlation between pathological lordosis of the spine and sacroiliac joint dysfunctions was found. A proper lordosis was observed in 4 (26%) patients, decreased in 8 (53%) and increased in 1 (7%) patient. Scoliosis, i.e. a lateral curving of the spine, was present in 2 (14%) patients (fig. 7). A statistically significant correlation was found between pain and increased abdominal press (coughing, sneezing and tenesmus). Those ailments were present in 13 (86%) patients (fig.8).

The functional test of standing flexion as a test of the functions of the sacroiliac joints was positive in all the patients from the group (fig.12). The Laseque's test

which was supposed to differentiate between sciatica and hip joint pain showed that over a half, 12 (80%), of the patients felt pain in the lower part of the spine when raising the lower limb over the angle of 45°, the remaining 3 (20%) patients felt pain when raising the limb to the 45° angle (Fig.10).

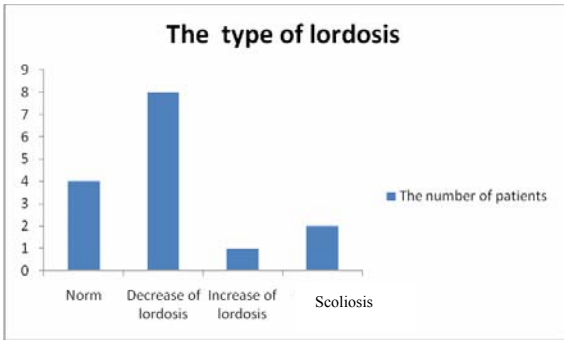

Fig. 7. *Type of lordosis*

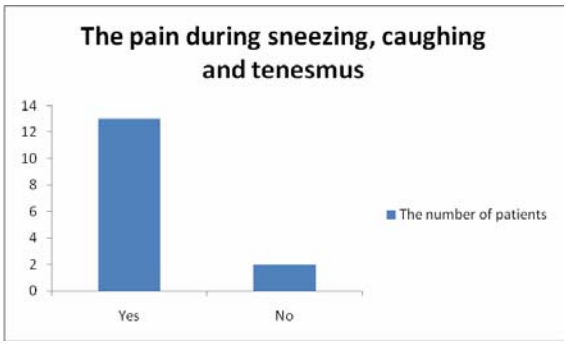

Fig. 8. *Aggravation of pain in the sacroiliac join during sneezing, caughing and tenesmus*

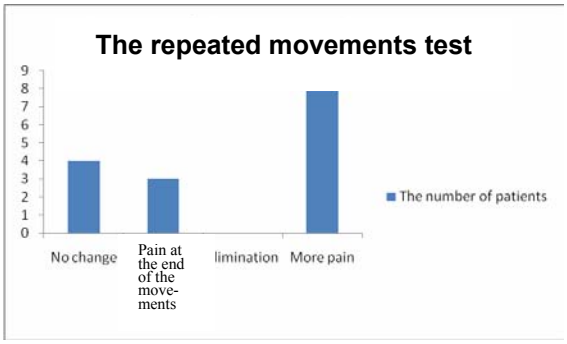

Fig. 9. *Values of the repeated movements test in the patients who took part in the study*

Based on the Patrick's test, which differentiates between pain in the hip joint and the sacroiliac joint, limited adduction and abduction of the hip joint was observed in 11 (75%) of the patients and in 4 (25%) only a limitation of abduction was found. No patient met the norm (Fig.11).

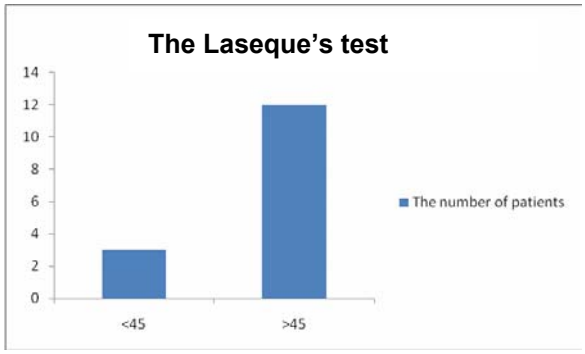

Fig. 10. *Laseque's test in the study group*

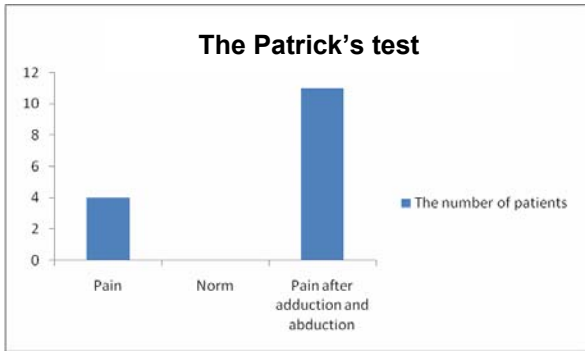

Fig. 11. *Patrick's test values in the study group*

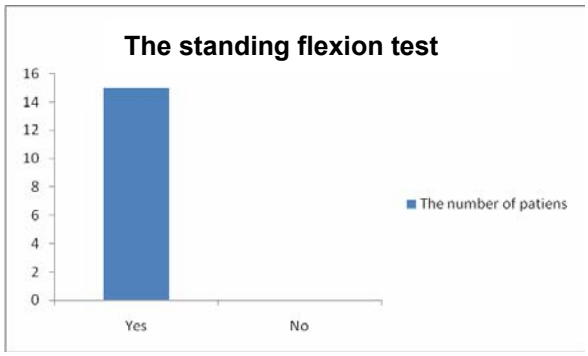

Fig. 12. *Values of the standing flexion test in the study group*

The repeated movements test for current pain symptoms in the fibular and frontal areas showed an aggravation of the symptoms during overstraightening in 8 (53%) patients and 3 (21%) patients felt pain at the end of the movement. 4 (26%) patients did not feel any change during the conducted test (fig. 9).

The functionality test of sacroiliac ligaments was positive in 11 (73%) patients, 4 (20%) of which felt pain during the test. The test proved negative in one patient (7%) from the tested group (fig. 13).

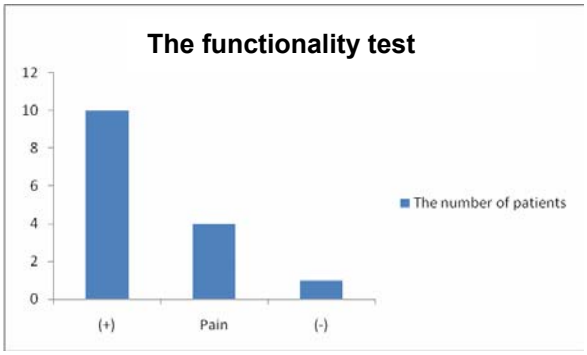

Fig. 13. *Values of the functionality test of sacroiliac ligaments in the study group*

DISCUSSION

In the subject literature there are only few reports regarding the role of the sacroiliac joint in the formation of low back pain. Studies regarding this syndrome usually concern one chronic pain treatment, classification of this syndrome, its diagnostics and the algorithm of therapeutic procedure. Moreover, it is the therapy that is still discussed in the Polish and English literature of the subject.

According to Dziak the sacroiliac joint should not be rehabilitated in any way as it is a structure with a small mobility and the movements of the sacrum against the hip bone involve no muscles that are the basis of any therapeutic and rehabilitation treatment [3]. Duckworth, on the other hand, claims that the physiological movement of the sacroiliac joint is present and it is based on the rotation of the sacrum around the sacroiliac ligament.

The studies conducted by our team show that the sacroiliac joint plays an important role in the development of osseoarticular ailments. All of the patients who took part in our study and had a dysfunction of this joint suffered from such ailments.

The pathological mechanism is based on a long-term blockage which leads to disorders of the balance of tensions between the muscles responsible for the statics and the muscles which are responsible for the active movements (also movements of the torso), and then to spinal disorders, osteophytes and as a result, low back pain.

Despite better and better diagnostic methods and orthopedic treatment this problem is still growing [5] Pain in the lumbosacral area is difficult in the diagnostic and therapeutic sense. The etiology of the ailment is often ambiguous, there is no link with an injury, inflammation or growth process, no neurological deficits [1]. In order to choose a proper therapeutic procedure it is necessary to locate more precisely the pathology in the osseoarticular area. Low back pain can appear in the form of 3 types of disorders: structural, dysfunctional and postural [3, 8]. The diagnostics of the lumbosacral area uses not only functional tests but also intra-articular injections of an anesthetic, a fluoroscopic control. It is a highly specialized method used only in 30% of the patients. It makes possible to asses if the pain really originates in the sacroiliac joint or in a different structure [3]. An inadequate mobility of the sacroiliac joint coupled with an improper position exerts painful mechanic pressure on the tissues. Sacroiliac joints improperly distribute the pressure from the ground and the torso [5, 11]. The topography of the pain is very similar to the symptoms of radiculitis, especially in its acute phase when it comes to a blockage, i.e. a dysfunction, of the sacroiliac joints.

In our studies pain above 6 in the VAS scale hinders “normal” functioning, makes it impossible to conduct everyday activities or slows them down - this was reported by 65% of patients. Coughing and sneezing aggravates these ailments. This confirms the findings of Bessler and Neumann [7,10].

CONCLUSIONS

The conducted study shows that functional changes in the area of the sacroiliac joint affect the etiology of low back pain.

REFERENCES

1. Lewit K.: Terapia manualna w rehabilitacji narządu ruchu. ZL Natura, Kielce 2005.
2. Stodolny J.: Choroba przeciążeniowa kręgosłupa epidemią naszych czasów. ZL Natura, Kielce 2005.
3. Dziak A.: Bóle krzyża. PZWL, Warszawa 1990.
4. Rępała A.: Zespół bólowy kręgosłupa. PZWL, Warszawa 2003.
5. Kaltenborn F.M.: Lokalizacja objawów w obrębie kręgosłupa i kończyn. Wyd. Rolewski, Toruń 2002.
6. Arkuszewski Z.: Podręcznik medycyny manualnej. Atlas zabiegów-miednica, kręgosłup lędźwiowy, kręgosłup piersiowy, żebra. ELIPSA-JAIM, Kraków 2006.
7. Neumann H.D.: Medycyna manualna. PZWL, Warszawa 1992.
8. Ongley M.J., Klein R.G., Dorman T.A., Eek B.C., Hubert L.J.: A new approach to the treatment of chronic low back pain. Lancet, 1987; 2: 143-146.
9. Exelby L.: (2001) The locked lumbar facet joint: intervention using mobilisations with movement - case report. Manual Therapy 6(2), 116-121.

10. Bessler, J., Beyerlein, C. (2006). Der sichere Weg zur Diagnose - Untersuchung und Therapie des Iliosakralgelenks. Physiopraxis, 6, 20-24
11. Mulligan, BR. (2003). Self Treatments for Back, Neck and Limbs. Plane View Services Ltd. New Zealand.

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ORIGINAL ARTICLE / PRACA ORYGINALNA

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THE INFLUENCE OF ISCHEMIA AND REPERFUSION INJURY ON THE REACTIVITY OF ARTERIES INDUCED BY ANGIOTENSIN II AND BAY K8644

WPŁYW NIEDOKRWIENIA I REPERFUZJI NA REAKTYWNOŚĆ NACZYŃ WYZWALANĄ PRZEZ ANGIOTENSYNĘ II I BAY K8644

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Head: dr hab. n. med. Grzegorz Grześk

Summary

Background: Vascular smooth muscle cells reactivity is regulated by many factors acting as constrictors or relaxants. The aim of this study was to investigate the role of ischemia/reperfusion (I/R) injury in reactions induced by angiotensin II (ANG II, agonist of metabotropic receptor AT1) and Bay K8644 (agonist of calcium channel).

Material and methods: Experiments were performed on perfused male Wistar rats' tail arteries. Contraction induced by ANG II and Bay K8644, mediated by intracellular (in Ca+-free PSS – FPSS) or extracellular calcium (in PSS, after previous emptying Ca^{2+} cellular stores), after I/R and in the presence of P 7208 (blocker of G protein – Bordatella pertussis toxin) or xestospongin C (XeC - IP3 receptor antagonist) was analyzed.

Results: ANG II induced increase of perfusion pressure using intracellular or extracellular calcium, while Bay K8644 induced contraction mediated only by extracellular calcium. Ischemia reduced, while reperfusion augmented response of the vascular smooth muscle cells to ANG II, but did not change effects of Bay K8644. P 7208 reduced reactions to ANG II mediated only by intracellular calcium. XeC reduced contraction induced by ANG II in FPSS. P 7208 did not change effects of Bay K8644.

Conclusion: I/R modulates vascular contraction induced by ANG II, but not by Bay K8644. Both intracellular and extracellular Ca^{2+} mediate the contraction induced by ANG II, while only extracellular Ca^{2+} mediate contraction induced by Bay K8644. Results suggest that G protein modulates effects of ANG II mediated by intracellular Ca^{2+}.

Streszczenie

Wstęp: Reaktywność mięśniówki gładkiej naczyń podlega regulacji przez wiele czynników kurczących i rozkurczających.

Celem pracy było określenie wpływu uszkodzeń związanych z niedokrwieniem / reperfuzją na skurcz wyzwalany przez angiotensynę II (ANG II, agonistę receptora metabotropowego AT1) i Bay K8644 (agonistę kanałów wapniowych zlokalizowanych w błonie komórkowej).

Materiał i metody: Badania przeprowadzono na perfundowanych tętnicach ogonowych szczurów, samców szczepu Wistar. Badano skurcz wyzwalany przez ANG II i Bay K8644 przy udziale wewnątrz- (i.c. Ca^{2+}) i zewnątrzkomórkowej (e.c. Ca^{2+}) puli jonów wapnia po I/R oraz w obecności P 7208 (toksyny krztuśca - blokera białka G) i ksestosponginy C (XeC - antagonisty receptora dla IP3).

Wyniki: ANG II wyzwala wzrost ciśnienia perfuzyjnego przy udziale wewnątrz- i zewnątrzkomórkowej puli jonów wapnia, podczas gdy skurcz wyzwalany przez Bay K8644 odbywa się jedynie dzięki napływowi wapnia do komórki. Niedokrwienie redukuje, a reperfuzja wzmaga reakcję tętnic na ANG II, ale nie zmienia działania Bay K8644. P 7208 redukuje efekt ANG II w warunkach i.c. Ca^{2+}. XeC znosi skurcz wyzwalany ANG II przy udziale

wewnątrzkomórkowej puli jonów wapnia. P 7208 nie wpływa na działanie Bay K8644.

W n i o s k i: I/R moduluje skurcz tętnic wyzwalany ANG II, ale nie wpływa na reakcje indukowane przez Bay K8644. W działaniu ANG II pośredniczy wewnątrz- i zewnątrzkomórkowa pula jonów wapnia, podczas gdy Bay K8644 stymuluje skurcz jedynie przy udziale jonów wapnia napływających do komórki. Wyniki sugerują, że białko G moduluje działanie ANG II wyzwalane jedynie przy udziale wewnątrzkomórkowej puli Ca^{2+}.

Key words: ANG II, Bay K8644, calcium ions, ischemia, reperfusion
Słowa kluczowe: ANG II, Bay K8644, jony wapnia, niedokrwienie, reperfuzja

INTRODUCTION

Reactivity of vascular smooth muscle is regulated by many contractile and relaxing factors. Angiotensin II is a metabotropic receptor agonist, triggers the smooth muscle contraction, activating angiotensin receptor AT1 and associated with it G protein [1]. The increase of pressure obtained is dependent on the free calcium ions in cytoplasm due to the rapid release from intracellular stores and the influx from the extracellular space through channels for calcium ions located in the cell membrane. Bay K8644 is a calcium channel agonist located in the cell membrane, which, stimulating the influx of calcium ions into the cell, activates the contraction of smooth muscles.

Calcium ions perform a significant role in regulating cells' functions. Cocreating the signals at the level of cell membrane, they act as a primary transmitter**,** while activating many enzymes they act as a secondary transmitter of intracellular information; they are essential in the process of muscle contraction, blood clotting and nerve activity. The level of free calcium ions in the cytoplasm is maintained within a very narrow range, due to which their physiological function of the signaling system in the cell is fulfilled [2]. The increase of calcium ion concentration also occurs in pathological situations, such as hypoxia or ischemia, when cells have a deficit of energy**.** [3]. Calcium ions are said to perform a role in the cell death by the apoptosis, ischemia/reperfusion or excitotoxicity [4, 5, 6].

The aim of the study was to determine the influence of disorders associated with ischemia and reperfusion on contraction triggered by ANG II and Bay K8644 with a particular emphasis on the importance of intracellular and extracellular calcium pool in these reactions.

MATHERIAL AND METHODS

Experiments were performed on isolated and perfused Wistar rats' tail arteries, weighing 250-350 g, anesthetized by urethane, injected intraperitoneally (dose 120 mg/kg i.p.). In order to study the arteries' reactivity to ANG II (10^{-10} - 3 x 10^{-6} M/l) and Bay K8644 (10^{-8} – 10^{-4} M/l) the experiments began by identifying the control CRCs for analyzed agonists.

In order to investigate the influence of I/R on vascular smooth muscle reactivity to selected agonists (ANG II, 30 nM/L; Bay K8644 30 μM/l), a hemostat was put on the proximal part of preparated artery for 30 or 60 minutes. No sooner than after this time, were arteries cut off, the cannula was mounted and then connected with perfusion system and a set to measure and register the perfusion pressure.

To assess the contribution of intracellular (i.c. Ca^{2+}) and extracellular (e.c. Ca^{2+}) pool of Ca^{2+} to the reactions triggered by investigated agonists in control conditions, after ischemia and reperfusion and in the presence of G protein blocker – pertussis toxin (P 7208; 110 ng/ml) and antagonist of receptor for IP3 - xestospongin C (20 μM/l), the experiments were carried out using two types of Krebs' fluid:

1. Ca^{2}+ free fluid – EGTA – Ca^{2+} free Krebs' fluid - FPSS – composition: NaCl (71,8mM/L), KCl (4,7 mM/L), $NaHCO_3$ (28,4 mM/L), $MgSO_4$ (2,4 mM/L), KH_2PO_4 (1,2 mM/L), glucose (11,1 mM/L) with the addition of EGTA (30 μM/L);

2. Ca^{2+} containing fluid - EGTA- Krebs' fluid (normal) – PSS - composition: NaCl (71,8mM/L), KCl (4,7 mM/L), $CaCl_2$ (1,7 mM/L), $NaHCO_3$ (28,4 mM/L), $MgSO_4$ (2,4 mM/L), KH_2PO_4 (1,2 mM/L), glucose (11,1 mM/L) with the addition of EGTA (30 μM/L), after emptying intracellular calcium pools.

The exponent of the vessel's contraction in the conducted experiments was the increase of perfusion pressure in the experiment system, at the specific transfer of perfusion fluid (around 1ml/min.). The results present the average values and standard deviation.

RESULTS

ANG II in a concentration-dependent manner (3 x 10^{-9} – 3 x 10^{-5} M/l) triggers the increase of perfusion pressure in tail arteries. EC_{50} value is determined as 2,79 (±0,12) x 10^{-8} (Fig. 1). The concentration - response curves for ANG II, drawn after I/R are shown in Figure 1.

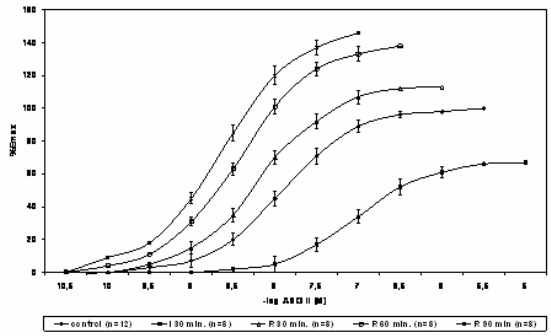

Fig. 1. *The influence of ischemia (I) / reperfusion (R) on CRCs for ANG II (mean ±SE). (%Emax - % of maximal perfusion pressure)*

Ryc. 1. *Wpływ niedokrwienia (I) oraz reperfuzji (R) na krzywe zależności efektu stymulacji od stężenia ANG II (wartości średnie ±SE). (%Emax - % maksymalnego ciśnienia perfuzyjnego)*

Bay K8644 in a concentration-dependent manner (10^{-8} – 10^{-4} M/l) triggers the increase of perfusion pressure in tails' arteries (Fig. 2). EC_{50} value is determined as 1,99 (±0,31) x 10^{-6}. In contrast to the experiments with ANG II, concentration - response curves for Bay K8644 after I/R do not undergo any significant alterations (Fig. 2).

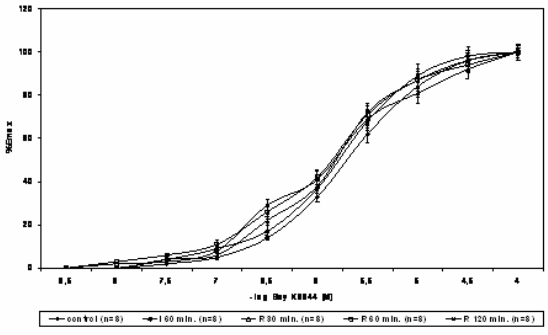

Fig. 2. *The influence of ischemia (I) / reperfusion (R) on CRCs for Bay K8644 (mean ±SE). (%Emax - % of maximal perfusion pressure)*

Ryc. 2. *Wpływ niedokrwienia (I) oraz reperfuzji (R) na krzywe zależności efektu stymulacji od stężenia Bay K8644 (wartości średnie ±SE). (%Emax - % maksymalnego ciśnienia perfuzyjnego)*

ANG II triggers the increase of perfusion pressure in FPSS and PSS, whereas the values are higher in PSS (Fig. 3). After ischemia a reduction of arteries' reaction to ANG II in both types of experiments was observed, but there was not observed any heightened contraction after reperfusion. Bay K8644 triggers a contraction only in PSS, which is not modulated by I/R. The values of perfusion pressure triggered by ANG II and Bay K8644 after I/R in FPSS and PSS are shown in Figure 3.

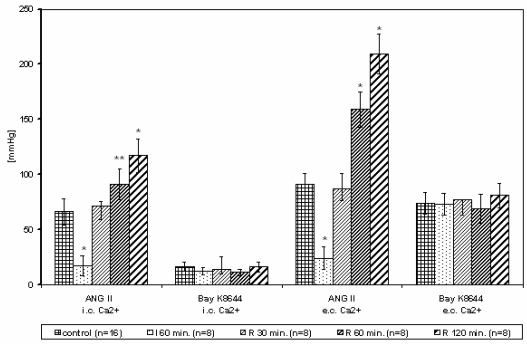

Fig. 3. *Perfusion pressure [mmHg] induced by ANG II and Bay K8644 after ischemia (I) / reperfusion (R), mediated by intracellular (i.c. Ca^{2+}) and extracellular (e.c. Ca^{2+}) calcium(mean ±SE). * p<0,0001 vs control; ** 0,05>p>0,0001 vs control*

Ryc. 3. *Ciśnienie perfuzyjne [mmHg] wyzwalane przez ANG II i Bay K8644 po niedokrwieniu (I) / reperfuzji (R), przy udziale wewnątrz- (i.c. Ca^{2+}) i zewnątrz-komórkowej (e.c. Ca^{2+}) puli jonów wapnia (wartości średnie ±SE). * p<0,0001 vs kontrola; **0,05>p>0,0001 vs kontrola*

In the presence of G-protein blocker - Bordatella pertussis toxin (P 7208) reactions triggered by ANG II are reduced only in FPSS. P7208 suppresses the modulating influence of I/R on arteries' contraction after ANG II in FPSS. However, pertussis toxin limits inhibitory effect of ischemia (p<0,05 vs experiments without P 7208) and the amplifying influence of reperfusion (p<0,0001 vs experiments without P 7208) on arteries' reactions triggered by ANG II (Fig. 4). P 7208 does not change the action of Bay K8644 also after I/R. Figure 4 shows the comparison of perfusion pressure values triggered by ANG II and Bay K8644 after 60 min of ischemia and 30, 60 and 120 min after reperfusion in the presence of Bordatella pertussis toxin.

A blockade of IP3 receptor by xestospongin C, causes suppression of the arterial reactions to ANG II in FPSS, whereas in FPSS a modulation of the reactions is observed after I/R (Fig. 5). XeC does not

affect the reactions of arteries to Bay K8644 in FPSS and PSS (Fig. 5).

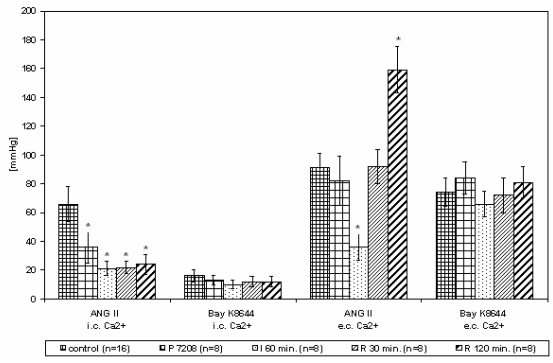

Fig. 4. *Perfusion pressure [mmHg] induced by ANG II and Bay K8644 after ischemia (I) / reperfusion (R), mediated by intracellular (i.c. Ca^{2+}) and extracellular (e.c. Ca^{2+}) calcium, in the presence of Bordatella pertussis toxin (P 7208) (mean ±SE). *p<0,0001 vs control*

Ryc. 4. *Ciśnienie perfuzyjne [mmHg] wyzwalane przez ANG II i Bay K8644 po niedokrwieniu (I) / reperfuzji (R), przy udziale wewnątrz- (i.c. Ca^{2+}) i zewnątrzkomórkowej (e.c. Ca^{2+}) puli jonów wapnia, w obecności toksyny krztuśca (P 7208) (wartości średnie ±SE). * p<0,0001 vs kontrola*

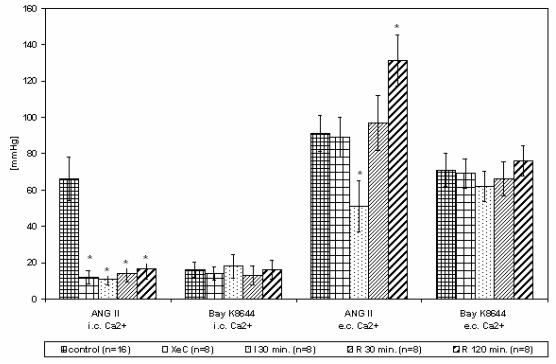

Fig. 5. *Perfusion pressure [mmHg] induced by ANG II and Bay K8644 after ischemia (I) / reperfusion (R), mediated by intracellular (i.c. Ca^{2+}) and extracellular (e.c. Ca^{2+}) calcium in the presence of XeC (mean ±SE). * p<0,0001 vs control*

Ryc. 5. *Ciśnienie perfuzyjne [mmHg] wyzwalane przez ANG II i Bay K8644 po niedokrwieniu (I) / reperfuzji (R), przy udziale wewnątrz- (i.c. Ca^{2+}) i zewnątrzkomórkowej (e.c. Ca^{2+}) puli jonów wapnia, w obecności XeC (wartości średnie ±SE). *p<0,0001 vs kontrola*

DISCUSSION

In the present study the contraction triggered in two experimental models: using angiotensin II – a metabotropic receptor agonist (AT1) and Bay K8644 - a dihydropyridine calcium channel agonist was analyzed. The study focused on the importance of calcium ions and the pathway: G protein - IP3. To determine the contribution of calcium ions (from intracellular stores and extracellular fluid), to the reactions of investigated agonists, experiments were carried out in a fluid without calcium (to evaluate the importance of intracellular pools) and in normal Krebs' fluid, after empting the stores of cellular calcium (assessment of extracellular pool participation). Then the effects of ischemia and reperfusion in these reactions were examined.

AT1 receptor is considered to be as a classic metabotropic receptor, functionally coupled via G proteins with PLC and IP3 synthesis. Smooth muscle contraction, according to the literature [7], is preceded by a biphasic increase of Ca^{2+} concentration. The first phase is to be IP3-dependent and associated with the release of calcium from the endoplasmic reticulum, while the second phase, the slow accumulation of Ca^{2+}concentration, is to be conditioned by the influx of Ca^{2+} ions from a particular extracellular space.

In the present study, the contraction of arteries triggered by ANG II was noticed, both through intra- and extracellular pool of calcium ions, but the reaction was stronger in PSS.

The experiments with pertussis toxin only partially confirmed the dependence of the function of AT1 receptor on G proteins. These experiments showed that only the increase of perfusion pressure obtained with the participation of intracellular IP3-dependent calcium pools remains in close association with protein G. The results indirectly indicate (because the contraction was measured and the level of calcium was not determined) that triggered by ANG II perfusion pressure depends on calcium influx from the extracellular pool and is partly independent of protein G. Different observations were made during the study of contraction triggered by metabotropic $alfa_1$ receptor, adrenergic by phenylephrine, or in which reduction of contraction was obtained in both types of experiments - in FPSS and PSS [8].

In contrast to ANG II, Bay K8644 triggers the increase of perfusion pressure only in the normal Krebs' fluid, as the action of Bay K8644 is the result of direct activation of dihydropyridine calcium channels located in the cell membrane. The reaction of the increased perfusion pressure induced by Bay K8644 uses only extracellular pool of calcium and is

independent of G proteins, which was confirmed also in the studies with pertussis toxin, which does not change the responses of arteries to Bay K8644. Similar observations are derived from Liu et al's experiments with the cardiac muscle cells [9, 10].

In order to confirm the partial dependence of the response of arteries to ANG II on G-protein pathway and activation of PLC and IP3, the experiments were conducted in normal and Ca^{2+} free Krebs' fluid. Numerous reports indicate an antagonistic influence of xestospongin C on IP3 receptor and calcium pump in SR [11, 12]. XeC suppresses only the reactions of the arteries to ANG II in Ca^{2+} free Krebs' fluid, but does not affect the reactions triggered by ANG II in normal Krebs' fluid, in which calcium channels located in the cell membrane (ROC) mediate. The mechanism of control of these channels is not fully understood. The performed experiments show that the opening of these channels due to AT1 receptor activation by ANG II is independent of G proteins.

Results of a series of experiments investigating the impact of I/R on reactions triggered by ANG II, prove that ischemia reduces and reperfusion increases the susceptibility of arteries to the agonist. Previous studies suggest that the inhibitory effect on contraction of coronary arteries is associated with the presence of endothelial nitric oxide synthesis and activation of cGMP [14]. Seascholtz et al's research suggests that G proteins are involved in the disorders caused by I / R, in the reactions triggered via α_1-adrenergic receptors [15]. Disorders between G proteins and protein-alpha G during I/R may be essential for the observed changes in vascular reactivity to contractile factors. In this study, both pertussis toxin and xestospongin C suppress the responses of arteries to ANG II in calcium free environment, moreover, they eliminate the inhibitory effect of ischemia and the amplifying effect of reperfusion on the contractile action of ANG II. Pertussis toxin and xestospongin C inhibit the modulating effect of ischemia and reperfusion in normal Krebs' fluid after emptying intracellular calcium ion pools.

The experiments with Bay K8644 show that ischemia and reperfusion do not affect the responses triggered by direct activation of dihydropyridine calcium channels by Bay K8644. Similar results were obtained in studies with depolarized concentration of KCl [16]. They stated that ischemia does not affect the responses of arteries to KCl. Thus, vasoconstriction triggered by both Bay K8644 and the depolarizing action of KCl is independent of G proteins.

CONCLUSION

The intra- and extracellular pool of calcium ions mediates in the action of ANG II, while Bay K8644 stimulates the contraction only with the involvement of calcium entering the cell. The reduction of ANG II-induced contraction by pertussis toxin suggests that G protein modulates the effect of ANG II triggered only with the involvement of intracellular Ca^{2+} pool.

Ischemia and reperfusion lead to modulation of the contraction of the arteries triggered by metabotropic agonist - ANG II, while the reactions induced by the agonist of calcium channels - Bay K8644 are not changed. No effect of ischemia and reperfusion on the reactions triggered by ionotropic agonist suggests that the modulating effect of ischemia and reperfusion concerns the coupling or uncoupling of G-protein with a receptor or effector.

REFERENCES

1. Gasparo M., Catt K. J., Inagami T., Wright J. W., Unger Th.: XXIII International Union of Pharmacology. The angiotensin II receptors. Pharmacol. Rev. 2000; 52(3): 415-472.
2. Karaki H., Ozaki H., Hori M., Mitsui-Saito M., Amano K., Harada K., Miyamoto S., Nakazawa H., Won K., Sato K.: Calcium movements, distribution, and functions in smooth muscle. Pharmacol. Rev. 1997; 49(2): 157-230.
3. Schäfer M., Bahde D., Bosche B., Ladilov Y., Schäfer C., Piper H. M. i Noll T.: Modulation of early [Ca2+]i rise in metabolically inhibited endothelial cells by xestospongin C. AJP: Heart and Circulatory Physiology 2001; 280(3): H1002-H1010.
4. Choi D. W. :Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci. 1998; 11: 465-469.
5. Fryer H. J. L., Knox R. J., Strittmatter S. M., Kalb R.: Excitotoxic death of a subset of embryonic rat motor neurons in vitro. J. Neurochem. 1999; 72(2): 500-513.
6. Jambrina E., Alonso R., Alcalde M., Rodríguez M. C., Serrano A., Martínez C., García-Sancho J., Izquierdo M.: Calcium influx through receptor-operated channel induces mitochondria-triggered paraptotic cell death. JBC Papers in Press. Published on February 5, 2003 as Manuscript M211388200.
7. Ji J., Benishin C. G., Pang P. K. T.: Nitric oxide selectively inhibits intracellular Ca++ release elicited by inositol trisphosphate but not caffeine in rat vascular smooth muscle. J. Pharmacol. Exp. Ther. 1998; 285, 16–21.
8. Słupski M., Szadujkis-Szadurska K., Szadujkis-Szadurski R., Grześk G.: The role of G protein and calcium (Ca2+)

during ischemia/reperfusion (I/R) on the reactivity of arteries. Transplantation 2008; 86(2): 737.
9. Liu P., Hopfner R. L., Xu Y. J, Gopalakrishnan V.: Vasopressin-evoked [Ca2+]i responses in neonatal rat cardiomyocytes. J. Cardiovasc. Pharmacol. 1999; 34(4): 540-6.
10. Liu P., Xu Y., Hopfner R. L., Gopalakrishnan V.: Phosphatidic acid increases inositol-1,4,5,-trisphosphate and [Ca2+]i levels in neonatal rat cardiomyocytes. Biochim. Biophys. Acta 1999; 1440(1): 89-99.
11. Smet de P., Parys J. B., Callewaert G., Weidema A. F., Hill E., de Smedt H., Erneux C., Sorrentino V., Missiaen L.: Xestospongin C is an equally potent inhibitor of the inositol 1,4,5-trisphosphate receptor and the endoplasmic-reticulum Ca(2+) pumps. Cell Calcium 1999; 26(1-2): 9-13.
12. Ozaki H., Hori M., Kim Y. S., Kwon S. C., Ahn D. S., Nakazawa H., Kobayashi M., Karaki H.: Inhibitory mechanism of xestospongin-C on contraction and ion channels in the intestinal smooth muscle. Br. J. Pharmacol. 2002; 137: 1207-1212.
13. Szadujkis-Szadurski L., Szadujkis-Szadurska K., Szadujkis-Szadurski R., Grześk G., Matusiak M., Wiciński M.: The role of endothelium in contraction of the vascular smooth muscle cells during ischemia/reperfusion. Basic Clin. Pharmacol. Toxicol. 2007 Vol. 101 suppl. 1 s. 64.
14. Seascholtz T. M., Gurdal H., Wang H. Y., Cai G., Johnson M. D., Friedman E.: Heterologous desensitization of the rat tail artery contraction and inositol phosphate accumulation after in vitro exposure to phenylephrine is mediated by decreased levels of G alpha q and G alpha i. J. Pharmacol. Exp. Ther. 1997; 283: 925-931.
15. Yan C.; Dongsoo K., Toru A., Bradford B. C.: Functional interplay between angiotensin II and nitric oxide: Cyclic GMP as a key mediator. Arterioscler. Thromb. Vasc. Biol. 2003; 23: 26.

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ORIGINAL ARTICLE / PRACA ORYGINALNA

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EFFECT OF ACETYLCHOLINE ON REACTIONS INDUCED BY 2 CONTRACTION AGENTS - ANGIOTENSIN II AND CAFFEINE

WPŁYW ACETYLOCHOLINY NA REAKCJE WYZWALANE PRZEZ 2 SUBSTANCJE KURCZĄCE - ANGIOTENSYNĘ II I KOFEINĘ

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S u m m a r y

B a c k g r o u n d : Many agents regulate tonus of vascular smooth muscle cells, acting as constrictors or dilatators. In this study the influence of acetylcholine (Ach) on reactions induced by two constrictory substances - angiotensin II (ANG II, agonist of metabotropic receptor AT1) and caffeine (agonist of ryanodine receptor) was investigated.

M a t e r i a l a n d m e t h o d s : Experiments were performed on perfused human superior mesenteric arteries, retrieved and conserved under the same conditions as transplanted organs. Perfusion pressure induced by ANG II and caffeine mediated by intracellular (in Ca^{2+}-free PSS – FPSS) or extracellular calcium (in PSS, after previous emptying Ca^{2+} cellular stores), in the presence of acetylocholine (Ach) and with addition of L-NNA (NOSe inhibitor), thapsigargine (Ca^{2+} dependant ATP-ase inhibitor), xestospongin C (XeC - IP3 receptor antagonist) or ODQ (CG inhibitor) was analyzed.

R e s u l t s : ANG II increased perfusion pressure in FPSS and in PSS, and the effect was reduced by Ach. In FPSS in the presence of XeC contraction induced by ANG II was diminished and the effect of Ach was not observed. In PSS XeC did not change reactions to ANG II. In both solutions in the presence of L-NNA and ODQ relaxing effect of acetylocholine was not observed. Caffeine induced the increase of perfusion pressure in FPSS and PSS . Thapsigargine lowered, while XeC did not change the perfusion pressure induced by caffeine. Reaction to caffeine was not changed by Ach.

C o n c l u s i o n : Our findings prove that relaxing effect of acetylocholine on muscular smooth muscle cells is connected with synthesis of nitric oxide in endothelium and activation of guanylyl cyclase. Our outcomes prove that contraction induced by caffeine is independent of IP3 - mediated calcium influx and extracellular calcium ions influx. Lack of relaxing effect of Ach on caffeine induced perfusion pressure indicates that contraction via ryanodine receptors is independent of nitric oxide signaling pathway.

S t r e s z c z e n i e

W s t ę p : Napięcie mięśniówki gładkiej naczyń jest regulowane przez wiele substancji kurczących i Rozkurczających. W pracy poddano analizie wpływ acetylocholiny na reakcje wyzwalane przez 2 substancje kurczące naczynia: angiotensynę II (ANG II, agonistę

receptora metabotropowego AT1) i kofeinę (agonistę receptora rianodynowego).

M a t e r i a ł i m e t o d y : Badania przeprowadzono na perfundowanych ludzkich tętnicach krezkowych górnych, pobranych i przechowywanych w tych samych warunkach jak narządy do przeszczepu. Badano skurcz wyzwalany przez ANG II i kofeinę przy udziale wewnątrzkomórkowej (w FPSS) i zewnątrzkomórkowej (w PSS, po uprzednim opróżnieniu magazynów komórkowych) puli jonów wapnia w obecności acetylocholiny (Ach) oraz po dodaniu L-NNA (inhibitora NOSe), thapsigarginy (inhibitora ATP-azy zależnej od Ca^{2+}), ksestosponginy C (XeC, antagonisty receptora dla IP3) lub ODQ (inhibitora rozpuszczalnej CG).

W y n i k i : ANG II wyzwala wzrost ciśnienia perfuzyjnego w FPSS i PSS, a to działanie jest redukowane przez acetylocholinę. W FPSS w obecności XeC istotnie zmniejsza skurcz wyzwalany ANG II i nie obserwuje się wpływu acetylocholiny, a w PSS XeC nie zmienia reakcji na ANG II. W obu roztworach po dodaniu L-NNA i ODQ nie odnotowano rozkurczającego działania acetylocholiny. Kofeina wyzwala wzrost ciśnienia perfuzyjnego w FPSS i PSS. Thapsigargina obniża , a XeC nie zmienia ciśnienia perfuzyjnego wyzwalanego kofeiną. Ach nie wpływa na działanie kofeiny.

W n i o s k i : ANG II wyzwala skurcz przy udziale wewnątrzkomórkowej i zewnątrzkomórkowej puli jonów wapnia. Doświadczenia wskazują, że rozkurczające działanie acetylocholiny na mięśniówkę gładką naczyń jest powiązane z syntezą tlenku azotu w śródbłonku i aktywacją cyklazy guanylanowej. Skurcz wyzwalany kofeiną jest niezależny od związanego z IP3 napływu wapnia i zewnątrzkomórkowej puli jonów wapnia. Brak rozkurczającego efektu Ach na działanie kofeiny wskazuje, że skurcz wyzwalany za pośrednictwem receptorów ryanodynowych jest niezależny od ścieżki sygnalizacyjnej związanej z tlenkiem azotu.

Key words: ANG II, caffeine, acetylcholine, contraction, calcium ions
Słowa kluczowe: ANG II, kofeina, acetylocholina, skurcz, jony wapnia

INTRODUCTION

Ca^{2+} ions perform both the building and functional-regulatory role in the body. They occur in very specific concentrations, which determines the normal function of the cell and the organism. The alteration of the concentrations affects cellular processes such as: contraction and relaxation of smooth muscle and striated muscle, synthesis and release of hormones, neurotransmitters, cell cycle, gene expression and apoptosis. The alteration of the concentration of Ca^{2+} ions in the cytoplasm of the cell can be carried out by their influx from the external pool or release from intracellular stores. The influx of Ca^{2+} ions from the extracellular pool is implemented, inter alia, by the Ca^{2+} channels - ROC - controlled receptors. Ca^{2+} ions can be stored in the cell in the endoplasmic reticulum (ER), mitochondria and the nucleus. The transport of Ca^{2+} ions between the cytoplasm and the ER takes place in both directions. Into the ER Ca^{2+} ions are transported by ATP - dependent Ca^{2+} pump (Ca^{2+} - ATP-ase), whereas into the cytoplasm by two types of channels. Depending on the triggering factor there are channels opened by: receptors for IP3- IP3R, and the ryanodine receptors - RyR [1, 2, 3]. One of the RyR receptor agonists is caffeine. The physiological factors activating RyR are: the level of cytosolic calcium ions (10^{-6} M/L) and ATP, while the increase of cytosolic calcium levels (10^{-9} M/L) leads to the inhibition of these receptors. Aforementioned Ca^{2+} channels ROC and IP3R are the elements of the pathways activated after stimulating the receptor for angiotensin II, leading to the smooth muscle contraction [4, 5, 6].

In this study the influence of acetylocholine (Ach) on reactions induced by two constrictory substances - angiotensin II (ANG II, agonist of metabotropic receptor AT1) and caffeine (agonist of ryanodine receptor) was analyzed.

MATERIAL AND METHODS

Experiments were performed on perfused human mesenteric superior arteries, retrieved and conserved under the same conditions as transplanted organs. To assess the contribution of intracellular and extracellular pool of Ca^{2+} to the reactions triggered by ANG II and caffeine, experiments were carried out using two types of Krebs' fluid:

1. Ca^{2+} free fluid – EGTA – Ca^{2+} free Krebs' fluid - FPSS
2. Ca^{2+} containing fluid - EGTA- Krebs' fluid (normal) – PSS, after emptying intracellular calcium pools.

Next, there was investigated the influence of rising concentrations of acetylcholine (Ach; 0,1 - 30 μM/L) on contractile reactions, which were triggered by ANG II (3 μM/L) and caffeine (100 μM/L) under controlling conditions and at the presence of L-NNA (NOSe inhibitor10 μM/L), thapsigargine (ATP-ase inhibitor Ca^{2+} dependent; 10nM/L), xestospongin C (XeC, receptor antagonist for IP3; 10 μM/L) or ODQ (soluble CG inhibitor; 3 μM/L).

The exponent of the vessel's contraction in the conducted experiments was the increase of perfusion pressure in the experiment system, at the specific transfer of perfusion fluid (around 1ml/min.). The results present the average values and standard deviation.

RESULTS

ANG II triggers an increase of perfusion pressure in FPSS (137 (± 12) mmHg) and PSS (157 (± 17) mmHg). The increasing concentrations of Ach lead to statistically significant reduction of perfusion pressure in both solutions (Figs. 1 and 2). In the presence of XeC in FPSS contraction triggered by ANG II is significantly reduced, and the perfusion pressure was 29 (± 9) mmHg (Fig. 1), whereas XeC does not change the reaction to ANG II in PSS, or the relaxing effect of Ach. (Fig. 2). There were no alterations of contraction induced by ANG II and acetylcholine's relaxing influence in both solutions after the addition of L-NNA and ODQ. Figures 1 and 2 present the influence of increasing concentrations of Ach on perfusion pressure triggered by ANG II in the presence of XeC, L-NNA and ODQ, respectively in FPSS and PSS.

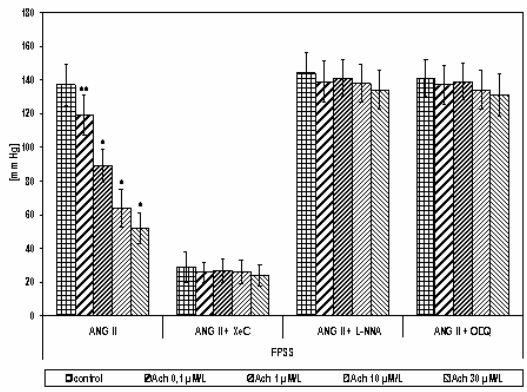

Fig. 1. *The influence of Ach on perfusion pressure [mmHg] induced by ANG II in FPSS, in the presence of XeC, L-NNA and ODQ (mean ±SE, n=9); * p<0,0001 vs control; ** 0,05>p>0,0001 vs control*

Ryc. 1. *Wpływ Ach na ciśnienie perfuzyjne [mmHg] wyzwalane przez ANG II w FPSS, w obecności XeC, L-NNA i ODQ (wartości średnie ±SE, n=9); * p<0,0001 vs kontrola; ** 0,05>p>0,0001 vs kontrola*

Caffeine triggers an increase of perfusion pressure in FPSS (93 (± 9) mmHg) and in PSS (87 (± 6) mmHg). Thapsigargine reduces contraction to 17 (± 6) mmHg in PSS, and XeC does not change the perfusion pressure triggered by caffeine. Ach does not affect caffeine's action in PSS and in FPSS (Fig. 3).

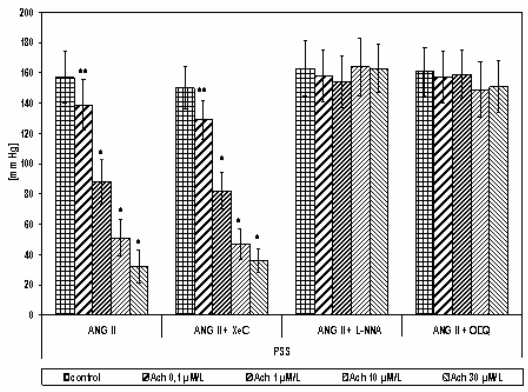

Fig. 2. *The influence of Ach on perfusion pressure [mmHg] induced by ANG II in PSS, in the presence of XeC, L-NNA and ODQ (mean ±SE, n=9); * p<0,0001 vs control; ** 0,05>p>0,0001 vs control*

Ryc. 2. *Wpływ Ach na ciśnienie perfuzyjne [mmHg] wyzwalane przez ANG II w PSS, w obecności XeC, L-NNA i ODQ (wartości średnie ±SE, n=9); * p<0,0001 vs kontrola; ** 0,05>p>0,0001 vs kontrola*

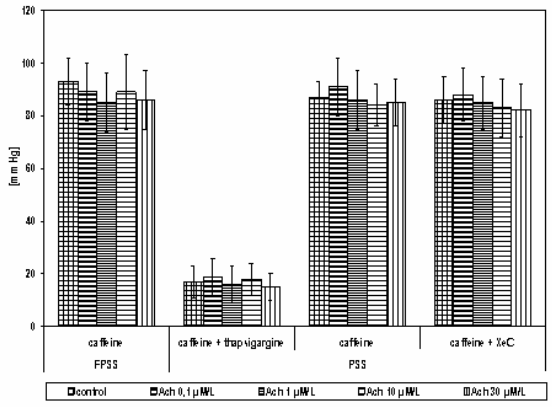

Fig. 3. *The influence of Ach on perfusion pressure [mmHg] induced by caffeine in PSS and FPSS, in the presence of thapsigargine and XeC (mean ±SE, n=9); * p<0,0001 vs control; **0,05>p>0,0001 vs control*

Ryc. 3. *Wpływ Ach na ciśnienie perfuzyjne [mmHg] wyzwalane przez kofeinę w PSS i FPSS, w obecności thapsigarginy i XeC (wartości średnie ±SE, n=9); * p<0,0001 vs kontrola; ** 0,05>p>0,0001 vs kontrola*

DISCUSSION

Ca^{2+} ions, which are the main activating signal, initiate contractile protein phosphorylation in the smooth muscle. [7, 8, 9]. The concentration of free Ca^{2+} ions in the cytoplasm is regulated via multiple

Ca^{2+} channels located in the cell membrane, Ca^{2+} pumps, compartments and buffer systems [1, 10, 11, 12, 13, 14, 15].

In this study the influence of Ach on reactions induced by two constrtictory substances - ANG II (agonist of metabotropic receptor AT1) and caffeine (agonist of ryanodine receptor) was investigated. In order to determine the significance of calcium ions (from intracellular stores and extracellular fluid), in the reactions of perfusion pressure increase, experiments were carried out in a fluid without calcium (to evaluate the importance of intracellular pools) and in normal Krebs' fluid, after empting the intracellular calcium store (assessment of extracellular pool participation).

The comparison of ANG II effect on arteries perfused with physiological Ca^{2+} free fluid containing EGTA with the arteries' reaction triggered by this peptide in physiological fluid containing EGTA and Ca^{2+} ions indicates that in the contraction triggered by ANG II there can be distinguished two components: the first one associated with PLC, IP3 and using an intracellular pool of Ca^{2+} ions, and the second one - using the extracellular pool of Ca^{2+} for the smooth muscle contraction. This observation is consistent with the literature [16] and the results of our experiments on rat tail arteries [17]. The contribution of PLC and IP3 to the increase of perfusion pressure triggered by ANG II in physiological fluid without Ca^{2+} ions, confirms the inhibitory effect on this increase by XeC, receptor antagonist for IP3.

Human superior mesenteric arteries previously constricted by ANG II and perfused with Ca^{2+} free physiological fluid, react by reducing the perfusion pressure in Ach concentration-dependent manner. The reaction of arteries to acetylcholine in the presence of L-NNA - NOSe inhibitor or ODQ - CG inhibitor, as it was described above, is completely eliminated. In these conditions, the performance of both aforementioned inhibitors confirms the relationship between the diastolic effect of Ach and the synthesis of NO and the activation of CG_s. These series of experiments suggests the participation of Ca^{2+} ions and the associated components of IP3 in the effect of Ach on the vascular endothelium.

In the next experimental cycle the Ach's impact on the arteries which were constricted by caffeine was examined. In the preliminary study it was found that arteries placed and perfused by calcium free physiological fluid react by the increase of perfusion pressure. The contraction of similar strength is triggered by caffeine in the physiological fluid containing calcium ions. In these conditions XeC remains without an influence on the increase of perfusion pressure triggered by caffeine, which confirms the independence of caffeine activity of PLC and IP3. Thapsigargine, an inhibitor of ATP-ase-dependent Ca^{2+} suppresses the response of arteries to caffeine. Arteries constricted by caffeine do not respond to Ach with relaxation .

In the experiments on isolated rat aorta it was found that Ach, identically to presented studies of human mesenteric artery, does not induce the relaxation of arteries constricted by caffeine [16]. In the outcomes of our research on human isolated chorionic arteries of placenta we state that the arteries do not react to nitrates and 8Br-cGMP [18, 19]. The studies cited confirm the independence of the contraction triggered by caffeine of the signaling system associated with the PLC, IP3, IP3R and lack of response to the diastolic function of Ach, NO and 8Br-cGMP. Moreover, the experiments conducted on human mesenteric arteries perfused with Ca^{2+} free fluid in the presence of thapsigargine - Ca^{2+}- ATP- ase inhibitor, indicated that after emptying the intracellular pool of Ca^{2+}, the contractions triggered by caffeine are completely eliminated.

In this study the contraction initiated by the interaction of ANG II with metabotropic receptors AT1 was compared with the contraction triggered by caffeine activating ryanodine receptors RyR. ANG II and caffeine trigger the increases of perfusion pressure in an environment devoid of Ca^{2+} ions. As our research shows, in contrast to the contraction triggered by ANG II, the increase of perfusion pressure triggered by caffeine, is independent of IP3.

The alterations of $[Ca^{2+}]_i$, and the increases of perfusion pressure induced by AT1 receptors' activation, which are functionally coupled with G proteins, independently of intracellular, IP3-dependent pool of Ca^{2+} ions, cause also the opening of ROC type calcium channels located in cell membrane. The importance of this second mechanism of ANG II action, independent of IP3, is indicated by the fact that in the liquid containing Ca^{2+} ions and in the presence of XeC, ANG II still causes an increase in perfusion pressure. In addition, ANG II also activates mechanisms sensitizing the Ca^{2+} ions. The activation of these mechanisms can be conditioned by the effect on RhoA, Rho-kinase and PKC, whose importance in the

reactions of contraction is confirmed by numerous data from the literature [20, 21, 22, 23, 24].

The alterations in the response of the mesenteric superior arteries to ANG II caused by XeC confirm the participation of PLC, IP3 and IP3R synthesis located on the Ca^{2+} channels of endoplasmic reticulum and the involvement of the processes in the signaling system of the peptide.

CONCLUSIONS

Experiments show that the relaxing effect of acetylcholine on the vascular smooth muscle is associated with the synthesis of nitric oxide in the endothelium and the activation of guanylyl cyclase. The experiments done in FPSS suggest that the Ach action associated with the endothelial muscarninic receptors depends on IP3, and the extracellular pool of calcium ions is sufficient to act on the pathway: endothelium - NOS - nitric oxide. The caffeine-triggered contraction is independent of calcium influx associated with IP3 and extracellular calcium pools.

The lack of relaxing effect of Ach on caffeine action indicates that the contraction triggered by means of ryanodine receptors is independent of nitric oxide signaling pathway.

REFERENCES

1. Karaki H., Ozaki H., Hori M. i wsp.: Calcium movements, distribution, and functions in smooth muscle. Pharmacol. Rev. 1997; 49: 157–230.
2. Nauli S.M., Williams J.M., Akopov S.E., Zhang L., Pearce W.J.:Developmental changes in ryanodine- and IP3-sensitive Ca2+ pools in ovine basilar artery. Am. J. Physiol. Cell Physiol. 2001; 281: C1785-C1796.
3. Valdés J.A., Hidalgo J., Galaz J.L., Puentes N., Silva M., Jaimovich E., Carrasco M.A.: NF-B activation by depolarization of skeletal muscle cells depends on ryanodine and IP3 receptor-mediated calcium signals. Am. J. Physiol. Cell Physiol. 2007; 292: C1960-C1970.
4. Somlyo A.P., Somlyo A.V.: Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. 2003; 83: 1325.
5. Breitwieser G.E.: G protein–coupled receptor oligomerization: Implications for G protein activation and cell signaling. Circ. Res. 2004; 94: 17-27.
6. Mehta P.K., Griendling K.K.: Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am. J. Physiol. Cell Physiol. 2007; 292: C82-C97.
7. Fay F.S., Shlevin H.H., Granger W.C. Jr, Taylor S.R.: Aequorin luminescence during activation of single isolated smooth muscle cells. Nature 1979; 280: 506–508.
8. Morgan J.P., Morgan K.G.: Vascular smooth muscle: the first recorded Ca2+ transients. Pflügers Arch. 1982; 395: 75–77.
9. Neering I.R., Morgan K.G.: Use of aequorin to study excitation-contraction coupling in mammalian smooth muscle. Nature 1980; 288: 585–587.
10. Himpens R., Missiaen L., Casteels R.: Ca2+ homeostasis in vascular smooth muscle. J. Vasc. Res. 1995; 32: 207–219.
11. Van Breemen C., Chen Q., Laher I.: Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. Trends Pharmacol. Sci. 1995; 16: 98–105.
12. Karaki H.: Historical techniques: cytosolic Ca2+ and contraction in smooth muscle. Trends Pharmacol. Sci. 2004; 25: 388–393.
13. Lee C.H., Poburko D., Kuo K.H. i wsp.: Ca2+ oscillations, gradients, and homeostasis in vascular smooth muscle. Am. J. Physiol. Heart Circ. Physiol. 2002; 282: H1571–H1583.
14. McFadzean I., Gibson A.: The developing relationship between receptor-operated and store-operated calcium channels in smooth muscle. Br. J. Pharmacol. 2002; 135: 1–13.
15. [15] Poburko D., Kuo K.H., Dai J. i wsp.: Organellar junctions promote targeted Ca2+ signaling in smooth muscle: why two membranes are better than one. Trends Pharmacol. Sci. 2004; 25: 8–15.
16. Ji J., Benishin C. G., Pang P. K. T.: Nitric oxide selectively inhibits intracellular Ca++ release elicited by inositol trisphosphate but not caffeine in rat vascular smooth muscle. J. Pharmacol. Exp. Ther. 1998; 285, 16–21,
17. Szadujkis-Szadurski R., Szadujkis-Szadurska K., Tafil-Klawe M., Szadujkis-Szadurski L., Słupski M., Grześk G., Matusiak G.: The influence of ischemia/reperfusion injury on the reactivity of arteries induced by angiotensin II and Bay K8644. Basic Clin. Pharmacol. Toxicol. 2009 Vol. 105 suppl. 1 s. 51.
18. [18] Szadujkis-Szadurski L., Szadujkis-Szadurski R., Szadujkis-Szadurska K., Skublicki S., Szymański W.: Nitric oxide induces dilation of human chorionic arteries via inhibition of Rho-Kinase signalling. Fund. Clin. Pharmacol. 2004a; Vol. 18 suppl. 1 s. 77.
19. Szadujkis-Szadurski L., Szadujkis-Szadurski R., Szadujkis-Szadurska K., Skublicki S., Szymański W.: Nitric oxide modulates angiotensin II-evoked Ca2+-release and influx responses in human chorionic arteries. Fund. Clin. Pharmacol. 2004b; Vol. 18 suppl. 1 s. 77.
20. Matsui T., Amano M., Yamamoto T., Chihara K., Nakafuku M., Ito M., Nakano T., Okawa K., Iwamatsu A., Kaibuchi K.: Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. EMBO J. 1996; 15(9): 2208-16.
21. Kureishi Y., Kobayashi S., Amano M., Kiura K., Kanaide H., Nakano T., Kaibuchi K., Ito M.: Rho-associated kinase directly induces smooth muscle

contraction through myosin light chain phosphorylation. J. Biol. Chem. 1997; 272: 12257.
22. Uehata M., Ishizaki T., Satoh H., Ono T., Kawahara T., Morishita T., Tamakawa H., Yamagami K., Inui J., Maekawa M., Narumiya S.: Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature 1997; 389(6654): 990-4.
23. Rikitake Y., Liao J.K.: Rho GTPases, statins, and nitric oxide. Circulation Research 2005; 97:1232-1235.
24. Shiga N., Hirano K., Hirano M., Nishimura J., Nawata H., Kanaide H.: Long-term inhibition of RhoA attenuates vascular contractility by enhancing endothelial NO production in an intact rabbit mesenteric artery. Circ. Res. 2005; 96: 1014-1021.

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ORIGINAL ARTICLE / PRACA ORYGINALNA

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MODULATION OF THE CONTRACTILE EFFECT OF BAY K8644 ON HUMAN VASCULAR SMOOTH MUSCLE CELLS BY ACETYLCHOLINE AND CALCIUM IONS

MODULOWANIE SKURCZU LUDZKIEJ MIĘŚNIÓWKI GŁADKIEJ NACZYŃ WYZWALANEGO BAY K8644 PRZEZ ACETYLOCHOLINĘ I JONY WAPNIA

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Summary

B a c k g r o u n d : Contraction of the vascular smooth muscle cells is regulated by many signaling pathways. In this study the role of acetylcholine and calcium in modulation of vascular smooth muscle cells contraction induced by Bay K8644, the agonist of calcium channel, was investigated.

M a t e r i a l a n d m e t h o d s : Experiments were performed on perfused human superior mesenteric arteries retrieved and conserved under the same conditions as transplanted organs, and on perfused male Wistar rats' tail arteries. Perfusion pressure induced by Bay K8644, mediated by intracellular (in Ca+-free PSS – FPSS) or extracellular calcium (in PSS, after previous emptying of Ca^{2+} cellular stores) in the presence of sodium nitroprusside (SNP, donator of nitric oxide), NOSe inhibitor (L-NAME or L-NNA) or ODQ (soluble CG inhibitor) was analyzed. Then the effect of acetylcholine (Ach) on contraction was investigated.

R e s u l t s : Bay K8644 induced concentration dependent increase of perfusion pressure. Contraction of the vessels was observed only in PSS. SNP diminished perfusion pressure induced by Bay K8644. Acetylcholine reduced contraction induced by Bay K8644 in PSS, and the effect was dependent on concentration of Ach. In the presence of L-NNA or ODQ perfusion pressure induced by Bay K8644 was not reduced by Ach.

C o n c l u s i o n : Vascular contraction induced by Bay K8644 is independent of intracellular calcium. Our findings prove that relaxing effect of acetylcholine on muscular smooth muscle cells is connected with synthesis of nitric oxide in endothelium and activation of guanylyl cyclase.

Streszczenie

W s t ę p : Skurcz komórek mięśniówki gładkiej naczyń jest regulowany za pośrednictwem wielu ścieżek sygnalizacji wewnątrz- i międzykomórkowej.

Celem pracy było określenie roli acetylocholiny i jonów wapnia w modulowaniu skurczu wyzwalanego przez Bay K8644, agonistę kanałów wapniowych zlokalizowanych w błonie komórkowej.

M a t e r i a ł i m e t o d y : Badania przeprowadzono na perfundowanych ludzkich tętnicach krezkowych górnych, pobranych i przechowywanych w takich samych warunkach jak narządy do przeszczepu, oraz na perfundowanych tętnicach ogonowych szczurów, samców szczepu Wistar. Badano skurcz wyzwalany przez Bay K8644 przy udziale wewnątrzkomórkowej (w FPSS) i zewnątrzkomórkowej (w PSS, po uprzednim opróżnieniu magazynów komórkowych) puli jonów wapnia oraz po dodaniu nitroprusydku sodu jako donatora tlenku azotu (SNP), inhibitora NOSe (L-NAME lub L-NNA), lub ODQ (inhibitor rozpuszczalnej CG). Następnie analizowano wpływ acetylocholiny (Ach) na wybrane reakcje skurczu.

W y n i k i: Bay K8644 wyzwala zależny od stężenia wzrost ciśnienia perfuzyjnego. Reakcje skurczu naczyń zachodzą wyłącznie w PSS. SNP obniża ciśnienie perfuzyjne wyzwalane przez Bay K8644. Ach, w sposób zależny od stężenia, redukuje skurcz wyzwalany przez Bay K8644 w PSS. L-NNA i ODQ znoszą rozkurczające działanie Ach.

W n i o s k i: Skurcz naczyń wyzwalany przez Bay K8644 jest niezależny od wewnątrzkomórkowej puli jonów wapnia. Doświadczenia wskazują, że rozkurczające działanie acetylocholiny na mięśniówkę gładką naczyń jest powiązane z syntezą tlenku azotu w śródbłonku i aktywacją cyklazy guanylanowej.

Key words: Bay K8644, acetylcholine, calcium ions
Słowa kluczowe: Bay K8644, acetylocholina, jony wapnia

INTRODUCTION

The contraction of the vascular smooth muscle cells is regulated by many intracellular and extracellular signaling pathways. Calcium ions are a factor required for muscle contraction, which may be triggered by the release of calcium from intracellular stores and by influx of ions from the extracellular space. Bay K8644 is the agonist of calcium channel located in the cell membrane, which stimulates the influx of calcium ions into the cells and stimulates the contraction of smooth muscle. The role of calcium ions in cellular signaling system is made possible by keeping their concentration within a very narrow range [1]. Ca^{2+} ions initiate the phosphorylation of contractile proteins in smooth muscles [2]. The concentration of free Ca^{2+} ions in the cytoplasm is regulated via multiple Ca^{2+} channels located in the cell membrane, Ca^{2+} pumps, compartments and buffer systems [3, 4]. A few authors also point to the fact that cyclic nucleotides may also trigger smooth muscle relaxation with minimal reduction in the concentration of [Ca2 +]i [5, 6]. The aim of this study was to determine the role of calcium ions in the contraction triggered by Bay K8644 and explore the possibility of modulating the contraction by the signaling pathway leading to cGMP.

MATERIAL AND METHODS

Experiments were performed on perfused human mesenteric superior arteries retrieved and conserved under the same conditions as transplanted organs, and on perfused male Wistar rats' tail arteries.

In order to determine the reactivity of arteries to Bay K8644 (10^{-8} – 10^{-4} M/l) the study began with drawing the concentration – response curve. To assess the contribution of intracellular and extracellular pool of Ca^{2+} under control conditions and in the presence of sodium nitroprusside as a nitric oxide donor (SNP), NOSe inhibitor (L-NAME or L-NNA), or ODQ (soluble CG inhibitor), experiments were carried out using two types of Krebs' fluid: 1. Ca^{2}+ free fluid – EGTA – Ca^{2+} free Krebs' fluid - FPSS – composition: NaCl (71,8 mM/L), KCl (4,7 mM/L), $NaHCO_3$ (28,4 mM/L), $MgSO_4$ (2,4 mM/L), KH_2PO_4 (1,2 mM/L), glucose (11,1 mM/L) with the addition of EGTA (30 μM/L);

2. Ca^{2+} containing fluid - EGTA- Krebs' fluid (normal) – PSS - composition: NaCl (71,8mM/L), KCl (4,7 mM/L), $CaCl_2$ (1,7 mM/L), $NaHCO_3$ (28,4 mM/L), $MgSO_4$ (2,4 mM/L), KH_2PO_4 (1,2 mM/L), glucose (11,1 mM/L) with the addition of EGTA (30 μM/L), after emptying intracellular calcium pools.

Then the influence of increasing acetylcholine (Ach) concentration on particular contraction was investigated. Exponent of the vessel's contraction in the study was an increase in the pressure of the perfusate in the experimental system with a fixed perfusion fluid (about 1ml/min.). The results represent mean values and standard deviation.

THE RESULTS

In experiments conducted on rat tail arteries Bay K8644 induced concentration dependent increase(10^{-8} - 10^{-4} M/l) of perfusion pressure (Fig. 1).

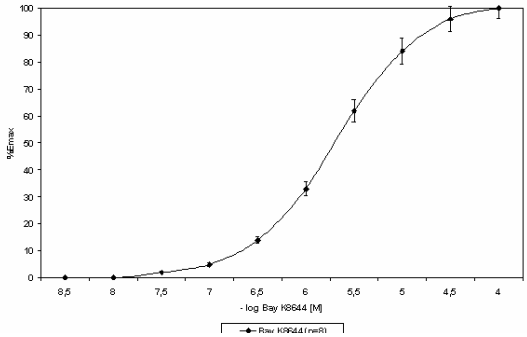

Fig. 1. *Concentration - response curve for Bay K8644 (mean ±SE) (%Emax - % of maximal perfusion pressure)*

Ryc. 1. *Krzywa stężenie - efekt dla Bay K8644 (wartości średnie ±SE) (%Emax - % maksymalnego ciśnienia perfuzyjnego)*

In an environment devoid of calcium (FPSS) the perfusion pressure was 16 (±4,1) mmHg, in PSS, after

emptying intracellular stores of this ion, the pressure increases to 74 (± 10) mmHg (Fig. 2). The addition of SNP, as a source of nitric oxide, significantly reduces the perfusion pressure triggered by Bay K8644. After the application of L-NAME (NOS inhibitor) and ODQ (soluble CG inhibitor), there were no alterations in the contraction triggered by Bay K8644 (Fig. 2).

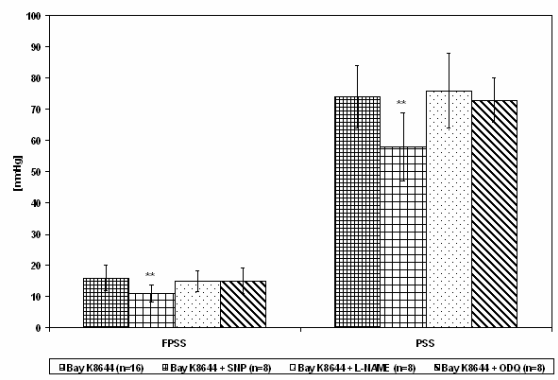

Fig. 2. *The effect of SNP (100 μM/L), L-NAME (300 μM/L) and ODQ (100 μM/L) on perfusion pressure of rat's tail artery [mmHg] induced by Bay K8644 (30 μM/L) in FPSS and PSS (mean ±SE). ** 0,05>p>0,0001 vs Bay K8644*

Ryc. 2. *Wpływ SNP (100 μM/L), L-NAME (300 μM/L) i ODQ (100 μM/L) na ciśnienie perfuzyjne tętnicy ogonowej szczura [mmHg] wyzwalane przez Bay K8644 (30 μM/L) w FPSS i PSS (wartości średnie ±SE). ** 0,05>p>0,0001 vs Bay K8644*

In the next series of experiments carried out on human superior mesenteric arteries, Ach, proportionally to concentration, reduces the contraction triggered by Bay K8644 in PSS (Fig. 3).

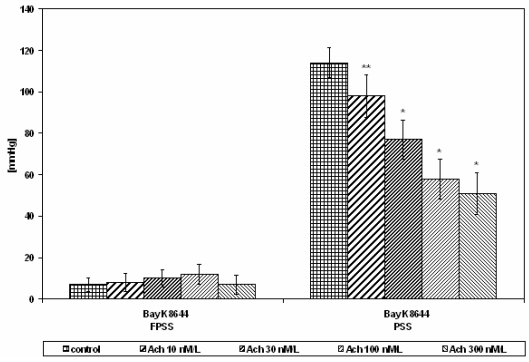

Fig. 3. *The effect of Ach (10 - 300 nM/L) on perfusion pressure of superior mesenteric arteries [mmHg] previously constricted by Bay K8644 (10 μM/L) in FPSS and PSS (mean ±SE, n=9). * p<0,0001 vs control; ** 0,05>p>0,0001 vs control*

Ryc. 3. *Wpływ Ach (10 - 300 nM/L), na ciśnienie perfuzyjne tętnic krezkowych górnych [mmHg] obkurczonych przez Bay K8644 (10 μM/L) w FPSS i PSS (wartości średnie ±SE, n=9). * p<0,0001 vs kontrola; ** 0,05>p>0,0001 vs kontrola*

L-NNA and ODQ abolish relaxing effect of Ach (Fig. 4).

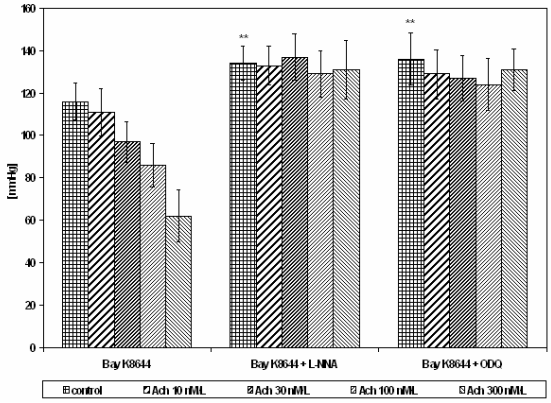

Fig. 4. *The influence of L-NNA (10 μM/L) and ODQ (3 μM/L) on modulatory effect of Ach (10 - 300 nM/L) on perfusion pressure of superior mesenteric arteries [mmHg] previously constricted by Bay K8644 (10 μM/L) in PSS (mean ±SE, n=9). ** 0,05>p>0,0001 vs control Bay K8644*

Ryc. 4. *Wpływ L-NNA (10 μM/L) i ODQ (3 μM/L) na modulujące działanie Ach (10 - 300 nM/L) na ciśnienie perfuzyjne tętnic krezkowych górnych [mmHg] obkurczonych przez Bay K8644 (10 μM/L) w PSS (wartości średnie ±SE, n=9). **0,05>p>0,0001 vs kontrola Bay K8644*

DISCUSSION

The study analyzed the importance of calcium ions in the contraction triggered by Bay K8644, dihydropyridine calcium channel agonist. Therefore, the experiments were made in the fluid without calcium (to evaluate the importance of intracellular pool) and the normal Krebs' fluid, having emptied stores of calcium in the cell (assessment of extracellular pool participation).

Then the possibility of contraction by modulating the signaling pathway CG → NO → cGMP was investigated, using the SNP as a nitric oxide donor, NOSe inhibitor (L-NAME or L-NNA), or ODQ (soluble CG inhibitor).

The role of Ca^{2+} ions is to trigger smooth muscle contraction, in the cardiac muscle and skeletal muscle. Ca^{2+} ions activate calmodulin in the smooth muscle and troponin in the skeletal muscle [7]. The resulting complex of calmodulin or troponin with Ca^{2+} ions, activates myosin light chains and triggers contraction [8].

The functions of smooth muscle Ca^{2+} ion oppose the action of cyclic nucleotides such as cAMP and

cGMP. Increase in intracellular [Ca^{2+}]i precedes and triggers the contraction of smooth muscle. Cyclic nucleotides cAMP and cGMP are synthesized inside the cell, respectively through the adenylyl and guanylyl cyclase [9]. The resulting cyclic nucleotides cAMP, cGMP in the signaling chain are further factors activating protein kinases PKA and PKG. They regulate intracellular Ca^{2+} levels and functions of ion channels [10, 11, 12]. The increasing level of cyclic nucleotides leads to a decrease of [Ca^{2+}]i and of Ca^{2+} sensitivity of smooth muscle and relaxation [11, 13]. PKG is responsible for the hyperpolarization by K + ion removal from the cell. Hyperpolarization inhibits Ca^{2+} influx into the cell. By contrast, PKA inhibits the interaction of the complex Ca^{2+} -calmodulin with MLCK.

Both cyclic nucleotides are degraded by phosphodiesterases [10].

Pathway CG → NO → cGMP interferes with the pathway triggering smooth muscle contraction, in which the triggering factor is the activation of the receptors functionally coupled with G proteins [10]. It increases the levels of Ca^{2+} leading to the activation of MLC-kinase and phosphorylation of myosin light chains, and then to the interaction of myosin filaments with actin and to contraction. The second pathway, associated with the synthesis of cGMP, is dependent on the synthesis of NO released by NOS activation. Nitric oxide activates the soluble form of guanylyl cyclase [14, 15]. In this way, NO inhibits vascular contraction, proliferation and migration and reduces the adhesion of platelets and inhibits inflammation in blood vessels [15, 16]. The results of studies already carried out on a cascade of reactions initiated by NO suggest that the chain of enzymes involved in the cascade can be regarded as a place for pharmacotherapeutic and genotherapeutic intervention [17, 18].

The experiments conducted in FPSS and PSS showed that Bay K8644 triggers the increase of perfusion pressure via calcium ions coming from the outside.

Similarly, other researchers claim that the increase in perfusion pressure triggered by Bay K8644 is independent of IP3 and directly depends on the agonist binding to the L-type Ca^{2+} channels and activation of these channels [19, 20]. This model of contraction is closely associated with the presence of Ca^{2+} ions in the perfusion liquid, which is the source of the increase in extracellular concentrations of ions in the cytoplasm, this type of contraction is completely independent of the intracellular pool of Ca^{2+} ions.

The experiments found that human mesenteric arteries and rat tail arteries placed and perfused by physiological fluid devoid of Ca^{2+} ions do not react to Bay K8644 increased perfusion pressure. Only after emptying a pool of intracellular Ca^{2+} ions and subsequent addition of Ca^{2+} ions in physiological fluid Bay K8644 triggers the increse of perfusion pressure. The studies conducted with rat tail arteries showed that SNP (a nitric oxide donor) results in a significant reduction of contraction induced by Bay K8644, whereas the NOS inhibitor (L-NAME) and ODQ (an inhibitor of soluble CG) did not change the actions of Bay K8644.

In the next stage of experiments, the effect of acetylcholine on superior mesenteric arteries constricted by Bay K8644 was explored. The increasing concentrations of acetylcholine lead to a decrease in arterial perfusion pressure. After adding the inhibitor NOSe (L-NNA), or ODQ (an inhibitor of soluble CG) the perfusion pressure triggered by Bay K8644 significantly increases and the arterial response to Ach is completely abolished.

In the experiments performed on perfused human mesenteric arteries constricted previously by Bay K8644, it was found that Ach, whose activity is dependent on endothelium, NO synthesis and activation of CGs, in a concentration-dependent way decreases the perfusion pressure, in which activation of the L-type Ca^{2+} channels mediates.

The studies conducted so far, indicate that depolarizing concentration of KCl and Bay K8644 contract smooth muscle by opening the L-type Ca^{2+} channels localized in the cell membrane, and increased concentration of free ions [Ca^{2+}] in cytoplasm [21, 22]. Also, our previous studies carried out with perfused rat tail arteries [23] showed that increases in perfusion pressure triggered by Bay K8644 and KCl are closely dependent on the presence of Ca^{2+} ions in the perfused liquid and are blocked by Ca^{2+} channel antagonists: nifedipine and diltiazem. Similar results were obtained by Iesaki and Wolin [24] stating that KCl (at depolarizing concentration of 30 mM / L) and Bay K8644 in an environment devoid of Ca^{2+} ions would not contract coronary artery smooth muscle cells of the ox. In these conditions, arterial responses to KCl and Bay K8644 are triggered by the addition of 1.5 mM/L $CaCl_2$.

One of the pathways considered for the prevention of cardiovascular disease is associated with the signaling system activated by natriuretic peptide and nitric oxide, in which cGMP performs the central role [25, 26]. This nucleotide, and the whole signaling system associated with cGMP can be used in the diagnosis and treatment of cardiovascular diseases [27, 28].

CONCLUSION

The contraction of the vessels triggered by Bay K8644 is independent of intracellular calcium pools. Experiments show that the relaxing effect of acetylcholine on the vascular smooth muscle is associated with the synthesis of nitric oxide in the endothelium and the activation of guanylyl cyclase.

REFERENCES

1. Karaki H., Ozaki H., Hori M., Mitsui-Saito M., Amano K., Harada K., Miyamoto S., Nakazawa H., Won K., Sato K.: Calcium movements, distribution, and functions in smooth muscle. Pharmacol. Rev. 1997; 49(2): 157-230.
2. Neering I.R., Morgan K.G.: Use of aequorin to study excitation-contraction coupling in mammalian smooth muscle. Nature 1980; 288: 585–587.
3. Karaki H.: Historical techniques: cytosolic Ca2+ and contraction in smooth muscle. Trends Pharmacol. Sci. 2004; 25: 388–393.
4. McFadzean I., Gibson A.: The developing relationship between receptor-operated and store-operated calcium channels in smooth muscle. Br. J. Pharmacol. 2002; 135: 1–13.
5. Abu-Soud H.M., Rousseau D., Stuehr D.: Nitric oxide binding to the heme of neuronal nitric oxide synthase links its activity to changes in oxygen tension. J. Biol. Chem. 1996; 271: 32515-32518.
6. Morgan J.P., Morgan K.G.: Alteration of cytoplasmic ionized calcium levels in smooth muscle by vasodilators in the ferret. J. Physiol. 1984; 357: 539–551.
7. Nakayama H., Chen X., Baines C.P., Klevitsky R., Zhang X., Zhang H., Jaleel N., Chua B.H.L., Hewett T.E., Robbins J. i wsp.: Ca2+- and mitochondrial-dependent cardiomyocyte necrosis as a primary mediator of heart failure. J. Clin. Invest. 2007b; 117(9): 2431-2444.
8. Inoue R., L.Jensen, Shi J., Morita H., Nishida M., Honda A., Ito Y.: Transient receptor potential channels in cardiovascular function and disease. Circ. Res. 2006; 99: 119-131.
9. Keef K.D., Hume J.R., Zhong J.**:** Regulation of cardiac and smooth muscle Ca^{2+} channels ($Ca_V1.2a,b$) by protein kinases. Am. J. Physiol. Cell Physiol. 2001; 281: C1743-C1756.
10. Zaccolo M., Movsesian M.A.: cAMP and cGMP signaling cross-talk: Role of phosphodiesterases and implications for cardiac pathophysiology. Circ. Res. 2007; 100(11): 1569-1578.
11. Bender A.T., Beavo J.A.: Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol. Rev. 2006; 58: 488-520.
12. Münzel T., Daiber A., Ullrich V., Mülsch A.: Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. Arterioscler. Thromb. Vasc. Biol. 2005; 25: 1551-1557.
13. Somlyo A.P., Somlyo A.V.: Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. 2003; 83: 1325.
14. Teixeira C.E., Priviero F.B.M., Todd J. Jr, and Webb C.R.: Vasorelaxing effect of BAY 41-2272 in rat basilar artery: Involvement of cGMP-dependent and independent mechanisms. Hypertension 2006; 47: 596-602.
15. Yetik-Anacak G., Xia T., Dimitropoulou C., Venema R. C., Catravas J. D.: Effects of hsp90 binding inhibitors on sGC-mediated vascular relaxation. Am. J. Physiol. Heart Circ. Physiol. 2006; 291: H260-H268.
16. Oberwittler H., Hirschfeld-Warneken A., Wesch R., Willerich H., Teichert L., Heinz K.H., Lehr E., Ding R., Haefeli W. E., Mikus G.: Significant pharmacokinetic and pharmacodynamic interaction of warfarin with the NO-independent sGC activator HMR1766. J. Clin. Pharmacol. 2007; 47: 70-77.
17. Yan C., Kim D., Aizawa T., Berk B.C.: Functional interplay between angiotensin II and nitric oxide: cyclic GMP as a key mediator. Arterioscler. Thromb. Vasc. Biol., 2003; 23: 26-36.
18. Martin E., Sharina I., Kots A., Murad F.: A constitutively activated mutant of human soluble guanylyl cyclase (sGC): Implication for the mechanism of sGC activation. PNAS 2003; 100: 9208.
19. Pesic A., Madden J.A., Pesic M., Rusch N.J.: High blood pressure upregulates arterial L-type Ca2+ channels: Is membrane depolarization the signal? Circ. Res. 2004; 94(10): e97-e104.
20. Pratt P.F., Bonnet S., Ludwig L.M., Bonnet P., Rusch N.J.: Upregulation of L-type Ca2+ channels in mesenteric and skeletal arteries of SHR. Hypertension 2002; 40: 214-219.
21. Piper A.S.. Large W.A.: Direct effect of Ca2+–calmodulin on cGMP-activated Ca2+-dependent Cl– channels in rat mesenteric artery myocytes. J. Physiol. 2004; 559: 449-457.
22. Matchkov V.V., Aalkjaer C., Nilsson H.: A cyclic GMP–dependent calcium-activated chloride current in smooth-muscle cells from rat mesenteric resistance arteries. J. Gen. Physiol. 2004; 123: 121.
23. Szadujkis – Szadurski L., Talar J., Wiśniewski K., Tomaszewski W., Łukowicz M.,Szadujkis – Szadurski R.: Modulujące efekty promieniowania laserowego na pulę zewnątrz i wewnątrzkomórkową Ca2+ i opór naczyniowy tętnicy ogonowej szczura. Fizjoter. Pol. 2002, 2 (1), 11-20.

24. Iesaki T., Wolin M.S.: Thiol oxidation activates a novel redox-regulated coronary vasodilator mechanism involving inhibition of Ca2+ influx. Arterioscler. Thromb. Vasc. Biol. 2000; 20: 2359.
25. Green A.K., Stratton R.C., Squires P.E., Simpson A.W.M.: Atrial natriuretic peptide attenuates elevations in ca2+ and protects hepatocytes by stimulating net plasma membrane Ca2+ efflux. JBC Papers in Press. Published on September 24, 2007 as Manuscript M707115200.
26. James S.K., Armstrong P.W., Barnathan E.S., Califf R.M., Lindahl B.: N-terminal pro brain natriuretic peptide and other risk markers for the separate prediction of mortality and subsequent myocardial infarction in patients with unstable coronary disease: a GUSTO IV substudy. Circulation 2003; 108: 275-281.
27. Morrow D.A., Cannon C.P., Jesse R.L., Newby L.K., Ravkilde J., Storrow A.B., Wu A.H.B., Christenson R.H.: Clinical characteristics and utilization of biochemical markers in acute coronary syndromes. Clinical Chemistry 2007; 53: 552-574.
28. Nakayama H., Harada N., Asano M., Nomura N., Saito T., Mishima A., Okajima K.: Atrial natriuretic peptide reduces Ischemia/reperfusion-induced spinal cord injury in rats by enhancing sensory neuron activation. J. Pharmacol. Exp. Ther 2007a; 322:582-590.

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ORIGINAL ARTICLE / PRACA ORYGINALNA

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MICROORGANISMS COLONIZING THE INTRACRANIAL CATHETERS IN PATIENTS WITH GLIOBLASTOMA MULTIFORME TREATED WITH STEREOTACTIC BRACHYTHERAPY

DROBNOUSTROJE KOLONIZUJĄCE CEWNIKI ŚRÓDCZASZKOWE U PACJENTÓW PODDANYCH STEREOTAKTYCZNEJ BRACHYTERAPII Z POWODU GLIOBLASTOMA MULTIFORME

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Summary

Introduction. Catheter-related infections are still a big problem in hospitals. The main sources of microorganisms colonizing catheters are the patient's and personnel's skin and the hospital environment. Education of medical workers is essential to prevent catheter-related infections. The purpose of this study was to assess the intracranial catheter tips colonization in patients with inoperable brain tumours glioblastoma multiforme treated with stereotactic brachytherapy.

Material and methods. A total of 234 intracranial catheter tips were analysed by semiquantitative (roll plate) and quantitative technique. Catheter colonization was defined as any semiquantitative culture yielding ≥ 15 CFU, or any quantitative culture yielding $\geq 10^3$CFU of bacteria (CFU - colony forming units). Any colony counts below these levels were considered to be negative. All isolated bacteria were identified by standard microbiological methods. We also tried to estimate the correlation between catheter colonization and infection.

Results. Thirty catheters (12.8%) were positive by quantitative method and 38 (16.2%) by roll plate method. Coagulase-negative staphylococci comprised 67.6 and 75.0% of all isolates. The most frequently identified microorganism was *Staphylococcus epidermidis*. Overall, 23.3 and 15.8% of quantitative and semiquantitative cultures, respectively, were polymicrobial. There was not any significant difference between the bacterial species found on the external and internal side of the catheters. None of the patients demonstrated any clinical manifestations of infection associated with catheters.

Conclusions. The results show that positive intracranial catheter tips cultures were rare in patients undergoing stereotactic brachytherapy. Bacteria isolated from catheter tips represented mainly skin flora.

Streszczenie

Wstęp: Zakażenia pochodzenia odcewnikowego ciągle stanowią poważny problem w szpitalach. Źródłem drobnoustrojów kolonizujących cewniki jest zazwyczaj skóra pacjenta i personelu oraz środowisko szpitalne. W zapobieganiu zakażeniom odcewnikowym największą rolę odgrywa edukacja personelu. Celem pracy była ocena kolonizacji cewników śródczaszkowych stosowanych w brachyterapii stereotaktycznej u pacjentów chorych na nieoperacyjne guzy mózgu glioblastoma multiforme.

Materiał i metody: Materiał do badań stanowiły 234 końcówki cewników śródczaszkowych. Do oceny kolonizacji zastosowano metodę półilościową (rolowania) i ilościową. Wzrost uważano za istotny, jeśli drobnoustroje występowały w liczbie ≥ 15 CFU i/lub $\geq 10^3$ CFU (CFU, ang. colony forming units, jednostki tworzące kolonie). Wzrost poniżej przyjętego progu uznawano za wynik ujemny. Wyhodowane drobnoustroje identyfikowano zgodnie ze standardowymi metodami obowiązującymi w laboratoriach

mikrobiologicznych. Podjęto również próbę oceny zależności między kolonizacją cewnika a występowaniem zakażeń.

W y n i k i : W metodzie ilościowej otrzymano 30 (12,8%), a w metodzie rolowania 38 (16,2%) posiewów dodatnich. Gronkowce koagulazo-ujemne stanowiły 67,6 i 75,0% izolowanych drobnoustrojów. Najczęściej izolowanym drobnoustrojem był *Staphylococcus epidermidis*. Wielobakteryjną kolonizację stwierdzono w 23,3 i 15,8% posiewów wykonanych odpowiednio metodą ilościową i półilościową. Profil drobnoustrojów kolonizujących zewnętrzną i wewnętrzną powierzchnię cewnika był podobny. U żadnego chorego nie zaobserwowano klinicznych objawów zakażenia związanego ze stosowaniem cewników.

W n i o s k i : Dodatnie wyniki posiewów końcówek cewników śródczaszkowych wśród chorych na glioblastoma multiforme występowały rzadko. Drobnoustroje kolonizujące cewniki stanowiła głównie flora fizjologiczna skóry.

Key words: intracranial catheters, colonization, coagulase-negative staphylococci

Słowa kluczowe: cewniki śródczaszkowe, kolonizacja, gronkowce koagulazo-ujemne

INTRODUCTION

The typical treatment of patients with glioblastoma multiforme involves surgical resection and post-surgical radiotherapy and chemotherapy in selected patients. In patients who do not qualify for surgical treatment and who suffer from post-surgical relapses, brain brachytherapy is applied. In this method, the radiation source is administered directly to the tumour tissue by means of catheters.

This study aimed at a quantitative assessment of microorganisms colonising the external catheters used in brain brachytherapy; an attempt was also made to assess the relations between the occurrence of microorganisms on the outer or inner surface of the catheter and potential local infections and neuroinfections.

MATERIALS AND METHODS

The procedure of implanting the non-porous CH 8 external catheter of polyvinyl chloride was carried out 1-2 days prior to the commencement of brachytherapy in a sterile operating room. The procedure was preceded by the planning of biopsy trajectory in a three-dimensional system for treatment planning, on the basis of a fusion of MRI and CT scans. The radiation dose was 15 Gy/izodose in 5 fractions at 24-hour intervals. If the distribution of the radiation dose was unsatisfactory, two external catheters were applied at the same time (Fig. 1-2). Directly before radiation, a sterile F6 internal catheter of polyvinyl chloride was inserted to the external catheter, stitched to the skin, and removed after each fraction (Fig. 3). Afterwards, a chamber dressing with crystalline penicillin was applied.

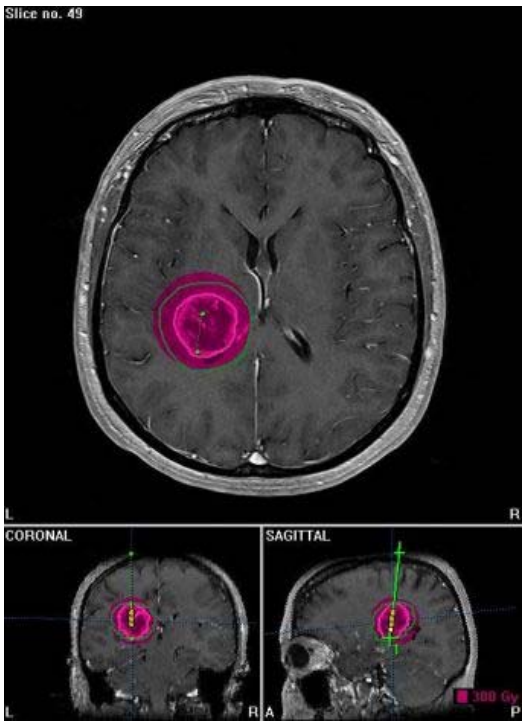

Fig. 1 *Planning of the biopsy trajectory (green line) and dose distribution (pink circle) for a deep located inoperable tumour*

Ryc. 1. *Planowanie trajektorii biopsji (zielona linia) i rozkładu dawki (różowe koło) głęboko położonego nieoperacyjnego guza*

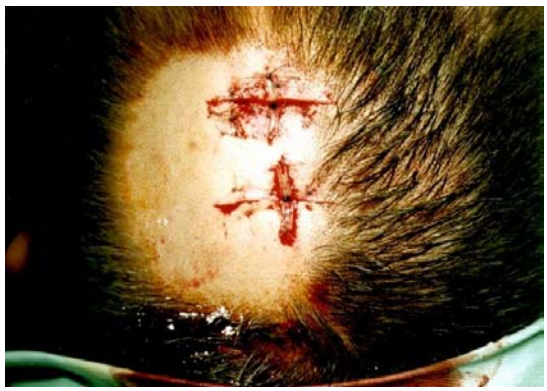

Fig 2. *Implantation of external catheters*

Ryc. 2. *Implantacja cewników zewnętrznych*

Fig. 3. *Patient irradiation. Visible internal catheter*
Ryc. 3. *Napromienianie pacjenta. Widoczny cewnik wewnętrzny*

Material for studies included tips of 234 internal catheters removed after completed radiation (after the fifth fraction) from 201 patients with malignant brain glioma, hospitalised in the years 2001-2005 in the Clinical Ward of Brachytherapy. As prophylaxis, all the patients were receiving ceftriaxone in the dose of 1g/day throughout the period in which the catheter was inserted. The catheters were removed after 5-7 days of treatment in the patients' room and the wound was stitched. The process of collecting a piece of a catheter for study is presented in Fig. 4-5. After collecting, the catheter was handed over to the Department of Microbiology within 15 minutes, where it was analysed using a semiquantitative (roll plate) technique according to Maki [1] – the catheter piece was rolled four times on the surface of a plate with Columbia agar + 5.0% ram blood (bioMérieux) – and a quantitative technique (from the inside diameter of the catheter) on the medium of Columbia agar + 5.0% ram blood, after shaking in trypticase soy broth (bioMérieux). Growth was regarded as significant if the number of microorganisms was $\geq$ 15 CFU/plate and/or $\geq 10^3$ CFU/ml [2,3]. The microorganisms were identified by means of manual and/or automated tests (bioMérieux), in accordance with the instructions for the Microbiology Department. The growth of microorganisms in the semiquantitative and quantitative method was demonstrated by stating the number of CFU/plate (colony forming units) and CFU/ml respectively.

The frequency of external and internal colonisation of the catheter surface was compared by means of a two-fraction test (frequency test), modified in such a way as to take into account small samples.

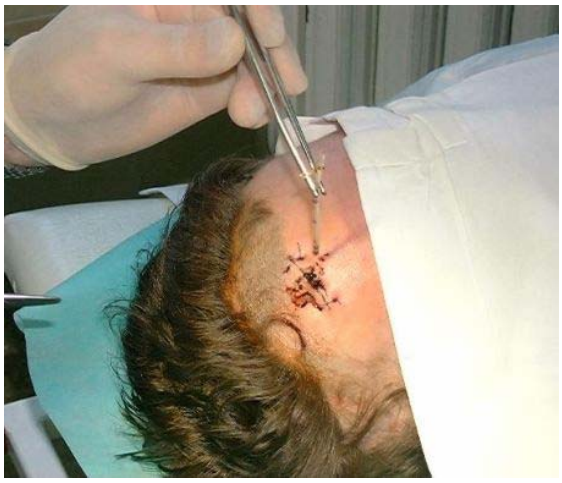

Fig. 4. *Removal of external catheter*
Ryc. 4. *Usunięcie cewnika zewnętrznego*

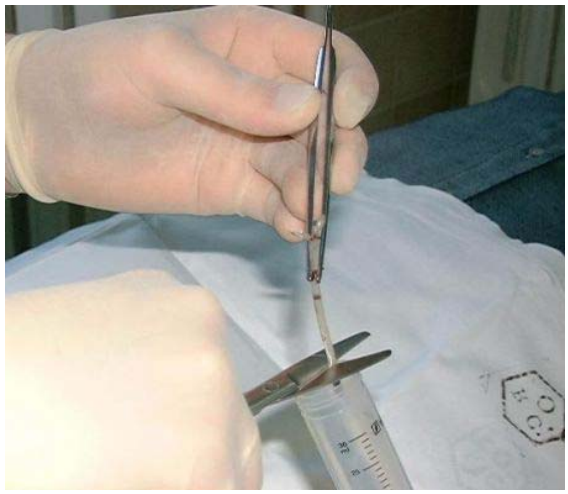

Fig. 5. *Collection of external catheter tip for microbiological examinations*
Ryc. 5. *Pobieranie fragmentu cewnika zewnętrznego do badań mikrobiologicznych*

RESULTS

Thirty cultures (12.8%) were positive by the quantitative method and 38 (16.2%) by roll the plate method, according to the accepted criteria. The microorganisms occurred mainly in monoculture, 23 (76.7%) and 32 (84.2%) positive catheters in the quantitative and semiquantitative method respectively. The following were isolated from the culture from the inside diameter of the catheter: *Staphylococcus epidermidis* (*S. epidermidis*) (n=10), *S. haemolyticus* (n=8), *S. xylosus* (n=2), *S. capitis* (n=1), *S. sciuri (n*=1*)*, other coagulase-negative staphylococci (n=3),

Corynebacterium xerosis (*C. xerosis*) (n=2), *C. jeikeium* (n=1), *Corynebacterium* group G1 (n=2), other *Corynebacteria* (n=6) and *Streptococcus* spp (n=1) (fig. 6). Using the roll plate method the growth of the following organisms was obtained: *S. epidermidis* (n=14), *S. haemolyticus* (n=8), *S. capitis* (n=1), *S. simulans* (n=2), *S. sciuri* (n=2), *S. hominis* (n=1), coagulase-negative staphylococci (n=5), *C. xerosis,* (n=3), *Corynebacterium* groups G1 (n=2) and other *Corynebacteria* (n=5) and *Enterococcus faecalis* (*E. faecalis*) (n=1) (fig. 7).

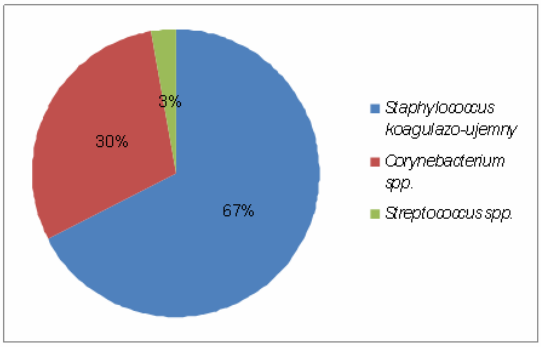

Fig. 6. *Microorganisms isolated from intracranial catheter tip samples – quantitative technique*
Ryc. 6. *Drobnoustroje izolowane z końcówek cewników śródczaszkowych – metoda ilościowa*

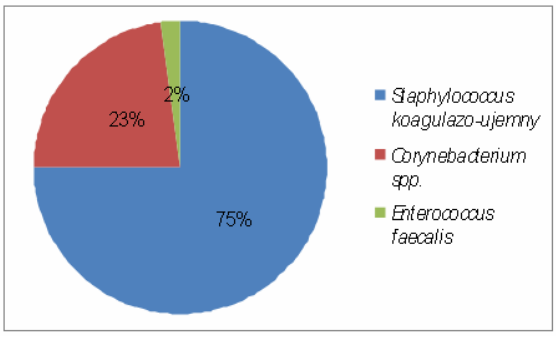

Fig. 7. *Microorganisms isolated from intracranial catheter tip samples – semiquantitative technique (roll plate)*
Ryc. 7. *Drobnoustroje izolowane z końcówek cewników śródczaszkowych - metoda półilościowa (rolowania)*

No clinical symptoms of local infection or features of neuroinfection connected with the application of catheters were observed in any patient.

DISCUSSION

The factors predisposing to catheter-related infections include the patient's general condition, age, basic disease, application of radio- and chemotherapy, as well as the material from which the catheter was made, the place of insertion, period of retaining the catheter and the quality of patient care [4-6]. The bacterial flora that colonises the catheters is quite typical. Gram-positive cocci, especially methicillin-resistant coagulase-negative staphylococci, predominate [3,7-9]. This is connected with the common occurrence of these microorganisms on skin and in the environment. The catheters are colonised as a result of the contact with patient's colonised skin or staff's colonised hand skin. The ability of staphylococci to adhere to surfaces made of plastic and to produce mucus forms favourable conditions for colonisation. Other authors report that gram-positive bacteria constitute 60.0-80.0% of all the microorganisms isolated from the end tips of various catheters [3,8,10-11]. The profile of microorganisms isolated in the study material did not differ from literature data. Coagulase-negative staphylococci predominated, and the leading species was *S. epidermidis*. This staphylococcus carries potential virulence factors. It produces substances that allow it to survive and spread in the body. It is one of the basic etiological factors responsible for infection of biomaterials [12-13]. In the material we studied no gram-negative rods or yeast-like fungi were detected. However, it ought to be emphasised that in the available reference material no publications were found on the bacterial flora and fungi that colonise catheters inserted directly into the brain tumour.

The roll plate (semiquantitative) method makes it possible to detect external colonisation on the surface of the catheter, whereas the quantitative study confirms internal colonisation [1-2]. Microorganisms more often colonised the external surface, but the difference was not statistically significant ($p>0.05$). The profiles of the microorganisms we isolated from the external and internal surface did not differ considerably. However, it is worth mentioning that external colonisation with negative cultures from the inside diameter of the catheter was observed in the case of *E. faecalis*.

Despite a growth of microorganisms that exceeded the accepted diagnostic threshold, no symptoms of local infection, such as reddening, pus, swelling, features of sepsis and neuroinfection, were observed. It would seem that the frequency of catheter colonisation demonstrated in this paper and the isolation of mainly physiological flora of skin may have been affected by the accepted pattern of care provided to patients with intracranial catheter and by observance of sterility

rules. The relatively short time of retaining the catheter and the small number of manipulations were also of importance. Moreover, all the patients qualified for radiation received, as prophylaxis, beta-lactam antibiotics: penicillin – locally in the chamber dressing and third-generation cephalosporin – intravenously. The antibiotic therapy was introduced 2 hours before the implementation of the catheter. Therefore, the impact of antibiotics on the study results cannot be eliminated.

The results obtained in this paper confirm that the intracranial catheters used in brain brachytherapy lasting for a few days in patients with glioblastoma multiforme are not a source of infection if appropriate rules and procedures are observed.

CONCLUSIONS

1. Intracranial catheters in patients with glioblastoma multiforme were rarely positive.
2. The microorganisms colonising the catheters included skin coagulase-negative staphylococci, *S. epidermidis* being predominant.
3. Catheter colonisation, even in a number exceeding the accepted diagnostic threshold, did not cause infections in the study group of patients.
4. Prophylactic application of third-generation cephalosporin is probably an effective method of preventing neuroinfections during brain brachytherapy.

REFERENCES

1. Maki D., Weise C, Sarafin H.: *A semiquantitative culture method for identifying intravenous-catheter-related infection*. N Engl J Med 1977, 296, 1305-9.
2. Brun-Buisson C. i wsp.: *Diagnosis of central venous-related sepsis. Critical level of quantitative tip cultures*. Arch Intern Med 1987, 147, 873-7.
3. Bouza E. i wsp.: *A European perspective on intravascular catheter-related infections: report on the microbiology workload, aetiology and antimicrobial susceptibility (ESGNI-005 Study)*. Clin Microbiol Infect 2004, 10, 838-42.
4. Zingg W. i wsp.: *Impact of a prevention strategy targeting hand hygiene and catheter care on the incidence of catheter-related bloodstream infections*. Crit Care Med 2009, 37, 2167-73.
5. Pawińska A.: *Naruszenie ciągłości tkanek – zakażenia zawiązane z terapią dożylną* [w:] Zakażenia szpitalne, Red: Dzierżanowska D., Jeljaszewicz J., α-medica press, Biesko-Biała 1999, 277-93.
6. Warren D. i wsp. *The effect of an education program on the incidence of central venous catheter-associated bloodstream infection in a medical ICU*. Chest 2004, 126, 1612-18.
7. Cirković I. i wsp.: *Colonization of central venous catheter in the intensive Care Units at the Institute of Cardiovascular Disease "Dedinje" - one year study.* Acta Chir Iugosl 2005, 52, 33-7.
8. Misiołek H. i wsp.: *Ocena posiewów bakteriologicznych zakończeń cewników zewnątrzoponowych oraz częstości występowania zakażeń miejscowych podczas stosowania analgezji zewnątrzoponowej w odcinku piersiowym-doniesienia wstępne*. KiTP 2007, 4, 278-85.
9. David A. i wsp.: *Central venous catheters and infections*. Minerva Anestesiol 2005, 71, 561-4.
10. Tomanović B., Morović V.: *Frequency and colonization rate of intravascular catheters.* Vojnosanit Pregl 2004, 61, 255-8.
11. Dzierżanowska D., Pawińska A.: *Posocznica odcewnikowa*. Przegl Epidemiol 2002, 56, 443-52.
12. Vadyvaloo V., Otto M.: *Molecular genetics of Staphylococcus epidermidis biofilms on indwelling medical devices*. Int J Artif Organs 2005, 28, 1069-78.
13. Stevens N.T., Tharmabala M., Dillane T.: *Biofilm and the role of the ica operon and aap in Staphylococcus epidermidis isolates causing neurosurgical meningitis*. Clin Microbiol Infect, 2008, 14, 719-22.

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ORIGINAL ARTICLE / PRACA ORYGINALNA

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THE OPINION OF NURSING STUDENTS ON THE SENSE OF PROFESSIONAL SATISFACTION, MOTIVES INFLUENCING THE CHOICE OF PROFESSION, THEIR EXPECTATIONS AND EXPRESSION OF CONCERNS

OPINIE STUDENTÓW KIERUNKU PIELĘGNIARSTWO DOTYCZĄCE POCZUCIA SATYSFAKCJI ZAWODOWEJ, MOTYWÓW WYBORU ZAWODU, OCZEKIWAŃ I PRZEJAWIANYCH OBAW

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S u m m a r y

I n t r o d u c t i o n . The aim of this study was to obtain the opinion of full-time and part-time nursing students on the sense of professional satisfaction, motives influencing choices of career, their expectations, and expression of concerns. An attempt was made to conduct a comparative analysis among students of two programmes : full-time and part-time.

M a t e r i a l s a n d m e t h o d s . The study was conducted in 2009 in a group of 164 first year nursing students of the full-time and part-time second-cycle programme in the Faculty of Health Sciences, Nicolaus Copernicus University (NCU) Collegium Medicum in Bydgoszcz. A diagnostic survey based on a self-constructed questionnaire was used.

R e s u l t s . Over 90% of the respondents displayed satisfaction in terms of the career choice and this is more frequently the case among part-time students. 30% of the full-time students and 25% of the part-time students declared that their career choice had been made purely by chance. 20% of the full-time students and 17.3% of the part-time students would not make the same career choice the second time. The main motive behind the career choice, according to the full-time students, was the constant demand for nurses on the job market (46.3%), according to the part-time students it was due to a vocation (50%). Approximately 90% of nursing students believe that the change in the system of education (nursing programme offered at an academic level) contributes to the growth of professional prestige. Over 90% of respondents are hoping for an increase in the prestige of the profession they have chosen. The greatest expectation associated with the nursing profession is connected with a rise in earnings (full-time students-95.1%, part-time students-83%). 95.1% of the full-time students and 14.6% of the part-time students fear job loss. A high level of professional responsibility was the most frequent fear amongst part-time students (72%) whereas this number was lower amongst full-time students (39%). 59.7% of nurses studying full-time and 52.4% of nurses studying part-time fear professional burn-out.

C o n c l u s i o n s . Vast majority of nursing students express satisfaction in relation to their choice of profession. A constant demand for nurses on the job market was indicated as the main motive behind the choice of profession (full-time students) and vocation (part-time students). The increase in self-esteem was the key motive influencing the decision of starting second-cycle studies. Nearly all respondents hope for a rise in earnings . Full-time students more frequently fear job loss and mobbing than part-time students. Just over a half of the respondents fear professional burn-out.

S t r e s z c z e n i e

W s t ę p . Celem badań było poznanie opinii studentów studiów stacjonarnych i niestacjonarnych na kierunku pielęgniarstwo, dotyczących poczucia satysfakcji zawodowej, motywów wyboru zawodu, oczekiwań i przejawianych obaw. Podjęto próbę analizy porównawczej na dwóch systemach kształcenia: stacjonarnym i niestacjonarnym.

M a t e r i a ł i m e t o d y . Badania przeprowadzono w 2009 roku na grupie 164 studentów I roku stacjonarnych i niestacjonarnych studiów drugiego stopnia na kierunku pielęgniarstwo Wydziału Nauk o Zdrowiu UMK Collegium Medicum w Bydgoszczy. Zastosowano metodę sondażu diagnostycznego z wykorzystaniem autorskiego kwestionariusza ankiety.

W y n i k i . Ponad 90% respondentów wykazuje zadowolenie z wybranego zawodu, częściej studenci studiów niestacjonarnych. Wybór zawodu raczej z przypadku deklarowało blisko 30% studentów studiów stacjonarnych i 25 % studentów studiów niestacjonarnych. Po raz kolejny nie podjęłoby decyzji o wybranym zawodzie około 20 % studentów studiów stacjonarnych i 17,3% studiów niestacjonarnych. Głównym motywem wyboru zawodu, jaki wskazali respondenci studiów stacjonarnych, było stałe zapotrzebowanie na pracę pielęgniarki (46,3%), zaś studiów niestacjonarnych – powołanie (50%). Około 90% studentów kierunku pielęgniarstwa uważa, że zmiana systemu kształcenia na poziomie akademickim przyczynia się do wzrostu prestiżu zawodowego. Ponad 90% badanych liczy na wzrost prestiżu w wybranym przez siebie zawodzie. Największe oczekiwanie związane z zawodem studiujące pielęgniarki/pielęgniarze wiążą z poprawą warunków płacowych (studia stacjonarne – 95,1%; niestacjonarne – 83%). Utraty pracy obawia się 95,1% studentów studiów stacjonarnych i 14,6% studentów studiów niestacjonarnych. Duża odpowiedzialność zawodowa była najczęściej wskazywaną obawą wśród studentów studiów niestacjonarnych (72%) i w 39 % przez studentów studiów stacjonarnych. Wypalenia zawodowego obawia się 59,7% pielęgniarek/pielęgniarzy studiujących systemem stacjonarnym i 52,4% studiujących w systemie niestacjonarnym.

W n i o s k i . Zdecydowana większość studiujących pielęgniarek/pielęgniarzy wykazuje zadowolenie z wybranego zawodu. Jako główny motyw wyboru zawodu respondenci wskazali: perspektywą stałego zapotrzebowania na pracę w tym zawodzie (studia stacjonarne) i powołanie (studia niestacjonarne). Wzrost poczucia własnej wartości był z kolei najważniejszym motywem podjęcia studiów drugiego stopnia. Prawie wszyscy respondenci oczekują poprawy warunków płacowych. Osoby studiujące w systemie stacjonarnym znacznie częściej obawiają się w przyszłości utraty pracy i mobbingu niż studiujące w systemie niestacjonarnym. Nieco ponad połowa respondentów obawia się wypalenia zawodowego. .

Key words: nursing profession, hope, fear, motive, satisfaction

Słowa kluczowe: nursing profession, hopes, worries, motives, satisfaction

INTRODUCTION

Nursing is gaining a higher social status as a profession. The primary determinant of this is a wide range of autonomy in undertaking new professional roles[1]. The change in the educational system (nursing programme offered at an academic level) shapes the image of nurses, preparing them to play various roles and become competent members of therapeutic teams. The work of a contemporary nurse is based on specialized knowledge, high qualifications, aspirations and is in the pursuit of autonomy and independence [2].

An important factor influencing the choice of career, including that of a nurse, is social prestige. Factors which define social prestige are, amongst others: high earnings , secure employment and prospects for promotion, level of education and gained qualifications, the range of social needs in relation to representatives of the profession, the range of autonomy and the hierarchy of values acknowledged by the community[2]. Social prestige may be a factor which attracts or discourages individuals from choosing and practising a certain profession [2]. Professional prestige is "respect, obedience and deference displayed in relation to a profession (occupational group), arising directly from the professional and economic position it occupies" [3]. Professional prestige – according to the Dictionary of Sociology and Social Science [4] – is " a diverse social assessment attributable to certain occupations. Knowledge which people have about the profession, and their perceptions of the profession are rather permanent; there is a much greater diversity in terms of values, which they attribute the profession with". The personality traits of a nurse are made up of characteristics such as: kindness, affection for others, generosity, thoughtfulness, empathy [2].

The aim of the research was to obtain the opinion of the full-time and part-time nursing students on the sense of professional satisfaction, motives influencing the choice of professional career, their expectations and expression of concerns. An attempt was made to

conduct a comparative analysis among students of two programmes : full-time and part-time.

MATERIAL AND METHOD

The research was conducted among a group of 164 first year nursing students of the full-time and part-time second-cycle programme from the Faculty of Health Sciences of NCU Collegium Medicum in Bydgoszcz. The research group comprised 82 full-time students and 82 part-time students. A vast majority of the respondents were women 161 (n=164). Study participants differed in terms of work experience in the nursing profession, marital status and place of residence. Among the full-time students – 46.3% had no work experience in the field, 41.5% had short-term work experience of up to 5 years (first months of work) and 12.2% stated they had between 11 and 20 years work experience in the field. Among the part-time students most of them (43.9%) stated they had worked in the field for 11-20 years, a little less (34.1%) had over 21 years of experience, 15.9% up to 5 years of experience and 6.1% between 6 and 10 years of experience. 75.6% of the part-time students were married or in a serious relationship while this number was 48.8% in terms of the full-time students. The remaining respondents were single. Nearly ¾ of the respondents lived in a big city.

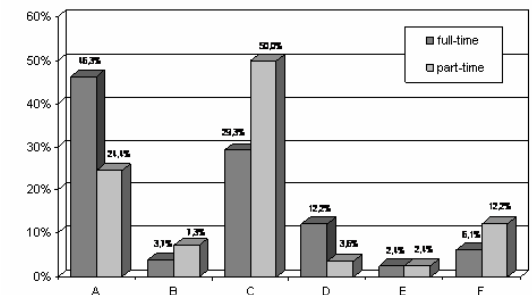
A self-constructed questionnaire was used in the study. It comprised 20 questions concerning, among others, work-experience, marital status, place of residence, professional satisfaction or its lack, motives influencing career choice and the decision to start second-cycle studies, expectations and fears connected with the profession. The research was conducted in 2009 after gaining the consent of the Bioethics Committee of NCU Collegium Medicum in Bydgoszcz (KB/562/2008). This research was voluntary and anonymous. A percentage distribution was used in the analysis process.

RESULTS

Most of the studying nurses are satisfied with their choice of profession. 40.3% of the part-time students and 31.7% of the full time students answered "definitely, yes" to this question and 58.5% of the part-time students and 59.8% of the full-time students answered "yes". 8.5% of the full-time students and 1.2% of the part-time students are not satisfied with their choice (answered "no"). None of the individuals who took part in the research answered "definitely not" to this question.

73.2% of the part-time students and 65.9% of the full-time students intentionally and consciously chose the nursing profession, whereas 25.6% of the part-time students and 29.2% of the full-time students admit to have made the choice purely by chance. 1.2% of the part-time students and 4.9% of the full-time students took up nursing studies due to the lack of vacancies in other fields of study. 30.5% of the part-time students and 24.4% of the full-time students would definitely make the same career choice. 56.1% of the full-time students and 51.2% of the part-time students declared that they would probably do the same. 19.5% of the full-time students and 14.6% of the part-time students would probably not make the same career choice a second time, whereas 3.7% of the part-time students would definitely not make the same decision. Majority of the respondents (92.7% of the part-time students and 87.8% of the full-time students) stated that their choice of career had nothing to do with family traditions.

When asked to indicate the main motive influencing their career choice the respondents stated that it was due to, in the case of full-time students: constant demand for nurses on the job market (46.3%), vocation (29.3%), because they had not been accepted to a different programme (12.2%), the change in the educational system (nursing programme offered at an academic level) (3.7%), family tradition (2.4%) and 6.1% of the respondents stated that their motive had been different (interesting career). Amongst the part-time students the stated motives were: vocation (50%), constant demand for nurses on the job market (24.4%), different motive (12.2%), the change in the educational system (nursing programme offered at an academic level) (7.3%), because they had not been accepted to a different programme (3.6%), family tradition (2.4%). Fig. 1.



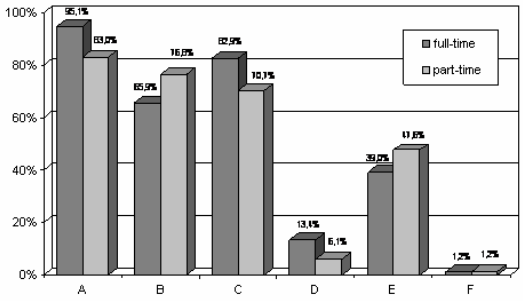
A – constant need for nurses
B – change in the higher education system
C – vocation
D – I was not admitted to other study programme
E – family tradition
F – other

Fig. 1. *The main motive for the choice of profession*

Self-esteem was the main motive behind the choice to start second-cycle studies for 57.4% (part-time students) and 53.7% (full-time students), demand for employees with a higher education – 19.5% (full-time students) – 2.4% (part-time students), testing their abilities – 17.1% (part-time students) and – 11% (full-time students), chances for job promotion – 13.4% (part-time students) and – 7.3% (full-time students), intense competition in the job market – 7.3% (part-time students) and 2.4% (full-time students), maintaining their current job – 3.7% (part-time students), maintaining the position of manager – 1.2% (part-time students), desire to upgrade their qualifications – 2.4% (part-time students) and 1,2% (full-time students).

When asked whether in their opinion "the change in the educational system (nursing programme offered at an academic level) influences the rise in professional prestige?" the respondents replied as follows: 45.5% (part-time students) and 40.2% (full-time students)- "yes, definitely", 47.6% (full-time students) and 41.5% (part-time students)- "probably yes", 13.4% (part-time students) and 12.2% (full-time students) – "probably not". According to the respondents, during the recent years, the social status of the nursing profession has increased: 20.7% (part-time students) and 6.1% (full-time students) stated "yes, definitely", 52.5% (full-time students) and 40.3% (part-time students) stated "probably yes". 40.2% (full-time students) and 36.6% (part-time students) stated that this was probably not the case and 2.4% (part-time students) and 1.2% (full-time students) said that this was definitely not the case. Nearly all nursing students count on an increase in prestige of their chosen professional career (97.6 % of the part-time students and 96.3% of the full-time students).

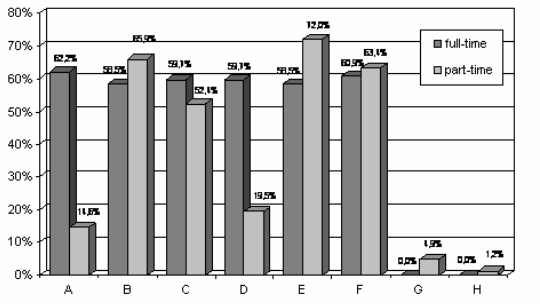
The hopes/expectations, in terms of career, expressed by nursing students pursuing second-cycle studies are: pay-rise (95.1% of the full-time students and 83% of the part-time students), an increase in the status of the profession (82.9% of the full-time students and 70.7% of the part-time students), responsibilities differentiated according to the level of education (76.8% of the part-time students and 65.9% of the full-time students), greater employment opportunities (47.6% of the part-time students and 39% of the full-time students), the prospect of working abroad (13.4% of the part-time students and 6.1% of the full-time students), no expectations (1.2% of the full-time students and 1.2% of the part-time students). Fig. 2.



A – increased salaries
B – different scope of competence – related to the level of education
C – increase of the profession's prestige
D – prospect of working abroad
E – better opportunities for employment
F – no expectations

Fig.2. *Expectations/hopes related to the chosen profession*

The respondents were also asked about their fears concerning the chosen profession. The full-time nursing students fear the following : job loss (62.2%), professional burn-out (59.7%), mobbing (59.7%), low earnings / low retirement fund (58.5%), high level of responsibility connected with the profession (58.5%), whereas the part-time students pointed to: high level of responsibility connected with the profession (72%), low pay / low retirement fund (65.9%), high workload and the related risk of medical error (63,45%), professional burnout (52.4%), mobbing (19.5%), job loss (14.6%), others (1.2%), no fears (4.9%). Fig. 3.



A – job loss
B – low salary/low pension
C – professional burnout
D – mobbing
E – great professional responsibility
F – big workload and risk of making a medical mistake
G – no concerns
H – other

Fig. 3. *Concerns about the nursing profession*

Some of the respondents admitted having thought about a change of career (34.1% / full-time students and 19.5% / part time students), but few made any attempts to do so. A vast number of nursing students

believe that it is easy for a nurse to change profession (41.5% of the part-time students and 39% of the full-time students).

Over a half of the respondents stated that they sometimes felt confused and had problems with the incoming information connected with ongoing changes (64.6% /full-time students and 52.4% / part-time students), the remaining individuals have no problem with this. Just over a half of the students have a sense of self-confidence (58.5% / full-time students and 54.9% / part-time students). Over 90% of both full-time and part-time students consider themselves to be creative. More than 80% of the respondents declared that they were able to cope with stress (86.6% of the part-time students and 81.7% of the full-time students). Vast majority of the respondents (86.6% of the part-time students and 81.7% of the full-time students) are willing and open to innovations appearing in today's rapidly changing world.

In the open ended question "What actions should be, in your opinion, taken to improve the image of the profession?" - the most frequently pointed out were: pay rise , an increase in the autonomy of the profession, differentiated responsibilities- according to the level of education, appropriate selection of the university candidates, responsible attitude of nurses (mutual respect between nurses), more respect from doctors, a change in the way nurses are presented in the media (showing nurses as educated, competent and responsible individuals), eliminating stereotypes e.g. "sister" etc.

DISCUSSION AND ANALYSIS OF THE RESULTS

An optimistic fact is that the vast majority of nurses continuing postgraduate courses, are people satisfied with the chosen profession. The fact that most of the respondents (about 80%) would make the same career choice once again indicates that these people's choice of profession was accurate. Czekirda et al from the Lublin District drew similar conclusions from their studies on the sense of professional satisfaction in nurses [5]. According to their research 74% of nurses stated that they were happy with their career choice and 21.9% were not satisfied with their choice [5]. A vast majority of nursing students seem to share the opinion that an improvement in the economic status is needed to increase the satisfaction of this work group. This is confirmed by research carried out by Zajkowski and Martinovich [6] conducted among nurses working in the primary health care centres in Poland and the United States. Polish nurses expect rise in earnings as an important determinant of professional satisfaction (62.2%), in turn – nurses in the U.S.A., when better paid, achieve a higher degree of professional satisfaction. In addition, they point to other important elements of professional satisfaction, such as, among others, the ability to communicate with the staff, especially doctors, the ability to upgrade their qualifications [6]. In studies by Czekirdy et al [5], nurses also indicate a lack of satisfaction with the monthly income and the deterioration of their financial situation. Low pay may be a serious source of stress in the workplace of nurses. Studies by Klimak et al [7] show that 100% of surveyed nurses point to this aspect.

The degree of professional satisfaction – according to Lipinska-Grobelny and Głowacka [8] depends on how "fit" an individual is for the job. If one is "fit" for the job there is a higher level of emotional and cognitive satisfaction. According to these authors [8], individuals who are very well "fit" for the job are, in a higher degree, characterized by a high level of satisfaction with work components such as: colleagues, managers, work content and working conditions, organization, development opportunity, salary - compared with those who fit the profession at a low and medium level.

It is noted that social changes (threat of unemployment) seem to influence the motives for studying nursing. A vast majority of full-time nursing students are pragmatic young individuals with no work experience. As the main motive behind their choice of occupation they pointed out, a constant need for nurses on the job market , they also seemed concerned about job losses in the future. The main motive of part-time students influencing their choice of occupation was vocation. In studies carried out by Czekirdy et al [5], the main motive behind the choice of the occupation was the desire to help people (51%). In turn, the research by Barczykowski et al [9] indicated that the main motive for studying nursing was the desire to increase professional qualifications (100%) and to obtain higher education (around 60%). Other nationwide studies conducted on a large research group (n = 1001) showed that the motives behind the choice of undergraduate nursing studies was the compatibility of interests with the chosen field of study, personality factors and value systems in life [10].

Intense competition on the job market results in other important risks, such as mobbing. Interestingly,

part-time nursing student ,mostly with extensive work experience, almost completely do not feel threatened by job loss and are less afraid of mobbing.

The occupational group of nurses is particularly vulnerable to the emergence of professional burnout. According to Maslach [11], the phenomenon of burnout threatens especially those who deal with helping others, dedicate themselves to others (such as doctors, nurses, teachers). Some reports [12] suggest that age, work experience and educational level have no significant effect on the level of burnout. In these cases the ability to relax outside the workplace and the use of active forms of recreation are desirable.

In the self-constructed questionnaire the following open ended question was asked: 'What action should be taken to improve the image of the profession?'- the surveyed nurses stated that they expected a better doctor-nurse relationship, mainly greater respect. Contemporary well-educated nurses want to be treated like equal partners in the therapeutic team and expect more autonomy in their profession.

An overwhelming majority of respondents expect an increase in the status and prestige of their profession. According to Sadurski et al [13], throughout the years 1958-1999 the prestige of the nursing profession in Poland increased from the 13th place (1958) in the ranking list (the criterion of prestige) to the 7th place (1999). Due to the honesty and integrity of the professionals, it is ranked at a very high position (second) in the hierarchy of professions (1998,2000,2006)[13]. According to the latest survey by CBOS (Public Opinion Research Center) from 2009, nurses – due to deference - hold a high 4th place on average when assessed in the hierarchy of 33 professions [14]. Coming before nurses, situated in the leading positions invariably compared with the last survey in 1999 are, university professors, firefighters and miners. In turn, appearing after nurses in this survey are; doctors, engineers working in factories, teachers, etc. A comparison of the average occupational prestige ratings for nurses from nationwide studies from 1999 (65) with those from 2008 (75) leads to the optimistic conclusion that the importance of this profession in the public opinion has considerably strengthened and this, in turn, has had a beneficial effect on improving the image of the profession. Results of these studies relate to the "current issues and events" project, executed in November 2008, comprised of a representative sample of 1050 adult Polish citizens [14]. These figures demonstrate the high status of the nursing profession in public awareness, mainly, according to the CBOS report of 2009 [14] , due to the high social utility of the profession. This profession is very useful, but difficult, highly responsible, demanding sacrifice in helping the sick / suffering, often linked with burdensome physical conditions (e.g. night work, etc.) and, unfortunately, still low-paid.

Nearly ¾ of the surveyed part-time students fear large professional responsibility, to a lesser extent, these concerns are manifested in the respondents studying full-time, which could confirm their solid professional background. Well prepared individuals probably show less concern. A large number of nurses felt undervalued in the work environment [7]. In the own study every second respondent stated that the motive for taking up second-cycle studies was to increase their self-confidence.

Andragogy which educates, directs, supports; preparing an individual for a life in the fast-changing reality, comes to help a professional nurse. Its task is to constantly influence the consciousness of contemporary people, preparing them for many challenges of modern times, helping to function in a world where there is a chance for development but also a number of risks (job loss, stress, mobbing, professional burnout, aggression) [15]. The task of teachers - educators is to shape creative and responsible individuals, who are in demand in contemporary times.

CONCLUSIONS

A vast majority of student nurses display satisfaction with the chosen profession. The main motive behind the choice of the profession was the prospect of permanent demand for nurses on the job market for the full-time students while , for the part-time students - vocation. The greatest expectations concern the rise in earnings . Full-time students fear losing their jobs (62.2%) and mobbing (59.7%) at a larger extent than part-time students (respectively 14.6% and 19.5%). Every second respondent sees a threat in the form of burnout.

REFERENCES

1. Wrońska I.: The role of socio-professional nurses. Study of modern nursing. Center of Medical Education, Warsaw, 1997.

2. Wrońska I., Mariański J.: The value of life of young people (nursing schools). Medical and Neurocentrum, Lublin 1999.
3. Olechnicki k., Załęcki P. Sociological dictionary. Publisher Graffiti BC, Torun, 1998.
4. Marshall, G. (ed.). Dictionary of sociology and social sciences. Educational Publishings OWN, Warsaw, 2004, p. 255, 256
5. Czekirda M. Pabis M. Jarosz, MJ: A sense of job satisfaction of nurses from the Lubelskie district. Nursing twenty-first century 2008, 2-3, (23-24), 10-15.
6. Zajkowska E., Martinovich, L.: Elements of professional satisfaction of primary health care nurses in Poland and the United States. Public Health 2005, 115 (3), 274-278.
7. Kimak K. Kimak K. Skorek.: Stress in the work environment of professional nurses. Public Health 2000, 110 (12): 427-431.
8. Lipinska-Grobelny, A., Glowacka K.: satisfaction with work and the relevance of the profession. Psychological Review of 2009.52, 2, 181-194.
9. Barczykowska E. Brick, B., Gierszewska M et al: Motives for studying nursing and the level of satisfaction with obtaining the degree of bachelor of nursing. In: Bartuzi Z. (ed.): Interdisciplinary dimensions of health sciences. CM UMK, Bydgoszcz 2007, 27-33.
10. Kądalska E. Fronczyk K.: Motives influencing the choice to undertake undergraduate nursing studies in Poland. Nursing XXI century, 2006, 1 / 2, (14 / 15), 111-114.
11. Maslach C. Burnout in multi-dimensional. In: Sek H. (ed.): career burnout. Causes and prevention. Educational Publishings OWN, Warsaw 2004, 13-32.
12. Cegła B., Dowbór-Dzwonka A., Filanowicz M et al: Prevalence of burnout in the team of professionally active nurses. In: Bartuzi Z. (ed.): Interdisciplinary dimensions of health sciences. CM UMK, Bydgoszcz 2007, 55-61.
13. Sadurska A et al: The prestige of the profession of nurses in Poland. Nursing XXI century, 2006, 4 (17), 53-55.
14. Current issues and events. Communication from the CBOS research. BS/8/2009. Warsaw, January 2009 (www.cbos.pl 23.11.2009).
15. Wujek T. (ed.): An Introduction to andragogy. Center for Vocational Education and Training of Personnel. Institute of Sustainable Technologies. Warsaw 1996.

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4. Praca powinna być napisana jednostronnie w programie Word (na jednej stronie może być do 32 wierszy, tj. 1800 znaków, margines z lewej strony – 4 cm), czcionką 12 pkt., interlinia – 1,5.
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8. Prace oryginalne powinny mieć następujący układ: streszczenie w języku polskim i angielskim, słowa kluczowe w j. polskim i angielskim, wstęp, materiał i metody, wyniki, dyskusja, wnioski, piśmiennictwo.
9. Tabele i ryciny należy ograniczyć do niezbędnego minimum. Tabele numerujemy cyframi rzymskimi. Tytuł tabeli w jęz. polskim i angielskim umieszczamy nad tabelą. Opisy wewnątrz tabeli zamieszczamy w języku polskim i angielskim.
10. Ryciny (fotografie, rysunki, wykresy itp.) numerujemy cyframi arabskimi. Tytuł ryciny w jęz. polskim i angielskim umieszczamy pod ryciną. Opisy wewnątrz rycin zamieszczamy w języku polskim i angielskim.
11. Odnośniki do piśmiennictwa zaznaczamy w tekście cyframi arabskimi i umieszczamy w nawiasie kwadratowym.
12. Streszczenie powinno mieć charakter strukturalny, tzn. zachować podział na części, jak tekst główny. Objętość streszczenia zarówno w języku polskim jak i angielskim – ok. 250 wyrazów.
13. Autor dostarcza pracę na dyskietce oraz 3 egzemplarze, w tym 1 kompletny, zgodny z dyskietką, zawierający nazwiska autorów i nazwę instytucji, z której praca pochodzi (patrz pkt. 5 i 9) oraz 2 egz. przeznaczone dla recenzentów bez nazwisk autorów, nazwy instytucji i innych danych umożliwiających identyfikację.
14. Na dyskietce w odrębnych plikach powinny być umieszczone:
   a) tekst pracy,
   b) tabele,
   c) ryciny (fotografie w formacie BMP, TIF, JPG lub PCX; ryciny w formacie WMF, EPS lub CGM),
   d) podpisy pod ryciny i tabele w formacie MS Word lub RTF.
15. Fotografie powinny mieć postać kontrastowych zdjęć czarno-białych na błyszczącym (ewentualnie matowym) papierze. Na odwrocie należy podać imię i nazwisko autora, tytuł pracy, numer oraz oznaczyć górę i dół.
16. Należy zaznaczyć w tekście miejsca, w których mają być zamieszczone ryciny. Wielkość ryciny: podstawa nie powinna przekraczać 120 mm (z opisami).
17. Piśmiennictwo – tylko prace cytowane w tekście (maksymalnie 30 pozycji) – powinno być ponumerowane i ułożone wg kolejności cytowania, każdy tytuł od nowego wiersza. Pozycja piśmiennictwa dotycząca czasopisma musi zawierać kolejno: nazwisko, inicjał imienia autora (ów) – maksymalnie trzech – tytuł pracy, tytuł czasopisma wg skrótów stosowanych w „Index Medicus", rok, numer tomu i stron. Przy cytowaniu pozycji książkowej (monografii, podręczników) należy podać nazwisko i inicjały imion autorów, tytuł dzieła, wydawcę, miejsce i rok wydania.

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