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THE ROLE OF TUMOR MICROENVIRONMENT AND IMPACT OF CANCER STEM CELLS ON BREAST CANCER PROGRESSION AND GROWTH

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ABSTRACT

Breast cancer is not only a mass of genetically abnormal tissue in the breast. This is a well-organized system of a complexheterogeneous tissue. Cancer cells produce regulatory signalsthat stimulate stromal cells to proliferate and migrate; then,stromal elements respond to these signals by releasing components necessary for tumor development that provide structuralsupport, vasculature, and extracellular matrices. Developing tumors can mobilize a variety of cell types from both local anddistant niches via secret chemical factors derived from cancercells themselves or neighboring cells disrupted by growing neo-plasm, such as fibroblasts, immune inflammatory cells, and endothelial cells. CSCs are a group of very few cells that are tumorigenic (able to form tumors) and are defined as those cellswithin a tumor that can self-renew and lead to tumorigenesis.BCSCs represent a small population of cells that have stem cellcharacteristics and are related to breast cancer. There are different theories about the origin of BCSCs. BCSCs are responsiblefor breast carcinoma metastasis. Usually, there is a metastaticspread to the bones, and rarely to the lungs and liver. A phenomenon that allows BCSCs to make the transition from epithelialto mesenchymal expression and thus avoid the effect of cytotoxic agents is the epithelial-mesenchymal transition (EMT).During this process, cells change their molecular characteristicsin terms of loss of epithelial characteristics taking the mesenchymal phenotype. This process plays a key role in the progression,invasion, and metastasis of breast tumors.

Keywords: *Cancer stem cell, tumor microenvironment, breast cancer stem cell, resistance to conventional therapy.*

INTRODUCTION

The tumor is a tissue mass resulting from its abnormal growth. Conventionally, this mass is classified as a benign, malignant, and so-called tumor in situ. Until the formationof a tumor, cells go through specific stages of metaplasia and dysplasia. However, not always do metaplasia and dysplasia finally result in the creation of a neoplasm (1-3).

Analogously to the above, breast cancer is formed as a result of breast tissue cells' abnormal growth. Breast cancer is not only a mass of genetically abnormal tissue in thebreast. This is a well-organized system of complex heterogeneous tissue (4). This heterogeneity in the tissue and understanding of cancer as a heterogeneous disease that helpsto understand disease progression and treatment failure (5). Cancer cells produce regulatory signals that stimulate stromal cells to proliferate and migrate; then, stromal elements respond to these signals by releasing componentsnecessary for tumor development that provide structuralsupport, vasculature, and extracellular matrices (6). It isincreasingly appreciated that tumor stroma crosstalk is animportant event for cancer initiation, growth, and progression (7). Developing tumors can mobilize a variety of celltypes from both local and distant niches through production of chemical factors derived from cancer cells themselves or neighboring cells disrupted by growing neoplasm,such as fibroblasts, immune and inflammatory cells, andendothelial cells. This assortment of cells and moleculestogether comprises the tumor microenvironment (TME)(8). TME is composed of extracellular matrix (ECM) andmany distinct cell types, including carcinoma associated fibroblasts (CAFs), tumor associated macrophages(TAMs-M2), cancer stem cells (CSCs), mesenchymal stemcells (MSCs), myofibroblasts, smooth muscle cells, endothelial cells and their precursors, pericytes, neutrophils, eosinophils, basophils, mast cells, T and B lymphocytes, natural killer cells (NK), and antigen presenting cells (APC) such as macrophages and dendritic cells (Figure 1). These non-tumor cells have important roles not only in tumor initiation, progression, and metastasis but also in therapeutic resistances (9-11).

In breast cancer, the most frequent component of tumor stroma is CAFs. There are many hypotheses about theorigin of CAFs (12). The dominant role of CAF in tumor tissues is to increase the expression of matrix metalloproteinase-14 (MMP14) and MMP9 activity, which promote tumor invasion and metastasis (13, 14). Besides the origin,these cells differ by expressing different surface markers which are mainly dependent on the tissue origin. In breastcancer, important CAF markers are fibroblast activation protein (FAP) and a combination of platelet-derived growthfactor-α and β receptor (PDGFR- α and β) and α-smooth muscle actin (α-SMA) (13). However, some studies have demonstrated that CAF can promote tumor progressionin other ways. It has been demonstrated that CAF-derivedCCL2 increases number of breast cancer stem cells (CSCs)which promotes metastasis (15).

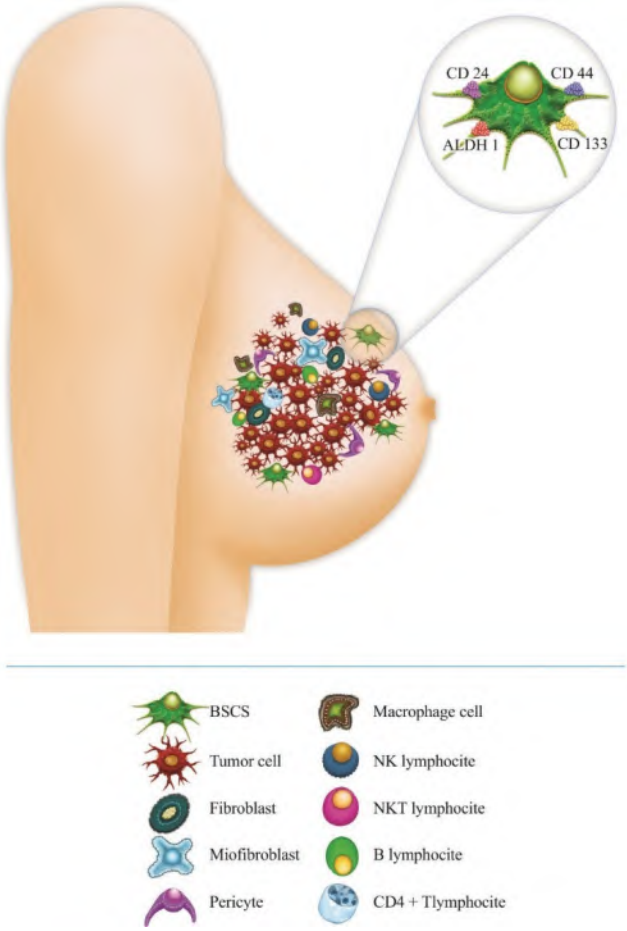
Many immune cells, such as macrophages, NK cells, regulatory T cells (Tregs), myeloid-derived suppressor cellshave also been implicated in breast cancer development (16). Macrophages can alter their polarization state from M1 to M2 (17). "Alternatively-activated" M2 macrophages produce anti-inflammatory cytokines like IL-10 and express non-inflammatory chemokines CCL17, CCL18, CCL22, CCL24 and have pro-tumorigenic functions (18). TAMs are mostly M2 macrophages populations that are either tissue-resident or derived from peripheral reservoirssuch as the bone marrow and spleen. The role of TAMsin breast cancer is to promote immunosuppression, neo angiogenesis, and tumor cell migration and invasion (19). TAMs accumulate in regions of hypoxia which regulate theexpression of M2-related genes that promote angiogenesis. By the production of vascular endothelial growth factor A (VEGF-A) and placental growth factor (PlGF), TAMs induce neo angiogenesis. TAMs-derived epidermal growth factor (EGF) and proteases, such as cysteine cathepsins, promote tumor progression and invasion (16). Studies show that a decrease of mammary tissue macrophages andan increase of TAMs in patients with breast cancer is a badprognostic sign (20).

The suppression and evasion of the host immune system during the progression of tumors can be achieved even through inhibition of effector immune cells or via stimulation of immunosuppressive cells. Myeloid-derived suppressor cells (MDSCs) and Treg cells suppress host immune system and contribute to tumorigenesis through enhancement of tumor immune evasion (21). MDSCs are immature myeloid cells witch derange tumor- associated antigen presentation, the polarization of macrophage, andthe activation of cytotoxic T cells and NK cells. Besides these functions, it has been shown that Treg cells can produce VEGF-A and induce neo angiogenesis. A high number of Treg cells in TME reduces the survival rate of breastcancer patients (17).

NK cells are important immune cells in anticancer immune response. NK cells control tumor initiation; however, they undergo crucial alterations during cancer progression (22). In tumor microenvironment, different factors have aneffect on the phenotype and function of these cells (23). There are two subpopulations of NK cells in tumor stroma, tumor-infiltrating natural killer cells (TINKs) and tumor-associated natural killer cells (TANKs). TINKs and TANKs have changed cytokine expression and increased levels of pro-angiogenic factors important for neo angiogenesis andtumor progression (19).

Myofibroblasts are cells with the characteristics of myoblasts and fibroblasts which have an important role inbreast cancer progression and invasion. Genes expressed in tumor myofibroblasts encode chemokines CXCL12 andCXCL14, important in breast cancer progression. CXCL12have a role in the earlier stages of breast tumorigenesis, while CXCL14 probably participate in inflammation (24).

Figure 1. Tumor microenvironment



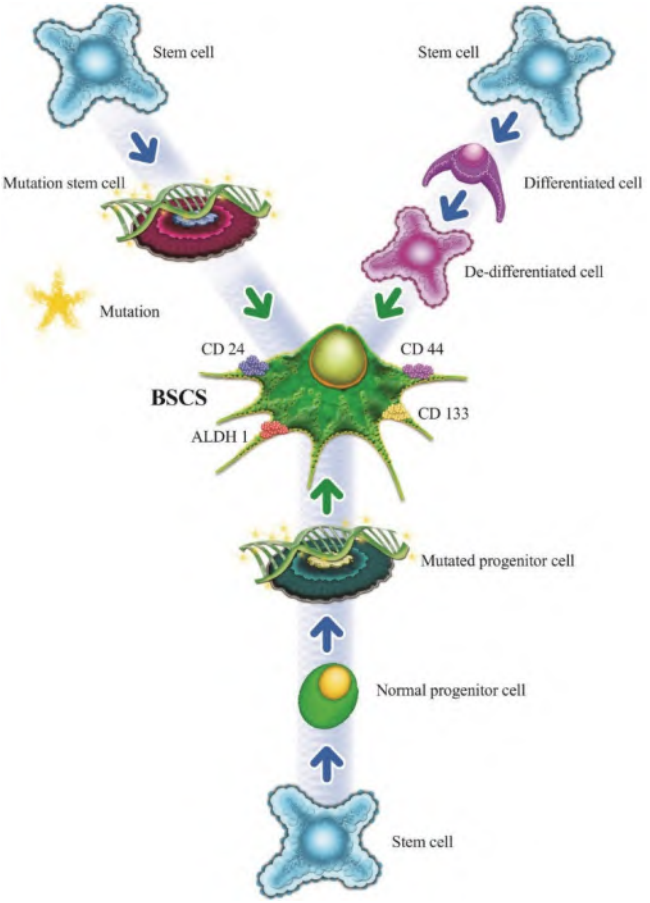
MSCs are multipotent cells that are capable of modulating tumor microenvironment and have an immunomodulatory function. Through these functions, these cells supportbreast cancer growth and progression (25). The immuno-modulatory function is dominantly immunosuppressive and includes changing of immune responses from Th1 to Th2 orinduction of Treg production and proliferation (26).

BCSCs have been associated with tumor initiation, progression, metastasis and resistance to conventional therapy. These small subpopulation of cells inside the tumor mass can be influenced by the other components of tumor microenvironment through complex interactions.T cells and CAFs from breast cancer microenvironment can both induce and inhibit BCSCs. Treg produce factors such as VEGF and TGF-β which promote cancer stemnessand BCSC expansion and effects of the tumor microvasculature and angiogenesis (27). Loss of Tissue Inhibitor of Metalloproteinases (TIMP) by CAF through activation of Notch signalling pathway and upregulation of typical BC- SCs markers increase the formation of distant metastasis. BCSCs express reduced levels of NK ligands what is BC- SCs mechanism for immune escape and also is connected with metastatic spread (28). Other cells including TAMs, MSCs and endothelial cells have effects on BCSCs throughnetworks of cytokines and growth factors. Notch, Hedgehog, Wnt, PI3K, NF-κB, and Jak/STAT stem cell regulatorypathways in breast cancer are most often dysregulated by signals from the tumor microenvironment. Notch signalling pathway regulate the selfrenewal of BCSCs. TAM-derived factors promote BCSCs selfrenewal and maintenance, as well, BCSC-derived factors induce protumor signals in TAMs (29).

Although, breast cancer tissue is composed from desciped cells, it has been noted that different tumor cells may show differences that can be reflected in the cell morphology, gene expression, metabolism, movement, proliferation,and metastasis (30). This functional heterogeneity among cancer cells has led to the creation of at least two models, which have been put forward to account for heterogeneityand differences in tumor-regenerative capacity: the cancerstem cell (CSCs) and clonal evolution models (31).

Under normal conditions, it has been observed that stem cells create a new stem cell and a progenitor cell. In this situation, the progenitor cell provides a differentiated cell. Either by the influence of direct mutations or the effect of external factors, cells enter into a dedifferentiation process and lose their specificity and, as such, these cells can lead to the formation of cancer stem cells (32). Also, certain cells may end the differentiation process before the progenitor cell whose mutations can still lead to CSCs (Figure 2).

Figure 2. Mechanisms of BCSCs occurrence.



It has been proven for many tumors that de novo mutations and events lead to the formation of BCSCs. Biological characteristics of BCSCs are various. Thanks to the specific mutation within tumor cells and its nature, BCSCs frequency, cell-surface phenotype, and drug sensitivity may vary. Also, tumor progression will depend on the tumor itself, i.e. its pathogenesis or a decisive challenge for chemotherapy, which is responsible for BCSCs biology (33).

BCSCs are a group of very few cells that are tumorigenic (able to form tumors) and are defined as those cells within a tumor that can self-renew and lead to tumorigenesis. There are two models of tumors development and growth described so far. One of these models is BCSCs model and it postulates a hierarchical organization of cells such that only a small subset is responsible for sustaining tumorigenesis and establishing the cellular heterogeneity inherent in the primary tumor (34).

On the other hand, the clonal evolution model claims that all cells within a tumor do their bit in varying degrees to maintain a tumor (35). In this model, a number of genetic and epigenetic changes occur over time, leading to the result that the most aggressive cancer cells are ultimately liable for breast tumor progression. The initial tumor cell evolution may occur by two methods: linear and branched expansion (36).

BREAST CANCER STEM CELLS (BCSCS)

Previous studies results demonstrated that the processes of breast tumors initiation, progression, and proliferation occur thanks to the small group of BCSCs which is able to self-renew and differentiate (37). Features of BCSCs are the result of the impact of complex molecular mechanisms or microenvironment (38, 39). Cytokines and their impact on the BCSCs microenvironment are responsible for tumor heterogeneity and the so-called plasticity of BCSCs (40).

BCSCs represent a small population of cells that have stem cell characteristics and are related to breast cancer. There are different theories about the origin of BCSCs. One of them states that improper regulation or mutations may lead to the transformation of normal stem cells into breast cancer stem cells (BCSCs) (41). According to another, the "misplacement somatic stem cell" theory, BCSCs may originate from misplacement of somatic stem cells *de novo* (42). Evidence shows that somatic cells can be considered the BCSC origin. There are studies that suggest there are intratumoral lineages differentiated from common progenitor cells (43). BCSCs were isolated from breast tumor tissue and the cell was characterized as CD44+/CD24/low Lin phenotype (44). CD44 is a cell surface glycoprotein and a specific receptor for hyaluronan. It is a crucial element for breast cancer adhesion, motion, migration, and invasion, and its interaction with osteopontin causes tumor progression. It has an important role in cell proliferation and tumor angiogenesis (38, 39). CD24, a second-surface glycoprotein expressed at low levels, increases tumor's ability to grow and metastasize (38). However, one report shows that CD44+CD24- is not expressed in all breast cancer cell populations (45). The results of some studies show that CSCs is to identify the presence of very important ALDH markers (46). Aldehyde dehydrogenase 1 (ALDH 1) consists of a family of cytosolic enzymes involved in the oxidation of intracellular aldehydes and oxidizes retinol to retinoic acid during stem cells differentiation. ALDH1 plays a role in stem cells differentiation and its activity forecasts poorer clinical outcomes (47). The other markers that have been used to identify BCSCs include CD133, CD49fhi, and CD61 (48, 49). Although the list of CSCs markers grows, some researchers do not consider these markers suitable for identifying CSCs.

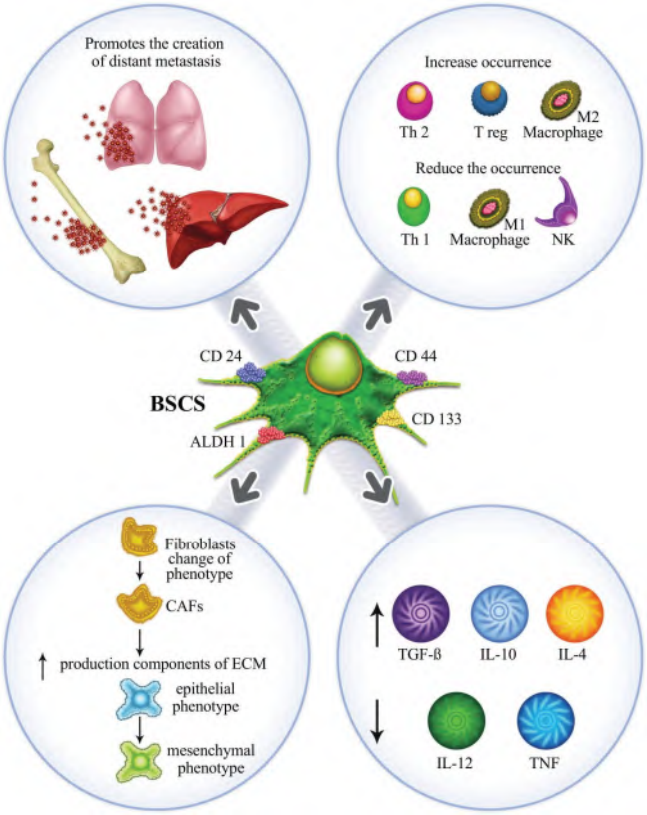
The immediate environment of the BCSCs consists of a group of various cells and molecules which together forming a BCSCs niche. This niche provides adequate physical and chemical conditions for the development of tumors (include fibroblast stimuli, immune cells, autocrine signals, and extracellular matrix (ECM) components, oxygen pressure, nutrients, and PH (50). Those cells produce cytokines such as interleukin IL-1, IL-6, and IL-8, CXCL12, CCL2, and growth factors such as platelet-derived growth factor (PDGF), TGF-β, TNF-α, EGF, vascular endothelial growth factor, and FGF that are responsible for tumor growth and progression (51). This is supported by studies which have shown that blockage of the IL-6 receptor can inhibit tumor metastasis and growth (52). The other results show that blockage of TGF-β with IL-8 inhibition increases the number of BC-SCs in triple negative breast cancer and prevents tumor formation in preclinical models (53). This and other studies show that the cell niche content can and should be an important point for a breast cancer targeted therapy (54). BCSCs are responsible for breast carcinoma metastasis.

Usually, there is a metastatic spread to the bone, and rarely to the lungs and liver (55). The basic molecules of the breast cancer target tissue are hyaluron and osteopontin that exhibit binding sites for the CD44 molecules in BCSCs (bone, brain, liver, and lung, bone marrow endothelium). Osteopontin is associated with a higher incidence of tumor metastasis and invasion (56).

A phenomenon that allows BCSCs to make the transition from epithelial to mesenchymal expression and thus avoid the effect of cytotoxic agents is called *epithelial-mesenchymal transition EMT* (57). During this process, cells change their molecular characteristics in terms of loss of epithelial characteristics taking a mesenchymal phenotype. This process plays a key role in the progression and invasion of metastasis breast tumors. Throughout EMT, some changes occur such as the shutdown of transcription and regulation of epithelial markers such as E-cadherin, and the appearance of mesenchymal markers such as vimentin, fibronectin, and N-cadherin. This leads to destabilization of structures and functions in these cells (58). This transformation leads to cancer cells migration and invasion. It has been found that malignant cells with mesenchymal characteristics are more resistant to therapy and EMT provides an increase in the number of cancer stem-like cells (59). BCSCs are also responsible for a large

number of breast cancer subsets and have a great clinical significance (60). Itis necessary to take into account the fact that tumor is heterogeneous and that the characteristics of BCSCs in one region may be an inadequate predictor for the outcome of the whole breast cancer (61). The results of many studies suggest the need for testing BCSCs as a prognostic factor for different types of breast cancer outcome (Figure 3).

Figure 3. Impact of BCSCs on tumor microenvironment and on progression and invasion of metastasis



RESISTENCE OF BCSCS TO CONVENTIONAL THERAPY

Recent studies suggested that BCSCs posses inbred chemo-and radiation-therapy resistance mechanisms which allow them survive. Resistance of BCSCs to conventional therapy is providen by several mechanisms such as DNA damage repair, cell cycle checkpoint proteins activation, activation of self-renewal pathways or avoidance of apoptosis (62). Radiation induce cell death through DNA damage. All cells respond to DNA damage by activationof detection and repair mechanisms which includes ATM (ataxia telangiectasia mutated) and the checkpoint kinases,Chk1 and Chk2, initiating cell cycle arrest, repair of DNA or apoptosis. BCSCs use these mechanisms more rapidly than non-stem cancer cells and avoid radiation-induced cell death (63). Other potential radioresistance mechanisms is activation of Wnt/β-Catenin signalling pathway which promotes DNA damage tolerance. Jagged- 1 expression and the Notch signalling pathway have also been implicated as playing roles in radioresistance. In the mammary gland, Wnt/β-catenin, Notch and Hedgehog (Hh) signalling pathways induce stem cell self- renewal and theyare potential targets for therapy (64).

ATP-binding cassette (ABC)-G2 transporters, such as breast cancer resistance protein (BRCP-ABCG2) and MDR-associated protein-1 (ABCB1/MDRR1), class ofdrug transporters are often the cause of multidrug resistance. These transporters are expressed on normal stem cells and cancer stem cells and they are capable of pumpingout of these cells different substances, including cytotoxic drugs (65). Some clinical studies have been shown that another possible reason for chemotherapy and radiation therapy resistance can be high expression of CD44 and lowexpression of CD24 on breast cancer cells (66).

However, the clinical relevance of BCSCs in human breast cancer is still under debate. Also, the question arisesas to whether there are any differences between BCSCs and tumor-initiating cells.

CONCLUSION

Role of BCSCs is remarkable in tumor progression and metastasis. Extensive interactions among cancer stem cells, their microenvironments, and other present cells initiate a cascade of growth factors and inducing elements, which in turn influence cancer stem cell role in breast cancer. This population is resistant to conventional therapies due to enhanced membrane transport by specific protein transporters, specific mechanisms of DNA repair, and ROS scavenging systems, and the ability to detoxify cytotoxic drugs. Transcriptional factors, signalling pathways, and tumor suppressor genes act to maintain and amplify a state of stability. More studies are needed to investigate each of these aspects of BCSCs. And finally, the BCSCs as a key point of breast cancer should be subjected to a study in order to individualize the therapy directed to the system of a given breast cell carcinoma.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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None.

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THE EFFECT OF ELECTROCHEMOTHERAPY ON BREAST CANCER CELL LINES

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ABSTRACT

Despite advances in treatment, breast cancer remains one of the leading causes of death, and obviously new approaches to the treatment are needed. Due to minimal side effects, unlike more aggressive forms of therapy such as chemotherapy and radiotherapy, the application of irreversible electroporation-electrochemotherapy represents a new modality in the treatment of cancer. Electrochemotherapy uses an electric field (375 V cm^{-1}) to allow increased absorption of chemotherapeutic drugs selectively in tumor cells. Accordingly, the total dose of these agents can be significantly reduced and numerous side effects can be avoided in this way. The Real Time Cell Analysis-RTCA-xCELLigence system was used to monitor the cytotoxic effects of the treatment. The results confirmed the justification of the use of paclitaxel in chemotherapy and showed cytotoxic effects of paclitaxel which were time and dose-dependent in both cell lines. When paclitaxel was administered in combination with an electric field, in both cell lines, the results showed a greater cytotoxic effect compared to the same treatment without electrochemotherapy. MCF-7 cells are more sensitive to electrochemotherapy treatment with paclitaxel compared to MDA-MB-231. Electrochemotherapy using paclitaxel in MCF-7 cells had a 6.4-fold higher cytotoxicity compared to the treatment only with paclitaxel . The results obtained support the current knowledge of the benefits of electrochemotherapy. It has been shown that electrochemotherapy can significantly increase the effects of paclitaxel in the tested cell lines. In this way, a very high concentration of chemotherapeutics in the targeted tissue was achieved, which represents localized chemotherapy.

Keywords: *Electrochemiotherapy, RTCA technology, breast cancer, cytotoxicity.*

INTRODUCTION

Despite advances in treatment, breast cancer remains one of the leading causes of death, and obviously new approaches to the treatment are needed (1). One of the modern modalities of treatment for this disease is electroporation. When biological tissue is exposed to very short voltage amplitude impulses, an electric field emerges (2). If the intensity of the generated electric field increases above the corresponding critical value, the cell membrane becomes permeable to the ions and molecules, which otherwise cannot pass into the cell. Such a modulation of cellular membrane permeability under the influence of an electric field is called electroporation or electropermeabilization and allows the transport of molecules and ions in the cell to be enhanced (3).

Electroporation can be a reversible or irreversible process depending on the ability of the cell to recover the integrity and function of the membrane (4). Irreversible or reversible electroporation is a new ablative technique for treating carcinoma. It uses ultra-short pulses, which destroy the homeostasis in the target tissue through the permeabilization of the plasma membrane of the carcinoma cells. Due to such changes in the membrane as well as ablative effects, the cell death occurs. Unlike thermal techniques, irreversible electroporation leaves the surrounding tissue untouched, leading to fewer side effects, lower morbidity rates, which results in a faster recovery of the patient (5,6).

Unlike aggressive forms of therapy such as chemotherapy and radiotherapy, the application of irreversible electroporation has just a minor side effects. Other local ablative techniques such as cryo-ablation, radiofrequency ablation (RFA), microwave ablation and high-intensity focused ultrasound are thermo techniques which can damage vital structures near the tumor such as blood vessels (7). The immunocytological evaluation of cells treated by irreversible electroporation shows the characteristics of necrosis and apoptosis (8). Programmed cell death - apoptosis is associated with tissue regeneration and does not cause a systemic immune response, therefore it is the most desirable effect. However, it must be noted that depending on different parameters (strength of the electric field, number of pulses, time interval, etc.) electroporation can also cause the coagulation necrosis (8).

The combination of reversible electroporation with chemotherapy has evolved to a new method which is used in the treatment of solid tumors known as electrochemotherapy (9). Electrochemotherapy uses an electric field to enable increased absorption of chemotherapeutic agents in tumor cells, selectively. This is the local treatment of the high-voltage electric field on the cells, and as a result, the permeability of the plasma membrane is increased. In this way, antineoplastic agents are easier to enter the cell and have an increased cytotoxicity. Accordingly, the total dose of these agents can be significantly reduced and numerous side effects can be avoided in this way.

Chemotherapy represents the use of cytostatics that usually affect the entire body in order to destroy cancer cells. In this way, cytostatic agents have great influence on the healthy, normal cells, especially rapidly dividing cells (bone marrow cells, the cells of the hair follicles, of the gastrointestinal tract, etc.). Thanking to the electropermeabilization of tissue, chemotherapy can become a local method for the selective removal of tumors. In combination with electroporation, the cytostatic acts only on the tissue that is exposed to the electric field, i.e. on the tissue between electrodes (10).

METHODS

Cultivation of cells

In this experiment, we used human breast cancer cell lines MDA-MB-231 and MCF-7 obtained from the American Type Culture Collection (ATCC). The cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C and then grown in 75 cm^2 culture bottles supplied with 15 ml of Dulbecco's Modified Eagle's medium-DMEM (with 10% Fetal Bovine Serum-FBS, with 100 units of ml-1 Penicillin and 100 μg ml-1 Streptomycin). Upon reaching the appropriate confluence of 80% of the cells, they were used for the experiment.

Conditions of electrochemotherapy

For the electrochemotherapy of cells, the apparatus Electroporation System, BTX-Harvard Apparatus, ECM 399 Generator was used. The apparatus consisted of ECM 399 Generator (45-0050), PEP (45-0212) and cuvettes with 1, 2 and 4 mm (distance between electrodes). In this paper, cuvettes with a distance between the electrodes of 4 mm were used. For electropermeabilization of adherent cells, an electropermeabilization assay was used: the cells were raised with trypsin-EDTA, and then resuspended in DMEM medium. The cells were then centrifuged for 5 min at 1000 rpm. The supernatant evaporated and the cells of the concentration of 1 x 10^6 cells / ml were resuspended by electropermeabilization buffer, transferred to the electropermeabilization cuvette and placed on the electroporation apparatus. The electropermeabilization buffer contained 250 mM sucrose, 10 mM phosphate buffer and 1 mM MgCl2, pH 7.4 (11). In addition to the above mentioned components, a corresponding concentration of Paclitaxel (0.01, 0.1, 1, 5, 10, 25 and 50 μM) was added to the buffer. The electropermeabilization of the cells was performed using a voltage of 375 Vcm-1.

Cell viability determination by xCELLigence technology (*Real Time **Cell** Analysis*-RTCA)

The xCELLigence system was used to continuously monitor the effects of electrochemotherapy on adherent cell lines - in real time. After the treatment, cells were seeded in E-plate 16 wells with incorporated gold electrodes. Through these electrodes, the device measured the electrical impedance, which was represented as the Cell Index (CI) and was complementary to the current cell status. This provided quantitative information on the number of cells, cytotoxicity,

viability, and cell morphology at any time. The percentage of viable cells after electrochemotherapy was the ratio of cell index (Cell Index - CI) of treated and untreated cells. The described method was explained in detail in Cvetković et al., 2017. IC50 values (treatment of electrochemotherapy that kills 50% of cells) were calculated from the dose-dependent curves obtained by computer program CalcuSyn.

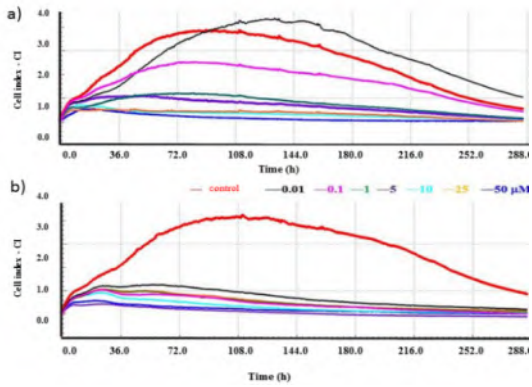
Statistical analysis

All experiments were done in triplicate, and the obtained results represented as the mean value of three independent experiments ± standard error. Student's T test, Independent test and ANOVA were used, with *p <0.01 being considered as statistically significant difference. The results were processed by SPSS (Chicago, IL) program for statistical results processing (SPSS for Windows, ver. 17, 2008).

RESULTS

Figure 1a shows the effect of electrochemotherapy treatment on MDA-MB-231 breast cancer cells, monitored in real time (xCELLigence, RTCA technology). The cytotoxic effect of paclitaxel is time-dependent and dose-dependent in this cell line. As the concentration of the drug is greater and the treatment time is longer, the cytotoxic effects are more pronounced in relation to control untreated cells. Figure 1b presents the cytotoxic effects of electrochemotherapy, that is, combined treatment of electroporation of the voltage of 375 V cm-1 and paclitaxel at all investigated doses. The results showed a significant cytotoxic effect of electrochemotherapy even when the lowest concentration (0.01 μM) was used. The cytotoxicity of the electrochemotherapy was significantly increased compared to treatment with paclitaxel alone, and the IC_{50} values were lower in the cotreatment (Table 1).

Figure 1. Real time monitoring of the cytotoxic effects of paclitaxel (a) and electrochemotherapy (b) on the breast cancer cell line MDA-MB-231.



The results are presented as the mean ± SE from 3 independent experiments.

Figure 2. Real time monitoring of the cytotoxic effects of paclitaxel and combined electrochemotherapy in the MDA-MB-231 breast cancer cell line, after 24 (a) and 72 hours (b) of the treatment.

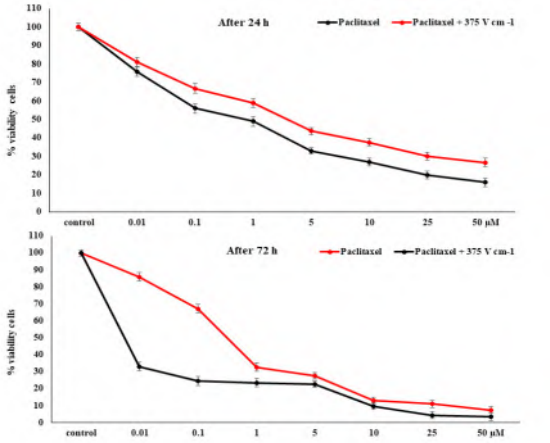
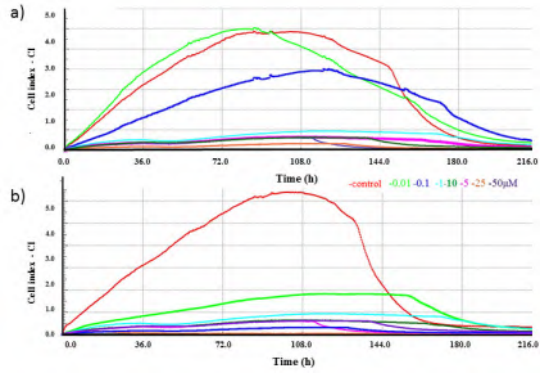


Figure 2a and b shows the effects of electrochemotherapy after 24 and 72 hours of the treatment. The cytotoxic effect of paclitaxel can be clearly seen when combined with electroporation, even in the administration of the smallest doses of this cytostatic.

The results are presented as the mean ± SE from 3 independent experiments.

Figure 3a shows the effect of paclitaxel antineoplastic drug treatment on MCF-7 breast cancer cells in real time. The treatment with the lowest concentration of paclitaxel (0.01 μM) did not show cytotoxic effect compared to control untreated cells. All other concentrations of paclitaxel show a cytotoxic effect that is time-and-dose-dependent in this cell line. As the concentration of the drug is greater and the treatment time is longer, the cytotoxic effects are more pronounced. Figure 3b presents cytotoxic effects of electrochemotherapy, i.e. combined treatment of paclitaxel and electroporation (voltage 375 V cm -1). The results showed a significant cytotoxic effect of electrochemotherapy even when the lowest concentration of paclitaxel (0.01 μM) was used, which was also shown by lower IC_{50} values in the cotreatment (Table 1).

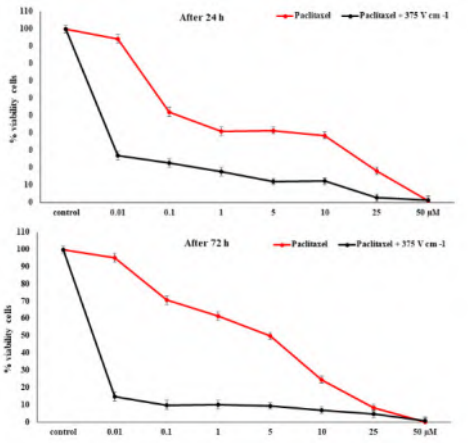
Figure 3. Real-time monitoring of cytotoxic effects of paclitaxel (a) and electrochemotherapy (b) in breast cancer cell line MCF-7.



The results are presented as the mean ± SE from 3 independent experiments. Figure 4 shows the cytotoxic effects of

paclitaxel and electrochemotherapy on the MCF-7 breast cancer cell line after 24 and 72 hours of the treatment. The results showed a markedly dose-dependent cytotoxic effect of paclitaxel after 24 hours of the treatment compared to untreated, control cells (a). This effect is significantly enhanced by combining paclitaxel with electroporation. After 72 hours of the treatment, mild cell recovery is observed in the use of lower concentrations of individual treatment of paclitaxel (up to 1 μM), while higher concentrations caused higher cytotoxicity on these cells. Combined treatment of paclitaxel and electroporation showed a very cytotoxic effect, in all applied concentrations, which can be concluded also on the basis of lower IC_{50} values in the co-treatment (Table 1).

Figure 4. Real-time monitoring of cytotoxic effects of paclitaxel and electrochemotherapy in MCF-7 breast cancer cell line after 24 (a) and 72 hours (b) of treatment.



The results are presented as the mean ± SE from 3 independent experiments.

Table 1. Cytotoxicity of Paclitaxel and Electrochemotherapy - IC_{50} values (μM) for MDA-MB-231 and MCF-7 cells after 24 and 72 hours.

			IC_{50}
MDA-MB-231	24 h	Paclitaxel	2.57 ± 0.26
		Paclitaxel +375 V cm^{-1}	0.39 ± 1.96
	72 h	Paclitaxel	0.37 ± 0.82
		Paclitaxel +375 V cm^{-1}	0.02 ± 0.001
MCF-7	24 h	Paclitaxel	0.70 ± 0.26
		Paclitaxel +375 V cm^{-1}	0.002 ± 1.9
	72 h	Paclitaxel	0.92 ± 0.82
		Paclitaxel +375 V cm^{-1}	0.05 ± 0.001

The results are presented as the mean ± SE from 3 independent experiments.

DISCUSSION

Electroporation can be used in an irreversible mode, when a permanent opening of the pore on the cell membrane is achieved, leading to cell death due to the loss of homeostasis and influx and efflux of the ions. This results in an ablative effect on the tissue, i.e. the treatment of the cancer cells (12). Also, electroporation can be used in reversible mode. The application of current of certain properties can cause temporary opening of membrane pores, allowing some macromolecules to enter into the cell, which cannot otherwise pass through the membrane. After that the pores close and the cells return to the original condition. If reversible electrotherapy is used to insert an antineoplastic agent, i.e. cytostatic, into the cell, then this type of therapy is called electrochemotherapy (9). However, this kind of therapy has not yet been widely accepted in clinical practice, despite its obvious advantages. First of all, the effect of local chemotherapy can be achieved using very low concentration of cytostatics resulting in a very high (and up to 100 times) concentration of cytostatics in the target region of the tissue, while the amount of cytostatics that go into free circulation is very small. This accomplishes a dual effect; first, the concentration of cytostatics in the treated tissue is significantly increased, and on the other hand, numerous, well-known systemic side effects, closely intertwined with the use of antineoplastic therapy, can be avoided allowing a very small amount of the drug to be distributed into circulation.

A small number of tumors, such as melanoma, urinary bladder tumors, etc. were the subject of the study of the effects of electrochemotherapy in the available literature (9). We performed the tests on the breast cancer cell lines. It is literally known that some antineoplastic agents have a significantly greater effect if used in electrochemotherapy than when used in standard chemotherapy regimens. One of these drugs is paclitaxel, for which it has been proven that such modus of therapy achieves a higher concentration in the tissue than in the standard application (9). Our studies have shown the cytotoxic effect of paclitaxel on MDA-MB-231 and MCF-7 breast cancer cells at all observed time intervals.

Electrochemotherapy with paclitaxel significantly increases cytotoxic effects in both cell lines. Our previous findings showed the high resistance of the MDA-MB-231 cell line to the effect of electroporation. Only in case of using high voltages, which at the same time had a very high cytotoxic effect on healthy lines, MDA-MB-231 cells showed a high degree of necrosis (8). A triple-negative, MDA-MB-231 metastatic breast cancer cell line was isolated from the pleura of a patient with breast cancer (13). It represents a good model system for analyzing potential forms of therapy for this type of cancer. The treatment with paclitaxel in combination with an electric field in these cells showed a 2.59-fold higher cytotoxicity compared to the application of this drug without electroporation, 72 hours after the treatment.

The MCF-7 metastatic breast cancer cell line, also isolated from pleura, is a good *in vitro* model for the study of

endocrine therapy mechanisms, since it has expressed estrogen and progesterone receptors (14). MCF-7 cells showed greater sensitivity to electrochemotherapy treatment with paclitaxel compared to MDA-MB-231. Thus, electrochemotherapy in these cells had a 6.40-fold rise in cytotoxicity compared to paclitaxel alone. Electrochemotherapy can be used as a single modality or in combination with surgical treatment. Its effectiveness in neoadjuvant modality has been demonstrated. Namely, certain tumors are surgically difficult to reach, due to the presence of important physiological structures (e.g. nerves and blood vessels) or because of potential damage to other organs, or for some other reason. Electrochemotherapy can be used to reduce tumors before surgical removal, i.e. to bring the tumor into a workable range, or translate it from an inoperable to an operable stage. Electrochemotherapy as an adjuvant therapy for the purpose of sterilization of possibly residual malignant cells after surgery, to our knowledge, has not been published in the literature so far (15,16). Another very important feature of electrochemotherapy treatment is that this method does not belong to the so-called thermal methods, which means that local heat generation is not a method of destruction of malignant cells. Thanking to that, electrochemotherapy can treat tumors that are closely related to important organs or structures such as blood vessels, which can be easily injured by other, thermal methods, and lead to bleeding that is very difficult to control. Also, there is no effect of heat transfer by blood in the vicinity of large blood vessels, which is characteristic of thermal methods (17,18).

CONCLUSION

The use of electrochemotherapy achieves the effect of localized chemotherapy. The use of controlled electrical impulses temporarily increases the permeability of the cell membrane and facilitates the entry of the chemotherapeutic agent into the tumor cells, while at the same time very little drug is released into free circulation, which reduces numerous and severe general side effects of chemotherapy.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REPRODUCTIVE HEALTH AND RISK FACTORS OF NON-COMUNICABLE DISEASE IN FEMALE STUDENT POPULATION (STEPWISE APPROACH)

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ABSTRACT

University students are a specific adolescent population which is preparing to take participation in different domains of a society as its integral and creative part. Chronic noncommunicable diseases have a major impact on women's reproductive health, so their adverse epidemiological situation has significant effects on reproductive health in general. Since non-communicable chronic diseases have been a growing burden on reproductive health, the aim of this paper is asses of reproductive behavior and risk factors of non-communicable disease in female student population on Faculty of Medical Sciences University of Kragujevac. This study was conducted as a prospective cross-sectional study. The sample includes 59 female students of The Faculty of Medical Sciences, University of Kragujevac. The study was conducted in three stages based on the methodology and instruments recommended by STEPwise Approach to Noncommunicable Disease Risk Factor Surveillance of the World Health Organization (STEPS). About 71.2 % of the participants reported that they had a sexual intercourse. In average, the respondents were 18 years old (SD±1.222) at the time of their first sexual intercourse. At that point, a little less than one third of them did not use any type of protection (31.7%). During the last sexual intercourse, about 65.5% did not use protection. About 22.5% of our subjects have morning glycemia with prediabetes values (glycemia cut off ≥ 5.5mmol/L). Hypercholesterolemia is present in 3.4% of the respondents (cut off ≥ 5,2mmol/l). Most respondents are eutrophic (18.8% preobese and 2.1% obese). The android obesity type is the least frequent (about 10%). This results indicates that female students has unhealthy habits in terms of their reproductive health and preventive measures. This activity indicaty a wide array of preventive action which will aim at preserving reproductive health and health in general.

Keywords: *Reproductive health, risk factors of non-comunicable disease, female student population.*

INTRODUCTION

University students are a specific adolescent population which is preparing to take participation in different domains of a society as its integral and creative part. Period of university education, in addition to biological and psychological maturation, represents the period when individuals undergo the process of inclusion into social communitie. At this life stage, young people are expected to acquire skills and abilities that will enable them to take on the most important roles in all social activities. The processes of inclusion into a society, as a rule, last until an individual meets a certain degree of social autonomy, responsibility and independence (1)

The World Health Organization defines health as a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity. The same applies to reproductive system, its functions and processes (2). Chronic noncommunicable diseases have a major impact on women's reproductive health, so their adverse epidemiological situation has significant effects on reproductive health in general. In addition to traditional and widely known diseases and conditions, haemostatic disorders (either as a propensity for hemorrhages or thromboses) have been increasingly recognized nowadays as significant indicators of reproductive health of female population.

The definitions of reproductive health are complex and frequently multi-layered. The most comprehensive are those which recognize that reproductive health implies responsible, safe and satisfying sex life, the ability to reproduce and make free decisions about personal reproduction (3).The studies have shown that reproductive health problems are one of the most frequent causes of morbidity among the youth and that sexually transmitted diseases (STDs), unwanted pregnancies and sexual abuse have become a global public health issue of great importance. More than 400 million people annually suffer from sexually transmitted diseases. This leads to a conclusion that prevention represents a fundamental process in preserving reproductive health (4).

In Serbia, cervical cancer has been occupying a significant place in the total structure of morbidity and mortality for a long period of time. With 1300 newly diagnosed patients and 500 deadly outcomes annually, this malign disease is the second leading and the fourth most fatal cancer disease in female population. Diachronically observing, the distribution of cervical cancer based on age has had a growing trend of occurrence after the age of 30 and the maximum frequencies among the age groups 45 - 49 and 70 - 74 years of life. The current data indicate unfavorable changes in age distribution where peak values of morbidity are moving towards younger age groups (5). These changes strengthen the effects on reproductive health. In addition, according to Cancer Registry of the Republic of Serbia for 2015, the age-adjusted incidence rate of cervical cancer was 18.1 and the mortality rate 6.1 per 100,000 women (6). These findings are devastating taking into consideration fact that cervical cancer is one of the most preventable malign diseases.

Since non-communicable chronic diseases have been a growing burden on reproductive health, the aim of this paper is asses of reproductive behavior and risk factors of non-communicable disease in female student population of Faculty of Medical Sciences University of Kragujevac by using the WHO STEPS approach.

MATERIAL AND METHODS

This study was conducted as a prospective cross-sectional study. The sample includes 59 female students of The Faculty of Medical Sciences, University of Kragujevac. The study was conducted in three stages based on the methodology and instruments recommended by STEPwise Approach to Noncommunicable Disease Risk Factor Surveillance of the World Health Organization (STEPS).

Step 1. This stage is a questionnaire-based assessment. This study used three questionnaires as instruments: a general standardized questionnaire, a questionnaire about sexual behavior and a questionnaire about cervical cancer prevention. The general questionnaire inquires about demographic and socioeconomic indicators. In addition, the participants filled in two more questionnaires. The first of the two inquired about their sexual behavior (e.g. the age of the first sexual intercourse, the use of contraception, sexually transmitted diseases, pregnancies, abortions, etc.) while the other inquired about their practices which are crucial in cervical cancer prevention (e.g. whether they had ever done a Pap test, when and where they did it the last time, what the results were, etc.).

Step 2. The second phase of the assessment refers to simple physical measurements which include anthropometric measurements (height, weight, circumference, BMI) and the measurements of arterial blood pressure (with measurement methods and interpretations of the obtained results in accordance with the WHO recommendations). The weights were measured with the same calibrated digital scale and the heights with the same calibrated stadiometer. Both the heights and the weights were measured while standing and wearing no shoes and only light clothes. The waist circumference values (WC) were measured with a plastic metric tape. The body mass indexes (BMIs) were calculated with the following formula: BMI = weight (kg) / squared height m^2. Based on the WHO classification, a BMI of 25 - 30 kg/m^2 is considered overweight, and BMI $\geq$ 30.0 kg/m^2 as obesity. Blood pressure was measured after spending at least five minutes in sitting position. We used an automatic sphygmomanometer and a tailored sized cuff. The arm was placed at the heart level and both feet were placed on the ground. When blood pressure was evaluated as high (systolic blood pressure (SBP) $\geq$ 140 mm Hg and diastolic blood pressure (DBP) $\geq$ 90 mm) the measurement was redone after 5 minutes.

Step 3. The third phase refers to biochemical analyses (fasting glycemia, total cholesterol). Glycemia and

cholesterol concentrations were measured with automatic analyzing devices suitable for the examinations on field. The values were determined as: glycemia cut off ≥ 5.5mmol/L and cholesterol cut off ≥ 5,2mmol/l (7).

The statistical analysis and assessment of the data was conducted with IBM Statistical Package for the Social Sciences (SPSS, software version 19.0). The data cleaning was performed to prevent any missing values, coding error or any illogical data values. The qualitative variables were presented as numbers and percentages and the continuous variables as means and standard deviations (SD).

Ethical approval was obtained from the Faculty of Medical Sciences Ethical Committee. The ethical standards are in accordance with international (the Helsinki Declaration) and national legislation. The necessary measures were taken to protect the privacy of the respondents and to ensure information confidentiality of individuals, with regard to the processing of personal data and on the free movement of such data, in accordance with the Law on Personal Data Protection (8), Law on Official Statistics (9) and the Directive (95/46/EC) of European Parliament on the protection (10). The researchers were obliged to administer a printed document to all participants which informed them about the study and their rights including to whom and how they could submit complaints if they felt that their rights were in any way compromised. The respondents were also required to sign an informed consent.

Statistical analyses

Statistical analysis was performed using SPSS for Windows 20.0 (*SPSS Inc. USA).* Continuous variables are summed as arithmetic means, medians and standard deviations, and categorical variables as proportions (percentages of categories).

RESULTS

STEP 1

Sexual health

About 71.2 % of the participants reported that they had a sexual intercourse. In average, the respondents were 18 years old (SD±1.222) at the time of their first sexual intercourse. At that point, a little less than one third of them did not use any type of protection (31.7%). During the last sexual intercourse, about 65.5% did not use protection. During the last 12 months, 11.1% students had sex with more than one partner. About 2.4% of students had an STD.

Cervical cancer prevention

The results show that 63.6% of the students have never done a Pap test. Among those who were tested, about 15.4% did a Pap test because pain or other symptoms were present and 23.1% never found out the results of their Pap test.

STEP 2

The results of our study show that the average weight of the respondents is 63.46± 9.847 kg, the average height - 167.03±5.6501 cm, the average waist circumference - 76.86± 6.710 cm and the average BMI - 22.717±3.061 kg/m2. Most respondents are eutrophic (18.8% preobesity and 2.1% obesity). The android obesity type is the least frequent (about 10%). The mean heart rate is 81.1±8.96 and arterial hypertension was found in 3.4% of the subjects.

STEP 3

About 22.5% of our subjects have morning glycemia with prediabetes values. Liporegulation is satisfactory; hypercholesterolemia is present in 3.4% of the respondents.

DISCUSSION

The results of our study indicate that 71,2% of our subjects had a sexual intercourse and that they were approximately 18 years old when they had their first sexual experience (SD±1.222). The 5.3% of them was pregnant at some point. Similarly, the study conducted in educational institutions in Illorin, Nigeria revealed that 77.6% of the female students had had a sexual intercourse with 98.6% of them not being married. Unwanted pregnancies were detected in 67.8% of the students and induced abortions in 63.5% (11). The Malaysian National Health and Morbidity Survey showed that 27% of the female population had already had sex before the age of 14. The same report revealed that 11% of females had had multiple sex partners. Another report showed that 8.3% of the students had sexual intercourse at the mean age of 15. In another study, the authors found that persons who had become sexually active at earlier age had more chance of having multiple sex partners. A study conducted in China showed that females who had had their first sexual intercourse before the age of 18 were more likely to have multiple partners in comparison to those whose first sexual experience had occurred at the age of 19 or more (12). A study that surveyed 630 students in three Italian cities revealed that just over a half of the students were sexually active with the mean age of the first intercourse of 15.6 years (SD±1.3) (13). A survey conducted on Portuguese adolescents indicated that 44 - 95% of the adolescent population was sexually active and that there was an increase in the age of the first intercourse which was 15.6 years at the time. They also found that early sexual activity commencement was related to tobacco and regular alcohol consumption. (14).

The results of our study indicate that about 31.7% of our subjects did not use any kind of protection during their first sexual debut and that about 65.5% did not use it during their last sexual intercourse. Contraception was most commonly obtained from medical workers (22.7%). During the last 12 months, 11.1% of the students had more than one sexual partner. Approximately 2.4% of the respondents had already had a sexually transmitted disease. The similar studies were conducted in other countries. In Nigeria, only 25.4% of the subjects used any contraception. Reportedly, friends and

relatives had been used as the most frequent sources of information about contraception (73.7%) and fear of side effects was the most common reason for nonuse (77.5%) (11). In Brazil, 94% of the surveyed young people were aware of the condom use in STD prevention but only 34% used it (12). In Bangladesh, the use of contraception among the women aged 10 - 49 rose from 49 - 61% from 1996 to 2011. During the same time period, the values rose from 33 - 47% among married adolescents [20]. In Malawi, the use of contraception among married women aged 15 - 49 rose from 13 - 46% from 1992 to 2010 (15). With Portuguese adolescents, condoms were the most commonly used contraceptive method during their first (76 - 96%) and subsequent (52 - 69%) sexual encounters. However, only one third of the subjects had visited a medical facility for contraception or STD counseling. (14) Condoms were also the most frequently used contraception type in Italian adolescents where coitus interruptus, natural family planning and no method were also commonly provided answers (13).

The results of this study show that 63.6% of the selected population has never done a Pap test while 15.4% have done it due to pain or other symptoms. The results also indicate that 23.1% has never found out the result of their testing. Similar results were obtained in other countries. For example, in South Africa, a cross-sectional study showed that 15% (22/147) of female university students aged 18 - 26 who had been sexually active and had heard about cervical cancer, had had a Pap test. Students who had had a Pap test had significantly higher average scores on knowledge, benefit and motivation, and self-efficacy in comparison to those who had never done it (16). A study conducted in Brasil on 437 female university students found that 30.4% of the students had no knowledge on the meaning of altered outcome and that 30% had never obtained their results from the doctors' offices (17). In Latin American countries the share of the recent Pap smear was below 55%. For example, in the Dominican Republic the proportion was 49% (95% CI, 49% - 50%), in Bolivia 42% (95% CI, 41% - 43%) and in Peru 52% (95% CI, 51% - 53%). The proportion of women unfamiliar with Pap smears grew in both Bolivia and Peru and the levels of knowledge were consistently higher in the latter country (18). The similar studies were also conducted in the neighboring countries. The screenings for cervical cancer in Hungary were about 74%. In Albania, the screening rate for women in their reproductive age (15 to 44 years) was the lowest in the region with only 3.2% (19).

Our STEP 2 results show that the average height of the respondents amounts to 63.46± 9.847kg and the average height to 167.03±5.6501cm. The results of the similar studies indicate that average weight and height values of the students in Mexico were 59.0±13.0kg and 159.9±60.0cm, respectively (20). In Iran, those values were 58.1±8.63kg and 159.4±59.88cm (21) and in Lebanon, 60.49± 9.96kg and 164.12±5.98cm (22). The average BMI value of the surveyed population in our study amounts to 22.717±3.061 kg/m^2. Most subjects are eutrophic (18.8% with preobesity and 2.1% with obesity). The android type of obesity is the least frequent (about 10%). Arterial hypertension has been found in 3.4% of the respondents.

A cross-sectional study conducted on 375 students attending Saint-Joseph University of Beirut aged 18 - 25 (both the medical science and social science campus included) revealed preobesity and obesity prevalence rates (20.6% and 8%, respectively) that are higher than our results. However, their values of the average BMI (22.45±3.47 kg/m^2) are similar to ours (22). The results of the study conducted in Turkey which included 650 medicine students evaluated the average BMI which is slightly lower than the results obtained in our study (20.89 ±1.6 kg/m^2) (23).

STEP 3 results of our study show that every fifth subject has morning glycemia at the level of prediabetes (22.5%). The lower prevalence of prediabetes with respect to our results was found among adolescent population in Qatar (4.2%) (24) and Lebanon (2.5%) where no participant had a diabetes (22). The prevalence rates of prediabetes and type 2 diabetes in adolescents and young adults (aged 12 - 19) have been increasing drastically in the United States (17.7% and 0.8%, respectively (25,26).

The primary limitation of this study is its cross-sectional design which does not enable inferences about potential causal relations between the explanatory variables and disorders of interest. Furthermore, self-reporting nature of the questionnaires also presents a limitation. Finally, our sample included a small group of students attending only one faculty. Since other universities are excluded, the generalizations are necessarily limited.

CONCLUSION

This study indicates that a huge number of female students has bad habits in terms of their reproductive health and preventive measures. On the other hand, weight and lipo statuses, as well as cardiovascular parameters are not disturbed in the third decade of life. This activity indicaty a wide array of preventive action which will aim at preserving reproductive health and health in general. The focus of the activity should be on both primary and secondary prevention. The activities of primary prevention should be based on intensifying the education about health in order to develop and master health-preserving skills in young adults. The activities of the secondary prevention should be focused on organized, systematic and more frequent screenings. These would enable early detections of unrecognized health disorders in asymptomatic disuse phases.

Table 1. Sexual health

Variables		n	%
Have you ever had a sexual intercourse?	yes	42	71.2
	no	17	28.8
How old were you when you had your first sexual intercourse?		18.08 ±1.222	
The first sexual intercourse was with:	a spouse	1	2.6
	someone you did not marry	37	94.9
	I don't know	1	2.6
Did you use protection during the first sexual intercourse?	yes	28	65.9
	no	14	31.7
When was the last time you had a sexual intercourse?	last week	18	43.9
	from a week to a month ago	11	26.8
	from a month to a year ago	10	24.4
	more than a year ago	2	4.9
How many partners did you have during the last 12 months?		1.06 ±0.34	
Did you have sexual intercourses with more than one partner during the last 12 months?	yes	3	11.1
	no	24	88.9
Did you use any protection during the last sexual intercourse?	condom	14	35
	pill	17	68
Where did you get the protection for unwanted pregnancies/infections?	from a store/machine	8	40
	from a medical worker	5	22.7
	from a friend	2	10.5
Have you ever had a sexually transmitted disease?	yes	1	2.4
	no	40	97.6
Have you ever asked for advise/treatment concerning STDs?	yes	1	7.1
	no	12	85.7
Have you ever had sexual intercourses with same-sex partners?	yes	1	1.8
	no	56	98.2
Have you ever been pregnant?	yes	3	5.3
	no	54	94.7
How old were you when you were pregnant?		25.3 ±4.041	
Have you ever had an abortion?	yes	1	12.5
	no	7	87.5

Table 2. Cervical cancer prevention

Variables		n	%
Have you ever done a Pap test?	yes	20	36.4
	no	35	63.6
How old were you when you were first tested for cervical cancer?		20.33±2.50	
When was the last time you were tested for cervical cancer?	less than a year ago	7	53.8
	from 1 - 2 years ago	2	15.4

Variables		n	%
	I don't know	4	30.8
Why were you tested?	a routine check-up	5	38.5
	a check-up after indeterminate abnormal results	1	7.7
	recommended by a medical worker	1	7.7
	for pain and other symptoms	2	15.4
	other	1	7.7
Where were you last tested?	a health center	2	20
	a private practice	5	50
	a hospital, clinic, clinical center	3	30
What was your result?	normal	10	76.9
	I don't know	3	23.1
Did you have check-ups due to your result?	yes	4	36.4
	no	6	54.5
	I don't know	1	9.1
Did you undergo treatment after your Pap results?	yes	1	8.3
	no	10	83.3
	I don't know	1	8.3
Why didn't you undergo treatment?	because I was not told to do so	4	50
	I don't know	4	50
What is the main reason you have never done a Pap test?	because I did not have time	1	16.7
	due to bad quality of health service	1	16.7
	I don't know	4	66.7

Table 3. Assesment of blood pressure

Variables		n	%
Has your blood pressure ever been measured by a medical worker?	yes	58	98.3
	no	1	1.7
The average systolic blood pressure:		115.68 ±12.69	
The average diastolic blood pressure:		71.37±8.30	
Has a medical worker ever informed you about your higher levels of blood pressure or hypertension?	yes	5	8.5
	no	54	91.5
Were you informed about it during the last 12 months?	yes	4	15.4
	no	22	84.6
Did you take any medication prescribed by a doctor for high blood pressure during the last two weeks?	yes	2	8.0
	no	23	92.0

Table 4. Biochemical measurement

Variables		n	%
Has your blood sugar level ever been measured by a medical worker?	yes	42	71.2
	no	17	28.8
The average blood sugar level:		5.27± 0.480	
Has a medical worker ever informed you about your higher blood sugar levels or diabetes?	yes	8	17.0
	no		39
	53.0		
Were you informed about it during the last 12 months?	yes	5	23.8
	no	16	76.2
Did you take any medication prescribed by a doctor for diabetes during the last two weeks?	yes	1	4.5
	no	21	95.5
Are you currently taking insulin prescribed by a doctor for your diabetes?	yes	1	4,5
	no	21	95.5

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CONFLICT OF INTERESTS

The authors fully declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SWIMMING ATTENUATES BLOOD PRESSURE AND OXIDATIVE STRESS IN HYPERTENSIVE RATS

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ABSTRACT

Hypertension presents one of the main risk factors for cardiovascular diseases which are the leading cause of morbidity and mortality worldwide. Structural and mechanical changes of the heart and blood vessels as well as overproduction of reactive oxygen species may occur due to the increased blood pressure. Therewith, the goal of our study was to estimate the effects and duration of swimming as a possible therapy approach on blood pressure and oxidative stress parameters in normotensive and hypertensive rats. The study was conducted on 60 male Wistar albino rats divided into two groups, normotensive and hypertensive rats. Each of these groups was divided into three subgroups according to the swimming protocol. The swimming training was kept constant (60 min/day, for five days a week) with two days of rest. After six or nine weeks of the swimming protocol, blood pressure and oxidative stress markers were measured. The control group rats were put in water for one minute a day, in order to avoid water-induced stress. Training significantly reduced systolic blood pressure in hypertensive rats, while diastolic pressure did not change in the group that swam six or nine weeks. The results showed that swimming increases the activity of all measured antioxidative parameters, while values of prooxidants varied depending on the training protocol. Our results confirmed that swimming, as an aerobic exercise, decreases blood pressure and has time-dependent positive system adaptations, especially on the antioxidant parameters.

Keywords: *Antioxidant protection, hypertension, oxidative stress, rats, swimming.*

INTRODUCTION

Despite significant progress in the pathophysiology understanding and available effective treatment strategies, hypertension is still the leading risk factor in developing many diseases, especially cardiovascular ones (1). Hypertension leads to numerous changes such as a structural and mechanical modification of the heart and/or blood vessels (2). Furthermore, hypertension has been related to an imbalance between oxidants and antioxidants in favor of oxidants, which consequently causes tissue damage, vascular disorders and diseases (3). The beneficial effects of exercise are highlighted in cardiovascular diseases and many authors emphasize its importance in the treatment of hypertension (4).

Regular physical exercise is considered to be one of the crucial factors for a healthy lifestyle due to its ability to diminish the risk of osteomuscular, endocrine, cardiovascular and immune system disorders (5). Exercise, especially aerobic, represents an important and necessary part of everyday life since it may prevent and treat various diseases and pathological conditions (6).

Mechanisms responsible for beneficial effects of training on blood pressure are not quite t revealed. Nevertheless, various papers have been speculating about peripheral mechanisms responsible for antihypertensive effect, such as vascular resistance and endothelium dependent relaxation (7). Previous investigations reported the role of oxidative stress during exercise in terms of enhancing the prooxidants. On the other hand, some researchers suggested that exercise may also enhance antioxidant enzymes activity. However, it should be taken into consideration that oxidative stress response to exercise can be affected by type, duration and frequency of training which is of great importance in stimulation of adaptive processes of the antioxidative system, especially in patients with cardiovascular problems (8, 9).

Swimming represents the aerobic type of exercise where the motion of the body and all muscles induces adaptation of the cardiovascular system. As a total-body workout, it increases flexibility and leads to an improvement in blood circulation, superior systolic and diastolic function and less cardiac fibrosis (1). Considering that swimming leads to suppression of the sympathetic nervous system and the renin-angiotensin system, as well as to lower vascular resistance, it should be recommended to patients with hypertension (2, 10).

In past few decades, an increasing number of scientists invested great efforts to find exercise which is ideal for patients with cardiovascular diseases. Given the fact that influence of oxidative stress in swimming is not still clarified, we aimed to reveal the effects and different duration of swimming training on blood pressure and systemic oxidative stress parameters in normotensive and salt-induced hypertensive rats.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were carried out in the Laboratory for Cardiovascular Physiology of the Faculty of Medical Sciences, the University of Kragujevac. It was approved by Ethics Committee of the institution as well as according to EU Directive for welfare of laboratory animals (86/609/EEC) and principles of Good Laboratory Practice (GLP).

Animals

Our research included sixty male *Wistar albino* rats (six weeks old) received from the Military Medical Academy, Belgrade, Serbia, placed under controlled conditions: temperature of 22 ± 1 °C with twelve hours automatic illumination daily. Food and tap water or solution of NaCl were available to rats which were randomly divided into two groups: normotensive (NT) and hypertensive (HT) animals, while each group consisted of three subgroups depending on the swimming protocol. Normotensive rats were separated into: normotensive rats subjected to swimming for six weeks (NT-6-ST, n=10); normotensive rats exposed to swimming for nine weeks (NT-9-ST, n=10) and sedentary control rats (NT-C, n=10).

To induce hypertension, rats from HT group were drinking 8% high sodium (NaCl) mixture for four weeks (11). Hypertension was assessed on the day after completing the swimming protocol by using the tail-cuff (*Rat Tail Cuff Method Blood Pressure Systems (MRBP-R), IITC Life Science Inc. USA*) (12). After the confirmation of hypertension, animals were divided into three subgroups according to swimming sessions: hypertensive rats exposed to six weeks of swimming (HT-6-ST, n=10); hypertensive rats subjected to weeks of swimming (HT-9-ST, n=10) and sedentary control rats (HT-C, n=10).

Swimming training protocol

Rats were practicing in a specially constructed glass swimming pool with following dimensions: 80 × 60 × 100 cm. Water temperature (37 ± 1°C) was preserved by an electric heater, while waves were made by pump. Animals were abstaining from food during the night prior to the swimming protocol. The swimming training protocol was chosen according to a recent investigation (13) and was maintained five days a week at 9:00–10:00 am for all exercise sessions. The adaptation protocol includes ten minutes of constant swimming exercise on the first day and slowly enhanced daily until reaching sixty minutes on the fifth day. After accomplishing the adaptation, rats were subjected to the continuous swimming protocol: one hour daily during five days with two days of rest. In order to accomplish similar water-induced stress, control group rats (NT-C and HT-C) were in water one minute per day for five days a week during all exercise sessions. Between every procedure animals rested for

two days. Supervisor was continuously present during swimming.

Biochemical analysis

Blood from all experimental groups was collected for the determination of redox status after establishing blood pressure. Quantification of the index of lipid peroxidation via reactive thiobarbituric substances (indirect, measured as TBARS), nitrites (NO_2^-), superoxide anion radical (O_2^-), and hydrogen peroxide (H_2O_2) were performed in plasma samples, while superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) were determined in erythrocytes samples. All biochemical analyses were carried out using the spectrophotometric method (UV-1800 Shimadzu UV spectrophotometer, Japan) by repeatedly confirmed methods used in our previous studies (14).

Determination of the index of lipid peroxidation measured as TBARS

Products of the reaction with thiobarbituric acid were used for determination of the index of lipid peroxidation. Briefly, 0.4 ml of plasma samples and 0.2 ml of 28% trichloroacetic acid were vortexed, incubated for fifteen minutes on ice and centrifuged (6000 rpm) for fifteen minutes. Afterwards, 0.4 ml of supernatant and 0.1 ml of 1% thiobarbituric acid were incubated at 100˚C for fifteen minutes and measured at 530 nm spectrophotometrically. The distilled water was used as blank control.

Determination of nitrites (NO_2^-)

Nitric oxide (NO) quickly resolves into nitrites/nitrates. Therefore, nitrites (NO_2^-) are used as an index of NO production via a spectrophotometric method. For NO_2^- determination in plasma 0.1 ml 3 N PCA (perchloride acid), 0.4 ml 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 ml plasma were put on ice for fifteen minutes, centrifuged for fifteen minutes at 6,000 rpm. After pouring off the supernatant, 220 µl K_2CO_3 was added. Detection of nitrites was performed at 550 nm. Distilled water was used as a blank probe.

Determination of superoxide anion radicals (O_2^-)

Superoxide anion radical concentration was measured using the NTB (Nitro Blue Tetrazolium) reagent in assay mixture (TRIS buffer) with plasma samples. Wavelength for determination of O_2^- was 530nm. Blank control was assay mixture.

Determination of hydrogen peroxide (H_2O_2)

Measurement of H_2O_2 is based on phenol red oxidation by H_2O_2 in a reaction catalyzed by horseradish peroxidase (HRPO). A total of 0.2 ml of sample was precipitated with 0.8 ml of freshly prepared phenol red solution, followed by the addition of 10 µl of (1:20) HRPO (made *ex tempore*). An adequate volume of distilled water solution was used in blank probes. The concentration of H_2O_2 was detected at 610 nm.

Determination of antioxidant enzymes (SOD, CAT)

SOD activity was measured by mixing 0.1 ml lysate, 1 ml carbonate buffer and 100 µl of epinephrine. Detection was performed at 470 nm. Distilled water was used as a blank probe. Isolated RBCs were washed three times with three volumes of ice-cold 0.9 mmol/l NaCl and hemolysates containing about 50 g Hb/l were used for the determination of CAT activity. Then 50 µl CAT buffer, 100 µl sample, and 1 ml 10 mM H_2O_2 were added to the samples. Spectrophotometric measurement was at 360 nm.

Determination of reduced glutathione (GSH)

Based on GSH oxidation via 5,5-dithiobis-6,2-nitrobenzoicacid, we determined the level of GSH spectrophotometrically. Combination of 0.1 ml 0.1% EDTA, 0.4 ml hemolysate, and 0.75 ml precipitation solution was mixed on the vortex machine and extracted on ice for fifteen minutes. Afterwards, the mixture was centrifuged on 4000 rpm for ten minutes. Measuring was performed at 412 nm. Distilled water was used as a blank probe.

Statistical analysis

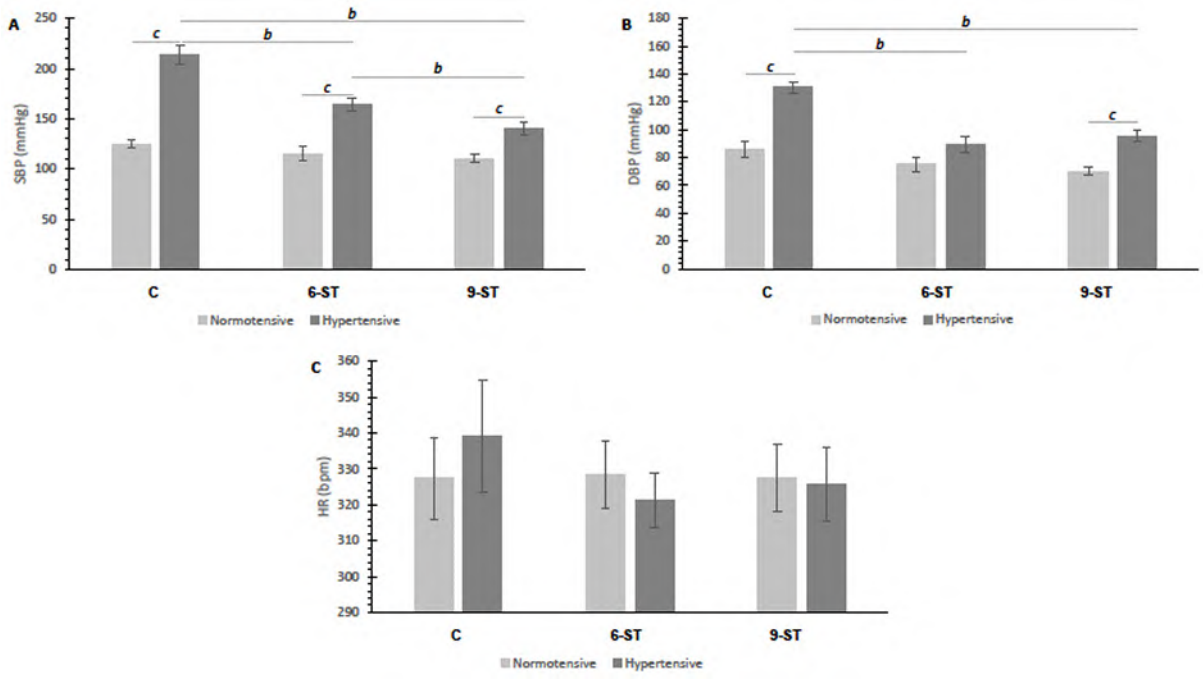
Statistical analyses were performed by using SPSS 23.0 software. Data are presented as the mean values ± standard deviations of the mean with statistical significance. The Shapiro–Wilk test was used for determination of normality of parameter's distribution. We used a parametric Friedman's ANOVA test or a non-parametric Mann–Whitney U test (Kruskal–Wallis test) for comparison of groups. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Blood Pressure and Heart Rate

Training did not significantly affect blood pressure in the NT group of rats, while hypertensive trained rats had a significantly reduced blood pressure compared to the sedentary group. Systolic blood pressure was significantly lower after nine weeks relative to six weeks of training (HT-9ST vs. HT-6ST). On the other hand, diastolic blood pressure didn't significantly change after the prolongation of training. Although the training significantly decreased the blood pressure of hypertensive rats, values were actually still significantly higher after six or nine weeks of swimming than in normotensive rats (Figures 1A, 1B). The heart rate was similar in all groups, regardless of blood pressure or training (Figure 1C).

Figure 1. Time-dependent swimming training-induced alterations in blood pressure: (A) systolic blood pressure; (B) diastolic blood pressure; (C) heart rate. Each bar represents the mean ± standard deviation, *a* statistically significant difference between normotensive rats; *b* statistically significant difference between hypertensive rats; *c* statistically significant difference between normotensive and hypertensive rats. ($p < 0.05$)



Systemic redox state

Levels of TBARS

The TBARS level was increased in the hypertensive sedentary group (HT-C) compared to the normotensive sedentary group (NT-C) while values of TBARS didn't significantly change comparing hypertensive and normotensive rats during different training sessions (Figure 2A).

Levels of NO_2^-

Hypertensive rats which swam six and nine weeks had a significantly increased level of NO_2^- compared to sedentary rats (HT-C). Statistically higher levels of NO_2^- were noticed in the hypertensive sedentary group relative to the normotensive sedentary group (NT-C vs. HT-C). Normotensive rats had lower level of NO_2^- than hypertensive rats who swam six as well as nine weeks (NT-6ST vs. HT-6ST and NT-9ST vs. HT-9ST) (Figure 2B).

Level of O_2^-

The level of O_2^- significantly decreased after nine weeks of swimming relative to sedentary normotensive rats as well as to hypertensive sedentary rats. Nine weeks of exercise significantly reduced the level of O_2^- relative to six weeks of swimming both in the normotensive and hypertensive group (NT-9ST vs. NT-6ST and HT-6ST vs. HT-9ST). In hypertensive rats, six weeks of swimming significantly reduced the level of O_2^- relative to the sedentary hypertensive group (HT-6ST vs. HT-C). Statistically higher levels of O_2^- were recorded in the hypertensive rather than in the normotensive sedentary groups (HT-C vs. NT-C) (Figure 2C).

Levels of H_2O_2

A significantly reduced level of H_2O_2 was observed after six and nine weeks of swimming comparing to sedentary normotensive and hypertensive rats. Exposure to nine weeks of training led to a significant reduction of H_2O_2 in both normotensive and hypertensive rats. Statistically increased levels of H_2O_2 in hypertensive relative to normotensive rats were observed in the sedentary groups, while after nine weeks of swimming, statistically lower levels were noticed in hypertensive comparing to normotensive rats (Figure 2D).

Activity of superoxide dismutase (SOD)

Swimming statistically increased SOD activity in both the normotensive and hypertensive groups after six (NT-C vs. NT-6ST and HT-C vs. HT-6ST) and nine (NT-C vs. NT-9ST and HT-C vs. HT-9ST) weeks of training relative to the sedentary group. Nine weeks of exercise induced a higher activity of SOD relative to six weeks of training. There was no difference in this parameter between hypertensive and normotensive rats (Figure 2E).

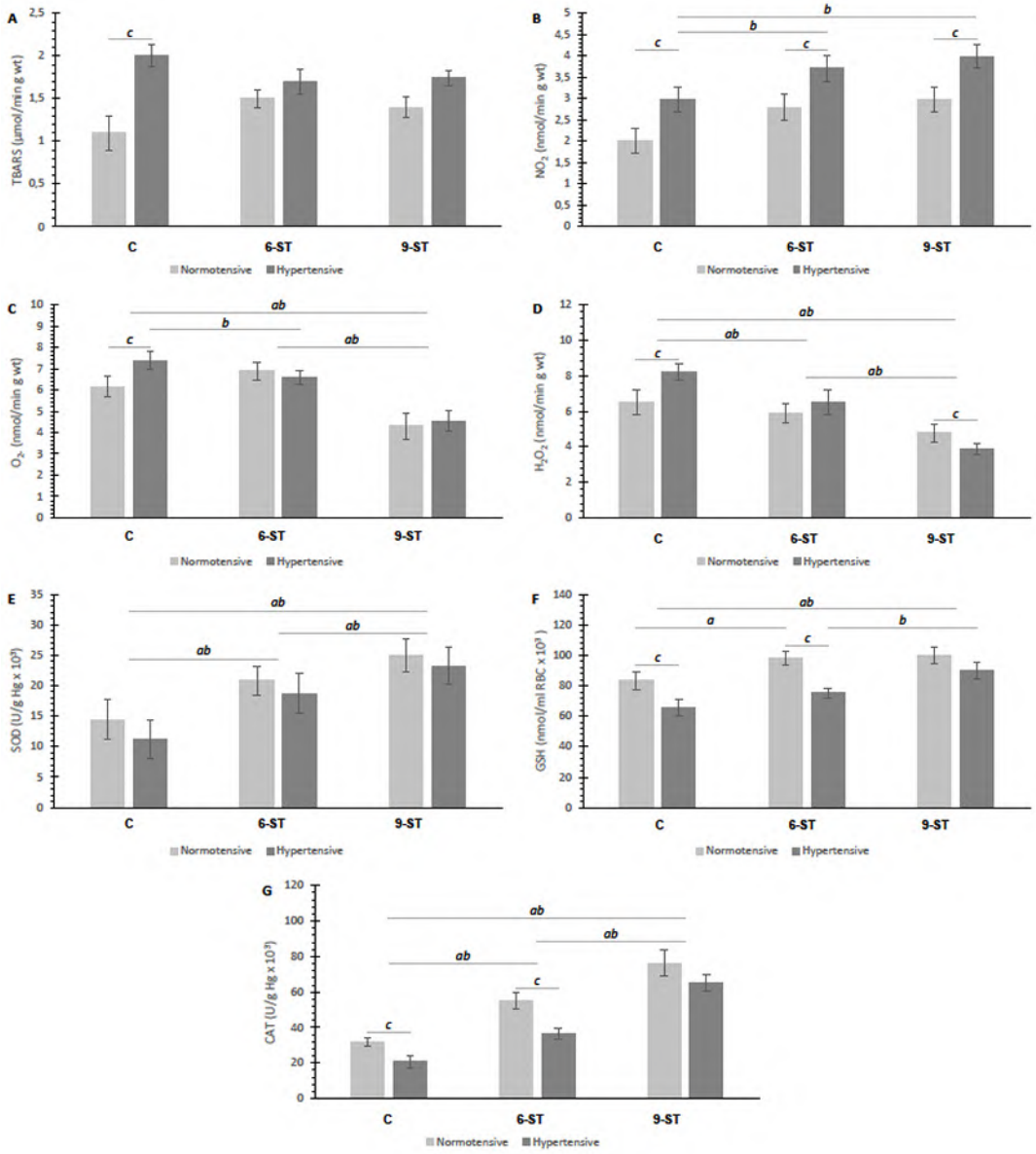
Levels of reduced glutathione (GSH)

Nine weeks of the swimming protocols significantly increased the value of GSH relative to the sedentary normotensive and hypertensive groups. Normotensive rats had a higher level of GSH after six weeks of training than in the control group. Higher levels were noticed in hypertensive rats after nine weeks of swimming compared to six weeks. Hypertensive sedentary rats as well as hypertensive rats after six weeks of swimming had statistically lower values of GSH compared to the normotensive group and six-week-trained normotensive rats (Figure 2F).

Activity of catalase (CAT)

The activity of CAT increased after six and nine weeks of training both in the normotensive and hypertensive group relative to the control group. Furthermore,the nine-week training protocol (NT-9ST, HT-9ST) had significantly higher values of CAT than the six-week training protocol (NT-6ST, HT-6ST). Sedentary and six-week-trained hypertensive rats had a statistically lower value of CAT than sedentary and six-week-trained normotensive rats (Figure 2G).

Figure 2. Time-dependent swimming training-induced alterations in systemic redox status: (A) TBARS; (B) NO_2; (C) $O_2^{\cdot}$; (D) H_2O_2; (E) SOD; (F) GSH; (G) CAT.
Each bar represents the mean ± standard deviation, *a* statistically significant difference between normotensive rats ($p < 0.05$); *b* statistically significant difference between hypertensive rats ($p < 0.05$); *c* statistically significant difference between normotensive and hypertensive rats ($p < 0.05$)



DISCUSSION

Swimming, as aerobic training, has been proposed as a convenient model for studying the physiological changes and stress response to exercise. In addition, it presents one of the non-pharmacological therapy approaches for treating hypertension (15). However, available information regarding the time-dependent benefits is deficient. Therefore, the present study aimed to estimate the effects of six and nine weeks of swimming protocols on systemic oxidative stress markers and blood pressure in hypertensive rats.

In the current study, we confirmed the previous findings that swimming lowers blood pressure which might be the consequence of vascular resistance reduction (16). Blood pressure, especially systolic blood pressure was decreased in correlation with training duration in hypertensive groups (HT-6ST and HT-9ST). These experimental findings indicate that swimming might be useful in a combination with suitable antihypertensive agents. Additionally, prolongation of exercise enhances the beneficial effect of swimming on arterial blood pressure which might be due to reduced sympathetic activity after physical activity. The findings from earlier preclinical and clinical investigations are in line with our results (17). Gilbert and co-workers (17) showed that physical activity significantly affected the endothelial dysfunction occurring in hypertension and observed that aerobic physical activity led the reduction of vasoconstriction in rats (18). Recent findings demonstrated that twelve weeks of swimming aerobic exercise is effective in evoking lower blood pressure thus improving vascular function and arterial rigorousness in prehypertensive or hypertensive subjects at the first stage (19).

Another part of our investigation was focused on the ability of swimming to affect the redox status of both hypertensive and normotensive rats. In that sense, we measured the markers of systemic oxidative stress and activity of antioxidative enzymes. Although numerous studies were mainly concentrated on the effects of anaerobic exercises (treadmill) on the redox status, we decided to use the swimming model, as a natural ability of rats (20). The results of our study clearly showed that over-production of ROS is linked to hypertension as well as that swimming training led to a decrease of almost all pro-oxidants and an increase of almost all antioxidants measured in the blood of both normotensive and hypertensive rats. Generally viewed, duration of swimming affected the values of redox markers. Actually, nine weeks of swimming indicated more benefits than six weeks of the training protocol. Although as a consequence of the adaptive cell response to exercise, an increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can occur (21), oxidative damage didn't happen in our investigation. That was probably due to the moderate intensity swimming which was long enough to allow the organism adjustment to stress.

Lipid peroxidation indicates the oxidative damage mainly to the membrane lipids thus, TBARS is expected to increase in hypertensive rats (22). Our results are in accordance with an earlier study carried out by Hu and authors (21), who didn't notice changes in the lipid peroxidation level in the rat liver and heart after seven days of swimming (23). Moreover, some authors (22) revealed no modifications in the levels of malondialdehyde (MDA) in male rats after eight weeks of swimming (24).

We noticed that swimming training reduced the NO_2^- level in a time-dependent manner, which was more pronounced in hypertensive than in normotensive rats. These results are in correlation with the previous findings suggesting that moderate exercise on the treadmill increased total nitrates/nitrites in spontaneously hypertensive rats, thus inducing relaxation and subsequent vasodilatation. In fact, this is one of the most commonly proposed mechanisms which explain reduction of blood pressure due to training (25). A large body of evidence indicates that aerobic exercise improves vascular function in the blood vessels of hypertensive patients and animals primarily through increase in NO production and/or decrease in NO inactivation by oxidative stress (26).

Reduced values of the prooxidants, especially O_2^- and H_2O_2 were supported by elevation of SOD and CAT activity. Actually, SOD eliminates O_2^- acceleration of its dismutation to H_2O_2, while CAT as a ferric hem protein can catalyze the degradation of H_2O_2 (21). Bearing in mind that O_2^- can react quickly with several radicals and iron-sulfur clusters in the protein, as well as that H_2O_2 has cytotoxic properties (27), the reduction of these free radicals directly indicates the benefits of swimming in hypertension. According to the several lines of evidence, a moderate physical exercise has antioxidative effects (28), but training duration necessary to achieve these benefits is still controversial. However, there are only few studies dealing with this problem in hypertension. In this study, it was found that six weeks of swimming were sufficient to increase statistically significantly SOD, GSH and CAT in healthy, respectively SOD and CAT in rats with hypertension. On the other hand, all of the measured antioxidative parameters were enhanced in both normotensive and hypertensive rats after nine weeks of swimming. Literature data revealed that the duration and intensity of physical activity are directly related to the antioxidant levels of enzyme activity (29). Additionally, based on our results, nine weeks of swimming were sufficient to achieve the antioxidant effects in hypertensive animals.

Our research illustrated that the moderate intensity swimming training reduced blood pressure values in hypertensive conditions, which was more featured in the nine-week swimming group. Lifestyle modifications in sense of starting swimming, afterdeveloping hypertension, may be beneficial as non-pharmacological co-therapy. Moreover, swimming has a positive influence on the system adaptation, especially in controlling the redox status, which is unbalanced in hypertension.

ACKNOWLEDGMENTS

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ETHICS APPROVAL

Research was approved by Ethics Committee of the institution. All research procedures were done according to EU Directive for welfare of laboratory animals (86/609/EEC) and principles of Good Laboratory Practice (GLP).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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PROPOSITION OF A SIMPLIFIED PROTOCOL AND NEW PARAMETER INTRODUCTION IN NMRI MICE ANHEDONIA INDUCTION

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ABSTRACT

A broad spectrum of research involving stress and stress protocols has long proven that a point of anhedonia, social defeat and learned helplessness can be achieved and observed – with anhedonia being a clinical symptom of Anxiety, Depression and Bipolar Affective Disorder. The aim of this study is the development of a simplified protocol for anhedonia induction in NMRI male mice in order to shorten the period of mice suffering and decrease complexity of the procedure for other researchers and introduction of new parameter in order to achieve better standardization of results. 21 male NMRI mice were introduced to 2 different stress protocols (one found in literature and one simplified) where cognitive-behavioral status was tested using the Sucrose Preference Test, Open Field Test, Grooming Pattern and histological examination of adrenal glands, and to propose a new protocol for fellow researchers. Results observed include the successful induction of anhedonia proven by Sucrose Preference Tests, Barbering effect and microhemorrhage of the adrenal glands. Simplified protocol showed superiority compared to the one found in literature. Simplified protocol showed higher efficiency and reduced amount of work during testing phase. Introduction of NMI as a new parameter during behavioral evaluation resulted in better standardization of measured SPT values that incorporates common knowledge of mammal physiology. A big developmental step was the introduction of the Normalised Mass Index to even out mass fluctuations and differences in basal metabolism, which we recommend to other researchers and institutions.

Keywords: *Chronic stress protocol; anhedonia; anxiety; depression; NMRI mice.*

INTRODUCTION

In the boom of mood disorders such as depression, anxiety and bipolar affective disorder it comes to no surprise that stress besides genetic and environmental variables is held accountable as a major source triggering those illnesses (1-4). With this being said stress can be subdivided into psychological, physical, physiological and environmental stress (1, 5-6). Each of them is a plausible stressor in humans and can lead to mood changes, anhedonia, energetic changes, difficulty in concentration and memory, disrupted circadian circuits, decreased or increased sensation of hunger and finally suicidal thoughts (7). A common belief for above mentioned symptoms is now that humans far derived way of living, such as light exposure during night, are major contributing features that deepen not only the anhedonic state, but also anxiety-relating behavior and further explains why mood disorders are continuously on the rise (2, 8-10).

Some of the most important indicators for stress are Cortisol and Corticosterone in the human and Corticosterone in the rodent. Both of these steroid hormones follow a circadian rhythm by peaking in the morning and lowering down slowly throughout the day, reaching its minimum during the night and are used as markers as both of them regulate Gluconeogenesis and suppression of immunity during stressful events amongst many other important functions (5). Glucocorticoids, which are produced in the adrenal cortex, are part of the hypothalamic-pituitary-adrenal axis (HPA axis) system. This system includes the hypothalamus, which via secretion of corticotropin releasing factor (CRF) stimulates secretion of adrenocorticotropic hormone (ACTH) in the anterior pituitary gland (6, 11).

Heim et al. (12) suggests that a chronic exposure to stress, especially during childhood, determines vulnerability and leads to a hypersecretion of CRF from the hypothalamus and therefore causes a down-regulation of CRF receptors in the adenohypophysis, which induces anxiety and depression-like symptoms. Based on previous findings it is known that receptors of the HPA-axis can undergo epigenetic modifications during stressful periods and the three-hit concept further explains that based on genetic variables, experiences in early and later life coping with stress leads to vulnerability or resilience and finally as far as to the development of a mental disorder (13-15). This concept comprises the cumulative stress hypothesis (stress and misfortune adding up and leading to disease) and the mismatch hypothesis (mismatch between early and later life leading to the development of a mental disorder) (14).

It is important to mention that the HPA axis influences the HPG axis (the hypothalamic-pituitary-gonadal axis), especially during stress. Higher glucocorticoids hereby mean a suppression of gonadotropin releasing hormone (GnRH), gonadotropins and gonadal function (16-17). The impact of chronic stress on the testes depends if it was applied pre-puberty or during adulthood. Pre-puberty stressed mice show hereby reduced tubular compartment with the danger of reduced fertility. Meanwhile, changes in adult mice are reversible. Most common pathohistology seen in testes after stress exposure is vacuoles in the seminiferous epithelium and degeneration of primary spermatocytes (17).

Although the communication problem between animals and humans does not provide us with insight into the animals mind, animal models have become a valid model for mood disorders as anhedonic state, learned helplessness, social defeat and grooming behavior can be observed and/or measured (3, 18). The animals state of coat can in a figurative sense be applied to human appearance and is therefore commonly used for interpretation of the degree of anxiety, depression, obsessive-compulsive disorder, etc (3, 19). Sucrose Preference Test, Open Field Test, Forced Swimming Test and more have been widely recognized and acknowledged in mood disorder studies (3). There are several protocols used for anhedonia induction in NMRI mice in order to study mechanisms of depression, anxiety, medications, etc. Most of them require complex alternation of stressors during several weeks in order to reach detectable state of anhedonia.

The aim of this study is the development of a simplified protocol for anhedonia induction in NMRI Mice in order to shorten the period of mice suffering and decrease complexity of the procedure for other researchers and introduction of new parameter in order to achieve better standardization of results.

METHODS AND MATERIALS

Animals

Experimental animals were treated in coordinance with Ethical norms of Pasteur Institute Novi Sad (permission number 01-35/3). A total of 21 male NMRI mice were used for the development and evaluation of a simplified protocol for anhedonia induction. Hereby three cages (27 x 20,5 x 14 cm) with 7 mice each were utilized. Age of mice at the beginning of the experiment was 4 weeks for positive control group (CRTL+), experimental (EXP) and between 7 and 8 weeks for negative control group CRTL-. The control group was reused as part of the Replacement, Refinement and Reduction Guideline for animal research. Room temperature was kept constant at 23±2 °C. Mice were habitated according to a 12-hour dark-light circadian rhythm and each group was kept in separate room. Mice were kept on a rodent pellet diet and acified water was provided ad libitum except during Sucrose Preference Test. The bedding was changed accordingly to need which varied depending on the stress protocol but minimally once a week. Aggressiveness between mice and rats was tested in a week-long pilot before starting the study, as one stressor specifically required for exposure to one male Wistar rat. After group division by randomization mice were given 7 days before starting the experiment to acclimatize within their group.

Stress Procedure

CRTL- group was non-stressed and used for reference values in SPT tests, weight measurements and grooming patterns, whereas both experimental groups underwent several stressors. The experiment lasted for 28 days, after which EXP and CRTL+ mice were humanely euthanized (Figure 1).

CRTL+ group followed a well-developed Chronic Stress Protocol found in literature (3) that included Rat Exposure, Restraint (Immobilization), Water Emergency, Forced Swimming Test and Tail Suspension (Figure 1).

Meanwhile EXP group underwent a newly designed protocol which consisted of a combination of Immobilization, Dim Lighting, Rat Exposure, Forced Swimming Test, Sound, Tail Suspension and Bedding with Cat Odor (Figure 1).

After the 28 day EXP group reached anhedonic state and stress protocol was over. Mice were kept one more week before being humanely euthanized by neck dislocation. Their adrenal glands were taken and examined using histological routine techniques in order to register potential morphological changes.

Immobilization

Immobilization Chambers (10,5 x 3 cm, polyethylene with cork screw) were always washed with hot water after usage as to remove shed hair and the smell of urine and feces.

Forced Swimming Test (FST)

Forced Swimming Test was done in a polyethylene cylindrical container using water at room temperature at a height of around 12,5 cm.

Bedding with Cat Odor

Fifty grams of bedding with cat odor was placed on top of conventional rodent bedding and when it was dismissed another fifty grams was added.

Sound

When sound was used as a stressor white radio noise or normal radio sounds were turned on in a separate laboratory, which mice were previously transported to.

Dim Lighting

As with the previous stressor Dim lighting was carried out in our laboratory by applying light from a dim light source over night.

Tail Suspension

When undergoing Tail Suspension mice were taped to a pole by using laboratory labeling tape. Tail climbing could not always be completely avoided.

Testing

Sucrose Preference Test, Open Field Test, Body weight and Grooming were used to measure stress effect, anhedonia and anxiety in mice following literature protocols (3). Adreanal glands were histologically analyzed.

Sucrose Preference Test (SPT)

Twelve hours prior to testing of Sucrose Preference, mice were derived of food and water. Then both water and a mixture of 1% sucrose solution was provided and left for 24 hours. Water and Sucrose Solution Intake was measured and Sucrose Preference was concluded by using the formula:

$$SPT = \frac{Sucrose\ solution(g)}{Sucrose\ solution(g) + water\ (g)} \times 100$$

If sucrose preference was higher than 65% mice were termed resilient, if it was lower than 65% they were termed susceptible.

Furthermore a “Normalised Mass Index” (NMI) was calculated using the following formula:

$$NMI = \frac{Sucrose\ solution\ (g)}{Sucrose\ solution\ (g) + water\ (g)} \div mean\ weight\ of\ mice\ (g) \times 100$$

NMI was introduced within this research to reduce error considering that mean weight fluctuated for both experimental groups and therefore had impact on their Basal Metabolism requirements. The NMI was not previously found in literature.

Open Field Test

Open Field Test (OFT) was examined over a time frame of four minutes by recording the arena via camera (Canon EOS D700) and analysed using computer software ToxTrac v2.83 to examine mobility, average speed and number of bolus dropped per mice. [20] OFT was conducted in arena with base dimensions 40cm x40cm of opal white color and transparent acrylic walls high 35cm. Arena was cleaned with 70% ethanol after recording of each mouse.

Body weight measuring

Mice body weight was measured the day before each Sucrose Preference Test in the morning period and calculated as average body weight per group. Body weight changes over the course of time were compared between groups using Wilks' Lambda Multivariance Analysis.

Grooming Behaviour

Grooming behavior was checked three times throughout the experiment by putting a see-through, clear acrylic glass cover over the cage and videotaping the mice in their habitat. The grooming itself was judged by the strokes, patterning of bouts, duration and overall state of coat.

Histological examinations of adrenal glands

Adrenal glands were fixated in buffered 10% formalin (pH 7.4), dehydrated using routine ethanol-xylene sequence and embedded in paraffin (Histowax, Gotenburg) at 60°C. Histological slides were cut on Leica RT350 rotary microtome, stained by Eriochrome-cyanine R & Eosin technique according to Stefanovic et al. [21] and photographed by camera mounted on Leica DM microscope.

Statistical analysis

Data was analyzed using the SPSS software (IBM, USA). Pearson Correlation was used for comparison and evaluation of Normalised Mass Index regarding SPT values. Wilks' Lambda Multivariante test was used for comparison of average body weights between groups during experiment. Two-tailed Student t-test was used for comparison of parameters aquired through Open Field Test. Statistically significant difference was considered at $p \leq 0.05$.

Complications

Both rat exposure (group CRTL+) and bedding with cat (group EXP) odor seemed to be minor stressors for mice. After initial shock, consensual grooming was seen in the interaction of rat and mice. The rat was later replaced. Cat bedding was completely dismissed by EXP mice, as they hid the cat sand under their normal bedding.

One mouse was eaten by its comates in Protocol EXP during the second Sucrose Preference Test. It was later concluded that this was due to the fact that the mouse itself had scratched open its nose during immobilization (air holes) and this way created a wound. The mouse was removed and tests were normally continued.

RESULTS

Stress effect on body weight

Using Wilks' Lambda Multivariance Analysis and Bonferroni post hoc test have found statistically significant difference in average body weight in the course of experiment between CTRL – on the one side and CTRL + and EXP on the other, which is caused by implementation of 3R principle (Figure 1).

Anhedonia induction

Regarding SPT values, experimental group was only one that reached near-anhedonic state during 28-day-course of experiment(Figure 3). Despite measured values, a need for adequate standardization was noticed due to average body mass difference, therefore Normalised Mass Index was introduced (Figure 4), which showed correlation with measured SPT values; 0.969;0.896 (Table 1) . Although, positive control group was very near the cutoff point of 65% on 21st day of experiment, a week later their results showed higher values in contrast to experimental group. Negative control group showed constant SPT values during the whole course of the experiment.

Table 1. Correlation of NMI and SPT values

	NMI_exp	SPT_exp	NMI_ctrl +	SPT_ctrl +
NMI_exp	1	0.969*	0.773	0.411
SPT_exp	0.969*	1	0.805	0.471
NMI_ctrl +	0.773	0.805	1	0.896
SPT_ctrl +	0.411	0.471	0.896	1

Open Field Test (OFT)

Results obtained from OFT include average speed, average mobility and total fecal boli dropped by individual animal in one group. Student's t-test showed no statistical difference between groups in average speed and mobility. In contrast, there is a significant difference between CTRL + and EXP group in dropped fecal boli per animal (p=.34), where average number of fecal bolus in CTRL+ group is 4.3 and 1.0 in EXP group.

Figure 1. Depiction of Stress Protocols CRTL-,
CRTL+ and EXP and time line when Sucrose Preference Test (SPT),
Open Field Test (OFT), Grooming (G) and Weight Measurement (W) was carried out.

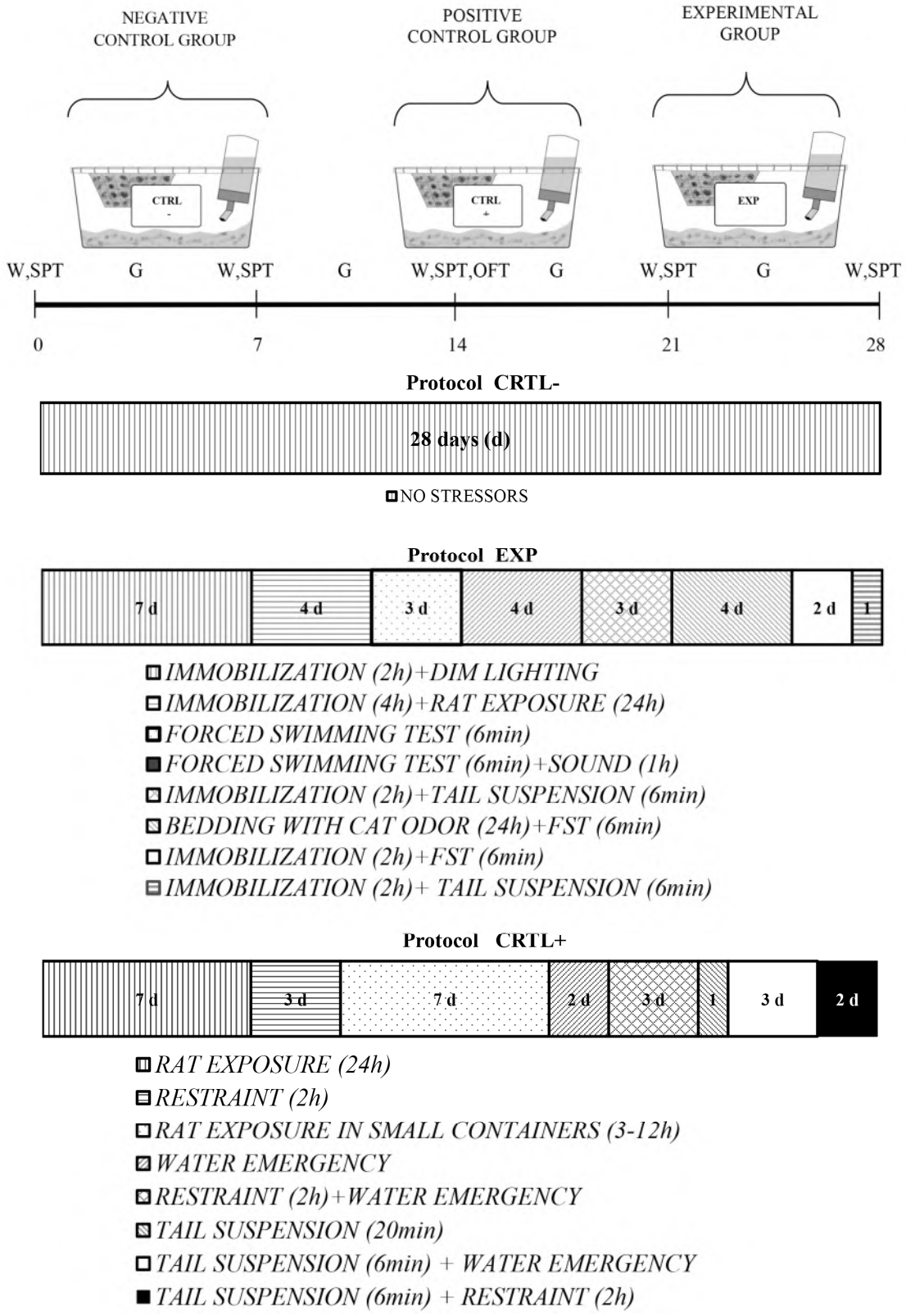
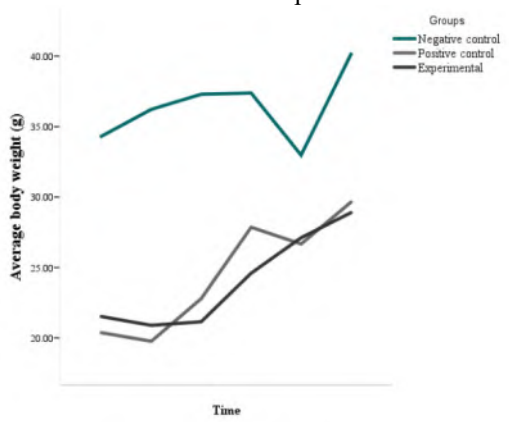
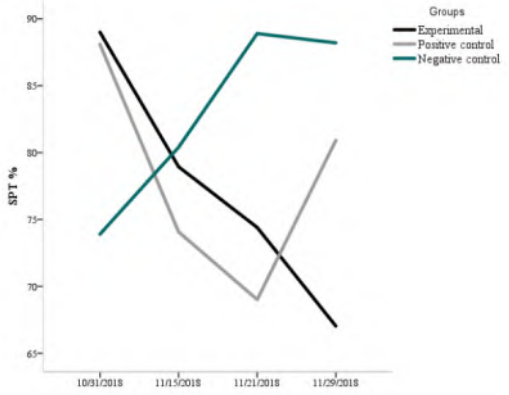


Figure 2. Average body weight over the course of the experiment.



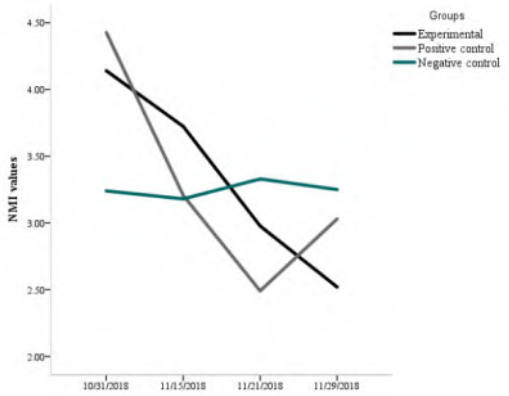
Statistically significant difference in CTRL - group is present due to implementation of 3R principle

Figure 3. SPT values.



EXP group was the only one that reached near-anhedonic state

Figure 4. NMI values.



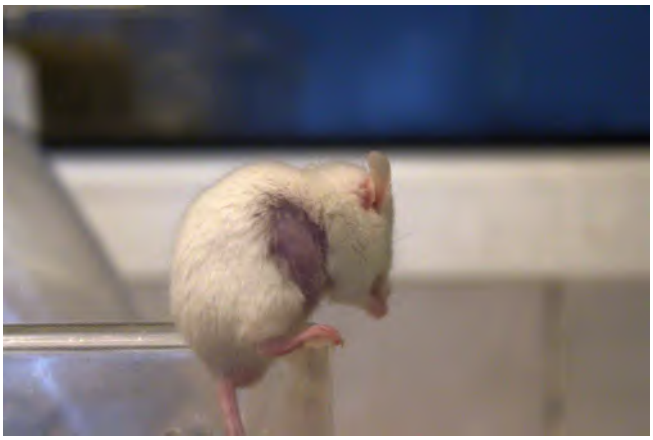
EXP group is still having the lowest score. In contrast to SPT values, CTRL – group is showing a more constant trend through NMI

Grooming Behaviour

After first week of experiment, one of the most prominent features in the EXP group was the occurrence of the Barbering effect with an exposure of the ventral abdominal surfaces in two mice and indication of exposure of the neck in another one. (Figure 5) It is worth mentioning at this point that their whiskers (crucial sensory organ) and the snout was intact. This Barbering effect disappeared in week four, when 'milder' stressors such as cat bedding were used. Nevertheless, state of coat deteriorated tremendously.

For CRTL+ the coat worsened immensely in the last two weeks of the experiment by thinning out. Stress (FST, Immobilization, etc) led to hyperactivity and excessive grooming with missing strokes and/or decreased or increased duration. Rat exposure was an exception as heterogrooming was seen until the rat was exchanged. The question if anhedonic, stress-evoked mices grooming prolonged or shortened was not uniform in neither of the groups. In a non-stress-evoked, resting state mistakes were seen either as a prematurely terminated bout or as a bout with skipped transitions. A prematurely terminated bout often led to the immediate restart of a new cephalocaudal bout or continuation of the already started one after a few seconds.

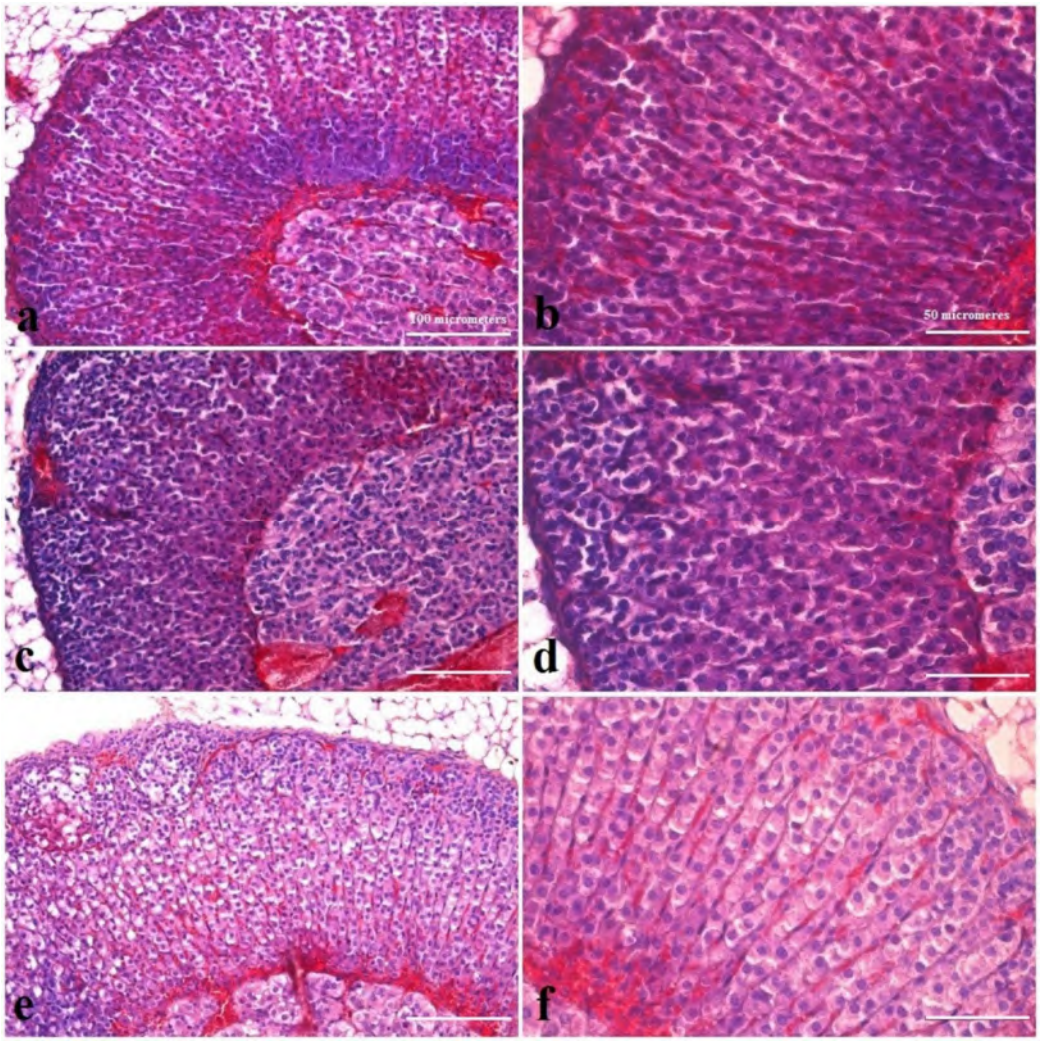
Figure 5. Barbering effect seen in EXP group



Histological Findings

Histological examination of adrenal glands revealed dilatation of blood vessels in CTRL+ (Fig.6a-b) and EXP group (Fig. 6e-f) compared to CTRL- group (Fig 6c-d). There is a noticeable hyperemic zone between the medulla and cortex in both stress-induced groups that can be analog to nontraumatic adrenal hemorrhage in humans.

Figure 6. Adrenal glands (ECR&E; x200;x400)



a-b: Hyperemia with microhemorragia in the zone between medulla and cortex and dilatation of cortical blood vessels in the four mice of CTRL+ group . c-d: Normal histological finding in CTRL – group, absence of hyperemia, microhemorragia and dilated blood vessels . e-f: Four mice of the EXP group shows similar findings as CTRL+ group.

DISCUSSION

The aim of this study was to create a simplified protocol for anhedonia induction in order to minimize suffering of mice and reduce the amount of work needed to achieve the anhedonic effect. Regarding the SPT findings, highlights observed were hereby the occurrence of the Barbering effect as a sign of anxiety and adrenal microhemorrhage due to disruption of the HPA axis.

One of the most outstanding occurrences was the Barbering effect observed in EXP group. NMRI mice are known to show high levels of grooming with frequent manifestations of the Barbering effect (approximately 80 – 100% of the time). This has been commonly interpreted as a way of mice to show social dominance, especially in same-sex cages and more commonly in males, and affecting their snouts and whiskers (the Dalila effect). The coping hypothesis furthermore states that inadequate housing leads to this effect (22-24). However, close observation and recording of EXP group lead to the clear result that the Barbering effect in this experiment was not due to hetero-grooming, but due to self-grooming, that neither snout not whiskers were affected and that the inadequate housing was not to blame for this occurrence, as CRTL+ was exposed to the exact same living conditions, but was completely unaffected. The only difference between the experimental groups was in fact the stress protocol that they underwent. Interpretation of this stress-evoked Barbering from a behavioral standpoint can be attributed to subdivisions of Obsessive Compulsive Disorder (OCD) such as Trichotillomania which constitutes as compulsive hair pulling due to increased self grooming or Anxiety Disorders. These are both related to elevated anxiety levels and can be seen in humans as nail biting, hair pulling and skin picking. Interestingly

enough, the appearance of the symptom of anhedonia contributed to Major Depression Disorder (MDD) has been said to lead to decreased self-grooming and as a consequence to bad hygiene (19). A possible explanation therefore could be strain specific differences - NMRI mice naturally show higher grooming levels and are naturally more prone to Barbering. Further research needs to be done for better understanding of Barbering effect in relation to strain specific characteristics and behavioral impact.

A downfall during this study was the cannibalism observed during the second Sucrose Preference Test in experimental group EXP. This unfortunately occurred after immobilization as the mouse had a wound on its nose and neither food nor water were supplied overnight. The next morning, when water and sucrose solution were added, the mouse was found dead with parts of its head eaten. The question hereby is if the 12-hour period with food and water derivation is too long to bear for animals? It has to be mentioned that besides this incident no mouse was harmed during Sucrose Preference Testings and although this period itself can be classified as a stressor, it proved to be both bearable for mice and practical for veterinary assistance. This could of course be shortened to a 6-hour derivation phase with a following 8 hour Sucrose Preference Test, which could be question to further research.

Another fail was bedding with cat odor as a stressor as mice dismissed it completely. This came to a surprise since there is scientific research on trace amine-associated receptors (TAAR) in mice noses, especially trained for smells of predators, as is cats urine (25). The most probable answer to this is that there was not enough cat bedding in the cage or that the smell wasn't strong enough. The boundaries should be tested, as filling the entire cage with bedding with cat odor could overpower mice receptors. A possible solution could be supplying a tissue that has been used to pet a cat and putting it into the cage. As no reaction was seen during this experimentation, we have to further ask ourselves if this can provide a form of psychological stress as in social defeat.

Results of the Open Field Test were unclear. It has been proven that there is a negative relationship between ambulation and defecation, meaning that anxious mice will show reduced locomotion with increased stimulation of the autonomic nervous system, which in turn increases defecation. The latter has been often been linked to emotionality or the presence of an affective state (3, 26-27). In this experiment, however, speed and mobility didn't show statistically significant difference, yet boli drop did. Hereby CRTL+ group showed increased boli drop and EXP group decreased boli drop with both of them showing a deviation from the norm – even though into two opposite realms. Although there are no data for comparison regarding NMRI mice, same results were obtained in the study by Fedotova et al. in prenatally stressed male Wistar rats (28).

A rare revelation that was seen in positive control and experimental group are microhemorrhagic zones between the medulla and cortex. This occurrence can be linked to nontraumatic adrenal hemorrhage in humans, which is a rare stress-induced condition. The stress that leads to the manifestation of nontraumatic adrenal hemorrhage in humans is said to be due to surgery, sepsis, burns, hypotension or pregnancy (29-30). Mice in this experiment underwent several different stressors of different families (physical, physiological, environmental, psychological), with most of them leading to shock and/or crisis in mice. There are several factors deemed responsible for this incident. Firstly, mice used were very young. Adrenal hemorrhage is more likely to occur in neonates than in adults and although mice weren't neonates, they were still more susceptible to adrenal damage than adults. Second, some stressors might have led to hypoxia (f.e. immobilization). Third and most importantly, it seems undeniable that a disruption of the HPA axis is to some degree responsible for adrenal hemorrhage (6, 11, 29-30).

Further research should focus on strain difference, as well as germ and germ-free animals, and its entanglement when it comes to Grooming pattern, Open Field Test and Sucrose Preference Test. Furthermore, more emphasis should be put onto the study of different mouse strains and stressors and their entanglement.

CONCLUSION

Compared to anhedonia-inducing stress protocol found in literature, the simplified protocol showed higher efficiency and reduced amount of work during testing phase. Introduction of NMI as a new parameter during behavioral evaluation resulted in better standardization of measured SPT values that incorporates common knowledge of mammal physiology. Regarding our difficulties with the comparison of OFT results with other studies there is a need for improvement and standardization of tests used for behavioral status of mice.

ETHICS APPROVAL

All research procedures were carried out in strict accordance with the European Union Directive for the welfare of laboratory animals (No. 2010/63/EU).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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None.

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THE RELATIONSHIP BETWEEN THE INCIDENCE OF CORONARY HEART DISEASE AND ETHNIC MINORITIES

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ABSTRACT

The study aimed at the determination of risk factors, their relationship with the development of stenosing lesions of the coronary arteries in different ethnic groups in Kazakhstan. Primary coronary angiographies of n=640 patients diagnosed with coronary heart disease (CHD) (2017-2019) have been analysed (Almaty, Kazakhstan). The patients were subdivided into: Kazakhs (n=338) and Russians (n=302). In the Russian group, the chance of arterial hypertension incidence was higher (44% and 33%, p<0.05). In the Russian group, the percentage of obstructive CHD was higher than in the Kazakhs (66% and 57%, p<0.05). There was association between obstructive CHD and risk factors such as male sex, diabetes, smoking, and diastolic blood pressure (DBP) in the Kazakhs (p<0.05). In the Russian group, the relationship between development of CHD and age, level of total cholesterol and high-density lipoprotein (HDL) was higher (p<0.05). There is an association between smoking, diabetes, sex, DBP and the development of CHD in Kazakhs. In the Russian group, CHD was associated with risk factors such as older age, dyslipidaemia and arterial hypertension. There were significant ethnic differences in the risk factors and CHD, in the Russian group the probability of development of obstructive CHD was higher. There was an association between smoking, diabetes, sex, DBP and the development of CHD in Kazakhs. In the Russian group, CHD was associated with risk factors such as older age, dyslipidaemia and arterial hypertension. These finding indicate the need to develop differentiated programmes for the screening, preventive measures for different ethnic groups.

Keywords: *Ischemic heart disease, coronary angiography, risk factors, ethnicity.*

INTRODUCTION

According to the World Health Organization (WHO), cardiovascular diseases such as CHD and strokes, are among the leading causes of death worldwide (1).

The development of CHD has been associated with a range of cardiovascular risk factors, including overweight, arterial hypertension, impaired carbohydrate metabolism, dyslipidaemia, smoking, age, male gender, heredity and physical inactivity (2-5). Taking into account the multinational composition of the inhabitants of many countries, ethnicity as a risk factor for the development of CHD remains an open question (6-9).

Up to date, there is a number of reports on the role of ethnic differences as a risk factor of CHD (10-14). For example, European descents in the United States have a less aggressive type of coronary artery disease (according to coronary angiography). It can be explained by a lower incidence of some risk factors for the development of CHD compared to nationalities from other parts of the world (for example, South Asia, India, and Bangladesh). In Israel, there is a more aggressive type of coronary artery disease among the Arab population compared to the non-Arab population. It has been associated with greater exposure of Arabs to certain risk factors and a lower social level. It was shown that some ethnic groups are vulnerable to cardiovascular diseases due to different susceptibility to various cardiovascular risk factors, which is especially important for countries with a multi-ethnic composition of the population (15-17).

Today Kazakhstan is a multi-ethnic state. According to statistical sources, more than 130 nationalities live in the territory of Kazakhstan. In the overall proportion, the main ethnic groups are represented by Central Asians (Kazakhs 61.3%) and Slavs (Russians 23.7%), while representatives of other nationalities make up a small share of residents of the Republic (13.2%) (18-20).

Up to date, a number of studies were carried out on ethnic differences in Kazakhstan for risk factors such as alcohol consumption and smoking (21, 22). It includes the research within the framework of the international study "Intrepid" conducted in Russia, Kazakhstan, and Kyrgyzstan (23). This study encompasses the investigation of risk factors such as arterial hypertension, smoking and overweight. However, these studies did not consider the influence of factors such as dyslipidaemia, the presence of type 2 diabetes mellitus and the state of the coronary arteries that play a critical role in the pathogenesis of CHD (24).

Apart from that, it must be noted that the influence of gender on the development of cardiovascular diseases has been widely discussed in the scientific literature as well (25). Thus, the study of these aspects can provide an insight into the vulnerability of representatives of different ethnic groups to certain risk factors for CHD. In fact, many risk factors for cardiovascular diseases are modifiable (23). So the study of the risk factors, including ethnicity, might help in the early detection and prevention of stenosing coronary lesions.

The objective of this study was to determine the role of risk factors, including ethnic origin and gender in the development of stenosing lesions of the coronary arteries among the population of Kazakhstan.

MATERIALS AND METHODS

Data collection

The prospective cohort study was conducted at the clinic of JSC "Central Clinical Hospital", one of the largest multidisciplinary clinics in Almaty, Kazakhstan.

The dataset was carried out according to the CONSORT criteria [24], from 2017 to 2019. Inclusion criteria were: verified diagnosis of coronary artery disease, primary coronary angiography, and ethnicity identified as Kazakh or Russian.

Patients were diagnosed with coronary artery disease according to standard clinical criteria (26, 27). Of the total number of cases (n = 1,628), 61% (n = 988) of patients were not included in the study for the following reasons: 9% (n = 146) did not meet the criteria by nationality, 52% (n = 842) were previously implanted with a stent and/or had undergone coronary artery bypass grafting. Thus, the study included n = 640 (39%) cases, which were classified by ethnicity: Kazakhs and Russians (Figure 1).

The following demographic characteristics were determined: gender, average age and nationality. Nationality was determined by the passport data of the patients. According to age indicators, patients were divided into 5 age categories: 30-39, 40-49, 50-59, 60-69 and ≥70 years.

We also studied indicators of body mass index (BMI) and the presence of bad habits such as smoking. Smokers (at the time of coronary angiography) were defined as those who smoked at least 1 cigarette per day for at least 1 year, or who quit smoking less than 6 months before hospitalization.

In terms of BMI, a value < 25.0 was regarded as normal body weight, a BMI of 25.0-29.9 was defined as overweight, and a value ≥ 30 as obesity.

Among the comorbidities, the presence of arterial hypertension, obesity, dyslipidemia and type 2 diabetes mellitus was screened for.

Arterial hypertension was confirmed by anamnestic data and registration of systolic blood pressure (SBP) ≥140 mmHg. and/or diastolic blood pressure (DBP) ≥90 mmHg (28).

The criteria for dyslipidemia for patients with coronary artery disease were the levels of total cholesterol (TC) above 4.5 mmol/L, triglycerides (TG) above 1.7 mmol/L, low-

density lipoprotein (LDL) above 2.6 mmol/L, HDL below 1.0 mmol/L in men and 1.2 mmol/L in women (29-31).

The presence of type 2 diabetes mellitus was determined mainly by anamnestic data and the use of hypoglycemic therapy. If necessary, the diagnosis was made on the basis of fasting glucose tests (≥ 6.1 mmol/L for venous blood), glucose tolerance test (≥ 10 mmol/L for venous blood), glycated haemoglobin (HbAlc ≥ 6.5%) (32, 33).

According to the type of hospitalization, the subjects were divided into planned and emergency patients. To assess the state of the coronary vessels, during coronary angiography, the lesion of at least one epicardial coronary artery with stenosis of ≥50% was considered hemodynamically significant, which was determined by computer-digital analysis (34).

Statistical analysis

Statistical processing of results was carried out using the SPSS program, version 21.0, IBM (USA). The selected methods were chi-square for nominal values and frequency indicators, Student's t-test for comparing mean values of interval scales, and binary logistic regression analysis to determine the likelihood of developing stenosing coronary lesions. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Demographic and clinical characteristics of patients with CHD

Demographic and clinical characteristics of patients with CHD were presented in Table 1. N = 640 out of 1,628 patients were included in the study, of which 338 (53%) were Kazakhs and 302 (47%) Russians. The average age of Russians was 65.6 ± 10 years, while this indicator for Kazakhs was 61 ± 1.5 years ($p = 0.001$).

Among the studied age groups in Kazakhs (n = 123; 36%) and in Russians (n = 119; 39%), the highest frequency of coronary artery disease diagnosis falls in the age group 60-69 years. However, there is no statistically significant difference ($p > 0.05$). In the age group ≥70 years, the number of ischemic heart disease cases was significantly higher in Russians (33%) in comparison with Kazakhs (22%) ($p > 0.05$).

In terms of gender, the number of males prevailed in both groups: 66% (n = 424) compared with 34% of females (n = 216) ($p = 0.001$).

By BMI, normal body weight (<25) was determined only in 16% (n = 105) of cases. Overweight was found in almost half the cases (n = 313; 49 %), where this degree of BMI (25-29.9) among Kazakhs (52%) was higher in comparison with Russians (46%). However, BMI ≥30 was more often recorded in Russians (38%) in comparison with Kazakhs (31%) ($p > 0.05$).

Arterial hypertension was recorded in 38% (n = 245) of the patients studied (n = 640). In Russians, hypertension was detected in 44% (n = 133) of cases, which was significantly higher than in Kazakhs (33%; n = 133) ($p = 0.005$). The average SBP and DBP in Russians were comparatively higher (135.0 ± 17.0 and 82.0 ± 8.2 mmHg) in contrast to these indicators in Kazakhs (130.0 ± 16.7 and 80.0 ± 8.2 mmHg), respectively ($p = 0.02$).

Type 2 diabetes was registered in almost 25% of the cases (n = 152). Type 2 diabetes was found in 26% (n = 89) Kazakhs and in 21 % (n = 63) Russians ($p = 0.10$).

There were no statistically significant differences in the presence of DLP in Kazakhs and Russians with indicators equal to 89% and 87%, respectively ($p = 0.60$). According to the indices of the mean values of lipid metabolism, the level of HDL in Kazakhs (1.2 ± 0.4 mmol/L) was significantly higher than in Russians (1.0 ± 0.4 mmol/L) (p = 0.001). Significant differences between Kazakhs and Russians in the mean values of total cholesterol (4.8 ± 1.1 and 4.9 ± 1.1 mmol/L; p = 0.10), LDL (3.1 ± 1.0 and 3.2 ± 0.95 mmol/L; p = 0.19) and TG (1.6 ± 0.9 and 1.5 ± 0.9 mmol/L; p = 0.17) were not detected.

The proportion of smoking patients among Kazakhs and Russians had almost identical indicators (n = 77; 23% and n = 71; 24%; $p > 0.05$).

By the type of hospitalization, the number of planned cases was higher among Kazakhs compared to Russian (67.5% vs. 57%, $p = 0.02$).

According to the results of coronary angiography, significant stenosis of the coronary arteries (≥50%) was more often determined among Russian patients (n = 198; 66%) in comparison with Kazakhs (n = 193; 57%) ($p = 0.03$).

The results of the study of coronary arteries affected by stenosis showed that single-vessel lesions in Kazakhs amounted to 19.5% (n = 66) and in Russians 23% (n = 70) ($p > 0.05$). Two-vascular lesions in the Kazakh group amounted to 18% (n = 61), and in the Russian group 20% (n = 59) ($p > 0.05$), and three-vascular lesions in Kazakhs amounted to 19.5% (n = 66) and in the Russian group 23% (n = 69) ($p > 0.05$).

National differences of the study participants depending on clinical characteristics and gender

National differences of the patients depending on clinical characteristics and gender were indicated in Table 2. In the studied ethnic groups, according to the average age indicator, women were significantly older than men: the average age was 60.6 ± 10.7 years for Kazakh men and 63.7 ± 9.9 years for Kazakh women ($p = 0.016$), and it was 63.5 ± 9.9 years for Russian men and 68.4 ± 10 years for Russian women ($p = 0.001$) .

In terms of lipid metabolism in Kazakh women, the average level of HDL was significantly higher than in Kazakh men (1.1 ± 0.4 and 1.3 ± 0.4 mmol/L; $p = 0.005$). For the rest of the mean values of cholesterol metabolism in Kazakhs, no gender differences were found. In the Russian group, women had significantly higher average total cholesterol levels and HDL cholesterol than men: for Russian women, the total cholesterol level was 5.2 ± 1.2 mmol/L, and for men it was - 4.7 ± 1 mmol/L ($p = 0.001$); the level of HDL in women was 1.1 ± 0.5 mmol/L, and in men it was - 0.9 ± 0.3 mmol/L ($p = 0.001$). No gender differences were found in mean LDL and TG values.

In the Russian group, the average BMI in women was significantly higher than in men (30.6 ± 5.8 and 28.7 ± 4.7 kg/m^2; $p = 0.002$). In the Kazakh group, there were no significant differences in BMI between men (28.7 ± 4 kg/m^2) and women (28.9 ± 4.7 kg/m^2) ($p > 0.05$).

Mean blood pressure indicators did not show significant gender differences in either ethnic group ($p > 0.05$).

The relationship of risk factors with obstructive CHD

Relationship between stenosing coronary lesions and risk factors was presented in Table 3. In the Kazakh group, stenosing was related to risk factors such as male gender ($p = 0.014$), type 2 diabetes mellitus ($p = 0.001$), smoking ($p = 0.001$) and DBP ($p = 0.036$). In the Russian group, stenosing was related to age ($p = 0.011$) and average levels of total cholesterol ($p = 0.014$) and HDL ($p = 0.001$).

Table 1. Demographic and clinical characteristics of patients with CHD

Characteristics	Ethnicity		Total N=640 n (%)	*p*
	Kazakhs	Russians		
	N=338 (53) n (%)	N=302 (47) n (%)		
Age, years				
30-39	7 (2)	3 (1)	10 (1)	
40-49	43 (13)	14 (5)	57 (9)	
50-59	92 (27)	65 (22)	157 (25)	
60-69	123 (36)	119 (39)	242 (38)	
≥70	73 (22)	101 (33)	174 (27)	
Average age, SD	61.0 ± 10.5	65.6 ± 10.0		0.001
Gender				
Male	251 (74)	173 (57)	424 (66)	0.001
Female	87 (26)	129 (43)	216 (34)	
Body mass index				
<25	57 (17)	48 (16)	105 (16)	
25-29.9	175 (52)	138 (46)	313 (49)	
>30	106 (31)	116 (38)	222 (35)	
Co-morbidities				
Arterial Hypertension	112 (33)	133 (44)	245 (38)	0.005
Diabetes Mellitus type 2	89 (26)	63 (21)	152 (24)	
Dyslipidemia	300 (89)	264 (87)	564 (88)	
Smoking				
Current smokers	77 (23)	71 (24)	148 (23)	
Laboratory findings				

Characteristics	Ethnicity		Total N=640 n (%)	*p*
	Kazakhs N=338 (53) n (%)	Russians N=302 (47) n (%)		
LDL ± SD	3.1 ± 1.0	3.2 ± 1.0		
HDL ± SD	1.2 ± 0.4	1.0 ± 0.4		0.001
Cholesterol ± SD	4.8 ± 1.1	4.9 ± 1.1		
TG ± SD	1.6 ± 0.9	1.5 ± 0.9		
Blood pressure				
SBP ± SD	130.0 ± 16.7	135.0 ± 17.0		0.001
DBP ± SD	80.0 ± 8.2	82.0 ± 8.2		0.02
Type of hospitalization				
Urgent	110 (32.5)	131 (43)	241 (38)	
Elective	228 (67.5)	171 (57)	399 (62)	0.02
Stenosis of the coronary arteries				
≥50%	193 (57)	198 (66)	391 (61)	0.03
<50%	145 (43)	104 (34)	249 (39)	
Number of affected coronary vessels				
Non-obstructive disease	145 (43)	104 (34)	249 (39)	
1-vessel disease	66 (19.5)	70 (23)	136 (21)	
2-vessel disease	61 (18)	59 (20)	120 (19)	
3-vessel disease	66 (19.5)	69 (23)	135 (21)	

Table 2. National differences of the patients depending on clinical characteristics and gender

Characteristics, M ± SD	Kazakhs		p	Russians		p
	Male n=251	Female n=87		Male n=173	Female n=129	
Average age*	60.6 ±10.7	63.7 ± 9.9	0.01	63.5 ± 9.9	68.4 ±10.0	0.001
Average BMI*	28.7 ± 4.0	28.9 ± 4.7		28.7 ± 4.7	30.6 ± 5.8	0.002
LDL	3.2 ± 1.1	3.1 ± 0.8		3.1 ±1.0	3.3 ± 1.0	
HDL*	1.1 ± 0.4	1.3 ± 0.4	0.005	0.9 ± 0.3	1.1 ± 0.5	0.001
Cholesterol *	4.7 ± 1.3	4.9 ± 0.9		4.7 ± 1.0	5.2 ± 1.2	0.001
TG	1.6 ± 0.9	1.5 ± 0.7		1.5 ± 0.8	1.6 ± 1.0	
SBP	130 ± 16	131 ± 18		133.7 ± 17.0	136.0 ± 17.8	
DBP	81 ± 8	80 ± 9		81.9 ± 7.4	82.8 ± 9.2	

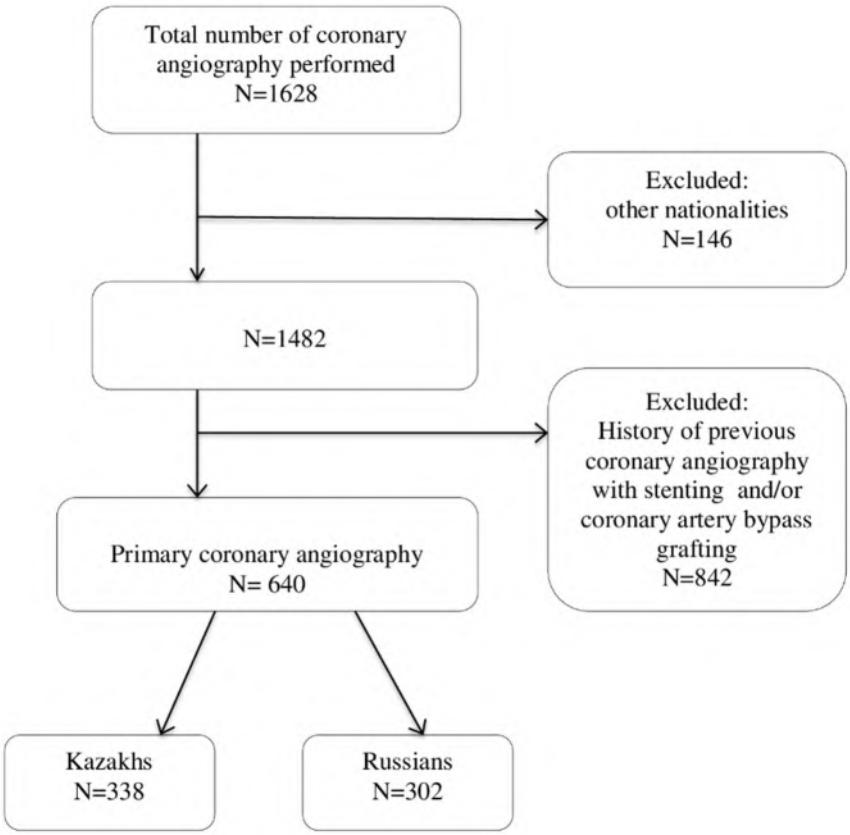
*mean ± SD

Table 3. The relationship of risk factors with obstructive CHD

Characteristics	Kazakh		p	Russians		p
	Non-obstructive (145)	Obstructive (193)		Non-obstructive (104)	Obstructive (198)	
Average age ± SD	60.5 ± 10.8	62.1 ±10.4		64.8 ±10.5	66.0 ± 10.2	0.01
Male sex	94 (65)	157 (81)	0.01	48 (46)	125 (63)	
Arterial Hypertension	47 (32)	65 (34)		40 (38)	93 (47)	
Diabetes Mellitus type 2	25 (17)	64 (33)	0.001	20 (19)	43 (22)	
Dyslipidemia	130 (90)	170 (88)		91(88)	173 (87)	
Overweight	119 (82)	162 (84)		85 (82)	167 (84)	

Characteristics	Kazakh		p	Russians		p
	Non-obstructive (145)	Obstructive (193)		Non-obstructive (104)	Obstructive (198)	
Current smokers	17 (12)	60 (31)	0.001	17 (16)	54 (27)	
Average BMI ± SD	28.9 ± 4.5	28.6 ± 4.0		29.9 ± 5.2	29.4 ± 5.4	
LDL ± SD	3.1 ± 0.9	3.2 ± 1.1		3.3 ± 0.9	3.2 ± 0.9	
HDL ± SD	1.2 ± 0.4	1.1 ± 0.4		1.3 ± 0.5	0.9 ± 0.3	0.001
Cholesterol ± SD	4.8 ± 1.2	4.7 ±1.2		4.9 ± 1.3	4.8 ± 1.1	0.01
TG ± SD	1.5 ± 0.8	1.6 ± 0.9		1.5 ± 1.0	1.5 ± 0.8	
SBP ± SD	130.0 ± 16.8	130.0 ±16.6		132.1 ± 17.2	136.2 ± 17.5	
DBP ± SD	81.5 ± 7.6	80.0 ± 8.7	0.03	82.5 ± 8.3	82.0 ± 8.2	

Fig. 1. Flow chart of study participants' recruitment.



DISCUSSION

The prevalence of major risk factors of cardiovascular disorders has geographic and ethnic variability. It has been demonstrated that the contribution of one or another risk factor to cardiovascular morbidity and/or mortality in different populations can differ significantly (35-38).

The aim of our study was to determine the differences in traditional risk factors between the two main ethnic groups living in the Republic of Kazakhstan: Russians and Kazakhs. The study analyzed the association between risk factors and the state of the coronary blood system based on angiography.

The widespread use in routine medical practice of modern methods of revascularization results in changing the initial picture of coronary arteries. In this regard, we excluded from observation patients with a known state of the coronary arteries.

In our study assessment criteria of the structure of risk factors consist of: lipid metabolism disorders (88%), overweight (83%), male sex (66.3%), arterial hypertension (38%), diabetes mellitus (23.8%) and smoking (23%).

According to our obtained results the Russian group, patients were on average older compared to Kazakhs. In the Russian group there is an association between age and the incidence of coronary artery disease (especially in women). Considering the relatively low percentage of patients aged ≥70 years in the Kazakh vs. Russian group, the higher mortality from coronary heart disease in younger age categories is not excluded.

It has been thought that the cardiovascular risk in women is less than in men. This may be due to the fact that estrogen regulates the cardiovascular inflammatory response and metabolism, as well as the survival and hypertrophy of cardiomyocytes and stem cells by activating the estrogen receptor (ER), and the pleiotropic effects of estrogen on the cardiovascular system are often beneficial (39). However, according to the international study INTERHEAT, females develop the first signs of morbidity only later than in men (on average 10 years) (3). After this, the incidence of ischemic heart disease is steadily growing among women. The results of our study indicate that the incidence of coronary artery disease among women in the Russian group is higher than in the Kazakh. According to other studies (25), this can be explained by the fact that with age, there is a certain levelling of risk factors between men and women (in our case, in the Russian group).

According to our observations, arterial hypertension is a more pathognomonic risk factor for the Russian and Kazakh group, and in average blood pressure, which are significantly higher in the Russian group than in the Kazakh group. No significant gender differences were found in average BP in either ethnic group. These findings coincide with the data of previous studies (40, 41). This circumstance is most likely complex and multicomponent. The peculiarities of the way of life (first of all, a diet with a large amount of salt) adopted by the Russian population are one of these factors, since genetically Russians living in these conditions are less adapted to such characteristics. And the degree of lifestyle acceptance and satisfaction can vary from country to country (40, 41).

Lipid metabolism disorders are similar in Kazakhs (88.8%) and Russians. According to our data, the main differences relate to the average HDL values, which are significantly higher in the Kazakh group than in the Russian group. By gender division, the highest average level of HDL is observed in Kazakh women; in Russian men this indicator is the lowest, while Russian women and Kazakh men are approximately the same. This circumstance suggests that it is the HDL level that is the decisive factor in the development of coronary artery disease. The relatively high level of HDL in Kazakhs may be associated with a nomadic cultural and historical feature (42, 43). For example, nomads ate primarily meat and much less plant-based food (44), which developed a kind of adaptation mechanism (45-47).

In terms of our observations, no ethnic differences were found for a factor like overweight. But it can be noted that in the Russian group, in terms of the average BMI, women are included in the category of obesity compared to men. In the Kazakh group, the average BMI of both men and women is almost the same and is classified as overweight. According to previous studies, overweight indicators progress with age and women are more prone to obesity than men (41), which we observe in the Russian group of our study. An earlier study examined the effect of a low-calorie diet and aerobic exercise on cardiovascular risk factors and predicting the risk of coronary heart disease among obese African Americans (48). After a 6-month program including a low-salt and fat diet and aerobic exercise, according to the Framingham risk calculator, the 10-year risk decreased from 6% to 4% in women and from 16% to 13% in men. This was achieved by improving BMI (kg/m2), waist circumference, blood pressure, LDL and HDL (48). The implementation of similar programs that take into account the cultural characteristics of minority groups can significantly improve the state of the cardiovascular system and reduce the risk of coronary heart disease in the population at risk.

Apart from that, no ethnic differences were detected between the Kazakh and Russian groups in terms of risk factors such as diabetes mellitus and smoking. According to previously published reports, in rural areas, the Russian population smokes more than the Kazakh (21, 41). In our case, the absence of a difference may be associated with the urban conditions of the observed population.

Analysis of coronary angiography showed that representatives of Russian nationality are more susceptible to stenosing coronary lesions than Kazakhs. The results demonstrated that the non-obstructive type of CHD predominates in the Kazakh group that may be associated with damage of the microvasculature or a vasospastic variant of CHD (49, 50). Besides, it is impossible to exclude the factor of over-diagnosis in the Kazakh group.

According to the results of our study, the chance of detecting obstructive CAD in the Kazakh population is higher in males. In addition, we observed the presence of such risk factors as diabetes mellitus, smoking, and dependence on the level of DBP among Kazakhs.

For the Russian group, the chance of detecting stenosing lesions of the coronary system increases with age, depending on the level of total cholesterol and HDL cholesterol.

Considering that type 2 diabetes is a systemic, metabolic disease, the absence of obstructive lesions in persons suffering from it in any ethnic group, in our opinion, is a matter of time and depends on the duration and severity of diabetes mellitus (51).

In a study conducted in Iran, when studying the risk factors for CAD development, it was noted that the severity and risk factors of CAD vary among different ethnic groups in this country (52). Other studies also note the need for additional research to fully understand the differences in CVD risk, prevention and treatment to improve outcomes in our increasingly diversified population, with a high focus on raising awareness of practitioners on this issue (53).

To summarize, we identified ethnic differences in risk factors such as age, gender, and hypertension with mean SBP and DBP, as well as in HDL. It must be noted that in the Kazakh group patients tended to be younger. In addition, the male population dominated in the Kazakh group. Moreover, the average level of HDL was higher in Kazakhs (especially among women).

The Russian group was significantly older. The incidence of hypertension was more common for Russians. In this group, the average indicators of SBP and DBP were higher, compared with the Kazakh group. The average level of HDL was lower, particularly in men.

We hypothesize that due to climatic, geographical, social and cultural-historical conditions of Kazakhstan, both nationalities developed similar adaptive mechanisms. Therefore, the ethnic differences in this study were not clearly pronounced in comparison with previously published reports.

Nevertheless, the observed differences can make an additional contribution to the optimization and improvement in the diagnosis and treatment of CHD. It could be done through more aggressive and effective screening, treatment and controlling of risk factors.

Ethnic differences were determined by factors such as gender, age, and arterial hypertension, HDL levels. In the Russian group, in comparison with the Kazakh, the probability of detecting stenosing lesions of the coronary arteries during angiographic examination was higher. The results showed that for the Kazakh group, the following risk factors play an important role in the development of CHD: smoking, diabetes mellitus, and male sex. At the same time, age, lipid metabolism indicators (total cholesterol and HDL) and arterial hypertension are more important for the Russian group. These findings indicate the need to develop differentiated programmes for the screening, treatment and prophylactic measures for different ethnic groups of the population, thereby ensuring optimal treatment for all.

This one-time study was carried out at one medical institution. We did not consider risk factors such as heredity, physical activity, diet, level of apolypo-proteins due to incomplete information. Also, limitations include an incomplete reflection of the severity of coronary atherosclerosis, data on indicators such as lipid spectrum and blood pressure, which can dynamically change under the influence of diet and/or drug correction. Unfortunately, we did not have the possibility of long-term monitoring of these indicators, therefore, data were provided at the time of the initial angiographic examination.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Local Ethics Committee of the S.D. Asfendiyarov Kazakh National Medical University, Almaty, Republic of Kazakhstan (No. 5 (82) of 04.24.2019). Informed consent was obtained from all patients..

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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IMAGE AND LABORATORY ASPECTS OF CAROTID ATHEROSCLEROSIS

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ABSTRACT

Carotid atherosclerosis is a main risk factor for ischemic stroke. Plaque instability is determined by the morphological characteristics of the plaque and can be characterized by immunological biomarkers. The study aimed to examine the connection between serum levels of hs-CRP, fibrinogen, ICAM-1, VCAM-1 and carotid atherosclerosis and the different types of atherosclerotic plaques imaged by ultrasound and magnetic resonance. The study involved 120 patients with carotid atherosclerosis and 33 patients without carotid atherosclerosis. Blood samples were collected to analyze the serum level of hs-CRP, fibrinogen, ICAM-1 and VCAM-1. The ultrasound analysis included detection of atherosclerotic plaques in the internal carotid arteries, measurement of artery stenosis in percentage and determination of plaque types by the classification of Gray-Weales/Gerolacus. A small subset of 30 patients with carotid atherosclerosis performed 3T magnetic resonance imaging. Atherosclerotic plaques were classified into 8 types based on the modified MR classification of the American Heart Association. Significantly higher serum levels of hs-CRP ($p < 0.001$) and fibrinogen ($p = 0.018$) were observed in patients with carotid atherosclerosis compared to patients without atherosclerosis. Criterion values for hs-CRP > 4.13mg/l and for fibrinogen > 3.6 g/l were associated with the presence of carotid plaques with accuracy of 70%. No relation was observed between the investigated biomarkers, the artery stenosis and the types of atherosclerotic plaques determined by ultrasound and magnetic resonance diagnostic methods. Hs-CRP and fibrinogen are reliable serum markers whose increased serum concentrations are connected with the presence of carotid atherosclerosis.

Keywords: *Carotid atherosclerosis, biomarkers, image diagnostics, ultrasound, MRI.*

INTRODUCTION

Atherosclerosis of the internal carotid artery is a main risk factor for ischemic stroke (1). Atherogenesis is modified by immunological processes and specific biomarkers can characterize them (2). A histological classification of atherosclerotic plaque was proposed by American Heart Association (3). Morphological structures like large lipid-rich necrotic nucleus, plaque hemorrhage, thin fibrous capsule with surface irregularities determine the instability of the plaques (4). Non-invasive diagnostic methods such as ultrasound and magnetic resonance imaging can show plaque morphology and distinguish vulnerable plaques.

The study aimed to examine the connection between serum levels of hs-CRP, fibrinogen, ICAM-1 and VCAM-1 and carotid atherosclerosis and the different types of atherosclerotic plaques imaged by ultrasound and magnetic resonance.

MATERIAL AND METHODS

An observational, cross-sectional study has been conducted for 6 months (from November 2017 till May 2018) in the Clinic of Neurology, UMHAT "St.George"– Plovdiv, Bulgaria. It enrolled consecutively 153 patients that were sent for ultrasound examination of the carotid arteries in the Ultrasound Laboratory of the Clinic. The inclusion criteria were all indications for ultrasound investigations of the carotid artery such as acute ischemic stroke or primary stroke prevention because of risks factors such as history of hypertension, carotid atherosclerosis, atrial fibrillation, diabetes mellitus and cardiovascular diseases. The exclusion criteria were autoimmune diseases, cancer and inflammatory diseases that could influence the biomarkers. Two main groups were formed: patients with carotid atherosclerosis (120 patients) and patients without carotid atherosclerosis (33 patients). The study protocol was approved by the Local Ethics Committee (31.03.2016/protocol №2). All subjects provided written informed consent prior to their participation in the study.

Laboratory investigations

Blood samples from all patients were collected to analyze the serum level of hs-CRP, fibrinogen, ICAM-1 and VCAM-1. The sample were taken in the morning between 6-8 a.m. as atraumatically as possible on the next day after the ultrasound investigation. For the patient with an acute stroke, blood samples were taken during the first 24 hours after the onset of the stroke. The high sensitive C-reactive protein was determined immunoturbimetrically using an automated OLIMPUS AU400 clinical chemistry analyzer, Beckman Coulter USA. The measurement of hs-CRP is in mg/L. Serum fibrinogen was measured coagulometrically by an automated Sysmex Cs 2000 analyzer. The fibrinogen test is in g/l with a reference range of 2-4g/l. Serum concentrations of VCAM-1 and ICAM-1 were measured by ELISA analysis using commercially available CE brand kits (Bender Med Systems,GmbH). There are no definite data in the literature on the reference serum values of VCAM-1 and ICAM-1.

Image investigations

The ultrasound examinations were performed with the ultrasound machine Philips ClearVue 500. The analysis included the detection of atherosclerotic plaques in the internal carotid arteries, the measurement of artery stenosis in percentages using the local method and the determination of plaque types according the classification of Gray-Weales / Gerolacus (5). A small subset of 30 patients with ultrasound proven atherosclerosis in the internal carotid artery performed 3T magnetic resonance imaging. MR scans were done on a 3T MR imaging system (Discovery MR750; GE Healthcare) with a 24-channel head and neck coil. A standardized protocol was used, which includes 4 different contrast-weighted images in the axial plane - 3D time of flight magnetic resonance angiography with high resolution (HR art 3D TOF MRA), T1-weighted (T1W), proton density weighted (PDW), T2 -weighted (T2W) images and a sagittal modality T1 3D Cube. Both carotid bifurcations were identified by 2D MR PC (Phase contrast) coronary angiography. Using 2D MR PC-MRA as a localizer, the four impulse sequences (HR art 3D TOF, T1-W, T2-W, PD) were performed on bilateral carotid arteries in a transverse plane 2 cm proximal and 2 cm distal to the carotid bifurcation. T1W, PDW, T2W and T1 3D Cube were FSE (Fast spin echo) sequences in which was used the Black Blood (BB) plaque imaging technique. ECG triggering was used in T1W, T2W and PDW. For T1 and PD weighted images, ECG triggering was one cardiac cycle (time for one R-R interval), and for T2 weighted images, it was two cardiac cycles. Fat suppression (FS) to reduce the signal from subcutaneous adipose tissue was used in T1W, T2W, T1 3D Cube in all patients and in PDW in some patients. For each sequence, the signal of the adjacent sternocleidomastoid muscle was used as a reference zone, which compares the signal intensity of the major plaque component. Atherosclerotic plaques were classified into 8 types based on the modified MR classification of the American Heart Association (6).

Statistical analysis

The data were analyzed through the statistical software IBM SPSS version 27 (2020) and Medcalc version 21.1 (2021). Continuously measured variables were described through the mean values and standard deviations when normality was observed, and between group comparisons were performed using the t-test for independent samples. Variables that were not normally distributed were described with the medians and interquartile range (IQR) and compared between groups through the Mann-Whitney U test. Potential connections between biomarkers and different types of atherosclerotic plaques were examined through Spearman rank-order correlation analysis. Binary and categorical data were presented in frequencies and percentages and associations were established through the Chi-square test and Fisher's

exact test. Receiver operating characteristic (ROC) curve was used to establish the potential of biomarkers in the diagnosis of different atherosclerotic plaque. All statistical tests were two-tailed and performed at level of significance *alpha* = 0.05.

RESULTS

Demographic and clinical data

The study involved 120 patients with carotid atherosclerosis (CA) of mean age 67.10±9.80 years and 33 patients without CA of mean age 57.75±10.52, with a significant age difference ($p < 0.001$) (Table 1). The sex distribution was similar with no significant differences between the patients with CA and without CA ($p = 0.077$). A significantly higher proportion of the patients with CA had hypertension ($p = 0.039$) and cardiovascular diseases ($p = 0.005$) in comparison with the patients without CA. The groups did not differ in the proportions of atrial fibrillation ($p = 0.202$), diabetes ($p = 0.590$) and stroke ($p = 0.434$).

The serum hs-CRP level was significantly higher in the patients with CA compared to the patients without CA, $p < 0.001$ (Figure 1a). The proportion of patients with risk levels of hs-CRP > 3mg/l was 60.00% in the CA group versus 36.40% in the group without CA, $p = 0.018$. Significantly higher fibrinogen levels were observed in the patients with CA, $p=0.012$ (Figure 1b). The proportion of the patients with fibrinogen > 4g/l was 54.00% in the group with CA versus 27.27% in the patients without CA, $p = 0.026$. ICAM-1 and VCAM-1 did not show a significant association with the presence of CA.

ROC curve analysis showed an acceptable level of diagnostic accuracy of hs-CRP in distinguishing patients with CA from patients without CA (AUC = 0.702, $p < 0.001$). The optimum criterion cut-off value was estimated as hs-CRP > 4.13 mg/l, associated with 59.33% sensitivity and 78.79% specificity. Fibrinogen showed a similar level of diagnostic potential (AUC = 0.793, $p < 0.001$), criterion value > 3.61 g/l, sensitivity 56.77% and specificity 84.00% (Table 2 and Figure 2).

Carotid artery stenosis

The percentage of carotid artery stenosis ranged between 5% and 100%, with a mean value of 40.70±28.09% and a median of 40%. No significant association was observed between the percentage of carotid vessel stenosis and the serum levels of the biomarkers: hs-CRP ($r^s = 0.001$, $p = 0.993$); fibrinogen ($r^s = -0.010$, $p = 0.920$); ICAM-1 ($r^s = - 0.025$, $p = 0.820$); VCAM-1 ($r^s = -0.014$, $p = 0.901$) (Figure 3).

Biomarkers versus the different types of carotid plaques determined by ultrasound and MRI diagnostic methods

Gray-Weale/Gerolacus classification

In the group with CA, the Gray-Weale/Gerolacus US plaque types showed the following distribution: Type I - 4.2% (n =5); Type II - 15.8% (n=19); Type III - 40% (n=48); Type IV- 26.70% (n = 32); Type V - 13.30% (n=16). None of the biomarkers showed a significant association with the Gray-Weale/Gerolacus types: hs-CRP ($r^s = 0.028$, $p = 0.760$); fibrinogen ($r^s = -0.078$, $p = 0.468$); ICAM-1 ($r^s = -0.073$, $p = 0.479$); VCAM-1 ($r^s = -0.202$, $p = 0.066$) (Figure 4).

AHA MRI classification

AHA MRI classification of the carotid plaques was performed for 30 CA patients, 18 male and 12 female, yielding the following distribution of plaque types: Type I & II – 3.30% (n = 4); Type III – 1.7o% (n = 2); Type IV & V – 9.20% (n = 11); Type VI – 3.30% (n = 4); Type VII – 5.00% (n = 6); Type VIII – 2.50% (n=3).

The serum levels of the target biomarkers did not show a significant association with the AHA plaque types: hs-CRP (rs = -0.075, $p = 0.697$); fibrinogen (rs = 0.199, $p = 0.300$); ICAM-1 (rs = - 0.293, $p = 0.122$); VCAM-1 (rs = -0.262, $p = 0.171$) (Figure 5).

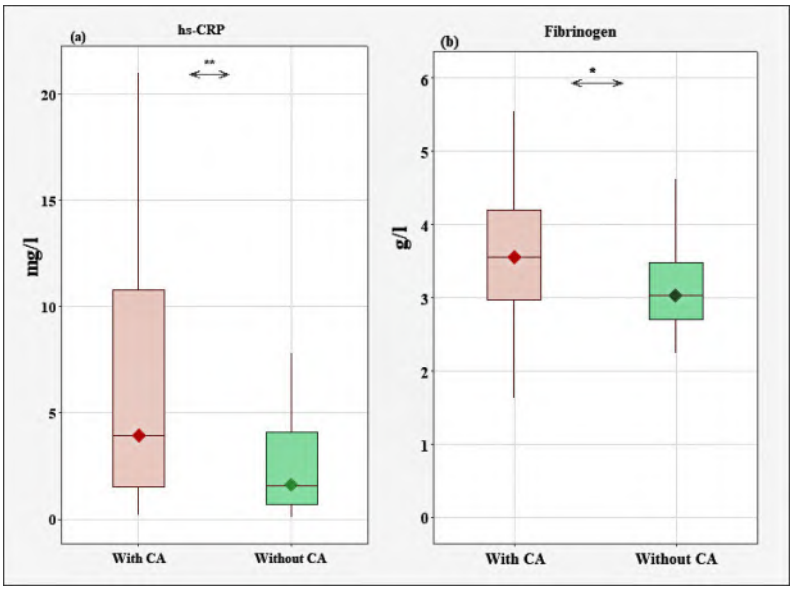
Table 1. Demographic and clinical data

Variables	Group		p
	With carotid atherosclerosis (n = 120)	Without carotid atherosclerosis (n = 33)	
Age			
o Mean (SD)	67.10 (9.80)	57.75 (10.52)	$< 0.001^t$
o Min.-Max.	41 to 88	38 to 78	
Sex			
o Male	69 (57.50%)	13 (39.40%)	0.077^f
o Female	51 (42.50%	20 (60.60%)	
Hypertension n (%)	105 (87.50%)	24 (73.00%)	$0.039^{\chi 2}$

Variables	Group		p
	With carotid atherosclerosis (n = 120)	Without carotid atherosclerosis (n = 33)	
Atrial fibrillation n (%)	23 (19.00%)	3 (9.00%)	0.202 χ^2
Cardiovascular diseases n (%)	62 (51.70%)	8 (24.20%)	0.005 χ^2
Diabetes n (%)	40 (33.30%)	9 (27.30%)	0.590 χ^2
Stroke n (%)	65 (54.16%)	15 (45.45%)	0.434 χ^2
hs-CRP mg/l			
o Median (IQR)	3.93(9.27)	1.57(3.38)	0.005 U
o Min.-Max.	0.21 to 314.78	0.11 to 52.84	
o n(%) > 3mg/l	72 (60%)	12 (36.40%)	0.018 f
Fibrinogen g/l			
o Median (IQR)	3.56 (1.22)	3.03 (0.78)	
o Min.-Max.	1.63 to 7.32	0.66 to 5.23	0.012 U
o n(%) > 4g/l	65 (54.00%)	9 (27.27%)	0.026 f
ICAM-1			
o Median (IQR)	457.50 (201.25)	417.00 (133.50)	0.168 U
o Min.-Max.	164.00 to 981.00	176.00 to 671.00	
VCAM-1			
o Median (IQR)	1032.50 (1267.50)	1020.00 (1246.25)	0.319 U
o Min.-Max	395.00 to 7705.00	300.00 to 2465.00	

t- t-test for independent samples; f – Fisher's exact test; $\chi 2$ – chi-square test; U – Mann-Whitney

Figure 1: Significantly higher hs-CRP levels (**panel a**) and fibrinogen levels (**panel b**) in the patients with CA (**b**)



** - p < 0.01; *- p < 0.05

Table 2. ROC curve results for the diagnostic potential of hs-CRP and fibrinogen

Biomarker	AUC 95% CI	SE	p	Criterion value	Sensitivity	Specificity
hs-CRP	0.702 (0.623 to 0.774)	0.05	<0.001	>4.13mg/l	59.33%	78.79%
Fibrinogen	0.703 (0.616 to 0.789)	0.05	<0.001	> 3.61 g/l	56.77%	84.00%

Figure 2. Roc curves for the diagnostic potential of serum hs-CRP (panel a) and fibrinogen (panel b) in distinguishing patients with CA from patients without CA

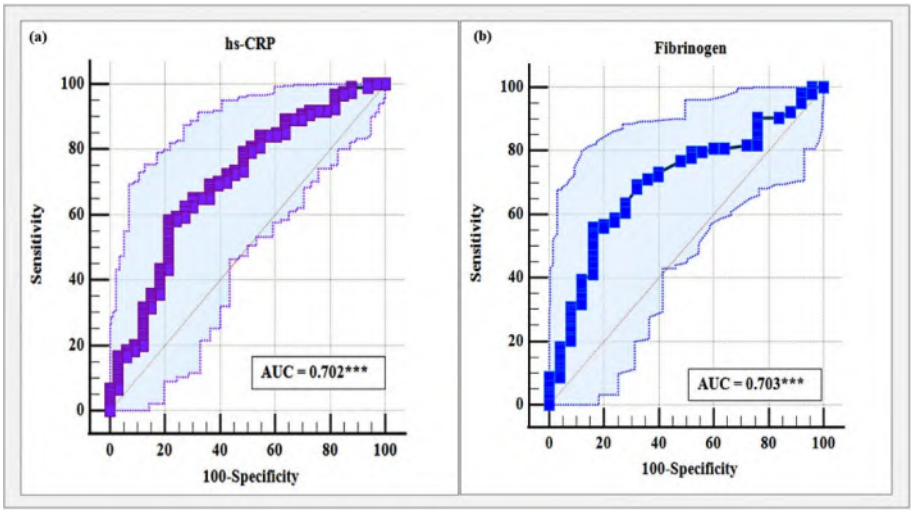


Figure 3. Scatter plots show no association between % stenosis and serum levels of hs-CRP (**panel a**), Fibrinogen (**panel b**), ICAM-1 (**panel c**) and VCAM-1 (**panel d**)

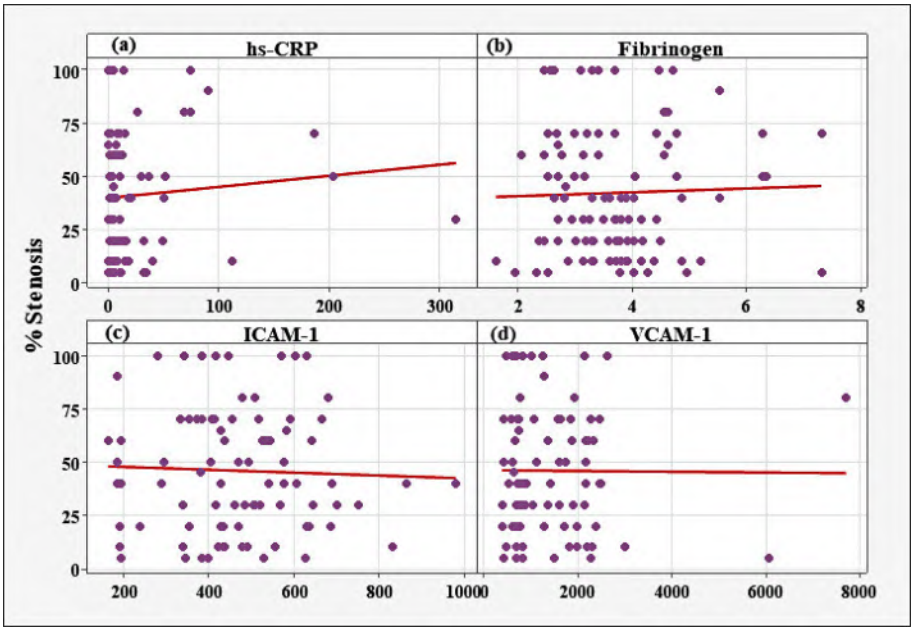


Figure 4. Scatter plots show no association between Gray-Weale/Gerolacus US plaque types and serum levels of hs-CRP (**panel a**), Fibrinogen (**panel b**), ICAM-1 (**panel c**) and VCAM-1 (**panel d**)

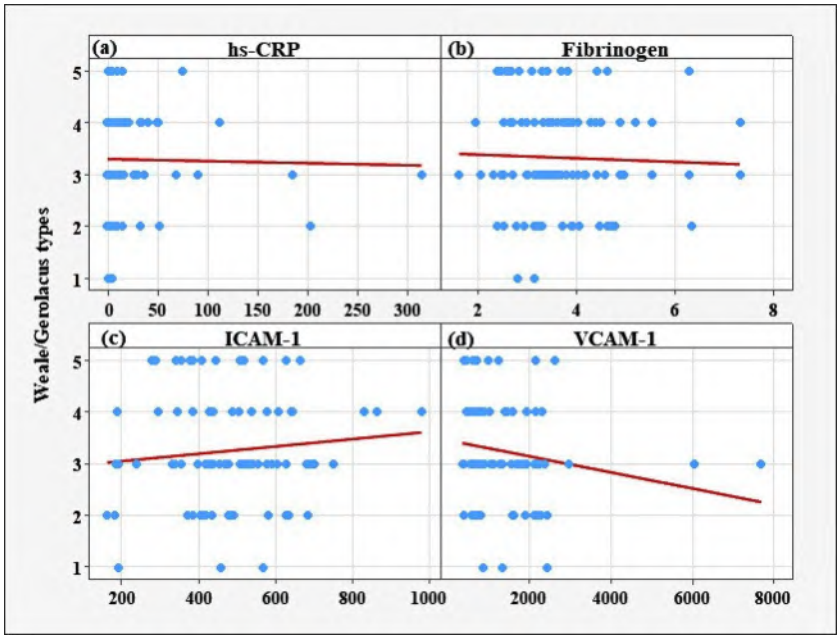
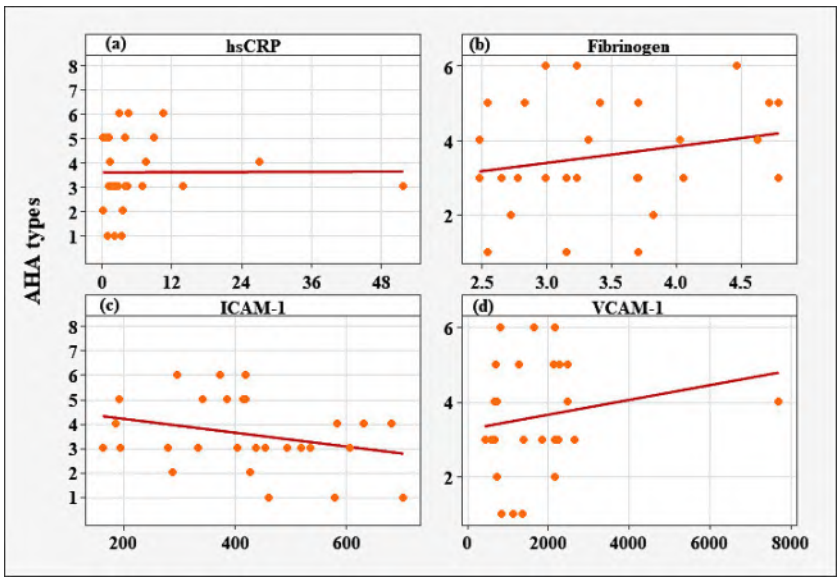


Figure 5: Scatter plots show no association between AHA MRI plaque types and serum levels of hs-CRP (**panel a**), Fibrinogen (**panel b**), ICAM-1 (**panel c**) and VCAM-1 (**panel d**)



DISCUSSION

The demographic data in our study showed that the mean age of patients with CA is significantly higher compared to patients without CA. We connect this fact with the aged- related changes. With increasing of age, the plaque burden and the incidence of atherosclerosis-related events accelerates and the morphology of atherosclerotic plaques changes toward vulnerable plaques (7, 8).

About the comorbidity we found that a significantly higher proportion of the patients with CA had hypertension and cardiovascular diseases (ischemic heart disease, cardiomyopathy, heart failure, valve defects, etc.). We think that further investigations should be done to analyze this connection.

In our study we chose to investigate several serum biomarkers (hs-CRP, fibrinogen, ICAM-1, VCAM-1) associated with the processes of vascular dysfunction and atherosclerosis (9-11). To determine their relationship with carotid atherosclerosis we performed investigations in several directions. First we performed a comparative analysis between patients with established CA and without CA on the level of hs-CRP, fibrinogen, ICAM-1 and VCAM-1. The serum levels of hs-CRP ($p < 0.001$) were significantly higher in the patients with CA. These data confirm the results obtained by other authors for the association of hs-CRP with carotid atherosclerosis (12-14). Most of the cited studies used the approved by the American Heart Association hs-CRP value above 3 mg / l. In their study, Puz et al [14] determined hs-CRP above 5 mg / l as a marker for the presence of carotid atherosclerosis. Studies in carotid atherosclerosis and ischemic stroke also confirmed the importance of hs-CRP as a diagnostic biomarker, but no standardized value was determined (15, 16, 17). If we used the proposed by AHA risk level we found that the proportion of patients with hs-CRP > 3mg/l was 60.00% in the CA group versus 36.40% in the group without CA ($p = 0.018$). We used the ROC curve analysis to find an acceptable level of diagnostic accuracy for hs-CRP in distinguishing patients with CA from patients without CA (AUC = 0.702, $p < 0.001$). The level of hs-CRP > 4.13 mg/l showed 59.33% sensitivity and 78.79% specificity.

Fibrinogen was the other biomarker that was significantly increased in patients with CA ($p=0.012$). The analyze about the distribution of patients with fibrinogen values > 4.0 g / l also showed a higher proportion in patients with atherosclerosis (54.00%) compared to those without atherosclerosis (27.27%). Increased fibrinogen levels could be observed in atherosclerosis with different localization - coronary heart disease, peripheral arterial insufficiency and carotid atherosclerosis (18). In order to find an acceptable value for fibrinogen associated with the presence of CA we again used the ROC curve analysis. Fibrinogen showed a level of diagnostic potential (AUC = 0.793, $p < 0.001$) with criterion value > 3.61 g/l (sensitivity 56.77% and specificity 84.00%).

Cell adhesion molecules (CAM) are immunoglobulin-like structures with a proven role in endothelial dysfunction and atherosclerosis (11, 19, 20). Increased expression was found in ischemic heart disease and in particular in acute coronary syndrome [21]. Debing et al. [12] studied the serum VCAM-1 levels in 180 patients with carotid atherosclerosis and compared the data with healthy controls and looked for additional dependencies with symptomatic carotid stenosis. The results of the study revealed that VCAM-1 could serve as markers for carotid atherosclerosis but not to identify plaque at risk for symptomatic conversion. Our analyzes of the potential association between ICAM-1, VCAM-1 and carotid atherosclerosis showed that CAM levels varied between patients, without a statistically significant trend. The measured serum values of ICAM-1 and VCAM-1 did not show a connection with the presence of carotid atherosclerosis.

In our study no significant association was observed between the percentage of carotid stenosis and the serum levels of the selected biomarkers. Lack of such connection was also reported by Puz et al. (14), and Debing et al. (12). In a study of 104 patients, Benbir et al. (22) found a connection between hs-CRP and the severity of carotid atherosclerosis. In the study, patients were divided into 4 categories - patients without plaques, with increased IMT, with plaques below 50% stenosis and with plaques above 50% stenosis. According to the authors, increased hs-CRP values and more pronounced atherosclerotic changes were independent risk factors and their interaction increased the risk of cerebrovascular accident. Musialek at al. (23) investigated several circulating biomarkers in 300 patients with ultrasound assessed carotid stenosis ≥50% who were referred for potential revascularization. They observed that the levels of high-sensitivity C-reactive protein were higher ($p=0.04$ and $p=0.07$, respectively) in the symptomatic stenosis group compared to the asymptomatic stenosis group. Sabeti et al. (24) monitored fibrinogen levels in 1,268 patients with atherosclerotic plaques of the carotid arteries. Patients performed periodic ultrasound assessment at 3 and 6 month intervals, and were divided into 6 groups depending on the percentage of arterial stenosis (from 0% to 29%, from 30% to 49%, from 50% to 69%, from 70% up to 89%, from 90% to 99% and total occlusion). In order to determine the activity of inflammatory processes in atherosclerosis, a comparison was made with other inflammatory markers. The results of the study showed increased fibrinogen levels with the progression of atherosclerosis, but this association seemed to be nonspecifically related to the extent of the inflammatory process in atherosclerotic disease rather than to specific properties of fibrinogen.

The morphological characteristics of the plaque are important in determining their instability and risk of causing cerebrovascular accidents (25, 26). In our study, we used established Gray-Weale/Gerolacus ultrasound scale (5) and the Modified AHA Classification for MRI (6). With these diagnostic methods, plaque structures such as calcification, fibrous cap, plaque hemorrhage and lipid-rich necrotic nucleus could be identified with moderate to good sensitivity and specificity (27). Weiss and al. (28) explored MRI properties of large arteries an their association with serum markers of inflammation. The authors thought that MRI of large arteries could provide a new approach to investigate the contribution of inflammation in atherogenesis. van Dijk and al. (29) investigated the association between fibrinogen and fibrinogen γ' and atherosclerotic plaque morphology in symptomatic carotid artery stenosis. Presence of plaque ulceration, intra plaque hemorrhage (IPH) volume and lipid-rich necrotic nuclei (LRNC) volume was determined by Multidetector-Row Computed Tomography and Magnetic Resonance Imaging. The author found that fibrinogen and fibrinogen γ' were inversely associated with IPH volume and LRNC volume, independent of inflammation.

The results about the hs-CRP are controversial. Puz et al. (14) found no correlation between hs-CRP values and ultrasound morphological characteristics of unstable carotid

plaques (hypoechoic, with an irregular surface and stenosis above 70%). Lombardo et al. (30) observed higher values of hs-CRP in ultrasound assessed complex atherosclerotic plaques with the following characteristics - heterogeneous with irregular and ulcerated surface.

There are a small number of studies comparing CAM and the imaging characteristics of atherosclerotic plaques. Rohde et al. (31) monitored the levels of CAM in patients with carotid atherosclerosis, finding a positive relationship between their serum concentrations and the ultrasound index IMT. Shindo et al. (32) studied patients with unstable atherosclerotic plaques of the carotid arteries diagnosed by magnetic resonance imaging. They found a positive correlation between the magnetic resonance characteristics of unstable plaques and the serum values of several pro-inflammatory factors, one of which is VCAM-1.

In our study no significant association was observed between the levels of hs-CRP, fibrinogen and ICAM-1 and VCAM-1 and different types of atherosclerotic plaques determined by the Gray-Weale / Gerolacus ultrasound classification and the modified MP classification of the American Heart Association.

CONCLUSIONS

Our data showed significantly higher serum levels of hs-CRP and fibrinogen in the patients with carotid atherosclerosis. A criterion value for hs-CRP > 4.13mg/l and for fibrinogen > 3.6 g/l can be applied in the diagnosis of carotid plaques in patients with accuracy of 70%. We found no correlation between serum levels of selected biomarkers and arterial stenosis and plaque morphology presented by ultrasound and MR diagnostic methods.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the Local Ethics Committee (31.03.2016/protocol №2). All subjects provided written informed consent prior to their participation in the study.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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None.

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EFFECT OF THE ACUTE TOTAL GAMMA RADIATION IN A SUBLETHAL DOSE ON THE BIOPHYSICAL PROPERTIES OF RED BLOOD CELLS, LIPID PEROXIDATION, ANTIOXIDANT SUPPLY AND HEMOCOAGULATING PROPERTIES OF ERYTHROCYTES

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ABSTRACT

The aim of the investigation was to study the effect of acute, total gamma-irradiation in a sublethal dose on the biophysical properties of erythrocytes, the intensity of lipid peroxidation, antioxidant supply and hemocoagulating properties of erythrocytes. The experiments were carried out on 11-12-week-old age guinea pigs, males and females in equal numbers. The animals were exposed to a single total radiation at a dose of 4.5 Gy (sublethal dose, LD 50/30). The studies were carried out on the 7th day after exposure to radiation (at the height of radiation sickness). The development of radiation damage was accompanied by intense erythropoiesis and the appearance of erythrocytes with a high resistance to hemolysis and an increased sedimentation rate. After acute gamma irradiation, depletion of the antioxidant system was noted. It manifested in a decrease in the activity of superoxide dismutase of erythrocytes by 19.7% ($p<0.01$) and the concentration of serum ceruloplasmin by 21.5% ($p<0.01$). The content of thiobarbituric acid reactive substances (TBARS) and their accumulation while the incubation of erythrocytes remained within the normal range. The erythrocytes of the irradiated animals exhibited increased procoagulant and decreased antiheparin activity, which reflects conformational changes in highly radiosensitive fatty acid chains of phospholipids in their membranes. A decrease in the fibrinolytic activity of erythrocytes in irradiated animals was found.

Keywords: *Gamma-irradiation, erythrocytes, lipid peroxidation, antioxidants, hemocoagulation.*

INTRODUCTION

The hematopoietic system is very sensitive to the action of ionizing radiation, and the wide representation of hematopoietic tissue in the body determines its obligatory damage at any type of radiation exposure (1). Under the influence of ionizing radiation, there is a violation of both the structural organization of cells and an increase in the oxidation of lipids of biomembranes (2, 3). At the same time, the erythrocyte is a radio-resistant cell, as it does not have DNA and is not capable of RNA synthesis, but this feature decreases their ability to post-irradiative reparation. The main radiation target is plasma membrane of an erythrocyte. Under ionizing irradiation, reactive oxygen and nitrogen forms, which activate enzymes (nicotinamide adenine dinucleotide phosphate oxidase, lipoxygenases, nitric oxide synthase, cyclooxygenases) involved in damage to cell membranes are formed (4). All physiological mechanisms of damages of hemostasis erythrocyte link are not clear now, that is important to take into account the presence of post-irradiative hemopoesis depletion.

One of the manifestations of acute radiation sickness is a violation of hemostasis, which is manifested by bleeding. Modern research shows that disseminated intravascular coagulation plays a very important role in the death of people and animals from radiation damage in relatively low doses. The mechanisms of such development should be investigated (5). However, to date and the role of damaged erythrocytes in impaired hemostasis remain almost unexplored (6, 7).

The aim of this investigation was to study the effect of acute, total gamma radiation in a sublethal dose on the biophysical properties of erythrocytes, the intensity of lipid peroxidation, antioxidant supply and hemocoagulating properties of erythrocytes.

MATERIALS AND METHODS

Animals

The experiments were performed on 11-12-week-age guinea pigs, 441.7±9.8 g weigh, males and females in equal numbers, kept separately. These animals are one of the best models, including for the study of radiation damage, since they do not synthesize ascorbic acid, similar to humans (8). Laboratory animals were kept in a vivarium of the Ukrainian Medical Stomatological Academy (UMSA is Poltava State Medical University after 05.05.2021) in a room that did not contain specific pathogens, with a natural lighting cycle at a constant temperature (21±1ºC) and humidity (50%±10%), on a standard diet.

Compliance with Ethical Standards

This study was carried out in accordance with the national "General Ethical Principles of Animal Experiments", which is consistent with the provisions of the European Convention for the Protection of Vertebrate Animals used for experiments or other scientific purposes (Strasbourg, March 18, 1986). This experiment was approved by the UMSA Ethics and Bioethics Commission.

Experimental procedure

Animals were divided into two groups, each of 10 animals. The first group was intact animals (healthy animals that were not exposed to any effects). Animals of the second group were subjected to a single total exposure at a dose of 4.5 Gy (sublethal dose, LD 50/30).

It is known that radiosensitivity is quite adequately characterized by a radiation dose that causes 50% death of certain mammal species. The average LD 50/30 for a guinea pig is 4.5 Gy, and the greatest degree of granulocytopenia (maximum first emptying) characterizing the height of acute radiation syndrome occurs 7-8 days after acute exposure (9, 10).

Radiation was carried out in the installation "Agat-R". ^{60}Co was used as a source of ionizing radiation. The radiation dosage received by each animal was determined according to the calculation results, based on the dosage rate measurements by a type 270I2 clinical dosimeter. The measurement error of the dosage was within the limits of 8-10%.

The studies were carried out on the 7th day after the previous exposure (in the midst of radiation sickness) (11). The biological material was taken under hexenal anesthesia at a dose of 120 mg/kg intraperitoneally.

Tissue study

The objects of the study were whole blood, plasma, serum and whole red blood cells of experimental animals.

Evaluation parameters and biochemical estimations.

The number of red blood cells was determined in an automatic counter PCE 210; hemoglobin was measured by a MiniGem 540 hemoglobinometer.

To study the electrokinetic properties of red blood cells a modified method of fractional erythrocyte sedimentation rate was used (12).

Peroxidation resistance of erythrocytes was determined by the Jager F.C. method. (14). The following blood parameters have been studied: the resistance of erythrocyte membranes to hydrochloric hemolytic (13), the accumulation of thiobarbituric acid reactive substances (TBARS) in red blood cells (15), the activity of superoxide dismutase (16) and catalase in red blood cells (17), the content of ceruloplasmin in blood serum (15), conjugated dienes in blood serum (15), total serum lipids (15), low density lipoprotein and very low density lipoprotein (LDL and VLDL) and serum cholesterol (15).

When studying the hemocoagulating and fibrinolytic properties of red blood cells a standard platelet-free substrate plasma was used. In one sample, whole erythrocytes from

animals after irradiation were added to the substrate plasma. In the second sample, whole erythrocytes of intact animals were added to the substrate plasma. Plasma substrate with physiological saline served as a control in the study. The effect on recalcification time, thrombin time and plasma fibrinolytic activity was determined (18).

All the values were expressed as mean ± standard error of mean (SEM). The data was analyzed by Student's T-test. The analysis of the normality of the distribution of indicators was carried out using one-sample Kolmogorov-Smirnov Test. The data was analyzed by Student's T-test. All p value less than 0.05 were considered to be statistically significant. The statistical analysis was performed using SPSS (Version 13.0).

RESULTS

A radiation dose of 4.5 Gy 6 days after exposure did not significantly affect the number of circulating red blood cells (4.37±0.08×1012 / L in intact animals versus 4.41±0.28× 1012 / L in irradiated animals, p>0.5).

Given the significant role of the surface charge in maintaining the structural and functional integrity of red blood cells, the reaction of erythrocyte sedimentation in irradiated animals was investigated. 15 minutes after, the height of the plasma column during erythrocyte sedimentation in irradiated animals exceeded the same value in the intact group by 45.2 % (1,80±0,12 mm versus 1,27±0,14 mm, p<0.05). At the 30th and 45th minutes, the values in the experimental and the control groups were close to each other, and only at the 60th minute a sharp acceleration of erythrocyte sedimentation with an increase in plasma column height during erythrocyte sedimentation was of 68.4 % in the irradiated animals in comparison with the intact group (8,00±0,82 mm versus 4,75±0,42 mm, p<0.01).

The changes in the process of erythrocyte agglutination, which depends on the electric charge of the cells, by the 60th minute indicates the destruction of the electrostatic system of red blood cells, as a result the transport and exchange function of the entire bloodstream decreases and the risk of erythrocyte blood clots increases.

The development of radiation damage was accompanied by a change in the resistance of red blood cells to acid hemolysis, which may be associated with a qualitative change in the composition of red blood cells. The total duration of the hemolysis process, the onset time of the hemolysis maximum and the destruction time of the most stable forms of red blood cells significantly increased in combination with a decrease in the total number of decaying red blood cells in irradiated animals. The total duration of the hemolysis process increased by 27.3% in irradiated animals (p<0.01), as well as the time of hemolysis maximum increased by 27.1% (p<0.02), the number of destroyed red blood cells in hemolysis decreased by 36,8% in relation to the same number in irradiated animals (p<0.05) (Table 1).

In irradiated animals, the erythrogram maximum is shifted to the right, which, apparently, is associated with a sharp rejuvenation of the erythrocyte pool and indicates an abnormally highly stable erythrocyte entering the vascular bed, and the flattening of the erythrogram reflects the dysregulation of erythropoiesis.

Thus, intense erythropoiesis and the appearance of red blood cells with high resistance were noted in irradiated animals already on the 7th day.

After acute gamma radiation, depletion of the antioxidant system was observed, which manifested itself in a decrease in the activity of erythrocyte superoxide dismutase (SOD) by 19.7 % (p<0.01) and serum ceruloplasmin concentration by 21.5 % (p<0.01). The content of TBARS and their accumulation during the incubation of erythrocytes remained at the level of the intact group (Table 2).

An increase in the number of total serum lipids of the irradiated animals was noted by 38.7 % (p<0.05), while the content of low and very low density lipoproteins under the influence of radiation did not change.

It can be assumed that the changes obtained are caused by the release of phospholipids of cell membranes, including erythrocyte membranes, which underwent structural modification during lipid peroxidation (LPO). In turn, the release of phospholipids with pronounced thromboplastic properties affects the state of red blood cell hemostasis (Table 3).

Red blood cells of irradiated animals reduced plasma recalcification time more significantly than red blood cells of intact guinea pigs. Moreover, the thromboplastic activity of red blood cells of irradiated animals decreased during washing, whereas it did not change in intact guinea pigs. The erythrocyte supernatant shortened the recalcification time in the same way in both groups of animals. The erythrocytes of animals exposed to acute gamma radiation had less antiheparin activity than the erythrocytes of the intact group. When washing, the antiheparin activity of erythrocytes in intact and irradiated animals was unchanged.

The red blood cells of animals of both studied groups had a pronounced fibrinolytic effect. However, the fibrinolytic activity of the erythrocytes of the irradiated animals was 15.0 % (p<0.02) less than in intact animals, which may be due to the increased activity of antiplasmin and inhibitors of plasminogen activation in the erythrocyte stroma. The fibrinolytic activity of the washed red blood cells of both groups of animals did not differ significantly. The supernatant did not have a pronounced effect on the rate of the euglobulin clot lysis.

Table 1. Effect of sublethal gamma radiation on the resistance of red blood cells to acid hemolysis in guinea pigs

The studied indicators	**Intact animals, n= 10**	**Animals after radiation, n= 10**
Total duration of the hemolysis process (min)	5.23±0.12	6.66±0.26 p<0.01
Time of hemolysis maximum (min)	3.25±0.08	4.13±0.14 p<0.02
Number of destroyed red blood cells in hemolysis maximum (%)	19.50±2.24	12.31±2.37 p<0.05
Destruction time of the most stable forms of red blood cells (min)	4.61±0.28	5.36±0.12 p<0.05

Note: p is the significance indicator of differences between indicators of intact and irradiated animals.

Table 2. Effect of sublethal gamma radiation on peroxidation and blood lipid metabolism in guinea pigs

The studied indicators	**Intact animals, n= 10**	**Animals after radiation, n= 10**
Spontaneous erythrocyte hemolysis (% hemolysis)	**3.04±0.17**	**3.03±0.29 p>0.05**
Diene conjugates (μmol/L)	**35.22±1.65**	**38.78±1.67 p>0.05**
The level of TBARS before the incubation of red blood cells, (μmol/L erythrocyte)	**10.65±1.13**	**10.97±1.36 p>0.05**
The level of TBARS after 1.5 hours of incubation of erythrocytes (μmol/L erythrocyte)	**12.32±0.81**	**12.21±0.25 p>0.05**
The increase in TBARS during the incubation of red blood cells (μmol/L erythrocyte)	**3.08±0.78**	**3.61±0.73 p>0.05**
Superoxide dismutase (U)	**0.76±0.03**	**0.61±0.04 p<0.005**
Catalase index	**1.65±0.16**	**1.61±0.16 p>0.05**
Ceruloplasmin (mg/L)	**44.70±2.97**	**33.50±2.89 p<0.02**
Cholesterol (mmol/L)	**1.27±0.15**	**1.19±0.11 p>0.05**
Total lipids (g/L)	**1.68±0.25**	**2.33±0.11 p<0.02**
LDL and VLDL (g/L)	**1.33±0.36**	**1.17±0.08 p>0.05**

Note: p is the significance indicator of differences between indicators of intact and irradiated animals.

Table 3. Effect of gamma radiation at a sublethal dose on the hemocoagulating and fibrinolytic properties of red blood cells in guinea pigs

The studied indicators	C	Intact animals, n= 10	Animals after radiation, n= 10
The plasma recalcification time with the addition of red blood cells (s)	**146.03±2.08**	**76.03±3.27 p<0.001**	**65.87±2.67 p<0.001 p_1<0.02**
Plasma recalcification time with the addition of washed red blood cells (s)	**146.03±2.08**	**74.37±2.42 p<0.001**	**80.75±2.65 p<0.001 p_1<0.05**
Plasma recalcification time when supernatant is added (s)	**146.03±2.08**	**78.13±2.29 p<0.001**	**76.62±3.59 p<0.001 p_1>0.05**
Thrombin time of plasma with the addition of red blood cells (s)	**44.01±1.19**	**21.01±2.27 p<0.001**	**32.02±2.53 p<0.001 p_1<0.001**
Thrombin time of plasma with the addition of washed red blood cells (s)	**44.01±1.19**	**24.13±1.23 p<0.001**	**30.13±2.29 p<0.001 p_1<0.05**
Thrombin time of plasma with the addition of supernatant (s)	**44.01±1.19**	**28.63±1.34 p<0.001**	**30.80±1.25 p<0.001 p_1>0.05**
Euglobulin plasma clot lysis time with the addition of red blood cells (min)	**298.24±6.07**	**227.41±9.68 p<0.001**	**261.52±6.10 p<0.001 p_1<0.001**
Euglobulin plasma clot lysis time with the addition of red blood cells (min)	**298.24±6.07**	**225.00±8.94 p<0.001**	**217.40±4.72 p<0.001 p_1>0.05**
The time of lysis of the euglobulin plasma clot with the addition of supernatant (min)	**298.24±6.07**	**294.02±0.29 p>0.05**	**281.10±9.47 p>0.05 p_1>0.05**

Note: C is plasma control (substrate plasma + physiological saline); p is the significance indicator of differences between the indicators of substrate plasma and plasma with the addition of red blood cells; p_1 is the significance indicator of differences between indicators of intact and irradiated animals.

DISCUSSION

According to our data, after acute sublethal irradiation of animals, on the 7th day, the appearance of cells with high resistance to hydrochloric acid hemolytic and an increase in the erythrocyte sedimentation rate associated with a decrease in the charge of their membranes were revealed. Most researchers consider the decrease in the surface charge of erythrocytes after exposure to ionizing radiation as a result of structural rearrangement of the membrane (19-21).

The consequence of irradiation is the activation of LPO and profound changes in the conformation of membrane proteins, including the aggregation of membrane proteins with the formation of -S-S-bridges, which affects the mechanical properties of membranes and their resistance to chemical hemolysis (22).

The observed increase in erythropoiesis at the height of radiation sickness can be explained by the mobilization of reserves of erythrocyte production (23). An increase in the pool of proliferating hematopoietic cells, as well as the acceleration of cell differentiation, bypassing some “normal” stages of their maturation, can be considered as an additional source of enhancing erythropoiesis (24).

In our research, depletion of the antioxidant system was also noted under exposure at a sublethal radiation dosage. It

manifested in a decrease in the activity of superoxide dismutase (SOD) of erythrocytes and the concentration of serum ceruloplasmin without changing the concentration of primary and secondary lipid peroxidation products.

Data on the unchanged amount of conjugated dienes and the concentration of TBARS under total irradiation at a sublethal dose do not contradict the literature.

After radiation, the amount of antioxidant phospholipids in the bloodstream increases and the intensity of free-radical autooxidation of lipids in tissues decreases (25). In turn, it was found that the humoral products of activation of stress-realizing systems - catecholamines and steroid hormones - have antioxidant activity (26, 27). Their hypersecretion can be considered as a response to LPO activation, which develops through a negative feedback mechanism. However, a long-term excess of their normal level in circulation by 5-10 times and more causes the secondary activation of LPO (4).

The absence of an LPO outbreak on the 7th day after sublethal irradiation may be associated with a weakening of the "respiratory activity" of leukocytes. Thus, a number of authors noted the inhibition of the phagocytic activity of neutrophils at the height of radiation sickness, and the release of glucocorticoids at the earliest stages after irradiation inhibits the respiratory burst of neutrophils (28, 29).

In turn, the suppression of the "respiratory explosion" in polymorphonuclear leukocytes is accompanied by a decrease in the power of the pentose phosphate cycle, which can also be observed in erythrocytes (30).

Since the source of the reducing equivalents of the cell antioxidant system is the pentose phosphate pathway, the observed decrease in the SOD activity in erythrocytes can be associated with a possible inhibition of the pentose cycle, a decrease in the level of 02•-, which acts in relation to the enzyme as an inducing and activating factor (31, 32).

The noted decrease in the concentration of the main plasma antioxidant, ceruloplasmin, may be associated with inhibition of the release of the leukocytes endogenous mediator, which is responsible for the synthesis and release of protein reactants of the active phase, including ceruloplasmin (33).

An increase in the concentration of blood serum total lipids with a constant content of low and very low density lipoproteins may be the result of an increase in the amount of high density lipoproteins. A similar antiphase change in the amount of high and low density lipoproteins was observed in works by Serkiz et al. (34).

The studies show that the observed post-radiation changes in erythrocytes affect the structural rearrangement of the cell membrane, accompanied by a change in the conformation of membrane molecules and disruption of the enzyme systems of erythrocytes. Erythrocytes of irradiated animals exhibited increased procoagulant and decreased antiheparin activity, which reflects conformational changes in highly radiosensitive fatty acid chains of phospholipids in their membranes.

The observed decrease in the fibrinolytic activity of erythrocytes in irradiated animals is possibly explained by the increased activity of antiplasmins and inhibitors of plasminogen activation in the erythrocytes stroma, as well as by the fact that at the height of radiation sickness, the plasminogen proactivators and activators enter the plasma from erythrocytes intensively; the activation of the fibrinolytic chain of hemostasis system is observed during this period (35).

CONCLUSIONS

In summary, the evidence of the participation of radioresistant specialized cells (erythrocytes) in functional disorders in the organism after irradiation was obtained.

It was found that on the 7th day after a single sublethal irradiation of animals, intense erythropoiesis and the appearance of red blood cells with high hemolysis resistance and an increased sedimentation rate were observed. An increase in procoagulant and a decrease in the fibrinolytic activity of erythrocytes, an antioxidant system stress, aimed at maintaining lipid peroxidation, which at this time does not exceed the normal level, despite pronounced signs of impaired function of erythrocyte membranes, were observed.

Further study of the mechanisms of oxidative stress and hemostasis in radiation-induced tissue damages will provide the opportunity to better develop preventive and therapeutic strategies in the future.

ETHICS APPROVAL

This study was carried out in accordance with the nationalGeneral Ethical Principles of Animal Experiments, whichis consistent with the provisions of the European Conventionfor the Protection of Vertebrate Animals used for experiments or other scientific purposes (Strasbourg, March 18,1986). This experiment was approved by the UMSA Ethicsand Bioethics Commission.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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AGE AND GENDER DIFFERENCES IN ORBITAL MEASUREMENTS WITHIN SERBIAN POPULATION IN KRAGUJEVAC REGION OF THE REPUBLIC OF SERBIA

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ABSTRACT

Orbital measures are not only important parameters in planning ophthalmologic and aesthetic surgical procedures, but also significant anthropology and forensic medicine markers. Using computer tomography and subsequent multiplanar reconstruction we analyzed orbits from 75 Serbian healthy volunteers, examined in the Clinical Hospital of Kragujevac. The subjects were subclassified in age categories, namely 24-39, 40-59, 60-69 and 70-85 years of age, as well as by genders. Taken as a whole population, regardless of age, women had smaller orbital height and width and smaller volumes than men, but similar orbital indices, thus proving once again the importance of the orbital index for comparisons. Additionally, both biorbital and interorbital distances were higher in men than in women. Comparing age groups, biorbital and interorbital distances increased with aging in males, but remained constant in females. The opposite was true for orbital indices which increased significantly with age in females, but remained constant in males. Taken together, our results indicate the presence of age- and gender-related differences in orbital measures within ethnically and geographically homogenous population. It would be interesting to examine subjects from other regions to confirm the patterns reported here.

Keywords: *Age, gender, multiplanar reconstruction, orbit, Serbia.*

INTRODUCTION

The orbital cavity contains eyeball, optic nerve, and accessory ophthalmic elements such as muscles, ligaments, orbital fat body, blood vessels and nerves. It is one of the most complex structures on the skull. The orbit is approximately pyramidally shaped, with its base oriented frontally, and its peak converging backwards and medially to sella turcica (1). Anatomical parameters of the orbit are used in anthropology and forensic medicine, for race and gender predictions, as well as in reconstructive and esthetic surgery. The shape of the orbit is determined by various genetic and acquired features of the cranial and facial bones, and its morphometric characteristics are variable and depend on age, gender, race and ethnicity (1-5).

One of the most consistent findings, and a critical factor in anthropological and forensic studies, is the sexual dimorphism of the orbit (6-8). Various studies on different ethnical groups described the orbit of males as larger in comparison to females (6). However, sexual differences between orbits vary depending on racial and ethnical backgrounds. For example, Aboriginal and Japanese females have larger orbital height than the males, and the orbit of South-African females is more oval than in males (1). Another interesting feature of orbital measures is their asymmetry. Statistically, as approximately 90% of people in any given population have the dominance of the left forebrain hemisphere, the right orbital cavity is mostly larger than the left one (7, 9-11).

The first parameter to describe the orbit in vivo was orbital volume. Its measurements were introduced in the nineteenth century by Gyat, and the methods to measure orbital volume in vivo have changed much over the years (8, 12). Today, most of our methodology is based on the analysis of computer tomography (CT) and magnet resonance (MR) images, although no gold-standard method for orbital volume measurements exists (14, 15). Orbital volume in most adults, independent of measuring method, falls between 20-30 ml (1, 2, 6, 7). Another important parameter for facial morphometry, the orbital index, was introduced by Paul Broca in the late nineteenth century. It is defined as the fraction of the orbital height, as numerator, and the orbital width as denominator, multiplied by 100. Based on the orbital index, there are 3 broad categories of orbital cavities, which mirror racial division: 1) large (megaseme) orbits - with the orbital index of 89 and more, present mainly in the yellow race, with the round shaped orbital aperture; 2) intermediate (mesoseme) - orbital indices that range from 83 to 89, common feature of the white race, and 3) small (microseme) - the orbital index of less than 83, seen in the black race, with the rectangularly shaped orbital aperture (13, 16). The orbital index has great attraction for studying, as it describes the shape of the face and varies among races, regions within the same race, and ethnic groups (17).

The aim of our present study was to determine the morphometric characteristics of orbital cavity in Serbian population of Kragujevac region, to examine if there are differences in examined parameters between male and female examinees, as well as within different age groups. It is, thus, a basic anthropometric study aimed not only at increasing knowledge about local population in Kragujevac region of Serbia, but also at drawing conclusions about the variability of orbital parameters in any given population. Our results imply that even within a homogenous population, the values of key orbital parameters change over time, and we report gender-related difference in several parameters.

MATERIALS AND METHODS

Design of study

This is a retrospective, descriptive, non-randomized observational anthropometric study. We used data from patients' skull images, archived in the hospital system for data archiving of the Department of Diagnostic Radiology, Clinical Center of Kragujevac during a time span of more than 5 years, from January 2010 to November 2015.

Protocol of study

The computer tomography images were obtained on 64-slice MDCT scanner (Aquilion 64, Toshiba, Japan). The scans were performed in the axial plane, with subsequent multiplanar reconstruction. The subjects were laid on their back, with arms extended downwards. The head restraint was used. We present the results from 75 subjects (50 male and 25 female), aged from 24 to 85 years. All subjects used in this study had no pathological changes of the skull and soft tissue elements within it and they were referred to this examination for various reasons. Orbits were examined as part of a broader inspection (whole head, face, paranasal cavities). All subjects were of Serbian ethnicity and lived in the greater Kragujevac region of the Republic of Serbia.

Scanning parameters were: 120 kVp, 500 mAs, gantry rotation of 0.75 sec, pitch 0.5 mm, slice thickness of 0.5 mm and 0.4-0.6 mm reconstruction thickness. Analysis of all images and MDCT data was performed on a Vitrea 2 workstation ver.4.1.14.0 (Vital Images). All measurements were performed by two independent radiologists blinded to patient's personal data, using commercially available software (Imaging Software ver.4.1.14.0, Vital-Images). To estimate inter-observer reliability, the intra-class correlation coefficient (ICC) was used, and the ICC values of more than 0.8 were considered acceptable for the study.

Standard anatomical points were determined and used for the measurement of the orbital width, height, biorbital and interorbital diameters. Orbital width — the distance between the dacryon (the point where frontal, lacrimal and maxillary bones intersect forming the medial border of the orbit) and ectoconchion (the point of intersection of the anterior surface of the lateral border of the orbit); this line divides the orbit along its vertical axis into two parts (5); orbital height-the distance between the superior and inferior orbital borders; the line which defines it is perpendicular to its width and similarly divides the orbit into two parts, but along the horizontal

axis (5); biorbital width — the distance between left and right ectoconchion (5); interorbital width — the distance between right and left dacryon (5). Based on these measurements, the software automatically calculated orbital index — orbital height/orbital width × 100 (9), and orbital volume (8). Measurements were performed on coronal plane using 3D images reconstructed from raw CT axial plane images. The groups for comparisons were based on age and gender. Age categories were formed based on the equal distribution of patients within age classes, and thus comprised a group of adults and young adults (24-39 years), middle-aged (40-59), mature or old adults (60-69 years and aged individuals (over 70 years).

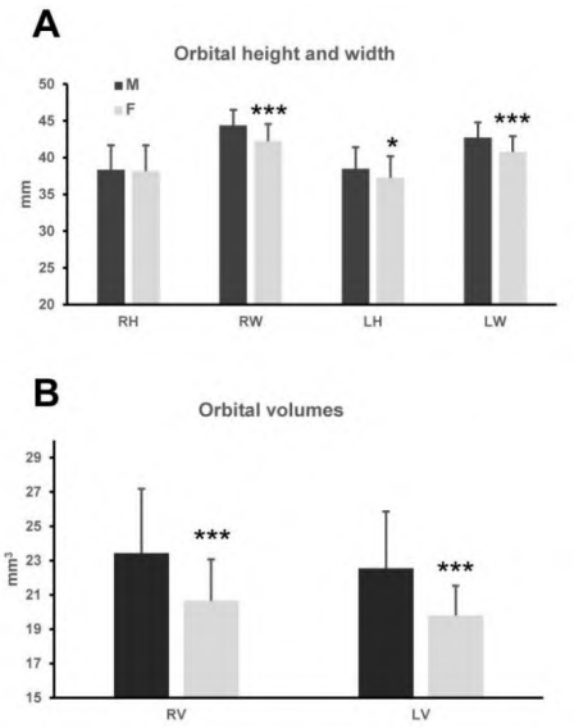
Statistical analysis

All data are presented as the mean values ± standard deviation. Statistical analysis was performed using a parametric statistical test, as the data had normal distribution and equal variance. Two-way analysis of variance (ANOVA) with factors "age category" and "gender" was used to compare the groups, followed by Holm-Sidak post-hoc multiple comparisons analysis. Within the same gender, differences between the age groups were determined using one-way ANOVA, followed by Holm-Sidak post hoc. The probability value to accept differences between the groups as significant was set at 0.05.

RESULTS

We examined the orbits of 50 male and 25 female volunteers, using CT scanner and multiplanar reconstruction. The subjects were age-matched, with the average age of 55.8 ± 17.5 and 58.4 ± 15.7 for male and female groups, respectively ($p = 0.5$; two-way ANOVA). As sexual dimorphism has previously been reported for orbital measures (6), we analyzed our results using two-way ANOVA, with factors "gender" and "age". The height of the right orbit (38.34 ± 3.33 mm in males and 38.17 ± 3.51 mm in females) was not significantly different between male and female subjects. However, the width of both orbits (44.38 ± 2.13 and 42.73 ± 2.05 mm in males and 42.26 ± 2.29 and 40.76 ± 2.14 mm in females for the right and left, respectively; $p < 0.001$ for both) and the height of the left orbit (38.47 ± 2.93 mm in males and 37.25 ± 2.94 mm in females; $p = 0.023$) were significantly higher in males than in females (Fig. 1A).

Figure 1. Orbital measurements in male (M, black bars) and female (F, gray bars) examinees

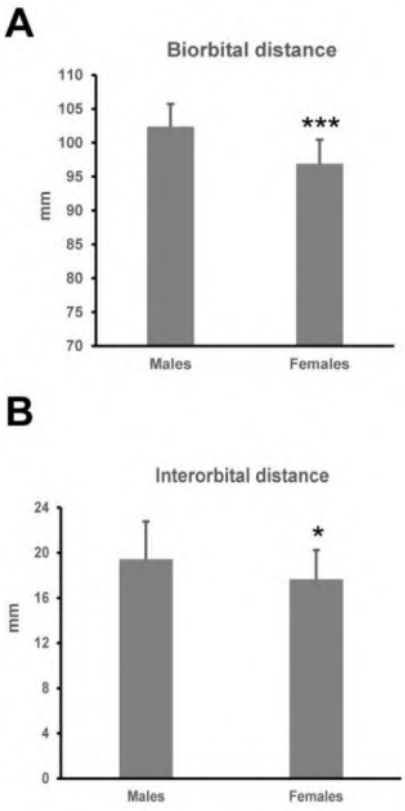


Shown are mean values + SD for orbital height and width (A), and orbital volume (B), in male and female examinees. Two-way ANOVA with Holm-Sidak post-hoc, * $p < 0.05$; *** $p < 0.001$. R (right), L (left) H (height), W (width), V (volume).

Both the right orbital volume (23.43 ± 3.75 mm^3 in males and 20.66 ± 2.42 mm^3 in females; $p < 0.001$), and the left one (22.54 ± 3.32 mm^3 in males and 19.8 ± 1.73 mm^3 in females; $p < 0.001$) were significantly larger in males than in females (Fig. 1B). Orbital indices, however, for both the right (86.5 ± 7.29 in males and 90.4 ± 6.43 in females; $p = 0.43$) and the left orbit (90.2 ± 7.2 in Serbian and 91.4 ± 6 in females; p =

0.78) did not significantly differ between males and females. As for the distances, both biorbital distance (102.37 ± 3.37 mm in males and 96.89 ± 3.58 mm in females; $p < 0.001$) and interorbital distance (19.41 ± 3.35 mm in males and 17.65 ± 2.58 mm in females; $p = 0.033$) were both significantly larger in males than in females (Fig. 2A,B).

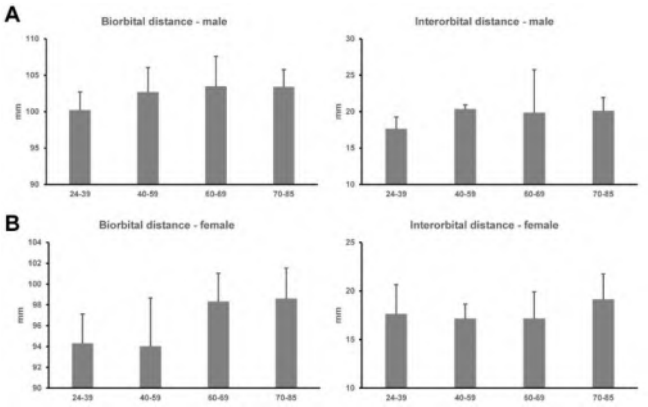
Figure 2. Biorbital and interorbital distances in male and female examinees



Shown are mean values + SD for biorbital (A), and interorbital distance (B), in male and female examinees. Two-way ANOVA with Holm-Sidak post-hoc, * $p < 0.05$; *** $p < 0.001$.

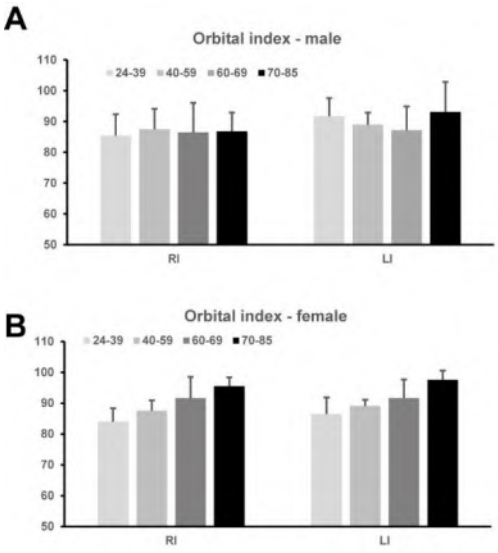
As our study was conducted over a relatively short period of time, we divided the subjects into age groups, to estimate if there is a difference in people born in different decades, thus implying a change in orbital parameters over time. The age group brackets were determined based on the criteria to have similar numbers of examinees within each group. Interestingly, among males the only parameters significantly different between the age groups were biorbital and interorbital distances, which were both higher in older subjects (Fig. 3A). These parameters, however, remained constant in females (Fig. 3B). Conversely, both right and left orbital indices had a clear trend of increasing with increasing age in female (Fig. 4B), but not in male examinees (Fig. 4A).

Figure 3. Biorbital and interorbital distances in male and female examinees by age groups



Shown are mean values + SD for biorbital (A), and interorbital distance (B), in male (left panels) and female (right panels) examinees. One-way ANOVA with Holm-Sidak post-hoc. R (right), L (left), I (index).

Figure 4. Orbital inices in male (A) and female (B) examinees by age groups



Shown are mean values + SD. One-way ANOVA with Holm-Sidak post-hoc.

DISCUSSION

Here we used computed tomography as the most frequently applied method for the evaluation of the orbital anatomy to demonstrate the gender- and age-related differences of orbital measures within the relatively homogenous Serbian population. Our results indicate significant differences between male and female examinees in the orbital height, width, volume, as well as biorbital and interorbital distance, but the orbital index remained unaffected by gender. This adds to the evidence that orbital index is constant within a homogeneous population and can be used to standardize the results (17). The difference between males and females in the orbital height, width, volume, as well as orbital distance, but

not orbital index, were confirmed by various studies on different ethnic and racial backgrounds, with male orbits having significantly larger measures than female (10, 16, 21, 22). This appears to be a constant feature of the human race, in congruence with other body measures that are larger in men (18). The results of our study also confirmed the earlier findings in variation of orbital dimensions between the left and right side (13, 23). The observed parameters of the orbital height and width had a tendency to be higher on the right side, leading to the higher values of the left orbital index (right: 87.75 ± 7.2, left: 90.6 ± 6.8; p = 0.0135, *t* test). This is in accordance with the earlier findings and widely accepted theory that the skull and the face right/left asymmetry with higher values of the right orbital measures are the consequence of the brain asymmetry and the dominance of the left hemisphere (11, 23). Interestingly, the right orbital index falls well within mesoseme category, typical for the European and Caucasian race (23), whereas the left orbits were at the lower end of megaseme category (1, 13, 16, 17). This underlines the conclusion that ethnicity is an important determinant of the orbital measures. It noteworthy in this context, that ethnic Serbs have a mixed pool of genes brought to this region by various conquerors, including those with the strong Asian racial background (mainly Turkish and Hun people), thus the megaseme tendency for the left orbit is likely due to genetic mix caused by migrations (1, 10).

Another remarkable set of data in our study concerns age-related differences. The age groups were formed based on the equal distribution of examinees within each group, but with the aim to remain biologically sound. Thus or subjects were either full-developed adults and young adults (24-39 years), middle-aged (40-59 years), older adult (60-69 years) and aged individuals (>70 years) We would hesitate to interpret these results as the changes in orbital size that progress with aging, as we did not follow the same subjects over time, although this remains one possible interpretation. Another possibility is that over period of several decades, Serbian population undergoes changes in orbital measures. Here, a clear trend of increasing size of biorbital and interorbital distances was established for older males, whereas no such trend was present in females. For the orbital index, the situation was exactly the opposite - practically linear increase is present for both left and right orbital index in females, while there is little or no variation in males (Fig. 4). This is consistent with the hypothesis that Serbian orbits had a tendency to become smaller during the last 80 years. These differences in orbital measures in different age groups could be interpreted as another gender specificity i.e. that the population changes over time are gender-specific, as reported previously (3, 7, 16, 21, 23).

CONCLUSION

Morphological measurements of the orbit are important to obtain better practical knowledge and understanding of the difference between genders, race and ethnic groups, which are essential for surgical procedures, forensic medicine evaluations and anthropological studies. We conclude that within the Serbian population there are significant gender-related differences in almost all orbital measures, with males having larger orbits and distances between them. This is, to our knowledge, the first study addressing gender and age related difference in anthropometric parameters of the orbital cavity in Serbian population.

CONFLICTS OF INTEREST

The authors confirm that there is no conflict of interest regarding the publication of this article.

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GARLIC THE WONDER ADJUVANT IN MEDICINAL FIELD

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ABSTRACT

Plant derived compounds are drawing attention in curing and treating variety of ailment and diseases. This increase in popularity of natural products has renewed interest in garlic, which has been used by human for centuries. It has been found that garlic pulp contains more than 200 chemical compounds and numerous garlic molecules can still be explored, extracted, synthesized and optimized. As in market various preparations of garlic are available which include tablets made from dried and powdered clove, oils and liquid extracts however, it would also be interesting to explore the effect of different forms of garlic extract on standard drug therapy especially when used as an adjuvant therapy. In this review a report on the pharmaceutical preparation which has used extracted compounds from garlic or its derivatives as a main constituent is compiled, so that it could be useful to increase our knowledge about the therapeutic effect of garlic and could improve our future experimental and chemical plans. We performed a systematic review of literature using term garlic. In this report a comprehensive investigation has been conducted on garlic which includes various scientific aspects about it by which researchers from various disciplines could be directed to put efforts toward discovering the benefits of garlic on human health. Garlic and its extracts had a wide range of applications even against resistant organisms to serve as powerful anti-microbial agent. Therefore, research is needed to refine the pathophysiological mechanisms of action of garlic and its utility in the treatment of various diseases by developing more stable and suitable formulations. The development of Garlic as a commercial anti-biotic has come to a halt. Although its efficiency is scientifically proven, it has only been used as a dietary supplement or traditional medicine.

Keywords: *Ailment, clove, garlic, treatment, utlity.*

INTRODUCTION

Garlic is famous as allium, da-suan, la-suan, rustic treacle, stinking rose, poor man's treacle, nectar of the gods, camphor of the poor and also known by other common names like Rasonam, Lasan, Vellulli, Vallai-pundu, Seer, Ullippoondu, Maharu (1) is a bulbous herb having botanical name-Allium sativum belongs to family Lillaceae (2) is a native to central Asia and northeastern Iran. Garlic had been used by some civilizations against the bad evils, in ancient times and also as a therapeutic medicinal plant in many other places. It has considered as a traditional medicinal herb based on the experiences passes from generation to generation whose description is available in Rig-veda 41 and some old existing corpus also support its uses in Chinese, Egyptian, French and Ayurvedic medicine (3, 4). Throughout history, worldwide garlic has been using both as a spice and medicine (5). Now day garlic has attracted more attention as a modern medicine due to its broad-spectrum therapeutic effect with minimal toxicity (6).

CHEMICAL CONSTITUENTS OF GARLIC

Garlic has more than 500 species in 30 genera (7), is a good source of anti-oxidants and contains at least 33 sulfur compounds, several enzymes, vitamin B, flavonoids and certain minerals (8). Garlic contains 17 amino acids e.i. lysine, histidine, arginine, aspartic acid, threonine, glutamine, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tryptophan, serine and phenylalanine (9). Garlic's pungent odor and many of its medicinal effects (10) are due to presence of high concentration of sulfur compounds than any other *Allium* species. Allicin (diallyl thiosulfinate or diallyldisulfide) is the most biologically active compounds in garlic and Alliin (S-allylcysteine sulfoxide) is the most abundant sulfur compound which is a colorless, odorless and water-soluble compound which is present at 10 and 30 mg/g in fresh and dry garlic, respectively (11, 12).

Garlic and onion contain, Allicin ($C_6H_{10}OS_2$) which is a volatile compound. Alliin (L-(+)-S-Allyl cystein sulfoxide) is an amino acid which, under the action of the alliinase enzyme, converts to allyl sulfenic acid (2-propene sulfenic acid), an unstable and highly reactive compound at room temperature (13). Then, two allyl sulfenic acid molecules condense spontaneously to form allicin with the elimination of water molecule (Figure-1).

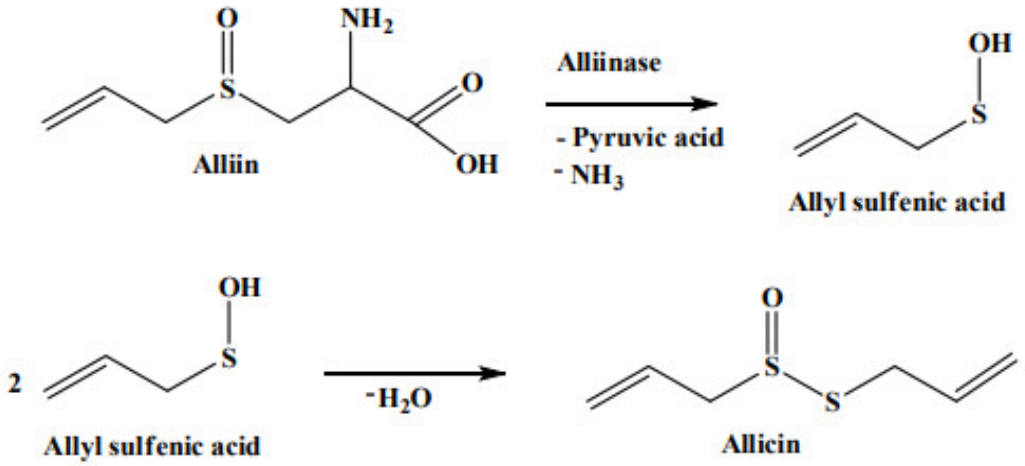

Figure 1. Synthesis of Allicin

Allicin decomposes in the presence of air and water producing mainly "diallyl disulfides" (responsible for the characteristic odor of garlic). This same degradation process occurs in the body, and it is associated with the characteristic odor in breath after garlic ingestion (13).

Table 1. Chemical constituents found in garlic bulb (14, 15)

S. No	Compound	Amount (ppm)	S. No	Compound	Amount (ppm)
1	1,2-Dimercaptocyclopentane	2.4	33	Fiber	7000-39,000
2	1,3- Dithiane	0.08-3	34	Glutamic acid	8050-19,320
3	2-Vinyl-4H-1,2-dithiin	2-29	35	Glycine	2000-4800

S. No	Compound	Amount (ppm)	S. No	Compound	Amount (ppm)
4	3,5-Diethyl-1,2,4-trithiolane	0.15-43	36	Histidine	1130-2712
5	3-Vinyl-4H-1,2-Dithiin	0.34-10.65	37	Iron	15-129
6	Alanine	1320-31,168	38	Isobutyl-isothiocyanate	0.14 - 25
7	Allicin	1500-27,800	39	Isolucine	2170-5208
8	Allin	5000-10,000	40	Leucine	3050 -7392
9	Allyl – propyl – disulfide	36 – 216	41	Lysine	2730 - 6552
10	Aluminium	52	42	Magnesium	240-1210
11	Aniline	10	43	Manganese	5.4-15.3
12	Arginine	6340– 15, 216	44	Methyl-allyl-disulfide	6-104
13	Ascorbic acid	100 – 788	45	Methyl-allyl-sulfide	0.5-4.6
14	Aspartic acid	4890– 11,736	46	Methyl-allyl-trisulfide	6-279
15	Beta-carotene	0.17	47	Methyl-propyl-disulfide	0.03-0.66
16	Biotin	22	48	Niacin	4-17
17	Boron	3 – 6	49	Nickel	1.5-1.7
18	Caffeic acid	20	50	Nicotinic acid	4.8
19	Calcium	180 – 4947	51	P-coumaric acid	58
20	Carbohydrates	274000-851000	52	Phenylalanine	1830 - 4392
21	Chromium	2.5 – 15	53	Phosphorus	880 - 5220
22	Cobalt	0.5 – 100	54	Potassium	3730-13,669
23	Copper	4.8 – 9.7	55	Proline	1000-2400
24	Cystine	650 – 1560	56	Propenethiol	1-41
25	Diallyl-disulfide	16 – 613	57	Protein	35,000-179,000
26	Diallylsulfide	2 – 99	58	Protodegalactotigonin	10
27	Diallyl-trisulfide	10 – 1061	59	Protoeruboside-B	100
28	Dimethyl-difuran	5 – 30	60	Quercetin	200
29	Dimethyl-disulfide	0.6 – 2.5	61	Riboflavin	0.5-3
30	Dimethyl-trisulfide	0.8-19	62	Scordinine-A-1	67-30,000
31	Fat	2000-12,000	63	Scordinine-A-2	250-8000
32	Ferulic acid	27	64	Scordinine-B	800

EFFECT OF GARLIC'S FORM ON ITS ACTIVE CONSTITUENT

Traditionally, Garlic was used in its raw form, but now days it is also used in heated, dehydrated and aged, form. Heat is used for dehydrating the plant to form garlic powder but at high temperatures alliinase is deactivated and hence cannot react with alliin to form allicin (16). This explains why cooked garlic has a mellower flavor than raw garlic. Only freshly crushed garlic has hydrogen sulfide, which is suspected to have significant cardio-protective effects as a vasodilator (17).

Allicin content can be retained in the powder to some extent if the cloves are frozen before being pulverized; acetone removes the water and alliin and alliinase remain separate yet intact until water is added, at formation point of allicin. Alliinase is not destroyed during dehydration process of forming

powder in comparison to heat by which more than half of the alliin is lost. Alliinase is deactivated by the acidic environment of the stomach. It has also concluded that when dehydrated garlic powder is exposed to simulations of the gastrointestinal fluids the production of allicin is decreased by 99% presumably due to the lack of allinase. This is further matter of research that dehydrated garlic powder when taken in a capsule with an enteric coating whether it is protected or not from stomach acid. These studies also show that manipulating garlic's form leads to changes in the active constituents and could lead to data inconsistencies in studies (18, 19).

USES OF GARLIC

Therapeutic uses (20)

Traditionally, it has been employed to treat: infections, wounds, diarrhea, rheumatism, heart diseases and diabetes.

Experimentally, it has been shown to exert: anti-lipidemic, anti-hypertension, anti-neoplastic, anti-bacterial, immune-stimulant and hypoglycemic actions.

Clinically, it has been evaluated to treat number of conditions including: hypertension, hypercholesterolemia, intermittent claudication, diabetes, rheumatoid arthritis, common cold, arteriosclerosis and cancer (20).

Garlic, from crushed to capsules, is consumed throughout the world, despite the widespread use of garlic for various purposes, the increasing craze on health maintenance and use of natural/ herbal products, garlic is being used for variety of formulations in various forms such fresh garlic, garlic oil, extracts of garlic or its chemical constituents (21). Furthermore, garlic has pharmaceutical effects and used to cure vast conditions including blood pressure, cholesterol (22, 23) and cancer (24, 25). Moreover, garlic is also used as hepato-protective (20, 26), anti-protozoal (27, 28), anti-viral (29, 30), anti-oxidant (31), anti-microbial (20, 32) and anti-fungal. Further, it is also used to treat wounds (33), diabetes (34), asthma, arthritis, sciatica, lumbago, backache, bronchitis, chronic fever, tuberculosis, rhinitis, malaria, obstinate skin disease including leprosy, leukoderma, discoloration of the skin and itches, indigestion, colic pain, enlargement of spleen, piles, fistula, fracture of bone, gout, urinary diseases, kidney stone, anemia, jaundice, epilepsy, cataract and night blindness. Garlic products are used as sources of medicine in many ways in human beings in their day-to-day life (35, 36).

Table 2. The medicinal spectra of garlic compounds (37)

Pharmacological activity	Chemical compounds of garlic contributed activity	Pharmacological activity	Chemical compounds of garlic contributed activity
• Anticoagulant	Ajoene	• Antiparasitic	Allicin-alliin
• Antihypertensive	Selenium germanium	• Antibiotic	Allicin-alliin
• Antimicrobial	Selenium germanium	• Antimycotic	Allicin-alliin, Ajoene
• Antiviral	Allicin- Ajoene	• Hypolipemic	Diallyl disulfide
• Antioxidants	Selenium, germanium	• Antiaging	Selenium, diallyl disulfide
• Antitumour	Selenium, germanium	• Humoural immunity	Germanium allicin
• Detoxification of heavy metals	Selenium allyl mercaptan germination	• Vitamins	Thiamine, vitamins A and C
• Natural killer cell activity and other kind of cell mediated immunity • Anti-inflammatory activity • Hepatoprotective activity • Digestive system protection	Selenium, germanium Ethyl linoleate, garlic 14-kDa protein, allicin Garlic oil, DADS and LAFGE DADS, DAS and allicin	• Complement activity • Modulating immune system • Cardiovascular protection • Anti-cancer activity • Anti-diabetic activity	Magnisium, calcium Polysaccharides, garlic oil Polyphenols, S-1-propylenecysteine, alliin and allyl methyl sulfide Lipid bioactive compounds, allicin, DATS and Z-ajoene LAFGE and garlic oil

DOSAGE

A commercial garlic product should provide a daily dose equal to at least 4000 mg (one to two cloves) of fresh garlic. The cloves may be diced and mixed with wildflower honey for palatability. This dosage translates to at least 10 mg alliin or a total allicin potential of 4000 ug. In dried form this would be 300 mg of garlic powder tablet (standardized to 1.3 percent alliin or 0.6 percent allicin yield) two to three times per day or 7.2 g of aged garlic extract per day. In tincture form

from fresh bulb as a 1:2 in 95 % alcohol, the dosage can be 40 drops up to six times per day (38).

TYPES OF GARLIC FORMULATION

1. *Antiacne garlic gel*: It was developed and evaluated for the anti-acne activity containing garlic juice against *P. acnes* to facilitate topical usage (39).
2. *Sustained release tablet: Allium Sativum* tablets were formulated by wet granulation using acacia, and gelatin in order to enhance efficacy and improve patient compliance (40).
3. *Garlic Extracts:* Extracts are the preparations of crude drugs which contain all constituents which are soluble in the solvent. The following extracts have been prepared -
   A. *Garlic extract for skin care*: The physiological effects of a formulation containing garlic extract as compared to the base formulation on skin care were compared. In conclusion, the garlic formulation had high inhibitory activities for tyrosinase and elastase, thus suggesting that garlic may have beneficial properties as a material for cosmeceuticals (41).
   B. *Aqueous extract of garlic* as a pessary*: It* was formulated using the pouring method and cocoa butter as a base (42).
   C. *Pharmaceutical formulation of garlic and turmeric dried crude extract and their synergistic anti-fungal activity:* The effectiveness of these natural products towards *Candida albicans* causes Candidiasis (fungal infection) was identified towards their pharmacological and toxicity aspects. The agar disc diffusion method was used to study the anti-fungal activity of their ethanolic extracts. As delivery agents, cream and gel formulations demonstrated good stability test results. Furthermore, both plants showed synergistic effects (43).
4. *Garlic Oil Preparations:*
   A. *Garlic essential oil nanoemulsion:* The nanoemulsion of garlic essential oil by ultrasonic emulsification was developed and the results showed that it can be used for developing natural nano acaricide (44, 45).
   B. *Garlic oil nanoparticles with enhanced anti-microbial activities:* Garlic oil (GO) colloidal nano-particles (NPs) were prepared by combining GO with poly lactic-co-glycolic acid (PLGA) polymer by single emulsion/solvent evaporation (SE/SE) method with 70-80% of more anti-bacterial activity compared with GO in bulk form (46).
   C. *Formulation, development and evaluation from garlic oil macerate:* Ajoene is one of the active constituents of garlic is highly unstable, it remains stable only in oil macerate form. For this garlic oil is obtained by two methods steam distillation and cold maceration by this its antibacterial as well as anti-fungal properties can be preserved (47).
   D. *Anti-fungal soap of garlic oil:* The formulated soap of garlic oil is typically effective against athlete's foot and jock itch (48).
   E. *Preparation methods for monodispersed garlic oil microspheres in water using the microemulsion technique and their use as anti-microbial:* The purpose of the present work is to develop and evaluate an oil-free microemulsion system. Microemulsions were prepared with ethoxylated hydrogenated castor (Cremophor RH40) as surfactant, n-butanol (or ethanol) as co-surfactant, oleic acid-containing garlic oil as oil phase, and ultrapure water as water phase. In addition, the anti-microbial activity (*in vitro)* against *Escherichia coli* and *Staphylococcus aureus* was assessed. The experimental results show that a stable microemulsion region can be obtained when the mass ratio of surfactant to co-surfactant is respectively, 1:1, 2:1 and 3:1 especially when the mixture surfactants of RH40/n-butanol 2/1 (w/w) is used in the microemulsion formulation. The area of o/w microemulsion region is 0.089 with the particle size 13.29 to 13.85nm and garlic oil encapsulation efficiency 99.5%. The prepared microemulsion solution exhibit remarkable anti-bacterial activity against *S. aureus* (49).
5. *A novel microparticulate formulation with Allicin in situ synthesis:* Spray drying was used to obtain a powder that releases allicin (Alliin and alliinase, served as precursors for allicin production, and were encapsulated separately into microspheres) and the in situ synthesized allicin was made available under safe and reproducible conditions for pulmonary application (50).
6. *Formulation Savings: Liquid Extracts in Replacement of Dry Powders? Garlic and Onion Powder:* Liquid garlic and onion extracts can offer as much as 40 to 80 percent savings over dehydrated powders. Because liquid extracts are more concentrated than powders, they allow for significantly lower usage rates and can also provide additional savings by reducing inventory, shipping and quality testing (51).
7. *Garlic: A natural antibiotic as liquid and cream formulations:* It has been found that allicin has potent activity against vancomycin-resistant enterococci (VRE) *in vitro.* In another study, it has been found that allicin liquid and cream formulations were highly potent against clinical isolates of methicillin-resistant *staphylococcus aureus* (MRSA) (52).
8. *Anti-oxidant potential of garlic and turmeric mixture (Traditional Indonesian formulation):* The combination of anti-oxidant activity of garlic bulb water extract and turmeric ethanol extract had been examined *in vitro* using DPPH (2,2-diphenyl-1-picrylhydrazyl) method and activity of the extract and its combination had been examined using lipid peroxidation method *in vitro* and in Swiss Webster female rats *ex vivo* which showed higher anti-oxidant activity *in vitro* compared to each extract, but in *ex-vivo* study showed similar effect (53).
9. *Garlic extract versus cryotherapy in the treatment of male genital warts:* The aim of this clinical study was to compare the garlic extract effect with cryotherapy in the treatment of male genital warts (54).

10. *Formulation of garlic oil-in-water Nanoemulsion: antimicrobial and physicochemical aspects:* In this work, a nanoemulsion containing garlic essential oil (GEO) was formulated to cover and protect the volatile compounds of GEO and it has been found that the formulated nanoemulsions had a stronger effect against Gram-positive bacterium (*Staphylococcus aureus*) than Gram-negative bacterium (*Escherichia coli*) (55).
11. *Development and assessment of stable formulations containing two herbal anti-microbials: Allium sativum L. and Eruca sativa miller seed oils:* Emulsions of both oils were prepared by the bottle method using water, Tween 80 and Span 80 and were evaluated for creaming index (CI), droplet size, and turbidity to determine rHLB. Utilizing determined rHLB, creams were formulated using a combination of two surfactants, Span 60: Brij 58 (1:2.333) at three different concentrations (2, 4 and 6%) (56).
12. Garlic oil: It is a volatile oil (essential oil) derived from garlic. It is usually prepared using steam distillation, and can also be produced via distillation using ether. It is used in cooking and as a seasoning, a nutritional supplement, and also as an insecticide. Steam-distilled garlic oil has around 900 times the strength of fresh garlic and around 200 times the strength of dehydrated garlic (57).
13. *Formulation and Evaluation of odour-free garlic powder:* A gastro retentive floating matrix tablet (FMT) from garlic powder (GP) was prepared using wet granulation technique and non-enteric film coating was applied to mask GP odor (58).
14. *Herbal Anti-dandruff Shampoo Containing Garlic Loaded Solid Lipid Nanoparticles:* These were formulated by using garlic as an anti-fungal agent. The ALL - SLNs (allicin - solid lipid nanoparticles) were formulated by hot homogenization method and evaluated by using different parameter. It is more effective for the treatment of dandruff on scalp and hair with no side effect (59).
15. *Garlic powder is an herbal formulation used in ayurvedic system of medicine for the treatment of platelets aggregation:* The interaction of garlic with heparin has been studied in influence to platelets aggregation effect of standard drug in various parameters. No significant toxicity was observed during toxicity study (60).
16. *Efficacy of new EC (emulsifiable concentrate) formulation derived from garlic creeper (Adenocalymma alliaceum Miers.) against anthracnose and stem end rot diseases of mango:* Different leaf extracts of Garlic creeper (Adenocalymma alliaceum Miers.) using water and solvents were prepared and they were screened for their anti-fungal activity against Colletotricum gloeosporioides Penz. and Botryodiplodia theobromae Pat. causal agents of mango post harvest diseases viz., anthracnose and stem end rot respectively. The extract was partially purified and formulated as ADENOCAL 60 EC for the management of post harvest diseases of mango fruits (61).
17. *Formulation and evaluation of herbal ointment for Antimicrobial activity:* The present work is to formulate and evaluate the ointment of garlic bulb extract for anti-microbial activity. The benzene extract was prepared by the Soxhalation method. The formulation shows more zone of inhibition against *Bacillus subtilis* (62).
18. *Formulation, development and evaluation of cream containing natural essential oils having mosquito repellent property:* A mosquito repellent cream naturally obtained from medicinal plants instead of commonly available synthetic insecticides and repellents such as N-Diethyl-3-methylbenzamide (DEET), which are carcinogenic and non-eco-friendly. Essential oils of Tulsi, Clove, Garlic, Kapoor kacheri and Lemongrass were used in the cream formulation and evaluated for various parameters. It was concluded that the formulated mosquito repellent cream using essential oil is natural, safe, effective, usable for the skin and stable too (63).
19. *Aqueous preparation of Allium Sativa (garlic) on erythrocyte osmotic fragility in Wistar rats: in vivo and in vitro studies:* The effects of garlic on the osmotic fragility of red blood cells in albino rats were assessed *in vivo* and *in vitro*. In the *in vivo* studies, five albino rats weighing between 150-200 g composed each of three study groups. Group A were administered 150 mg/Kg body weight aqueous garlic preparation; Group B 75 mg/Kg body weight aqueous garlic and 75 mg/Kg body weight garlic preparations and Group C served as the control and were administered distilled water. The treatment regimens were orally administered thrice a week, for a period of four weeks by gavages. The *in vitro* erythrocyte osmotic fragility was also evaluated in 12 Wistar rats that were not pre-treated with garlic. The same observation was made in the *in vitro.* It is concluded that garlic increases the osmotic fragility of red blood cells in albino rats (64).
20. *Garlic used as an additive in pesticides:* Many people have expressed concerns about the harmful effects of chemical pesticides and show interest in organic farm products. Thus, to address these concerns there is a need to reduce the use of chemical pesticides and supplement with relatively lesser toxic like natural additives such as garlic (Allium sativum) and hot pepper (Capsicum frutescens) which exhibit synergistic effects on the neem product. Studies also reveal some relatively great effectiveness when these extracts are mixed with or applied alternatively with bio-pesticides such as *Bacillus thuringiensis* (Bt) (65).
21. *Formulation of garlic capsules:* Garlic gelatin capsules were prepared, they are used as a preventative for age-related vascular changes, for the treatment of arteriosclerosis, colds, coughs, fevers, high blood pressure, high cholesterol, infections, intestinal parasites, inflammation of the mouth, inflammation of the pharynx, and for those with a tendency towards infection. They are effective as a supportive to dietary measures for elevated lipid levels in the blood. They are useful as an anti-bacterial and anti-infection agent also helps to reduce cough, flu, and respiratory ailments (66).

CONCLUSION

Garlic and its extracts had a wide range of applications even against resistant organisms to serve as powerful anti-microbial agent. Therefore, research is needed to refine the pathophysiological mechanisms of action of garlic and its utility in treatment of various diseases by developing more stable and suitable formulations. The development of Garlic as a commercial anti-biotic has come to a halt. Although its efficiency is scientifically proven, it has only been used as dietary supplement or traditional medicine. In this report a comprehensive investigation has been conducted on garlic which includes various scientific aspects about it by which researchers from various disciplines could be directed to put efforts toward discovering the benefits of garlic on human health.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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RESTORATION OF MEIBOMIAN GLAND FUNCTIONALITY WITH NOVEL MESENCHYMAL STEM CELL-DERIVED PRODUCT "DERIVED-MULTIPLE ALLOGENEIC PROTEINS PARACRINE SIGNALING (D-MAPPS)": A CASE REPORT

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ABSTRACT

Meibomian gland dysfunction (MGD) results in the increased tear film osmolarity and leads to the development of dry eye disease. Results obtained in several experimental and clinical studies suggested that mesenchymal stem cells (MSCs) could promote repair and regeneration of injured meibomian glands. We recently developed a new biological product "derived-Multiple Allogeneic Proteins Paracrine Signaling (d-MAPPS)" which activity was based on the effects of immunosuppressive and trophic factors secreted by MSCs. Herewith, we report a case of MGD treated by d-MAPPS containing eye drops, demonstrating therapeutic potential of d-MAPPS in regeneration of injured meibomian glands and in the attenuation of MGD. D-MAPPS containing eye drops significantly attenuated MGD-related symptoms (foreign body sensation, burning, pain in the eye and eye fatigue) and remarkably improved quality of life. The analysis of meibomian glands demonstrated restoration of meibomian gland morphology, structure and function, after the 3-weeks of d-MAPPS based therapy. MGD patient did not report any adverse effects related to the d-MAPPS administration, indicating that d-MAPPS containing eye drops were safe for intraocular application.

Keywords: *Meibomian gland dysfunction; therapy; mesenchymal stem cells; d-MAPPS*

INTRODUCTION

Meibum is an oily substance which, due to the high content of phospholipids, cholesterol and wax esters, protects the ocular surface against microbial pathogens and environmental hazards (1). It is produced by meibomian glands, holocrine sebaceous glands located in the upper and lower eyelids (2). Through the production of lipid-rich meibum that reduce aqueous tear evaporation, meibomian glands provides tear film stability and protects the ocular surface against desiccation (3). Accordingly, both congenital and acquired meibomian gland dysfunction (MGD) results in the increased tear film osmolarity and leads to the development of evaporative dry eye disease (DED) (4).

Meibomian gland dropout and altered meibum secretion were usually seen in the patients suffering from MGD (5). Hyperkeratinization of the ductal epithelium and obstruction of the meibomian gland orifice, accompanied by the stasis of the gland, cystic dilatation and atrophy of the excretory acini, have been considered as the most important pathogenic mechanisms responsible for the development of MGD (6). Additionally, as suggested by the several recently published studies, dysfunction of PPARγ gene and the depletion of meibomian gland stem cells might be also involved in the pathogenesis of MGD (5). PPARγ is highly expressed in meibomian gland acinar cells where regulates lipogenesis and meibum composition (7). Therefore, PPARγ dysfunction results in altered lipid synthesis, higher protein/lipid ratio, increase in meibum viscosity and tear film instability (5, 7). Meibomian gland stem cells are small population of self-renewable cells located at the interface between the ductal and acinar basal epithelium (8). Throughout life, ocular surface and meibomian glands undergo many periods of stress and injury that provoke meibomian stem cells to proliferate and differentiate into meibocytes in order to re-establish their number and function (5). Accordingly, aging and stress-driven exhaustion of meibomian stem cells leads to the meibomian gland dropout, resulting in the development of MGD (5).

Dryness, grittiness, scratchiness, soreness, irritation, and burning are the most usually symptoms reported by the patients suffering from MGD (9). Currently, there is no cure for MGD and the treatments are directed towards improving the symptoms rather than towards eliminating the cause of the disease (10). Therefore, new therapeutic approaches should be focused in the modulation of the main pathological mechanisms which are responsible for an altered function of meibomian glands.

Results obtained in several experimental and clinical studies suggested that mesenchymal stem cells (MSCs), self-renewable stem cells that reside in almost all postnatal tissues, including the corneal stroma, trabecular meshwork and periorbital fat of the eye, could be considered as new therapeutic agents in the treatment of MGD (11). MSC-based therapy significantly improved tear volume and tear film stability which resulted in the attenuation of MGD and DED (11). Beneficial effects of MSCs were attributed to the immunomodulatory and regenerative properties of their secretome (12). MSCs produce large amount of immunosuppressive factors that may suppress inflammation-driven injury of meibomian glands and, at the same time, MSCs secrete trophic factors which support proliferation of meibocytes and promote regeneration of injured epithelial cells (11).

In line with these findings, we recently developed a new biological product “derived-Multiple Allogeneic Proteins Paracrine Signaling (d-MAPPS)” which activity was based on the effects of immunosuppressive and trophic factors secreted by MSCs (13). D-MAPPS efficiently suppressed generation of inflammatory phenotype in activated peripheral blood mononuclear cells and promoted regeneration of injured corneal epithelial cells (14). Continuous administration of d-MAPPS containing eye drops significantly alleviated ocular discomfort and pain in 131 DED patients during the 12-month follow-up (15). Herewith, we report a case of MGD treated by d-MAPPS containing eye drops, demonstrating therapeutic potential of d-MAPPS in regeneration of injured meibomian glands and in the attenuation of MGD.

CASE REPORT

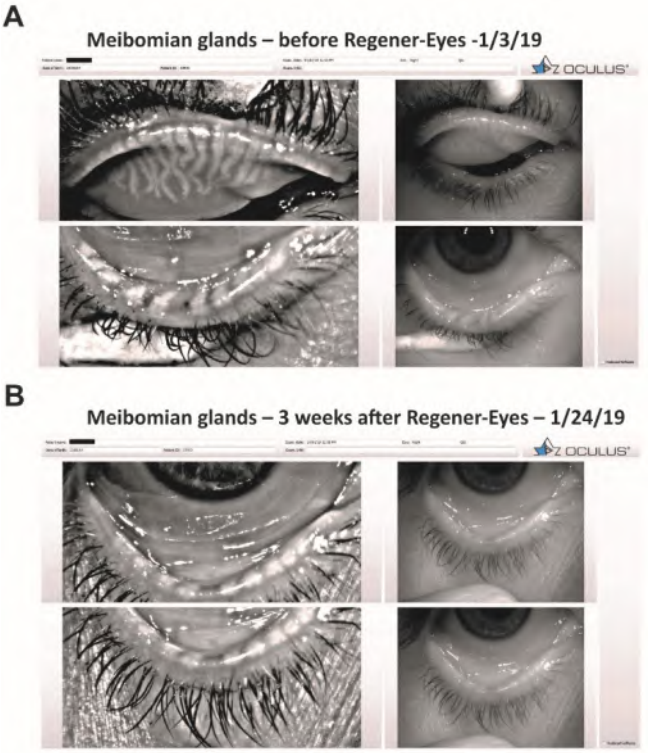
A 55-year-old woman reported symptoms characteristic of MGD: dryness, scratchiness, tearing and burning. She noted that her symptoms first occurred several years prior to her visit and were increasing in severity during the last few months. Although during the past few years she used different types of artificial tears, ointments and intense light pulse therapy, the symptoms become worsen and affected her daily activities. She reported no history of autoimmune and chronic inflammatory diseases and denied use of medications.

At the time of visit, a MGD patient reported foreign body sensation and the pain in the eyes, which were accompanied with grittiness, soreness, irritation, burning and eye fatigue. The slit lamp examination revealed inflamed and rough eyelids with mild telangiectasia. Lid margin debris, which was easily wiped away with a cotton-tipped applicator, was also observed, while pouting material from the meibomian gland orifices were not seen in MGD patient.

D-MAPPS containing eye drops significantly attenuated MGD-related symptoms and remarkably improved quality of life, during the 3-weeks of follow up. The analysis of meibomian glands demonstrated restoration of meibomian gland morphology and structure, after the 3-weeks of d-MAPPS based therapy. As it is shown in Figure 1A, before application of d-MAPPS containing eye drops, meibomian ducts of MGD patient were dilated while meibomian glands were enlarged and tortuous with abnormal structure. Morphology of meibomian glands was significantly improved after 3 weeks of d-MAPPS based therapy (Figure 1B). Meibomian glands were observed as hypoilluminescent grape-like clusters showing normal morphology and structure. Similarly,

hyperilluminescent ducts and underlying tarsus indicated beneficial effects of d-MAPPS containing eye drops (Figure 1B).

Figure 1. Restoration of meibomian gland morphology and structure after the 3-weeks of d-MAPPS-based therapy

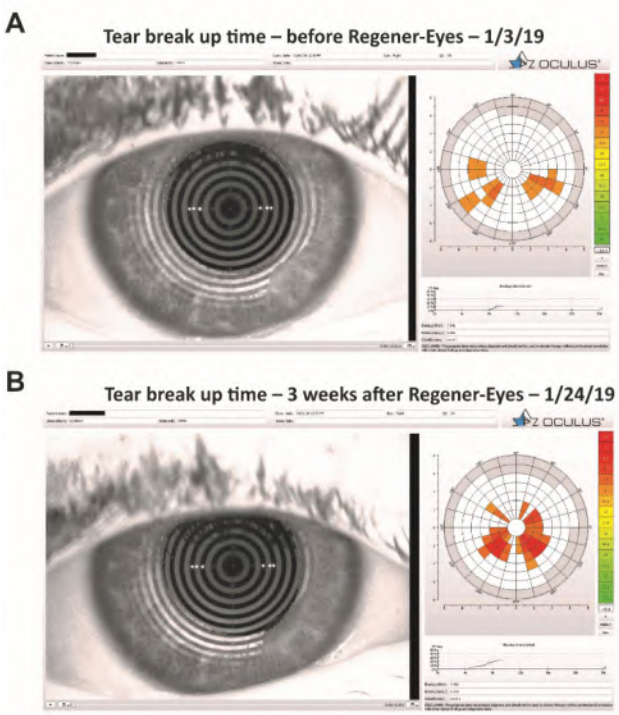


Meibomian ducts of MGD patient were dilated and meibomian glands were enlarged and tortuous with abnormal structure before d-MAPPS-based therapy (A). Meibomian glands had normal morphology with and structure 3-weeks of d-MAPPS-based therapy. (hypoilluminescent grape-like clusters of glands with hyperilluminescent ducts and underlying tarsus) (B).

The tear film breakup time (TBUT) was measured in order to confirm the efficacy of d-MAPPS based therapy. Due to the altered contents of lipids, tear film in patient suffering from MGD was unstable and rapidly evaporated (4). TBUT measured tear film stability and was used to indirectly assess the function of meibomian glands (16). TBUT determines the time elapsed from the last complete eyelid blink until appearance of the first dry spot on the cornea (16). As it is shown in Figure 2B, significantly improved TBUT was noticed 3 weeks after d-MAPPS based therapy, confirming d-MAPPS induced restoration of meibomian gland function.

Complications such as ocular pain, persistent bleeding, and infections were not observed during or after the administration of d-MAPPS containing eye drops. Additionally, MGD patient did not report any adverse effects related to the d-MAPPS administration, indicating that d-MAPPS containing eye drops were safe for intraocular application.

Figure 2. Improved function of meibomian glands after the 3-weeks of d-MAPPS-based therapy



Tear film breakup time (TBUT) measured in a patient suffering from MGD before d-MAPPS-based therapy (A). Significantly improved TBUT noticed 3 weeks after d-MAPPS-based therapy, suggesting d-MAPPS-induced restoration of meibomian gland function (B).

DISCUSSION

MSCs are, due to their potential to provide trophic support to the injured meibomian glands and due to their ability to suppress detrimental T cell-driven immune response in the eye, considered as potentially novel agents in cell-based therapy of MGD (11). As demonstrated by Beyazyıldız and colleagues (17), administration of MSC containing eye drops significantly alleviated DED symptoms in experimental rats. MSCs express various number of chemokine receptors which enable them to migrate to the site of injury and inflammation both after local and systemic application (18). Accordingly, topical administration of MSCs resulted in their higher presence both in injured meibomian glands and in inflamed conjunctival epithelium (17). MSC based therapy significantly increased presence of secretory granules and promoted regeneration and expansion of goblet cells, which resulted in improved tear volume and tear film stability in MSC treated DED rats (17). Additionally, MSCs were capable to increase tear production, resulting in alleviation of eye inflammation and DED-related symptoms in a mouse model of Sjögren's syndrome (19).

However, results obtained in several animal models suggested that transplanted MSCs, in response to the growth factors produced in the local microenvironment, may differentiate into undesired tissues, mainly bone and cartilage (20, 21).

Although MSCs have low expression of human leucocyte antigens (HLA), several studies reported allogeneic immune responses in HLA mismatched recipients of allogeneic MSCs (22-25). Therefore, safeness of MSCs in cell-based therapy of autoimmune and degenerative diseases is still a matter of debate (26).

Majority of MSC-mediated beneficial effects in alleviation of inflammatory eye diseases relied on immunosuppressive and regenerative capacity of MSC-derived factors (15). Accordingly, with aim to avoid safety concerns related to unwanted differentiation of transplanted MSCs (26), we designed d-MAPPS, an ophthalmic MSCs-derived soluble product, which contained MSC-derived immunomodulatory factors that are capable of suppressing detrimental immune response in the eye, and to alleviated MGD (sTNFRI, sTNFRII, IL-1Ra) (13).

TNF-α and IL-1β play important pathogenic role in the development and progression of intraocular inflammation, including DED and MGD (27-29). An elevated concentration of these inflammatory cytokines in serum samples and tears of patients suffering from MGD and DED correlated with the severity of symptoms and clinical parameters (30). Soluble TNF receptors (sTNFRI and sTNFRII) suppress TNF-α-driven inflammation in the eye (31). Accordingly, an up-regulation of sTNFR1 in inflamed ocular surfaces provide protection to the corneal epithelium and ocular surface (31). In similar manner, MSC-derived IL-1Ra, a naturally occurring cytokine, acts as a competitive inhibitor of IL-1β and attenuates IL-1βdriven inflammation in the eye by preventing accumulation of circulating leucocytes in injured corneal epithelium (32). IL-1Ra binds to IL-1 receptor on endothelial cells and prevents pro-inflammatory events initiated by IL-1β:IL-1R interaction, including enhanced influx of neutrophils, macrophages, and lymphocytes in inflamed eyes (33). Furthermore, MSCs, in IL-1Ra dependent manner inhibited activation of inflammasome and reduced consequent production of IL-1β in macrophages, leading to the attenuation of corneal injury, acute and chronic inflammatory diseases in the eye, including DED and MGD (32). In line with these findings, we assume that high concentration of sTNFRI, sTNFRII and IL-1Ra in d-MAPPS containing eye drops and consequent inhibition of TNF-α and IL-1βdriven inflammation may be crucially responsible for enhanced regeneration of meibomian glands and alleviation of MGD in d-MAPPS treated patients.

ACKNOWLEDGMENT

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethicalstandards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary writtenand informed consent was obtained from the patient prior to enrollment in the study.

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SUBGLOTTIC TRACHEAL STENOSIS, RESECTION, AND RECONSTRUCTION: A CASE REPORT

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ABSTRACT

Post-intubation stenosis are the most frequent indications for tracheal resection and reconstructions. They are mostly caused postintubation inflated cuff and after distal tracheostomy. 16-year-old female was admitted to thoracic surgery department, General hospital Vienna with the diagnosis of an impossible weaning with a tracheostomy in place. The pre-operative bronchoscopy and MSCT of the neck evaluation revealed a total occlusion of the trachea below the cricoid arch and reaching distally to the level of the tracheostomy (total length approx. 3cm) by means of an acquired tracheostomy-associated tracheal stenosis (Myer-Cotton IV°). The distal trachea was unaffected. Thus, the indication for a surgical repair was set. Tracheal resection through a cervical incision was performed. The pre-existing tracheostomy as well as the stenotic segment was resected (resection length approx. 3.5cm) and a cricotracheal end-to-end anastomosis was performed. Subglottic resection of the trachea is rare, if conducted a good selection of patients performed precise surgical procedures with the support of anesthesia is considered by some to be the procedure of choice for the treatment severe (>70% luminal obstruction).

Keywords: *Trachea, subglottic stenosis, resection.*

INTRODUCTION

Post-intubation stenosis are the most frequent indications for tracheal resection and reconstructions. They are mostly caused postintubation inflated cuff and after distal tracheostomy. These injuries can be in the upper, middle, and distal trachea.

ANATOMY

Functionally, the trachea serves principally as a conduit for ventilation. Anatomically, these features are unpaired nature, unique structural rigidity, short length, relative lack of longitudinal elasticity, proximity to major cardiovascular structures and segmental blood supply. The adult human trachea averages 11.8cm in length (range, 10to 13cm) from the infracricoid level to the top of the carinal spur. Typically, 18 to 22 cartilaginous rings occur within this length, approximately two rings per centimeter. The blood supply of the human trachea is segmental, largely shared with the esophagus and derived principally from multiple branches of the inferior thyroid artery above and the bronchial arteries below.

CASE PRESENTATION

16-year-old female was admitted to thoracic surgery department, General hospital Vienna with the diagnosis of an impossible weaning with a tracheostomy in place. She was injured in January 2016. after traffic accident with traumatic brain damage. After stabilization, they performed distal tracheostoma and nutritive gastrostomy. The pre-operative bronchoscopy and MSCT of the neck evaluation revealed a total occlusion of the trachea below the cricoid arch and reaching distally to the level of the tracheostomy (total length approx. 3cm) by means of an acquired tracheostomy-associated tracheal stenosis (Myer-Cotton IV°). The distal trachea was unaffected. Thus, the indication for a surgical repair was set (Figure 1,2,3).

Figure 1. Preoperative bronchoscopy

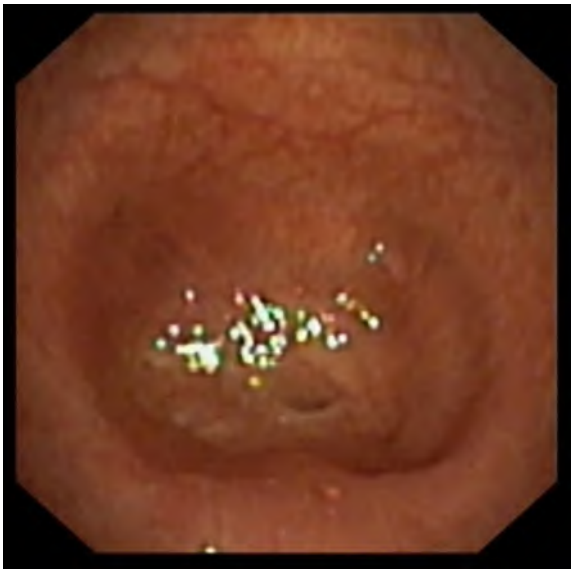


Figure 2. MSCT of the Neck

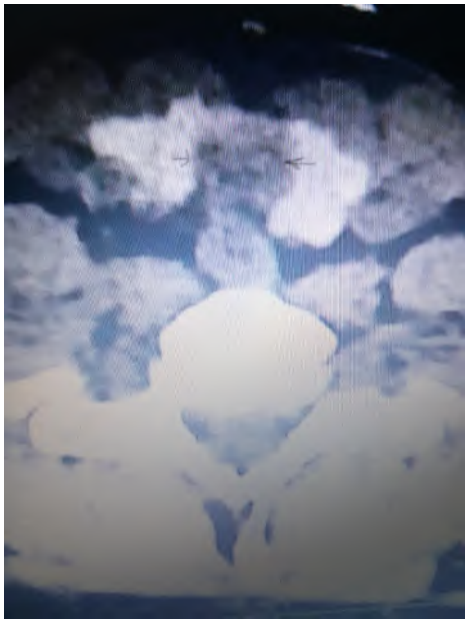
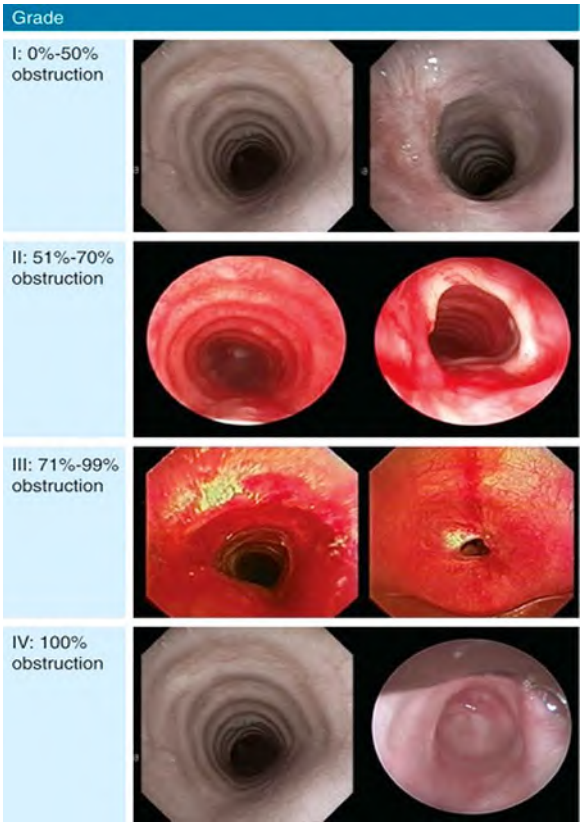


Figure 3. Myer-Cotton subglottic classification. Source: www.google.com.



Myer-Cotton staging system: Grade I - less than 50% obstruction, Grade II - 51% to 70% obstruction, Grade III - 71% to 99% obstruction, Grade IV - no detectable lumen or complete stenosis. Most useful for mature, firm, circumferential stenosis confined to subglottic. Grade IV are incompatible with life if not have tracheostomy tube (2).Tracheal resection

through a cervical incision was performed. The pre-existing tracheostomy as well as the stenotic segment was resected (resection length approx. 3.5cm) and a cricotracheal end-to-end anastomosis was performed (Figure 4,5,6,7,8). The patient was transferred to the intensive care unit for further surveillance during the early postoperative course. The further postoperative course was uneventful. Perioperative antibiotic treatment was initiated with Tazonam and Teicoplanin according to the microbiological examinations. Oral feeding could be started again additional to the parenteral feeding.The postoperative mucous clearance and breathing capacity reached the highest level of satisfaction. A bronchoscopy before discharge showed bilateral normal vocal cord function, a widely open tracheal lumen, and a well-healed anastomosis. The patient was discharged in good general condition after seven days (Figure 10).

Figure 4. Look before incision

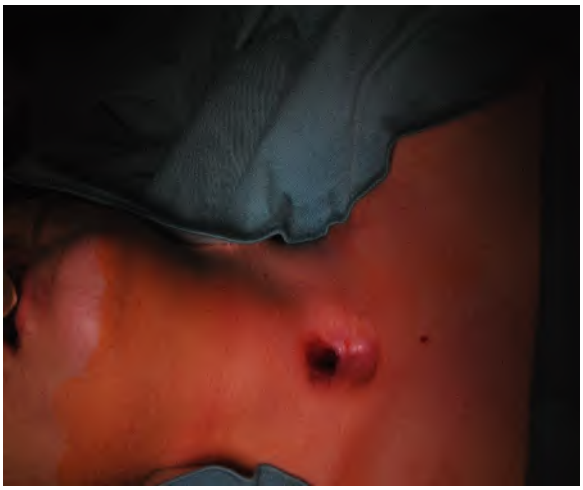


Figure 5. Tracheostomy and stenosis

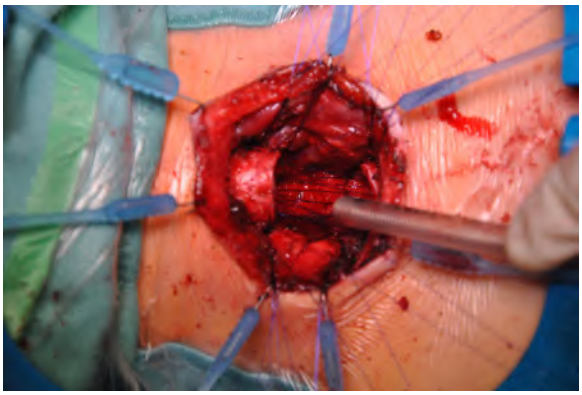


Figure 6. Placement end to end stitches

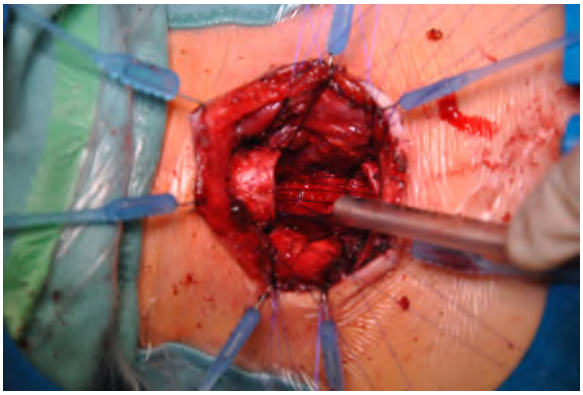


Figure 7. End of the operation

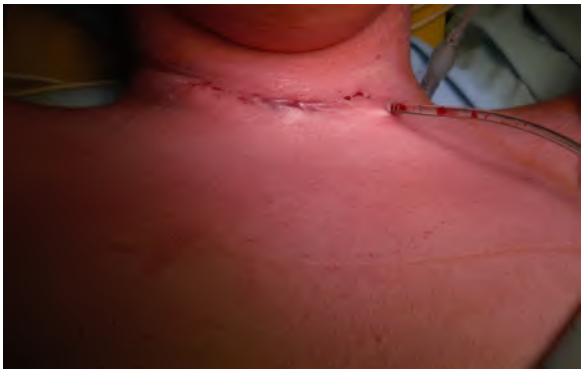


Figure 8. Resection length approx. 3.5cm

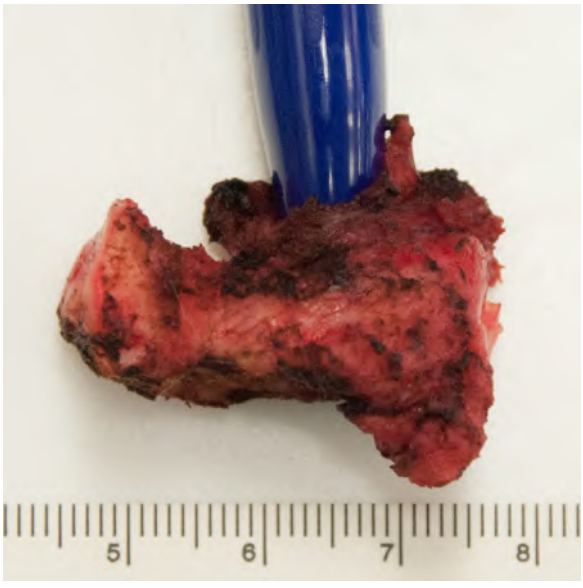
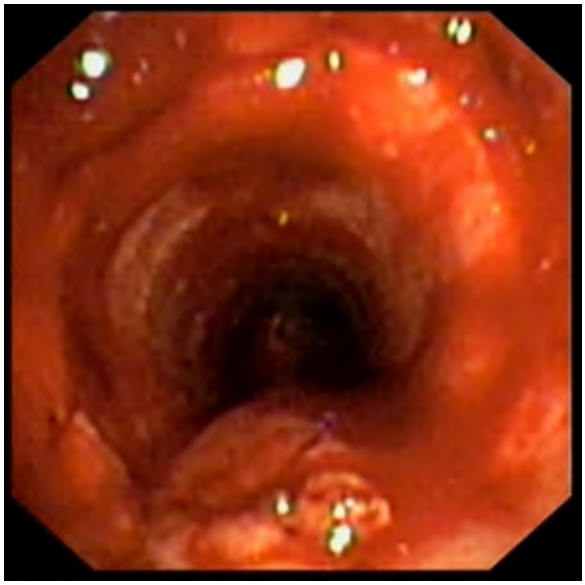


Figure 9. Bronchoscopy view of anastomosis



DISCUSSION

The most desirable treatment of benign tracheal obstruction is resection and reconstruction. More patients can be successfully operated after completing the treatment of primary disease. If patient has a serious psychiatric and neurological deficit due to postoperative cooperation it is best to postpone the surgery.

Non-operational methods for the treatment of benign stenosis are dilatation, stents but often must be repeated, because it does not solve stenosis and tracheal wall turns into scar tissue.

Toty and colleagues pointed out that laser treatment can lead to cure only in granuloma, also easily removed by bronchoscopy and thin web like stenosis. This stenosis is rare. The principal effect of the laser in these lesions has been to delay definitive treatment and sometimes to worsen the lesion (3).

Tracheal resection should not be undertaken if active infection or inflammation at the site of surgery is present. These conditions will most likely lead to restenosis or, worse, wound dehiscence (4).

Tracheal mobilization and release procedures allow to be resected longer segments with primary end to end anastomosis. To facilitate tensionless closure of the tracheal defect, cervical neck flexion can add up to 6 cm. Other methods to add length include extreme transcervical mobilization of the trachea and mainstem bronchi and the suprahyoid laryngeal release. Both provide 1.0–2.0 cm while minimizing the swallowing difficulties of other release procedures. Transthoracic mobilization of the right hilus with division of the pulmonary ligament, intrapericardial dissection of pulmonary vessels, and division of intracartilaginous tracheal ligament allows an additional 3–4 cm length. Combined cervicomediastinal approach may be required for stenosis >5–6 cm, if stenosis is within the thoracic inlet, or if adequate mobilization is not achieved with a cervical approach (5).

Our patient has not morbidity, quality of tracheal wall was good. Adequate mobilization and release procedures are implemented. Careful patient selection is crucial for good outcomes following tracheal resection. Risk factors are age less than 17 years, reoperation, increased length of resection. Diabetes is a surprisingly important risk factor for anastomotic complication, with an odds ratio of 3 (6). Results of inadequate treatment listed in other series included neurologic dysphagia, cardiac decompensation requiring postoperative ventilation, tracheoesophageal fistula, anastomotic separation, restenosis.

Klepetko et al. planned a two-stage tracheal allotransplantation following bilateral lung transplantation for COPD in a patient with long-segment tracheal stenosis. During the lung transplant procedure, they wrapped the donor trachea in the recipient's omentum, sutured it to the abdominal wall and left it there for 6 months to allow neovascularization. The patient then underwent cricotracheal resection and reconstruction; however, primary end-to-end anastomosis was achievable and therefore the tracheal graft was unnecessary. The tracheal graft was harvested, and examination revealed a stable trachea with viable cartilage covered by respiratory epithelium and excellent neovascularization of the tracheal wall (7). Recent work with tissue-engineered prostheses has resulted in optimistic results in animal models. There have also been optimistic results with the use of sheep marrow stromal cells cultured onto a mesh in order to engineer cartilage for a functional tracheal replacement. Jaquet et al. has worked with prefabricated vascularized mucosa-lined composite grafts with cartilaginous support and mucosal lining in rabbits with success. Tissue engineering of cartilage may also be a viable method of tracheal prosthesis. However, a viable tissue-engineered tracheal substitute which can be used dependably in humans will require many more years of refinement prior to mainstream application. This technique, however, shows much promise for the future of tracheal replacement because initial results have been promising, biocompatibility is best, and no immunosuppression is necessary (8). The reconstruction materials can be subdivided into synthetic grafts, autografts, allografts, and bioengineering constructs. Reconstruction of tracheal defects greater than half of the tracheal length was not possible until recently. Numerous publications on animal experimental techniques, and rare human case reports show few successful outcomes. During the last five years, new reconstructive options have emerged: autograft of composite flaps mimicking tracheal architecture and bioengineered tracheal constructs (9). Tracheal reconstruction techniques have recently progressed and replacing a long segment of trachea can be envisaged for the future. Current clinical and translational studies have yet to identify the most effective strategy for tracheal replacement. Further studies to identify the mechanisms of epithelialization and cartilage repopulation are necessary. Trials comparing varying scaffold

and cell seeding techniques with the application of uniform, comprehensive characterization as well as protocolization of interventions will help homogenize data for improved outcome metrics (10).

CONCLUSION

Post-intubation stenosis is the most frequent tracheal obstruction. Non-operational methods for the treatment of benign stenosis did not give good results. Tracheal resection should not be undertaken if active infection or inflammation at the site of surgery is present. Tracheal mobilization and release procedures allow to be resected longer segments with primary end to end anastomosis. Subglottic resection of the trachea is rare, if conducted a good selection of patients performed precise surgical procedures with the support of anesthesia is considered by some to be the procedure of choice for the treatment of severe (>70% luminal obstruction).

ETHICS APPROVAL AND CONSENT TOPARTICIPATE

The study was conducted in accordance with the ethicalstandards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from the patient prior to enrollment in the study.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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