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CUPRINS

PROF. DR. FRANCISC SCHNEIDER MD, PHD, DHC AT THE AGE OF 80: PERFORMANCE, VALUE, EXCELLENCE

On the 80th birthday of distinguished Prof. Dr. Francisc Schneider MD, PhD, DHC we would like to pay tribute and to summarize the remarkable didactic and scientific accomplishments of this great Romanian physiologist, as a sign of appreciation and respect to creation and excellence.

He was born on July 27, 1933 in Timisoara, where he attended the primary and secondary school (1940-1951) and later the University of Medicine (1951-1957), while between 1957 and 1960 he was a country doctor in Certeze-Negreşti Oaş, a place where he later became a commune *Honorary Citizen*. Between 1960 and 1998 he was a faculty member and a researcher at the Physiology discipline of the Faculty of Medicine within the University of Medicine and Pharmacy "Victor Babeş" based in Timişoara, going through all the university stages, by passing all exams, from preparatory, assistant, head of works, conferentiary to professor, head of department, PhD coordinator and research contract manager. As MD and senior physician, he was head of the Clinical Physiology and Functional Exploration Laboratory within the Timişoara County Hospital (1972-1990) and Manager of the Timişoara Institute of Hygiene (1996-1998). He is founding editor-in-chief of the *Fiziologia-Physiology* magazine, the *Official Journal of the Romanian Society for Physiological Sciences* as of 1991 and until the present day. It is virtually the only Romanian physiology review, which remained viable after 1900, professional and dynamic, also present in the IDB - International Data Bases.

A close collaborator of the founding-rector Prof. Dr. Aurel Ardelean, he wisely and competently supported the development of the Arad medical university education, by supporting the establishment of the Faculty of Medicine from "Vasile Goldiş" Western University (VGWU) based in Arad. Starting with 1991 he organized and coordinated as university professor the Arad physiology education and scientific research, editing, alongside his collaborators, physiology courses of a high academic standard, coordinating PhD theses and leading in his capacity of manager the activity of the Centre for Research in Applied Physiology of VGWU Arad. Actively anchored in the Arad university, academic and cultural life, he was pro-rector of VGWU Arad (1999-2002), he continued the cooperation with the University of Medicine and Pharmacy "Victor Babeş" based in Timişoara, in particular the Physiology Department (collaborators and students), he promoted and contributed to projects, contracts, congresses and regional collaborations - DKMT (Danube-Criş-Mureş-Tisa) Euro-region, which includes the western part of Romania (the Timiş, Arad, Caraş-Severin and Hunedoara districts), the Bács-Kiskun, Csongrád, Jász-Nagykun-Szolnok and Békés counties in Hungary and the autonomous Vojvodina province in Serbia.

He is a titular member of the *Academy of Medical Sciences*, a titular member of the *European Academy of Sciences and Arts* - Salzburg, Austria, a member of the *Physiological Society* - London, England and an honorary member of the *Hungarian Society of Physiological Sciences*. Some of his distinctions include: the "Ratz Samuel" Medal of the *Hungarian Society of Physiological Sciences*, *Academia Brasiliera Medal* "Em tributo pe la luta no integracao dos noscos dois paises Brasil e Romania", *Doctor Honoris Causa* of the University of Medicine and Pharmacy "Iuliu Haţieganu" Cluj-Napoca, the *CNCSIS Honorary Diploma* – the National Council of Scientific Research 2003, the *Chrystal Trophy at the Medical Life Gala* 2010, *the Silver Medal "*20 years of institutional history" of UVVG Arad (2010) and Professor Emeritus (2011).

He has a vast and valuable editorial activity, increasing by the year:

- 207 scientific papers published in the country and abroad, 22 of which are ISI-Thomson-Reuters ranked;
- 21 published didactic books on physiology (general, per apparatus and system, fundamental and clinical);
- 9 scientific monographs published alone or in collaboration, of which we mention: *Broncho-motricity* (1998), in collaboration with A. Petroiu and G. Mihalaş, awarded with the "Daniel Danielopolu" Prize of the Romanian Academy; *Modern mathematics in contemporary medicine* (2001), in collaboration with M. A. Roz, G. I. Mihalaş, D. Renti; *Introduction into Clinical Physiology* (2002); *Clinical Physiology of the Venous System* (2003), in collaboration with I. R. Şişka and J. A. Avram, Kluwer Acad. Publ., Boston, MA, awarded with the "Daniel Danielopolu" Prize of the Romanian Academy; *Diet Biology and the Physiological Nutrition* (2008), in collaboration with A. Ardelean and L. Moş;
- 8 literary books (essays, evocations, history of physiology): *Medical and Paramedical Essays* (1998); *Arad Breaths* (2003); *Memories from the Country Above* (2004); *With the South-West Wind* (2006); *Knowingly* (2009); *Childhood under Three Reigns* (2009); *The „Goldiş" University Members* (2010); *Physiologists* (2011).

With a simultaneously analytic and systemic, complex and global, open, receptive thinking, in keeping with the novelties and current scientific transformations, he introduced and promoted highly important concepts in physiological sciences and bio-medicine, cardinal for the future of human knowledge:

- *the inseparable connection between fundamental-preclinical physiology and clinical physiology,* the connection being substantiated by bio-mathematical laws, biophysical and biochemical processes (together with Prof. Dr. Gheorghe I. Mihalaş and Prof. Dr. Georgeta Mihalaş) and the mechanisms of molecular biology (together with Prof. Dr. Aurel Ardelean and Conf. Dr. Daniela Motoc);
- *the necessary and obligatory connection between normal and pathological physiology (physiopathology, pathophysiology),* by identifying the normal connection bridges *(adaptology:* a concept introduced with I. Hăulică and later developed with Petru Derevenco, Dan Riga and Sorin Riga) and pathological ones *(stressology, maladaptation:* concepts further developed through collaborations with Petru Derevenco, Dan Riga and Sorin Riga);
- *dynamic concepts of physiom and physiomics* (introduced in Romania at the same time with I. Hăulică and later developed through Bucharest collaborations with Sorin Riga and Dan Riga) and the numerous pleas for *functional genomics*.

The force of this prodigious scientific activity is strengthened by the respect of Prof. Dr. Francisc Schneider for the forefathers and the cult of the masters – brilliant figures of Romanian physiology, alongside the cult of truth, a must for a valuable researcher *(The master is the one who shows us what is possible in the other of impossible* - Paul Valery; *And then, despite the flaws and mistakes, scientists have more or less the same soul, they all profess the cult of truth by itself, for them science is a religion* - Charles Richet).

Prof. habil. Dr. Dan RIGA, MD, PhD, DHC
Prof. habil. Dr. Dan RIGA, MD, PhD, DHC
Titular members of the Academy of Romanian Scientists

REVIEW

HETEROGENEITY AMONG CANCER CELLS: SO FAR, MANY DECOYS, FEW VALIDITIES

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ABSTRACT

The model of clonal evolution and the premise that the majority of tumor cells are capable to propagate and cause the tumor growth were the cornerstone of antineoplastic treatments: destroying cancerous cells. This approach has been elicited by the cancer stem cell (CSC) hypothesis. According to this, a rare population of tumor cells own stem cells characteristics, are involved in growth, resistance and recurrence of the tumor. They also are not affected by traditional therapies on the basis of their long-term growth; thereby it is essential to establish a selective targeting therapy of these cells. As captivating this assumption sounds, as laborious it is to testify it. Variances of xenotransplantation data, the lack of specificity of CSC markers, the molecular signaling pathways which underlie the CSC behaviour are still ambiguous issues that have to be elucidated.

Key words: cancer stem cells, heterogeneity, signaling pathways

INTRODUCTION. HYPOTHESES...

The cellular background (the chemical modifications of chromatin, or the epigenetic state) in which a transforming genetic lesion occurs (cell of origin) may contribute to the complexity of cancer. The potential importance of the cell of origin and the transformability of multiple cell types raises questions about hierarchical relationships between cells and the properties of tumor-initiating cancer stem cells (thought to be cells within a tumor that can self-renew and give rise to heterogeneous populations of cancer cells that constitute the tumor).

By tradition, cancer has been considered as a disease in which environmental or endogenous occurrences induce mutations to critical oncogenes and tumor suppressor genes within a normal cell. The clinical manifestations of cancer appear when these mutations result in the cell's tranformation to a more primitive, highly proliferative state from which the leukemia or solid tumor develops by clonal expansion (1). This standard model of clonal (stochastic) evolution asserts that tumours develop as a consequence of accumulated mutations and selection of clones over a period of time, resulting in a heterogeneous group of malignant cell in which cells of the dominant population (selected and expanded because of their growth benefit) have more or less equal ability for tumour regeneration and repopulation (2). Even so, this pattern does not slightly explain the long developmental latency of many cancers and metastases, the mechanism by which the initiating events produce cellular dedifferentiation and cellular immortality, or the genesis of the functional and phenotypic diversity of the cells within the tumor itself.

Recently, developing proofs promote the idea of originating of the tumor from the transformation of tissue stem cells by mutations that cause a dysregulation of the normal mechanisms that control stem cell growth and proliferation (3,4,5).

Each organ possess a small niche populated with adult stem cells. Some of their characteristics are eloquent: these cells are long-lived and multipotent; they remain quiescent within the niche environment for long periods of time; the asymmetric cell division gives rise to one stem cell, termed self-renewal, and one differentiated progeny; they are resistant to apoptosis; they have enhanced telomerase and DNA repair activities and also possess membrane-bound ABC transporters that leave out xenobiotics (this fact confers cell resistance to the pharmacological agents effect) (4,6,7). These abilities of adult stem cells could unveil them to harmful factors in order to accumulate mutations that may arise in malignant transformation (8). Cancer stem cells are displaying mostly the same cell surface markers as their normal equivalents, but show uncontrolled proliferation, possibly due to a alteration of responsiveness to negative growth regulators (6,9,10). These statements emerged to the cancer stem cells (hierarchy model) hypothesis, which is not a new idea, especially regarding myeloid leukemia and some germ lineage neoplasm (12,13).

The CSC theory has been released many years ago in patients with chronic myeloid leukemia in which clonal hematopoiesis including both the myeloid and erythroid lineages was described (14). First malignant cells capable of reprising the disease in NOD/SCID mouse were isolated from some acute myeloid leukemia cases. These cells also possessed hematopoietic stem cells phenotype and self-renewal potential (15).

The debate between the obscure aspects of these hypotheses is more enhanced than ever. Nevertheless, for the interest of a good understanding and approach, it is more than favorable to look inside the issues from all points of view and to not exclude any assumption. One single argument supports this: it seems

Received 15th of April 2013. Accepted 20th of May 2013. Adress for correspondence: Laura Cernat, MD PhD student, "Victor Babes" University of Medicine and Pharmacy Timisoara, Eftimie Murgu Square No. 2A, RO-3000041, Timisoara, phone/fax: +40256220479; e-mail: mlaura_73@yahoo.com

that cancers that follow the stem cell model are also subject to clonal evolution; during progression, various burdens could result in the emergence of new clones through genetic and epigenetic alteration inside the cancer stem cells population (16,17). There are evidences for functional diversity within the stem cell population of the tumor (for example, a subset of CSC population which expresses the multidrug resistance transporters ABCB1 and ABCG2) (18,19). The existence of a different clone of CSC may compromise the targeted therapy against the CSC population.

Intertumor, but primarily intratumor heterogeneity - a critical and strategic reality?

Differences in driver mutations and cell-of-origin among patients raise the question of whether similar hierarchies of tumorigenic and nontumorigenic cells, with similar markers, are conserved among patients with similar cancers. This question addresses both to leukemic disease (AML) and to solid cancers. Several studies ((20) (21)) revealed inconsistent data regarding links between leukemic activity and cells isolated depending on presence/absence of some surface markers (patients with AML, comparison of CD34+CD38-, CD34+CD38+, CD34-CD38+, CD34-CD38- leukemia-initiating cells: the results showed that leukemogenic activity is not usually restricted to the CD34+CD38- fraction and there is heterogeneity among patients in leukemogenic cell phenotype). Same story for solid tumors. Al-Hajj et al. demonstrated that CD44+CD24-/low markers could not delineate tumorigenic from non tumorigenic breast cancer cells (22).

The conclusion is indisputable: it is mandatory to evaluate the heterogeneity among patients by testing CSC markers in a significant number of patients, in order to validate the assessment at clinical level.

Last years, regarding the malignancies study, many experimental bodies of evidence promoted the idea of an hierarchical structure of heterogeneous tumor cells. The intratumoral heterogeneity is due to the presence of inflammatory cells, cancer-associated fibroblasts, the distance of tumour cells from the vasculature (23). But it seems that this is not all. In this population lies a small number of cancer stem cells (CSC). This small population expresses some particular surface markers, has higher capacity to develop tumour an *in vivo* xenograft and forms spheres in non-adherent culture plates (24). Certain cancers (including several germ cell cancers, breast, brain and colon cancers and some leukemias) have been recognized for decades to include neoplastic cells that differentiate into post-mitotic derivatives. At the same time, recent studies showed that a small population of cancer cells shifts the neoplastic diseases after the transplantation into immunocompromised mice; these cells exhibit markers which are not present on neoplastic cells from bulk cell population (22,25,26).

Thus, these ideas boosted the cancer stem cells hypothesis based on hierarchically structure of tumour in quite same manner as normal tissues. As normal stem cells differentiate into phenotypically diverse progeny with limited proliferative potential, it is argued that cancer stem cells also undergo epigenetic changes analogous to the differentiation of normal cells, forming phenotypically diverse nontumorigenic cancer cells that compose the bulk of tumoral cells (27). Furthermore, there is a promoted idea according to which CSC generate cell progeny hierarchically organized into transit amplifying (TA) and terminally differentiated (TD) cells (16) (Figure 1).

TA cells possess more rapid though limited proliferation, low self-renewal and restricted differentiation and increase the number of differentiated cells produced by one CSC division. Although both CSC and TA cells divide and produce the same outputs - a spectrum of differentiating progeny - they differ in their ability to proliferate and maintain an undifferentiated state for an extended period of time (28).

According to some authors, TD cells are representing the terminal differentiation and are accompanied with loss of both proliferation and tumorigenicity; there are findings that suggest the possibility of using the nuclear reprogramming as a therapeutic solution in neoplasms (29,30).

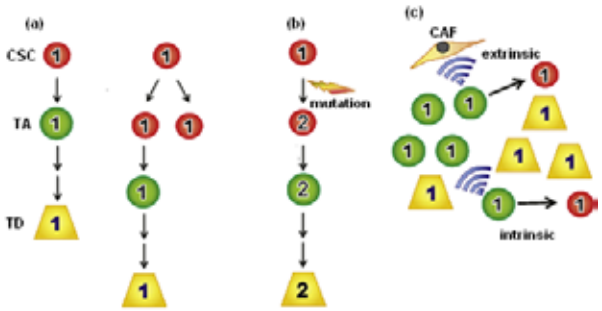

Fig.1. [adapted from (16)]. (a) CSC undergo self-renewal as asymmetric division, resulting in cells with stem properties and transit amplifying cell (TA); TA cells undergo terminal differentiation (TD) after a number of cell divisions; the symmetrical division of CSC leading to daughters CSC; (b) following a mutation, CSC may develop a clonal population with different genetic properties; (c) TA cells and TD cells could relapse to stem cell condition beneath of influence of signaling from tumor stromal cells (including CAFs)

Questions and controversies are more than present. Skeptic authors question about the xenograft models as gold-standard assays for CSC. Injecting human CSCs into immunocompromised mice exclude the existing of stem cells niche and gives rise to questioning whether these cells are initiating tumor or metastasis. Attempting to study a certain population of cancer stem cells *in vitro* does not recapitulate the microenvironment and may negatively affect observation outcomes. Moreover, cancer stem cell phenotype may be a context-specific event, showing up only in certain patient samples at certain ages (17).

Nevertheless and although controversial, there are some correlations that have to be considered; among them, the influence of microenvironmental factors on cancer cells looks relevant. The presence of multiple surface molecules (IL-1, IL-6, CD44, CD24, TNFα, IL-4, IL-10 etc.) which promote strong interactions of CSC with tumor microenvironment contributes to CSC growing, withstanding chemotherapy and evading immune vigilance (31,32). Furthermore, it is shown on some cancer cell

lines that several microenviromental factors contribute to modulation of stemness of cancer cells (33) (Table I).

Table I. Microenviromental modulating factors of cancer cells stemness [adapted from (33)]

Factor	Cancer cell line	Effect of stemness
Extracellular ATP	Gliomas	↓
High energy metabolites (lactate, ketones)	MCF-7 (breast)	↑
Hypoxia	Prostate, brain, breast, cervix, lung, colon, liver, kidney	↑
Hepatocyte growth factor	Colon	↑
VEGF	Skin,gliomas	↑
Nitric oxide	Gliomas	↑
Retinoic acid	Gliomas	↓
ROS	Breast	↑
Conditioned medium	DU145 (prostate)	↑ or ↓ (depending on the media)

However, in terms of clinical issues, the presence of CSCs responsible for therapy failure is the foundation of devising CSC targeted therapy; in addition, tumor microenvironment targeted therapy will notably improve clinical outcomes.

EMT and cell signaling pathways: keystones in embryonic development & concealed instruments in tumorigenesis and cancer cells heterogeneity

It is already shown that, due to their plasticity, cancer cells are able to modify their phenotype and to acquire new capabilities. The tumor microenvironment has its crucial role in this occurrence.

The epithelial-mesenchymal transition (EMT) is a critical cellular process that entitles polarized, and immobile epithelial cells to shift to motile mesenchymal cells. During early stages of embryogenesis mesenchymal cells arise from the primitive epithelium. The cellular phenotype undergo major changes (34): morphological changes from a cobblestone-like monolayer of epithelial cells with an apical-basal polarity to dispersed, spindle-shaped mesenchymal cells with migratory protrusions; changes of differentiation markers from cell-cell junction proteins and cytokeratin intermediate filaments to vimentin filaments and fibronectin, some integrins; conversion of stationary cells to motile cells that can invade through extracellular matrix. EMT could be induced under pathological conditions in adult tissues, including the tumor invasion and metastasis.

The signaling pathways involved in EMT during embryogenesis are present and effective also in tumor evolution (Figure 2).

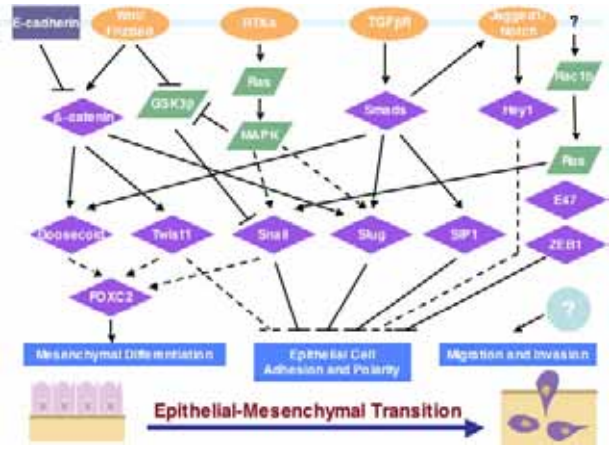

Fig.2. The molecular signaling pathways involved in EMT (34)

Some authors state that, after undergoing EMT, cancer cells gain CSC markers, highly self-renewal capacity, augmented tumor formation (11). Due to its importance in tumor progression, the factors and signals that regulate and control EMT have to be explored and understood. Thereby, hypoxia and TGF-β (provided by tumor microenvironment) are found to promote undergoing to CSC phenotype, the upregulating of stem cell factors (OCT4, NANOG), increase of self-renewal capability (11,36,37).

Hedgehog, Notch, Wnt, and PTEN are known pathways that control the self-renewal, proliferation, and survival of both normal and cancer stem cells. Mutations leading to the constitutive activation of one or more of these pathways are observed in most aggressive cancers.

The Hh (Hedgehog) signaling pathway is a crucial mediator of normal tissue development. Aberrant activation of the Hh pathway in cancer is caused by mutations in the pathway (ligand-independent) or through Hh overexpression (ligand-dependent) (38,39). One xenograft model study showed that Hh pathway inhibition could result in tumor growth inhibition; these effects were mediated through the stromal microenvironment, consistent with a paracrine signaling mechanism (40). A quite narrow evidence showed a link between Hh signaling and CSCs, such as the polycomb gene Bmi-1, which may regulate stem cell self-renewal by control of Gli transcription factor (41).

There is an increasing interest in studying Notch activity and therapeutic targeting because of the implication of this signaling pathway in regulation of survival and proliferation both of "bulk" cancer stem and CSCs and also in angiogenic activation (42,43).

The Wnt pathway is involved in vertebrate limb development and regeneration; it promotes self-renewal, proliferation, and transient differentiation of normal stem cells, maintains the adult stem cell populations of tissues with rapid cellular turnover such as hair follicles, mammary gland, skin, and intestinal lining (44).

The Wnt/*beta*-catenin signaling pathway is complex and cross-talks with other signaling pathways. Regurarly, this pathway is constitutively activated in tumors arising in these adult stem cell niches (45). It is also known the contribution to the malignant transformation of cancer stem and progenitor cells from stomach, pancreas, liver, prostate, brain, and lung, some leukemias (multiple myeloma and chronic myelogenous

leukemia) (19,46,47).

Wnt include a family of 19 extracellular glycoproteins that bind to the Frizzled (Fz) family of receptors, consequently activating a pathway that inhibits the proteolytic degradation of *beta*-catenin (44). This allows the accumulation of cytoplasmic beta-catenin and its translocation to the nucleus where it promotes gene transcription through the Tcf/Lef transcription factors (56). In the absence of Wnt activation, the proteins Axin and adenomatous polyposis coli protein (APC) form a complex with glycogen synthase kinase 3 (Gsk3) that promotes the phosphorylation of cytoplasmic *beta*-catenin that, alternately, dirrects *beta*-catenin for proteolytic degradation.

Activation of the Wnt pathway requires the association of Fz with its coreceptor, LDL receptor-like protein 5/6 (Lrp5/6) (44).

Formation of the Wnt/Fz/Lrp5/6 complex activates the phosphoprotein Dishevelled (Dvl), an integral component of the Fz receptor that, in turn, recruits Axin/Gsk3, thus dissociating the Axin/APC/Gsk3 complex. Lrp5/6 is sequentially phosphoryled by Gsk3 and casein kinase 1γ (Ck1γ) (57). Phosphorylated Lrp5/6 binds to Axin/Gsk3, forming a complex that stabilizes *beta*-catenin by preventing its phosphorylation.

The Wnt signaling has been divided historically in to the canonical pathway (including Wnt1, Wnt3A and Wnt8) and the noncanonical pathway (including Wnt5A and Wnt11). The canonical pathway is associated with the downstream activation of *beta*-catenin and its subsequent accumulation in the cell nucleus.

In the absence of Wnt signaling, *beta*-catenin is rapidly degraded in the cytoplasm. Also, nuclear levels of *beta*-catenin are kept low by its interaction with APC and Axin, both of which are involved in transporting *beta*-catenin back to the cytoplasm.

The members of Wnt signaling behave as positive and negative factors, alike all oncogenic signaling pathways (Figure 3).

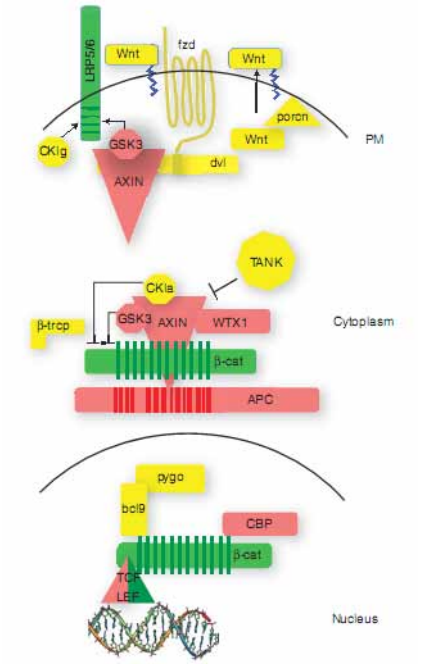

Fig.3. Tumor suppressors and oncogenes in the Wnt pathway (48); Oncogenes – green; Tumor supressors – red

APC (a tumor supressor) appears to be the most frequent mutated/lost function protein in colo-rectal tumors. Mutations in *beta*-catenin have been described for colon, endometrial and prostate tumors, endometrioid tumors of the ovary and hepatocellular carcinomas and are followed by increased *beta*-catenin stabilization and activity (49) (50). Other authors revealed that the upregulation of HER3, activation of HER2/HER3 signaling and enhancement of cell growth by androgen receptor activation was dependent on *beta*-catenin; thus Wnt signaling was proposed as a mechanism for the potentiation of EGF receptor tyrosine kinase family signaling in ER-/Her2+ breast cancers (51).

According to some authors, it seems that Wnt signaling is somehow connected with stem cells from tissue niche and also with CSCs. It is the case of intestinal crypt stem cells which, coming next after the deletion of APC gene, are capable to form benign adenoma (52). It is also shown the subordination of sustainment of cutaneous CSCs of the Wnt pathway (53). More appreciable evidences arise from hematopoetic cancer studies that confirmed the impact of *beta*-catenin run down without recognisable genetic lesions and the involving of Wnt signaling in CSCs maintenance (54).

A very intriguing article advanced the idea that Wnt signalling activity is a marker for colon CSCs and is regulated by the microenvironment; furthermore, same authors showed that bulk, differentiated and non-clonogenic cancer cells were reprogrammed to express CSC markers and tumorigenic ability after stimulation with myofibroblast-derived factors (55). Again, one more reason for approaching as an overall picture in which the complex regulation of CSCs phenotype and function consist of intrinsic factors (as Wnt signaling) but also of extrinsic influences (tumor microenvironment).

CONCLUSIONS. ARISING QUESTIONS.

Emerging evidence suggest that cancer stem cells (CSCs) are an essential target for effective cancer therapy because CSCs drive tumor growth, chemo-resistance, tumor relapse, and metastasis. Although many studies have focused on the genes and pathways involved in stemness, characterization of the factors in the tumor microenvironment and the intrinsic factors that regulate CSCs is lacking. Current methods for isolation and study of CSCs rely on cell surface markers found to be enriched in populations with stem cell-like properties. But controversial issues did not hesistate to come out. However, it is clearly that cancers contain a hierarchy of epigenetically distinct populations of tumorigenic and nontumorigenic cells. Using tests as sphere formation, CSCs models *in vitro*, xenotransplantation models are required to clarify the complicate depict about the frequency of tumorigenic cells, the degree of hierarchical organization and the dimension to which markers can distinguish tumorigenic from nontumorigenic cells; it is also necessary to expose the differences between cancers, and even between patients with the same cancer. At the same time, signalling pathways in CSCs will need to be thoroughly described, keeping in mind the signalling cross-talk. There are already emerging targeted therapies

against CSCs which act on cellular cycle or on pro-apoptotic steps from siganling pathways, monoclonal antibodies directed to some surface molecules, etc. The continuous developing of appliances and assays for CSC study will allow revealing of tumor-initiating events, environmental influences, nutrition aspects. Finally, let's not forget about the niche that is crucial in tumor growth, metastatic formation, protection of CSCs. All these lead to same goals: best efficiency, less toxicity, non-harming normal tissues, patients cancer-free.

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HETEROGENITATEA CELULELOR TUMORALE: PANA IN PREZENT, MULTE CAPCANE, PUTINE CERTITUDINI

REZUMAT

Obiectivul major al terapiei antineoplazice (distrugerea tumorii) se bazeaza modelul de dezvoltare clonala a celulelor tumorale ai pe capacitatea acestora de propagare si de crestere tumorala. Aceasta abordare este pusa la incercare de o ipoteza diferita: existenta celulelor stem tumorale; acestea se afla in numar redus in masa tumorala si sunt responsabile de dezvoltarea tumorii, rezistenta la terapie si de recaderi. Celulele stem tumorale nu sunt influentate de terapia conventionala datorita ritmului lent de crestere. Astfel sunt necesare variante terapeutice efective asupra acestor celule. Totusi, exista multe necunoscute in acest sens; lipsa unor markeri cu specificitate ridicata, insuficienta cunoastere a mecanismelor moleculare ce stau la baza comportamentului acestor cellule, rezultate variate ale experimentelor *in vivo* pe animale de laborator, sunt doar cateva dintre neclaritatile ce trebuie elucidate.
Cuvinte cheie: celule stem tumorale, heterogenitate, cai de semnalizare

THYMECTOMY FOR MYASTHENIA GRAVIS: SPECIAL FEATURES

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ABSTRACT

Thymectomy is part of the surgical treatment for myasthenia gravis. However, the role of oral medication cannot be neglected, and neither oral medication can be interrupted. From this point of view, a study of certain features in terms of thymectomy efficacy versus corticotherapy, the age, the disease onset, may be referred. With all of this thymectomy still remains an important link in the overall therapeutic arsenal of myasthenic ill.

Keywords: thymectomy, myasthenia, variables, conservative therapy, benefits

INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disorder of neuromuscular transmission with an impact in the biomechanical function of the striated muscle. The deficiency in acetylcholine receptors (AChR) plays an important role in the disease's pathogenesis.

A current treatment option includes:

- Symptomatic treatment (anticholinesterase agents)
- Chronic immunomodulating treatments (glucocorticoids and other immunosuppressants)
- Rapid immunomodulatory treatment (plasmapheresis, intravenous immunoglobulin)
- Surgical treatment (thymectomy)

The decision and the reasoning for thymectomy in patients with MG comprise some particularities, such as:

- The role played by the thymus in pathogenesis of myasthenia
- The thymic abnormalities detected in the most patients with MG with autoantibody against AChR:
- Hyperplasia (60-70 %), thymoma (10-15%)
- In plus a computed tomography and magnetic resonance imaging of the chest/mediastinum is as well a diagnostic tool.

The indication for thymectomy is suitable in all patients with thymoma, regardless of the status of MG (generalized, bulbar, ocular) (1-2).

Thymectomy in absence of thymoma is generally beneficial for those with generalized MG and AChR antibodies. There still remain other unanswered remarks regarding thymectomy.

SURGICAL APPROACH

The aim of thymectomy is to remove as safely possible the thymic tissue, mediastinal and cervical adipose tissue which may contain variable amounts of thymic tissue as well can be considered as a source of autoantibodies (3).

Four operator options approach are available:

- Trancervical thymectomy
- Minimmaly invasive thymectomy (videoscopic→ robotic assisted)
- Classical transteranl thymectomy
- Combined transcervical/transsternal thymectomy

This situation requires some warnings because the patients with myasthenia gravis have in general a higher risk for thymectomy when the respiratory function is compromised. However the pre/paraoperative mortality was significantly reduced by using the new thymectomy procedures and surgical protocols (4). In any case, there will always be controversies about which surgical technique is more effective in MG. The surgical technique used in all patients in this present study was unilateral thoracoscopic thymectomy with left or right approach, depending on the appearance of the thymus in computed tomography examination. After transferring the patient from the intensive care unit to the department of surgery, the neurological evaluation was performed in order to appreciate the myasthenic deficit and to resume the oral medication (5).

The postoperative criteria were:

- Missing of clinical complications
- The normal physical examination
- The absence of worsening of myasthenic deficit
- A correct respiratory function

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MATERIALS AND METHODS

The lot of patients which was taken into study was 87m/13f, between 17-51 years old. The lot of operated patients had 6m/44f, and the lot of unoperated patients had 7m/43f. The statistical methods used in analyzing the desired variables were determined by the proposed goals. As we already mentioned were studied non-thymectomized/thymectomized patients variables. With regards on the characterization from statistically point of view of the two groups of MG patients we used statistical indicators of central tendency (arithmetic average, the median, confidence interval for the mean), scattering indicators such as: standard deviation, dispersion, maximum value, minimum value, amplitude).

Currently selected variables were:

- Patient's particularities (age)
- Surgical intervention
- Follow-up of patients (complications of corticotherapy)

The results were expressed as: distribution of the patients according to their age; the type of the onset; corticotherapy complications in the thymectomized/non thymectomized patients.

RESULTS

1. Patient's age repartition

As we can see from the Table I and Figure 1, no statistically significant difference is present, which is confirmed by the match design criteria of the patient's lot repartition according with their age.

Table I. Patients' age distribution

No.	Age	Patients			
		Surgery		Non-surgery	
		No.	%	No.	%
1	>20	3	6.0%	6	12.0%
2	20 - 29	17	34.0%	22	44.0%
3	30 - 39	18	36.0%	12	24.0%
4	40 - 49	11	22.0%	8	16.0%
5	< 49	1	2.0%	2	4.0%
TOTAL		50	100%	50	100%

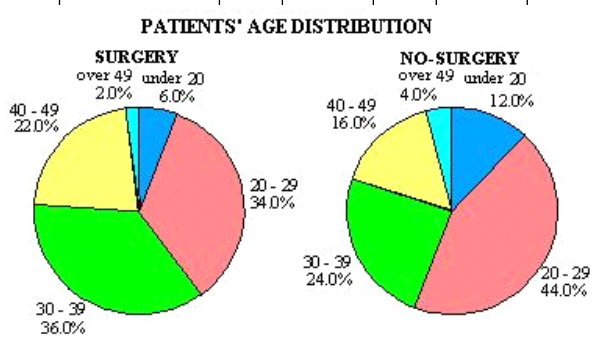

Fig.1. Patients' age distribution

2. Onset

Neither in the terms of the onset, there are no distribution differences between groups, as the Table II and Figure 2 reveals.

Table II. Patients' onset distribution

Onset * Crosstabulation surgery				
Count				
		Lot		
		MGNT	MGT	Total
Onset	Bulbar	9	14	23
	Cephalic	0	1	1
	Ocular	29	23	52
	Spinal	12	12	24
	Total	50	50	100

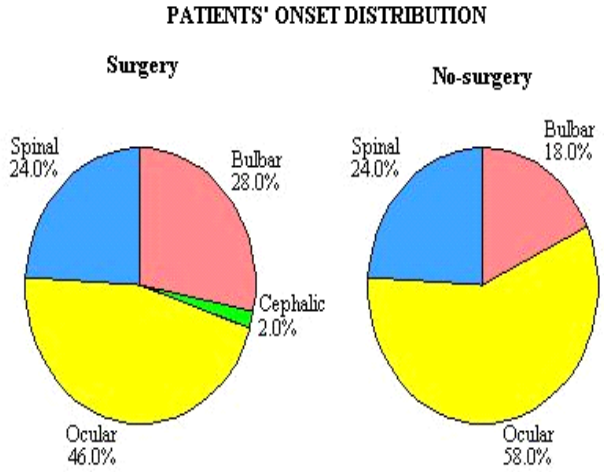

Fig. 2. Patients' onset distribution

3. Corticotherapy complications

From the Table 3 and Figure 3 we conclude that the Chi-square test performed in order to compare the status frequency of the two patients groups analyzed, were statistically significant.

Table III. Complications of corticosteroid therapy in thymectomized/non-thymectomized myasthenia gravis patients

Complications	Thymectomy	No-thymectomyi
HTA	9.10%	11.30%
Dyslipidemia	34%	43.40%
Diabetes mellitus	5.14%	7.14%
Osteoporosis	8.60%	10.70%
Cataract	7.80%	13.60%
Cushing	3.40%	5.86%
Without complication	31.96%	8%

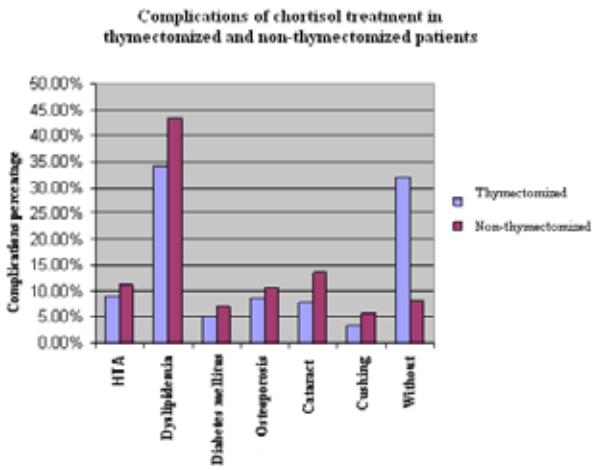

Fig. 3. Complications of corticosteroid therapy in thymectomized/non-thymectomized myasthenia gravis patients

CONCLUSIONS

MG is a rare autoimmune disease of the myoneural junction, but growing in frequency in the last years in all age groups. The thymectomy benefit is not an immediate one. Thymectomy remains the important link in the complex treatment of MG with long-term benefit effects, conditioned by a complete removal of the thymus containing adjacent tissue. The benefits of thymectomy are proved by the clinical course of the disease with fewer relapses and milder symptomatology.

Few authors argue that there are no differences between the patients treated conservatively and the thymectomized ones. The conservative treatment shouldn't be stopped after thymectomy and we consider that must be conducted for patient benefit.

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TIMECTOMIA PENTRU MIASTENIA GRAVIS: CARACTERISTICI SPECIALE

REZUMAT

Timectomia face parte din tratamentul chirurgical al misteniei gravis. Cu toate acestea, rolul medicației orale nu poate fi neglijat și nici medicația orală nu poate fi întreruptă. Din acest punct de vedere se pot supune atenției studiului anumite particularități în ceea ce privește eficacitatea timectomiei versus corticoterapie, vârsta și tipul de debut al bolii . Totuși, timectomia rămâne veriga importantă în arsenalul terapeutic general al bolnavului miastenic.

Cuvinte-cheie: timectomie, miastenie, variabile, tratament conservator, beneficii

INSIGHTS INTO MELANOMA HISTOLOGICAL ASPECTS, PROGRESSION AND PROGNOSTIC

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ABSTRACT

Melanoma, a malignant tumor of melanocytes, is the most aggressive form of skin cancer and its frequency is increasing in all Caucasian skin populations. The strongest risk factors are family history of melanoma, multiple pre-existent melanocytic nevi, previous melanoma, previous nonmelanoma skin cancer. Based on the histological features, primary cutaneous melanomas have been classified in four types. The major histologic subtypes of melanoma are superficial spreading melanoma, nodular melanoma, lentigo melanoma and acral lentiginous melanoma. Early diagnosed, melanoma has a good prognosis, beeing completely cured in the early stage by operation. In order to facilitate the early recognition of potentially curable cutaneous malignant melanoma, the ABCDE acronym is a easy tool provided. The Clinical features described are: Asymmetry, Border irregularity, Color variegation, Diameter greater than 6 mm and Evolving, which recognize the dynamic nature of the skin malignacy. During the melanomogenesis process, histological changes are accompanied by biological events, molecular lesions and genetic alterations. Starting with the formation of benign nevus, and then progressing to the junctional melanocytic hyperplasia and then aberrant differentiation, a mutation of BRAF gene and activation of the MAPK signaling pathway take place. Progression to the radial growth phase is accompanied by loss of CDKN24 and loss of PTEN. The switch from radial growth phase to vertical growth phase is the most crucial step in melanoma tumorigenesis, commonly associated with subsequent metastatic disease. Loss of E-cadherin and expression of N-cadherin, mutation in BRAF and NRAS genes are events that mark this progression. Also associated events are loss of two transcription factors, activator protein-2α (AP-2α) and cAMP response element binding protein (CREB). The metastatic melanoma is the last stage, when the metastatic tumor enter the lymphatic and blood circulation and metastasize to other organs like lymph nodes, brain, liver or lung. Galentin-3 and Galentin-1, are two members of galentin family which are directly corelated with the metastatic potential of melanomas. The review seeks to examine melanoma progression and some molecular changes that accompanied the process. Also, a histologic classification and prevention and early recognition of melanoma are beeing discussed.

Key words: melanoma progression, radial growth phase, vertical growth phase, early recognition, molecular changes

GENERAL CONSIDERATIONS

Skin cancer, one of the most common types of cancer, could become a significant public health problem over the coming years. From all skin cancers, melanoma is the most aggressive form of skin cancer and its frequency is increasing in all Caucasian skin populations, particularly in the 30–50 age bracket (1). Although melanoma is the rarest form of skin cancer (4%), it is responsible for more than 80% of deaths from skin cancers (2-4), due to its high capacity for invasion and metastasis, affecting deeper skin layers (5-6). Despite of public health initiatives, the incidence of melanoma continues to increase.

Melanoma is a malignant tumor of melanocytes, the pigment-producing cells, called melanin. Melanoma is formed either from neoplasms of isolated melanocytes or from pigmented nevi (7). Melanocytes origins derive from the neural crest (8-10), being differentiated during embryonic development (11). Although the function of melanocytes from skin is most characterized, melanocytes are cells collated in the skin, hair follicles, stria vascularis of the inner ear, and uveal tract of the eye (11). Melanin sintesis is carried out by the tyrosinase family of proteins, including tyrosinase and the tyrosinase-related proteins TRP-1 and TRP-2, as a physiological defense response against keratinocytes apoptosis and DNA damage caused by free radicals generated by UV radiation. This protection occurs due to photoprotective capacity of eumelanin, with antioxidant and radical scavenging properties. Melanin is distributed around keratinocytes and partially protects them, but when its protective capacity is exceeded, damaged keratinocytes are eliminated by apoptosis. Unlike keratinocytes, melanocytes exhibit a resistance to apoptosis. After exposure to UVB, they rather proliferate and produce melanin in order to keep their protective role. This resistance is considered important in the transition from normal melanocytes to melanoma cells (6). The role of melanogenesis in melanoma resistance was

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demonstraded by Brozyna et al., who showed that pigmented cells with higher tyrosinase activity and melanin content, exhibited lower sensitivity to ionizing radiation compared to unpigmented ones (6, 12).

A strong hereditary link for melanoma coexist in addition to skin pigmentation, melanoma being a disease most associated with light skin pigmentation, showing an incidence of 10 times higher among Caucasians than Africans (13). Regarding the risk factors for developing melanoma, they were classified as strong risk factors and additional risk factors. The strongest risk factors are family history of melanoma, multiple pre-existent melanocytic nevi (benign or atypical), previous melanoma, previous nonmelanoma skin cancer (basal cell carcinoma, squamous cell carcinoma). Amoung additonal risk factors exposure to ultraviolet radiation, immunosuppression, air skin that burns easily, blistering sunburn can be mentioned (5, 14, 15).

Unlike non-melanoma skin cancers, which occur in an older age, more than 60 years, the frequency of melanoma is greatest between 20 and 45 years. The incidence is higher in women than in men, but mortality is higher in men because men tend to develop lesions in less noticeable areas like the back, and is therefore diagnosed later. Also men can develop melanoma on abdomen and stomach. In women often occurs on the lower legs (16). Although more than 95% of melanomas are found in the skin, the tumors can occur in any tissue that contains melanocytes, including noncutaneous sites such as the oral mucosa, nasopharynx, paranasal sinuses, tracheobronchial tree, vulva, vagina, anus, urinary tract, central nervous system, and eye (17).

A HISTOLOGIC CLASSIFICATION OF MELANOMAS

Based on the histological features, primary cuteneous melanomas have been classified in four types. The major histologic subtypes of melanoma are superficial spreading melanoma, nodular melanoma, lentigo melanoma and acral lentiginous melanoma (5, 18-20). Other types of melanomas are mucosal melanomas which arises in mucosal tissues in the respiratory, digestive, and reproductive tracts and uveal melanomas and uveal melanoma witch arises from melanocytes in the choroid, ciliary body, or iris of the eye (21).

Smoller described the diagnostic histological criteria of the major histological subtypes of melanoma, organizing the description by architectural and cytologic features and examining each subtype in term of epidermal findings and dermal findings (20). The most common type is *superficial spreading melanoma (SSM)*, representing almost 75 % of melanoma. Superficial spreading melanoma affects the trunk and extremities, and its lesions are often variegated in color (18). Characterized by the lateral spreading of malignant melanocytes within the epidermis, superficial spreading melanoma, as its name says, tends to start by spreading out across the surface of the skin, known as the radial growth phase (5, 20). Microscopic features of superficial spreading melanoma are: poor circumscription of melanocytes, single melanocytes predominating over nests of melanocytes, haphazard and aberrant distribution of melanocytes within the epidermis, the presence of melanocytes above the basal layer (Pagetoid spread) and dyscohesive nests of melanocytes (20). Lack of maturation, mitotic activity, brisk and asymmetrical host, inflammatory response and occasional focal fibrosis with neovascularization are features of the dermal component of superficial spreading melanoma, according to Smoller description.

Nodular melanoma (NM) is the most agressive form of melanoma because it tend to grow rapidly in thicknes, penetrating the skin deeply entering in a vertical growth phase, corelating with a higher rate of metastatis, wich lead to a poor prognosis of this subtype of melanoma (5, 20). Malignant melanoma of the nodular type is an example of a primary tumor without a precursor developmental stage such as a radial-growth phase (19). Nodular melanoma occurs most often on the chest or back and it's incidence is corelated with middle-aged adults. The tumor is most often darkly pigmented; it may be very dark brown–black or black and enlarge rapidly (18). Histologicaly, nodular melanoma had a signifiant differention of superficial spreading melanoma. The tumor often have a symmetrical architectural pattern, beeing well circumscribed, and no lateral extension of the intraepidermal component. Also, ulceration is common feature in this subtype. Redarding the epidermal component, epitheloid melanocytes with abundant cytoplasm, vesicular nuclei and prominent nucleoli can be observed. The dermal melanocytes are enlarged and have proeminent and eosinophilic nucleoli (20).

Lentigo melanoma is a subtype of melanoma which usually occurs on sunlight-exposed skin in elderly patients, on face and neck (18). More often, it grows very slowly and can remain in a non-invasive form for years (5). Lentigo melanoma is the in situ phase of lentigo malignant melanoma, the melanoma that invase the dermis. The lentigo melanoma epidermis is athophic it is characterised by confluent melanocytes along the dermal epidermal jonction. The cells are hyperchromatic and small, with multinucleoli., dense and often unapparent (20). Lentigo maligna melanoma has irregular margins (18).

A rare subtype of melanoma, *acral lentiginous melanoma* arise from melanocytes in the skin but specifically on the part which have less UV exposure such as palms, soles, and subungual surfaces or around the toenails (5, 21). It often demonstrates massive invasion when the vertical growth phase occurs (18).The melanocytes are present as nests and single cells along the dermal epidermal junction with extensive Pagetoid upward migration. The dermal component is characterised by hyperchromatic melanocytes, single cells and nest. Also, it is corelated with aggregation around blood vessels and a marked tendency to track down eccrine structures (20).

Beside the histologic differention between these subtypes of melanoma, there are differention at the genomic level, these melanomas being characterised by distinct oncogenic mutation and distinct pattern of DNA copy number alteration. Several genes that have different alteration are: BRAF, NRAD, KIT, GNAQ/GNA11 and BAP1 (21).

PREVENTION AND EARLY RECOGNITION OF MELANOMA

Because melanoma incidence and mortality still continue to increase, prevention and early recognition of melanoma are crucial in reducing morbidity and mortality and recently has become a continuing public health priority in order to improve melanoma's prognostic (22). Early diagnosed, melanoma has a good prognosis, beeing completely cured in the early stage by operation, which is dependent on the vigorous spreading of tumors (5, 23). In order to provide a easy tool for the early recognition of potentially curable cutaneous malignant melanoma, in 1985, Friedman et al, developed the ABCD acronym for melanoma screening (23, 24). The ABCD acronym refers to some parameters wich can be useful to interpret the pigmented lesions and diferentiate the benign nevi from cutaneous melanoma. The Clinical features described are: **A**symmetry, **B**order irregularity, **C**olor variegation, and **D**iameter greater than 6 mm. Lately, this acronim was improved, with the addition of another parameter **E** from Evolving, which recognize the dynamic nature of the skin malignacy (22), with a big importance in recognition of nodular melanoma. The evoling lesions are defined as those lesion wich change in size, shape, symptoms, surface or shades of color. Thise lesions are a good candidate for a further examination and biopsy. The ABCDE have the greatest diagnostic accuracy when used in combination, a concept that should be kept in mind when evaluating pigmented lesions 6 mm or less in diameter (22).

Another screening test is the ,revised seven-point checklist' advocated by MacKie (25), aslo known as the Glasgow 7-point checklist (22, 26). This checklist has been less widely adopted than the ABCD criteria (22). The 7-point checklist includes three major indicators and 4 minor indicator. The three major indicators are change in size, change in shape, and change in colour. The four minor signs are inflammation, crusting or bleeding, sensory change, and a diameter of more than 7 mm, indicator which are observed less frequently (22, 25-27).

Strategies aimed at improving the early diagnosis of malignant melanoma will need to be directed at the general public as well as their doctors. These tools are usefull tools of evaluation for nondermatologists - clinicians and laypersons - in differentiating common moles from cancer and were not meant to provide a comprehensive list of all melanoma characteristics. The specialist evaluation may result in further workup of pigmented lesions via dermoscopy, biopsy, or both (22) Dermoscopy (also called epiluminescence microscopy) is a method of evaluation that can access the subsurface structures of the skin for in vivo examination, with a diagnostic accuracy to 70–95% (23). Nevertheless, there are some limitations of dermoscopy due to the lack of some specific dermoscopic features that some pigmented lessions have. These limitations lead to the conclusion that is important to indentify the interacting mechanisms which control the early growth of skin cancer in order to develop a ore accurate diagnostic techniques (23).

PROGRESSION OF MELANOMA

The transformation of melanocytes in melanoma is a pathological process is called melanomogenesis. This process is associated with changes at cellular level (proliferation, epithelial-mesenchymal transitions, etc.). The interest in undestanding melanoma progression with an established criteria for the histologic diagnosis of cutaneous malignant melanoma rise at the late 60', when Clark proposed a staging model for the progression of melanoma on the basis of skin invasion levels (28). The model emphasizes the histopathological changes that occur in the progression of melanoma (14). Subsequently, Breslow and Macht evidenced that the major prognostic indicator for melanoma is the depth of invasion (thickness) of the tumour, known as the Breslow thickness (from the top of the granular cell layer of the epidermis to the deepest extension of the tumor) (4,18, 23, 26, 29). Microscopic measurement of the deepest levels of melanoma involvement in the skin provides a useful indication of the associated prognosis (18). There is a define association between progressive depth of invasion and a worsened prognosis (30). The Clark's model describes histologic changes grouped in six sequential stages starting with the normal melanocytes and finishing with the malignant melanoma (31 – 32). The melanocytic lesions in the first three steps are nonmalignant while the next three stepts represent the malignant process of melanoma (33). In the multistep tumorigenesis, the histological changes are acompaniated by biological events, molecular lesions and genetic alteration that involve inactivation of tumour suppressor genes, activation of oncogenes, and defects in housekeeping genes such as mismatch repair (MMR) genes (14, 32 - 34).

The initial event is a proliferation of structurally normal melanocytes leading to the benign nevus. The benign nevus are composed of neval melanocytes. At this stage, the growth of nevus is limited and self-controled, rarely progresses to cancer probably due to oncogene-induced cell senescesce (14, 31 - 32, 35). The genetic changes at this first step of the tumorigenesis are still largely unknown, earliest genetic changes appearing to involve mutations of the melanocytes of the melanocytic dysplastic nevi phase, according to Hussein's review (32). However, Pollock et al. found that about 80 % of the diverse nevi (congenital, intradermal, compound, dysplastic) show a mutation of BRAF gene and activation of the mitogen-activated protein kinase (MAPK) signaling pathway. BRAF encodes a serine/threonine kinase that acts in the MAPK pathway to transduce regulatory signals from RAS to MEK1/2 (36). Activation of MAPK has an important role in melanocyte proliferation. Thaking into consideration that most of benign nevus don't progress to cancer, the conclusion is that BRAF activation alone is insufficient for the development of melanoma and there are required additional molecular events to become malignant (14, 36).

In order to continue in the tumorigenesis process, a focal proliferation of the melanocytes from the melanocytic nevus takes place. The naevus cells proliferate to the dermo-epidermal junction. Here they establish a lentigenous melanocytic hyperplasia, which is a junctional melanocytic hyperplasia. Some of the nevi remain stable at this stage but if the process continues,

cells grow and start the developmental flaw, called aberrant differentiation, with the resultant of so called melanocytic nuclear atypia or melanocytic dysplasia (31-32). These Melanocytic dysplastic nevi are considered risk factors for cutaneus malignant melanoma because they are the melanoma's precursor. Anyway, not all the melanocytic dysplastic nevi progress to melanoma, most of them are teminal lesions (31, 34, 37-38). As in the initial benign nevi, the growth of melanocytic dysplastic nevi si limited and self-controlled. The melanocytic dysplastic nevi may arise from preexisting benign nevi or as new lesions (14) and are categorized into sporadic and familial dysplastic nevi (34, 38). These lesions features are clinically, architecturally, and cytologically atypic. The clinical features include asymetry, irregular borders, multiple colors and increasing diameters. The histological feature is random and discontiguous cytologic atypia (14, 37). At the molecular level, the abnormalities of this stage affect the cell growth, DNA repair and susceptibility to cell death. The changes incude loss of cyclin-dependent kinase inhibitor 2A (CDKN24) in 25-40 % of melanoma, CDKN24 beeing a single gene that encodes two tumor-suppressor proteins, p16INK4A and p19ARF and loss of phosphatase and tensin homologue (PTEN) in 25-50 % of melanomas(14, 39). You et al. showed that the colloboration of PTEN and INK4A/ARF (an inhibitor of CDK4) lead to skn cancer in invo (14, 40). Genetic changes that take place at this stage of melanoma evolution are complex and involve allelic loss, microsatellite instability (MSI), and alterations of tumour suppressor gene (TSGs), mismatch repair (MMR) proteins, oncogenes, and some growth factors, due to Hussein report (32).

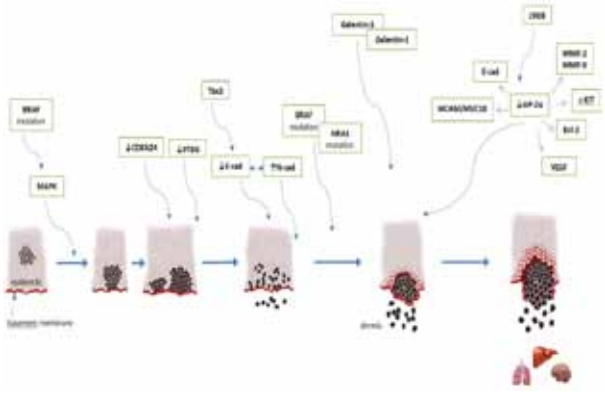

Fig. 1. Melanoma progression and mechanisms involved: starting with the formation of benign nevus, and then progressing to the junctional melanocytic hyperplasia and then aberrant differentiation, a mutation of BRAF gene and activation of the MAPK signaling pathway take place. Progression to the radial growth phase is accompanied by loss of CDKN24 and loss of PTEN. The switch from radial growth phase to vertical growth phase is the most crucial step in melanoma tumorigenesis, commonly associated with subsequent metastatic disease. Loss of E-cadherin and expression of N-cadherin, mutation in BRAF and NRAS genes are events that mark this progression. Also associated events are loss of two transcription factors, activator protein-2α (AP-2α) and cAMP response element binding protein (CREB). Galentin-3 and Galentin-1, are two members of galentin family which are directly corelated with the metastatic potential of melanomas. The metastatic melanoma is the last stage, when the metastatic tumor enter the lymphatic and blood circulation and metastasize to other organs like lymph nodes, brain, liver or lung.

The next step is radial growth phase (RGP), a characteristic phase of flat which is considered the first stage of cancer in the melanocytic system (32). Tumors at this stage of evolution are considered safe, with a superficial and nondestructive invasion. The term suggested for this stage is microinvasive melanoma because at this stage melanoma is not associated with intradermal proliferation or tumor nodule formation (41). Tumors in the radial growth phase don't have the competence for metastasis (33, 41), even the cells have partial growth autonomy, with the ability to grow both indefinitely and independently. In order to proceed to the vertical growth phase (VGP), cells need to acquire more growth autonomy (32). Weder et al. have demonstrated that RGP cells are not tumorigenic in vivo when injected subcutaneously in immunocompromised mice, but VGP and metastatic cells (42). The radial growth phase is characterized by the net enlargement of the tumor at its periphery, along the radii of an imperfect circle (31). In the radial growth phase, tumors grow locally in the epidermis, with an increase in the population of melanoma cells at the expense of the interstitial liquid, through which the chemical factors diffuse (23, 42-43), being considered primary melanoma aroused in situ, before invading the dermis passing through the basal laminae (23). Some biologic events in the radial growth phase are the decreased differentiation, unlimited hyperplasia and clonal proliferation (14). According to the ABCDE acronym, asymmetry, border contour irregularities, color variegation, and diameter increase over time (evolving), are characteristics of the radial growth phase (41). In the later stages of radial growth phase evolution, called invasive radial growth phase, single cells or very small groups of cells start to penetrate the dermis, with a distinctive form of invasion of the papillary dermis (28, 43-44). This capacity to invade into the dermis is considered a form of invasion across the basement membrane (41). However, in the dermis these cells don't form a tumorigenic nodule and don't compress or obliterate preexisting structures (41). Molecular changes have to happen for the cells in the radial growth phase acquire a metastatic potential.

Vertical growth phase is another step in Clark model of melanoma progression. The progression of radial growth phase of cutaneous melanoma in the vertical growth phase is the most crucial step in melanoma tumorigenesis, because it is commonly associated with subsequent metastatic disease (19). The progression consists in accumulation of growth autonomy (32). A new population of cells within the melanoma appears at this stage, tumors that degrade the extracellular matrix and invade the dermis (42). In the earliest of the vertical growth phase, melanoma is characterized by the tumor nodule formation, formed by the mitotically active cells (41). Melanoma cells in vertical growth phase have heterogeneity, termed polyclonism. This polyclonism refers to morphologically distinctive subpopulations comprising the tumorigenic component of primary human melanomas that frequently occur in this stage and may be manifested by distinctive zones of divergent cell size, shape, pigmentation, or stromal reaction (41, 45). In the transition from the radial growth phase to vertical growth phase multiple events have been identified. Changes occur at specific stages and can interfere with critical

molecules for initiating the tumor development, angiogenesis, and survival (46).

Alteration in level of adhesion molecules, loss of E-cadherin (epithelial) which are surface molecules that form homophilic and heterophilic interactions on surrounding cell (39), in particular and expression of N-cadherin (neural) are two events that mark the progression from the radial-growth phase to the vertical-growth phase of melanoma (14, 23, 47). In case of melanoma these homophilic and heterophilic interactions contribute to the invasion, migration, cells surviving in circulation due to the aggregation of cancer cells to each other or to host stroma cells or cell from the circulatory system (39). E-cadherin expression in melanocytes and keratinocytes permits the association between the two cells. When E-cadherin switch to N-cadherin, a dissociation of melanocytes from keratinocytes results and melanoma cells start to invade the dermis (39). Rodriques et al. showed that E-cadherin expression is directly repressed by Tbx3 protein, a member of the T-box transcription factors family. Tbx3 is a transcription repressor, overexpressed in melanoma and it contribute in melanoma invasiveness and transition from radial growth phase to vertical growth phase by down-reglating of E-cadherin expression (47).

Mutation in the BRAF gene which started in the initial step of the tumorigenesis process continue to increase and are accompaniated by mutation in the NRAS gene as melanoma progress from an in-situ (RGS) to an invasive stage (VGP), as Greene et al reports (48). BRAF and NRAS mutation conduct to amino acid substitutions within the NRAS and BRAF oncogenic proteins. These substitution lead to a constitutive activation of growth signaling through the mitogen-activated protein kinase pathway (49).

Once the basement membrane and extracellular matrix are degraded and melanoma cells acquire the capacity to form a tumor and infiltrate more extensively into the dermis and subcutaneous tissues, the melanoma is considered to be malignant (43). The metastatic melanoma is the last stage, when the metastatic tumor enter the lymphatic and blood circulation and metastasize to other organs like lymph nodes, brain, liver or lung (14, 42, 46). Only a small subset of cells from the primary tumor have the acquired molecular changes to complete the metastatic cascade, because a condition for a tumor to metastasize, is the cells ability to survive the circulation, arrest in the capillary bed of the distant organ, enter the parenchyma, grow within the organ microenvironment, and initiate angiogenesis (46).

Recently the role of galentin family in melanoma is studied because the melanoma progression has been correlated with a deregulation of the galectin family of carbohydrate-binding proteins. Proteins from the galentin family have the ability to bind to glycosylated proteins such integrins, fibronectin, laminin, NCAM, EGRF (50) which made them crucial in many biological processes such cell-cell adhesion, inhibiton of cell receptor internalization, induction of T-cell apoptosis, induction of angiogenesis (46, 50). The most involved in melanoma progression galentins are galentin-3 and galentin-1, which are directly corelated with the metastatic potential of melanomas (51). Galentin-3 binds to many cells surface proteins in order to initiate adhesion and metastasis. Also, galentin-3 enhance cell agregation in melanoma and protects them during the circulation in circulatory system. In addition, it increase the activity of matrixmetalloproteinases (MMPs) and increases the secretion of MMP-2 contributing at the basement membrane degradation during the transition from RGP to VGP. Another role of this protein is in angiogenesis, increasing neovascularization by modulating the endothelial cell response to VEGF and bFGF (46). Expressed by the immune cells and endothelial cells , galentin-1 increases tumor aggresion by binding to T—cells membrane glycoproteins and inducing apoptosis (46, 52) and also it is responsable for the melanoma resistance to therapy: chemotherapy, radiotherapy, and immunotherapy (53).

The switch from RGP to VGP and further to metastatic melanoma is also associated with the loss of two transcription factors, activator protein-2α (AP-2α) and cAMP response element binding protein (CREB). Dereglation of c-KIT, MCAM/MUC18 melanoma cell adhesion molecule, E-cadherin, VEGF, Bcl-2, MMP-2, MMP-9 and p21 are consequences of AP-2α loss (39). Melnikova et al. demostrated that down- regulation of AP-2α, during melanoma progression is attributed by the activation of CREB, nominating it as a "major regulator" of melanoma progression (39, 54).

Beeing the most aggressive form of skin cancer, melanoma is the subject of many studies in order to find effective treatments. Despite modern results in the research, the results of conventional therapy of metastatic melanoma in the advanced stages remain unsatisfactory due to the apoptosis resistance that tumor cells acquire (7). The standard treatment is not well defined, there is much controversy at this level (55-56). However the reference treatment in this fiels is considered to be Dacarbazine (57-58). Dacarbazine treatment can be supplemented with other techniques. However Lev et al. showed that repeated exposure of melanoma cells with a weak aggressive phenotype to dacarbazine, leads to a more aggressive phenotype with larger tumors in vivo (56). Researcher devote much attention to cancer prevention and early detection and to the development of less toxic treatments. In this context new classes of compounds are being explored, in particular plant-derived molecules, including pentacyclic triterpenes. All these issues and developments in research requires reproducible and related models of human pathology. Animal models are used to test new drugs in the treatment of melanoma are also useful for a better understanding of the molecular mechanisms involved in the pathology of cancer (8, 59).

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INTROSPECȚIE ÎN ASPECTELE HISTOLOGICE, PROGRESIA ŞI PROGNOSTICUL MELANOMULUI

REZUMAT

Melanomul, o tumoră malignă a melanocitelor, este cea mai agresivă formă de cancer cutanat şi incidenţa lui creşte în toate populaţiile caucaziene. Cei mai puternici factori de risc sunt istoricul familial, multiplii nevi melanocitari preexistenţi, prezenţa anterioară a unui melanoma sau a altor forme de cancer non-melanomice. Subtipurile majore ale melanomului sunt melanomul superficial, melanomul nodular, melanomul lentigo şi melanomul acral lentiginos. Diagnosticat precoce, melanomul are un prognostic bun, fiind complet curabil în stadiile timpurii prin operaţie. Cu scopul de a facilita recunoaşterea precoce a melanoamelor cutanate maligne cu potenţial de vindecare, acronimul ABCDE este un instrument prevazut. Caracteristicile clinice descrise sunt Asimetria, Bordura, Culoarea, Diamentrul mai mare de 6 mm şi Evoluţia, care recunoaşte natura dinamică a malignităţii. În timpul procesului de melanomogeneză, schimbările histologice sunt însoţite de eventimente biologice, leziuni moleculare şi alterări genetice. Începând cu formarea nevilor benigni, progresia spre hiperplazia melanocitică joncţională şi apoi spre diferenţierea aberantă, au loc mutaţii în gena BRAF şi activarea căii de semnalizare MAPK. Progresia spre faza de creştere radială este însoţită de pierderea CDKN24 şi a PTEN. Trecerea de la faza de creştere radială spre cea de creştere verticală este cel mai important pas în procesul tumorigenezei, asociat în mod comun cu boala metastatică ulterioară. Pierderea E-caderinei şi expresia N-caderinei, mutaţii ale genelor BRAF şi NRAS sunt evenimente care marchează această progresie. De asemenea, evenimente asociate sunt pierderea a doi factori de transcripţie, AP-2α şi CREB. Melanomul metastatic este ultima fază, când tumorile metastatice intră în circulaţia sanguină şi limfatică şi metastazează în alte organe precum noduli limfatici, creier, ficat sau plămâni. Galentin-3 şi Galentin-1 sunt doi membri ai familiei galentinelor direct corelaţi cu potenţialul metastatic al melanomului. Această trecere în revistă încearcă se examineze progresia melanomului şi câteva schimbări moleculare care însoţesc procesul. De asemenea, sunt discutate clasificarea histologică, prevenţia şi recunoaşterea timpurie.
Cuvinte cheie: progresia melanomului, faza creşterii radiale, faza creşterii verticale, recunoaşterea timpurie, schimbări moleculare

PREVALENCE OF POLYGLANDULAR AUTOIMMUNE SYNDROME TYPE II IN A GROUP OF CHILDREN WITH THYROID DISEASES AND DIABETES MELLITUS

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ABSTRACT

Background&Aims: Polyglandular autoimmune syndrome (PAS) type II is the most common of the immunoendocrinopathy syndromes. It is characterized by the obligatory occurrence of autoimmune Addison disease in combination with thyroid autoimmune diseases and/or type 1 diabetes mellitus. Primary hypogonadism, myasthenia gravis, and celiac disease also are commonly observed in this syndrome. The purpose of this study is to determine the prevalence of PAS type II in a group of children with thyroid diseases and diabetes mellitus (DM). **Methods:** The studied group was of 83 children with DM type 1 (71 girls and 12 boys), aged between 7 and 17 years. The methods of investigation were represented by clinical, imaging, biochemical, hormonal and immunological parameters. **Results:** The prevalence of PAS type II in the study group was 6.02 % (80% girls and 20 % boys, $p = 0.57$, $X^2 = 3.6$). In all cases was a triple association: adrenal insufficiency, chronic autoimmune thyroiditis and type 1 diabetes. We don't have symptomatic cases of Addison's disease in children group, but only 5 cases of asymptomatic Addison's disease identifying through the presence of specified antibodies. **Conclusions:** In the children group, PAS type II comprises 3 diseases: adrenal insufficiency, chronic autoimmune thyroiditis and type 1 diabetes; we don't find significance difference between girls and boys**.** Because Addison's disease was asymptomatic, identifying through the presence of specified antibodies, if we have a children with two or more autoimmune disease, we must investigate him for another possible autoimmune disease.

Keywords: diabetes mellitus, thyroid disease, polyglandular autoimmune syndrome type II, children

INTRODUCTION

PAS II is the most common form of PAS. It is characterized by the presence, in the same patient, a two or more of the following diseases: Addison's disease, type 1 diabetes, autoimmune thyroid disease, primary hypogonadism, celiac disease and myasthenia gravis (5).

It is estimated that the prevalence is 1/200,000, and the incidence of about 1 - 2/10000/year, that is 10-20 times higher than that of PAS I. Diagnosis is indicated, usually between 20 to 60 years and it is about three times more common in women than in men (4).

In PAS II etiopathogenesis are involved the genetic predisposition, environmental factors and specific auto antibodies. On a background of genetic predisposition acting environmental factors can trigger the autoimmune process and lead to the disease (1, 2).

Regarding genetic predisposition (6) transmission mode of PAS II is polygenic, autosomal dominant with incomplete penetrance. An important role are gene polymorphisms of HLA system (5), located on the short arm of chromosome 6. Most diseases are associated with HLA haplotypes following: A1, B8, DR3 (DQA1 * 0501, DQB1 * 0201) and DR4 (DQA1 * 0301, DQB1 * 0302) (2, 7). Lately it seems that in genetic susceptibility to PAS II is involved CTLA gene polymorphism („cytotoxic T lymphocyte antigen-4") (8).

The main environmental factors which may trigger the autoimmune process in PAS II are: congenital rubella early, intake of cow's milk (DM type 1), gliadin (celiac disease), methimazole (anti-insulin auto antibodies), penicillamine (myasthenia gravis), anti CD-52 monoclonal antibodies (used in the treatment of multiple sclerosis) (Graves-Basedow disease), and interferon (hypothyroidism) (8).

Auto antibodies are serological markers of immune process, ongoing, at endocrine gland and predict subsequent occurrence of clinical manifestations. Some of them have pathogenetic role: anti-acetylcholine for myasthenia gravis, and anti-TSH receptor, for Graves-Basedow disease (7).

In addition to the three common endocrinopathies (Addison disease, type 1 diabetes and autoimmune thyroid disease), the clinical manifestations of patients can include other autoimmune diseases, endocrine and non-endocrine: celiac disease, vitiligo, seriousness, IgA deficiency, primary hypogonadism, hypoparathyroidism, stiff man syndrome, alopecia, pernicious anemia, pituitary gland and myasthenia gravis. Frequently, can be positive only specific organ auto antibodies, both at the patients and their relatives without clinical signs to be present (3).

Various endocrinopathies not begins at the same time, between the diagnosis of two successive diseases of the syndrome can go even 20 years. The PAS II occurrence prob-

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ability in a patient with a particular autoimmune endocrinopathy varies depending on the prevalence of the disease and family history. In the case of isolated thyroid disease, common in the population, and in the absence of family history of PAS II, the risk for other endocrinopathies is very lower. On the other hand, in the rare cases of autoimmune diseases, such as Addison's disease, the hypothyroidism probability is much higher, of 14-21%. Frequent hypoglycemia or reduced insulin requirements in a patient with Type 1 diabetes must draw attention to the possibility of developing adrenal insufficiency, hypothyroidism or gluten enteropathy (8).

The diagnosis is established, usually by highlighting autoimmune syndrome components. Based on the symptoms and clinical manifestations of each case is recommended metabolic, hormonal and immunological determination which must confirm the diagnosis.

Treatment is represented by the hormonal replacement for endocrine insufficiencies, being specific to each disease (2). If it is established early, patients have a good evolution and do not develop complications. There are some therapeutic particularities, given by certain associations. For example, patients with Addison disease and hypothyroidism should be treated first for adrenal insufficiency because if it is administered initially thyroid hormones may be trigger adissonian crisis. In the case of non endocrine autoimmune diseases, with unfavorable evolution it can try immunosuppressive therapy. In recent years, in the patients with PAS II are being tested a number of preparations, such as anti-CD20 antibody (Rituximab) and anti-CD3 monoclonal antibody, from which are expected benefit in the prevention of Type 1 diabetes (8).

MATERIAL AND METHOD

INVESTIGATED POPULATION

The group of children was represented by 83 subjects aged between 7-17 years. All children from the study group had Type 1 diabetes. In the studied group, the gender distribution of the children was 5.9/1, represented by 71 girls (85.54%) and 12 boys (14.45%).

METHODS OF INVESTIGATION

The methods of investigation were represented by **clinical data** - case history, current status, **imagistic-** thyroid ultrasound, **biochemical** - ***for glycemic balance***: fasting blood glucose, glycosylated hemoglobin, ***investigation of the thyroid gland***: TSH, FT_4, FT_3, thyroid antibodies, ***investigation of the adrenal gland:*** cortisol, 21-hydroxylase antibodies, ***gonadotropins:*** FSH, LH and appropriate sex hormones (testosterone, estradiol), ***investigation of celiac disease***: antitissue transglutaminase antibodies, ***investigation of pernicious anemia***: complete blood count with mean cell volume and vitamin B_{12} levels.

Determination of plasma glucose was performed by enzyme technique with glucosooxidasis. Normal values were taken between 70 - 110 mg%; diabetes mellitus - values equal or over 126 mg%, impaired glucose tolerance - values between 110 - 125 mg% and the OGTT at 2 h between 140 - 200 mg% and impaired fasting glucose - values between 110 - 125 mg% and OGTT at 2 h under 140 mg%.

Determination of HbA1c was achieved through the DiaStat for measuring HbA1c reported to the total HbA.

To determine ***the TSH level in plasma, the free fraction of triiodotironin (FT_3), and the plasma free fraction of thyroxin (FT_4)*** were performed a quantitative method ARCHITECT; witch is an immunological method, Chemilumnescent Microparticle Immunoassay (CMIA). Normal values were following: TSH = 0.465 - 4.68 Miu/ml, FT_3 = 3.69 -10.4 pmol/l, FT_4 = 10 - 28.2 pmol/l.

To obtain ***the level of cortisol*** was performed the technique IMMULITE / IMMULITE 1000, an imunometric method, in solid phase, competitive, of chemiluminescent, Immuno Chemilumino Enzymometric assay (ICEM). It was considered normal: a.m. 5 - 25 microgram/dl.

FSH level was measured quantitatively by the ARCHITECT method; a Chemilumnescent Microparticle Immunoassay. Reference values: determined with ARCHITECT test (Table I).

Table I. The reference values for FSH

Population field	mIU/ml
Women:	
- Follicular phase	3.35 – 21.63
- Ovulating phase	4.97 – 20.82
- Luteal phase	1.11 – 13.99
- Postmenopausal	2.58 – 150.53
Men	1.37 – 13.58

LH level was measured quantitatively by the ARCHITECT method; a Chemilumnescent Microparticle Immunoassay. Reference values: determined with ARCHITECT test (Table II).

Table II. The reference values for LH

Population field	mIU/ml
Women:	1.26 – 10.05
- Follicular phase	2.57 – 26.53
- Ovulating phase	18.06 – 90.23
- Luteal phase	0.67 – 23.75
- Postmenopausal	
Men	1.09 – 92.45

Testosterone was determinate by ELISA method. The references values are depending by age and gender:

Adults:

– men: 0.019 - 0.145 nmol/L;

– women in fertile period: < 0.014 nmol/L;

– pills: 0.001 - 0.0069 nmol/L;

– postmenopausal: 0.0003 - 0.0058 nmol/L.

Estradiol was determinate by immunochemical with electrochemiluminiscent detection method (ECLIA). The references values are depending by age and gender, and at women also

with the menstrual cycle period and pregnancy (Table III).

Table III. The reference values for estradiol

Age and gender	References values (pmol/L)
Adults – **Women** • Follicular phase	46.0 - 607
• Ovulating phase	315 - 1828
• Luteal phase	161 - 774
• Postmenopausal	<18.4 - 201
– **Men**	28.0 - 156
Pregnancy (first quarter)	789 – 15781
Children (1-10 years) • girls	22.0 - 99.1
• boys	<18.4 - 99.1

The immunological parameters were represented by autoimmune thyroid markers - antibodies (antiTPO and antiTg antibodies).

To determine ***serum levels of antiTPO antibodies*** it was used the kit AxSYM antiTPO, an immunological method (Microparticle Enzyme Immunoassay) (MEIA). Normal values: antiTPO antibodies <35 IU/ml.

To determine ***serum levels of antiTg antibodies*** it was used the kit AxSYM antiTg, a MEIA method as well (Microparticle Enzyme Immunoassay). Normal values: antiTg antibodies <55 IU/ml.

To determine ***21-hydroxylase (anti 21-OH antibodies) antibodies level*** it was used the radioimunodetermination method combined with a technique of imunoprecipitation, based on human 21-OH marked with I 125 reacting with the antibodies anti 21-OH from the samples test and forming immune complexes that precipitated with the solid-phase of protein A. Normal range: <1 IU/ml

Antitissue transglutaminase antibodies were determinate by ELISA method.

References values: IgA, IgG: <10 U/mL: negative; ≥10 U/mL: positive.

Vitamin B_{12} levels were determinate by immunochemical with electrochemiluminiscent detection method (ECLIA). References values: 191 - 663 pmol/L (for European population).

Determination of complete blood count was achieved with automatic method: electric impedance method. Normal values (for children): erythrocytes = 4 - 5.5 mil/mm^3, leucocytes = 4500 – 11000 mil/mm^3, plateled = 150000 – 450000/mm^3, hematocrit (Ht): 32 – 44 %, hemoglobin (Hb): 9.5 – 15.5 g/dl.

Constants and red cell indices are calculated automatically, depending on the values of Hb, Ht and red blood cells (RBC) count. Normal values**:** mean corpuscular volume (MCV) = 80 - 100 fl, mean corpuscular hemoglobin concentration **(**MCHC) = 32 - 36 g Hb/100 ml erythrocytes, mean corpuscular hemoglobin (MCH) = 27 - 32 pg.

Thyroid ultrasound was performed in all cases and allowed us to measure thyroid volume, thyroid study and the changes in parenchyma's density.

An increased density, uniform, characterizes normal thyroid parenchyma easily distinguished from the neck muscles that are hypo dens.

Inflammatory processes and autoimmune pathology appears hypo dens**.** The scale was assessed as being discreet +, moderate ++ and marked +++.

In the autoimmune thyroid disease the parenchyma of the gland appears hypo dens.

Chronic autoimmune thyroid disorder appears with a hypoecogenity of the parenchyma and normal or increased thyroid volume.

STATISTICAL ANALYSIS

For statistical analysis we used Microsoft Excel and POP Tools from Microsoft Office 2003 and EPI 2000 program. To measure the quantitative variables were determined average (A) and standard deviation (SD), and to assess the gender differences we used the unpaired t test and ANOVA test, considering statistically significant a $p < 0.05$.

RESULTS AND DISCUSSION

In the case of children group, the main autoimmune endocrine associations were the following (Table IV).

Table IV. The prevalence of autoimmune endocrine diseases in children and adolescents group with type 1 diabetes

Associated autoimmune endocrine diseases	n	%
ACT	49	59.03%
Graves-Basedow disease	4	4.82%
ACT + asymptomatic Addison disease	5	6.02%

From the 83 cases of children and adolescents, in 5 cases with type 1 diabetes and ACT was associated asymptomatic Addison's disease.

Asymptomatic Addison's disease was diagnosed in 5 children by dosing 21-OH antibodies in 37 children from the study. From the 5 patients with positive titers, 4 were females and 1 male. The titers were between 1 to 2 IU/ml in 4 patients, only 1 patient presented titers of 2-3 IU / ml.

If are associated more endocrine autoimmune diseases, the term is polyglandular autoimmune syndrome (PAS). Autoimmune polyglandular syndromes comprise a group of autoimmune diseases characterized by inability of endocrine glands to produce their hormones. These endocrine abnormalities tend to occur together. It is estimated that about a quarter of patients with evidence one gland hypofunction present another endocrine diseases. Therefore it is recommended that in the case of one endocrine glands hypo function to assess the function of other endocrine glands.

There are several classifications of autoimmune polyendocrinophathies, but was universally accepted classification of Neufeld and Blizzard (Table V).

Table V. PAS classification (9)

PAS type	Associated disease
PAS I	Chronic mucocutaneous candidiasis I, chronic autoimmune hypoparathyroidism, autoimmune Addison's disease
PAS II	Autoimmune Addison's disease, autoimmune thyroid disease and / or type 1 diabetes (Addison's disease should always be present)
PAS III	Autoimmune thyroid disease + other autoimmune diseases (excluding autoimmune Addison's disease, autoimmune chronic hypoparathyroidism, autoimmune chronic candidiasis)
PAS IV	2 or more organo-specific autoimmune disease (which are not included in the type 1, 2 or 3).

PAS II was present in 5 cases. Initially PAS II covered PAS IIa (Smith syndrome) (Addison's disease associated with autoimmune thyroiditis) and PAS II b (Carpenter syndrome) (Addison's disease associated with type 1 diabetes). It was later revealed the possibility of a triple association: adrenal insufficiency, chronic autoimmune thyroiditis and type 1 diabetes.

In the case of PAS II onset is usually with adrenal insufficiency (AI), in time associating DM type 1; there are cases in which the diagnosis is performed simultaneously for AI and Type 1 diabetes or Type 1 diabetes diagnosis can precede their ICSR (9).

To endocrine immunopathies may be associated with a variable incidence no endocrine organ-specific systemic diseases. In our studied group, the patients don't present any endocrine organ-specific autoimmune diseases (celiac disease, vitiligo, alopecia, pernicious anemia).

The medium interval between onset age of type 1 diabetes and the occurrence of ACT was 8.59 ± 3.24 years.

No case presented the first endocrine immunopathy ACT, followed by type 1 diabetes.

The prevalence of PAS type II in the study group was 6.02 % (80% girls and 20 % boys, $p = 0.57$, $X^2 = 3.6$). In United States, approximately 14 – 20 people per million populations are affected by polyglandular autoimmune syndrome type II. Observations have revealed, however, that the disease is much more prevalent if sub clinical forms are included (3).

Regarding the gender, we don't find significance difference between boys and girls, but in United States the female – to – male ratio of polyglandular autoimmune syndrome type II is 3 – 4:1 (3). Normally, the polyglandular autoimmune syndrome type II occurs in the third or fourth decade of life.

In all cases was a triple association: adrenal insufficiency, chronic autoimmune thyroiditis and type 1 diabetes. We don't have symptomatic cases of Addison's disease in children group, but only 5 cases of asymptomatic Addison's disease identifying through the presence of specified antibodies.

Among patients with type 1 diabetes mellitus, thyroid autoimmunity and celiac disease coexist with sufficient frequency to justify screening. Measuring annual TSH levels in individuals with type 1 diabetes is recommended as cost – effective.

Clinical history and examination suggesting evidence of more than 1 endocrine deficiency should prompt testing, to include serum autoantibody screening and an evaluation of end - organ function.

Evaluation of end – organ function is necessary to confirm the diagnosis in patients with positive auto antibodies. Even if these antibodies are negative, still perform testing if clinical suspicion is high, because the sensitivity of these assays is not perfect. Are recommended the following tests to be performed annually: gonadotropins, TSH, free thyroxin (T_4) and free triiodothyronine (T_3) if is necessary, adrenocorticotropic hormone (ACTH), fasting blood glucose, complete blood count (CBC) with mean cell volume (MCV) and vitamin B_{12} levels.

CONCLUSIONS

In the children group, PAS type II comprises 3 diseases: adrenal insufficiency, chronic autoimmune thyroiditis and type 1 diabetes; we don't find significance difference between girls and boys**.** Because Addison's disease was asymptomatic, identifying through the presence of specified antibodies, if we have a children with two or more autoimmune disease, we must investigate him for another possible autoimmune disease.

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PREVALENTA SINDROMULUI AUTOIMUN POLIGLANDULAR TIP II IN GRUPUL DE COPII CU BOALA TIROIDIANA SI DIABET ZAHARAT

REZUMAT

Introducere: Sindromul poliglandular autoimun tip II (SPA tip II) este cel mai frecvent dintre sindroamele endocrine autoimune. Se caracterizează prin prezenţa obligatorie a bolii Addison autoimune în combinaţie cu boala autoimună tiroidiană şi/sau diabetul zaharat tip 1. În cadrul acestui sindrom se pot asocia şi hipogonadismul primar, miastenia gravis, precum şi boala celiacă. Scopul acestui studiu este de a determina prevalenţa SPA tip II într – un grup de copii cu afecţiuni autoimune tiroidiene şi diabet zaharat (DZ). **Metode:** Grupul de studiu a fost reprezentat de 83 de copii cu DZ tip 1 (71 fete şi 12 băieţi), cu vârsta cuprinsă între 7 şi 17 ani. Metodele de investigaţie au fost reprezentate de determinarea unor parametrii clinici, imagistici, biochimici, hormonali şi imunologici. **Rezultate:** Prevalenţa SPA tip II în grupul de studiu a fost de 6,02% (80% fete şi 20% băieţi, $p = 0,57$, $X^2 = 3,6$). În toate cazurile s-a întâlnit o triplă asociere: insuficienţa corticosuprarenaliană, tiroidita cronică autoimună şi diabetul zaharat tip 1. Nu am avut cazuri de boală Addison simptomatică, doar 5 cazuri de boală Addison asimptomatică, identificate prin prezenţa anticorpilor specifici. **Concluzii:** În cazul grupului de copii studiat, SPA tip II a cuprins 3 afecţiuni: insuficienţa corticosuprarenaliană, tiroidita cronică autoimună şi diabetul zaharat tip 1; nu am constatat diferenţe semnificative statistic între fete şi băieţi. Deoarece boala Addison a fost asimptomatică, identificată prin prezenţa anticorpilor specifici, dacă avem un copil cu una sau mai multe afecţiuni tiroidiene, trebuie să-l investigăm şi pentru alte posibile afecţiuni autoimune.

Cuvinte cheie: diabet zaharat, boală tiroidiană, sindrom poliglandular autoimun tip II, copii

THE SHORT-TERM EFFECT OF ERYTHROPOIETIN ON HEMOGLOBIN DURING ISCHEMIA REPERFUSION INJURY IN RATS

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ABSTRACT

Aim of this experiment study was the erythropoietin testing, on rat model and certainly on ischemia reperfusion protocol. The beneficial or non effect of that molecule was studied hematological on hemoglobin level.

Material and methods: 40 rats were used of mean weight 247.7 g. Hemoglobin levels (hl) were measured on these time points: on 60 min after reperfusion (groups A and C), and on 120 min after reperfusion (groups B and D), A and B without but C and D with erythropoietin administration.

Results were that erythropoietin administration does not increase importantly the hl by 0.305 gr/dl [-0.4277628 g/dl - 1.037763 g/dl] (P = 0.4047), accordant also with paired t-test (P = 0.5008), reperfusion time does not increase importantly the hl by 0.3849999 g/dl [-0.3436895 g/dl - 1.113689 g/dl] (P = 0.2916), accordant also with paired t-test (P= 0.3998) and interaction of erythropoietin administration and reperfusion time does not increase importantly hl by 0.1863636 g/dl [-0.2553986 g/dl - 0.6281259 g/dl] (P = 0.3984).

Conclusions are that erythropoietin administration, reperfusion time and their interaction has not important short – term effect on recovery pathophysiology of hl.

Key words: erythropoietin, hemoglobin, reperfusion

INTRODUCTION

Tissue ischemia and reperfusion remains one from main causes of damage (permanent or transient) with serious implications on near organs and certainly on patients' health. The use of erythropoietin is a well established knowledge a lot of years ago. However, even if important progress has been made, satisfactory answers have not been given yet in fundamental questions, as, by what velocity this factor acts, when should it be administered, and in which dosage. The particularly satisfactory action of erythropoietin in stem blood cells recovery was noted by already performed experiments. It was realized that this certain factor has been tried in ischemia/reperfusion experiments, after international literature (PubMed - Medline) careful examination. However, just few relative reports were found, not covering completely this particular object of action velocity. Also, a lot of publications concerned trial of such particular other molecules of growth factors "family" in which the studied molecule also belongs to.

AIM

Aim of present experimental study was the trial of erythropoietin in rat animal model and certainly in ischemia/reperfusion protocol. It was studied hematological the beneficial or no action of that particular molecule on hemoglobin levels (hl).

MATERIALS AND METHODS

Experimental groups

40 Wistar rats of mean weight 247.7 g [Std. Dev: 34.99172 g] were used, min weight ≥ 165 g and max weight < 320 g. They were naturalized in laboratory for 7 days before experimentation. They had free access in water and food. They were accidentally separated in the following experimental groups (10 animals in each group). The experiment was acute, that is, the animal use was completed by following experimentation time expiry as awakening and preservation did not exist.

1 - Ischemia for 45 min and afterwards reperfusion for 60 min (group A).

2 - Ischemia for 45 min and afterwards reperfusion for 120 min (group B).

3 - Ischemia for 45 min and afterwards immediate erythro-

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poietin IV administration and reperfusion for 60 min (group C).

4 - Ischemia for 45 min and afterwards immediate erythropoietin IV administration and reperfusion for 120 min (group D).

The molecule erythropoietin dose was 10 mg/Kg body weight of animals.

The experiment was beginning by pre-narcosis and general anesthesia administration in animals. Their electrocardiogram and acidometry were continuously monitored. The vessels concerning blood supply were prepared so as their flow to be excluded by forceps. After exclusion, the protocol of ischemia/reperfusion was applied, described more in experimental groups. The molecules were administered immediately after reperfusion through inferior vena cava (catheterization had been preceded at experiment beginning, after general anesthesia establishment).

The hl measuring was performed on these time points:

1 - on 60 min of reperfusion (groups A and C),

2 - on 120 min of reperfusion (groups B and D).

Performance

Introduction into general anesthesia was becoming by initial IM administration of 0.5 cc compound, constituted by 0.25 cc xylazine, [25 cc, 20mg/cc] and 0.25 cc ketamine hydrochloride [1000, 100mg/cc, 10cc]. 0.03 cc butorphanol [10mg/cc, 10cc] anesthesia was administered s.c. before laparotomy. Continuous oxygen supply was administered during whole experiment performance. Ischemia was caused by clapping inferior aorta for 45 min after laparotomic access. Reperfusion was achieved by removing clapping and inferior aorta patency re-establishment.

Control groups

20 control rats (controls: 1 - 20) mean weight 252.5 g [Std. Dev: 39.31988 g] suffered by ischemia for 45 min and then reperfusion.

Group A

Reperfusion which lasted 60 min concerned 10 controls rats of mean weight 243 g [Std. Dev: 45.77724 g], mean hl 13.86 g/dl [Std. Dev: 0.8771166 g/dl] (Table I).

Group B

Reperfusion which lasted 120 min concerned 10 controls rats of mean weight 262 g [Std. Dev: 31.10913 g], mean hl 14.52 g/dl [Std. Dev: 1.111355 g/dl] (Table I).

^{Erythropoietin} group

20 rats (L: 1 - 20) of mean weight 242.9 g [Std. Dev: 30.3105 g] suffered by ischemia for 45 min and then reperfusion in the beginning of which 10 mg erythropoietin /kg body weight were IV administered.

Group C

Reperfusion which lasted 60 min concerned 10 L rats of mean weight 242.8 g [Std. Dev: 29.33636 g], mean hl 14.44 g/dl [Std. Dev: 1.407283 g/dl] (Table I).

Group D

Reperfusion which lasted 120 min concerned 10 L rats of mean weight 243 g [Std. Dev: 32.84644 g], mean hl 14.55 g/dl [Std. Dev: 1.139444 g/dl] (Table I).

Table I. Weight and hemoglobin mean levels and Std. Dev. of groups

Groups	Variable	Mean	Std. Dev
A	Weight Hemoglobin	243 g 13.86 g/dl	45.77724 0.8771166
B	Weight Hemoglobin	262 g 14.52 g/dl	31.10913 1.111355
C	Weight Hemoglobin	242.8 g 14.44 g/dl	29.33636 1.407283
D	Weight Hemoglobin	243 g 14.55 g/dl	32.84644 1.139444

Weight comparison

Weight comparison of each one from 4 rats groups initially was performed with other one from 3 remained groups applying statistical paired t-test (Table II).

Some weight correlations resulted statistically important. Any emerging important difference among hl, will be investigated whether owed in the above mentioned important weight correlations.

Hemoglobin levels (hl) comparison

Hl comparison of each one from 4 rats groups initially was performed with other one from 3 remainder groups applying statistical paired t-test (Table II).

Table II. Statistical importance of mean values difference for groups (DG) after statistical paired t test application

DG	Variable	Difference	p-value
A-B	Weight Hemoglobin	-19 g -0.66 g/dl	0.2423 0.2422
A-C	Weight Hemoglobin	0.2 gr -0.5800001 g/dl	0.9900 0.3868
A-D	Weight Hemoglobin	0 g -0.69 g/dl	1.0000 0.0791
B-C	Weight Hemoglobin	19.2 g 0.0799999 g/dl	0.2598 0.8569
B-D	Weight Hemoglobin	19 g -0.0299999 g/dl	0.1011 0.9637
C-D	Weight Hemoglobin	-0.2 g -0.1099998 g/dl	0.9883 0.8852

Applying generalized linear models (glm) with dependant variable the number of hl and independent variables the erythropoietin administration or no, the reperfusion time and their interaction, results in: 1) erythropoietin administration does not increase importantly the hl by 0.305 g/dl [-0.4277628 g/dl - 1.037763 g/dl] (P= 0.4047), accordant also with paired t-test (P= 0.5008), 2) reperfusion time does not increase importantly the hl by 0.3849999 g/dl [-0.3436895 g/dl - 1.113689 g/dl] (P= 0.2916), accordant also with paired t-test (P= 0.3998), and 3) interaction of erythropoietin administration and reperfusion time does not increase importantly hl by 0.1863636 g/dl [-0.2553986 g/dl - 0.6281259 g/dl] (P= 0.3984).

Reviewing the above and Table II, the Table III turns up concerning the increase influence of erythropoietin in connection with reperfusion time.

Inserting weight as independent variable in glm model, a non important correlation results with the hl (p= 0.4019).

Table III. The increase influence of erythropoietin in connection with reperfusion time

			p-value	
Increase	**95% c. in.**	**Reperfusion time**	**t-test**	**glm**
0.5800001 gr/dl	- 0.5216883 g/dl - 1.681689 g/dl	1h	0.3868	0.2833
0.305 gr/dl	- 0.4277628 g/dl - 1.037763 g/dl	1.5h	0.5008	0.4047
0.0299999 gr/dl	- 1.027464 g/dl - 1.087464 g/dl	2h	0.9637	0.9531

DISCUSSION

Barshishat-Kupper M. et al. (1) administered captopril for 7 days before mice (total body irradiation) TBI which ablates early and late-stage erythroid progenitors, resulted in earlier EPO induction and activation. That short-term mice suppression of radiation-induced EPO immediately after TBI is favorable for erythroid recovery. Weltert L et al. (2) recorded patients with isolated coronary vessel disease mean hemoglobin 15.5% higher in erythropoietin group (P < .05), on postoperative day 4. This group required 0.33 units of blood per patient, whereas the controls required 0.76 units per patient (risk ratio 0.43, P = .008). No adverse events related to erythropoietin administration were recorded. Gardin C et al. (3) used high-dose erythropoietin to reduce or suppress red blood cell transfusions needs in selected subgroups of myelodysplastic syndromes MDS. Ferber A et al. (4) did not detect rise in plasma erythropoietin (EPO) until 4 to 6 hours after the initiation of hypoxia. One hundred low-risk pregnancies with a normal fetal heart rate at admission to labor and delivery were eligible for participation. Deliveries for "non-reassuring fetal status (acute hypoxia)" comprised the study group. All other deliveries served as controls. Umbilical cord blood was prospectively collected for blood gas analysis, NRBC counts, EPO. A significant association between elevated NRBC counts and EPO (P values <.01) was demonstrated. Stepwise regression analysis identified EPO as independent variables associated with elevated NRBC counts at birth (P values <.0001). A significant increase in NRBC counts was noted in study patients. There was no difference in EPO between groups. The NRBC counts were elevated in fetuses who were delivered for "non-reassuring fetal status" with EPO being normal. Fujishiro N et al. (5) studied the long-term response of mammals to hypoxia in the erythropoietin production increase with the consequent increase in red blood cells. Vatansever U et al. (6) used 69 high-risk neonates; 37 control group healthy term infants. Three blood samples were obtained from each infant within 12 h (initial), 3 days and 7 days after birth to measure NRBC counts and EPO levels. The initial NRBC counts were significantly lower in the control groups compared with the study groups (P = 0.002). While there was no significant difference between patients with good and poor outcome in terms of serum EPO concentrations of initial samples, a significant difference existed in terms of NRBC counts (P = 0.038) in the circulation of neonates associated with states of relative hypoxia. Singbartl G et al. (7) shown erythropoietin to be effective both in the reversal of anaemia in patients with end-stage renal failure and to increase the volume of autologous red blood cells donated preoperatively as well as to decrease the units of homologous blood transfused. Hara H et al. (8) examined serial changes in erythropoietic precursors in the femur, spleen, and blood of mice prepared with bleeding, erythropoietin injections. The transient decline in the femoral erythropoietic burst-forming units BFU-E coincided with the temporary increase in the splenic and blood BFU-E. A more pronounced increase in erythrocytic colony-forming units CFU-E was noted in the femur and spleen of these mice. Next, we examined the proliferative state of the erythropoietic precursors in the marrow and spleen. In the marrow and spleen of normal mice, the BFU-E and CFU-E in the DNA synthetic phase was about 36 and 74%, respectively. These results indicate that the serial changes in the number of BFU-E represent migration of BFU-E from marrow to spleen rather than BFU-E proliferation. Marrow CFU-E increased in anemic mice and decreased in polycythemic mice without changes in their proliferative state. It is possible that the target of erythropoietic stimulation in mice may be cells at maturational stages intermediate between BFU-E and CFU-E. Shoji S et al. (9) demonstrated that although darbepoetin-α DPO-α has a greater advantage than recombinant human erythropoietin rHuEPO (P = 0.030), both maintained hemoglobin level in the target range between 10.0 and 11.0 g/dL in hemodialysis (HD) patients. Emans ME et al. (10) correlated neutrophil-gelatinase associated lipocalin NGAL levels inversely with baseline EPO levels but they were decreased in response to short-term erythropoietin stimulating agent (ESA) treatment. There was no correlation with baseline reticulocyte hemoglobin content (CHr) levels in combined chronic heart failure and chronic kidney disease CKD/ CHF patients. Chung M et al. (11) suggested that CHr (with cutoff values of <27 or <28 pg) have better sensitivities and specificities to predict iron deficiency, better predictive ability for a response to IV iron treatment than classical markers in HD CKD patients, reducing potential harms from IV iron treatment. Golfam M et al. (12) observed no significant decline in serum haemoglobin or erythropoietin among patients with localized prostate cancer treated with radiotherapy alone. That change was followed by a significant decline (p < 0.001) in serum hemoglobin at 3-6 months among patients treated with radiotherapy combined with total androgen blockade (tab) either short-term (≤6 months) tab (group 2) or long-term (≥2 years) (group 3). They also observed a small but statistically significant increase in serum erythropoietin (p < 0.001) in group 2 and group 3 after 6 months of tab. Sureshkumar KK et al. (13) did not show any clinically demonstrable beneficial effects of high-dose erythropoietin-a EPO-α versus placebo given intra-arterially during the early reperfusion phase in deceased-donor kidney (DDK) transplant recipients in

terms of reducing the incidence of delayed graft function (DGF) or improving short-term allograft function (P=0.24) having similar levels of hemoglobin at multiple times points soon after transplantation. Kojima E et al. (14) noted improved hemoglobin levels and reduced erythropoietin-stimulating agent after home hemodialysis HHD. Martinez-Vea A et al. (15) observed mean Hb levels increased ($p < 0.0001$) in anemic predialysis patients with chronic kidney disease CKD and hemoglobin (Hb) levels < 11 g/dl treated with EPO for 6 months. Pottgiesser T et al. (16) showed that following a hypoxic intervention with a beneficial Hb outcome (increase by 5.5% after 26 nights altitude camp), there may be a high probability of a rapid Hb decrease (decrease by 3.0% at day 9 and decrease in serum EPO (-34%) at 2 d, after return to sea level) upon return to normoxic conditions. Wang FD et al. (17) indicated that 2 U/ml recombinant human erythropoietin rhEPO may possess curative effect for anemia of chronic disease (ACD) disease. During short-term follow-up of treated patients with multiple myeloma the Hb level is stable, the influence of patients serum on hepcidin mRNA of Hep-3b cells decreases, which shows the stabilization of disease and amelioration of ACD patient status. Taniguchi N et al. (18) showed no significant changes of hemoglobin after 24h and 48h short-term `low-dose EPO' 6,000 IU in patients with a first ST-elevated acute myocardial infarction (AMI) undergoing percutaneous coronary intervention (PCI) who was randomly assigned to EPO or placebo (saline) treatment. Ishikawa Y et al. (19) did not note difference between groups received perioperative oral nutrition (ON) with branched chain amino acids BCAA (BCAA group) or a usual diet (control group) in serum hemoglobin levels after operation among patients with either malignant or benign liver tumors. Among patients with hepatitis, serum erythropoietin (EPO) levels on POD 3, 5, and 7 were slightly but not significantly higher in the BCAA group than in the control group. Among patients with non-hepatitis, serum EPO levels on POD 3, 5, and 7 were significantly higher in the BCAA group than in the control group. Higher EPO levels might be beneficial in protecting liver cells from ischemic injury and preventing intraoperative hemorrhage associated with lower perioperative levels of alanine aminotransferase and aspartate aminotransferase in serum. Tsubakihara Y et al. (20) established a target Hb level in PD and ND patients of 11 g/dL or more, and recommended 13 g/dL as the criterion for erythropoiesis stimulating agent (ESA) therapy dose reduction/withdrawal. Cohen RS et al. (21) evaluated the short-term clinical effect of subcutaneous erythropoietin injections with an average weekly dose of 3926 units in patients with heart failure anemia with preserved ejection fraction (55%+2%) to affect a rate of rise in hemoglobin but not to exceed 0.4 g/dL /weekly during a 3-month study. Weltert L et al. (22) did not found significant difference in mean preoperative hemoglobin levels, but on postoperative day 4, mean hemoglobin was 15.5% higher in erythropoietin group ($P<0.05$) between patients presenting with a diagnosis of isolated coronary vessel disease randomized to either high-dose erythropoietin therapy for very short-term administration or a control group. Kusaba T et al. (23) observed the Hgb value of patients having increased significantly (10.3 + 1.2 to 10.6 + 1.4 g/dl) 2 months after switching from epoetin-a to darbepoetin-a so as to control the hemoglobin (Hgb) value between 10 and 12 g/dl. Darbepoetin-a increased the Hgb value effectively in HP in this short-term analysis. Bradbury BD et al. (24) attributed to confounding-by-indication for higher doses the short-term mortality related to high EPO doses associated with 90-day mortality risk (Risk Difference, RD = 3.0%) in unadjusted analyses but after adjustment for confounding (RD = 1.5%) following Hb < 11 g/dL for HP who received care for >4 consecutive months. Silver MR et al. (25) demonstrated that de novo every-other-week (Q2W) median darbepoetin-a 60 - 80 microg was effective in correcting and maintaining Hb levels > 11 g/dL in a median time of 5 weeks in erythropoiesis-stimulating agent (ESA)-naïve chronic kidney disease CKD subjects not receiving dialysis. Capelli JP et al. (26) did not prove that higher erythropoietic stimulating agent dose levels were associated with higher mortality rates. Although, locally, there was a 69-fold increase, nationally was a 4-fold increase, they indicated that individually higher Hgb and albumin levels are associated with increased survival, and when higher Hgb levels are in association with high albumin levels, the survival rates and hospitalizations are synergistically improved. Li SX et al. (27) showed that hemoglobin of two pure erythroid aplasia patients obviously increased after treatment with rhbeta-EPO 6 000 U by subcutaneous injection for 3 times per week combined with AMF. Fauchère JC et al. (28) did not result in significant differences in hemoglobin of very preterm infants (gestational age: 24 to 31 weeks) after recombinant human erythropoietin or NaCl 0.9% treatment given intravenously 3, 12 to 18, and 36 to 42 hours shortly after birth. Winslow RM et al. (29) found the two ways of O_2 transport regulation hemoglobin involvement: a long-term adjustment in red cell mass mediated by erythropoietin (EPO), a response to renal oxygenation, and a short-term, rapid-response adjustment mediated by hemoglobin oxygen affinity. Homoncik M et al. (30) alleviated the decrease in hemoglobin combining ribavirin antiviral therapy with erythropoietin (EPO) treatment ($p < 0.0001$). González AJ et al. (31) noted significantly increased overall EPO levels at 12 (70%; $P = 0.0001$) and 24 h (72%; $P = 0.0001$) above baseline concentration following exposure to moderate 2200 m altitude, and thereafter, decreased EPO concentration at 48 h, but a significant increase in Hb levels (4.6 + 4%; $P = 0.0001$) was observed at the end of the experiment, suggesting negative feedback in male endurance athletes. Lalle M et al. (32) showed a median level of 0.73 g/L hemoglobin increase and a mean level of > 1 gr/L hemoglobin increase from baseline in patients affected by solid tumors who received epoetin-a 40000 U once weekly. Cusick SE et al. (33) noted that vitamin A reduced erythropoietin (by 194.7 mIU/mL; $P = 0.011$) concentrations and increased the reticulocyte production index (by 0.40; $P = 0.041$) after 72 h than baseline in severely anemic (hemoglobin < 70 g/L) preschool children assigned to receive either vitamin A (100,000 or 200,000 IU depending on age) or the antimalarial drug sulfadoxine pyramethamine (SP) plus daily hematinic syrup for 90 d. Lu XC et al. (34) increased the hemoglobin values in peripheral blood after 5 x 0.4 g amifostine plus 3 x 6,000 U rh-EPO treatment per week in 2 aged MDS-RCMD patients. Sapojnikov M et al. (35) showed improvement in arterial stiffness when Hb is

corrected after 3 months with 2000 units rHuEpo treatment intravenously followed by 80 to 120 s/c units/kg/body weight, with dosage titration according to Hb level in patients with chronic renal failure anemia who were not receiving dialysis. Casino FG et al. (36) screened all maintenance hemodialysis (HD) patients with Hb < 11 g/dL at a single unit, to establish the presence/absence of any common cause associated with inadequate response to epoetin treatment, reaching the Hb target (> 11 g/dL) in at least 85% of all patients in the unit. Sohmiya M et al. (37) reported that short-term continuous subcutaneous infusion (CSI) of recombinant human growth hormone (rhGH) increased plasma erythropoietin levels and hemoglobin concentrations in patients with adult GH deficiency for 1 year. Katz SD (38) invented a subnormal compensatory rise in endogenous erythropoietin levels in response to anemia is one contributory factor in chronic heart failure after randomized trials with recombinant human erythropoietin therapy in anemic patients with chronic kidney and concomitant heart disease. Shander A (39) found that from critically ill patients randomized to receive 40,000 units of exogenous erythropoietin had significantly greater increases in hemoglobin than placebo. Warady BA et al. (40) found a significant decreased dose of r-HuEPO (234.0 to 157.6 U/kg per week, P=0.046) and an increased CHr (29.2 to 30.1 pg, P=0.049) only in i.v. iron, these changes were not significantly different from those experienced by iron-replete pediatric patients (aged >1 to <20 years) in the oral iron group up to 16 weeks, with end-stage renal failure in patients receiving hemodialysis for >2 months. Katz SD et al. (41) found that darbepoetin-a is a glycosylated derivative of erythropoietin with a prolonged half-life that may allow less frequent dosing in chronic heart failure CHF populations. Rocha VL et al. (42) counted final hemoglobin values of prematurity anemia newborns who did not receive erythropoietin significantly lower than those of newborns who received either seven daily doses of 100 U/kg erythropoietin per week; or two 350 U/kg erythropoietin doses per week. There was no significant difference between 2 last patients groups. The administration of 700 U/kg/week erythropoietin in premature infants with gestational age up to 33 weeks and birthweight up to 1550 g and postnatal age between 10 and 35 days stimulates erythropoiesis. David RB et al. (43) examined the hypothesis that long-term hypoxic stimuli are less efficient than short-term stimuli in stimulating Epo production in perinatal pigs. They found that in nearly fully developed fetuses and in new-born piglets, the concentration of Epo mRNA did not increase upon bleeding and did not observe significant changes in gene Epo expression. In 2- and 5-week-old piglets, bleeding was associated with a 12-15-fold increase in kidney Epo mRNA evoking increased translation of Epo mRNA into the protein hormone. Małyszko J et al. (44) resulted in a significant hemoglobin increase significantly after 3-month 2,000 U erythropoietin therapy subcutaneously three times a week on continuous ambulatory peritoneal dialysis (CAPD) than healthy volunteers control group. Borawski J et al. (45) induced increases in hemoglobin after a 4-week course of recombinant human erythropoietin (rHuEpo) therapy. Bolaños L et al. (46) suggested that hemodialysis patients in the maintenance phase of EPOrHu administration would obtain further benefit in terms of serum hemoglobin level ($p < 0.05$), with a continuous intravenous serum ferric gluconate regimen than intermittent group, at least in the short term of 16 weeks. Kato A et al. (47) found significantly increased hemoglobin levels after 4 weeks of intravenous infusion of 40 mg of iron during the first ten HD sessions and remained increased until the end of the study ($p<0.01$) in HD patients with iron deficiency resistant due to iron deficiency and adjusted for the rHuEPO dosage to maintain hemoglobin levels >10.0 g/dl. They found gradually increased hemoglobin levels in patients receiving 40 mg of iron injected once a week for 10 weeks until the end of the study ($p<0.01$), without difference in the final hemoglobin values between both groups. Lin JL et al. (48) supposed that a 240-mg intravenous iron challenge during a 2-week period might be a simple, accurate, and straightforward method to detect a functional iron deficiency status in anemia end-stage renal disease hemodialysis patients undergoing erythropoietin therapy increasing hemoglobin at least 0.2 g/dl after a 2-week intravenous iron trial. Aksoy MC et al. (49) showed that short-term and low-dose recombinant human erythropoietin usage strongly stimulates the bone marrow but without any significant differences between that group and placebo in terms of early postoperative hemoglobin level for patients scheduled for a total hip arthroplasty. Sezer S et al. (50) concluded that CAPD treatment has a short-term outcome superior to that of HD in terms of better control of anemia, seeing a significant increase in mean haemoglobin and a decrease in erythropoietin dose. Bohl D et al. (51) indicated that the continuous delivery of high amounts of autologous erythropoietin induced a sustained stimulation of β-minor globin synthesis and a stable improvement of erythropoiesis in β-thalassemic mouse model but only short-term erythroid precursors programmed to HbF in humans. Wong PN et al. (52) seemed that rHuEPO is effective in increasing the Hb level in CAPD patients. Gupta A et al. (53) stabilized erythropoietin on regular doses, since hemoglobin did not change significantly upon month 6, by both intravenous (i.v.) iron dextran or after oral iron supplements at maintenance hemodialysis patients. Díez JJ et al. (54) observed an increase of the hemoglobin concentration maintained at about 12 g/dL throughout the study period in uremic patients undergoing continuous ambulatory peritoneal dialysis (CAPD) than 10 normal controls after long-term rhEPO administration. Gargano G et al. (55) found hemoglobin values remaining normal at all patients operated for gynecological tumors receiving r-HuEPO subcutaneously in a dose of 200 IU/kg thrice weekly during the week before and after autologous blood donation (400 ml).

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EFECTUL PE TERMEN SCURT AL ERITROPOIETINEI ASUPRA HEMOGLOBINEI IN TIMPUL ISCHEMIEI DE REPERFUZIE LA SOBOLANI

REZUMAT

Scopul acestui studiu experimental a fost testarea eritropoietinei pe un model animal de sobolan, folosind un protocol de ischemie-reperfuzie cardiac. Efectele benefice sau lipsa efectului acestei substante au fost studiate din punct de vedere hematologic prin evidentierea nivelului hemoglobinei.

Materiale si metode: au fost folositi 40 de sobolani cu greutate medie de 247,7 g. Nivelul hemoglobinei (hl) a fost masurat la urmatoarele interval in timp: la 60 min dupa reperfuzie (grupurile A si C), la 120 min dupa reperfuzie (grupurile B si D), grupurile A si B fara administrare de eritropoietina, grupurile C si D cu administrare de eritropoietina.

Rezultatele au aratat ca administrarea de eritropoietina nu duce la cresteri importante ale hl, doar cu 0.305 g/dl [-0,4277628 g/dl – 1,037763 g/dl] (P = 0,4047), in concordant cu “testul t” pereche (P = 0,5008); timpul de reperfuzie nu creste semnificativ hl, doar cu 0.3849999 g/dl [-0,3436895 g/dl – 1,113689 g/dl] (P = 0,2916), in concordanta cu “testul t” pereche (P = 0,3998); interactiunea dintre administrarea de eritropoietina si timpul de reperfuzie nu duce la cresteri importante ale hl, doar cu 0,1863636 g/dl [-0,2553986 g/dl – 0,6281259 g/dl] (P = 0,3984).

Concluzia acestui studiu este ca administrarea de eritropoietina, timpul de reperfuzie si inter-relatia dintre acesti doi parametric nu are efecte importante pe termen scurt asupra fiziopatologiei de recuperare a nivelului de hemoglobina.

C**uvinte cheie:** eritropoietina, hemoglobina, reperfuzie

COMPARATIVE STUDY CONCERNING THE EFFICIENCY OF PHYSICAL TRAINING AT HYPERTENSIVE PATIENTS

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ABSTRACT

Arterial hypertension is a condition that causes about half of all deaths caused by strokes and heart failure with a prevalence in Romania of about 40% of the adult population. Practicing physical exercises represents a key factor, preventive and curative in fighting hypertension.

Purpose: The determination of the cardiovascular parameter change and arterial stiffness after constantly practicing physical and coordinated activities in the form of physical exercises, at hypertensive patients.

Material and methods: The study comprise 64 patients with first and second stage hypertension, according to the ESC/ESH 2007 guidelines, with the average age of 45.6 years. The patients were divided into two groups: group A-study group: 45 patients, that performed a physical training programme for 3 months, and group B - control group: 29 patients. We have investigated the effects of physical training on arterial elasticity - aortic pulse wave velocity (PWVao), and the evolution of the tension parameters: the systolic blood pressure (SBP), the diastolic blood pressure (DBP), the mean arterial pressure (MAP) and the pulse pressure (PP). These parameters were evaluated before and after a physical training programme of 3 months.

Results: At the end of the training programme, we have obtained changes regarding the tension parameters and arterial elasticity. PWVao significantly decreased in the study group with 0.8 m/s ($p<0.001$) and significantly increased in the control group by +0.7 m/s ($p<0.001$). MAP significantly decreased in the study group with -6.2 mmHg ($p<0.001$) and significantly increased in the control group by 8.7 mmHg ($p<0.001$). SBP significantly decreased in the study group with -10.2 mmHg ($p<0.001$) and significantly increased in the control group by 15.6 mmHg ($p<0.001$). DBP decreased in the study group with -3.2 mmHg ($p<0.010$) and increased in the control group by 5.2 mmHg ($p=0.065$). PP significantly decreased in the study group with -5.9 mmHg ($p<0.001$) and significantly increased in the control group by + 9.9 mmHg ($p=0.007$).

Conclusions: The decreased parameters presented above highlight the major effect that coordinated physical exercise has on the arteries, permanently controlled and continuously executed in order to improve their elasticity with immediate influence on physical adaptation to physical training and long-term effects on blood pressure: its decrease (maintaining and even reducing arterial age).

Keywords: physical aerobic exercises, arterial elasticity, pulse wave velocity, blood pressure.

INTRODUCTION

Arterial hypertension represents an important cause of morbidity and disability of the productive adult population and is also a risk factor for various diseases: coronary atherosclerosis, heart failure, strokes, chronic renal failure, leading to a decrease in the average lifetime.

According to the SEPHARD II study in Romania, the prevalence of arterial hypertension (HTN) is of 23.1% between 35-44 years, 49.7% between 45-54 years, 65.8% between 55-64 years and 81%≥65 years, increasing with age, independent of the gender and residential area of the patient (1).

The arterial stiffness is an independent indicator of cardiovascular morbidity and mortality at hypertensive patients being a generic term that describes the elasticity of the arterial wall (2). The arterial rigidity, in particular that of the aorta, is a major risk factor in the development of cardiovascular disease, and correlates with the overall mortality. The loss of arterial stiffness, determines a pulse pressure increase, an independent marker of cardiovascular risk, especially at elderly persons (3).

For the evaluation of the arterial elasticity the pulse wave velocity (PWV) must be determined. PWV is accepted as a simple, direct and non - invasive method of examining arterial stiffness, currently considerated an independent predictor of cardiovascular mortality (4).

Age plays a dominant role in altering vascular stiffness, pulse pressure and pulse wave velocity being genetically conditioned and influenced by lifestyle: sedentary, food, stress. Physical exercise contributes substantially to improving vascular elasticity and the marked reduction of the arterial stiffening processes induced by sedentary and improper lifestyles (5).

It is proved that regular physical exercises have a good effect in lowering blood pressure at hypertensive patients. In

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controlled clinical studies, it has been demonstrated that by performing regular exercises, you can decrease the systolic blood pressure by 5-15 mmHg (an average decrease of 5.3/4.8 mmHg)(6).

The lack of physical movement, inactivity in everyday life and especially the lack of coordinated exercise, as an integrated way of life existence, (regular exercises for at least 2-3 times per week) contribute greatly to the installation of chronic diseases with reflection on some vital organs, one of them being the arterial hypertension, whose negative influence over time substantially reduces lifetime (7).

PURPOSE

The determination of the cardiovascular parameter change (systolic blood pressure, diastolic blood pressure, mean arterial pressure, pulse pressure) and arterial stiffness (pulse wave velocity) after constantly practicing physical and coordinated activities in the form of physical exercises, at hypertensive patients.

The evaluation of the hemodynamic parameters and vascular elasticity: systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP) and pulse wave velocity (PWV), was made by an personalized physical exercise programme training lasting three months and assessed objectively through a suitable standardized apparatus that measures these parameters objectively.

The determinations were made in several steps:

- initial measurements were made at all patients included in the physical study programme,
- at the patients belonging to the control group, the measurements were made at the beginning and at the end of the study (3 months) during which they have not performed physical exercises in the study,
- current measurements of pulse and blood pressure before and after every workout
- initial measurements were repeated at the end of the study programme for 3 months, for both groups: study and control.

OBJECTIVES

- comparative study of hypertensive patients with or without a coordinated physical programme performed
- voluntary participation in an individualized and coordinated physical training for a period of 3 months,
- identification of the hemodynamic parameters and arterial stiffness.
- establishment of an efficient, uniform and graded physical training programme for patients with hypertension.
- to improve the physical performance at hypertensive patients included in the group,
- to lower the cardiovascular risk for the persons included in the study group.
- the greater adherence of patients to an active life, to a cardiovascular rehabilitation programme.

MATERIAL AND METHOD

We have selected 64 patients with first and second stage hypertension, according to the ESC/ESH 2007 guidelines, with the average age of 45,6 years. These patients had controlled tension values and did not suffer any therapeutic changes of the treatment scheme with at least two months before the start of the physical training programme. The patients were divided into two groups: group A-study group: 45 patients and group B – control group: 29 patients.

The patients involved in the study were selected from the point of view of the inclusion/exclusion criteria, as well as from the point of view of the engagement in the physical training programme.

The inclusion criteria

Patients with blood pressure values without treatment that had a systolic arterial blood pressure between 140-180 mmHg and a diastolic arterial blood pressure of 85-110 mmHg. The patients were kept under tension control (average systolic arterial blood pressure ≤ 140mmHg and/or diastolic arterial blood pressure ≤ 90 mmHg) through proper medication in order for them to avoid presenting risks to the physical exercise programme. Cooperative patients and those willing to participate in physical training, were people whose physical activity enables them to be put to physical effort testing.

Exclusion criterias

Patients with uncontrolled blood pressure values, uncooperative patients, people who can't be put to physical effort testing and/or physical training. Patients who refused to participate for various reasons at the physical exercise programme. Patients with various degrees of physical disabilities. Patients with severe cardio-vascular diseases associated with hypertension.

The haemodynamic parameters followed in the study are: *systolic blood pressure, diastolic blood pressure, heart rate, mean arterial pressure, pulse pressure, aortic pulse wave velocity*.

The physical effort test plays an important role in the evaluation and implementation of physical training. Depending on the heart rate and exercise intensity reached at the physical effort testing, the physical exercise training programme is determined (8). Due to the large experience in this type of physical effort, the use of ergometry at ergocycle exercises was preferred (9).

We used the TensioMed *Arteriography* device evaluated as having a good performance in the assessment of the parameters regarding its supposed purpose (10). This device offers us information about the arterial function with a pulse wave analysis and measurement of the arterial stiffness. Patients are not allowed to talk or sleep during the measurement. All the patients were tested in a dorsal position, by applying the sleeve at about the same level as the arm of each study participant. For the initial and final assessments, the measurements were made at the same time of the day and in the same position. Before the evaluation the patients had to follow some standard measures of rest, smoking, alcoholic beverages (11).

The statistical analysis was performed using the Microsoft Office Excel XP and the SPSS v.17 program. For the numeric

variables we calculated the central tendency and the dispersion indicators and we presented them as histograms and line charts; the differences between the independent variables were analyzed using the ANOVA test followed by the unpaired-t parametric significance. The differences between the variables originating from the same patients were analyzed using the paired-t test.

The patients from the control group did not participate in the physical training programme.

The patients from the study group (considered suitable) performed a physical training programme for 3 months as follows:

- 3 physical training sessions/week, adapted for people with essential arterial hypertension without cardiovascular related diseases, following a complex protocol based on the individual characteristics of each patient,

- the duration of the sessions was 50 minutes and consisted of:

• warm-up for 12 to 15 minutes,

• the actual physical exercise 25-30 with an intensity of aproximately 80% of the cardiac frequency (CF) achieved during the physical effort test,

• 8-10 minutes recovery after the physical effort, *stretching exercises to* reduce neuromuscular excitability at mild intensity.

- the type of physical exercises performed: dynamic, aerobic, endurance physical exercises as well as effort capacity development (cardio-respiratory) and isometric exercises performed with low weights.

- the intensity of the physical effort performed corresponded to the 80% percent of the intensity reached at the physical effort testing performed at the start of the training programme. The keeping of this intensity was achieved by maintaining about 80% of the maximal heart rate regarding age and 80% of the maximum systolic arterial blood pressure allowed, both achieved during the physical effort testing performed beforehand.

At the end of training the diastolic and systolic arterial blood pressure and the heart were checked using the tensiometer.

The physical exercise room was equipped with: treadmills; ergometer training bicycles with a resistive electromechanical torque; elliptical bicycles; complex systems of resistive torques (gravitational and elastic) for the arms, hands, legs and torso (abdomen); wall bars, medicine balls, barbells and light dumbbells, sand bags; synchronous asynchronous defibrillator; emergency kit with emergency intubation and medication.

After two weeks of physical training, from the initial group of 45 patients, one was excluded (drop out), because he changed his residence.

RESULTS

The patients were reviewed at the end of the three months of training. In the following tables the most significant changes I have obtained have been presented for both groups - study and control:

Table I. Pulse wave velocity

Variable	Initial	After 3 months	$p^{semnif.}$
Pulse wave velocity **PWVao** (m/s) **Study**	9.7 ±2.17	8.9±2.06	**<0.001**
Pulse wave velocity **PWVao** (m/s) **Control**	9.2 ±2.14	9.9 ±2.06	**<0.001**

Average value ± standard deviation

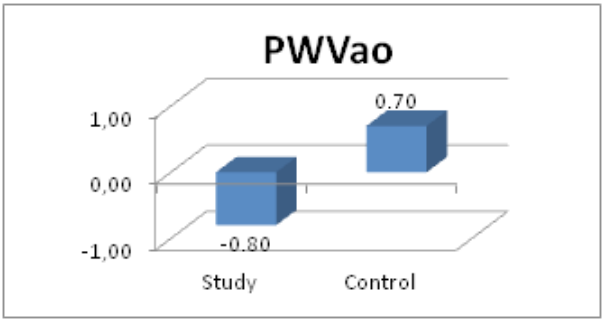

Fig. 1. The PWVao average value distribution at the initial moment and the final moment, after 3 months for both groups

There is a significant decrease in the pulse wave velocity (PWVao) of -0.8 m/s after 3 months of physical training ($p<0.001$, $\alpha=0.001$) in the study group and a significant increase of +0.7 m/s ($p<0.001$, $\alpha=0.001$) in the control group.

Table II. Systolic blood pressure

Variable	Initial	After 3 months	$p^{semnif.}$
Systolic blood pressure **SBP** mmHg **Study**	136.5±15.22	126.3±13.38	**<0.001**
Systolic blood pressure **SBP** mmHg **Control**	131.1 ±20.49	146.7±22.43	**<0.001**

Average value ± standard deviation

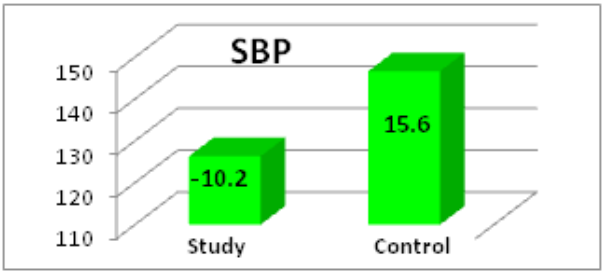

Fig. 2. The SBP average value distribution at the initial moment and the final moment, after 3 months for both groups

The systolic blood pressure (SBP) significantly decreased after 3 months of training in the study group, in statistical terms by -10.2 mmHg ($p<0.001$, $\alpha=0.001$) and significantly increased in the control group + 15.6 mmHg ($p<0.001$, $\alpha=0.001$).

Table III. Diastolic blood pressure

Variable	Initial	After 3 months	$p^{semnif.}$
Diastolic blood pressure **DBP** mmHg **Study**	84.0±10.18	79.8±10.61	**0.010**
Diastolic blood pressure **DBP** mmHg **Control**	81.9 ±15.74	87.2±14.70	0.065

Average value ± standard deviation

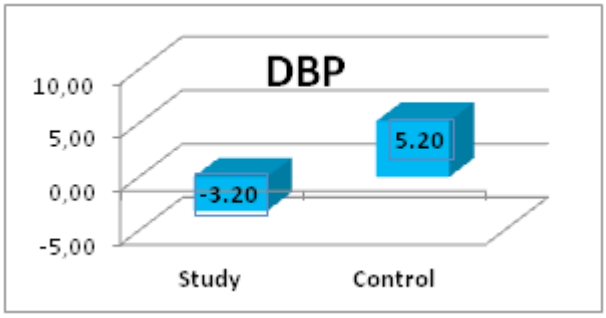

Fig. 3. The DBP average value distribution at the initial moment and the final moment, after 3 months for both groups

A significant decrease of -3.2 mmHg of the diastolic blood pressure (DBP) was noticed in the study group after ending the three months of physical exercise training (p=0.01, α=0.05), but in the control group even if the increase was by +5.3, it has no significance value regarding the survey.

Table IV. Mean Arterial Pressure

Variable	Initial	After 3 months	$p^{semnif.}$
Mean Arterial Pressure **MAP** mmHg **Study**	101.5±11.13	95.3±10.69	**<0.001**
Mean Arterial Pressure **MAP** mmHg **Control**	98.2 ±16.91	106.9±15.70	**<0.001**

Average Value ±Standard Deviation

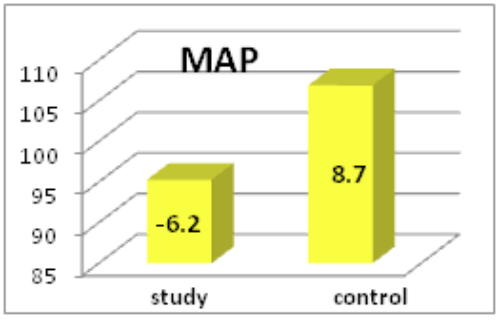

Fig. 4. The MAP average value distribution at the initial moment and the final moment, after 3 months for both groups

In the study group, the Mean Arterial Pressure (MAP) significantly decreased statistically after 3 months by -6.2 mmHg (p<0.001, α=0.001). In the control group, the MAP significantly increased by +8.7 mmHg (p<0.001, α=0.001).

Table V. Pulse pressurre

Variable	Initial	After 3 months	$p^{semnif.}$
Pulse Pressure **PP** mmHg **Study**	52.4 ±10.42	46.5±9.51	**0.001**
Pulse Pressure **PP** mmHg **Control**	49.5 ±9.64	59.4 ±17.22	**0.007**

Average Value ±Standard Deviation

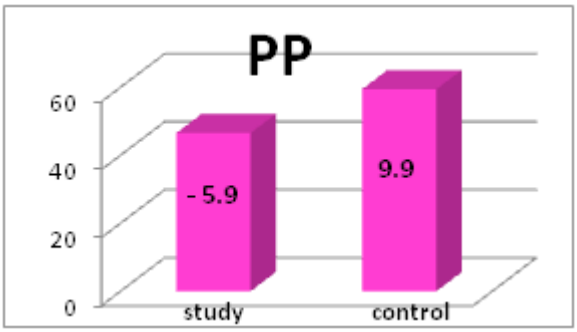

Fig. 5. The PP average value distribution at the initial moment and the final moment, after 3 months for both groups

We have obtained significant results regarding the pressure pulse value (PP) which decreased by -5.9 mmHg, under the action of physical training (p=0.001, α=0.01) in the study group, but in the control group, the value significantly increased with +9.9 mmHg (p=0.007, α=0.01).

DISCUSSION

At the end of the physical exercise training, the study group improved their arterial tension and elasticity parameters in contrast with the control group as follows: the pulse wave velocity in the aorta (PWVao) significantly decreased in the study group with -0.8 m/s (p<0.001) and significantly increased in the control group by +0.7 m/s (p<0.001), the systolic blood pressure (SBP) significantly decreased in the study group with -10.2 mmHg (p<0.001) and significantly increased in the control group by +15.6 mmHg (p<0.001), the diastolic blood pressure (DBP) decreased in the study group with -3.2 mmHg (p = 0.010) and increased in the control group with +5.2 mmHg (p0.065), the pulse pressure (PP) significantly decreased in the study group with -5.9 mmHg (p = 0.001) and significantly increased in the control group by +9.9 mmHg (p = 0.007), the mean arterial pressure (MAP) significantly decreased in the study group with -6.2 mmHg (p< 0.001) and significantly increased in the control group by +8.7 mmHg (p < 0.001).

Although the pulse pressure (PP) has an average variation assessed in an absolute value, the effect of this parameter as a reflection of arterial elasticity introduced significant changes

to the time course of blood pressure, due to the micro-vascular effects at endothelium molecular level (12). According to the last ESC/ESH guide a high PP is one of the important factors of evaluation of cardiovascular risk, associated with the asymptomatic organ damage.

All the results obtained in this study completes the development of the idea issued by past ESC/ESH studies, that demonstrated the fact that aerobic physical exercise contributes in a beneficial way to the improvement of vascular elasticity, low blood pressure and therefore leading to the life extension of hypertensive patients (13,14,15).

It seems that the aerobic physical exercise acts in a complex way on the blood vessels and in addition to the direct action on them, it also has a dual action with other risk factors that diminish them, especially on the lipid metabolism and on the mechanisms involved in the atheromatous deposits . Effect of interval training programme on pulse pressure in the management of hypertension: a randomized controlled trial

PWVao is the noninvasive method best correlated with the arterial stiffness (16, 17), and the reduction of this parameter with -0.8 m/s confirms the expected aim of this study regarding that controlled physical effort decrease arterial stiffness over time.

The beneficial influence of coordinated physical effort was felt on the pulsatile component of blood pressure (pulse pressure) and as the pulse pressure represents an independent predictor from the cardiovascular disease in the asymptomatic population (18), it can be concluded that physical training favorably influences the vascular compliance of elastic arteries and thus slowing the vascular stiffening process (19,20).

Sedendary people who do not practice systematic physical movement or effort are inclined to a more rapid deterioration of the arterial wall elasticity (21). Hypertensive patients who live a sedentary life, have a higher probability of getting strokes than trained people, due to the stiffness of the vascular system (22).

Vascular aging represented by the arterial stiffness over time was positively influenced by the physical effort programme, and this was objectively appreciated by the PWVao with a significantly decreases of -0.8m/s and the PP with a significantly decreased of 5,9 mmHg, evaluated in the study.

Also during the cardiovascular rehabilitation programme, no physical incidents and major cardiovascular changes were reported, that could have affected the smooth process of the specific exercises included in the study.

The study opened a new horizon regarding lifestyle changes making the patients become more responsive to the importance of physical movement in their existence, making them aware of the fact that physical training is beneficial both for their health as well as for their physical and mental tone.

CONCLUSIONS:

The decreased parameters presented above highlight the major effect that coordinated physical exercise has on the arteries, permanently controlled and continuously executed in order to improve their elasticity with immediate influence on physical adaptation to physical training and long-term effects on blood pressure: its decrease (maintaining and even reducing arterial age).

Physical training program is an effective non - pharmaceutical method in delaying the arterial stiffness for hypertensive patients. The exercise training program has proven effective in improving the physical performance, being also considered a safe method for reducing the cardiovascular risk.

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STUDIU COMPARATIV PRIVIND EFICIENȚA ANTRENAMENTULUI FIZIC LA PACIENȚII HIPERTENSIVI

REZUMAT

Hipertensiunea arterială reprezintă o afecţiune care provoacă în jur de jumătate din totalul deceselor de accident vascular cerebral şi insuficienţă cardiacă cu o prevalenţă în România de aproximativ 40% din populaţia adultă. Exerciţiul fizic practicat constant reprezintă un factor determinant, preventiv şi curativ în lupta cu hipertensiunea arterială

Scop: Determinarea modificărilori parametrilor cardiovasculari şi de rigiditate arterială în urma practicării activitităţii fizice constante şi coordonate sub forma exerciţiilor fizice, la pacienţi hipertensivi.

Material şi metode: Am selectat 64 de pacienţi cu hipertensiune arterială de stadiul unu şi doi, conform ghidurilor ESC/ESH 2007 cu media de vârstă de 45,6 ani. Pacienţii au fost repartizaţi în două loturi: lotul A-studiu: 45 de pacienţi şi lotul B – control: 29 de pacienţi. În grupa de studiu, pacienţii au fost introduşi într-un program de exerciţii fizice aerobe pe o perioadă de 3 luni. Am constatat eficienţa antrenamentului fizic asupra elasticităţii arteriale - viteza undei pulsatile aortice (PWVao) şi evoluţia parametrilor tensionali: tensiunea arterială: sistolică (TAS), diastolică (TAD), medie (TAM), presiunea pulsului (PP). Parametrii ce au fost analizaţi înainte şi după un program de antrenament fizic de 3 luni.

Rezultate: La sfârşitul programului de antrenament fizic am obţinut schimbări ai parametriilor tensionali şi ai elasticităţii arteriale. PWVao în lotul de *studiu* scade semnificativ cu -0,8 m/s (p <0.001), versus lotul de *control* care creşte semnificativ cu +0,7 m/s (p < 0,001). TAM în lotul de *studiu* scade semnificativ -6,2 mmHg (p < 0,001), iar în lotul de *control* creşte substanţial cu +8,7 mmHg (p <0.001). TAS în lotul de *studiu s*-a diminuat deasemenea semnificativ cu –10,2 mmHg (p <0.001), vs. lotul de *control* care creşte semnificativ cu +15,6 mmHg (p < 0,001). TAD în lotul de *studiu* a scăzut cu -3,2 mmHg (p 0.010**)** vs. lotul de *control care* a crescut cu +5,2 mmHg (p 0.065). PP în lotul de *studiu* a scăzut semnificativ cu -5,9 mmHg (p 0.001), vs lotul de *control* unde a crescut semnificativ cu +9,9 mmHg (p 0.007).

Concluzii: Scăderea parametrilor prezentaţi mai sus pun în evidenţă efectul major asupra arterelor al exerciţiilor fizice coordonate, controlate permanent şi executate gradat în sensul îmbunătăţirii elasticităţii lor, cu influenţa imediată asupra adaptării fizice la antrenamnet şi efecte pe termen lung asupra tensiunii arteriale: scăderea ei (menţinerea şi chiar diminuarea vârstei arteriale).

Cuvinte cheie: exerciţii fizice aerobe, elasticitatea arterială, viteza undei pulsatile, tensiunea arterială.

BILE SYNTHESIS PECULIARITIES FOLLOWING CHANGES IN THE FUNCTIONAL STATE OF THE ENDOTHELIN RECEPTORS

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ABSTRACT

Objectives: ET-1 regulates variety of biochemical processes in liver. However, there is not a clear view concerning endothelin receptors participation in regulation of qualitative and quantitative characteristics of liver secretory function. The purpose of this study was to evaluate the choleretic effect of ET-1 and to determine the role of **ET_A** receptors functional state in mediating the effect of ET-1 on bile and its organic components secretion. Material and method: Endothelin-1 and BQ-123 were intraportally injected. Bile flow, bile acid concentration and content, hydroxylation and conjugation coefficients were estimated. Results: Secreted bile volume was decreased under the effect of endothelin-1and BQ-123, although this decrease was more prolonged and profound in BQ-123 treated animals. Concentration of taurocholates, glycocholic acid and free bile acids was increased in the endothelin treated rats.WhenBQ-123 was administered an increase in GCDCA+GDCA and the taurin-conjugated bile acids concentration was found, whereas free bile acids concentration altered reversely. Coefficient of hydroxylation was diminished when entholelin receptors were blocked. Activation of endothelin receptors by exogenous endothelin-1 intensified bile acids biosynthesis via "neutral pathway", involving microsomal oxidation enzymes. Conclusion: Endothelin receptors blockade eliminated the regulatory function of endogenous endothelin and caused a shift in bile acids synthesis to mitochondrial enzymes through "acidic pathway".

Key words: Endothelin-1, BQ-123, bile acid, coefficient

INTRODUCTION

Regulatory peptide endothelin-1(ET-1), the predominant isoform of endothelin, is a potent vasoconstrictor agent that was originally isolated from bovine aortic and pulmonary endothelium. The translation of preproendothelin-1 mRNA resultsin formation of a big ET-1precursor processig into ET-1(1) activates G_i-protein–coupled 7-transmembrane domain receptors. There are different types of ET receptors thatwith various levels of expression are distributed in divers tissues in human and animal organs (1,2). ET_A receptor is selective for ET-1, whereas ET_B receptor reveals similar affinities for all isopeptides (2,3). Endothelin-1 has been recognized not only as a vasoconstrictor but also as a multifunctional agent. This peptide through activation of ET_A receptors on intrahepatic vascular smooth muscle cells (4), the common bile duct (5), Kupffer cells (6) and hepatocytes (7) elicits different pathophysiological effects. Moreover, wide variety of biochemical processes in liver including, glycogenolysis, gluconeogenesis and hemodynamic action are all regulated by endothelin (8).There are some investigations confirming inhibitory effect of ET-1on bile secretion. According to Isales and co-authors (9) ET-1 induces a dose-dependent decrease in bile flow in isolated perfused rat liver, while Tanaka et al (10) applying similar experimental model, demonstrated that low dose ET-1 increased bile acid-dependent bile secretion. However, these results do not permit to have a clear view concerning endothelin receptors participation in regulation of qualitative and quantitative characteristics of liver secretory function.

The purpose of this study was to evaluate the choleretic effect of ET-1 and to determine the role of ET_A receptors functional state in mediating the influence of ET-1 on bile formation and biliary organic components secretion *in vivo* in rats.

MATERIAL AND METHODS

Study was conducted inacute experiments on 35 linear (male, 180-250g) Wistar rats, obtained from the Institute of Gerontology, Academy of Medical Sciences of Ukraine (Kyiv, Ukraine) after 18 hours of food deprivation. Rats were anaesthetized with ethyl urethane (100mg/100g rat body weight) which is sufficient for 3-4 hours of acute experiment.Common bile duct was then cannulated with polyethylene catheters to collect bile samples and register choleresis changes following laparotomy, respectedly.Endothelin-1 (Sigma, USA, 0.1 μg/100g rat body weight) and ET_A-receptors antagonist, BQ-123 (Sigma, USA, 6μ g/100gratbody weight) were separately dissolved in 100 μl 0.9% sodium chloride and injected into the portal vein. According to previous studies, endothelin-1 in this concentration causes clear liver vascular movement that is an evidence for the active interaction between peptide and endothelin-1 receptors.

The animals of the control group were intraportally injected

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with0.9% sodium chloride (100 µl/100g rat body weight).Secreted bile was measured by µl/g rat body weight every 10 minutes during 3 hours trial.

Free and conjugated bile acids were divided and determined in each sample by thin-layer chromatography method that has been initially patented by Veselsky et al and has been completely described by Parchami Ghazaee and colleagues (11). We divided mixture of bile acids into following fractions: taurocholic acid (TCA), taurochenodeoxycholic acid+taurodeoxycholic acid (TCDCA+TDCA), glycocholic acid (GCA), glycochenodeoxycholic acid+glycodeoxycholic acid (GCDCA+GDCA), cholic acid (CA), chenodeoxycholic acid (CDCA) + deoxycholic acid (DCA). Bile acids concentration and content were estimated by mg% and µg/g rat body weight. Bile acid hydroxylation and conjugation coefficients were determined by calculating the ratios of 3-hydroxycholates to dihydroxycholates and conjugated to free bile acids contents.

All experiments were performed after entherahepatic circulation abortion, following stabilization of bile flow for 30 minutes at the beginning of experiment.

Stability of rat body temperature was controlled by interarectal thermometer.

Statistical analysis was performed using statistical package "Statistica". Data were expressed as means±SD.Student's t-test for normally distributed values (Shapiro-Wilks W test) was used to compare variables between groups (p<0,05 was considered significant).

The study protocol was approved by the institutional review board and ethics committee of Faculty of Biology, National Taras Shevchenko University of Kyiv.

RESULTS

In experiment on control animals we observed a gradual reduction in secreted bile volume by 13.4% during 3 hours of the experiment (from 0.298±0.09 µl/g in the first 10 minute interval to 0.258±0.11 µl/g in the last one). As illustrated in Figure 1, under the effect of endothelin there was a considerable retardation in the level of bile secretion. Maximum choleresis reduction was registered in 40 minutes following endothelin-1 injection by 15.6% (p<0.05) compared to control. Afterwards, a gradual bile flow restoration was observed and its level reached control values towards the end of the experiment. However, its level was lower than in the first 10 minute interval by 8.6% (Fig.1).

Biochemical analysis of the half-hour bile samples evidenced that endothelin-1 exhibited a diverse effect on concentration of different bile acids in rats. Although concentration of TCAwas gradually reduced during three-hours of the experiment both in control and ET-1treated animals, its level in the sixth half-hour sample was higher by 9.3% (p<0.05) in the second group (141.5±2.3 mg% in control versus 154.7±2.8 mg% in ET-1treated animals). Whereas, in ET-1treated total amount of TCDCA+TDCA was insignificantly higher in majority of bile samples compared to control rats.

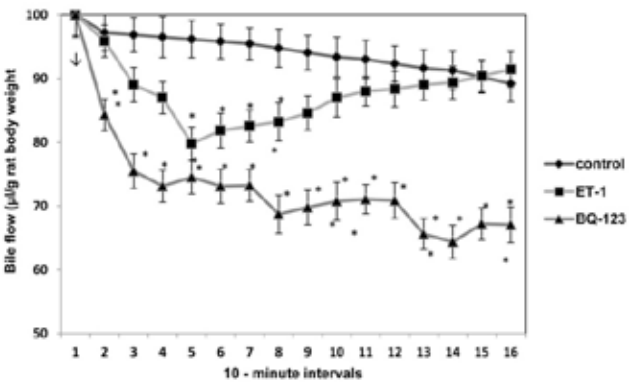

Fig.1. Effects of endothelin-1 and BQ-123 on choleresis, Mean±SEM; n=35, *p<0.05 as compared to control rats

The most drastic changes were observed inGCA concentration followingET-1 administration. Although concentration of GCA in control animals was gradually reduced during whole time of the experiment, it exceeded in the second half-hour toward the end of the experiment when ET-1 was applied. Particularly, GCA concentration was increased by 12.3% (from 131.3±3.4 mg% to148.3±4.1 mg%; p<0.01), 19.7% (127.1±3.7 mg% to 152.1±4.3 mg%; p<0.01) and 16.3% (125.3±3.5 mg% to 145±7 mg%; p<0.01) in the fourth, fifth and sixth half-hour samples. At the same time, under the effect of ET-1 only a tendency for reduction in GCDCA+GDCA concentration was observed.

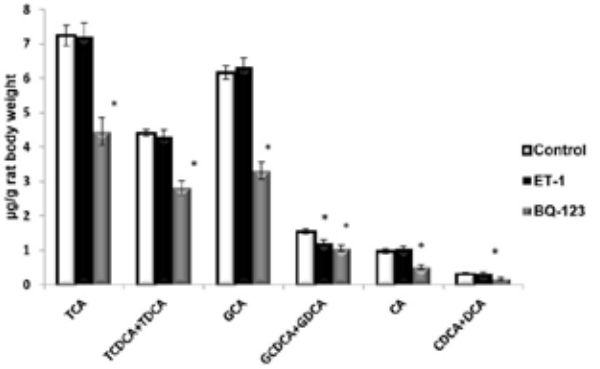

Fig. 2. Bile acidscontent in endothelin-1 and BQ-123 treated rats; Mean±SEM; n=35; (M+m);*p<0.05 compared to control.

Endothelin-1 caused an increase in CA concentration. Maximum changes were observed in the third and sixth half-hour bile samples by 20.6% and 19.8% (P>0.05). Whereas, CDCA+DCA Concentration in ET-1 treated animals was next to control values (Fig.2).

Peculiar changes in bile secretion were revealed when ET_A-receptors were blocked withET-1 antagonist, BQ-123. It suppressed the action of both endogenous and exogenous

endothelin-1 via this subtype of receptors. These functional changes in ET_A-receptors state led to more profound and prolonged decrease in choleresis. Bile secretion decreased by 15.7% ($p<0.05$) immediately10 minutes following BQ-123 injection. Further choleresis reduction was observed in the next two ten-minute periods toward the end of the third period. It decreased by 26.9% ($p<0.05$) compared to the initial state and 24.2% ($p<0.05$) compared to the control level. Maximum bile flow reduction was observed in minute 140by 35.6% ($p<0.05$), compared to the initial state (Fig.1).

Chromatography analysis depicted considerable changes in qualitative composition and quantitative content of the bile acids in BQ-123injected animals. The most marked increase in GCDCA+GDCA concentration was found in the fifth and sixth half-hour bile samples by 56% ($p<0.05$) and 60.6% ($p<0.05$) compared to the control values. At the same time, concentration of GCA remained almost the same. Concentration of taurin-conjugated cholates was characterized by fluctuated changes during BQ-123 injection. Significant changes were only observed when it was enhanced; TCA concentration in the fifth and the sixth half-hour samples was higher than control by 17.5% ($p<0.05$) and 25.3% ($p<0.05$). Also, the level of TCDCA+TDCA concentration was increased by26.9% ($p <0.05$) and 35.5% ($p <0.05$). It is important to note that free bile acids concentration simultaneously, conversely altered.

Table I. Effect of endothelin-1 and BQ-123 on hydroxylation and conjugation coefficients of bile acids in rats

interval		3-hydroxy-cholates mg%	Dihydroxy cholates mg%	Hydroxylation coefficient	Conjugated bile acids mg%	Free bileacids mg%	Conjugation coefficient
30	Control ET-1 BQ-123	327.6±3.8 330.5± 4.7 315.3± 4.8	151±2.7 136.9± 2.9 142.3 ±3	2.2 2.4 2.2	443.1± 5.6 434.3± 6.5 428± 5.3	30.6± 2.7 32.8 ±2.9 30.1± 2.8	14.5 13.2 14.2
60	Control ET-1 BQ-123	330.1±4.1 331.4 ±5.2 335.7± 5.1	144.2±2.4 140.7±3.2 168.8±3.3	2.3 2.4 1.9	447.2± 4.7 433.8± 6.3 476.9± 4.7	30.4± 2.5 33.5± 3.1 27.6± 3.1	14.7 12.9 17.3
90	Control ET-1 BQ-123	319.5±3.6 327.2± 4.8 326.8 ±4.4	137.8±2.2 136.9± 2.7 161.8± 2.7	2.3 2.3 2	428.7± 5.2 430.5± 5.9 461.3± 5.5	29.6± 2.1 33.3± 2.7 27.2± 2.4	14.5 12.4 17
120	Control ET-1 BQ-123	311.6±3.4 329.4± 3.6 331.8± 3.8	131.2±2.3 131.3± 2.5 163.3 ±2.6	2.4 2.5 2	415.6± 4.8 429.8± 5.4 468.3± 5.2	27.2 ±2.2 30.6± 2.5 26.8± 2.7	15.3 14.1 17.5
150	Control ET-1 BQ-123	294.9±3.2 331.3± 3.4 332± 3.7*	122±1.8 123.3± 2.2 161.8± 2.5	2.4 2.6 2	391.5± 4.5 427.8± 4.7 460.3± 4.2	25.4± 1.9 27.2± 2.3 23.5± 2.5	15.4 15.7 19.6
180	Control ET-1 BQ-123	285±2.9 321.6± 3.5 324.2± 3.7*	115.3±1.9 122.4 ±2.3 163.9± 2.4	2.5 2.6 1.9	375.9± 3.8 416.5± 4.3 464.3± 4.4	24.1± 2.1 28.1± 1.8 23.8 ±1.9	15.6 14.8 19.5

ET-1: endothelin-1, * $p<0.05$ compared to control

Dihydroxycholic acids concentration in the most bile samples was significantly higher in BQ-123 treated animals compared to control. This effect is clearly revealed in hydroxylation coefficient when endothelin receptors were blocked (Table I).

DISCUSSION

It is of great importance to clarify the bile synthesis peculiarities by evaluating the particular poly enzymatic systems efficiency in liver, which provide bile acid hydroxylation and amino acid conjugation processes, altering the bile colloidal system properties.

Determination of hydroxylation and conjugation coefficients, as well as separate bile acid content under the effect of endothelin-1 and BQ-123, indicated significant disturbances in bile formation process.

The results revealed that endothelin-1 intensified biosynthesis of both free and glycine conjugated 3-hydroxycholates, synthesis of which is closely associated with the microsomal oxidation enzymes activity (neutral pathway), and so depends on tissue supply with oxygen. This agrees with thesis that oxygen consumption by the liver tissue is reducedafterendothelin-1 administration (12,13, 14).

Following BQ-123 injection the coefficient of bile acids hydroxylation was reduced from 2.2 in the first sample to 1.9in the last one, whereas in control group it was increased from 2.2 to 2.5.This evidences intensification of dihydroxycholates biosynthesis in ET_A-receptors blockade that is confirmed by a significant improve in their glycine and taurine conjugated concentration. It is known that biosynthesis of the initial chenodeoxycholic acid in liver is realized by active participation of the mitochondrial enzymes that in our investigation is supported by a significant increase in its conjugates under the effect of BQ-123. Therefore, ET_A-receptors blockade points out an important role of endogenous endothelin-1 in neurohumoral regulation of bile organic components biosynthesis.

Study on rats has demonstrated that endothelin-1 and BQ-123 actively influence bile acids biosynthesis efficiency according to these metabolites content in bile. Endothelin-1 in applied dose promoted both biosynthesis of glycocholic acid and content of majority of bile acids in whole time of the experiment, whereas endothelin receptors antagonist caused a reversed effect.

It is important to note that significant and long-lasting choleresis retardation under the influence of BQ-123 is due

to partial removal of the bile acids from osmotic and diffusion processes in bile formation following a considerable decrease in their biosynthesis in hepatocytes.

We conclude that endothelin receptors activation by exogenous endothelin-1 provokes short-term choleresis retardation which is accompanied by intensification of bile acids biosynthesis via "neutralpathway", involving microsomal oxidation enzymes. The last is evidenced by alteration of 3-hydroxycholates to dihydroxycholates ratio and increasing of glycocholic acid content. Endothelin receptors blockade with BQ-123, which eliminates the regulatory function of endogenous endothelin, causes a sharp and long-lasting decrease in bile flow, simultaneously, shifting process of bile acids synthesis mainly to "acidic pathway", involving mitochondrial enzymes that is confirmed by enhancing the concentration of both free and conjugated dihydroxycholates.

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PARTICULARITATILE SINTEZEI BILIARE CA URMARE A MODIFICARILOR STATUSULUI FUNCTIONAL AL RECEPTORILOR PENTRU ENDOTELINA

REZUMAT

Obiective: ET-1 regleaza o varietate mare de procese biochimice la nivel hepatic. Cu toate acestea, nu exista o definire clara a participarii receptorilor pentru endotelina in reglarea caracteristicilor cantitative si calitative ale functiei secretorii hepatice. Scopul acestui studiu a fost de a evalua efectul coleretic al ET-1 si de a determina rolul statusului functional al receptorilor ET_A in medierea efectelor ET-1 la nivelul secretiei biliare si la nivelul secretiei componentelor organice ale acesteia. Materiale si metode: Endotelina-1 si BQ-123 au fost injectate la nivel intra-portal. Au fost estimati urmatorii parametri: fluxul biliar, concentratia si continutul acizilor biliari, precum si coeficientii de hidroxilare si conjugare. Rezultate: Volumul secretiei biliare a fost scazut sub efectul endotelinei-1 si a BQ-123, cu toate ca aceasta scadere a fost mai prelungita si mai accentuate la animalele tratate cu BQ-123. Concentratia taurocolatilor, acidului glicocolic si a acizilor biliari liberi a crescut la sobolanii tratati cu endotelina. Administrarea BQ-123 a dus la o crestere a GCDCA+GDCA si a concentratiei acizilor biliari conjugati cu tarina, in timp ce concentratia acizilor biliari liberi a suferit modificari in sens opus. Coeficientul de hidroxilare a fost scazut in cazul blocarii receptorilor pentru endotelina. Activarea receptorilor pentru endotelina prin administrarea exogena a endotelinei-1 a dus la intensificarea biosintezei de acizi biliari prin "calea neutra", care implica enzimele de oxidare microzomale. Concluzie: Blocarea receptorilor pentru endotelina elimina functia reglatoare a endotelinei endogene si determina schimbarea sintezei acizilor biliari la nivelul enzimelor mitocondriale prin "calea acida".

Cuvinte cheie: Endotelina-1, BQ-123, acizi biliari, coeficient