ISSN 2537-6373 (Print)
ISSN 2537-6381 (Online)

The Moldovan Medical Journal

The Publication of the Scientific Medical Association of Moldova

Frequency – 4 per year
Category – B+

Vol. 62, No 1
March 2019

Welcome to the Moldovan Medical Journal!

The Moldovan Medical Journal is an international scientific double-blind peer reviewed periodical edition, 4 per year, of the Scientific Medical Association of the Republic of Moldova designed for specialists in the areas of medicine, dentistry, pharmacy, social medicine and public health. From its debut the journal has striven to support the interests of Moldovan medicine concerning the new concepts of its development.

The Editorial Board warmly welcomes both the readers of and the authors for the journal, all those who are enthusiastic in searching new and more effective ways of solving numerous medicine problems. We hope that those who want to make their contribution to the science of medicine will find our journal helpful and encouraging.

Boris Topor, MD, PhD, Professor
Editor-in-Chief

Editorial Board

Advisory Board

Emeritus Members of the Advisory Board

TABLE OF CONTENTS

ORIGINAL ARTICLES

REVIEW ARTICLES

Editorial Staff

Ludmila Martinenko, English Corrector, Telephone: +37322205209
Irina Litvinenko, Editorial Secretary, Telephone: +37332205877

Address of the Editorial Office
192, Stefan cel Mare Avenue, Chisinau, MD-2004, the Republic of Moldova
Phone: +37322 205209. Mobile: +37379429274
www.moldmedjournal.md
editor@moldmedjournal.md
Index for subscription – 32130

Printing House "Tipografia Sirius"
2, A. Lapusneanu str., Chisinau, MD-2004
the Republic of Moldova
www. sirius.md

ORIGINAL ARTICLE

DOI: 10.5281/zenodo.2589990
UDC: 612.017:616.24-002.5-085.015.8

Level of cytokines in patients with pulmonary drug susceptible and resistant tuberculosis

Evelina Lesnic, MD, PhD, Associated Professor

Department of Pneumophthisiology, Nicolae Testemitsanu State University of Medicine and Pharmacy
Chisinau, the Republic of Moldova

Corresponding author: evelinalesnic@yahoo.com
Manuscript received January 10, 2019; revised manuscript February 18, 2019

Abstract

Background: Cytokines are the regulators of the immune response in tuberculosis: TNF-alpha and CXCL8 (IL-8) are involved in the granuloma formation, IL-10 inhibits the inflammation; some chemokines increase the liver production of the acute phase proteins (APPs). The aim of the research was to assess the serum level of IL-8, TNF-alpha, IL-10, C-reactive protein (CRP), ceruloplasmin and fibrinogen in patients with drug-sensitive and multidrug resistant tuberculosis (MDR-TB).

Material and methods: A prospective case-control study, which included 51 patients, distributed in 2 groups: the 1st study group (N=24 new cases with drug-sensitive TB) and the 2nd study group (N=27 new cases with MDR-TB) according to sex and age were compared with the control group (N=36 healthy individuals).

Results: Serum concentration of IL-8 was elevated up to 13 times, TNF-alpha up to 4 times and IL-10 up to 2 times in study groups, compared with the reference value of the control group. Fibrinogen concentration was elevated up to 2 times in study groups compared with the control group and CRP up to 3 times compared with conventional value. Ceruloplasmin was statistically higher in the drug-sensitive TB and mildly elevated in MDR-TB group.

Conclusions: Proinflammatory biomarkers are more elevated than the anti-inflammatory response, without differences among groups regarding drug sensitiveness.

Key words: tuberculosis, immunity, biomarkers.

Introduction

Evolution of tuberculosis is conditioned by the Mycobacteria tuberculosis (Mtb) virulence, the organism's protective mechanisms and capacity to maintain the infection in latent state [1]. Mtb is an intracellular pathogen with a high capacity to escape from the immune host defenses. About 1,7 billion people, 23% of the world's population are infected with Mtb, but only 10% of infected individuals will develop active tuberculosis, more frequently pulmonary forms [2]. The delay between the infection and the evolution towards an active disease differs due to the complexity of the immune suppressive risk factors [3]. In the first two years after the infection 5% of infected individuals will fall ill and 5% in a later period of life. People co-infected with HIV have a 5-15% per year risk to fall ill with tuberculosis [4].

The most important innate immune cells involved in the Mtb infection are: macrophages, dendritic cells and natural killer cells [5, 6, 7, 8]. These immune cells express a range of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), Nod-like receptors (NLRs) and C-type lectin receptors [9]. The activation of PRRs will stimulate the host defense functions: phagocytosis, autophagy, apoptosis and inflammatory cascade activation [9, 10]. Following the entrance in the pulmonary alveolus the Mtb infects the 2nd type pneumocytes and polymorphonuclear neutrophils. After the death of the infected cells, Mtb is phagocyted by the alveolar macrophages, which are the first immune cells involved in the innate immunity and in the recruitment of the monocyte-derived macrophages during the early stage of the tuberculous infection [11]. Among the innate immune cells, the macrophages play the major role in the host resistance against Mtb through multiple ways: production of the oxygen free radicals, nitric oxides, cytokines, phagosome acidification and autophagy of the infected cells [12, 13, 6, 14].

Before the onset of the proinflammatory cytokine cascade, the innate immune cells recognize Mtb through the pathogen-associated molecular patterns (PAMPs). The PAMPs molecules are glycolipids, lipoproteins and carbohydrates, which are encountered in the Mtb walls [9]. The recognition of the PAMPs by the PRRs of the alveolar macrophages induces the production of the proinflammatory cytokines, more expressed IFN-gamma, IL-12, IL-1β and macrophage inflammatory protein-1α [15, 6, 16]. There are two types of macrophages involved in the innate resistance: classically activated macrophages (CAM) – M1 and alternatively activated macrophages (AAM) – M2. The CAMs are induced by the interleukins secreted by the T lymphocytes

and have a high bactericidal activity. The alternatively activated macrophages (AAMs) have a reduced antigen processing capacity and are induced by the cytokines produced by the T helper 2 lymphocytes: IL-4, IL-13, IL-10 and TGF-β through the ligation with PAMPs and early secretory antigen 6 (ESAT-6) derived from Mtb [15, 17, 6]. The role of AAMs is to maintain the balance between the active mycobacterial growth and disease evolution by suppressing the T helper 1 lymphocytes [18]. Foam cells are the fat-laden alternatively activated macrophages identified in the tuberculous granuloma and their formation is triggered by the uptake of the modified low density lipoproteins into the monocyte-derived cells. Recently another population of macrophages involved in granuloma was described and defined as termed myeloid suppressor cells (MSCs). MSCs suppress T-cells responses through the secretion of the anti-inflammatory cytokine IL-10 and transforming growth factors [17]. Other innate-like cells involved in the immune defense against tuberculosis are: mucosal associated T cells, CD-1 restricted lymphocytes and natural killer T cells. It was established the role of the airway epithelial cells and mast cells in the early immune response against Mtb invasion [19].

The dendritic cells are important innate immune cells responsible for the recruitment of different cells to the site of infection due to their capacity to present the antigens to T lymphocytes from the lymph nodes, where the cell mediated immune response is primarily developed. During the primary infection, the ligation of the dendritic cell receptors by mannose-capped lipoarabinomannan of the Mtb reduces the production of the anti-inflammatory cytokine IL-10, which diminishes their maturation and decreases the production of other stimulator cytokines. The polymorphonuclear neutrophils are also involved in the innate immunity by the production of the free oxygen radicals, initiation of the inflammatory process and constitution of the granuloma, through the secretion of chemokines IL-8 and MCP-1 [20, 16, 19, 21].

The hallmark of the Mtb infection is the granuloma formation. It is a histopathological structure developed by the host to contain the infection in the latent state and to eliminate the mycobacteria. Tuberculous granuloma is composed by a large spectrum of modified macrophages: multinucleated giant cells (Langhans giant cells), epitheliod cells and foam cells, surrounded by a crown of T lymphocytes. In the centre of the granuloma is placed a necrotic region with a characteristic caseation appearance. It was recognized that the cell apoptosis is bactericidal against the Mtb and promotes the antigen presentation, but the caseous necrosis releases the Mtb and contributes to progression of the inflammation and tissue damage [7, 8, 22].

The recognition of the PAMPs by the alveolar macrophages induces the production of the inflammatory cytokines: IFN-gamma, IL-12, IL-1β and macrophage inflammatory protein-1α [6, 16]. The main proinflammatory chemokines involved in the granuloma formation are TNF-alpha and IFN-gamma. The producers of the IFN-gamma are CD4+ lymphocytes (T helper 1), CD8+ lymphocytes (T suppressor) and natural killer cells. The role of IFN gamma consists in the activation of the macrophages and the production of the nitric oxide. The TNF-alpha is produced mainly by the CD4+ lymphocytes (T helper 1) and macrophages [23]. The TNF-alpha role in the granuloma formation consists in the activation of macrophages and chemokines production [15, 14]. The cytokine IL-10 is produced mainly by the B lymphocytes and AAMs [17]. The major role of IL-10 consists in the polarization of macrophages into the alternatively activated form [19]. The AAMs are induced by the cytokines IL-4 and IL-13, which are produced by the T helper 2 lymphocytes. The AAMs produce and secrete the IL-10, TGF-β and arginase [18]. The chemokine CXCL8 (IL-8) is a proinflammatory chemokine produced mainly by the macrophages and infected epithelial cells of the respiratory tract, which have the major role in the recruitment of the T lymphocytes (CD3+, CD4+ and CD8+ cells) and other immune cells into the infection site [18]. The ligation of the IL-8 to Mtb increases the ability of the neutrophils and macrophages to phagocyte and to kill bacilli [24].

Under the influence of the chemotactic agents, such as IL-1, IL-6, IL-8 and TNF-alpha secreted by the innate immune cells, such organs as the liver, especially the parenchimal hepatocytes, increases the production and secretion of the acute phase proteins (APPs) [25, 26, 27]. Biochemically, the APPs are polypeptides. Other systems involved in the production of the APPs are endothelial cells, connective tissue and epithelial cells. The most important APPs are C-reactive protein, haptoglobin and serum amyloid [25, 27]. However, several types of APPs were identified and classified into positive and negative. The positive APPs are considered a part of the innate immune system produced with the aim to destroy or inhibit microbes [28]. The concentration of the positive APPs (C-reactive protein, mannose-binding protein, complement factors, feritin, ceruloplasmine, serum amyloid A, haptoglobin and fibrinogen) increases when the infectious stimulus appears [25, 26, 27, 29]. Other APPs proteins give a negative feedback on the inflammatory response: alpha-2-macroglobulin, serpins, coagulation factors [28, 26]. The coagulation factors are parts of the innate immune system due to the increasing vascular permeability for phagocytes (neutrophil granulocytes and macrophages) and chemotactic agents [26]. The maximum concentration of APPs is revealed within 24 to 48 hours after the inflammatory injury. The decline of the AAPs level is identified due to feedback regulations that will limit the concentration in 4-7 days after the initial stimulus, if no further stimulus occurs. If the hepatic receptors are triggered continuously, the high level of the AAPs can become chronic. Chronic infection, which is revealed in the long lasting tuberculosis process increases the concentration of the AAPs, however the level is lower than in an acute infection perceived in nonspecific infections [25, 26, 27, 29]. Following the evaluation of the scientific review, we could not find data about the serum concentration of the cytokines and acute phase proteins in tuberculosis caused by Mtb with different types of resis-

tance. The aim of the study was to assess the differences in the serum level of the pro-inflammatory chemokines IL-8, cytokine TNF-alpha, anti-inflammatory cytokine IL-10 and acute phase proteins: c-reactive proteins, ceruloplasmine and fibronogen in patients with drug susceptible and drug-resistant tuberculosis.

Material and methods

It was realised a prospective research evaluating the biomarkers of the immune system in 87 cases, of which 24 were new cases with drug susceptible pulmonary tuberculosis included in the 1st study group and 27 were MDR-TB patients which were included in the 2nd study group. The groups were compared between them and were compared with a control group (CG) composed of 36 healthy persons assessed according to the clinical and biochemical criteria. The research reported ethics committee approval (nr. 14 of 21/11/2017) and patients' consent was obtained. Patients were diagnosed in the medical specialized institutions of Chisinau during the period 01.01.2016-31.08.2016. Including criteria in the study group were: age more than 18 years patients diagnosed with pulmonary tuberculosis, "new case" type, the diagnosis was confirmed through the conventional microbiological methods (microbiological examination and molecular genetic test of the sputum). The study investigation schedule included information about sex, age, radiological aspects, microbiological patient's status, results of the drug susceptibility test, treatment regimen and adverse drug reactions. The including criteria in the control group were: age more than 18 years, conventionally healthy persons according to the clinical examination, blood test (complete blood count) and biochemical tests (liver transaminases, bilirubin test, hepatitis virus serological tests, HIV serology). The immunological investigation of patients was performed during the intensive phase of the treatment. The 36 healthy persons from the control group were investigated in ambulatory conditions.

The BosterBio manufecturer was chosen for the acquisition of the ELISA Kits and antibodies. The kits were provided with 96 wells per kit. For the assessment of the serum level of the IL-8 was used the ELISA Kits and antibodies for human IL-8 PicoKine. The assay results ranged from 7,8 pg/ml till 500 pg/ml of serum or plasma. The serum level of the IL-10 was used in the ELISA Kit Human IL-10 PicoKine with the assay range between 3,4 pg/ml till 250 pg/ml. The level of TNF-alpha was assessed using the kit Human TNF-Alpha PicoKine with the assay range between 7,8 pg/ml till 500 pg/ml. Statistical analysis was carried out by the comparative assessment of the quantitative and qualitative peculiarities of the selected patients using the Microsoft Excel XP programme. Accumulated material was systematized in simple and complex groups. For the testing of significant differences between the studied indices of the compared samples it was performed the statistic non-parametric T test at the significant threshold $p<0,05$.

Results

While distributing patients, according to the biological characteristics was established a similar rate of men and women in all three groups, with the predomination of men in the same proportion, which was accepted as a condition that permitted the comparability of the results between the selected samples. The same proportion of young persons aged less than 44 years was established in all groups. All enumerated conditions permitted the comparability of the laboratory data (tab.1).

Table 1

Segregation of patients in sex and age groups

Biological segregation	Parameters	1st SG (N=24)	2nd SG(N=27)	CG (N=36)
		N (%)	N (%)	N (%)
Sex stratification	Men	14 (58%)	18 (67%)	24 (67%)
	Women	10 (42%)	9 (33%)	12 (33%)
Stratification in age groups	18-44 years	18 (75%)	21 (77%)	29 (81%)
	≥45 years	6 (25%)	5 (23%)	7 (19%)

Detected by passive way, using standard tools (microbiological examination and chest X-ray) for the investigation of the symptomatic patients, were 15 (62%) cases from the 1st SG and 17 (63%) cases of the 2nd SG. The main proportion of both study groups was constituted from the patients with pulmonary infiltrative TB: 22 (91%) in the 1st SG and 24 (89%) cases in the 2nd SG. Radiological investigations identified the lung destruction in all selected TB patients. Microbiological status was positive in all patients and drug susceptibility testing permitted their distribution according to the obtained drug resistance results. Standard treatment for drug-sensitive TB was administrated in patients from the 1st SG and standard treatment for MDR-TB in patients from the 2nd SG. There were no major adverse drug reactions identified in the selected patients.

The assessed proinflammatory biomarkers constituted the serum concentration of the chemokine IL-8 and the cytokine TNF-alpha. The ELISA assay established that the concentration of IL-8 was up to 13 times more elevated in the groups diagnosed with tuberculosis. The concentration was higher even in the MDR-TB patients where the statistical threshold was not achieved. The concentrartion of the cytokine TNF-alpha was up to 4 times more elevated in the MDR-TB group and two times more elevated in the drug-sensitive TB group compared with the healthy individuals included in the control group and achieved a high statistical threshold in both study groups compared with the control group. Additionally, it was established a more elevated concentration in the MDR-TB group compared with the drug-sensitive TB group, achieving a high statistical threshold (tab. 2).

The assessed anti-inflammatory biomarker constituted the serum concentration of the cytokine IL-10. The concentration was statistically more elevated in both study groups of patients with tuberculosis compared with the control group (tab. 3).

Table 2

The serum level of the proinflammatory cytokines in the patients with drug-sensitive TB and MDR-TB

Cytokines	Parameters	1st SG (N=24)	2nd SG(N=27)	CG (N=36)
		M±SD	M±SD	M±SD
IL-8	Assay range pg/ml serum	15.54±9.37 ○	16.05±7,68 ◊	1.68±1.16
	Compared to the control group	1339	1383	100
TNF-alpha	Assay range pg/ml serum	141.03±66.15 ●○	278.93±247.91 ◊	65.77±12.09
	Compared to the control group	216	427	100

Note: Values are mean ± SD. The percentage was assessed comparing the study groups with the reference value of the control group (100%). Comparison between study groups – ● p<0.001, comparison between the 1st SG and CG – ○ p<0.001, comparison between the 2nd SG and CG – ◊ <0.001.

Table 3

The serum level of the anti-inflammatory cytokine IL-10 in the patients with drug-sensitive TB and MDR-TB

Cytokines	Parameters	1st SG (N=24)	2nd SG(N=27)	CG (N=36)
		M±SD	M±SD	M±SD
IL-10	Assay range pg/ml serum	0.08±0.04 □	0.08±0.02 ▪	0.06±0.011
	Compared to the control group	133	133	100

Note: Values are mean ± SD. The percentage was assessed comparing the study groups with the reference value of the control group (100%). Comparison between the 1st SG and CG – □ p<0.05, comparision between the 2nd SG and CG – ▪< 0.05.

The serum concentration of some acute phase proteins in tuberculosis patients established a statistically higher concentration of the ceruloplasmin in the drug-sensitive TB group and mildly elevated concentration in the MDR-TB group. It can be explained by a longer evolution of the MDR-TB which contributed to an intensive negative feedback, which diminished the hepatic synthesis of the ceruloplasmin. The serum level of fibrinogen was statistically more elevated in both groups with tuberculosis compared with the reference value of the control group, without difference among groups. The concentration of the C-reactive protein was detectable in a three times more elevated concentration than the normal conventional threshold (less than 6 mg/dL). The investigation was not performed in the control group, due to the conventional negative results in the healthy individuals (tab. 4).

Discussion

While distributing the patients, according to the sex and age, it was determined the predomination of men at economic, reproductive age (18-44 years) in both study groups, as well as in the control group, which was accepted as a condition for the comparability of the results. Detected, using standard microbiological examination and chest X-ray investigation, were two thirds of both study groups. Similar data were obtained in the national studies [30, 31]. The majority of both study groups was diagnosed with pulmonary infiltrative TB with lung destruction. Microbiological status was positive in all patients and drug susceptibility testing permitted their distribution according to the obtained drug resistance results. Standard treatment for the drug-sensitive TB was administrated in patients from the drug-sensitive TB group and standard treatment for MDR-TB in patients from the MDR-TB group. The regimens were used according to the WHO recommendations [4, 32].

Our immune biochemical research established a similar high level of the chemokine IL-8 in patients with drug-sensitive TB and MDR-TB. Other studies established high levels of IL-8 in tuberculosis and infections with mycobacteria other than tuberculosis [21]. The research of Ameixa C. proved the down-regulation of IL-8 secretion from Mtb infected monocytes by IL-10 [33]. Our clinical study estab-

Table 4

The serum level of the acute phase proteins ceruloplasmine and fibrinogen in the patients with drug-sensitive TB and MDR-TB

Cytokines	Parameters	1st SG (N=24)	2nd SG(N=27)	CG (N=36)
		M±SD	M±SD	M±SD
Ceruloplasmine	Assay range mg/ml serum	911.31±210.71 □	852.11±256.1	724.3±27.8
	Compared to the control group	125	117	100
Fibrinogen	Assay range ng/ml serum	4.00±1.11 ●	4.12±0.87 ●	2.24±0.48
	Compared to the control group	178	183	100
C-reactive protein mg/dL		21.87±19.57	18.57±16.99	NA

Note: Values are mean ± SD. The percentage was assessed comparing the study groups with the reference value of the control group (100%). Comparison between study groups and the control group – □ p<0.001, comparison between the 1st SG and CG – ● p<0.05. NA – non available.

lished thirteen times higher concentration of the IL-8 compared with IL-10, without differences between the study groups and it was not proved the regulation of Il-8 on the IL-10 concentration.

The concentration of the cytokine TNF-alpha was 4 times higher in the MDR-TB group and two times higher in the drug-sensitive TB group compared with the healthy group. Elevated concentration of the cytokine TNF-alpha was identified in multiple studies, which proved the boosted capacity of the macrophages to phagocytose and kill Mtb, when level of TNF-alpha is increased [15, 14]. However, similar researches identifying the differences between the concentration of the TNF-alpha in the drug-sensitive TB and MDR-TB were not found. The concentration of IL-10 was two times higher in both groups of patients at an analogical threshold. However, the concentration of IL-10 was not elevated at a similar height as IL-8 and TNF-alpha. Some studies established that an increased IL-10 level contributes to the survival of the Mtb in the infected host [33, 15, 17, 34].

The serum concentration of the positive acute phase proteins in selected patients established a statistically higher concentration of the ceruloplasmine in the drug-sensitive TB and a non-statistically higher concentration in the MDR-TB group. While identifying high concetration of active phase reactants, fibrinogen and ceruloplasmine Cernat R.I. established a straight correlation between those two active phase reactants. His research established high concentration of the Cu, Fe and Zn and the Cu-binding protein – ceruloplasmin before the initiation of the treatment [35]. However, similar studies to show the differences among drug susceptible and MDR-TB patients were not performed. Because an important protein is involved in the protection against oxidative stress, ceruloplasmin could be appreciated more as antioxidant than as proinflammatory biomarker [36, 37, 29]. Fibrinogen was found in an increased concentration in both groups and was established as a biomarker of tuberculous process by multiple researchers [38]. C-reactive protein was elevated in both groups of patients without differences among them and was interconnected with the increased concentration of fibrinogen and ceruloplasmin [27].

Conclusions

1. The proinflammatory chemokine IL-8 assessed in the serum through the ELISA assay established that the concentration of the IL-8 was 13 times higher in patients with both types of tuberculosis, without differences among drug-sensitive TB or MDR-TB.

2. The proinflammatory cytokine TNF-alpha was two times higher in the drug-sensitive TB and four times higher in the MDR-TB, being obtained at a high statistical threshold between groups. It can be connected with the extensiveness of the destructive process.

3. The anti-inflammatory cytokine IL-10 was two times higher in both groups of patients compared with the healthy individuals without any differences among groups.

4. The serum concentration of acute phase proteins: fibrinogen and C-reactive protein was revealed in a statistically higher concentration in both groups of patients, compared with the healthy individuals, without any differences among groups.

5. Ceruloplasmine serum level was higher in the drug-sensitive TB and non-statistically higher concentration in the MDR-TB group.

6. The proinflammatory cascade is more activated, than the anti-inflammatory response, without differences between drug susceptible and MDR-TB. CXCL8 (IL-8) and TNF-alpha can be assessed as proinflammatory biomarkers of tuberculosis, with no regard to the drug susceptibility. The serum levels of the anti-inflammatory cytokine IL-10 and acute phase proteins are mildly increased, without differences between the drug-sensitive and MDR-TB.

References

1. Delogu G, Sali M, Fadda G. The biology of mycobacterium tuberculosis infection. Mediterr J Hematol Infect Dis. 2013;5(1):e2013070. doi: 10.4084/MJHID.2013.070.
2. World Health Organization. Systematic screening for active tuberculosis: Principles and recommendations. Geneva: WHO; 2011. 133 p.
3. Goldberg MF, Saini NK, Porcelli SA. Evasion of innate and adaptive immunity by Mycobacterium tuberculosis. Microbiol Spectr. 2014;2(5). doi: 10.1128/microbiolspec.MGM2-0005-2013.
4. World Health Organization. Companion handbook to the WHO guidelines for the programmatic management of drug-resistant tuberculosis. Geneva: WHO; 2014. 447 p.
5. Guerra C, Johal K, Morris D, Moreno S, Alvarado O, Gray D, et al. Control of Mycobacterium tuberculosis growth by activated natural killer cells. Clin Exp Immunol. 2012;168(1):142-52.
6. Kleinnijenhuis J, Oosting M, Joosten LA, Netea MG, Van Crevel R. Innate immune recognition of Mycobacterium tuberculosis. Clin Dev Immunol. 2011:405310. doi: 10.1155/2011/405310.
7. Korb VC, Chuturgoon AA, Moodley D. Mycobacterium tuberculosis: manipulator of protective immunity. Int J Mol Sci. 2016;17(3):131. doi: 10.3390/ijms17030131.
8. Kuroda M, Sugimoto C, Cai Y, et al. High turnover of tissue macrophages contributes to tuberculosis reactivation in simian immunodeficiency virus-infected Rhesus macaques. J Infect Dis. 2018;217(12):1865-74.
9. Killick KE, Ni Cheallaigh C, et al. Receptor-mediated recognition of mycobacterial pathogens. Cell Microbiol. 2013;15(9):1484-95.
10. Mortaz E, Adcock IM, Tabarsi P, et al. Interaction of pattern recognition receptors with mycobacterium tuberculosis. J Clin Immunol. 2015;35(1):1-10.
11. Saiga H, Shimada Y, Takeda K. Innate immune effectors in mycobacterial infection. Clin Dev Immunol. 2011;2011:347594.
12. Blomgran R, Ernst JD. Lung neutrophils facilitate activation of naïve antigen-specific CD4+ T cells during Mycobacterium tuberculosis infection. J Immunol. 2011;186(12):7110-9.
13. Gagneux S. Host-pathogen coevolution in human tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2012 Mar 19;367(1590):850-9. doi: 10.1098/rstb.2011.0316.
14. Shi J, Sun BH, Zhou L, Wang X. Role of IL-10 and TNF-α during mycobacterium tuberculosis infection in mureine alveolar macrophages. Genet Mol Res. 2016;15(3). doi: 10.4238/gmr.15037819.
15. Cavalcanti VN, Brelaz MC, Neves JK, et al. Role of TNF-alfa, IFN Gamma and IL-10 in the development of pulmonary tuberculosis. Pulm Med. 2012; Article ID:745483. doi: 10.1155/2012/745483.
16. Lerner TR, Borel S, Gutierrez MG. The innate immune response in human tuberculosis. Cell Microbiol. 2015;17(9):1277-85.
17. Jang S, Uematsu S, Akira S, Salgame P. IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is pre-

dominantly dependent on TLR2-mediated recognition. J Immunol. 2004;173(5):3392-7.
18. Sica A, Erreni M, Allavena P, Porta C. Macrophage polarization in pathology. Cell Mol Life Sci. 2015;72:4111-26.
19. Liu CH, Liu H, Ge B. Innate immunity in tuberculosis: host defense vs pathogen evasion. Cell Mol Immunol. 2017;14(12):963-75.
20. Krupa A, Fol M, Dziadek B, et al. Binding of CXCL8/IL-8 to Mycobacterium tuberculosis modulates the innate immune response. Mediators Inflamm. 2015;2015:124762. doi:10.1155/2015/124762.
21. Zhang Y, Broser M, Cohen H, et al. Enhanced interleukin-8 release and gene expression in macrophages after exposure to Mycobacterium tuberculosis and its components. J Clin Invest. 1995;95(2):586-92.
22. Sia JK, Georgieva M, Rengarajan J. Innate immune defenses in human tuberculosis: an overview of the interactions between mycobacterium tuberculosis and innate immune cells. J Immunol Res. 2015;2015:747543.
23. Winslow GM, Cooper A, Reiley W, Chatterjee M, Woodland DL. Early T-cell responses in tuberculosis immunity. Immunol Rev. 2008;225:284-99.
24. Dallenga T, Schaible UE. Neutrophils in tuberculosis–first line of defence or booster of disease and targets for host-directed therapy? Pathog Dis. 2016;74(3). pii: ftw012. doi: 10.1093/femspd/ftw012.
25. Pepys MB, Baltz ML. Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A proteins. Adv Immunol. 1983;34:141-212.
26. Robson SC, White NW, Aronson I, Woolgar R, Goodman H, Jacobs P. Acute-phase response and the hypercoagulable state in pulmonary tuberculosis. Br J Haematol. 1996;93(4):943-9.
27. Salazar J, Martinez MS, Chavez-Castillo M, et al. C-reactive protein: an in-depth look into structure, function and regulation. Int Sch Res Notices. 2014;2014:653045. doi: 10.1155/214/653045.
28. Abbas A, Lichtman A, Pillai S. Basic immunology: functions and disorders of the immune system. 5th ed. Philadelphia: Elsevier; 2015. 352 p.
29. Uriu-Adams JY, Keen CL. Copper, oxidative stress, and human health. Mol Aspects Med. 2005;26(4-5):268-98.
30. Lesnic E, Paladi C, Niguleanu A, Ciubotaru V, Sirbu P, Curocichin G. Segregation of tuberculosis patients by social, demographic and economic features on the model of Chisinau city and the role of the community support. Curierul Medical (Chişinău). 2016;59(4):11-17.
31. Lesnic E, Ustian A, Niguleanu A, Malic A, Paladi C. Social features of patients with pulmonary tuberculosis. [Tuberculosis, lung diseases, HIV-infection] (Kiev). 2016;25(2):36-40.
32. World Health Organization. Guidelines for treatment of drug-susceptible tuberculosis and patient care. Geneva: WHO; 2017. 56 p.
33. Ameixa C, Friedland JS. Down-regulation of interleukin-8 secretion from Mycobacterium tuberculosis-infected monocytes by interleukin-4 and -10 but not by interleukin-13. Infect Immun. 2001;69(4):2470-6.
34. Lopes RL, Borges TJ, Zanin RF, Bonorino C. IL-10 is required for polarization of macrophages to M2-like phenotype by mycobacterial DnaK (heat shock protein 70). Cytokine. 2016;85:123-9.
35. Cernat RI, Mihaescu T., et al. Serum trace metal and ceruloplasmin variability in individuals treated for pulmonary tuberculosis. Int J Tuberc Lung Dis. 2011;15(9):1239-45. doi: 10.5588/ijtld.10.0445.
36. Arshya B, Nirmaladevi K, Deepalarhmi P, et al. Serum ceruloplasmin albumin ratio as a biochemical marker to assist the diagnosis, treatment and prognosis of pulmonary tuberculosis patients. Natl J Basic Med Sci (India). 2014;6(1):2-5.
37. Hellman NE, Gitlin JD. Ceruloplasmin metabolism and function. Annu Rev Nutr. 2002;22:439-58.
38. Famodu AA, Ajaya OI, Awodu OA, Nguepi P. Effect of pulmonary tuberculosis on plasma fibrinogen and fibrinolytic activity. Haema. 2005;8(3):323-6.

Declaration of conflict of interests
Nothing to declare

DOI: 10.5281/zenodo.2589994
UDC: 616.717.2-001.5-089.22:615.465

The stable-functional osteosynthesis of clavicular fractures with external fixator

Gheorghe Rosu, MD, PhD

Department of Trauma, Emergency Medicine Institute, Chisinau
District Hospital Rascani, the Republic of Moldova

Corresponding author: tns_gl@yahoo.com
Manuscript received November 06, 2018; revised manuscript February 01, 2019

Abstract

Background: Over the past two decades has been paid insufficient attention to the treatment of patients with clavicle fractures. Analysis is of the recent available literature and the personal practice allow us to have a special position to this issue, solving the problem of the clavicle fractures treatment with a new and more efficient method.
Material and methods: For a better deduction and appreciation of the fracture fixation methods results we established a task based on the effectiveness evaluation of the external fixator utilization as a way for a stable osteosynthesis of the fragments performance. It was tested experimentally the efficacy of clavicle fragments fixation with different fixators. The experiment was performed at 30 anatomical specimens of clavicle aged from 38 to 70 years. The mechanical force of the osteosynthesised fragments fixation at flexion and stretching was appreciated in the Alfred G. Amsler German machine with a deviation from 0 to 50 Kn/cm and the capacity of a unit equal to 0.5 Kn/cm.
Results: After the application of the stable-functional osteosynthesis method in clavicle fractures treatment with the external fixator's help, the experimental samples prove objectively the qualities of the proposed fixator, which has an essential priority in the mechanical force of fixing the fragments compared to the traditional fixators used in most cases at the patients with clavicle fractures.
Conclusions: The new method of stable-functional osteosynthesis of the clavicle fractures by using the external fixator, which is simple in assembly and manufacture, excludes the shortcomings of the traditional methods of surgical treatment and allows its application at any level of specialized surgical aid.

Key words: osteosynthesis, external fixation, clavicular fractures.

Introduction

During the past two decades, according to the accessible literature, have been published few scientific papers meant to reflect all aspects of the clavicle fractures treatment problems. Being necessary to substantiate the above mentioned, it is relevant to bring into discussion the fact that in the treatment of patients with clavicle fractures, are used about 300 different constructions, devices, methods which may justify the difficulties faced by doctors when it comes to choose the right method of treatment [1,2,3,4,7,8,9,16,17,18,].

The traumatological practice has a lot of methods for the patient's treatment with both orthopedic and surgical clavicle fractures. According to some authors, the orthopedic method is often used in the clavicle fractures treatment and allows in most cases to achieve good results. Other authors consider that the use of various methods of immobilization is incommode, complicated and difficult to support by the patients, while not ensuring a stable fixation of the fragments [5,6,10,11,19,20].

When analyzing the orthopedic clavicle fractures treatment it can be said that the problem is not so easy to solve. None of the orthopedic treatment methods may exclude the secondary displacements of fragments. As a result, there is an increased number of complications and consequently, unsatisfactory results. At the present stage, it remains indisputable that in cases of ineffective orthopedic treatment or other indications, both absolute and relative, it is possible to go back to the surgical treatment. As a basis for surgical intervention serve the: complicated fractures with neurovascular trunk injury or the danger of their trauma; the ineffectiveness of fragments reduction or retrieval by orthopedic methods; the danger of skin perforation by the fragment and open fractures[12,13,14,15].

The diversity of fixers and constructions highlights the technical difficulties faced by practitioners during surgery. Some of the fixators (metal rod, brooches, screws) are providing a fragments stable fixation and may lead to complications (the migration of the rod or wires, secondary movements, vicious reinforcements, slowing down consolidations, pseudoarthroses, osteomyelitis) and can require additional application of external immobilization. Other machines, fixators mostly increase the stability of osteosynthesis and avoid further immobilisation. However, surgery itself is much more traumatic and all methods require a surgical re-insertion to remove the fixator [25,26,27,28,32,33].

According to the literature, unsatisfactory results from surgical treatment are found in 1.8-30.6% of the patients, mostly returns to slowing down consolidations and pseudoarthroses. The application of compressive devices resulted in some qualitative changes in the clavicular fractures treatment. They lack a number of shortcomings of other treatment methods, offering the possibility of reducing the number of unsatisfactory results to 2.7-3.0% [29,30,31,33,34,35].

However, the clavicular fractures osteosynthesis with existing external fixation devices is only possible in specialized traumatology clinics, appropriately equipped and with high-qualified doctors. Due to these causes, the frequency of its performance in the arsenal of clavicular fracture treatment is insignificant. Therefore, the idea of developing a

safer, less traumatic method in the surgical treatment of clavicular fractures, which would exclude traditional method shortcomings and could be applied at any level of specialized surgical help, is timely and actual.

These proposals are the subject of investigations of the developments contained in this scientific study which has as a purpose the solving of the of clavicle fractures treatment problem.

Biomechanical study

For the purpose of the effectiveness evaluation of the external fixator utilisation as a way for a stable osteosynthesis realization of fragments, has been tested experimentally the effectiveness of the clavicular fragments fixation with different fixators. It was estimated the mechanical force of the the osteosyntesised fragments fixation with the following fixators:

1) The external fixator proposed by us;
2) The rod with 125 mm in lentgh, 2.5 mm in thickness, made of stainless steel of IXI8H9T mark, realized by Bogdanov's example;
3) Two brooches made from stainless steel of IXI8H9T mark, diameter 1.8 and 2.2 mm, based on Kirschner and Elizarov' s example.

The experiment was performed on 30 anatomical specimens of clavicle aged from 38 to 70 years. The location and character of the trajectory in the experimental samples fractures have been identified for each studied fixator.

The location and character of the trajectory of the clavicular fractures in the experimental samples are represented in table 1.

Table 1

The location and character of the trajectory of the clavicular fractures

Location of the fracture	The trajectory character	The number of cases
Third medium	Oblique	12
Third medium	Transversal	6
Third medium	Transversal with oblique trajectory	3
The acromial	Oblique	3
The third-medium region – acromial portion	Oblique	3
The third-medium region – acromial portion	Transversal	3
Total		30

During the experiment the osteosynthesis was performed strictly according to the surgical methods with the same bone material (10 clavicles) and different fixators, thus confirming a comparatively higher degree of assessment and objectivity.

The mechanical fixative force of the osteosynthesised fragments, at the flexure and stretching, was appreciated at the German machine "Alfred G. Robert" brand with deviation from 0 to 50 kN/cm and a capacity of a unit equal to 0.5 kN/cm.

The osteosynthesised clavicula with different fixators has been submitted to the flexure and the stretching on the particular specialized device holder, the pressure gauge which indicates the flexure, continually, being performed a stretch until a fragments diastase equal to 5 mm, concomitant tending to fixate the indicated force on the general pressure gauge of the machine. The mechanical force of the osteosynthesis fragments fixation at the torsion was assessed at the second company machine "Alfred G. Amsler" with deviations from 0 up to 25 Kn/cm and a capacity equal to 0.5 Kn/cm.

The osteosynthesised clavicula was subjected to the torsion being fastened into special brackets of the articular ends of them, therefore, attached fragments were twisted up to 45 degrees, dropping the indicated force on the pressure gauge of the device.

The osteosynthesised clavicula using the proposed external fixator was submitted to the experimental evaluation (30 experimental samples) of mechanical fixative force of the osteosyntesised fragments:

At flexure – 10 experimental samples;
At distraction – 10 experimental samples;
At torsion – 10 experimental samples.

The osteosynthesised clavicula with the rod (mark IXI-8H9T) was subjected to experimental evaluation (30 experimental samples) of mechanical fixative force of the osteosynthesised fragments:

At torsion – 10 experimental samples;
At stretching – 10 experimental samples;
At flexure – 10 experimental samples.

The osteosynthesised clavicula with two brooches (mark IXI8H9T) of the proposed external fixator has been submitted to the experimental appreciation (30 experimental samples) of mechanical fixative force of the osteosynthesised fragments:

At torsion – 10 experimental samples;
At stretching – 10 experimental samples;
At flexure – 10 experimental samples.

Estimation of mechanical force of osteosynthesised fixated fragments at the "Alfred G. Amsler" German device.

The result of the biomechanical study

In total, 90 experimental samples were carried out and their results are presented in the tables 2, 3, 4.

Table 2

The estimation results of experimental samples of the mechanical fixative force of osteosynthesised fragments with an external fixator

Number of the clavicula	The flexure up to 5 mm	The stretching up to 5 mm	The twisting at 45 degrees
1	8.0	7.0	0.3
2	12.0	7.0	0.3
3	15.5	7.0	0.4
4	24.5	5.0	0.22
5	20.5	3.9	0.4
6	22.0	3.0	0.25

Table 3

The estimation results of experimental samples of mechanical fixative force of the osteosynthesised fragments with the rod with 125 mm in lentgh, 2.5 mm in thickness, made of stainless steel of IXI8H9T mark, realized by Bogdanov's example

Number of clavicula	The flexure up to 5 mm	The stretching up to 5 mm	The twisting at 45 degrees
1	8.9	1.2	0.13
2	6.5	2.0	0.11
3	5.5	1.8	0.2
4	9.4	3.3	0.2
5	7.5	4.1	0.18
6	11.5	3.0	0.12
7	13.0	6.7	0.16
8	9.0	5.2	0.18
9	5.0	6.5	0.14
10	4.5	1.0	0.11
M-m	8.29-2.0	4.81-2.89	0.178-0.05

Table 4

The results of experimental samples estimation of mechanical fixative force of the osteosyntesised fragments with two brooches made from stainless steel of IXI8H9T mark, diameter 1.8 and 2.2 mm, based on Kirschner and Elizarov' s example

Number of clavicula	The flexure up to 5 mm	The stretching up to 5 mm	The twisting at 45 degrees
1	2.7	3.5	0.1
2	1.2	2.7	0.08
3	1.4	2.8	0.12
4	3.0	2.2	0.07
5	1.4	2.0	0.2
6	2.0	1.2	0.2
7	1.5	2.5	0.13
8	1.3	1.5	0.1
9	1.8	5.0	0.16
10	2.0	1.5	0.6
M-m	1.83-0 609	2.21-1.5	0.13-0.05

The results of the experimental samples were subjected to statistical synthesis after the normative methodology (tab. 5). In the result of the synthesis we obtained arithmetic environmental parameter (M) and probable error parameter (m).

Table 5

Comparative results of mechanical fixative force of osteosynthesised fragments with different fixators

The fixator type	Flexure in kN/cm	stretching in kN/cm	Torsion in kN
External Fixator	M = 16.47 m = 5.31	M = 5.49 m = 1.74	M = 0.382 m = 0.091
Rod length 125 mm, thickness 3.5 mm	q	M = 4.81 m = 2.8876	M = 0.178 m = 0.054
Two brooches	M=1.83 m=0.609	M=2.71 m=1.518	M=0.126 m = 0.054

It was analysed the mechanical fixative force of the fragments at the flexure, stretching and torsion with different fixators (fig. 1, 2 , 3).

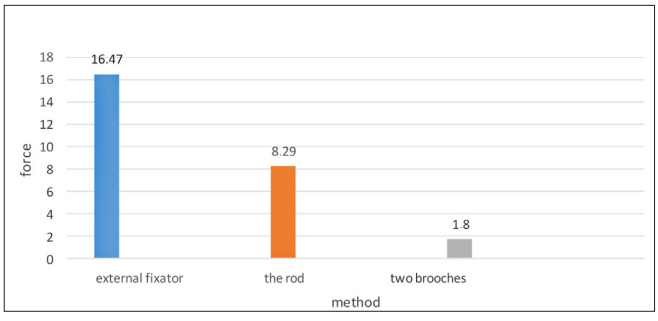


Fig. 1. The comparability of the mechanical fixative force of the fragments with different fixators at the flexure.

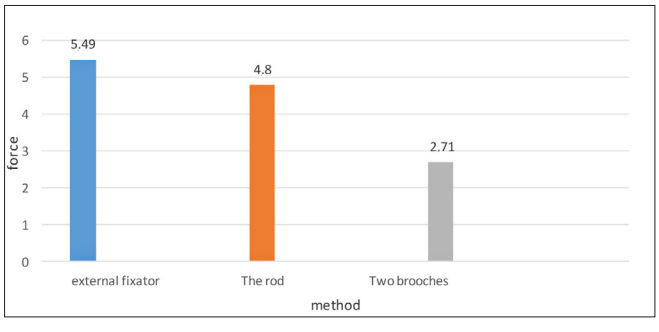


Fig. 2. The comparability of the mechanical fixative force of the fragments with different fixators at the stretch.

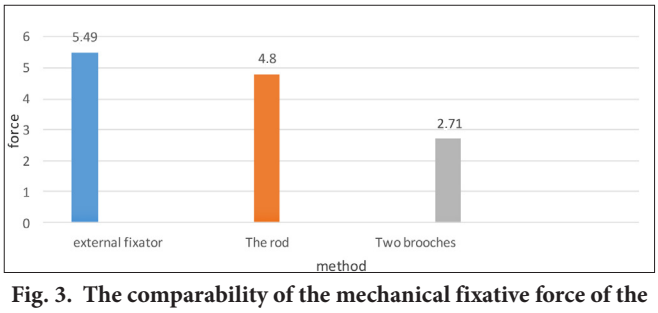


Fig. 3. The comparability of the mechanical fixative force of the fragments with different fixators at the torsion.

Conclusions

In this article the comparative calculations of reliability difference results were performed after Student's criteria:

Therefore, the comparativity of the mechanical fixative force of osteosynthesised fragments after calculated norm "t", which corresponds to the probability P<0.05 indicates an essential prospective of the external fixator, where:

t – the calculated norm Student's criteria for the comparison string;

P – the truthfulness of the difference.

On the basis of those exposed, we may conclude the following:

1. The fixative force of the osteosynthesised fragments with the external fixator at the flexure is 3 times greater than the fixative force of the osteosynthesised fragments

with the rod mark IXI8H9T and than the fixing force with 2 wires made by using the Kirschner and Elizarov's example – 6.7 times (P <0.01).

2. The fixative force of the osteosynthesised fragments with the external fixator at the stretch is 3 times greater (P< 0,01) than the fixative force of the osteosynthesised fragments with mark IXI8H9T and than the fixing force with 2 wires made by using the Kirschner and Elizarov's example by 4.3 times(P < 0, 05).

3. The fixative force of the osteosynthesised fragments with the external fixator at the torsion is 1.9 times greater (P<0.001) than the fixative force of the osteosynthesised fragments with mark IXI8H9T and than the fixing force with 2 wires made by using the Kirschner and Elizarov's example – 2.7 times (P <0.001).

Therefore, the performed experimental samples argue objectively the qualities of the proposed external fixator which has an essential priority in mechanical fixative force of the fragments in comparison with traditional fixators, used in clinic in most cases with clavicular fractures.

References

1. Golser K, Sperner G, Thoni H, Resch H. Early and intermediate results of conservatively and surgically treated lateral clavicular fractures. Aktuelle Traumatol. 1991 Aug;21(4):148-52.
2. Karaoglu S, Duygulu F, Kabak S, Baktir A. Results of conservative treatment of displaced mid-clavicular fractures in adults. Acta Orthop Traumatol Turc. 2002; 36(1):7-11.
3. Kotelnicki JJ, Bote HO, Mitts KG. The management of clavicle fractures. JAAPA. 2006 Sep;19(9):50, 53-4, 56.
4. Lazarides S, Zafiropoulus G. Conservative treatment of fractures at the middle third of the clavicle: the relevance of shortening and clinical outcome. J Shoulder Elbow Surg. 2006 Mar-Apr;15(2):191-4.
5. Nikiforidis P, Babis GC, Vayanos ED. Treatment of pseudarthrosis of the clavicle by bone plate with screw fixation and autologous spongiosa graft. Rev Chir Orthop Reparatrice Appar Mot. 1996;82(2):125-9.
6. Nordgvist A, Petersson CJ, Redlund-Johnell I. The natural course of lateral clavicle fracture. 15 (11-21) year follow-up of 110 cases. Acta Orthop Scand. 1993 Feb;64(1):87-91.
7. Nordgvist A, Petersson CJ, Redlund-Johnell I. Mid-clavicle fractures in adults: end result study after conservative treatment. J Orthop Trauma. 1998 Nov-Dec;12(8):572-6.
8. Nowak J, Holgersson M, Larsson S. Sequelae from clavicular fractures are common: a prospective study of 222 patients. Acta Orthop. 2005 Aug;76(4):496-502.
9. Pedersen MS, Kristiansen B, Thomsen F, Torholm C. Conservative treatment of clavicular fractures. Ugeskr Laeger. 1993 Nov 22;155(47):3832-4.
10. Robinson CM, Cairns DA. Primary nonoperative treatment of displaced lateral fractures of the clavicle. J Bone Joint Surg Am. 2004 Apr;86-A(4):778-82.
11. Eskola A, Vainionpaa S, Myllynen P, Patiala H, Rokkanen P. Outcome of clavicular fracture in 89 patients. Arch Orthop Trauma Surg. 1986;105(6):337-338.
12. Fann CY, Chiu FY, Chuang TY, Chen CM, Chen TH. Transacromial Knowles pin in the treatment of Neer type 2 distal clavicle fractures: a prospective evaluation of 32 cases. J Trauma. 2004 May;56(5):1102-5; discussion 1105-6.
13. Flinkkila T, Ristiniemi J, Lakovaara M, Hyvonen P, Leppilahti J. Hook-plate fixation of unstable lateral clavicle fractures: a report on 63 patients. Acta Orthop. 2006 Aug;77(4):644-9.
14. Fuch M, Losch A, Sturmer KM. Surgical treatment of the clavicle – indication, surgical technique and results. Zentralbl Chir. 2002 Jun;127(6):479-84.
15. Fung M, Kato S, Barrance PJ, et al. Scapular and clavicular kinematics during humeral elevations: a study with cadavers. J Shoulder Elbow Surg. 2001;10(3):278-85.
16. Hill JM, McGuire MH, Crosby LA. Closed treatment of displaced middle-third fractures of the clavicle gives poor results. J Bone Joint Surg Br. 1997 Jul;79(4):537-9.
17. Iannotti MR, Crosby LA, Stafford P, Grayson G, Goulet R. Effects of plate location and selection on the stability of midshaft clavicle osteotomies: a biomechanical study. J Shoulder Elbow Surg. 2002;11(5):457-62.
18. Jackson WF, Bayne G, Gregg-Smith SJ. Fractures of the lateral third of clavicle: an anatomic approach to treatment. J Trauma. 2006 Jul;61(1):222-5.
19. Jin CZ, Kim HK, Min BH. Surgical treatment for distal clavicle fractures associated with coracoclavicular ligament rupture using a cannulated screw fixation technique. J Trauma. 2006 Jun;60(6):1358-61.
20. Otverchenko GN, Zhanaspaev AM. K voprosu lecheniia perelomov kliuchitsy metodom skeletnogo vytiazheniia [About the treatment of clavicle fractures with skeletal traction]. In: Nauchnye trudy Rizhskogo Nauchno-Isledovatel'skogo Instituta Travmatologii i Ortopedii. T. 12 [Scientific papers of the Riga Research Institute of Traumatology and Orthopedics. Vol. 12]. Riga; 1976. p. 165-166. Russian.
21. Fishchenko PIa, Zhanaspaev AM. Lechenie perelomov kliuchitsy metodom skeletnogo vytiazheniia [Treatment of clavicle fractures with skeletal traction]. Ortop Travmatol Protez. 1980;(2):61-2. Russian.
22. Bil'kevich AA, Rakhimov KR. Migratsatsiia metallicheskogo sterzhnia [Metal rod migration]. Zdravokhranenie Kazakhstana. 1979;(2):71. Russian.
23. Bukach VA. Migratsiia spitsy Kirshnera v legkoe posle osteosinteza perelomov kliuchitsy [Migration of Kirchner's needles to the lung after osteosynthesis of clavicle fractures]. Zdravookhranenie Belorusii. 1980;(2):68. Russian.
24. Kalashnikov RN. Anatomicheskie osobennosti kliuchitsy v sviazi s osteosintezom [Anatomical features of the clavicle due to the osteosynthesis] . Ortop Travmatol Protez. 1977;(9):78-79. Russian.
25. Kapitanskii IS, Beliakov AA. Sravnitel'naia otsenka metodov lecheniia perelomov kliuchitsy [Comparative evaluation of the methods of clavicle fractures treatment]. Ortop Travmatol Protez. 1996;(10):60-63. Russian.
26. Morgan K, Simpson Dzh. Clavicle fracture mechanism. Clinical and biomechanical analysis. J Bone Joint Surg Am. 1989;70B(3):461-464.
27. Rokvell T. External fixation of the clavicle over a fracture or nonunion at the adults. J Bone Joint Surg Am. 1989:692-695.
28. Skoblin AP, Bom KB, Rekhlitskii AIa, Grabovoi AS. Perelomy i vyvikhi kliuchitsy [Fractures and dislocations of the clavicle]. Kiev: Zdorov'ia; 1973. 128 p. Russian.
29. Sushko GS. Lechenie perelomov kliuchitsy metodom vneochagovogo osteosinteza [Treatment of clavicle fractures using extrafocal osteosynthesis]. In: Lechenie ortopedo-travmatologicheskikh bol'nykh v statsionare i poliklinike metodom chreskostnogo osteosinteza, razrabotannym KNIIEKOT: vsesoiuznaia nauchno-prakticheskaia konferentsiia; 27-28 Ianv. 1982; Kurgan [Treatment of orthopedic and trauma patients in the hospital and policlinic by the method of transosseous osteosynthesis developed in KNIIEKOT: union scientific practical conference; 1982 Ian 27-28]. Kurgan; 1982. p. 93-95. Russian.
30. Tatti EIa, Melitser RI, Oshchukova SM. Ustroistvo dlia vpravleniia i fiksatsii akromial'nogo kontsa kliuchitsy [Device for restoring and fixing the acromial end of the clavicle]. Ortop Travmatol Protez (Khar'kov). 1993;(2). Russian.
31. Khodzhaev RR, Ibragimov ST. Modifikatsiia metoda vnutrikostnogo osteosinteza spitsei Kirshnera pri perelomakh kliuchitsy [Modification of the method of intraosseous osteosynthesis with a Kirschner needle for fractures of the clavicle]. Med Zh Uzbekistana. 1978;(11):58-62. Russian.
32. Ciuffoletti V, Bailee L. L'osteosintesi di clavicula con filo-vite d Ghisellini [Osteosynthesis of clavicle with phylum of Ghisellini]. Minerva Ortop. 1980;31(3):93-94. Italian.
33. Fontana G, Magliulo V. Il trattamento delle fratture del terzo esterno della clavicola mediante sindesmopessi conraco-clavicolare [Treatment of fractures of the external third of the clavicle using conraco-clavicular syndesmopexy]. Minerva Ortop. 1973;24(1-2):39-42. Italian.
34. Graner G. Zur operativen Behandlung von Frakturen und Pseudarthrosen der Klavikula [For surgical treatment of fractures and pseudarthrosis of the clavicle]. Zbl Chir. 1974;99:27. German.
35. Jablon M, Sutker A, Post M. Irreducibile fracture of the middle third of the clavicle. J Bone Joint Surg Am. 1979;61(2): 296-8.

DOI: 10.5281/zenodo.2589998
UDC: 611.36.018:57.085.2

Hepatocytes isolation from adult rats for liver recellularization

***Mariana Jian,** BioD; **Vitalie Cobzac,** MD, PhD Applicant; **Victoria Vartic,** MD;
Viorel Nacu, MD, PhD, Professor

Tissue Engineering and Cells Cultures Laboratory
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova

*Corresponding author: jian.mariana@usmf.md
Manuscript received January 10, 2019; revised manuscript March 01, 2019

Abstract

Background: Currently hepatocytes obtaining is prerequisite to create the necessary conditions for medical research, because it is an important tool in developing of new strategies in tissue engineering domain, which represents obtaining functional organs in laboratory conditions.
Material and methods: The study was made on adult Wistar rats liver with body weight 274.66± 2.52 g (n=3) which were used for hepatocytes extraction by perfusion through the upper cave vein with combination of type II collagenase and type I dispase and Hank's 0.9 mM MgCl2, 0.5 mM EDTA and 25 mM HEPES (HiMedia, India).
Results: The cells were counted with trypan blue 0.25% in hemocytometer and cultured in William's E medium (HiMedia, India) with 2 mM L-glutamine, 5% fetal bovine serum (Lonza, Belgium), antibiotic antimycotic solution (HiMedia, India), 100 nM dexamethasone and 100 nM insulin, with 2.5 x 10^5 cells per well in 12-well plates. After isolation were obtained 324, 48 ± 1, 25 x 10^6 hepatocytes, with a viability of 94.7 ± 0.9 % which indicates a high yield of cells viability.
Conclusions: The hepatocyte isolation method by liver perfusion with the combination of collagenase-dispase is feasible for obtaining a large amount of functional hepatocytes intended for the recellularization *in vitro* of decellularized liver scaffolds. The yield and viability of hepatic cells could be increased by enzymatic digestion of liver tissue using combination of collagenase/dispase solution due to the less cytotoxic effect.

Key words: hepatocytes, cell separation, cell survival, collagenases, dispase, in vitro techniques.

Introduction

Liver is the organ with a large functional metabolic profile, being a subject of an impressive number of biochemical investigations. Its main function in the body is the transformation of nutrients into physical and chemical forms that are used in the body, and on the other hand they are excreted. What is important is that the liver functions in the liver are only performed by parenchymal cells (hepatocytes). They constitute 90 – 95%, the majority, from the total weight of the other types of cells and 2/3 of the total cell population. This relative morphological uniformity of the liver as well as the linkage between hepatocytes with its organo-specific function make it possible to use parenchymal cells as a model for investigating various biochemical, biophysical, pharmacological and physiological processes that happen in the liver [1, 2, 3, 4]. The sources of hepatocytes may be from the liver that has been rejected after transplantation, portions of liver resected from patients with cirrhosis or other hepatic diseases and portions of liver resected from healthy patients [5, 6, 7, 8, 9, 10]. The hepatocytes isolated from rats, mice or human liver are a useful technique for studying liver function *in vitro* as well as for the recellularization of decellularized hepatic scaffolds. Also, the hepatocytes obtained after isolation have a major utility to be transplanted, as cell therapy of hepatic diseases for correction of metabolic disorders of the liver in the absence of donors liver for transplantation [11, 12, 13, 14, 15, 16, 17, 18].

Due to multitude possibilities for using isolated hepatocytes, there are various hepatocyte isolation protocols. What is important, is that they should be allowed for obtaining a higher number of hepatocytes with good viability and purity, preservating *in vitro* all functions specific for them *in vivo* [19].

The aim of our study is the isolation and cultivation of viable hepatocytes in large amounts from the liver of an adult rat for the *in vitro* liver recellularization.

Material and methods

The study was done on the liver from Wistar adult rats with body mass 274.66±2.52 g from which the hepatocytes were extracted. Before the isolation process, the substrate for hepatocytes adhesion was prepared. With 3-4 hours prior to initiating the experiment the cell culture surfaces of the culture plates or culture flasks were coated in a thin layer with Type I collagen solution at a concentration of 40 µg / ml. They were subsequently washed with HBSS (Hanks' balanced salt solution) without calcium and Mg (HiMedia, India) and allowed to dry in a Nuve 090 laminar flow hood. The hepatocytes were isolated by superior cava vein perfusion with 0.05% type I collagenase (HiMedia, India), 0.1% type I dispase (HiMedia, India) and HBSS without calcium and Mg (HiMedia, India) with 0.9 mM $MgCl_2$, 0.5 mM EDTA (Ethylenediaminetetraacetic acid) and 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HiMedia, India) after washing the intrahepatic blood vessels with Buffer I solution. Prior to initiating the hepatocyte isolation process Buffer I and II solutions were heated

in the water bath at 42° C. The work surface was sterilized with 70% alcohol after which it was left under UV rays for at least 15 minutes. During the perfusion process a 75 watt incandescent light lamp was set at 25-30 cm above the perfused liver area to maintain the temperature at around 37° C, in order to avoid hepatocytes death caused by temperature lowering during liver perfusion with solutions which become colder. Also, to maintain temperature of perfused liver, to prevent drying of its surface and to remove unwanted particles that precipitate from the air, with a syringe the liver surface was wetted continuously with warm 0.9% NaCl solution.

The isolated hepatocytes were counted with triptan blue in the hemocytometer and the biochemical parameters such as ALT (Alanine aminotransferase), glucose, total protein and glucose-6-phosphatase were determined with the Elitech kit.

Results

Isolation of hepatocytes from the liver of adult rat is required to study the recellularization liver process *in vitro*. Prior to sacrificing the animals 5000 IU of heparin was injected intraperitoneally. The general anesthesia was performed with 60 mg/kg ketamine and 5 mg/kg xylazine, then was removed the fur with a trimmer and it was processed with 70% alcohol. With scissors, the toracoabdominal wall was removed and a suprahepatic portion of the inferior cave vein was channeled with 18 G plastic catheter during persisting cardiac contractions, for keeping hepatocytes alive. We used a two step perfusion by collagenase/dispasse solution for hepatocytes isolation from adult Wistar rat liver through the superior vena cava with Buffer I solution (Hank's 0.9 mM $MgCl_2$, 0.5 mM EDTA and 25 mM HEPES) through a 0.22 mm filter for 4-5 minutes at a speed of 15 -20 ml/min. At the same time the inferior vena cava was ligated and the portal vein was sectionated and every 2 minutes it was clipped and released for decompression. This procedure is repeated 6-8 times (fig. 1). The second step was performed by Buffer II solution (collagenase/dispase) liver perfusion at a rate of 25-30 ml/min until it became flaccid, it took about 10-12 minutes, with clamping and realising of the portal vein every 2-3 minutes. Later the liver was extracted from the abdominal cavity and underwent mechanical disintegration for hepatocyte release (fig. 2). It was placed under a laminar flow hood in a Petri box with William E and 5% FBS nutrient solution placed on the ice for 5 minutes and was removed the liver capsule and the hepatocytes were released by shaking. With a pipette the released cells were collected and filtered through a 100 µm strainer placed on a 50 ml tube. After isolation, was appreciated the cells viability, that was 92%. Some of them were used for biochemical parameters tests, and some were preserved to be used in recellularization of decellularized liver matrices.

The cells were counted with 0.25% blue trypan solution in haemocytometer and apreciated the cellular viability. Then the cells were cultured in William E (HiMedia, India) medium with 2 mM of L-glutamine, 5% fetal bovine serum (Lonza, Belgium), antifungal antibiotic solution (HiMedia, India), 100 nM deaxmethasone and 100 nM insulin. The cells were seeded in 12-well plates by 2.5 x 10^5 cells per well and incubated at 37°C with 5% CO_2. Following the hepatocytes isolation were obtained a total of 324, 48±1, 25 x 10^6 cells with a viability of 94.7± 0.9%, indicating a high yield of viable cells (fig. 3).

The hepatocyte isolation is a very delicate process. During the hepatocyte isolation, it is necessary to obtain a maximum number of cells and to preserve their morphology and functionality. The biochemical parameters of the hepa-

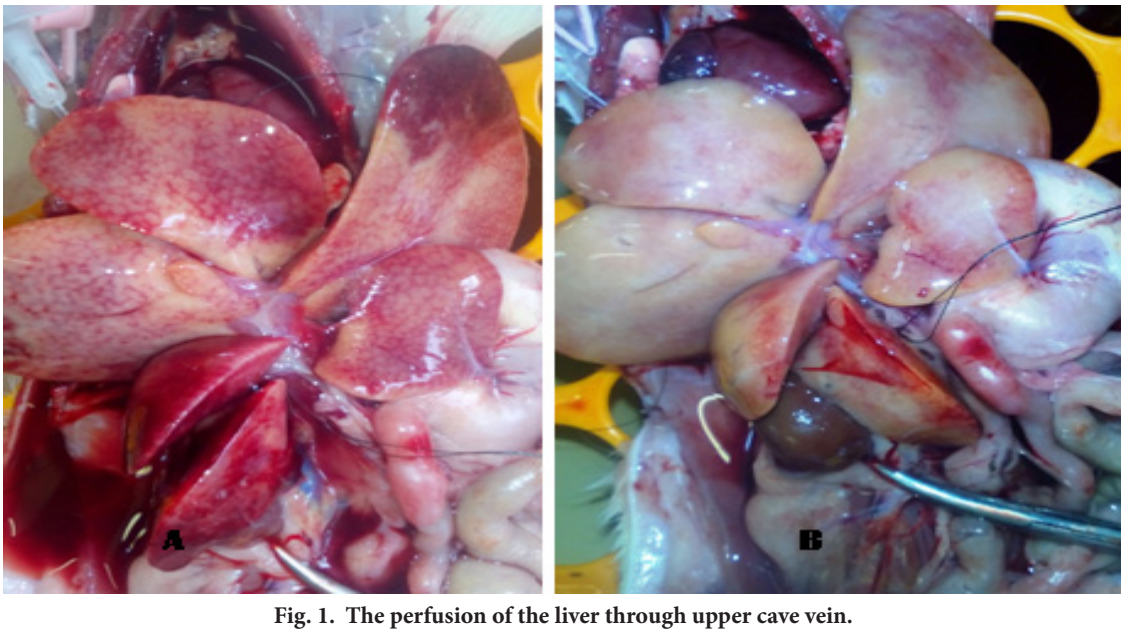


Fig. 1. The perfusion of the liver through upper cave vein.
A – with Hank's solution with 0.9 mM $MgCl_2$, 0.5 mM EDTA and 25 mM HEPES at a rate of 15-20 ml/min,
B – with Hank's solution and type II collagenase / type I dispase displacement with speed of 25 ml/min.

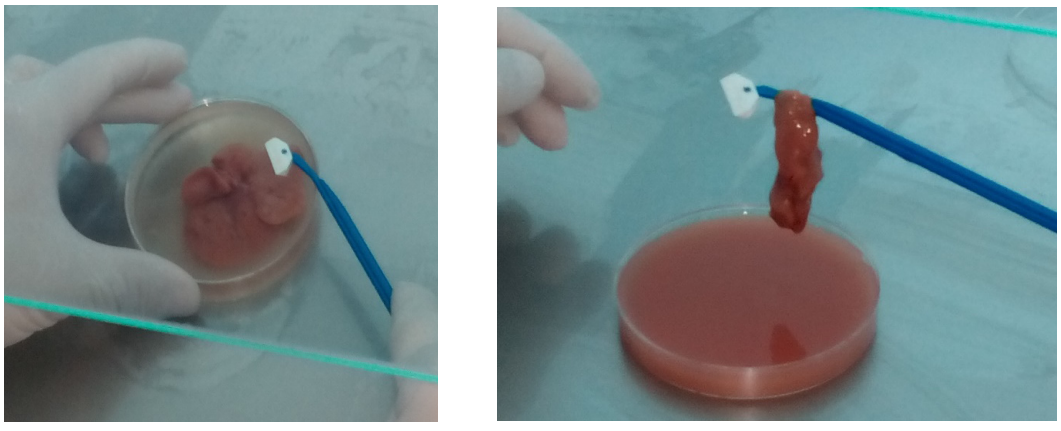


Fig. 2. The mechanical disintegration of the liver (A), liver carcase after isolation of the hepatocytes (B).

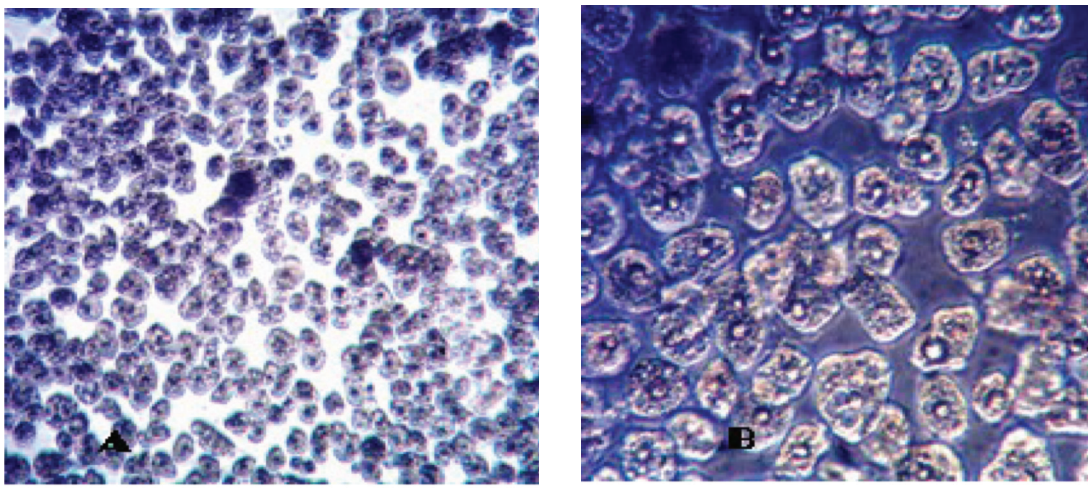


Fig. 3. Liver cells visualized with phase-contrast inverted microscope KZD: A – x10, B – x40.

tocytes that were analyzed show gluconeogenesis process, glucose-6-phosphatase, which is found only in hepatocytes and alanine aminotransferase, and as well as the content of glucose and total protein (tab. 1).

Table 1

Functional – metabolic properties of isolated hepatocytes in culture

Biochemical parameters	Catalytic activity
ALT, u / L	17.83 ±1.06
Glucose-6-phosphatase, u / L	3.41±0.46
Biochemical parameters	**Concentration**
Glucose, mM / L	4.16±0.10
Total protein, g / L	61.86±2.09

Discussion

Since hepatic tissue for liver isolation is limited, improvements in hepatocyte production and cryopreservation protocols are necessary to maintain cell viability during cultivation over a longer period of time and to prevent the hepatocyte number reduction after thawing [20, 21, 22].

According to the literature, there are many protocols for the isolation of hepatocytes with high viability and purity, but they depend on the type of collagenase used which determines significant differences in hepatocyte viability after digestion [23].

In the majority of the methods, collagenase is used to isolate hepatocytes from liver tissue. But Ricky H. Bhogal et al. used a combination of collagenase / protease / hyaluronidase and deoxyribonuclease [24].

Another study by MN. Berry shows that after buffered perfusion containing 0.05% collagenase and 0.10% hyaluronidase the isolated liver cells are viable and the form and function correspond to liver cells *in situ* with the presence of cytoplasmic vacuolization in a low number of cells and loss of potassium that are the only signs of cell lesion [25]. In another study in which the hepatocytes were isolated only by collagenase they showed a viability of 53% [26].

An effective change in our method was the insignificant reduction of collagenase concentration in 100 ml of buffer with approximately 400U to diminish, even insignificantly, the cytotoxic effect of collagenase on hepatocytes and the addition of solution of Dispase I enzyme which is not so cytotoxic at a concentration of 1U/ml.

In the hepatocytes grown medium were added supplements necessary for effective cellular respiration, since hepatocytes are highly specialized cells with intense biochemi-

cal activity requiring high energy consumption for protein, lipid and carbohydrate metabolism. These are: Na, selenium, transferin, bovine albumin, dexamethasone, aminoplasmol, hepatic amino acid complex, growth factors for hepatocytes and fibroblasts. It is important to note that the nutrient medium used for hepatocyte cultivation was changed every 2 days. During the medium changing within the first 4 hours, it should be taken into account that the hepatocytes are fragile and can easily be damaged by direct contact, so they are only pipetted from the side of the well.

Also is very important the supply of oxygen solutions, for which the oxygenator hose was introduced during the infusion first in Buffer I, and then in Buffer II solutions. It was programmed at 0.5 L/min. After strict following the listed steps, the viability of the hepatocytes is 94.7 ± 0.9%, compared to cell viability of 10% to 85%, which was obtained for use only for the detachment of hepatocytes from collagenase.

Conclusions

1. The high hepatocyte viability in dynamics is a priority and the sustained hepatocyte growth is determined by adding supplements necessary for effective cellular metabolism in the cell culture medium used.

2. The hepatocyte isolation method by liver perfusion with the combination of collagenase-dispase is feasible for obtaining a large amount of functional hepatocytes intended for the recellularization *in vitro* of decellularized liver scaffolds.

3. The yield and viability of hepatic cells could be increased by enzymatic digestion of liver tissue using combination of collagenase/dispase solution due to the less cytotoxic effect.

References

1. Hang H, Shi X, Gu G, Wu Y, Ding Y. A simple isolation and cryopreservation method for adult human hepatocytes. Int J Artif Organs. 2009 Oct;32(10):720-7.
2. Li WC, Ralphs KL, Tosh D. Isolation and culture of adult mouse hepatocytes. Methods Mol Biol. 2010;633:185-96. doi: 10.1007/978-1-59745-019-5_13.
3. Sasaki K, Kon J, Mizuguchi T, Chen Q, Ooe H, Oshima H, Hirata K, Mitaka T. Proliferation of hepatocyte progenitor cells isolated from adult human livers in serum-free medium. Cell Transplant. 2008;17(10-11):1221-30.
4. Wallace K, Fairhall EA, Charlton KA, Wright MC. AR42J-B-13 cell: an expandable progenitor to generate an unlimited supply of functional hepatocytes. Toxicology. 2010 Dec 30;278(3):277-87. doi: 10.1016/j.tox.2010.05.008. Epub 2010 Jun 1.
5. Akhter J, Johnson LA, Gunasegaram A, Riordan SM, Morris DL. Hepatocyte transplantation: a review of laboratory techniques and clinical experiences. Surgeon. 2007 Jun;5(3):155-64.
6. Alexandrova K, Griesel C, Barthold M, Heuft HG, Ott M, Winkler M, Schrem H, Manns MP, Bredehorn T, Net M, Vidal MM, Kafert-Kasting S, Arseniev L. Large-scale isolation of human hepatocytes for therapeutic application. Cell Transplant. 2005;14(10):845-53.
7. Baccarani U, Sanna A, Cariani A, Sainz-Barriga M, Adani GL, Zambito AM, Piccolo G, Risaliti A, Nanni-Costa A, Ridolfi L, Scalamogna M, Bresadola F, Donini A. Isolation of human hepatocytes from livers rejected for liver transplantation on a national basis: results of a 2-year experience. Liver Transpl. 2003 May;9(5):506-12.
8. Hsu HC, Matsuno N, Machida N, Enosawa S. Improved recovery of hepatocytes isolated from warm ischemic rat liver by citrate phosphate dextrose (CPD)-supplemented Euro-Collins solution. Cell Med. 2013 May 14;5(2-3):97-101. doi: 10.3727/215517913X666521. eCollection 2013 Nov 10.
9. Sahi J, Grepper S, Smith C. Hepatocytes as a tool in drug metabolism, transport and safety evaluations in drug discovery. Curr Drug Discov Technol. 2010 Sep;7(3):188-98.
10. Tanaka K, Soto-Gutierrez A, Navarro-Alvarez N, Rivas-Carrillo JD, Jun HS, Kobayashi N. Functional hepatocyte culture and its application to cell therapies. Cell Transplant. 2006;15(10):855-64.
11. Baccarani U, Adani GL, Sainz M, Donini A, Risaliti A, Bresadola F. Human hepatocyte transplantation for acute liver failure: state of the art and analysis of cell sources. Transplant Proc. 2005 Jul-Aug;37(6):2702-4.
12. Bartlett DC, Newsome PN. Hepatocyte cell therapy in liver disease. Expert Rev Gastroenterol Hepatol. 2015;9(10):1261-72. doi: 10.1586/17474124.2015.1073106. Epub 2015 Jul 27.
13. Iansante V, Mitry RR, Filippi C, Fitzpatrick E, Dhawan A. Human hepatocyte transplantation for liver disease: current status and future perspectives. Pediatr Res. 2018 Jan;83(1-2):232-240. doi: 10.1038/pr.2017.284. Epub 2017 Dec 6.
14. Fox IJ. Hepatocyte transplantation. Gastroenterol Hepatol (NY). 2014 Sep;10(9):594-6.
15. Maruyama M, Totsugawa T, Kunieda T, Okitsu T, Shibata N, Takesue M, Kurabayashi Y, Oshita M, Nakaji S, Kodama M, Tanaka N, Kobayashi N. Hepatocyte isolation and transplantation in the pig. Cell Transplant. 2003;12(6):593-8.
16. Hamooda M. Hepatocyte transplantation in children with liver cell failure. Electron Physician. 2016 Oct;8(10):3096-101. doi: 10.19082/3096.
17. Terry C, Dhawan A, Mitry RR, Hughes RD. Cryopreservation of isolated human hepatocytes for transplantation: State of the art. Cryobiology. 2006 Oct;53(2):149-59. Epub 2006 Jun 21.
18. Vidal I, Blanchard N, Alexandre E, Gandillet A, Chenard-Neu MP, Staedtler F, Schumacher M, Bachellier P, Jaeck D, Firat H, Heyd B, Richert L. Improved xenogenic hepatocyte implantation into nude mouse liver parenchyma with acute liver failure when followed by repeated anti-Fas antibody (Jo2) treatment. Cell Transplant. 2008;17(5):507-24.
19. Severgnini M, Sherman J, Sehgal A, Jayaprakash NK, Aubin J, Wang G, Zhang, Chang L, Peng CG, Yucius K, Butler J, Fitzgerald K. A rapid two-step method for isolation of functional primary mouse hepatocytes: cell characterization and asialoglycoprotein receptor based assay development. Cytotechnology. 2012 Mar;64(2):187-95.
20. Liu XL, Li LJ, Chen Z. Isolation and primary culture of rat hepatocytes. Hepatobiliary Pancreat Dis Int. 2002 Feb;1(1):77-9.
21. Mitry RR, Hughes RD, Dhawan A. Progress in human hepatocytes: isolation, culture & cryopreservation. Semin Cell Dev Biol. 2002 Dec;13(6):463-7.
22. Takesue M, Maruyama M, Shibata N, Kunieda T, Okitsu T, Sakaguchi M, Totsugawa T, Kosaka Y, Arata A, Ikeda H, Matsuoka J, Oyama T, Kodama M, Ohmoto K, Yamamoto S, Kurabayashi Y, Yamamoto I, Tanaka N, Kobayashi N. Maintenance of cold-preserved porcine hepatocyte function with UW solution and ascorbic acid-2 glucoside. Cell Transplant. 2003;12(6):599-606.
23. Lee SM, Schelcher C, Demmel M, Hauner M, Thasler WE. Isolation of human hepatocytes by a two-step collagenase perfusion procedure. J Vis Exp. 2013;(79). doi: 10.3791/50615.
24. Bhogal RH, Hodson J, Bartlett DC, Weston CJ, Curbishley SM, Haughton E, Williams KT, Reynolds GM, Newsome PN, Adams DH, Afford SC. Isolation of primary human hepatocytes from normal and diseased liver tissue: a one hundred liver experience. PLoS One. 2011;6(3):e18222.
25. Berry MN, Friend DS. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. J Cell Biol. 1969 Dec 1;43(3):506-20.
26. Mitry RR, Hughes RD, Aw MM, Terry C, Mieli-Vergani G, Girlanda R, Muiesan P, Rela M, Heaton ND, Dhawan A. Human hepatocyte isolation and relationship of cell viability to early graft function. Cell Transplant. 2003;12(1):69-74.

DOI: 10.5281/zenodo.2590001
UDC: 616.24-002.5(478)

Tuberculosis characteristics and risk factors in urban compared with rural patients

[1]**Aliona Tihon,** MD, PhD, Associate Professor; [1]**Ovidiu Tafuni,** MD, PhD, Associate Professor;
[2]**Alina Malic,** MD, PhD, Assistant Professor; [3]**Radu Niguleanu,** MD, PhD, Associate Professor;
[2]**Tatiana Osipov,** MD, Assistant Professor; [2]**Evelina Lesnic,** MD, PhD, Associate Professor

[1]Department of General Hygiene, [2]Department of Pneumophtisiology, [3]Department of Morphopathology
Nicolae Testemitsanu State University of Medicine and Pharmacy Chisinau, the Republic of Moldova

Corresponding author: evelinalesnic@yahoo.com
Manuscript received January 23, 2019; revised manuscript March 01, 2019

Abstract

Material and methods: A retrospective selective, descriptive study of socioeconomic, epidemiological peculiarities, case-management, diagnosis and microbiological characteristics of 694 patients with tuberculosis registered in Chisinau in 2016 was performed. Among them 581, had an urban residency and 112 rural residency.

Results: Residents from rural population and young persons in urban areas were most affected. Socioeconomic vulnerability predominated in both subpopulations; however, the gravity was more represented in the urban group. Lower level of education and tuberculosis contacts were more dominating in the rural group. Comorbidities, HIV infection were more frequently identified in the urban group, but destructive forms – in the rural patients. Low treatment outcomes were more frequently established in the rural group.

Conclusions: Risk factors for tuberculosis in urban subpopulation were: unemployment, lack of health insurance, homelessness, comorbidities, HIV infection. In rural population prevailed the following risk factors: low school education and tuberculosis contact.

Key words: tuberculosis, risk factors, outcome.

Introduction

Tuberculosis is one of the 10 causes of death worldwide and is a leading cause of death in HIV infected people worldwide [1, 2, 3]. The disease represents a serious public health problem in the Republic of Moldova (RM), affecting the most active economic age group of the population. According to the published data by the Moldovan National Centre for Management in Health during the period 2013-2015 it was registered an important decline of the incidence (with 22.4/100.000) in Chisinau: 2013 – 94.1/100.000, 2014 – 81.7/100.000 and 2015 – 71.7/100.000 population, prevalence (with 25.4/100.000) 2013 – 125.6/100.000, 2014 – 108.8/100.000 and 2015 – 100.1/100.000 population also the mortality 2013 – 10.6/100.000 and in 2014 – 6.9/100.000 population. During the same period of time in the RM the total incidence decreased: in 2013 – 125.6/100.000, 2014 – 108.8/100.000 and 2015 – 100.1/100.000 population, the prevalence (with 23.3/100.000) in 2013 – 109.7/100.000, 2014 – 97/100.000 and 2015 – 86.4/100.000 population, also the mortality 2013 – 10.5/100.000 and in 2014 – 8.8/100.000 population. An important difference between epidemiological indices registered in urban and rural localities was established. In the rural localities of the RM the total incidence was by 25% more elevated than in urban localities: 103.6 /100.000 in 2014 and 90.6/100.000 in 2015 compared with 78.4/100.000 in 2014 and 67.2/100.000 in 2015. A similar trend was identified regarding the incidence of the new cases, which were more elevated (by 18%) in rural localities compared with urban areas. The incidence of the new cases diminished by 18% from 91.5/100.000 in 2013, 85.4 /100.000 in 2014 and 73.5/100.000 in 2015 in the rural localities and by 19.7% in an urban population: from 72.3/100.000 in 2013, 62.1/100.000 in 2014 and 52.6/100.000 in 2015. More evidently diminished the prevalence in the urban localities (by 21%) 115/100.000 in 2013, 99/100.000 in 2014 and 90.4/100.000 population in 2015 compared with 6% in the rural areas: 207.3/100.000 in 2013, 199/100.000 in 2014 and 201.7/100.000 population [4].

In this epidemiological context, it must be exposed that the territory of the RM extends about 350 km from North to South and 150 km from West to East and is distributed in 56.7% of agricultural land, 13.6% of forests, 1.4% of urban localities and 7.6% of rural localities. From an administrative point of view, the territory of the RM is organized in 1.682 localities, classified in 5 municipalities, 61 cities, 916 villages and 659 communes (several villages), integrated in 32 districts and the autonomous territorial unit Gagauzia. The population of the RM was 3.550.900 people, including 1.476.100 urban residents and 2.074.800 rural persons in 2016. The urban population constituted 41.3% and the rural – 58.7% of the entire population of the RM. The urban settlements account for 1.4% of the RM territory, with an average density of 128 inhabitants/km^2. The rural settlements account for 7.6% of the RM territory, with a total number 1.614 of rural localities. Some of them have formed a population less than 10.000 inhabitants, with an

average density of the rural population being 7 inhabitants/km^2 [5].

The regional system of the RM was founded on the basis of legislative acts: the law of the regional development no. 438-XVI of 28.12.2006, the National Strategy for Regional Development (decision no 158 of 04.03.2010) and the regulatory framework that establishes the institutions which are responsible for the regional development (decision no 127 of 08.02.2008) [6, 7]. The regional development of the RM is realized according to the regional policy that aims to perform the territorial cohesion, which means to reduce the gaps between urban and rural localities, between the center and the periphery of the RM [7]. Assessing the health care disparities it was established that the rural households spent for health more than the urban family groups: 5.9% vs. 5.4% in 2014, 6.6% vs. 6.5% in 2015 from the total family income [8]. It is one of the major indices that showed a reduced accessibility for public health services of the rural population [9]. The poverty rate in rural areas is 5 times higher than in urban areas [8]. It was found that 86% of the poorest people from the RM live in villages [10]. Several social categories are among the poorest persons: families which are dependent only on agricultural activities, families with a lot of children, the elderly, persons with low levels of education attainment and lack of professional skills [11].

Distributing the population, according to the area of their habitation (house) it was established that in urban localities one person possesses 19.4 meters^2 and in rural areas – 23 meters^2/person [12]. Even if the area of the rural household is larger per capita, the quality and endowment are below the basic needs, which include electricity, natural gas, heating system, safe water and sewerage, as well the telecommunication (phone line) [12]. The urban areas have a better developed infrastructure, but the rural areas have an inhomogeneous distribution of the basic needs. All exposed data demonstrated the cause of the discrepancy of the public health indicators between urban and rural localities. While analyzing the statistical reports were established multiple healthcare problems and socially determined morbidities in rural localities: malnutrition, hipovitaminosis, severe anemia, tuberculosis, scabies, pediculosis. [14]. Residents of rural communities are more exposed to polluted water and to the consumption of dangerous food, due to the infestation of the land with wastewater and pesticides [13]. Current researches demonstrated a high level of the rural residents poisoning by poor management of the pesticides, fungicides, herbicides and insecticides. Due to the unequal distribution of the natural gas system, the residents of rural areas of the RM use fossil fuels (wood, coal and oil derivatives), which increase the risk of indoor air pollution with toxic gases such as carbon monoxide and other combustion gases involving the risk of involuntary poisoning and death [14].

Current political and economic trends are based on the reduction of the accessibility to the public healthcare services and increasing of the private sector offers [15]. A significant proportion of people from rural localities have no compulsory insurance policy, lack of education and high risk habits associated with the alcohol and tobacco consumption and unhealthy diet [16, 17]. The poor management of the healthcare, human resources, a big distance between the villages and primary health care institutions multiplied the barriers for the accessibility of the rural population to low price healthcare services [9, 14, 18, 19, 20]. Enumerated conditions aggravated by the social, economical vulnerability contributed to the extension of the tuberculosis in the rural population.

So, **the aim** of the study was to assess the tuberculosis features and risk factors of patients, residents of the urban sectors and rural localities of Chisinau city.

Objectives were: 1. Assessment of the socioeconomic and epidemiological risk factors of patients with tuberculosis and comparing them according to the urban and rural residence. 2. Evaluation of the case-management, diagnosis type, radiological aspects and microbiological characteristics of tuberculosis patients and comparing them according to the urban and rural residence. 3. Identification of the risk factors for tuberculosis, according to the urban and rural residence.

Results and discussion

According to the data obtained from the monitoring and follow-up of the case system during the period of 2016 were registered 694 tuberculosis cases among all residents of Chisinau, which included 581 patients from the urban sectors and 112 from rural communes: Bacioi, Bic, Braila (Bacioi), Bubuieci, Budesti, Tohatin, Cruzesti, Ciorescu, Codru, Colonita, Singera, Ghidighici, Stauceni, Gratiesti, Truseni, Vadul lui Voda and Vatra [12].

While distributing selected patients, according to the sex it was established the statistical predominance of men, with the highest rate in the rural group. So, men were 82 (73.2%) in the rural group and 392 (67.5%) in the urban group with a male/female ratio 2.1/1 in the urban group and 2.3/1 in the rural group. Repartition of the patients into age groups, according to the WHO recommendation identified that the largest subgroup in the urban group was between 25 and 34 years old – 136 (23.4%) patients, followed by those who were between 35 and 44 years old – 125 (21.5%) patients, between 45 and 54 years old – 115 (19.7%) patients and between 55 and 64 years old – 100 (17.2%) patients. In a minor proportion were represented patients younger than 24 years old – 41 (7.1%) cases.

In the rural group predominated patients who were between 35 and 44 years – 33 (29.4%) patients, followed by those who were between 45 and 54 years – 23 (20.5%), also between 55 and 64 years – 22 (19.6%) patients. In a minor proportion of patients were included young groups, who were between 25 and 34 years old – 19 (16.9%) and younger than 24 years – 10 (8.9%) cases. While comparing the groups was identified the predominance of the young subgroup of 25-34 years old in the urban group compared with the rural group: 136 (23.4%) vs. 19 (16.9%) patients, and older adults in the rural compared with the urban group: between 35 and 44 years old – 33 (29.4%) vs. 125 (21.5%) patients,

between 45 and 54 years – 23 (20.5%) vs. 115 (19,7%) patients and between 55 and 64 years old 22 – (19.6%) vs. 100 (17.2%) patients. Distribution of patients in three age groups established that young adults who were less than 34 years old predominated in the urban group – 177 (30.4%) vs. 29 (25.9%) patients from the rural group, also older adults, more than 55 years accounted for 164 (28.3%) patients in the urban vs. 27 (24.1%) patients in the rural group. The patients who were included in the subgroup between 35 and 54 years old were in a similar proportion of 56 (50%) vs. 240 (41.3%) patients. No statistical threshold was achieved comparing patients between the age subgroups (tab. 1).

Table 1

Distribution of patients by sex, age and demographic data

Indices	Sex Age groups	Urban group N=581 (P%)	Rural group N=112 (P%)	P value
Sex	Men	392 (67.5)	82 (73.2)	>0.05
	Women	189 (32.5)	36 (26.8)	>0.05
Age groups	18-24 years	41 (7.1)	10 (8.9)	>0.05
	25-34 years	136 (23.4)	19 (16.9)	>0.05
	35-44 years	125 (21.5)	33 (29.4)	>0.05
	45-54 years	115 (19.7)	23 (20.5)	>0.05
	55-64 years	100 (17.2)	22 (19.6)	>0.05
	+65 years	64 (11.1)	5 (4.5)	**<0.01**

Note: Applied statistical test: paired simple T-test, P – probability.

When distributing patients, according to the economic status, it was established that employed persons, which were contributing to the health budget by paying taxes predominated in the urban group – 137 (23.6%) vs. 21 (18.7%) patients and patients with health insurance policy predominated in the rural group – 68 (60.8%) vs. 289 (49.7%) patients in the urban group. Unemployed patients made up the majority of both groups and predominated in the rural group – 72 (64.2%) vs. 305 (52.4%), however, the patients without health insurance predominated in the urban group – 292 (50.2%) vs. 44 (39.2%) cases. It is explained by the fact that Moldovan citizens from rural localities, owners of the agricultural land have health insurance offered by the state [23]. Disease disabled patients, retired and students predominated in the urban compared with the rural group. The highest proportion among them were retired patients – 74 (12.7%) vs. 9 (8%), followed by disease disabled – 53 (9.1%) vs. 8 (7.1%) and students –12 (2.0%) vs. 2 (1.7%) cases in urban compared with the rural group (tab. 2).

Assessment of the educational level, demonstrated that most of the patients from both groups had secondary education, however, in the rural group there were 59 (52.7%) vs. 229 (39.4%) patients with secondary education in the urban group. Technical vocational education and bachelor studies predominated in the urban group – 162 (27.8%) vs. 19 (16.9%) in the rural group and respectively 47 (8.1%) cases in the urban group vs. 2 (1.8%) in the rural group. Primary and incomplete secondary education had each fourth patient in both groups – 143 (24.6%) in the urban group vs. 29 (25.9%) in the rural group. So, lower level of education statistically predominated in the rural group – 88 (78.5%) vs. 372 (64.1%) patients in the urban group. Exposed data are revealed in the table 3.

Table 2

Distribution according to the socioeconomic data

Indices	Economic state	Urban group N=581 (P%)	Rural group N=112 (P%)	P value P value
Economically stable	Employed	137 (23.6)	21 (18.7)	>0.05
	Insured	289 (49.7)	68 (60.8)	<0.05
Economically vulnerable	Disease disabled	53 (9.1)	8 (7.1)	>0.05
	Retired	74 (12.7)	9 (8)	>0.05
	Students	12 (2.0)	2 (1.7)	>0.05
	Unemployed	305 (52.4)	72 (64.2)	<0.05
	Lack of health insurance	292 (50.2)	44 (39.2)	<0.05

Note: Applied statistical test: paired simple T-test, P – probability.

Table 3

Distribution according to the last graduate level

Education	Urban group N=581 (P%)	Rural group N=112 (P%)	P value
Primary & incomplete secondary education	143 (24.6)	29 (25.9)	>0.05
Secondary education	229 (39.4)	59 (52.7)	**<0.01**
Secondary technical vocational education	162 (27.8)	19 (16.9)	**<0.01**
Bachelor studies	47 (8.1)	2 (1.8)	**<0.001**

Note: Applied statistical test: paired simple T-test, P – probability.

The major social characteristics of patients from the three groups were caused by the vulnerable economic state and living in poor conditions. Living under the poverty threshold predominated in patients from the rural localities – 36 (32.1%) vs. 165 (28.4%) cases from the urban group. The extreme poverty, caused by homelessness statistically predominated in the urban group – 130 (22.4%) vs. 17 (15.2%) cases in the rural group. History of migration during the last year was identified in a similar proportion of 59 (10%) patients from the urban vs. 11 (9.8%) cases. History of imprisonment was established in a similar proportion of 33 (5.8%) in the urban group vs. 5 (4.5%) in the rural group.

Close infectious contact with a member of a family who was previously diagnosed statistically predominated in the rural group – 16 (14.3%) compared with the urban group – 38 (6.5%). It could be explained by the fact that most of the sources of infection in the urban population are not identified, however, in the rural localities the infectious contact is efficiently managed due to a fewer number of population. Comorbid patients statistically predominated in the urban group – 258 (44.4%) vs. 24 (21.4%), more evident due to the high prevalence of the HIV infection – 59 (10.1%) in the urban group vs. 3 (2.6%) cases in the rural group. Harmful habits with health consequences such

as chronic alcoholism predominated in the urban group – 54 (9.2%) vs. 6 (5.4%), as well as the drug use – 10 (1.7%) vs. 1 (0.9%) patients in the rural group, as well as mental disorders – 11 (1.8%) vs. 1 (0.9%) case. Chronic respiratory and gastrointestinal diseases (including hepatitis) diseases predominated in the urban group – 37 (6.4%) vs. 6 (5.4%) and respectively 51 (8.7%) vs. 5 (4.4%) cases. No other statistical differences were detected among groups regarding the associated diseases (Table 4).

While distributing patients, according to the registered type of case it was identified that new cases predominated in the rural group – 70 (82) vs. 355 (61.1%) patients in the urban group. Each fourth patient in every group had a relapse – 137 (23.6%) patients in the urban group and 28 (25%) patients in the rural group. At a similar rate were patients included in the treatment after a previous "lost to follow-up" – 12 (10.7%) patients in the urban vs. 57 (9.8%) patients in the rural group and after a "treatment failure" – 29 (4.9%) patients in the urban vs. 2 (1.8%) patients in the rural group. Diagnosed and transferred from abroad to the RM were 3 (0.5%) patients. Data were demonstrated in the table 4.

Table 4

Distribution according to the risk groups

Category	Risks factors	Urban group N=581 (P%)	Rural group N=112 (P%)	P value
Socioeconomic	Poverty	165 (28.4)	36 (32.1)	>0.05
	Homelessness	130 (22.4)	17 (15.2)	**<0.05**
	Migration	59 (10)	11 (9.8)	>0.05
	History of detention	33 (5.8)	5 (4.5)	>0.05
Biological	Close contact	38 (6.5)	16 (14.3)	<0.05
	Associated diseases	258 (44.4)	24 (21.4)	<0.001
	HIV-infection	59 (10.1)	3 (2.6)	<0.001
	Diabetes	10 (1.7)	1 (0.9)	>0.05
	Chronic alcoholism	54 (9.2)	6 (5.4)	>0.05
	CRD	37 (6.4)	6 (5.4)	>0.05
	GID	51 (8.7)	5 (4.4)	>0.05
	Mental disorders (excluding IVDU)	11 (1.8)	1 (0,9)	>0.05
	Neoplasm	7 (1.2)	0	>0.05
	Renal diseases	5 (0.8)	1 (0.9)	>0.05
	Immune suppressive treatment	3 (0.5)	0	>0.05
	Drug users	10 (1.7)	1 (0.9)	>0.05
	Others	12 (2.0)	0	>0.05

Note: Applied statistical test: paired simple T-test, P – probability; NA-non available, CRD-chronic respiratory diseases, GID-gastrointestinal diseases.

Studying case-management, it was identified that the general medical staff was involved in the detection of most of the patients from the both groups and more perceptibly in the rural group – 45 (40.1%) vs 181 (31.1%) patients from the urban group. Screening of the people with high risk performed by the general practitioners detected more frequently patients from the urban group – 63 (10.8%) vs. 10 (8.9%) cases. Pulmonologists detected more frequently symptomatic patients from the urban group – 146 (25.1%) vs. 21 (18.7%). It is the consequence of the lack of the specialized medical staff, which manages the patients from the rural localities. High risk groups screening performed by pulmonologists detected more frequently patients from the rural group – 10 (8.9%) vs. 33 (5.6%) cases. Directly for hospitalization into a specialized institution came more frequently urban residents – 158 (27.1%) vs. 26 (23.2%) patients from the rural localities. So, it can be deducted that specialized hospital is more accessible for urban residents than for rural people. Death cases were more frequently detected in tuberculosis people from the rural localities – 5 (4%) than from urban districts of Chisinau – 12 (2%). Information is exposed in the table 5.

Table 5

Case-management characteristics of tuberculosis patients

Health level	Detection ways	Urban group N=581 (P%)	Rural group N=112 (P%)	P value
PHC	Detected by GPs symptomatics	181 (31.1)	45 (40.1)	>0.05
	Detected by GPs screening of HRG	63 (10.8)	10 (8.9)	>0.05
Ambulatory specialized level	Detected by SP symptomatics	146 (25.1)	21 (18.7)	>0.05
	Detected by SP screening of HRG	33 (5.6)	10 (8.9)	>0.05
Hospital level	Direct addressing	158 (27.1)	26 (23.2)	>0.05
Others	*Postmortem*	12 (2)	5 (4)	>0.05

Note: Applied statistical test: paired simple T-test, P – probability; GP-general practitioner, SP-specialist, HRG-high risk group.

Identifying the clinical, radiological forms of pulmonary tuberculosis it was established that pulmonary tuberculosis was diagnosed in a similar proportion in both groups – 546 (93.9%) vs 106 (94.6%) patients, as to extrapulmonary forms of tuberculosis – 31 (5.4%) vs. 6 (5.4%) patients. Generalized tuberculosis was established only in the urban group – 4 (0.6%) patients. Pulmonary infiltrative tuberculosis was identified in a similar proportion in both groups – 495 (85.2%) in the urban group vs. 97 (91.6%) patients in the rural group. Disseminated pulmonary tuberculosis was established more frequently in the rural group – 5 (4.7%) vs 18 (3.3%) patients from the urban group. Destructive forms of pulmonary tuberculosis were identified in a higher proportion in both groups, however, the destructive process in both lungs was statistically more frequently identified in the rural group – 36 (32.1%) vs. 97 (16.9%) patients (tab. 6).

When assessing the laboratory features of the enrolled pulmonary tuberculosis patients, it was identified that one third of the entire sample was microscopic positive for acid-fast-bacilli, 162 (27.8%) patients in the urban vs. 38 (33.9%) patients in the rural group. A similar proportion of patients was identified to have positive bacteriological results

Table 6

Microbiological features of tuberculosis patients

Index	Radiological features	Urban group	Rural group	P value
		N=581 (P%)	N=112 (P%)	
Clinical forms of TB	Pulmonary TB	546 (93.9)	106 (94.6)	>0.05
	Extrapulmonary	31 (5.4)	6 (5.4)	>0.05
	Generalized	4 (0.6)	0	>0.05
Clinical forms of pulmonary TB	PIT	495 (85.2)	97 (91.6)	>0.05
	PDT	18 (3.3)	5 (4.7)	>0.05
	FCVT	33 (6.1)	4 (3.7)	>0.05
Localization of destruction	One lung	171 (29.4)	38 (33.9)	>0.05
	Both lungs	97 (16.9)	36 (32.1)	<0.001
Microbiological features	AFB positive	162 (27.8)	38 (33.9)	>0.05
	MBT culture positive	155 (26.7)	35 (31.2)	>0.05
	GeneXpert MTB positive	227 (39.1)	51 (45.4)	>0.05
	GeneXpert MTB/Rif sensible	142 (24.4)	17 (15.2)	**<0.05**
	GeneXpert MTB/Rif resistent	85 (14.6)	34 (30.4)	**<0.01**
	MBT culture positive AFB positive	97 (16.6)	26 (23.2)	>0.05
	MBT culture positive AFB positive GeneXpert positive	81 (13.9)	23 (20.5)	>0.05
	MDR-TB	51 (8.8)	13 (11.6)	>0.05

Note: Applied statistical test: paired simple T-test, P – probability; PIT-pulmonary infiltrative tuberculosis, PDT-pulmonary disseminated tuberculosis, FCVT-fibro-cavernous tuberculosis. Applied statistical test: paired simple T-test, P – probability.

at cultivation on solid Lowenstein-Jensen or liquid MGIT BACTEC media: 155 (26.7%) patients in the urban vs. 35 (31.2%) patients in the rural group. The molecular genetic assay was performed in all cases, but positive results were obtained more frequently in the rural group – 51 (45.4%) vs. 227 (39.1%) patients in the urban due to a high proportion of cases with destructive forms of tuberculosis. Sensitive to rifampicin were more frequently identified patients from the rural group – 142 (24.4%) vs. 17 (15.2%), however, resistant to rifampicin have been more frequent cases in the rural group – 34 (30.4%) vs. 85 (14.6%) cases in the urban group. Microscopically positive for AFB and cultivation on the conventional media proved to be *Mycobacterium tuberculosis* (MTB) more frequently in the rural group – 26 (23.2%) vs. 97 (16.6%) in the urban group, as well microscopic positive for AFB, culture positive for MTB and GeneXpert MTB Rif positive assay were 23 (20.5%) patients from rural group vs. 81 (13.9%) patients from the urban group.

The standard treatment for new drug-susceptible tuberculosis in the RM has been used since 2000, lasts 6 months and consists of two phases with four first-line drugs: isoniazid (H), rifampicin (R), ethambutol (E) and pyrazinamide (Z) in the intensive phase and two first-line drugs: isoniazid and rifampicin in the continuation phase. For previously treated cases was used a regimen which lasts 8 months: 3 months with H, R, E, Z and streptomycin and 5 months with H, R and E. Patients with rifampicin-resistance or MDR-TB were treated with second-line drugs for 18 months or more divided in two phases. The regimen composition during the intensive phase lasts 6 months and includes kanamycin (Km) or capreomycin (Cm), levofloxacin (Lfx), para-amino salicylic acid (PAS), ethionamide (Eto), cycloserine (Cs) and pyrazinamide (Z) and for continuation phases during 12-18 months – Lfx, PAS, Etho, Cs and Z.

The standard treatment for drug susceptible tuberculosis was used for the treatment of a similar proportion of patients from both groups: 530 (91.2%) patients from the urban group and respectively 99 (88.4%) patients from the rural group. Every third patient from the urban group and every fifth patient from the rural group was treated as previously treated cases – 226 (38.9%) patients from the urban group and 60 (37.5%) patients from the rural group. Even the rate of MDR-TB was in average similar in both groups, only a minor proportion of patients from both groups was treated as drug-resistant patients: 51 (8.8%) patients from the urban group and 13 (11.6%) patients from the rural group. It is important to emphasize that the standard treatment for MDR-TB could be started only if the therapeutic compliance of the patient is established and the clinical tolerance is acceptable (tab. 7).

Table 7

Types of the cases according to the history of the anti-tuberculosis treatment

Case type	Outcome	Urban group	Rural group	P value
		N=581 (P%)	N=112 (P%)	
Never treated before	New case	355 (61.1)	70 (62.5)	>0.05
Previously treated	Relapse	137 (23.6)	28 (25)	>0.05
	Recovered after default	57 (9.8)	12 (10.7)	>0.05
	Recovered after failure	29 (4.9)	2 (1.8)	<0.05
Types of the drugs	First-line anti-TB drugs	530 (91.2)	99 (88.4)	>0.05
	Second-line anti-TB drugs	51 (8.8)	13 (11.6)	>0.05

All the patients were managed and treated with the standard treatment for tuberculosis. First-line anti-tuberculosis drugs were used in 531 (91.4%) patients from urban group vs. 13 (11.7%) patients from the rural group. Successfully treated were more frequently patients in the rural group – 80 (71.4%) vs. 373 (64.2%) patients in the urban group. The low therapeutic outcome was more frequently established in the urban group, such as "lost to follow-up" – 45 (7.7%) vs. 5 (4.5%) cases in the rural group, died – 69 (11.9%) vs. 10 (8.9%) patients in the rural group. Still continuing the treatment was almost each tenth patient in both groups (tab. 8).

Table 8

Treatment outcome of tuberculosis patients

Outcome	Urban group N=581 (P%)	Rural group N=112 (P%)	P value
Treatment success	373 (64.2)	80 (71.4)	>0.05
Treatment failure	9 (1.5)	0	>0.05
Lost to follow-up	45 (7.7)	5 (4.5)	>0.05
Death	69 (11.9)	10 (8.9)	>0.05
Still continuing	63 (10.8)	10 (8.9)	>0.05
Diagnosed excluded	22 (3.8)	6 (5.4)	>0.05

Note: Applied statistical test: paired simple T-test, P – probability.

An important research outcome represents the odds ratio (OR) and the attributable risk (AR), which are indices for identifying the priority interventions in the frame of high risk groups from every type of the subpopulation [22]. The values were calculated represented only for risk factors which predominated and exposed a statistical difference between the groups. It was established that the risk factors for tuberculosis in urban patients were linked with the sociovulnerability: unemployment, associated lack of health insurance, homelessness or lack of the residence visa, comorbidities and the immune suppressive condition – HIV infection. Attributable risk revealed the hierarchy of risks in urban population: HIV infection, comorbidities, homelessness, lack of health insurance and unemployment. In rural population the risk factors for tuberculosis were low level of the school education and tuberculosis contact. Related to this was identified lung destruction in both lungs, as a hallmark of the late detection of tuberculosis process. When leveling the risk factors it was established that more relevant was the tuberculosis contact followed by the low level of education (tab. 9).

Table 9

Risk factors for tuberculosis

Risk factors		OR	AR (%)
Social economical features	Unemployment	1.63 (1,07-2,48)	**18**
	Lack of insurance	1.56 (1,03-2,35)	**18**
	Homelessness	1.61(0,928-2,794)	**31**
	Low secondary education	2.06 (1,27-3,35)	**17**
Epidemiological and comorbidties	Close contact	2.32 (1,27-4,44)	**57**
	Associated diseases	2.9 (1,81-4,73)	**52**
	HIV-infection	4.1 (1,26-13,4)	**80**
Disease related	Both lungs involvement	2.36 (1,5-3,71)	**52**

Note: OR – odds ratio; AR – attributable risk.

The relation between tuberculosis indices and demographic particularities was widely studied [24, 25, 26, 27, 11, 28]. Globally, the epidemics of tuberculosis is much higher in urban areas than in rural localitites, because almost one half of the world's population lives in cities [29, 25, 26]. Our research identified high indices of tuberculosis in the rural subpopulation than in urban areas. It can be explained by the complexity of risk factors, which reflects the barriers for accessing the healthcare services of the rural population [30, 31, 32]. Several studies identified a poor quality of healthcare in private system which manages patients with tuberculosis [33, 26, 34]. In the RM the specialized institutions offer a standard approach, which corresponds to the international recommendations and national regulations [35, 21, 36]. The uncontrolled urbanisation is associated with extension of drug resistance and poor treatment outcome [37, 38, 39]. Our research established more increased rate of the drug resistance in the rural population. It can be explained by a deeper investigation of tuberculosis contacts in the rural areas. The uncontrolled urbanization is associated with lack of healthcare service at low price and expansion of the private sector [26, 27]. Our research established that the major proportion of patients was detected by public general practitioners and every fifth patient came directly to the hospital. No similar studies were conducted in the RM. Urbanization is associated with overcrowding, low level of sanitation and low socioeconomic state [40, 32, 41]. Our research identified also an important proportion of patients from both subpopulations, which were unemployed, homeless and without health insurance, however, their amount was more prevalent in the urban group which constituted risk factors.

Conclusions

Residents from the rural localities were more affected by tuberculosis compared with the urban population.

The age for acquiring tuberculosis was younger in urban population than in that from rural areas.

Socioeconomic vulnerability was extended in all patients with tuberculosis; however, the gravity was more evident in patients from the urban districts of Chisinau.

Low level of education predominated in the patients from rural localities.

Close contact with a sick patient predominated in the patients with tuberculosis from rural localities, which contributed to a higher rate of MDR-TB.

Associated diseases predominated in the urban group, more expressed was HIV infection.

Risk factors for tuberculosis in urban population were: unemployment and associated lack of health insurance, patient's homeless state, comorbidities and the immune suppressive condition – HIV infection. Risk factors for the rural population were tuberculosis contact and low level of the education.

References

1. World Health Organization. Global tuberculosis control 2011. Epidemiology, strategy, finances. Geneva: WHO; 2011. 258 p.
2. World Health Organization. Global tuberculosis report 2015. Geneva: WHO; 2015. 192 p.
3. World Health Organization. Global tuberculosis report 2017. Geneva: WHO; 2017. 249 p.
4. Centrul Național de Management în Sănătate al Republicii Moldova [National Center for Health Management of the Republic of Moldova]. Indicatori preliminari în format prescurtat privind sănătatea populației și activitatea instituțiilor medico-sanitare pe anii 2014-2015 [Preliminary indicators on population health and on healthcare institutions activity for 2014-2015]. Chisinau: The Center; 2016. 216 p. Romanian.
5. Biroul Național de Statistică al Republicii Moldova [National Bureau of Statistics of the Republic of Moldova]. Raport demografic 2017 [Demographic report 2017].
6. Guvernul Republicii Moldova [Government of the Republic of Moldova]. Hotărârea nr. 685 din 4.09.2013 privind Strategia Națională de Dezvoltare Regională pentru anii 2013-2015 [Decision no. 685 of 2013 September 4, on the National Strategy for Regional Development for the years 2013-2015]. Monitorul Oficial al Republicii Moldova. 2013 Sep 13;(198-204):art. no. 792. Romanian.
7. Guvernul Republicii Moldova [Government of the Republic of Moldova]. Legea nr. 438 din 28.12.2006 cu privire la dezvoltarea regională în Republica Moldova [Law no. 438 of 28 December 2006 on regional development in the Republic of Moldova]. Monitorul Oficial al Republicii Moldova. 2007 Feb 16;(21-24):art. no. 68. Romanian.
8. Pintea D, Gumene I. Analiza cheltuielilor destinate ocrotirii sănătății în Republica Moldova [Analysis of expenditures for health care in the Republic of Moldova]. Chisinau; 2016. 26 p. Romanian.
9. Berdaga V, Stefanet S, Bivol O. Access of the population of the Republic of Moldova to health services. Chisinau: Gunivas; 2010.
10. Government of the Republic of Moldova, UN Agencies in Moldova. The third Report on Millenium Development Goals. Republic of Moldova. Chisinau, 2013. 90 p.
11. World Health Organization. Commission on Social Determinants of Health. Action on the social determinants of health. Geneva: WHO; 2005. 50 p.
12. Biroul Național de Statistică al Republicii Moldova [National Bureau of Statistics of the Republic of Moldova]. Anuarul statistic al Republicii Moldova, 2017 = Statistical Yearbook of the Republic of Moldova, 2017. Chisinau: The Bureau; 2017. 486 p.
13. Centrul Național de Management în Sănătate [National Center for Health Management of the Republic of Moldova]. Sănătatea publică în Moldova, 2016: Raport [Public health in Moldova, 2016: Report]. Chisinau: The Center; 2016. Romanian.
14. National Bureau of Statistics of the Republic of Moldova. Results of the study of population health status and access to health services in the Republic of Moldova. Chisinau; 2016.
15. Ministerul Sănătății al Republicii Moldova [Ministry of Health of the Republic of Moldova]. Accelerarea reformelor: abordarea necesitatilor din domeniul sanatatii prin politici investitionale: Raport [Accelerating reforms: addressing the needs of the health area through investment policies: Report]. Chisinau; 2016. Romanian.
16. Bivol S, Vladicescu N; [Ministry of Health of the Republic of Moldova]. Vulnerabilitatea femeilor din Republica Moldova la HIV si SIDA: 2010 = Women's vulnerability to HIV and AIDS in the Republic of Moldova: 2010. Chisinau: Elan Poligraf; 2010. 138 p.
17. Lesnic E, Ustian A, Niguleanu A, Malic A, Paladi C. Social features of patients with pulmonary tueberculosis. [Tuberculosis, Lung Diseases, HIV Infection] (Kiev). 2016;25(2):36-40.
18. Centrul Național de Management în Sănătate al Republicii Moldova [National Center for Health Management of the Republic of Moldova]. Potențialul uman din sistemul sănătății implicat în acordarea asistenței medicale în ftiziopneumologie [The human potential of the healthcare system involved in the medical assistance in phthisiopulmonology]. Chisinau: The Center; 2011. Romanian.
19. Ciocanu M. Expertiza calitatii serviciilor spitalicești prestate persoanelor asigurate [Quality assessment of hospital services provided to insured persons]. Curierul Medical (Chisinau). 2007;(5):56-60.
20. Galan A. Expertiza Strategiei "Dezvoltarea resurselor umane pentru sănătate" și a Planului de Acțiuni în Republica Moldova: Raport final [Assessment of the strategy paper "Development of Human Resources for Health" and of the Action Plan in the Republic of Moldova: Final report]. Chisinau; 2011. 34 p. Romanian.
21. Domente L, Alexandru S, Iavorschi C, et al.; [Ministry of Health of the Republic of Moldova]. Tuberculoza la adult: Protocol clinic național [Tuberculosis in adults : National clinical protocol]. Chisinau: The Ministry; 2015, 140 p. (PCN-123). Romanian.
22. Spinei L, Lozan O, Badan V. Biostatistica [Biostatistics]. Chișinău; 2009. 186 p. Romanian.
23. Guvernul Republicii Moldova [Government of the Republic of Moldova]. Legea nr. 1585 din 27.02.1998 cu privire la asigurarea obligatorie de asistență medicală 30.04.1998 [Law no. 1585 of 27 February 1998 on compulsory health insurance]. Monitorul Oficial al Republicii Moldova. 1998 Apr 30;(38-39): art. no. 280. Romanian.
24. Kulzhabaeva A, Nabirova D, et al. Linkage between diagnosis and treatment of smear-positive pulmonary tuberculosis in urban and rural areas in Kyrgyzstan. Public Health Panorama. 2016;2(1):104-109.
25. Prasad A, Ross A, et al. A world of cities and the end of TB. Trans R Soc Trop Med Hyg. 2016;110:151-152. doi:10.1093/trstmh/trw004.
26. Rangan S, Gupte H, et al. Tackling tuberculosis in urban areas: experiences from Mumbai city. Health Administrator. 2006;15(1):72-79.
27. Sumaiya Rahman, Shohel Ahmed. To assess the tuberculosis situation in urban and rural areas of Bangladesh with special emphasis on the facility of treatment scenarios. Pub Health Res. 2017;7(3):73-77.
28. Shen X, Xia Z, et al. Tuberculosis in an urban area in China: differences between urban migrants and local residents. PLoS ONE. 2012;7(11):e51133. doi:10.1371/journal.pone.0051133.
29. Paquette K, Cheng MP, Kadatz MJ, et al. Chest radiography for active tuberculosis case finding in the homeless : a systematic review and meta-analysis. Int J Tuberc Lung Dis. 2014;18(10):1231-6.
30. Lesnic E. The assessment of different tuberculosis-related features in Moldova regions. Moldovan Medical Journal. 2017;60(3):3-10.
31. Lesnic E, Todorico L, Niguleanu A, et al. The socio-economic risk factors of tuberculosis in condition of a high migration in trans-border region. [Bukovinian Medical Herald] (Chernivtsy). 2016;20(4):109-111.
32. Tafuni O, Lesnic E. Condițiile de trai ale bolnavilor de tuberculoză din municipiul Chișinău în perioada rece a anului [The living conditions of patients with tuberculosis in the city of Chișinău in the cold period of the year]. Sănătate publică, Economie și Management în Medicină (Chisinau). 2016;(3(67)):138-141.
33. Ibiene Tobin-West Ch, Isodje Anastasia. Quality and rural-urban comparison of tuberculosis care in Rivers State, Nigeria. Pan Afr Med J. 2016;24:60. doi:10.11604/pamj.2016.24.60.8497.
34. Dye C, Lönnroth K, Jaramillo E, Williams BG, Raviglione M. Trends in tuberculosis incidence and their determinants in 134 countries. Bull World Health Organ. 2009 Sep;87(9):683-91.
35. Guvernul Republicii Moldova [Government of the Republic of Moldova]. Hotărârea nr.1171 din 21.12.2010 privind aprobarea Programului național de control a tuberculozei pentru anii 2011-2015 [Decision no. 1171 of 2010 December 21, on approving the National Tuberculosis Control Program for the years 2011-2015]. Monitorul Oficial al Republicii Moldova. 2010 Dec 31;(259-263):art. no. 1316. Romanian.
36. World Health Organization. Treatment of tuberculosis: guidelines. Geneva: WHO; 2014. 420 p.
37. Abubakar I, Crofts J, et al. Investigating urban-rural disparities in tuberculosis treatment outcome in England and Wales. Epidemiol Infect. 2008;136(1):122-127.
38. Almeida D, Rodrigues C, Udwadia Z, et al. Incidence of multidrug-resistant tuberculosis in urban and rural India and implications for prevention. Clin Infect Dis. 2003;36(12):e152-e154. doi: 10.1086/374931.
39. Centrul Național de Management în Sănătate al Republicii Moldova [National Center for Health Management of the Republic of Moldova]. Evaluarea costului catastrofal asociat tuberculozei drogrezistente în gospodăriile din Republica Moldova [Assessment of catastrophic cost associated with multidrug resistant tuberculosis in the Republic of Moldova]. Chișinău; 2017. Romanian.
40. Lobo MA, Paiva Neto HC, Bello LA. Spatial distribution of pulmonary tuberculosis in urban areas: a case from Belém, Brazil. Acta Biomedica Brasiliensia. 2016;7(2):1-14.
41. World Health Organization. The global plan to stop TB 2011-2015: transforming the fight towards elimination of tuberculosis. Geneva: WHO; 2010. 96 p.

DOI: 10.5281/zenodo.2590003
UDC: 618.19-006.6-074/-078:577.27

Tumor associated macrophages in breast cancer

Ecaterina Carpenco, MD, Assistant Professor

Department of Histology, Cytology and Embriology, Laboratory of Morphology
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova

Corresponding author: ecaterina.carpenco@usmf.md
Manuscript received February 01, 2019; revised manuscript March 04, 2019

Abstract

Background: Cancer research was focused on the studying of proper tumor cells for a long time. Despite the huge progress, there are still a lot of questions, that's why new molecular markers must be identified. These could reveal new information about tumorigenesis.
Material and methods: 15 cases of ductal invasive breast carcinomas have been analyzed and researched on tumor associated macrophages via immunohistochemistry. CD68 was used as a macrophage marker and $CD68^+$ cells were evaluated in tumor nest and peritumoral area, as well as hormone receptors (ER, PR) and HER2 protein.
Results: Most of tumors (10 cases out of 15/ 66.7%) were moderately differentiated (G2). The mean and std. error of mean of intratumoral $CD68^+$ cells were 2.0±0.2, of peritumoral $CD68^+$ cells – 1.4±0.2. Intratumoral $CD68^+$ cells registered higher scores than those located in the peritumoral area.
Conclusions: $CD68^+$ cells are more likely to be present in the tumor nest rather than in the peritumoral area. This research did not establish any significant correlations between intratumoral and peritumoral $CD68^+$ cells and patients' age, tumor grade, expression of ER and PR. The content of peritumoral $CD68^+$ cells inversely correlated with the number of $HER2^+$ carcinoma cells.

Key words: breast cancer, tumor associated macrophages, CD68, ER, PR, HER2.

Introduction

According to Globocan 2018, there were reported 2 088 849 of new cases of breast cancer (11.2% incidence), being the most frequent neoplasia in women and the leading cause of female cancer related death [1].

Treatment strategy depends on tumor progression and its morphological type. Molecular classification, based on the evaluation of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cytokeratin 5 (CK5) and epidermal growth factor receptor (EGFR), includes Luminal A, B, HER2 and triple-negative subtypes. This classification had a huge impact on individual prognosis, personalized treatment and response to the therapy. Despite the fact that many things about breast cancer have been discovered there are still a lot of questions. Molecular classification does not comprise and does not describe perfectly all of the entities, that's why new molecular markers must be identified. These could reveal new information about tumorigenesis [2, 3].

Cancer research was focused on the studying of proper tumor cells for a long time. But cancer cells do not act alone, tumor consists of both malignant and non-malignant elements like macrophages, lymphocytes, mast cells, fibroblasts, connective tissue fibers, nerve fibers. Among these, tumor associated macrophages represent the vast majority, sometimes more than 50%. Their importance should not be underestimated because they are able to control the immune response, cellular mobility and to stimulate/inhibit angiogenesis and lymphangiogenesis. Moreover, macrophages can modulate the drug resistance by various substances secreted into the microenvironment [4, 5].

Macrophages can be differentiated into 2 types depending on the chemical signals coming from the microenvironment: classically activated macrophages (M1) and alternatively activated macrophages (M2). M1 macrophages exhibit antitumoral activity because of their ability to activate type 1 helper T cells (Th1), recognize the cancer cells and phagocytose them. M2 macrophages on the other hand are involved in wound healing where they downregulate the inflammatory reactions, promote angiogenesis, recruit fibroblasts and regulate connective tissue remodeling. Thus, an increased number of M1 macrophages is associated with a lower tumor aggressiveness, whereas an increased number of M2 macrophages stimulates tumor growing and involves a poor prognosis [5, 6, 7].

The aim of this study was to determine the role of tumor associated macrophages in breast cancer pathology. We also researched on the localization of the macrophages (tumor nest/ peritumoral area) by studying the expression of CD68 marker and identified whether there does exist any correlation between $CD68^+$ cells and ER^+, PR^+, $HER2^+$ carcinoma cells. As a result, a single statistically significant negative correlation between $CD68^+$ cells and $HER2^+$ cells was established.

Material and methods

This research included 15 cases of ductal invasive breast carcinomas type NOS (not otherwise specified) collected

at Arad Clinical Hospital, Romania between 2013-2016. Patients ranged between 60 and 83 years old, mean of age being 69.6±2.1. All patients did not undergo chemo- or radiotherapy before surgery. The study was approved by the Ethics Committee of Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, Moldova (no. 33/ 37/ 12.02.2018).

Histological method. Specimens were obtained after surgery and fixed in 10% formalin. After the removal of fixative by washing with tap water, specimens were paraffin embedded (Paraplast High Melt, Leica Biosystems). Paraffin blocks were later used for creation of tissue microarrays by means of TMA Grand Master (3DHISTECH Ltd., Budapest, Hungary). Sections from these blocks were cut by using a Leica RM2245 microtome (Leica Biosystems, Newcastle UponTyne, UK) and mounted on glass slides (Surgipath X-tra Adhesive, Leica Biosystems, Newcastle UponTyne, UK).

Staining was accomplished by Leica Autostainer XL (Leica Biosystems, Newcastle UponTyne, UK). Mayer's hematoxylin (Merck, Germany) and aqueous eosin (Merck, Germany) were used. Slides were mounted automatically (Leica CV5030, Leica Biosystems, Newcastle UponTyne, UK). Tumor histology was reviewed by 3 pathologists and appropriate sections were selected for immunohistochemical stains.

Immunohistochemistry. Immunohistochemical staining was performed automatically by Leica Bond-Max (Leica Biosystems, Newcastle UponTyne, UK). Antigen retrieval was achieved by using of Bond Epitope Retrieval Solution 1 (pH 6) and 2 (pH 9) (Leica Biosystems, Newcastle UponTyne, UK). Primary antibody (ER, PR, HER2, CD68) was followed by 3% hydrogen peroxide (for endogenous peroxidase activity blocking). DAB (3, 3'- diaminobenzidine) was applied as a chromogen substrate for 10 minutes. Mayer's hematoxylin was the additional dye used for counterstaining (5 minutes). Then sections were placed in absolute alcohol for 5 minutes, dried and clarified in benzene for 5 minutes. Lastly, slides were mounted automatically (Leica CV5030, Leica Biosystems, Newcastle UponTyne, UK) using an ENTELLAN–like mounting medium (Leica CV Mount, Leica Biosystems, Newcastle UponTyne, UK) [8].

Methods of quantification. Hormone receptors (ER and PR) were evaluated according to Allred score. This score accounts the percentage of cells that test positive for hormone receptors, along with the intensity of staining [9]. HER2 protein was appreciated according to the recommendations of American Society of Clinical Oncology [10, 11].

CD68 is a glycoprotein found in lysosomes and to a lesser extent on the cell membrane. It is used for identification of macrophages, other members of the mononuclear phagocyte lineage and to describe the neoplasm of myeloid and macrophage/monocyte origin. Macrophages should show a moderate to strong cytoplasmic staining reaction [2, 12]. Quantification of brown stained macrophages was done by means of Axio Imager A2 microscope (Carl Zeiss, Germany). Sections were initially analyzed at a ×100 magnification in order to determine the most intensely stained regions. Then we analyzed intratumoral and peritumoral stroma, 2 microscopic fields for each one, at a ×200 magnification. The following score was applied: **"0"** – no staining observed; **"+1"** – up to 25% of $CD68^+$ cells; **"+2"** – 25-75% $CD68^+$ cells; **"+3"** – more than 75% $CD68^+$ cells. "+1", "+2" and "+3" were considered positive scores. The final value was the arithmetic mean of the values for the two fields.

Data analysis. A MS Excel 2010 database was used to store the data that were statistically analyzed by applying WinSTAT software. We considered a *p*-value of less than 0.05 as significant.

Results

Most of tumors (10 cases out of 15/66.7%) were moderately differentiated (G2). The other 5 cases (33.3%) were poorly differentiated (G3).

The mean and std. error of mean of ER^+ cells were 1.9±0.2; of PR^+ cells – 0.9±0.2; of $HER2^+$ cells – 0.5±0.2. Median's values were: "2" for ER, "1" for PR, "0" for HER2 (tab. 1).

Table 1

Patients' age, tumor grade, values of ER, PR, HER2, $CD68^+$ cells

	Age	Grade	ER	PR	HER2	CD68it	CD68pt
Valid cases	15	15	15	15	15	15	15
Mean	**69.6**	**2.3**	**1.9**	**0.9**	**0.5**	**2.0**	**1.4**
m	**2.1**	**0.1**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**
Minimum	60	2	0	0	0	0	1
Maximum	83	3	3	2	2	3	3
Median	**67**	**2**	**2**	**1**	**0**	**2**	**1**

Note: CD68it – intratumoral $CD68^+$ cells; CD68pt – peritumoral $CD68^+$ cells; m – Std. error of mean.

Table 2

The comparative analysis of patients' age, tumor grade and molecular markers

	Age	Grade	ER	PR	HER2	CD68it	CD68pt
Age							
rs		0.31	0.26	0.09	-0.06	0.22	-0.02
P		0.13	0.18	0.38	0.42	0.22	0.47
Grade							
rs	0.31		0.19	-0.30	0.00	0.40	-0.19
P	0.13		0.25	0.14	0.50	0.07	0.25
ER							
rs	0.26	0.19		0.39	0.32	0.18	-0.30
P	0.,18	0.25		0.07	0.12	0.25	0.14
PR							
rs	0.,09	-0.30	0.39		0.42	0.23	-0.24
P	0.38	0.14	0.07		0.06	0.20	0.20

HER2							
rs	-0.06	0.00	0.32	0.42		-0.25	**-0.53**
P	0.42	0.50	0.12	0.06		0.19	**0.02**
CD68it							
rs	0.22	0.40	0.18	0.23	-0.25		0.33
P	0.,22	0.,07	0.25	0.20	0.19		0.11
CD68pt							
rs	-0.02	-0.19	-0.30	-0.24	**-0.53**	0.33	
P	0.47	0.25	0.14	0.20	**0.02**	0.11	

Note: Age – patients' age; Grade – tumor grade; ER – estrogen receptor; PR – progesterone receptor; HER2 – human epidermal growth factor receptor 2; CD68it – intratumoral $CD68^+$ cells; CD68pt – peritumoral $CD68^+$ cells; rs – Spearman rank correlation; p – statistical significance. Statistically significant cases were marked in **bold**.

The mean and std. error of mean of intratumoral $CD68^+$ cells were 2.0±0.2; median was "2". In case of peritumoral $CD68^+$ cells, the mean and std. error of mean were 1.4±0.2; median was "1". Intratumoral $CD68^+$ cells registered higher scores than those located in the peritumoral area.

The comparative analysis of intratumoral, peritumoral $CD68^+$ cells and molecular markers (ER, PR, HER2) revealed a single statistically significant negative correlation between the expression of HER2 protein and peritumoral $CD68^+$ cells (rs = -0.53, p<0.02), (tab. 2).

Discussion

Breast cancer is a heterogeneous disease in terms of histology, therapeutic response, dissemination patterns to distant sites, and patients' outcomes. A plausible explanation for this scenario is, in part, that we still lack a complete picture of the biologic heterogeneity of breast cancers with respect to molecular alterations, treatment sensitivity, and cellular composition. Importantly, this complexity is not entirely reflected by the main clinical parameters (age, node status, tumor size, histological grade) and pathological markers (ER, PR and HER2), all of which are routinely used in the clinic to stratify patients for prognostic predictions and to select treatments [13]. In this study we partially approached macrophages, just one actor of this complex scenario.

CD68 is a pan-macrophage marker used as a marker for tumor associated macrophages. However, CD68 recognizes both tumoricidal M1 (classically activated) and anti-inflammatory M2 (alternatively activated) macrophages [5]. The term of macrophage activation was introduced by Mackaness in the 1960s in an infection context to describe the antigen-dependent, but non-specific enhanced, microbicidal activity of macrophages toward *BCG* (bacillus Calmette-Guerin) and Listeria upon secondary exposure to the pathogens [14]. M1 macrophages, or classically activated macrophages, are aggressive and highly phagocytic, produce large amounts of reactive oxygen and nitrogen species, and promote a Th1 response [11]. This is a macrophage response usually seen during microbial infections. M1 macrophages secrete high levels of IL-12 and IL-23, two important inflammatory cytokines. IL-12 induces the activation and clonal expansion of Th17 cells, which secrete high amounts of IL-17, and thus contribute to inflammation. In the context of cancer, classically activated macrophages are thought to play an important role in the recognition and destruction of cancer cells, and their presence usually indicates good prognosis. For a long time, M1 macrophages were thought to be the only functional macrophages and that anti-inflammatory molecules were inhibitory to their function. Now we understand that anti-inflammatory molecules did not inhibit macrophage function but provided an alternative activation of macrophages. M2 macrophages, or alternatively activated macrophages, are anti-inflammatory and are not capable of efficient antigen presentation. Expression of IL-10 by M2 macrophages promotes a Th2 response, and Th2 cells, in turn, upregulate the production of IL-3 and IL-4. IL-4 is an important cytokine in the healing process because it contributes to the production of the extracellular matrix. The tumor microenvironment significantly affects macrophage polarization. The process of polarization can be diverse and complicated because of the complex environment of IL-10, glucocorticoid hormones, apoptotic cells, and immune complexes that can interfere with the function of innate immune cells [15].

According to Weagel et al., the tumor mass contains a great number of M2-like macrophages and these can be used as a target for cancer treatment. Reducing the number of M2s or polarizing them towards an M1 phenotype can help destroy cancer cells or impair tumor growth [15]. Unfortunately, our study did not reveal which macrophages have registered higher scores in the intratumoral area: M1s, being able to destroy the tumor or maybe M2 macrophages, promoting tumor growth and repair?

CD163 could be the answer to our questions. CD163 is a scavenger receptor upregulated by macrophages in an anti-inflammatory environment and regarded as a highly specific monocyte/macrophage marker for M2 macrophages. This research could be continued by studying the specific M1 and M2 macrophages' markers, the localization of cells rather than merely the presence of tumor associated macrophages [6]. Results must be confirmed in a higher number of cases.

Conclusions

$CD68^+$ cells are more likely to be present in the tumor nest rather than in the peritumoral area. This research did not establish any significant correlations between intratumoral and peritumoral $CD68^+$ cells and patients' age, tumor grade, expression of ER and PR. The content of peritumoral $CD68^+$ cells inversely correlate with the number of $HER2^+$ carcinoma cells.

References

1. International Agency for Research on Cancer. GLOBOCAN 2018: Cancer incidence and mortality statistics worldwide and by region. Lyon: IARC; 2019 [cited 2019 Feb 12]. Available from: https://gco.iarc.fr/today/data/factsheets/cancers/20-Breast-fact-sheet.pdf.
2. Qiu SQ, Waaijer S, Zwager MC, de Vries EGE, van der Vegt B, Schröder CP. Tumor-associated macrophages in breast cancer: Innocent bystander or important player? Cancer Treat Rev. 2018;70:178-89.
3. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010;141(1):52-67. doi: 10.1016/j.cell.2010.03.015.
4. Schoppmann SF, Birner P, Stöckl J, Kalt R, Ullrich R, Caucig C, et al. Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. Am J Pathol. 2002;161(3):947-956. doi: 10.1016/S0002-9440(10)64255-1.
5. Medrek C, Pontén F, Jirström K, Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. BMC Cancer. 2012 Jul 23;12:306. doi: 10.1186/1471-2407-12-306. PubMed PMID: 22824040; PubMed Central PMCID: PMC3414782.
6. Choi J, Gyamfi J, Jang H, Koo JS. The role of tumor-associated macrophage in breast cancer biology. Histol Histopathol. 2018 Feb;33(2):133-145. doi: 10.14670/HH-11-916. Epub 2017 Jul 6. Review. PubMed PMID: 28681373.
7. Schoppmann SF, Fenzl A, Nagy K, Unger S, Bayer G, Geleff S, Gnant M, Horvat R, Jakesz R, Birner P. VEGF-C expressing tumor-associated macrophages in lymph node positive breast cancer: impact on lymphangiogenesis and survival. Surgery. 2006 Jun;139(6):839-46. PubMed PMID: 16782443.
8. Raica M, Cîmpean AM, Ceausu RA, Fulga V, Nica C, Rudico L, Saptefrati L. Hormone receptors and HER2 expression in primary breast carcinoma and corresponding lymph node metastasis: do we need both? Anticancer Res. 2014 Mar;34(3):1435-40. PubMed PMID: 24596391.
9. Breastcancer.org. [Internet]. Ardmore: The Organization; c2019 [cited 2019 Feb 10]. How to read hormone receptor test results. Available from: https://www.breastcancer.org/symptoms/diagnosis/hormone_status/read_results
10. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF; American Society of Clinical Oncology.; College of American Pathologists.. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol. 2013 Nov 1;31(31):3997-4013. doi: 10.1200/JCO.2013.50.9984. Epub 2013 Oct 7. PubMed PMID: 24101045.
11. Fulga V. Eterogenitatea tipurilor histologice a cancerului de san: origini, cauze si aplicare practica [The heterogeneity of histological types of breast cancer: origins, causes and practical application]. Chisinau: Impressum; 2016. 178 p. Romanian.
12. Pernick N. CD Markers: CD68. PathologyOutlines.com. [cited 2019 Jan 23]. Available from: http://www.pathologyoutlines.com/topic/cdmarkerscd68.html
13. Prat A, Perou CM. Deconstructing the molecular portraits of breast cancer. Mol Oncol. 2011;5(1):5-23. doi: 10.1016/j.molonc.2010.11.003.
14. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 2014;6:13. Published 2014 Mar 3. doi:10.12703/P6-13.
15. Weagel E, Smith C, Liu PG, Robison R, O'Neill K. Macrophage polarization and its role in cancer. J Clin Cell Immunol. 2015;6(4):338. doi: 10.4172/2155-9899.1000338.

Acknowledgements

This work was supported by CNFIS-FDI-2018-0459 grant offered by Romanian Ministry of Education and Research. Special thanks to the Department of Microscopic Morphology/ Histology, Angiogenesis Research Center, Victor Babes University of Medicine and Pharmacy, Timisoara, Romania.

DOI: 10.5281/zenodo.2590005
UDC: 615.375.035.2:618.2/.3

Inoffensivity of imupurin in pregnancy

Ina Pogonea, MD, PhD, Associate Professor; ***Carolina Catcov,** MD, Assistant Professor;
Victor Ghicavii, MD, PhD, Professor, Corresponding Academician

Department of Pharmacology and Clinical Pharmacology
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova

*Corresponding author: carolina.catcov@usmf.md
Manuscript received February 01, 2019; revised manuscript March 04, 2019

Abstract

Background: Determination of imupurin inoffensivity on embryogenesis, organogenesis and teratogenesis in rats.
Material and methods: The study of immupurin's safety during pregnancy was performed on 60 rats, divided into 4 groups. Animals from the control group were given 2 ml of physiological solution (NaCl, 0.9%), and those from the experimental groups – 2 ml of imupurin suspension, internally, 1000 mg / kg in different periods of pregnancy, to investigate embryotoxic, teratogenic and fetotoxic effect. The fetuses were monitored during the postnatal period, from birth to the age of 2 months, with appreciation of the physical development, the behavior and coordination of newborn movements, the evolution of body mass in dynamics, the teeth eruption, the appearance of the hair cover, the opening of the eyes, the ability to feed individually after removal from the female.
Results: The studies have shown that the pre-implantation and post-implantation indices in the control group were 4.1 and 3.8 respectively, and in experimental groups were 4.4 and 3.3. The number of live fetuses in the investigated groups was 12.1±1.5, which did not differ from the control group, whose live fetuses were 12.3±1.9. Postnatal period indices (teeth eruption, hair cover, and eye opening) were similar in all investigated groups and corresponded to the age of the rats.
Conclusions: Imupurin has been shown to have no embryotoxic, fetotoxic and negative effects on the postnatal period and may be recommended in pathologies accompanying pregnancy.

Key words: entomotherapy, imupurin, fetotoxicity, pregnancy embryotoxicity.

Introduction

Pathologies of allergic and immune genesis are nowadays widespread due to advanced technologies, also of using synthetic substances in the pharmaceutical, food and agricultural industries, and immunomodulatory drugs become a goal for many researchers [1, 2]. The fact that insects can produce substances that modulate the basic mechanisms of human immunity has been the basis for the synthesis from insects of new preparations with different pharmacological properties such as entomological preparations [3, 4, 2, 5], which by their lipoprotein and polysaccharide composition can be considered as compounds with an important immunostimulatory potential [6, 7].

Imupurin, an entomological preparation obtained from butterflies, species *Lepidoptera*, the *Lemantria* family [6, 3, 4] , due to the immunogenic amino acids and oligopeptides from its composition possesses marked immunomodulatory properties that are capable to stimulate immune system [7,8,9] . It is recommended for complex treatment of pathologies developed due to immune system disorders [8, 9, 10].

Pregnancy, physiological condition characterized by additional efforts of woman's immunity [11], which on the one hand must adapt to the new conditions of embryo and fetus presence, on the other hand, the immune system must provide effective protection against infections or reactivation of existing pathologies of the mother [12]. It is important to remember that pregnancy pathologies and a range of illnesses occurring during pregnancy evolve with more peculiarities and require medical treatment that needs double attention because the drugs can act on both the mother and the fetus [13]. In these situations, preparations which do not possess embryotoxic, fetotoxic or teratogenic effects are preferred.

Based on the above, we intend to investigate the embryotoxic, fetotoxic and teratogenic properties of imupurin in view of its inoffensivity in pregnancy [14].

Material and methods

Investigations of embryotoxicity, fetotoxicity and teratogenicity of imupurin were performed according to contemporary recommendations [15,16].

In the study were used 60 matured, reproductive age albino rats with a mass of 170-230 g, divided into four groups.

The studied substance (imupurin) was given endogastral, 1000 mg per kg. The frequency of administration was once daily at the same time, according to the following schedule: Group No 1 was intact and served as a control; they were given the saline sol. NaCl, 0.9% – 2 ml, internal use. The females from the group No 2 received imupurin from the 1st the day of pregnancy to the 6th, group No 3 – from the 6th day to the 16th, group No 4 – from the 16th day to the 20th day (tab. 1). Animals were monitored daily.

Table 1

Groups	Days of administration			
	Amount of the administered substance (mg) in suspension in 2 ml volume	1-6	6-16	16-20
I (control) (24 rats)	2 ml of saline solution, NaCl, 0.9%	+	+	+
II (12 rats)	Imupurin 1000 mg per kg	+	-	-
III (12 rats)	Imupurin 1000 mg per kg	-	+	-
IV (12 rats)	Imupurin 1000 mg per kg	-	-	+

Postnatal development of newborns was studied 24 hours from birth to 2 months old, and were appreciated the physical development, newborns behavior and movements coordination, evolution of body mass in dynamics, teeth eruption, the appearance of the hair cover, the opening of the eyes, the ability to feed individually after removal from the female 25 days after birth.

Results

The supervision of animals during experiences has not found deviations of behavior during pregnancy in females included in the study, compared with the control group. After imupurin administration, the rats became more active for 10 minutes, with subsequent behavioral restoration, feeding and use of water was common without differentiation from the control group. Examination of the skin, mucous membranes and hair cover did not show pathological changes. Once every 7 days they were weighed. The body weight of females on the average increased to 30 g in all groups. On the 20th day the animals were euthanized by dislocation of the cervical vertebrae and were determinated the following indices: embryonic mortality in pre- and post-implantation periods, developmental malformations, general retention of the development of the fetuses. Pre-implantation mortality was determined by the difference between the number of yellow bodies in the ovaries and the number of places implanted in the uterus. Later we determined the post-implantation index – by the difference between the number of implanted places and the number of live embryos.

The analysis of the investigated indices did not reveal significant deviations in the females from imupurin lots compared to the control regarding the number of yellow bodies, the number of implant sites, the number of live and dead females, the number of resorptions (tab. 2). No significant differences were found between experimental and control groups in mortality determination in the pre-implantation and post-implant period.

The number of newborns from the females who received imupurin corresponds to the number of fetuses born from the control group's females.

Table 2

Influence of imupurin on the embryotoxicity, teratogenicity and fetotoxicity parameters

Investigation indices	Imupurin	Control
Number of pregnant females	26	30
Number of yellow bodies	13.3 ± 1.6	13.9 ± 2.4
Number of implant sites	12.7 ± 1.3	13.3 ± 2.3
Number of live fetuses	12.8 ± 1.5	12.7 ± 1.9
Number of resorbtions	0.2 ± 0.4	0.3 ± 0.5
Number of dead fetuses	0.2 ± 0.4	0.3 ± 0.7
Mortality at preimplantation stage (%)	4.4	4.1
Mortality at postimplantation stage (%)	3.3	3.8
Fetuses weight	3.2 ± 0.29	3.3 ± 0.25

The weight of the fetuses in the investigated groups did not differ from the control and constituted an average of 3.2±0.3. Also, there were no significant differences in the number of fetuses per female, which was 12.1±1.5 in the study groups which received imupurin, once daily, compared to the control group. The mortality cases of the fetuses were few – 3.3% in the investigated group and 3.8% in the control group. Mortality during pre-implantation and post-implantation is not statistically significant (tab. 2).

In all study groups were not detected disturbances in the development of embryo-amniotic fluid which was transparent; fetal membranes were normally developed, well vascularized, without sclerosis. At the opening of the fetal membranes and the umbilical cord section, appeared the spontaneous breath of the fetuses. The skin was rosy, eyes and ears – covered. No external abnormalities of the skeleton and internal organs have been detected in the morphological research of the fetuses.

The postnatal behavior of rats undergoing study did not differ from those in the control group. After euthanasia of newborns, development abnormalities were not detected.

All rats were removed from natural food on the 26th day of life. Adaptation to artificial foods was in the early hours. Analysis of postnatal indices did not reveal significant deviations regarding the detachment of the ear pavilion, the teeth eruption, the appearance of the hair cover, the opening of the eyes (tab. 3).

Table 3

Appearance indices

The studied indices	Time of appearance
Detachment of the ear pavilion	Day 4
Tooth eruption	Day 8
The appearance of the hair cover	Day 11
Opening of eyes	Day 15

Conclusions

1. Entomological preparation did not influence the behavior of pregnant females throughout the pregnancy.

2. Imupurin did not show embryotoxic, teratogenic and fetotoxic properties.

3. The postnatal development of fetuses born to females which have been given imupurin was similar to the animals in the control group.

References

1. Catcov C, Pogonea I, Ghicavii V. Artropodele – o sursă bogată de medicamente [Arthropodes – a rich source of medicines]. Sănătate Publică, Economie și Management în Medicină (Chisinau). 2018;(4(78)):93-96. Romanian.
2. Ciuhrii M. Fauna în tratarea și alimentarea omului [Fauna in human treatment and alimentation]. 2006. Romanian.
3. Bacinschi N, Ghicavii V, Pogonea I. Peptide din insecte – sursă de preparate cu acțiune antimicrobiană [Peptides from insects – source of preparations with an antimicrobial action]. Revista de Știință, Inovare, Cultură și Artă „Akademos" (Chisinau). 2013;(4(31)):94-99. ISSN 1857-0461. Romanian.
4. Bacinschi N, Ghicavii V, Pogonea I. Peptide din insecte cu acțiune antivirală și antitumorală [Peptides from insects with antiviral and antitumoral action]. Anale Științifice ale USMF „Nicolae Testemițanu" (Chisinau). 2012;1:196-201. ISSN 1857-1719. Romanian.
5. Costa-Neto EM. Entomotherapy, or the medicinal use of insects. J Ethnobiol. 2005;25(1):93-114.
6. Bacinschi N. Preparate entomologice cu proprietăți imunotrope [Entomologic remedies with immunotrop properties]. Curierul Medical (Chisinau). 2010(6):3-8. Romanian.
7. Ghicavii V, Pogonea I, Bacinschi N, Ghinda S. Acțiunea entomopreparatelor asupra imunității umorale și celulare [Action of entomological drugs on humoral and cellular immunity]. Anale Științifice ale USMF „Nicolae Testemițanu" (Chisinau). 2013;1:236-239. ISSN 1857-1719. Romanian.
8. Ghicavii V, Pogonea I, Bacinschi N, et al. Optimizarea tratamentului complex al unor imunodeficiențe [Optimizing the complex treatment of some immonodeficiencies]. Chișinău: Medicina; 2011. 24 p.
9. Ivanov E, Pogonea I, et al. Evolutia maladiei HIV/ SIDA pe fondal de tratament cu un nou preparat entomologic – imupurin [Evolution of AIDS on the background of treatment with a new entomological preparation – imupurin]. Anale științifice ale USMF „Nicolae Testemitanu" (Chisinau). 2010;1:343-346. Romanian.
10. Pogonea I, Ghicavii V, Ghinda S. Noi medicamente imunocorectoare de origine entomologică [New immunocorective entomological drugs]. Buletinul Academiei de Științe a Moldovei. Științe Medicale. 2016;(1(50)):205-208. ISSN 1857-0011. Romanian.
11. Bonney EA. Immune regulation in pregnancy: a matter of perspective? Obstet Gynecol Clin North Am. 2016 Dec;43(4):679-698.
12. Rychlik KA, Sillé FCM. Environmental exposures during pregnancy: Mechanistic effects on immunity. Birth Defects Res. 2019 Feb 1. doi: 10.1002/bdr2.1469.
13. Malm H, Ellfolk M. Which drugs can be used during pregnancy? Duodecim. 2016;132(19):1781-9.
14. Konstantinova N, Pogonea I. Study of embryotoxic, fetotoxic and teratogenic properties of entomologic drugs. In: MedEspera 2012: 4th International Medical Congress for Students and Young Doctors; 2012 May 17-19; Chisinau; 2012. 274 p.
15. Vogel HG, ed. Drug discovery and evaluation: pharmacological assays. 3rd ed. Berlin: Springer; 2008. 2071 p. ISBN: 978-3-540-70995-4.
16. Habriev RU, ed. Rukovodstvo po experimental'nomu (doklinicheskomu) izucheniiu novykh farmakologicheskikh veshchestv [Manual on experimental (preclinical) study of new pharmacological substances]. 2nd ed. Moscow: Meditsina; 2005. 832 p. Russian.

DOI: 10.5281/zenodo.2590007
UDC: 618.291-07

Informativity of perinatal medical card in fetal growth assessment

*Eugenia Bogonovschi, MD, Undergraduate Student; Ion Bologan, MD, PhD, Associate Professor

Department of Obstetrics and Gynecology No 1, Nicolae Testemitsanu State University of Medicine and Pharmacy Chisinau, the Republic of Moldova

*Corresponding author: bogonovschi.eugenia@gmail.com
Manuscript received January 21, 2019; revised manuscript March 04, 2019

Abstract

Background: Failure to provide antenatal information for pregnant women as well as to complete the perinatal medical records at each visit may have negative effects on the child development.
Material and methods: A retrospective documentary review study was carried out, which included 100 women of fertile age. The questionnaire included 80 questions within the following rubrics: personal information about the patient, obstetrical-gynecological medical history, and perinatal medical card.
Results: The patients were divided into the following age groups <20 years – 10 patients (10.0%), 20-30 years – 67 patients (67.0%), 31-40 years – 21 patients (21.0%) and > – 2 patients (2.0%). The recommended weight gain range is ≤12 kg, whereas 59 patients (59%) reported the highest weight gain ≥ 20 kg and others showed a mean range of 12-20 kg. The assessment of parity's effects on fetal weight proved that multiparous women give birth to heavyweight newborns. Women in their second pregnancies make up 62%. Vaginal and cesarean births were registered in 88% and 12% of cases, respectively. Spontaneous abortions were reported in 20%, abortions on demand – 21%, and premature births in 5% of cases. The perinatal medical records were fully completed in 49.0% and partially – in 51.0% of cases.
Conclusions: The amount of perinatal medical card fulfillment has reached the lowest level, including the "gravidogram" that refers to the fetal growth charts.
Key words: gravidogram, fetal biometrics, perinatal medical card.

Introduction

Pregnancy is a normal, natural and healthy phenomenon in a woman's life. Both the pregnant woman and her family are responsible for her physical and emotional condition [1, 2, 3, 4]. The routine prenatal care may include a series of interventions designed to ensure an optimal fetal development. Pregnant women are provided with screening, prophylaxis and counseling. The medical care provided to the pregnant woman and her fetus throughout pregnancy is essential to prevent the early occurrence of any circumstances that might affect pregnancy outcomes so that they can be treated and monitored.

A mandatory standard for antenatal care is the Perinatal Medical Card (Form 113 / e), approved by Order of the Ministry of Health of the Republic of Moldova No 828, dated of 2011 October 31, that refers to "the approval of the Primary Medical Record Forms" [3, 4, 5]. The perinatal medical card is an evidence-based record book that is provided free of charge in the first prenatal visit [2, 3, 4]. The perinatal medical card is fulfilled by the family doctor or an obstetrician-gynecologist. The pregnant woman keeps the perinatal medical card that will be completed at each medical check-up throughout the entire gestational period [5, 6, 7, 8]. Therefore, the proper assessment of the fetal intrauterine growth is crucial for antenatal care. Fundal height measurement (FHM) has a medium diagnostic value in specifying fetuses of a small gestational age (SGA). The steady recording of FHM on the gravidogram increases the sensitivity and specificity of the method [5, 6, 9]. Ultrasound biometry is indicated in case of a suspected intrauterine growth restriction (IUGR), based on both FHM and gravidogram. Thus, the ultrasound parameters for diagnosis of IUGR are as follows: bi-parietal diameter (BPD), cranial circumference (CC), abdominal circumference (AC), and femoral length (LF). AC is the most advanced parameter in detecting IUGR of the fetus with a sensitivity of 61% and a specificity of 95%. FL is a prognostic indicator in severe cases of intrauterine growth restriction of vascular origin [5, 6, 7]. Each pregnant woman will undergo three ultrasound scans: at 12-14 weeks, 18-22 weeks, and 30-32 weeks [8]. Due to these examinations, the necessary ultrasound parameters for fetal development can be assessed within the given gestational term [5, 6, 7, 8]. A dynamic image ultrasonography of the fetal biometric parameters will be carried out additionally, in case if deviation from normal physical growth is recorded throughout the pregnancy and during the mandatory visits [10, 11]. This may precisely determine failure of a fetus to reach its pre-determined growth potential due to insufficient kinetics of intrauterine growth or abnormality resulting from the maternal-fetal disorders [12].

Preconception care is of great importance since it determines both the pregnancy outcomes and the health of the future child [10, 11]. It has been recognized that constant monitoring of physiological changes during pregnancy helps to prevent complications by early detection and emergency treatment, which are essential for maintenance of the pregnancy as a normal physiological process [13].

The purpose of this study is to assess fetal growth by using standardized fetal growth charts related to the gestational age, as well as the study results via FHM and fetal biometric parameters from ultrasound data.

Material and methods

A retrospective documentary study was designed and conducted on 100 women of childbearing age who were recruited from the patients in the postpartum period (2-12 days after birth) and admitted within the Department of Obstetrics No 1 and No 2 in the Municipal Clinical Hospital No 1, from March to July 2018. The questionnaire was impersonal and did not include any rubrics of personal information.

The questionnaire included 80 questions and was structured according to the following rubrics: patient personal data, obstetrical-gynecological anamnesis, perinatal medical cards. Simultaneously, 12 patients with ultrasound results were interviewed, of which only 5 patients presented the fetal biometric parameters assessed during the three antenatal mandatory visits: (BPD, CC, AC, FL) and the estimated fetal weight (EFW) at birth.

Primary data have been processed via Excel (from Microsoft Office 2010).

Results and discussion

According to their age, patients were divided into the following age groups: <20 years – 10 patients (10.0%), 20-30 years – 67 patients (67.0%), 31-40 years – 21 patients (21.0%) and > 40 years – 2 patients (2.0%), (fig. 1).

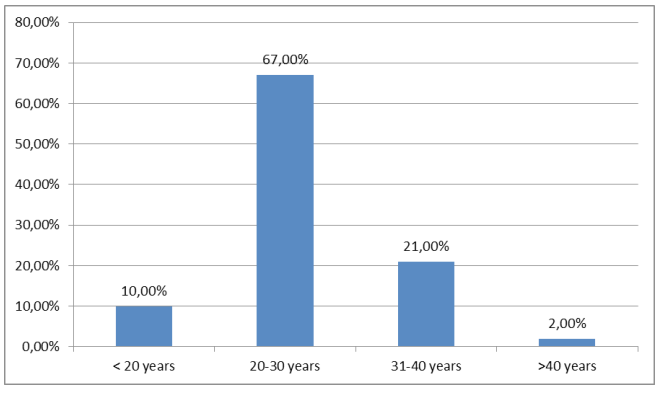


Fig. 1. Distribution of patients into age groups (%).

The distribution of interviewees according to the place of residence is as follows: 76 patients (76.0%) came from urban areas and 24 patients (24.0%) – from rural areas.

Twenty patients (20.0%) were reported to have a previous early miscarriage in anamnesis, 4 patients (4.0%) – late spontaneous abortion, and 5 cases (5.0%) resulted in premature death. Previous abortions on demand were also considered within the study. It should be noted that 21.0% of patients presented a history of abortions on demand (fig. 2).

The present study reported a weight gain within the recommended range (≤12 kg) in 14 patients (14.0%), an increased weight gain between 12 -20 kg was found in 27 patients (27.0%) and an overweight ≥ 20 kg – in 59 patients (59.0%), (fig. 3).

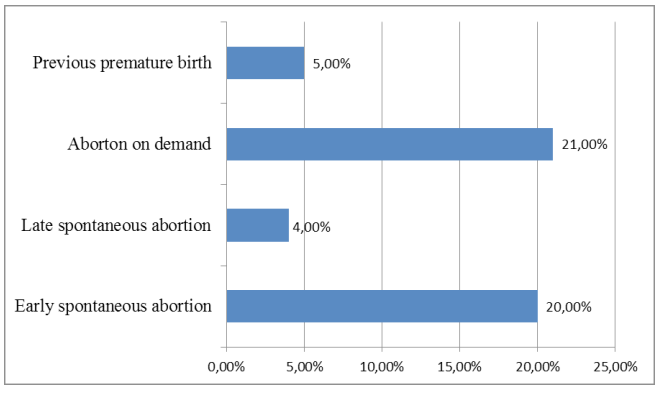


Fig. 2. The most common obstetrical complications in patients (%).

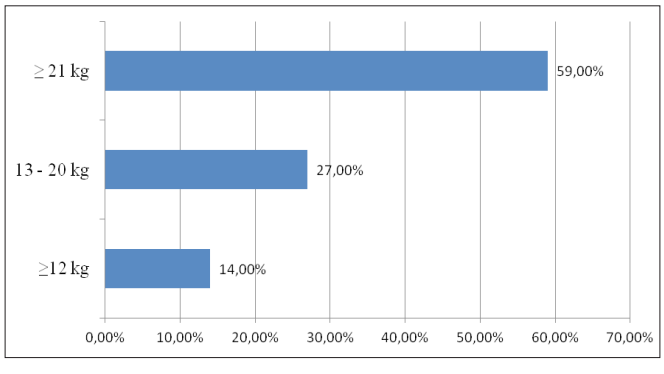


Fig. 3. The weight gain of the patients included in the study during the current pregnancy (%).

Most of the clinical studies that assessed the impact of parity on fetal weight have concluded that multiparous women give birth to heavier weight fetuses. Based on the research, we determined that 34 women were at their first birth (34.0%), 62 women were in their secondary pregnancy (62.0%), and the lowest rate was registered in multiparous women – 4 cases (4.0%), (fig. 4).

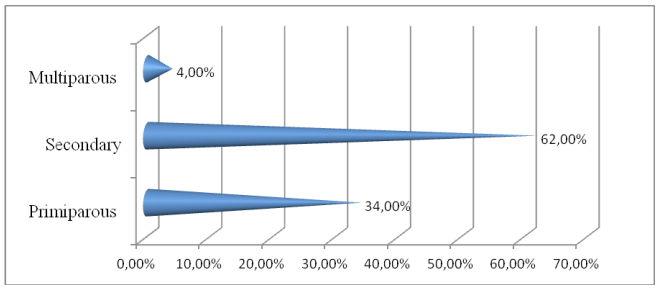
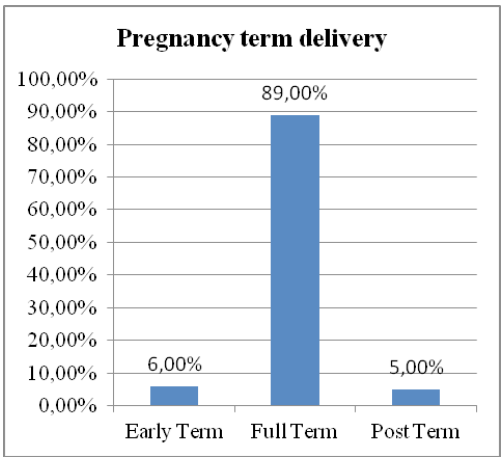


Fig. 4. Influence of parity on fetal weight (%).

According to the study, 88 cases resulted in natural birth and 12 women (12.0%) underwent caesarean section. Assessment of pregnancy term deliveries showed 89 (89.0%) full-term pregnancies, 6 cases (6.0%) resulted in premature childbirth and 5 cases (5.0%) reported birth at the gestational age of 41-42 weeks (fig. 5).



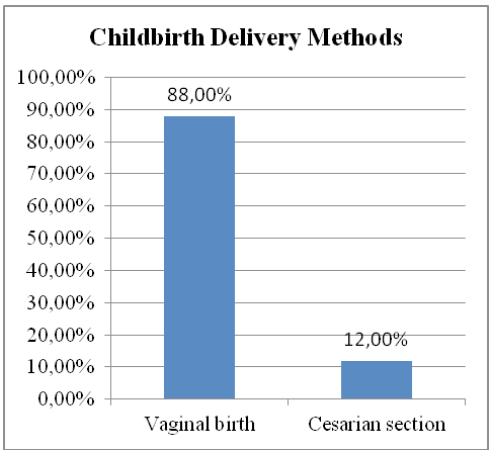


Fig. 5. The delivery term and types of childbirth (%).

The increase of the gestational age may increase both the mass index (MI) and the birth weight [14]. Studies of the fetal birth weight showed the highest rate in 84 (84.0%) newborns with the weight ranging from 2800 g – 3999 g, whereas 9 cases (9.0%) reported a fetal weight greater than 4000 g and 7 cases (7.0%) had less than 2800g at birth (fig. 6).

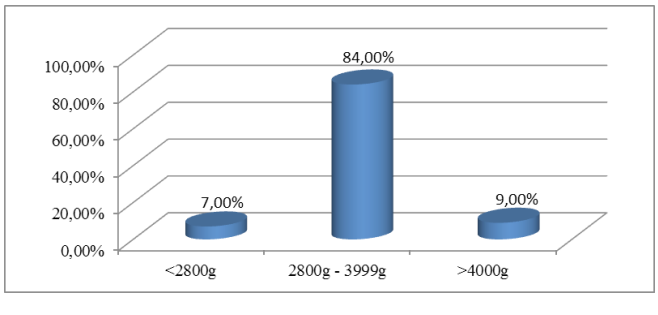


Fig. 6. Fetal birth weight (%).

The perinatal medical card is a mechanism for recording information related to antenatal follow-up and deliveries [15]. According to the amount of the perinatal medical card fulfillment, we found that 49.0% were satisfactorily fulfilled and 51.0% of them were partially or unsatisfactorily filled (tab. 1).

Table 1

The fulfillment of the gravidogram based on the perinatal medical cards used in the study

Gravidogram	Fulfillment of the gravidogram based on the perinatal medical cards	
	Absolute value	Percentage
Complete	85	85.0%
Incomplete	15	15.0%

The Ministry of Health recommends to carry out 6 antenatal care visits during pregnancy, of which 2 are standard visits to the obstetrician-gynecologist. It should be noted that WHO recommends at least 4 antenatal care visits for a normal pregnancy [2, 3, 15].

Table 2 shows the number of visits during pregnancy. It has been determined that most patients – 62 cases (62.0%) reported to have 6 antenatal visits, 26 patients (26.0%) – 5 visits, 10 patients (10.0%) – 4 visits and 2 patients (2.0%) – 3 visits (tab. 2).

Table 2

Number of antenatal care visits

Number of antenatal care visits	Absolute value	Percentage
1visit	-	-
2 visits	-	-
3 visits	2	2.0%
4 visits	10	10.0%
5 visits	26	26.0%
6 visits	62	62.0%

Proper assessment of the fetal intrauterine growth is the key task of the antenatal care [16, 17]. UFH measurement has a diagnostic value in predicting the fetal weight. It is essential to determine the gestational age and identify the abnormal growth rates in pregnant women since these may lead to a reduced infant mortality rate. The gestational age is assessed by using clinical criteria such as uterus size measurement, data regarding the last menstrual period, or ultrasound criteria. The last menstrual period data exhibits a rather high degree of errors since some pregnant women do not remember exactly the time of the last menstrual period or it did not last for 28 days in all cases. UFH shows a low net value since it can erroneously influence the height of the pregnant woman, some abdominal tumors or uterine fibromas [9]. Therefore, the ultrasound indices are still the most relevant ones [13, 16, 17]. The estimated fetal weight in relation to pregnancy term and actual birth weight was difficult to assess due to the lack / incomplete recording of all ultrasound data (BPD, CC, AC, FL) in the perinatal medical book.

The estimated fetal weight in relation to the clinical parameters of UFH and the abdominal circumference (AC) during 36-38 weeks of gestation according to the Iakubov formula: (UFH + AC) / 4 * 100 has also been considered.

This calculation formula has shown higher veracity than other formulas within a national study [9].

The estimated intrauterine weight of the fetus was as-

sessed only in 5 cases, which included the ultrasound parameters evaluated at mandatory antenatal visits and recorded in the medical record cards of these patients. Thus, the intrauterine growth charts were carried out individually for each case, whilst fetal growth assessment was related to 10-90 percentiles. In three cases out of five, the intrauterine weight was found within the 10-90th percentile in relation to the newborn's weight. The ultrasound is considered the method of choice in the diagnosis of fetal IUGR, having a specificity of 80-90%. However, ultrasonography also monitors the intrauterine growth process and allows the assessment of a growth abnormality and its degree of severity. Fetal IUGR is characterized by decreased parameters that define the process of intrauterine development of the fetus (the weight, waist, skull circumference, abdominal, thoracic, subcutaneous tissue and muscular mass). The estimated ultrasound parameters which provide the diagnosis IUGR of the fetus are as following: BPD CC, AC, and FL. Referring to fetal IUGR, the optimal assessment of the individual intrauterine growth rate is being considered, as well as both the mean fetal weight (MFW) at birth and the maternal factor. Therefore, two successive ultrasound examinations within about 14 days apart should be performed for the purpose of a reliable assessment of fetal growth dynamics [13, 16, 17].

The ultrasonic fetal cephalometry that is consecutively performed by assessing BPD (sensitivity – 89% and positive prognosis – 68%) and CC (sensitivity – 63% and positive prognosis-75%) is useful not only for detecting the risk of developing IUGR in fetuses in relation to individual intrauterine growth potential, but it also helps to differentiate between the symmetrical forms of the disease and asymmetrical ones (the method is sensitive in 94% of cases of symmetrical forms and 42% for the asymmetrical ones). Thus, the assessment was not properly performed due to the lack of ultrasound data, which should have been included within the specific rubric of the perinatal medical card.

Conclusions

1. The present study proved that the perinatal medical cards were satisfactorily completed in 49.0% of cases, and partially or unsatisfactorily fulfilled in 51.0% of cases.

2. Based on the study, we found that the pregnancy was completed at 37-40 weeks of gestation in 89 cases (89.0%), whereas the fetal weight ranged from 2800 g – 3999g in 84 cases (84.0%), greater than 4000 g in 9 cases (9.0%) and less than 2800 g in 7 cases (7.0%).

3. The gravidogram was satisfactorily fulfilled in 85.0% of cases within the current study.

4. It has been determined that the accuracy of the fetal weight according to the Iakubov formula made up 49.93%, thus allowing to identify indices under the 10th percentile and the 90th percentile for the gestational age in 30.61% of cases.

5. The study results proved the necessity of interpreting the perinatal fetal weight by using the ultrasound indices and their recording within individualized growth charts. The ultrasound examination should be integrated into the clinical context and for each case apart, as well as the population-specific growth curves should be thoroughly considered.

References

1. Centrul National de Studii pentru Medicina Familiei. Ingrijiri prenatale in sarcina cu risc scazut. Ghid de practica pentru medicul de familie [Routine prenatal care in low-risk pregnancies. Family Practice guidelines]. Bucharest: InfoMedica; 2005. p. 18. Romanian.
2. Stratulat P, Curteanu A. Fortificarea sistemului asistenței serviciilor perinatale de calitate [Enhancement of the quality perinatal-assisted care system]. Buletinul Academiei de Științe a Moldovei. Științe Medicale [Bulletin of the Academy of Sciences of Moldova. Medical Sciences]. 2007;(2/11):244-255. Romanian.
3. Principii de organizare și acordare a asistenței perinatale: Ghidul A Național de Perinatologie [Principles of organization and perinatal care assistance: National Guidelines for Perinatal Care]. 2nd ed. Chisinau; 2006. 165 p. Romanian.
4. [Ministry of Health of the Republic of Moldova]. [Order no. 828 of 2011 October 31, on the approval of the Primary Medical Forms]. [cited 2018 Aug 9]. Available from: http://old2.ms.gov.md/sites/default/files/legislatie/ordinul_nr._828_din_31.10.2011.pdf. Romanian.
5. [National Center for Health Management of the Republic of Moldova]. [Record forms] [cited 2018 Aug 9]. Available from: http://www.cnms.md. Romanian.
6. Rusu C, Stretean A. Ultrasound diagnosis of fetal macrosomia. Acta Medica Transilvanica. 2012;2(2):225-228.
7. Stratulat P, Curteanu A, Carauș T. Experiența utilizării curbelor individualizate de creștere intrauterină a fătului pentru diagnosticul problemelor de creștere fetală [The experience of using individualized intrauterine growth charts for the diagnosis of fetal growth disorders]. Buletinul Academiei de Științe a Moldovei. Științe Medicale [Bulletin of the Academy of Sciences of Moldova. Medical Sciences]. 2009;(1/20):235-240. Romanian.
8. National Institute for Clinical Excellence. Antenatal care: routine care for the healthy pregnant woman. London: NICE; 2003. (NICE Clinical Guideline, no. 6).
9. Corolcova N, Alaeldin M, Rusu C, Raetcaea V. Caracteristica comparativa a metodelor de calcul a masei probabile a fatului [Comparative characteristics of methods of calculating the estimated fetal weight]. Scientific Annals of the Nicolae Testemitsanu State University of Medicine and Pharmacy (Chisinau). 2008;9(5):37-40. Romanian.
10. [Ministry of Health of Romania, Romanian Association of Neonatology]. Determinarea vârstei de gestație la nou-născut [Determining the Age of Gestation in the Newborn]. Bucharest; 2011. 16 p. (Clinical Guide Collection for Neonatology, 01). Romanian.
11. Copilul.ro [Internet]. Bucharest; c2004-2019 [cited 2018 Aug 10]. [The length and weight of the fetus every week of pregnancy]. Available from: https://www.copilul.ro/sarcina/despre-sarcina/Lungimea-si-greutatea-fatului-in-fiecare-saptamana-de-sarcina-a11386.html. Romanian.
12. Alexander GR, Himes JH, Kaufman RB, Mor J, Kogan M. United States national reference for fetal growth. Obstet Gynecol. 1996;87(2):163-8.
13. Kiserud T, Piaggio G, Carroli G, Widmer M, Carvalho J, et al. WHO Fetal growth charts: a multinational longitudinal study of ultrasound biometric measurements and estimated fetal weight. PLOS Medicine. 2017;14(3): e1002284. doi:10.1371/journal.pmed.1002220.
14. Goldstein RF, et al. Association of gestational weight with maternal and infant outcomes: a systematic review and meta-analysis. JAMA. 2017;317(21):2207-2225.
15. Bologan I, et al. The impact of the perinatal medical card within the perinatal care service. In: [Proceedings of the 5th Congress of Obstetrics and Gynecology, with international participation; 2010 Oct 7-8; Chisinau, Republic of Moldova]. Chisinau: Ericon; 2010. p. 8-11.
16. Tucker J, Parry G, Fowlie PW, McGuire W. Organisation and delivery of perinatal services. BMJ. 2004;329(7468):730-732.
17. Gardosi J, Francis A. Controlled trial of fundal height measurement plotted on customised antenatal growth charts. Br J Obstet Gynaecol. 1999;106(4):309-17.

DOI: 10.5281/zenodo.2590011
UDC: 602.9:611.018.46:576.535

An efficient procedure of isolation, cultivation and identification of bone marrow mesenchymal stem cells

*Vitalie Cobzac, MD, PhD Applicant; Andrei Mostovei, MD, PhD, Associate Professor;
Mariana Jian, BioD; Viorel Nacu, MD, PhD, Professor

Tissue Engineering and Cells Cultures Laboratory
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova

*Corresponding author: vitaliecobzac@yahoo.com
Manuscript received February 01, 2019; manuscript revised March 05, 2019

Abstract

Background: Bone marrow mesenchymal stem cells (MSC) have a wide application in domain of Regenerative Medicine. Of a great importance is utilization of a suitable bone marrow extraction technique that can provide a sufficient number of MSC to perform laboratory tests without seriously affecting the health of the laboratory animal. At the same time, before using in researches and clinical application, the MSC needs to be identified.
Material and methods: The study was conducted in rabbits (n = 9), in which, from one iliac bone, by aspiration were taken 3.39 ± 1.27 ml of bone marrow. The nucleated bone marrow cells were separated through centrifugation using concentration gradient. The specific for stem cells culture medium was used, and MSC were multiplied during 2 passages. From the obtained MSC, $1x10^6$ cells were subject to differentiation by chondrocytes lineage for other 20 days. The obtained chondrocytes aggregates were morphologically examined by Hematoxylin–Eosin staining and specific cartilage staining with Safranin O and Toluidine blue/fast green.
Results: There was a strong correlation between the volume of collected bone marrow and the time required to achieve a 70-80% of MSC confluence (p=0.01). Also, the MSC isolated from bone marrow extracted from rabbit iliac bone were differentiated successful on chondrocyte line in all cases, confirmed through the specific cartilage staining with Safranin O and Toluidine blue/fast green (p<0,001).
Conclusions: The volume of 3.39 ± 1.27 ml of bone marrow, harvested from rabbit iliac bone is sufficient to obtain a large number of MSC for the laboratory tests *in vitro* and *in vivo.* As a standard method for MSC identification could be used just the capability of the cells to differentiate in the specialized cell, including chondrocytes.
Key words: mesenchymal stem cells, bone marrow, rabbits, cellular identification, iliac bone, autocells.

Introduction

Mesenchymal stem cells (MSC) are multipotent cells that can differentiate into different cell lines depending on the micromedium in which they are stored [1, 2, 3, 4, 5]. These cells had a wide utilization in researches for regeneration of bone tissue, cartilaginous tissue, tendon, meniscus, degenerative lesions of the locomotor apparatus, nervous system and internal organs [6, 7, 8, 9, 10, 11, 12, 13]. Methods of bone marrow harvesting from laboratory animals for isolation and cultivation of MSC for *in vivo* and *in vitro* tests are diverse. Most common method of bone marrow extractions is slaughtering of the animals with further jet flushing of diaphyses and epiphyses of long tubular bones [14, 15]. However, this method allows to use obtained stem cells for *in vitro* tests and allogeneic transplantation for *in vivo* tests. Another way is the aspiration of bone marrow from the metaphyseal areas of the long tubular bones after perforating the bone with a drill bit [16, 17, 18]. It requires a deep anesthesia and a lot of time for the procedure. This method allows experiments on the animals with autocells, but the deep anesthesia, the surgery that certainly will not be the last and the postoperative recovery period may endanger the experiments success due to suffering or even death of the animal [19]. Another way is performing of a short-term superficial anesthesia with local potency and harvesting the bone marrow from the iliac bone [2, 3], a similar procedure performed in humans [20]. A similar method is nucleated cells separation from iliac crest after resection [21], since this method involves extraction and shredding of a bone piece, followed by trypsin treatment, isolation only of MSC is compromised. It is a great risk that the culture can be contaminated with a large number of osteoblasts and fibroblasts, also the time needed for the procedure is bigger [22].

Material and methods

The study was performed on 9 house rabbits from 4 to 5 months old, 6 females and 3 males, with average weight of 3.78 ± 0.25 kg, in which, from one iliac bone were taken 3.39 ± 1.27 ml of bone marrow, followed by separation of nucleated cells with concentration gradient HiSep LSM 1077 (HiMedia, India). Then, the cells were cultured with mesenchymal stem cell expansion medium HiMesoXL (HiMedia, India) in incubator at 37°C, 5%CO_2 (SMART CELL, Heal Force) during the first 2 passages. In order to demonstrate the presence of MSC in culture, the MSC from the 2nd passage were differentiated into chondrocytes using the chondrocyte differentiation medium HiChondroXL (HiMedia, India). The differentiated chondrocyte aggregates were histologically examined by cartilage specific staining. The research on rabbits received a positive decision at the ethics committee meeting of 14.12.2016, No 31.

Cell media preparation

Culture medium for MSC was obtained by adding 11.4 ml of component B in 500 ml of component A of the medium for the MSC expansion HiMesoXL (HiMedia, India) and 5 ml of antimycotic antibiotic solution (HiMedia, India) [23].

The medium for chondrocyte differentiation is obtained by adding component B to 100 ml of component A of the chondrocyte differentiation medium HiChondroXL (HiMedia, India) and 1 ml of antimycotic antibiotic solution (HiMedia, India) [24].

The medium was prepared according to the manufacturer's instructions, with further sterilisation by filtration with 0.22 µm pores diameter PES filters (Sofra, China) and stored in the refrigerator at 4-8°C.

Isolation and cultivation of MSC from bone marrow harvested from the iliac bone

On the day of bone marrow harvesting, the animals were not fed, after weighing, they were anesthetized by intramuscular injection of 5 mg/kg xylazine and 2 mg/kg diazepam solutions. With a trimmer, the fur was removed from the dorsal part of the basin followed by aseptic processing with betadine and 70% alcohol solution. To potentiate the anesthesia, at the level of the iliac wing were injected 4 ml of 1% lidocaine. Was prepared a 5 ml syringe with 1250-2000 U heparin. A 18 G needle with trocar was used to perforate the first cortical of the iliac bone at the level of iliac wing. After bone perforation, the trocar was extracted and the bone marrow was aspirated with the heparinized syringe [2, 3] (fig. 1). The bone marrow went to laboratory for further processing and the animal was taken back to the vivarium.

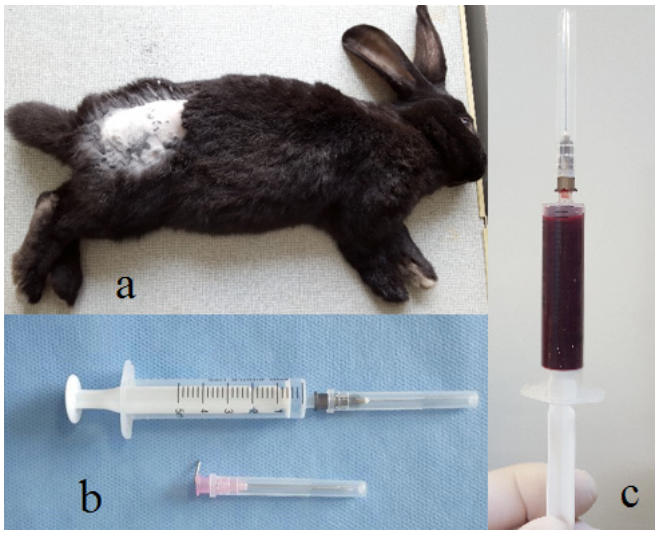


Fig. 1. Bone marrow harvesting from a rabbit iliac bone. Preparing the animal for bone marrow harvest (a), preparation of necessary tools for the procedure (b) and the harvested bone marrow in 5 ml syringe (c).

In the laboratory, the concentration gradient HiSep LSM 1077 (HiMedia, India) and PBS (Lonza, Belgium) preventively, were heated at 37^{0}C, in the water bath. In a 15 ml sterile tube the concentration gradient was poured in an equal volume with harvested bone marrow. The bone marrow was shifted in a 10 or 20 ml syringe containing the same volume of PBS (Lonza, Belgium). After homogenization, the PBS with bone marrow have been poured cautiously on the concentration gradient from the 15 ml tube without mixing. The tube was centrifuged at 400 x *g* for 15 minutes followed by removal of upper 2/3 of platelets and adipocytes layer, the mononuclear cells layer was collected in a separate tube along with 1/3 of the remaining overlying layer and the upper 1/3 of concentration gradient layer [25]. Then the tube was filled with PBS, followed by a careful pipetting and centrifuged at 170 x g for 10 minutes (fig. 2). The supernatant was removed and the centrifugation has been repeated after pipetting the cells with 10 ml of culture medium. After centrifugation the cells were resuspended in 5 ml culture medium, placed in a 25 cm^2 cell culture flask (Nunc, Denmark). The cells were cultivated in the incubator (SMART CELL, Heal Force) at 37°C with 5% CO_2, changing every 2-3 days of a half of the nutrition medium (fig. 3).

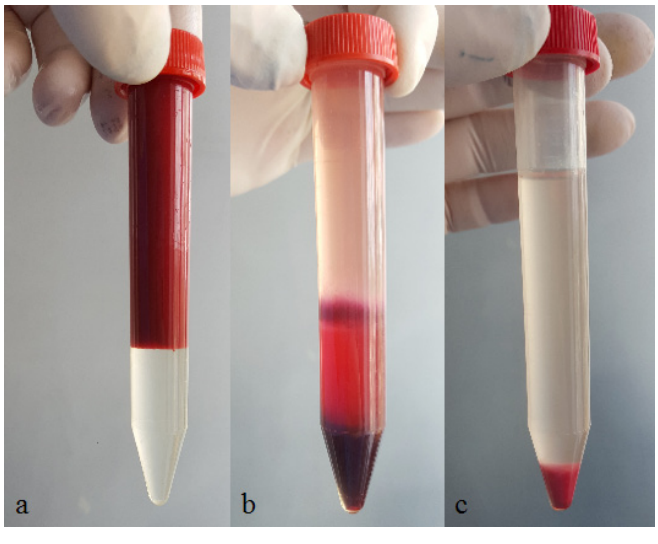


Fig. 2. Processing of harvested bone marrow. The 15 ml tube with bone marrow mixed with PBS located upon the concentration gradient in a ratio of 1: 1: 1 (a), separation in layers after centrifugation (b) and the bone marrow nucleated cell layer separated in another tube (c).

After a 70–80% confluence, the attached cells were washed twice with PBS , followed by addition of 2 ml trypsin–EDTA 0.25% solution into the flask. The flask was placed in the incubator for 3 to 5 minutes, after which 2–3 coups were applied to the flask, followed by visualisation under phase contrast microscope to evaluate the detachment of the cells. Trypsinization was stopped with 3 ml of soybean trypsin inhibitor (Lonza, Belgium). The cells suspension was centrifuged at 170 x *g* for 5 minutes. After supernatant decantation, 5 ml of culture medium were added and the cells gently pippeted. The cells were counted with haemocytometer and viability was assessed with 0.4% trypan blue (Sigma, UK). Then all cells were placed in 75 cm^2 cell culture flasks (Nunc, Denmark) at a density of 1x10^4 ± 1x10^3 cells/cm^2, with total culture medium change every 2

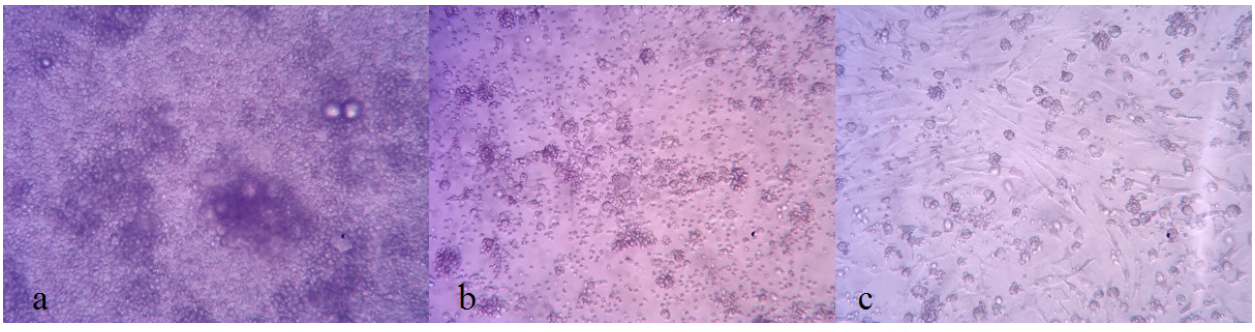


Fig. 3. Isolation of MSC from bone marrow. x60. Concentrate of bone marrow nucleated cells (a), appearance of fusiform cells attached to the cell culture flask bottom after 2 days of cells culture (b) and the 70–80% confluence of the MSC after 5 days of culture (c).

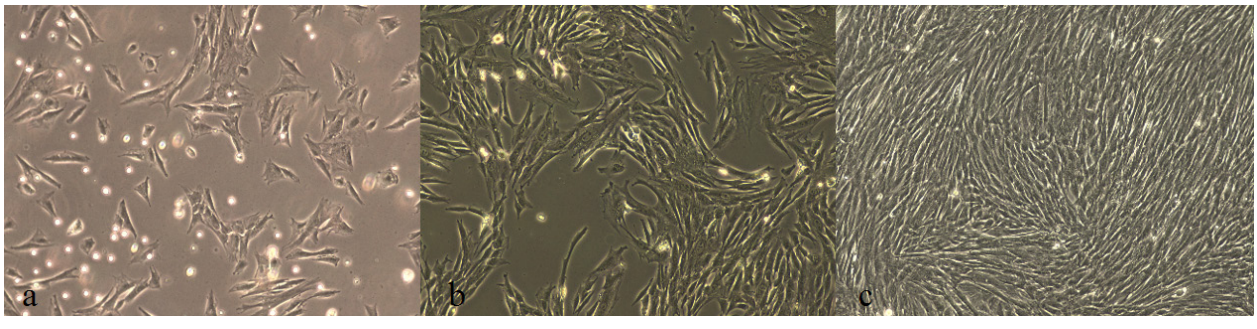


Fig. 4. MSC culture in the 2nd passage. x60. MSC attached to the culture surface with traces of bone marrow cells after 24 hours of culture (a), CSM on the 3rd day of culture (b) and the 90% confluence of MSC at the 5th day of culture (c).

days, until a 80-90% confluence (fig. 4). After trypsinization and cells counting with trypan blue (Sigma, UK), from each culture have been isolated $1x10^6$ cells and differentiated on chondrocytes lineage .The remained cells were frozen in concentration $5x10^5$ cells/ml with 10% DMSO (OriGen Biomedical, Germany) for future use.

Chondrocyte differentiation from MSC

The MSC differentiation potential is considered to be a functionally reliable criteria for their identification and their distinction from preadipocytes, preosteocytes or prechondrocytes [2, 23, 3]. Was used the chondrocytes line differentiation medium HiChondroXL (HiMedia, India).

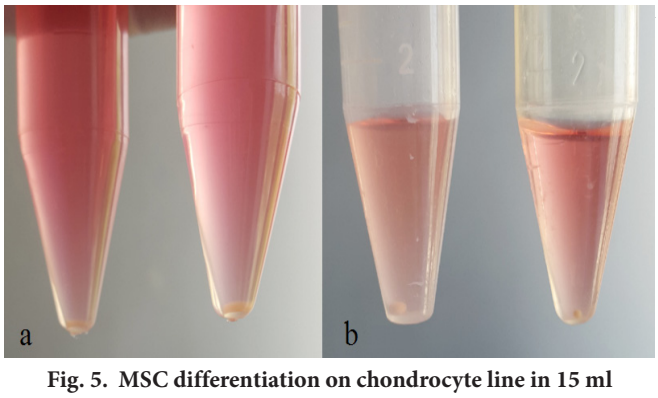


Fig. 5. MSC differentiation on chondrocyte line in 15 ml polypropylene tubes. The aspect of $1x10^6$ MSC before the differentiation on chondrocyte lineage (a) and chondrocytes aggregates (b).

In a 15 ml polypropylene tube were introduced $1x10^6$ cells/ml of MSC culture medium. The tube was centrifuged at 45 x *g* for 10 minutes, followed by supernatant elimination and addition of 1 ml of chondrocyte differentiation medium HiChondroXL (HiMedia, India). The cells were gently resuspended in medium and the tube was centrifuged again. The tube was placed into the incubator with a gently opened lid at 37°C, 5% CO2, without disturbing the cell pellets. The chondrocyte differentiation medium was changed every 48 hours for 20 days [17]. On the 5th–7th days of differentiation, at the bottom was observed formation of spherical or oval shape aggregates (fig. 5). After 20 days the aggregates were introduced into 10% buffered formaldehyde and stained with Hematoxylin–Eosin and specific staining for cartilage with Safranin O and Toluidine blue/fast green [26].

The statistical analysis of the obtained data was carried out using Excel and SPSS Statistics 17.0 programs.

Results

The time needed for bone marrow harvesting from the beginning of anesthesia is 36±3 minutes. During the bone marrow harvesting and after that, complications in experimental animals were not recorded. Once the cells from the 1st passage reached a 70–80% confluence they were trypsinized, thus, the average duration of the first passage cultivation was 7±1 days with a strong correlation between the volume of harvested bone marrow and the number of days required to achieve a 70–80% cells confluency (p=0.01). In Table 1 are presented the results of the MSC obtained from rabbit bone marrow resulted from cultivation in the first 2 passages.

In all cases the 2nd passage was cultivated for 5 days, sufficient time to achieve a 80–90% cellular confluence in all cases. So the average duration of cells cultivation during the first 2 passages was 12 ± 1 days, with a surprisingly 100% cell viability in all cases.

In the process of chondrocyte differentiation, cells aggregates were of irregular spherical shape attached to the bottom of the tube, which later detached and floated freely in the medium. Also in the first days of differentiation, the cells could be easily dispersed by pipetting, but after that they became floating aggregates, the cells were no more dispersed by pipetting. Though, the cells aggregates consisted of the same number of cells, they could have different sizes, which vary between 1.5 and 3 mm in diameter (fig. 5).

Table 1

MSC obtained from rabbit bone marrow resulted from cultivation in the first 2 passages

Rabbit body mass (kg)	Volume of harvested bone marrow (ml)	Procedure duration (min)	The 1st passage cultivation (days)	Number of cells from the 1st passage	Number of cells from the 2nd passage
3.2	4.5	40	6	775000	4250000
3.8	2.5	40	7	675000	3500000
3.6	4.0	35	6	750000	4200000
3.9	2.0	30	8	850000	4900000
3.2	1.5	35	8	800000	4550000
3.8	4.5	35	6	800000	4300000
3.6	5.0	35	5	725000	3800000
3.8	2.5	35	8	725000	4050000
3.9	4.0	40	6	625000	3500000
3.64±0.27	3.39±1.27	36±3	7±1	747222 ±68970	4116667± 464354.4

At the histological examination with Hematoxylin–Eosin, a rich cellularity of aggregates was determined, highlighted by a high density of cells and extracellular matrix formation (fig. 6).

At Toluidine blue/fast green staining, the obtained structure was intensely colored in purple and blue. As the cells are arranged in conglomerates, their nuclei can not be distinguished due to the overlapping of a large number of them, at the same time the blue color represents the cartilage extracellular matrix synthesized by chondrocytes (fig. 7).

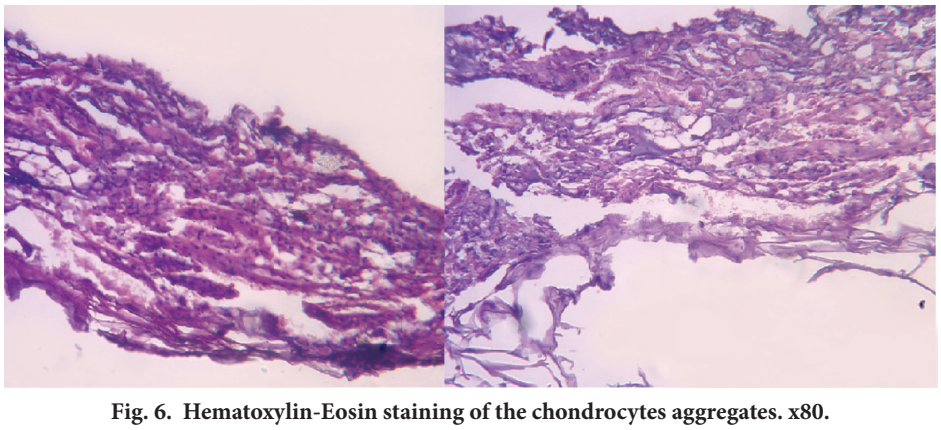


Fig. 6. Hematoxylin-Eosin staining of the chondrocytes aggregates. x80.

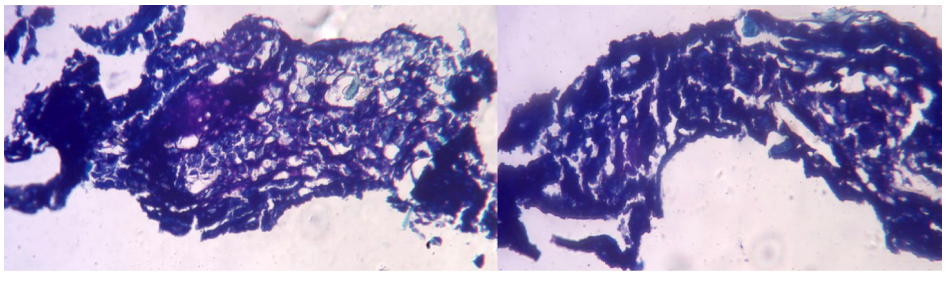


Fig. 7. Toluidine blue staining and Fast Green of formed aggregates. x80.

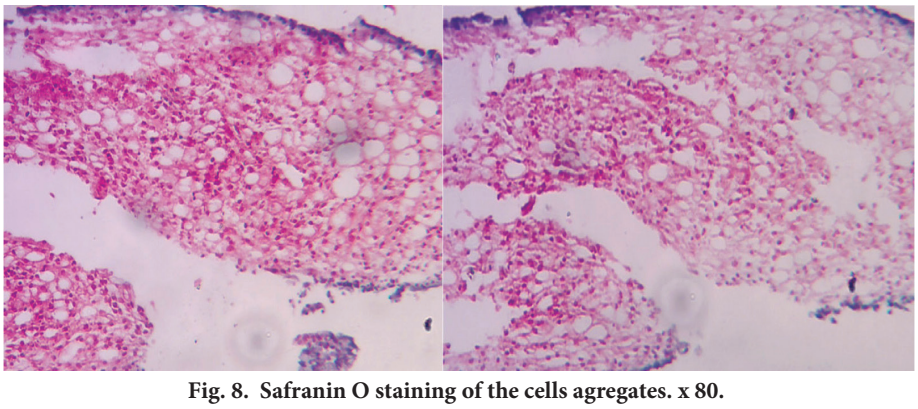


Fig. 8. Safranin O staining of the cells agregates. x 80.

After staining with Safranin O, a large number of darkened nuclei were determined and the extracellular matrix secreted by the cells was stained in red, this being specific to cartilaginous tissue (fig. 8).

The identification of obtained cells aggregates was positive at the specific staining for cartilaginous tissue in all cases (n=9) and is statistically significant (p<0.001).

Discussion

Bone marrow harvesting from rabbit iliac bone serves as an effective way to isolate and cultivate mesenchymal stem cells. This method allows performing *in vivo* tests with rabbit own MSC, without subjecting the animal to great suffering which could adversely affect the results of the experiments [19]. Numerous cases of MSC isolation from long tubular bones after rabbit sacrifice are described in the literature [14, 8, 15, 12], or aspiration of bone marrow from metaphyseal areas of long tubular bones, like femur or even tibia [16, 17, 12, 18] and nucleated cells separation from iliac crest after resection [21].

The iliac bone is smaller than the femoral bone, respectively the volume of harvested bone marrow will be smaller. However, the thickness of the iliac bone at the perforation site, in an adult rabbit, ranges between 4.3 and 4.8 mm (fig. 9), respectively the iliac bone can be easily penetrated with a 18G needle and the volume of harvested bone marrow may reach 4.5–5 ml, just from one side (fig. 1), without harming the animal's health and exposing it to risks which can cost us time and money [19]. At the same time, it must be taken into account that the obtained volume of bone marrow is more than enough to obtain in a relatively short period of time, 12±1 days, a number of 4116667±464354.4 cells, which is quite important.

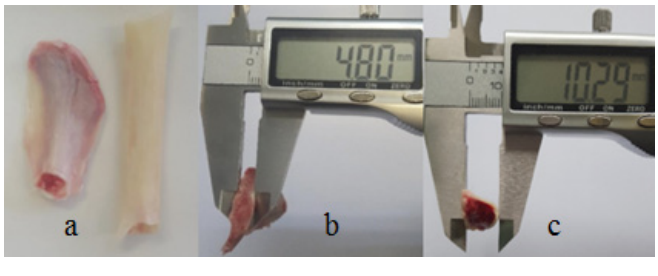


Fig. 9. The comparative dimensions of the iliac and femoral bones. Rabbit femoral and iliac bones (a). Assessment of iliac bone (b) and femoral bone (c) thickness.

According to literature data it is known that, in the bone marrow the number of MSC is very small, between 0.01 – 0.001% of total number of bone marrow nucleated cells [27], or 1/10000–1/100000 according to other sources [28, 29, 30]. MSC can be multiplied for 500 times during 50 generations, finally getting billions of cells [29, 30], also, from the 6th passage of *in vitro* culture, MSC lose their stem cells characteristics and the ability to differentiate [28, 30]. In other words, if we continue cultivating the obtained cells further in passages at a density of $8x10^3$ cells/cm^2, at the 5th passage we would have over 1 billion cells with differentiation potential. Therefore, even 1 ml of bone marrow taken from a single iliac bone in rabbits, represents a sufficient volume to get a large number of MSC capable to differentiate, for *in vitro* or *in vivo* tests in rabbits, which is groundless denied and ignored in the literature [15, 10, 4, 5, 16, 17, 12].

Stem cells identifying is an important step in working with them. At the moment, the most common ways to identify MSC are RT–PCR, cell differentiation by adipogenic, osteogenic and chondrogenic pathway [2, 3], flow cytometry [29, 3], immunofluorescence microscopy [28, 29]. In our research we identified bone marrow MSC only through chondrogenic differentiation pathway. Therefore, according to the criteria of the International Society of Cellular Therapy, MSC have not been fully identified, but in our opinion this is sufficient, because bone marrow MSC are multipotential cells with differentiation potential in specialized cells. However, the differentiation in chondrocytes line was confirmed by specific staining for cartilaginous tissue with Safranin O and Toluidine blue/fast green [31, 26].

Conclusions

The volume of 3.39 ± 1.27 ml of bone marrow, harvested from rabbit iliac bone is sufficient to obtain a large number of MSC for the laboratory tests *in vitro* and *in vivo*. As a standard method for MSC identification could be also the capability of the cells to differentiate in the specialized cells, including hondrocytes.

Disclaimer

The authors of this article did not benefit and will not benefit from the goods of commercial agents or manufacturers by using the media purchased from them.

References

1. Augello A, De Bari C. The regulation of differentiation in mesenchymal stem cells. Human Gene Ther. 2010;21(10):1226-38. doi:10.1089/hum.2010.173.
2. Gugjoo MB, Amarpal, Kinjavdekar P, Prasad AH, Ansari MM, Pawde MotiRam A, Taru GS. Isolation, culture and characterization of New Zealand white rabbit mesenchymal stem cells derived from bone marrow. Asian J Anim Vet Adv. 2015;10(10):537-548.
3. Ninu AR, Swapan KM, Shiva Kumar MU, Sandeep K, Sangeetha P, Deepka K, Saurabh G, Abhisek S, Naveen K. Isolation, proliferation, characterization and in vivo osteogenic potential of bone-marrow derived mesenchymal stem cells (rBMSC) in rabbit model. Indian J Exp Biol. 2017;55(2):79-87.
4. Panadero JA, Lanceros-Mendez S, Ribelles JL. Differentiation of mesenchymal stem cells for cartilage tissue engineering: Individual and synergetic effects of three-dimensional environment and mechanical loading. Acta Biomater. 2016;33:1-12. doi: 10.1016/j.actbio.2016.01.037.
5. Solchaga LA, Penick KJ, Welter JF. Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells: tips and tricks. Methods Mol Biol. 2011;698:253-278. doi: 10.1007/978-1-60761-999-4_20.
6. Ahmad Z, Wardale J, Brooks R, Henson F, Noorani A, Rushton N. Exploring the application of stem cells in tendon repair and regeneration. Arthroscopy. 2012;28(7):1018-29. doi: 10.1016/j.arthro.2011.12.009.
7. Cantz T, Manns PM, Ott M. Stem cells in liver regeneration and therapy. Cell Tissue Res. 2008;331(1):271-282. doi: 10.1007/s00441-007-0483-6.

8. Kalamegam G, Memic A, Budd E, Abbas M, Mobasheri A. A comprehensive review of stem cells for cartilage regeneration in osteoarthritis. Adv Exp Med Biol. 2018;1089:23-36. doi: 10.1007/5584_2018_205.
9. McLauchlan D, Robertson NP. Stem cells in the treatment of central nervous system disease. J Neurol. 2018;265(4):984-986. doi: 10.1007/s00415-018-8818-7.
10. Nauth A, Schemitsch HE. Stem cells for the repair and regeneration of bone. Indian J Orthop. 2012;46(1):19-21. doi: 10.4103/0019-5413.91630.
11. Lui PP. Stem cell technology for tendon regeneration: current status, challenges, and future research directions. Stem Cells Cloning. 2015;8:163-174. doi: 10.2147/SCCAA.S60832.
12. Walmsley GG, Ransom CR, Zielins RE, Leavitt T, Flacco SJ, Hu SM, Lee SA, Longaker TM, Wan CD. Stem cells in bone regeneration. Stem Cell Rev. 2016;12(5):524-529. doi: 10.1007/s12015-016-9665-5.
13. Zhao L, Kaye AD, Abd-Elsayed A. Stem cells for the treatment of knee osteoarthritis: a comprehensive review. Pain Physician. 2018;21:229-241. ISSN 1533-3159.
14. Liu H, Wei LK, Jian XF, Huang J, Zou H, Zhang SZ, Yuan GH. Isolation, culture and induced differentiation of rabbit mesenchymal stem cells into osteoblasts. Exp Ther Med. 2018;15(4):3715-3724. doi: 10.3892/etm.2018.5894.
15. Mohammed RH, Jasim AM, Almanseekanaa LH. Isolation and characterization of bone marrow mesenchymal stem cells from rat and rabbit: a modified method. Kerbala J Pharm Sci. 2012;(3):44-51.
16. Phedy, Dilogo IH, Jusuf AA, Kholinne E, Efendi Z. Iliac crest and femoral bone marrow as the source of plastic-adherent cells. Med J Indones. 2011;20:100-4.
17. Eça LP, Ramalho RB, Oliveira IS, Gomes PO, Pontes P, Ferreira AT, Mazzetti MP. Comparative study of technique to obtain stem cells from bone marrow collection between the iliac crest and the femoral epiphysis in rabbits. Acta Cir Bras. 2009;24(5):400-404.
18. Zhang W, Zhang F, Shi H, Tan R, Han S, Ye G, Pan S, Sun F, Liu X. Comparisons of rabbit bone marrow mesenchymal stem cell isolation and culture methods in vitro. PLoS One. 2014;9(2):e88794. doi: 10.1371/journal.pone.0088794.
19. Naderi MM, Sarvari A, Milanifar A, Boroujeni SB, Akhondi MM. Regulations and ethical considerations in animal experiments: international laws and islamic perspectives. Avicenna J Med Biotechnol. 2012;4(3):114-120.
20. Patterson TE, Boehm C, Nakamoto C, Rozic R, Walker E, Piuzzi SN, Muschler FG. The efficiency of bone marrow aspiration for the harvest of connective tissue progenitors from the human iliac crest. J Bone Joint Surg Am. 2017;99(19):1673-1682. doi: 10.2106/JBJS.17.00094.
21. Nacu V. Recuperarea defectului de dimensiune critică al osului parietal la iepure prin grefe compozite [Critical rabbit parietal bone defect reconstruction by composite grafts]. Curierul Medical (Chisinau). 2008;(6/306):66-72. ISSN 0310-1535. Romanian.
22. Shah KM, Stern MM, Stern AR, Pathak JL, Bravenboer N, Bakker AD. Osteocyte isolation and culture methods. Bonekey Rep. 2016;5:838. doi: 10.1038/bonekey.2016.65.
23. HiMedia Laboratories. HiMesoXL™ Mesenchymal Stem Cell Expansion Medium. Mumbai: The Lab; 2017 [cited 2019 Jan 24]. Available from: http://himedialabs.com/TD/AL512.pdf
24. HiMedia Laboratories. HiChondroXL™ Chondrocyte Differentiation Medium. Mumbai: The Lab; 2012 [cited 2019 Jan 24]. Available from: http://himedialabs.com/TD/AL523.pdf
25. HiMedia Laboratories. HiSep™ LSM 1077. Mumbai: The Lab; 2017 [cited 2019 Jan 24]. Available from: http://himedialabs.com/TD/LS001.pdf
26. Schmitz N, Laverty S, Kraus VB, Aigner T. Basic methods in histopathology of joint tissues. Osteoarthritis Cartilage. 2010;18 Suppl 3:S113-S116. doi: 10.1016/j.joca.2010.05.026.
27. Abdelwahid E, Kalvelyte A, Stulpinas Aurimas, Teixeira de Carvalho KA, Guarita-Souza LC, Foldes G. Stem cell death and survival in heart regeneration and repair. Apoptosis. 2016;21(3):252-268. doi: 10.1007/s10495-015-1203-4.
28. Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B. Aging of mesenchymal stem cell in vitro. BMC Cell Biol. 2006;7:14. doi: 10.1186/1471-2121-7-14.
29. Jones EA, Kinsey SE, English A, Jones RA, Straszynski L, Meredith DM, Markham AF, Jack A, Emery P, McGonagle D. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum. 2002;46(12):3349-60. doi: 10.1002/art.10696.
30. Nasef A, Fouillard L, El-Taguri A, Lopez M. Human bone marrow-derived mesenchymal stem cells. Libyan J Med. 2007;2(4):190-201. doi: 10.4176/070705.
31. Hoemann C, Kandel R, Roberts S, Saris DB, Creemers L, Mainil-Varlet P, Méthot S, Hollander AP, Buschmann MD. International Cartilage Repair Society (ICRS) Recommended Guidelines for histological endpoints for cartilage repair studies in animal models and clinical trials. Cartilage. 2011;2(2):153-172. doi: 10.1177/1947603510397535.

DOI: 10.5281/zenodo.2590015
UDC: 611.831.7

Types of extracranial branching of the facial nerve

Angela Babuci, MD, Assistant Professor

Department of Human Anatomy, NicolaeTestemitsanu State University of Medicine and Pharmacy
Chisinau, the Republic of Moldova

Corresponding author: angela.babuci@usmf.md
Manuscript received January 28, 2019; revised manuscript March 04, 2019

Abstract

Background: Interest in anatomical variability and individual specific features of the extracranial branches of the facial nerve, taking into consideration that nowadays, people are so concerned about their physical look, the knowledge of facial nerve peripheral branching is of high clinical significance. Even, if it is hardly difficult do discover something new, or unusual at the macromicroscopic level, we believe that reading this paper oncologists and oromaxillofacial surgeons will be surprised by anatomical variations of the peripheral braches of the facial nerve.
Material and methods: Our research project was carried out on 52 cadaveric semiheads that previously were fixed in 10% formalin solution: 36 male and 16 female; 25 of those samples were right and 27 left side semiheads. The bilateral pattern of dissection was carried out on 30 semiheads and unilateral dissection – on 22 semiheads. The dissected samples were photographed. Some samples were marked out with black papers for better contrast.
Results: The facial trunk on its exit from the sylomastoid foramen was descendent in 32 cases (61.54%), in 9 cases (17.3%) it ran horizontally and in 5 cases (9.62%) it had an ascending course, but in 6 cases (11.54%) the branches derived directly from the trunk in a fan-like fashion. According to the known classification of the facial nerve peripheral branching, the following percentage for each type was established: Type I (23.1%); Type II (7.7%); Type III (1.9%); Type IV (38.5%); Type V (7.7%); Type VI (9.7%). Few intermediate types have been marked out such as: Type II-III (3.8%); Type III-IV (3.8%), Type V-VI (3.8%). When dissecting the facial nerve we observed that the trunk in 37 cases divided into two primary braches and in 15 cases there were marked out diffuse types of branching.
Conclusions: A significant characteristics of the facial nerve is its variability and individual specific features. Among the well known classified types of peripheral branching of the facial nerve, there are intermediate types of its extracranial divisions.

Key words: facial nerve, trunk, landmarks, variability.

Introduction

Why we are so interested in reviewing the morphological peculiarities of the extracranial branches of the facial nerve, nowadays, when the modern medicine has the capacity to diagnose various impairments of the nervous system, including those of the facial nerve, using high technology methods of examination?

Of course, apparently, all the anatomical structures are well known and it is very difficult to discover something new, or unusual at the macromicroscopic level, but at the same time anatomy is an amazing science, that sometimes surprises us by its variability. According to our study, morphology may be rediscovered and new data can be supplied to the anatomical background.

Why facial nerve? It is a simple question, but the substrate of this question is deeply hidden inside the human body and it depends on individual character. Lately, too many people complain of physical and psychological extenuation and the number of those with chronic fatigue syndrome, characterized by extreme tiredness prevalence, is daily increasing.

When reading this paper, a question will arise in the reader's mind, what does the facial nerve have in common with the extreme tiredness? At a glance, there is nothing in common, but considering that tiredness and unhappiness will lead to the formation of specific facial wrinkles and corresponding physiognomy, as well as the fact that people of the 21st century are very concerned about their intellectual development, that should not be neglected, regards the physical aspect of an individual.

Unfortunately, our daily activity is accompanied by stress, environmental pollution, improper nutrition, exposure to various harmful factors, and one of the most substantial facts is related to the genetic changes of the bacterial flora and viruses, that are increasingly affecting central and peripheral nervous system. Taking into consideration that the facial nerve is highly susceptible to various harmful factors, we decided to review its morphology through applied anatomy.

Material and methods

Our study is a descriptive and retrospective one and according to international requirements for carrying out a research project on cadaveric material, it was approved by the Ethics Committee of Nicolae Testemitsanu State University of Medicine and Pharmacy of the Republic of Moldova.

The study was carried out on 52 adult semiheads, dissected at the Department of Human Anatomy of the named University.

The bilateral pattern of dissection was carried out on 30 semiheads and unilateral dissection was performed on 22 semiheads. For the fixation of the cadaveric material 10% formalin solution was used and the classic dissection method was applied.

As the purpose of our study was to establish the variants of branching of the extracranial part of the facial nerve, an incision of the skin of the median plan of the forehead, which continued on each side of the face around the eye, nose and lips to the end on the midline of the chin, was made. The skin of the face was removed and the landmarks of the facial nerve have been marked out. As soft tissue landmarks the posterior belly of the digastrics muscle and the stylomastoid artery were used and as bony landmarks we used the mastoid and styloid processes, the zygomatic arch, the posterior margin of the *ramus mandibulae*, the mandibular angle and mandibular margin [1, 2].

After identification of the facial nerve trunk the primary, secondary, tertiary and terminal branches of the facial nerve were dissected. The anatomical samples were examined and photographed. Some of them were marked out with black paper for better contrast. The obtained data were statistically processed.

Results

According to our dissecting practice, the most safety way to avoid the microtraumas of the facial nerve branches in surgery is to mark out at first the trunk of the nerve at its exit orifice, using as a landmark the mastoid process, posterior belly of the digastric muscles, stylomastoid artery and the posterior margin of the *ramus mandibulae*.

Another significant issue that should be kept in mind regards the trajectory of the facial trunk, when it exits the *Fallopian canal*. Along with descending course of the trunk, in the current study there were pointed out horizontal and even ascending pathways of the facial nerve trunk when it leaves the *Fallopian canal*. From the total number of samples, the facial trunk on its exit from the stylomastoid foramen was descendent in 32 cases (61.54%), in 9 cases (17.3%) it ran horizontally, in 5 cases (9.62%) it had an ascending course, but in 6 cases (11.54%) the branches derived directly from the trunk in a fan-like fashion.

Examining the dissected semiheads we tried to classify the sample according to known types described by specialty reference sources [3, 4].

When dissecting the facial nerve we observed that the trunk in 37 cases divided into two primary braches and in 15 cases there were marked out diffuse types of branching.

It was necessary to find out why it happens so that for some people are characteristic a lot of thin divisions of the facial nerve and various connections were formed between its branches and with the regional cranial nerves, but in other cases there were not so many secondary and tertiary divisions, but very few.

When examining the right and left sides of the face on the same individuals we came upon the conclusion that on the right side of the majority of samples the branching and the connections were more obvious and abundant, but on the left one, the divisions and connections of the primary branches were not so numerous.

Among the dissected samples, even in those cases when both semiheads belong to the same individual the divisions of the extracranial branches were different on each side of the face [5].

There barely could be found two, more or less, similar extracranial branching of the facial nerve, but nevertheless, all the dissected samples were classified according to known types of peripheral divisions of the facial nerve. An interesting issue concerning extracranial divisions of the facial nerve, that is worth mentioning, was that among the dissected samples there were identified some intermediate types of facial nerve branching.

The following types have been pointed out in our study and the percentage for each type was established: Type I (23.1%); Type II (7.7%); Type III (1.9%); Type IV (38.5%); Type V (7.7%); Type VI (9.7%) and the following intermediate types: Type II-III (3.8%); Type III-IV (3.8%), Type V-VI (3.8%). Some types of the extracranial part of the facial nerve divisions established during the study are presented in fig. 1, 2, 3 and 4 on page 44.

Discussion

The ethiogenesis of the peripheral facial nerve paralysis is controversial and highly diverse. Nevertheless, one factor that must be kept in mind by neurologists, and especially by neurosurgeons and oromaxillofacial surgeons, is the morphology of the facial nerve. Its motor branches have a superficial location on the face and they are highly susceptible to various injures of the face and to microtraumas in facial surgery such as parotid ablation, rejuvenating procedures, aesthetic surgery and other manipulations on the facial region.

The facial nerve is involved in pathology of other cranial nerves, but mainly in pathology of the vestibulochochlear one. The traumas of the head and neck, somatic diseases and metabolic disorders and a wide range of viruses, bacteria are the cause of facial nerve impairments [6, 7, 8].

The morphological and topographical peculiarities of the facial nerve, its relations to the regional anatomical structures and connections of its extracranial branches [5] with other cranial nerves, might be an explanation of the facial nerve involvement in different pathology.

It should be mentioned that in aesthetic surgery the most susceptible to iatrogenic injures are the branches of the temporal and frontal area, due to the fact that those branches are not so much ramified and few connections are characteristic for those branches. The high risk of frontotemporal branch lesion in facelift was pointed out by [9], and explanation was given by [10], who mentioned that the precise localization of the frontal division of the facial nerve is still problematic, for there were not established high fidelity landmarks that can be used for that purpose and [10] proposed to consider as landmarks the veins of the temporofrontal area.

For facial nerve is characteristic a high degree of variability [11] and that fact was marked out as well in all our samples. An important fact that is worth mentioning is that the variability of the extracranial branches of the facial nerve does not refer to different people only, but even in the

same individual the variability was highly marked out. In our study there barely could be found two more or less similar divisions of the facial nerve and that peculiarity should be considered by surgeons. Another significant moment that should be kept in mind in surgery is that prediction of the course and divisions of the facial nerve, even, if there was carried out a surgery on one side of the face, cannot be applied on the opposite side.

High risk of iatrogenic lesions of the extracranial branches of the facial nerve can be explained by multiple variations of its branches that may be accidently damaged in facial surgery, being one of the main causes of failure in parotid tumor ablation and other surgery of the facial region that may result in transitory peripheral facial paralysis. According to [12, 13, 14] the tight relationship between the facial nerve and the parotid gland is another high risk factor of iatrogenic microtraumas in parotid tumors surgery.

Another interesting fact was discovered regarding divisions of the marginal mandibular and cervical branches. It is known that the upper two thirds of the face receive corticonuclear pathways from both cerebral hemispheres, but the lower third from the contralateral side only. So, we tried to understand why in some cases the named above branches of the facial nerve give off a lot of secondary and tertiary divisions, but in other cases they run up to the innervated muscles almost without divisions. May be it depends on individual person's character and mimicry.

We leave this question open for future study. Some interesting things were found out in our study and as it was mentioned above anatomy is an amazing subject, but the facial nerve in our opinion is the most amazing and unpredictable cranial nerve.

Conclusions

Our study revealed a high variability of the extracranial branches of the facial nerve and we came to the conclusion that theoretically we can classify the types of facial nerve branching, but our results should be carefully used by practitioners, because the facial nerve is one of the most subjected to individual specific features, cranial nerve.

We can conclude that there barely could be found two samples with more or less similar extracranial branching of the facial nerve.

When examining the right and left sides of the face on the same individuals, we came to the conclusion that on the right side of the majority of samples the branching and the connections were more obvious and abundant, but on the left one, the divisions and connections of the primary branches were not so numerous.

Knowledge of types of branching of the extracranial part of the facial nerve is of clinical significance and should be kept in mind in parotid ablation, OMF surgery and in aesthetic surgery. A surgeon should always be aware that one of the characteristics of the seventh pair of cranial nerves is a high variability and of its extracranial branches that finally will determine the success or the failure of a facial surgery.

References

1. Davies JC, Agur AMR, Fattah AY. Anatomic landmarks for localization of the branches of the facial nerve. OA Anatomy. 2013;1(4):33.
2. Saylam C, Ucerler H, Orhan M, Ozek C. Anatomic landmarks of the buccal branches of the facial nerve. Surg Radiol Anat. 2006;28(5):462-7.
3. Standring S, editor. Gray's anatomy: the anatomical basis of clinical practice. 40th ed. Edinburgh: Churchill Livingstone/Elsevier; 2008. ISBN: 978-0-443-06684-9, ISBN: 978-0-8089-2371-8 (International ed.). p. 495.
4. Bergman RA, Afifi AK, Miyauchi R. Illustrated encyclopedia of human anatomic variation: Opus III: Nervous system variations in branching of facial nerve [cited 2019 Jan 28]. Available from: http://www.anatomyatlases.org.
5. Babuci A. Variations of connections of the parotid plexus branches: a cadaveric study. Mold J Health Sciences. 2015;5(3):21-26.
6. Gherman D, Moldovanu I, Zapuhlih G. Neurologie şi neurochirurgie [Neurology and neurosurgery]. Chisinau: Medicina; 2003. 528 p. Romanian.
7. Volk GF, Pantel M, Guntinas-Lichius O. Modern concepts in facial nerve reconstruction. Head Face Med. 2010;6:25. doi: 10.1186/1746-160X-6-25.
8. Gavriliuc M. Examenul neurologic [Neurological examination]. Chişinău; 2012. p. 31-38. Romanian.
9. Friel MT, Shaw RE, Trovato MJ, Owsley JQ. The measures of face-lift patients satisfaction: the Owsley Facelift Satisfaction Survey with a long-term follow-up study. Plast Reconstr Surg. 2010;126(1)245-257.
10. Sabini P, Wayne I, Quatela VC. Anatomical guides to precisely localize the frontal branch of the facial nerve. Arch Facial Plast Surg. 2003;5:150-152.
11. Baker DC, Conley J. Avoiding facial nerve injures in rhytidectomy. Plast Reconstr Surg. 1979;64(6):781-785.
12. Ataman T, Mîrşu C. Descoperirea nervului facial în porţiunea mastoidă [Disclosure of the facial nerve in the mastoid portion].O.R.L. [Otorhinolaryngology]. 1998;1-2:66-68. Romanian.
13. Cristea D, Maftei O, Debita M, Firescu D. Disecţia nervului facial în spaţiile profunde ale feţei. Manevră anatomo-chirurgicală obligatorie în patologia cervico-parotidiană [Dissection of the facial nerve in the deep spaces of the face. Mandatory maneuver in Surgical Anatomy of the cervico-parotid pathology]. Revista Română de Anatomie funcţională şi clinică, macro- şi microscopică şi de Antropologie [The Romanian Journal of Functional and Clinical Anatomy, Macro- and Microscopic and Anthropology]. 2004;3(3):26-30. Romanian.
14. Kirici I, Kilic C, Kazkayasi M. Topographic anatomy of the peripheral branches of the facial nerve. J Exp Integr Med. 2011;1(3):201-204.

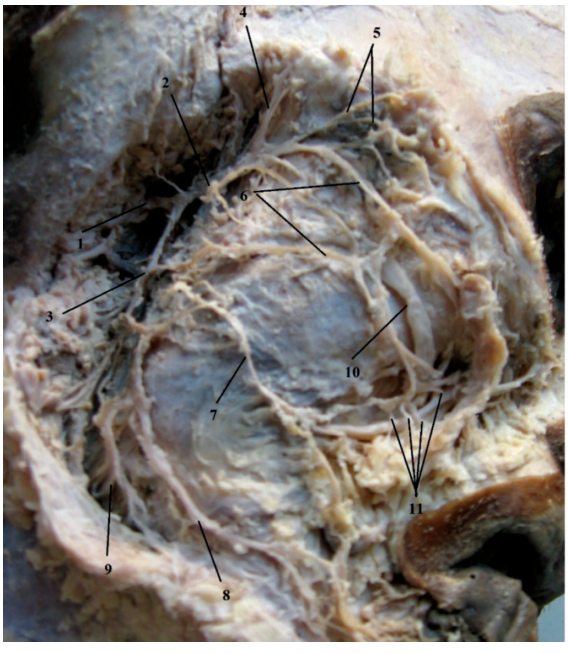


Fig. 1. Atypical branching of the extracranial part of the facial nerve. Type II.
1 – the main trunk of the facial nerve; 2 – temporofacial branch; 3 – cervicofacial branch; 4 – temporal branches; 5 – zygomatic branches; 6 – superior buccal branches; 7 – inferior buccal branch; 8 – marginal mandibular branch; 9 – cervical branch; 10 – parotid duct; 11 – terminal branches within the muscles of facial expression.

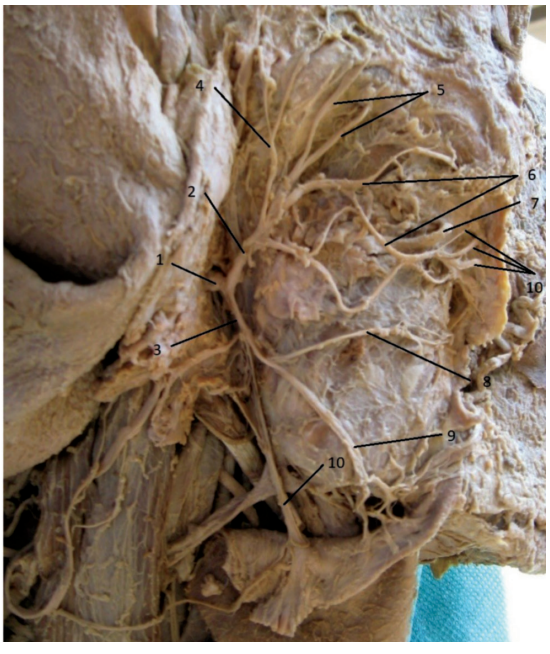


Fig. 2. Atypical branching of the extracranial part of the facial nerve. Type IV
1 – the main trunk of the facial nerve; 2 – temporofacial branch; 3 – cervicofacial branch; 4 – temporal branches; 5 – zygomatic branches; 6 – superior buccal branches; 7 – parotid duct; 8 – inferior buccal branch; 9 – marginal mandibular branch; 10 – cervical branch; 11 – terminal branches within the muscles of facial expression.

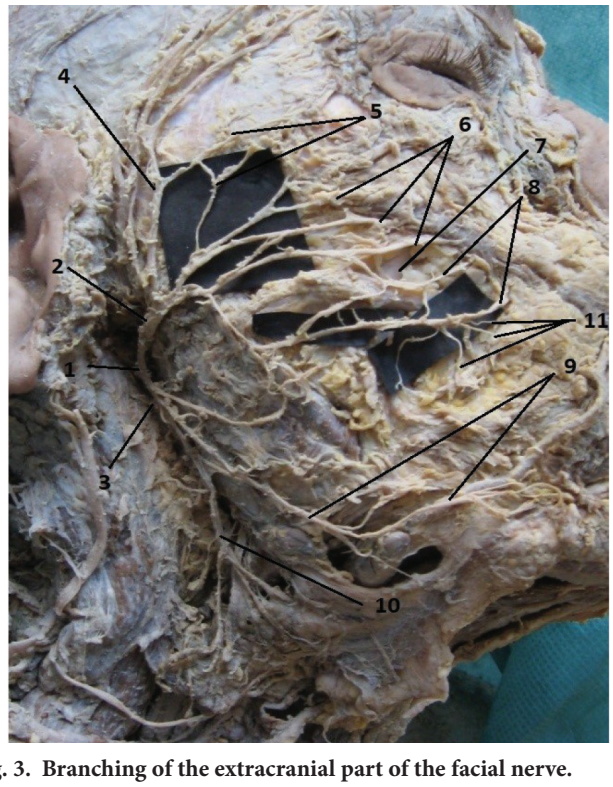


Fig. 3. Branching of the extracranial part of the facial nerve. Intermediate Type V-VI.
1 – the main trunk of the facial nerve; 2 – temporofacial branch; 3 – cervicofacial branch; 4 – temporal branches; 5 – zygomatic branches; 6 – superior buccal branches; 7 – parotid duct; 8 – inferior buccal branch; 9 – marginal mandibular branch; 10 – cervical branch; 11 – terminal branches within the muscles of facial expression.

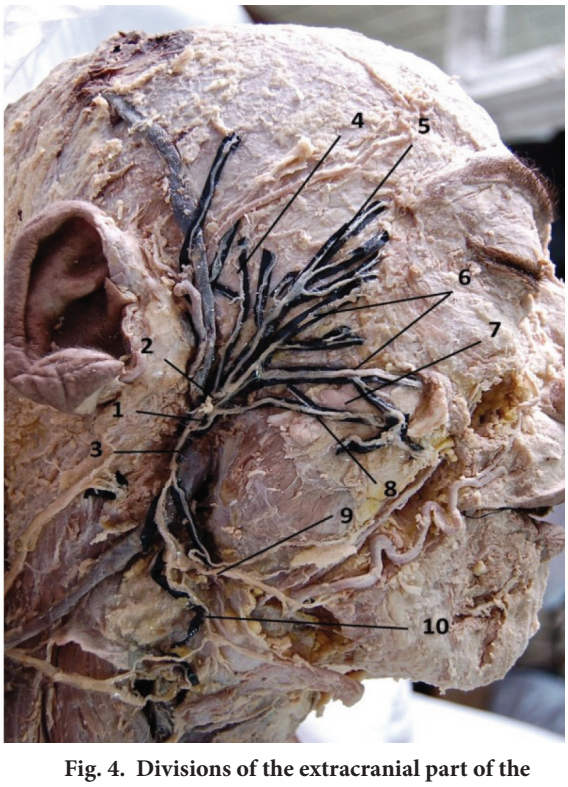


Fig. 4. Divisions of the extracranial part of the facial nerve. Type V.
1 – the main trunk of the facial nerve; 2 – temporofacial branch; 3 – cervicofacial branch; 4 – temporal branches; 5 – zygomatic branches; 6 – superior buccal branches; 7 – parotid duct; 8 – inferior buccal branch; 9 – marginal mandibular branch; 10 – cervical branch; 11 – terminal branches within the muscles of facial expression.

REVIEW ARTICLES

DOI: 10.5281/zenodo.2590020
UDC: 616.831.8-009.17

Lambert-Eaton myasthenic syndrome – a misdiagnosed condition

[1,2]**Paula Fala,** MD; [2]**Ina Cojocaru,** MD; [2]**Larisa Chetrari,** MD;
*[1,2]**Pavel Gavriliuc,** MD, PhD Applicant; [1,2]**Marina Sangheli,** MD, PhD, Associate Professor;
[1,2]**Vitalie Lisnic,** MD, PhD, Professor

[1]Department of Neurology No 1, Nicolae Testemitsanu State University of Medicine and Pharmacy
[2]Department of Neuromuscular Disorders and Polyneuropathies, Institute of Neurology and Neurosurgery
Chisinau, the Republic of Moldova

*Corresponding author: gavriliucpavel@gmail.com
Manuscript received December 18, 2018; revised manuscript February 05, 2019

Abstract

Background: Lambert-Eaton myasthenic syndrome (LEMS) is a rare disorder of the neuromuscular junction. Clinical features include proximal muscle weakness, markedly in the lower limbs, reduced deep tendon reflexes that can increase after exercise, and autonomic disturbances. The clinical picture as well as knowledge of the laboratory test that accompany LEMS will permit early recognition of the disease, that is crucial because it is often associated with malignancy, especially small cell lung cancer (SCLC). In this article we present a patient with proximal muscle weakness and typical changes on repetitive nerve stimulation, as well as a short literature review on the topic.

Conclusions: The diagnosis of LEMS is usually made on clinical grounds. The diagnosis is confirmed by electrophysiological testing, main features including decrement response on slow repetitive nerves stimulation (3Hz), and an increment of more than 100% in CMAP amplitude after brief exercise, or high frequency repetitive stimulation (30-50 Hz). Immunological panel assay with positive P/Q-type VGCC antibody is strongly suggestive of LEMS. While symptomatic treatment with 3,4 – diaminopyridine is available, one of the main priorities is evaluation for underlying malignancies in these patients, the most common being SCLC. Evaluation of patients with LEMS and no known cancer should start with CT of the chest, abdomen and pelvis. Brain imaging is recommended if focal neurological signs are present. If the initial evaluation of the patient is negative, repeated screening for malignancy after 6 months and up to two years is recommended.

Key words: Lambert-Eaton myasthenic syndrome, cancer, weakness, increment.

Introduction

The neuromuscular junction (NMJ) disorders are often seen in the clinical practice of general neurologists and other specialists. The prevalence of myasthenia gravis (MG), the most common NMJ disorder is 1:10.000 inhabitants [1]. At the same time, the spectrum of diseases affecting neuromuscular transmission at the synapse level is wide. If the classical postsynaptic disorder like MG is easily diagnosed, the presynaptic one is often misinterpreted. The presynaptic NMJ disorder like the Lambert-Eaton myasthenic syndrome (LEMS) is rare. It is an idiopathic or paraneoplastic autoimmune disorder of the presynaptic nerve terminal of the NMJ transmission. The relation between patients with LEMS and MG is 1:10 [2]. At the same time, the establishment of the correct diagnosis could lead to the search of the malignity when the last is at the treatable stage.

LEMS is a rare condition with the main clinical manifestation of skeletal muscular weakness, reduced reflexes and autonomic involvement. Specific clinical picture, laboratory and electrophysiological studies allow for early diagnosis, which is important as this syndrome is strongly associated with small cell lung cancer (SCLC) [3].

Pathophysiology of LEMS includes antibodies against P/Q-type voltage gaited calcium channels (VGCC) that reduce the amount of acetylcholine (Ach) released in the synaptic cleft [4].

We present a typical Lambert-Eaton syndrome.

A 58 year-old male, a truck driver, complaining of proximal muscle weakness and progressive gait disturbance (requiring a cane for walking at first presentation) for 2 months. Upon admission he had as well dry mouth, hypohidrosis. The patient noticed that he could stand up with difficulties in the cabin of the car. He complained of low back pain and his family physician diagnosed him with radiculopathy. He received treatment with Dexamethasone 8 mg and NSAIDs for 5 days consecutively with no significant improvement.

The patient's history is remarkable as he is a heavy smoker (138 pack-years, 60 cigarettes per day). The patient also suffers from a mild diabetes mellitus, arterial hypertension, and hepatic steatosis. At clinical examination he had a blood pressure 130/80 mmHg on antihypertensive medications, heart rate 75 beats per minute, in sinus rhythm, respiratory frequency 18 per min, and a BMI – 39.1 that qualifies as obesity class 2. Neurological examination revealed decreased muscle strength on MRC scale 4/5 in all limbs, but with a 3/5 in proximal muscle groups. He hardly could stand up from sitting position and had decreased deep tendon

reflexes, which improved after exercise. The sensory disturbances were suggestive of polyneuropathy with hypoesthesia in "gloves and socks" distribution and segmental L5 – S1 hypoesthesia. Neither pathological signs nor sphincter disturbances were found. Cerebellar tests were normal. Meningeal and elongation signs were negative.

MRI of the lumbar spine was performed that revealed intervertebral discs protrusions at the L2 – L3, L3 – L4, L5 – S1 levels.

Routine nerve conduction studies were performed which were within normal range. On EMG with slow (3Hz) repetitive nerve stimulation (RNS), decrement of 21% (fig. 1) was noted at rest, at the first examination, with a compound muscle action potential (CMAP) increase of more than 100% in amplitude after isotonic exercise for 30 seconds (fig. 2).

The history and physical examination of the patient, with proximal muscle weakness, increase in deep tendon reflexes after exercise, as well as increase of amplitude of more than 100% on repetitive nerve stimulation after isometric contraction, lead to the idea of a presynaptic disorder [5]. Given the history of the patient being a heavy smoker and clinical and electrophysiological changes suggesting a presynaptic disorder, the presumption of Lambert-Eaton myasthenic syndrome was made.

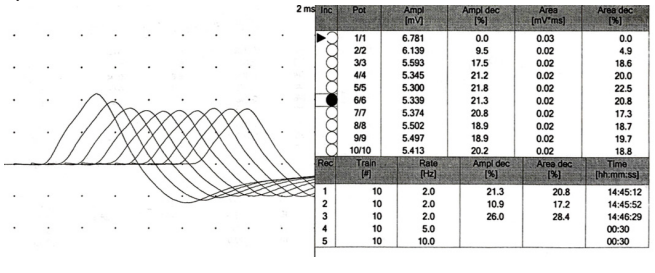


Fig. 1. Decrement of 21% at rest during slow (3 Hz) repetitive nerve stimulation of the left ulnar nerve.

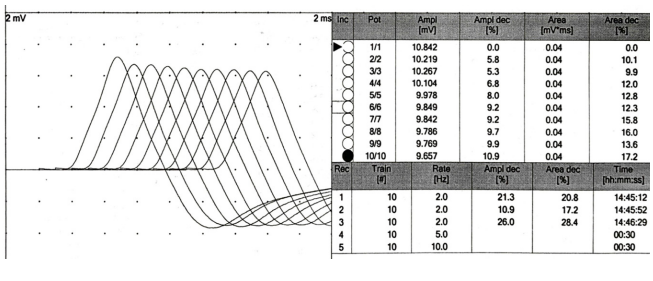


Fig. 2. Increment of more than 100% in CMAP amplitude following a 30 seconds isotonic exercise in the left ulnar nerve.

Computer tomography (CT) of the lungs was ordered to rule out neoplastic formations, and serological testing for specific antibodies was carried out.

The malignancy workup including CT of the lungs (fig. 3), oncological markers for CEA, PSA, CA 19-9 came back negative, while immunologic assay for anti VGCC antibodies came positive for both type N and PQ (tab. 1) that further confirmed our suspicions of Lambert-Eaton myasthenic syndrome.

During admission, plasma exchange was initiated with mild improvement of symptoms. When the immunologic results for anti VGCC came positive, treatment with Prednisone and Azathioprine was started to control the immune response, while 3,4-diaminopyridine was recommended for symptoms control.

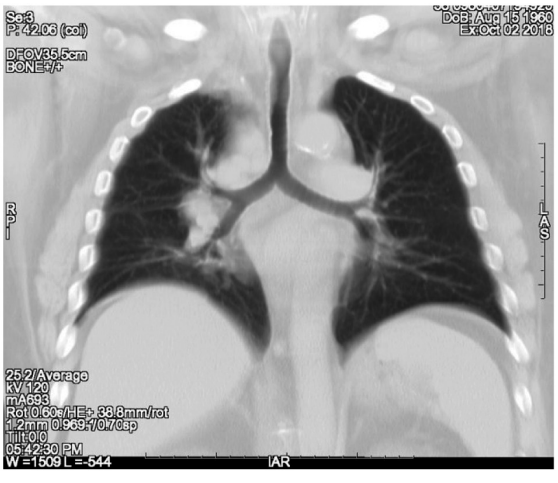


Fig. 3. Lung CT without pathological changes.

Table 1

Immunologic assay for anti VGCC (type N and PQ)	
Type N 20,9	Index < 10
Type PQ 248,9	pmol/L < 40

Discussion

Our patient addressed the first time for low back pain, and that remained his chief complaint even 2 months later after the initial episode, in spite of progressive weakness and difficulties in walking and getting up from a chair.

The most common causes of low back pain are degenerative disc disease, spondylosis, spinal stenosis, radiculopathy, and fractures. These mechanical conditions account for about 90% of cases in low back pain patients. Non-mechanical causes may include myopathy, myelopathy, plexopathies, neuropathies, neoplasms, vascular, gastrointestinal, and genitourinary infections [6].

The history and physical examination revealed proximal weakness greater than distal. The most common neuromuscular disorders causing this pattern of weakness include myopathies, acute/chronic inflammatory demyelinating polyradiculoneuropathy such as Guillain-Barre syndrome or diabetic plexopathy, disorders of the neuromuscular junction, and forms of Spinal Muscular Atrophy, most common Type III (Kugelberg–Welander disease). The differential diagnosis for neuromuscular junction disorders includes the most common post-synaptic disorders, MG and pre-synaptic disorders such as LEMS and botulism.

In our patient, back pain can be explained by the sequalae of LEMS. The proximal weakness and weakness of the supporting structures of the spine and more prominent in the lower back, contributed to an over stress of the lower spine that resulted in low back pain [7].

LEMS is a rare condition which results from an autoimmune attack against voltage-gated calcium channels at the presynaptic motor nerve terminal [8]. It is the second most

common neuromuscular junction disorder. This causes an abnormality of acetylcholine release at the neuromuscular junction. In LEMS the number of Ach quanta released from the presynaptic membrane is reduced, despite normal amount of Ach vesicles, normal presynaptic concentration, and normal postsynaptic Ach receptors. Lambert and Elmqvist described the unique features of this condition with normal miniature endplate potential amplitude, demonstrating normal postsynaptic sensitivity to acetylcholine and markedly reduced evoked endplate potential amplitude, suggesting a significant reduction in Ach release [9]. Ach release is increased by increasing calcium concentration but not potassium-induced depolarization.

LEMS is an autoimmune disorder with autoantibodies directed against voltage-gated calcium channels (VGCC). VGCC is a large transmembrane protein with many subunits and is the target of the antibodies. These antibodies interfere with normal function of the VGCC, thus reducing the normal flux of calcium required for release of acetylcholine [8].

Typical age of onset is of 50 years or more and is characterized by leg and/or general weakness, rarely muscle pain, arm weakness, diplopia and dysarthria [10]. Autonomic dysfunction such as xerostomia, hypohidrosis, blurred vision; constipation and orthostatic hypotension have been frequently observed [11]. Post exercise facilitation is another distinctive feature and is characterized by increase of the deep tendon reflexes and muscular strength after exercise. Cranial nerves are usually spared [12]. Respiratory symptoms may occur [13, 14].

There are two major forms of LEMS: paraneoplastic and non-paraneoplastic. It is most commonly associated with small cell lung cancer (SCLC) especially in heavy smokers, more frequently in males than in females [15, 16].

Electrophysiological testing includes routine motor and sensory nerve conduction studies, high-frequency repetitive nerve stimulation (RNS) and/or exercise testing that shows changes suggesting dysfunction of the presynaptic membrane in LEMS [17]. Needle electromyography is done to exclude motor neuron disease, and in selected patients, single fiber electromyography is performed as it is very sensitive for neuromuscular junction disorders, but not specific for a presynaptic localization [18].

Routine motor and sensory nerve conduction studies should be performed in at least two nerves, CMAP amplitudes usually are diffusely low or borderline, with normal latencies and conduction velocities. This response may increase after brief exercise [18].

RNS and exercise testing is done by either high-frequency (30-50Hz) RNS or slow (2-3 Hz) RNS stimulation and brief, 10 seconds, exercise. Exercise testing is better tolerated by the patients and is preferable to fast RNS unless the patient can not cooperate. An increment greater than 40% is abnormal. Most patients with LEMS will have an increment greater than 100%. Any increment between 40 and 100% is a sign of presynaptic disorder. Slow RNS (3Hz) may elicit a decremental response, however, after brief exercise, the baseline CMAP is significantly larger compared to pre-exercise CMAP [5, 19]. This peculiarity was registered at our patient.

Needle electromyography is usually normal in LEMS, but the action potential may be unstable and of low amplitude, sometimes polyphasic with normal or early recruitment pattern [18, 20].

Single-fiber EMG may be performed, and changes will be consistent with a neuromuscular junction disorder, like increased jitter and blocking, but it cannot differentiate LEMS from other disorders of the neuromuscular junction [21, 22, 23]. Serologic panel should contain P/Q calcium channel antibodies, creatine kinase and paraneoplastic markers [4, 24].

Differential diagnose should be made with MG [12], myopathies [25] and motor neuron disease [26]. MG involves the ocular and bulbar muscles to a greater extent whereas in LEMS the proximal lower extremities are primarily affected. In motor neuron disease muscle atrophy, hyperreflexia and pathological signs are the prominent features in contrast to LEMS where these clinical signs are not present. Electrophysiological and serological testing will differentiate from myasthenia gravis, myopathy or motor neuron disease.

Main priority in a patient with LEMS is to evaluate for malignancy that is found in about 50% of cases [27, 28]. In many patients, treatment of the underlying malignancy will improve the neurological symptoms. The most common tumor associated with LEMS is small cell lung cancer, especially among smoking patients which are 50 years or older [27]. Other malignancies associated with LEMS are Hodgkin lymphoma [29, 6], and rarely atypical carcinoid [30], thymic neuroendocrine carcinoma [31], malignant thymoma [32], and neuroblastoma [33].

Evaluation of patients diagnosed with LEMS and no known cancer, should start with CT of the chest, abdomen and pelvis. Brain imaging is recommended if focal neurological signs are present. If the initial evaluation of the patient is negative, repeated screening for malignancy after 6 months is recommended. Evaluations should be repeated until at least 2 years if no cancer is found [34].

Patients with paraneoplastic LEMS have a shortened life expectancy because of the progression of the associated neoplasm. Survival is correlated with the stage of the disease at presentation. A longer survival was observed in patients with SCLC that developed LEMS, the last one representing an independent predictor of prolonged survival [34].

Patients with non-paraneoplastic LEMS may have a normal or almost normal life expectancy, although a minority may remain disabled. Most of the deaths were not caused by LEMS but they might be related to complications of glucocorticoid therapy.

First line treatment in LEMS is 3,4 – diaminopyridine (3,4 – DAP) [35] that blocks the presynaptic voltage-gated potassium channels increase obtaining in result the release of ACh into the synaptic cleft. Long term immunosuppressive treatment with prednisone and azathioprine, plasma exchange or IVIg help ameliorate the symptoms. However, the aggressive immune suppression can lead to immunologic suppression of tumor growth and in this case, it is up to the physician whether aggressive therapy is safe [34].

At our patient the search for a malignancy didn't give an

indication of an underlying cancer. In spite of the fact that the patient is a heavy smoker we didn't get evidence of a SCLC. We will repeat the investigations, including chest CT, 3-6 months later. Treatment with azathioprine and corticosteroids slightly improved his condition.

Conclusions

LEMS is an acquired paraneoplastic or idiopathic disorder of the pre-synaptic membrane of the NMJ. Autoantibodies directed against voltage-gated calcium channels located on the pre-synaptic membrane lead to a decrease in release of acetylcholine in to the synaptic cleft. Clinical features include proximal muscle weakness, markedly in the lower limbs, reduced deep tendon reflexes that can increase after exercise, and autonomic disturbances. In about 50% of cases LEMS is associated with small cell lung cancer. Electrophysiological testing is important in the diagnostics of LEMS, main features including decrement response on slow repetitive nerves stimulation (3Hz), and an increment of more than 100% in CMAP amplitude after brief exercise, or high frequency repetitive stimulation (30-50 Hz). Immunological assay for antibodies directed against voltage-gated calcium channels P/Q or N type has a high specificity and is confirmatory of LEMS when present. Symptomatic Treatment includes 3,4 – diaminopyridine (3,4 – DAP) and immunosuppression, with intensive monitoring for malignancy, especially small cell lung cancer.

References

1. Mahadeva B, Phillips LH 2nd, Juel VC. Autoimmune disorders of neuromuscular transmission. Semin Neurol. 2008;28(2):212-27.
2. Nicolle MW. Myasthenia Gravis and Lambert-Eaton Myasthenic Syndrome. Continuum (Minneap Minn). 2016;22(6, Muscle and Neuromuscular Junction Disorders):1978-2005.
3. Sanders DB, Guptill JT. Myasthenia gravis and Lambert-Eaton myasthenic syndrome. Continuum (Minneap Minn). 2014;20(5 Peripheral Nervous System Disorders):1413-25.
4. Motomura M, Johnston I, Lang B, et al. An improved diagnostic assay for Lambert-Eaton myasthenic syndrome. J Neurol Neurosurg Psychiatry. 1995;58(1):85-7.
5. AAEM Quality Assurance Committee. American Association of Electrodiagnostic Medicine. Practice parameter for repetitive nerve stimulation and single fiber EMG evaluation of adults with suspected myasthenia gravis or Lambert-Eaton myasthenic syndrome: summary statement. Muscle Nerve. 2001;24(9):1236-8.
6. Lemal R, Chaleteix C, Minard P, Roche C, Bay JO, Tournilhac O, Lamy T. Large granular lymphocytic leukemia associated with Lambert-Eaton Myasthenic Syndrome: a case report. Leuk Res Rep. 2013 Apr 2;2(1):32-3.
7. Keesey JC. Clinical evaluation and management of myasthenia gravis. Muscle Nerve. 2004;29(4):484-505.
8. Lang B, Newsom-Davis J, Wray D, Vincent A, Murray N. Autoimmune aetiology for myasthenic (Eaton-Lambert) syndrome. Lancet. 1981;2(8240):224-6.
9. Lambert EH, Elmqvist D. Quantal components of end-plate potentials in the myasthenic syndrome. Ann N Y Acad Sci. 1971;183:183-99.
10. O'Neill JH, Murray NM, Newsom-Davis J. The Lambert-Eaton myasthenic syndrome. A review of 50 cases. Brain. 1988;111 (Pt 3):577-96.
11. Clark CV, Newsom-Davis J, Sanders MD. Ocular autonomic nerve function in Lambert-Eaton myasthenic syndrome. Eye (Lond). 1990;4 (Pt 3):473-81.
12. Wirtz PW, Sotodeh M, Nijnuis M, Van Doorn PA, Van Engelen BG, Hintzen RQ, De Kort PL, Kuks JB, Twijnstra A, De Visser M, Visser LH, Wokke JH, Wintzen AR, Verschuuren JJ. Difference in distribution of muscle weakness between myasthenia gravis and the Lambert-Eaton myasthenic syndrome. J Neurol Neurosurg Psychiatry. 2002;73(6):766-8.
13. Titulaer MJ, Wirtz PW, Kuks JB, Schelhaas HJ, van der Kooi AJ, Faber CG, van der Pol WL, de Visser M, Sillevis Smitt PA, Verschuuren JJ. The Lambert-Eaton myasthenic syndrome 1988-2008: a clinical picture in 97 patients. J Neuroimmunol. 2008;201-202:153-8.
14. Laroche CM, Mier AK, Spiro SG, Newsom-Davis J, Moxham J, Green M. Respiratory muscle weakness in the Lambert-Eaton myasthenic syndrome. Thorax. 1989;44(11):913-8.
15. Benatar M, Blaes F, Johnston I, Wilson K, Vincent A, Beeson D, Lang B. Presynaptic neuronal antigens expressed by a small cell lung carcinoma cell line. J Neuroimmunol. 2001;113(1):153-62.
16. Lennon VA, Lambert EH, Whittingham S, Fairbanks V. Autoimmunity in the Lambert-Eaton myasthenic syndrome Muscle Nerve. 1982;5(9S):S21-5.
17. Oh SJ, Kim DE, Kuruoglu R, Brooks J, Claussen G. Electrophysiological and clinical correlations in the Lambert-Eaton myasthenic syndrome. Muscle Nerve. 1996;19(7):903-6.
18. Preston DC, Shapiro BE. Neuromuscular junction disorders. In: Electromyography and neuromuscular disorders: Clinical-electrophysiologic correlations. 3rd ed. New York: Elsevier; 2013. p. 529.
19. Dumitru D, Amato AA. Neuromuscular junction disorders. In: Dumitru D, Amato AA, Zwarts MJ, editors. Electrodiagnostic medicine. 2nd ed. Philadelphia, PA: Hanley & Belfus; 2002. p. 1179-80.
20. Crone C, Christiansen I, Vissing J. Myopathic EMG findings and type II muscle fiber atrophy in patients with Lambert-Eaton myasthenic syndrome. Clin Neurophysiol. 2013 Sep;124(9):1889-92. Epub 2013 May 3.
21. Trontelj JV, Stalberg E. Single motor end-plates in myasthenia gravis and LEMS at different firing rates. Muscle Nerve. 1991;14(3):226-32.
22. Chaudhry V, Watson DF, Bird SJ, Cornblath DR. Stimulated single-fiber electromyography in Lambert-Eaton myasthenic syndrome. Muscle Nerve. 1991;14(12):1227-30.
23. Oh SJ, Ohira M. Single-fiber EMG and clinical correlation in Lambert-Eaton myasthenic syndrome. Muscle Nerve. 2013 May;47(5):664-7. Epub 2013 Mar 16.
24. Nakao YK, Motomura M, Fukudome T, Fukuda T, Shiraishi H, Yoshimura T, Tsujihata M, Eguchi K. Seronegative Lambert-Eaton myasthenic syndrome: study of 110 Japanese patients. Neurology. 2002;59(11):1773-5.
25. So YT. Immune-mediated neuropathies. Continuum (Minneap Minn). 2012 Feb;18(1):85-105.
26. Kim WK, Liu X, Sandner J, Pasmantier M, Andrews J, Rowland LP, Mitsumoto H. Study of 962 patients indicates progressive muscular atrophy is a form of ALS. Neurology. 2009;73(20):1686-92.
27. Payne M, Bradbury P, Lang B, Vincent A, Han C, Newsom-Davis J, Talbot D. Prospective study into the incidence of Lambert Eaton myasthenic syndrome in small cell lung cancer. J Thorac Oncol. 2010;5(1):34-8.
28. Wirtz PW, Lang B, Graus F, van den Maagdenberg AM, Saiz A, de Koning Gans PA, Twijnstra A, Verschuuren JJ. P/Q-type calcium channel antibodies, Lambert-Eaton myasthenic syndrome and survival in small cell lung cancer. J Neuroimmunol. 2005;164(1-2):161-5.
29. Argov Z, Shapira Y, Averbuch-Heller L, Wirguin I. Lambert-Eaton myasthenic syndrome (LEMS) in association with lymphoproliferative disorders. Muscle Nerve. 1995;18(7):715-9.
30. Gutmann L, Phillips LH 2nd, Gutmann L. Trends in the association of Lambert-Eaton myasthenic syndrome with carcinoma. Neurology. 1992;42(4):848-50.
31. Nalbantoglu M, Kose L, Uzun N, Gunduz A, Hallac M, Kiziltan ME, Akalin MA. Lambert-Eaton myasthenic syndrome associated with thymic neuroendocrine carcinoma, Muscle Nerve. 2015;51(6):936-8. Epub 2015 Mar 14.
32. Lauritzen M, Smith T, Fischer-Hansen B, Sparup J, Olesen J. Eaton-Lambert syndrome and malignant thymoma. Neurology. 1980;30(6):634-8.
33. Hajjar M, Markowitz J, Darras BT, Kissel JT, Srinivasan J, Jones HR. Lambert-Eaton syndrome, an unrecognized treatable pediatric neuromuscular disorder: three patients and literature review. Pediatr Neurol. 2014;50(1):11-7.
34. Titulaer MJ, Soffietti R, Dalmau J, Gilhus NE, Giometto B, Graus F, Grisold W, Honnorat J, Sillevis Smitt PA, Tanasescu R, Vedeler CA, Voltz R, Verschuuren JJ; European Federation of Neurological Societies. Screening for tumours in paraneoplastic syndromes: report of an EFNS task force. Eur J Neurol. 2011;18(1):19-e3.
35. Wirtz PW, Verschuuren JJ, van Dijk JG, de Kam ML, Schoemaker RC, van Hasselt JG, Titulaer MJ, Tjaden UR, den Hartigh J, van Gerven JM. Efficacy of 3,4-diaminopyridine and pyridostigmine in the treatment of Lambert-Eaton myasthenic syndrome: a randomized, double-blind, placebo-controlled, crossover study. Clin Pharmacol Ther. 2009;86(1):44-8. Epub 2009 Apr 8.

DOI: 10.5281/zenodo.2590026
UDC: 616.98:578.825.12

The role of cytomegalovirus in the development of opportunistic infections

***Elena Cirjau,** MD, Undergraduate Student; **Emilia Behta,** MD, Assistant Professor

Department of Microbiology and Immunology, NicolaeTestemitsanu State University of Medicine and Pharmacy
Chisinau, the Republic of Moldova

*Corresponding author: kirjeu@mail.ru
Manuscript received November 02, 2018; revised manuscript February 05, 2019

Abstract

Background: About one century ago, was found and described a new virus, which, due to its particular way of damaging cells, was called cytomegalovirus. Human is the only natural source of cytomegalovirus infection. The relevance is that it is a widespread pathology, and due to its ability to "disguise" in the human body, remains unnoticed until the "defect" appears in the body's immune system. It is especially dangerous for pregnant women, children and people with immunodeficiency. It is one of the most common infections that cause pathology in the fetus and newborns, which, in turn, leads to serious consequences, from disability to child death. There are many ways of cytomegalovirus transmission: airborne, parenteral, domestic contact, sexual and vertical (transplacentally, with aspiration of secretions from the birth canal and natural feeding). The virus is able to have a direct and indirect effect on the body. It is able to independently induce immunosuppression. The article describes the epidemiological data, pathogenesis, clinical manifestations, and modern methods of diagnosis, treatment and prevention of cytomegalovirus infection. Also, some diagnostic problems in immunosuppressive organisms are described.

Conclusions: Due to its consequences, namely, children`s disability, death and immunosuppressed people, cytomegalovirus has become a demographic problem. A high infection frequency indicates a low level of social development of the population. More public awareness is needed on the transmission and possible consequences of cytomegalovirus infection.

Key words: cytomegalovirus, opportunistic infections, immunosuppression.

Introduction

Cytomegalovirus (CMV) is an opportunistic pathogen that, after the primary infection, causes lifelong latent infection without any clinical manifestations in immunocompetent organisms. It causes severe diseases in weakened organisms, namely, newborns, patients receiving antitumor chemotherapy, transplant recipients and HIV patients. CMV has some mechanisms by which it avoids the detection and destruction of the host immune system [1]. A CMV is a ubiquitous virus. Numerous researchers all over the world have obtained data indicating that the population has a large number of virus carriers and individuals with a hidden form of CMV. The proof of human infection is the presence of CMV antibodies in the body. There is information about the wide spread of CMV in the human population, the upward trend, the associated morbidity, and its increasing importance in human pathology [2, 3, 4, 5, 6]. Most of the world population (65 to 90%) is infected with a pathogen throughout life. The infestation percentage depends on the socioeconomic status, geographical location and education [7]. It has been observed that seropositivity and virus carriage are more common in developing countries.

According to the decision of the WHO European Office, cytomegalovirus infection (CMVI) is included in the list of "new and mysterious diseases that determine the future of infectious diseases" [8]. The relevance of CMV infection is that it is a widespread pathology, and due to its ability to "disguise" in the human body, it remains unnoticed until the "defect" appears in the body's immune system. The increase in the number of people with immunodeficiency is of current interest. These include people who take immunosuppressants and cytostatics, patients who suffer from malignant neoplasms and HIV infection [3, 9]. Studies have shown that the development of CMVI during the treatment with cytostatics results in death in 80% of cases [10]. CMV is one of the most common infections which cause pathology in the fetus and newborns, which, in turn, leads to serious consequences, from disability to child death [11]. This indicates the presence of a demographic problem. The infection of the pregnant women during the first trimester of pregnancy is especially dangerous. Such cases are less common, but the fetus pathology is very pronounced [9, 12]. The exacerbation of chronic infection and superinfection with another strain of CMV is also dangerous [3]. It has been established that during blood transfusion and organ transplantation, CMV infection can also be transmitted.

History

In 1881, at a meeting of the Society of Physicians of the *Lower Rhine* region, the German pathologist H. Ribbert, for the first time described the histological picture of the neonatal nephritis with congenital syphilis. He found hypertrophied renal tubules with unusual giant cells containing nuclear inclusions. He suggested a protozoan etiology of the inclusion. In 1904 he described similar cells in other neonatal organs. In 1920–21 E. Goodpasture and P.Talbert

suggested that the etiology of this infection was viral, since the lesion was similar to herpes virus and chickenpox virus. The changes in the normal epithelial cells found in the liver, kidneys and lungs are due to chronic inflammation. This characteristic picture was called "cytomegaly". In 1925, for the first time, such cells were found in the body of an adult, viz. in a man who died as a result of complications of liver abscess and ulcerative colitis. In 1926 Cole and Kuttner found similar changes in the salivary glands of rats and gilts. The unsuccessful attempt to infect other animal tissues with infected human secretions proved the specificity of tropism specifically to the salivary gland epithelium. Fetterman (1952) found that cytomegalic inclusions can be discharged during the whole life, being found in the urine. In 1954 Smith isolated a pure virus culture from the salivary gland epithelium in rats, and later from the human salivary glands. Rowe found this virus in the adenoids of children. In subsequent years, intracellular inclusions characteristic of cytomegaly were found in the tissues of the liver, spleen, brain, retina of sick newborns and infants. Clinically, these changes manifested as hepatosplenomegaly, icterus, chorioretinitis, microcephaly with mental retardation, motor disorders, cerebral calcification. In 1960 Weller introduced a new nosology – cytomegalovirus infection. In 1960-1970, due to the active study of this pathogen, the modes of transmission (natural and artificial) were determined, and the fact that most people remain infected after initial contact [13, 14, 15].

Epidemiology

Human is the only natural source of CMV infection. The age of CMV infection depends on the geographical location, socioeconomic status, culture and education of the population. In developing countries most children become infected with CMV infection in the first years of life, therefore, in early youth, 100% of the adult population is seropositive. In developed countries, only 50% of young people of intermediate socioeconomic status are seropositive. This observation has important implications for the epidemiology of congenital CMVI. Inasmuch as a CMV- seronegative woman of childbearing age has a serious risk to be infected during pregnancy, this can cause a high probability of intrapartum infection of the fetus as well as the birth of a baby with some congenital infection symptoms. Cases of congenital CMVI in infants from mothers who have been infected before pregnancy are caused by reactivation or reinfection with another virus strain. Its main biological properties have a lifelong persistence (in the vascular endothelium, liver, spleen, salivary glands, brain and kidneys), and reactivation in the human immunosuppressed organism [7, 16, 17]. It is important to note that there are many ways of CMV transmission: airborne, parenteral, household contact, sexual and vertical (transplacentally, aspiration of secretions from the birth canal and breastfeeding). This infection is also called "kissing disease", as it is often transmitted through sexual contact, therefore it can be considered a problem of young people [18]. In turn, blood transfusion and organ transplantation are also important ways of CMV transmission. Thanks to blood screening, the number of cases of morbidity and mortality of premature babies was reduced due to transfusion-induced CMV infection [13]. According to some researchers, infectious diseases are detected in 50-60% of full-term newborns and 70% of premature newborns [19]. CMVI is one of the most common intrauterine infections that cause severe pathology, even the child death [11]. More than 50% of breast-fed infants of CMV-seropositive mothers are infected with CMV [20]. The uninfected children have a high risk of infection in kindergarten. According to some studies, about 80% of preschool children are CMV-seropositive. After all, the virus is easily transmitted to susceptible children through saliva, urine and other contaminated objects. Being infected in the kindergarten, children can transmit the infection to their parents [21, 22], which plays an important role in the epidemiology of CMV infections in young parents [23]. It is considered that the sexual transmission of CMV is prevalent in the adult population [18], but given the fact that the virus is found in saliva, vaginal secretions and semen, it is difficult to determine which particular way of transmission prevails. Serodiagnosis of donor and recipient blood before and after transfusion is evidence, that CMV is also transmitted parenterally. A research was conducted on blood screening before transfusion. It revealed that the rate of CMV transmission to premature newborns was reduced [13]. Often, the clinical manifestations of CMVI are found in previously infected individuals, which is the reactivation of the latent form or reinfection with a new strain. In HIV patients, cytomegalovirus affects the organs during the stages of progressive immunosuppression, viz, in the AIDS stages, which either do not receive or do not respond to antiretroviral therapy (ART). Other risk factors include the presence of associated opportunistic infections (pneumocystic pneumonia, toxoplasmosis, histoplasmosis, tuberculosis, etc.), high levels of CMV and HIV viremia in plasma [24].

Morphological and biological features of CMV

Cytomegalovirus: Herpesviridae family, Bethaherpesviridae subfamily, Cytomegalovirus genus [25]. Morphological and biological properties are similar to herpes simplex virus type 1. CMV belongs to the human herpes virus type 5. CMV has the largest double-stranded linear DNA genome from the entire Herpesviridae family. The virus consists of an icosahedral capsid covered with a tegument and a bilipid outer envelope [13, 26].The approximate size of spheric virion is 200-300 nm [26]. The cubic nucleocapsid contains 162 of capsomers. The surface and capsid glycoproteins are distinguished in the virus structure. The glycoproteins gB, gO, gN, gH, gL, located on the membrane surface, are involved in the attachment and penetration into the host cells. Protein gB is the initiator of attachment [27]. The remaining glycoproteins are involved in the cell-virus immune response [27]. The proteinkinase is found in CMV.

It phosphorylates ganciclovir (antiviral drug), which is very important in the treatment of CMVI. When a kinase mutates, the resistance to therapy develops [13, 28]. CMV is characterized by: low virulence, the ability to suppress cellular immunity, a long cycle of reproduction and the lowest cytopathogenic activity, in contrast to herpes simplex virus. Like other representatives of herpes viruses, CMV has the ability to infect mononuclear cells and lymphocytes. It replicates in cell culture (in vivo), but slowly [26].

CMV is able to encode more than 200 protein structures [13]. The functions of most of these proteins remain unclear, but it is known that they are divided into functional and structural proteins. Some functions of the functional proteins have been established: UL16 protein inhibits natural killer (NK) cells; UL24 induces cell cycle arrest; TRS1 inhibits the process of autophagy of affected cells; UL36 inhibits cell apoptosis; US2 destroys the components of the major histocompatibility complex (MHC) class 2, preventing the recognition of CD4 + lymphocytes; US3 prevents maturation and transport of MHC class 1 molecules; phosphoprotein 65 (pp65), located in the tegument, inhibits the cascade of interferon production, which provides an inborn antiviral immunity [26].

Pathogenesis

CMV has a direct effect on the body. Using the above surface glycoproteins, it attaches to specific receptors of the cytoplasmic membrane of the host cell [26]. In respect of the vital activity of the virus, the intracellular parasitization is necessary, as it has adapted itself to penetrate the cells through a few ways [26]. CMV penetrates through viropexis or fusion of capsid with cytoplasmic membrane. Being inside the cell, virus replication begins, which consists of 3 phases. The super early phase lasts 2-4 hours after infection. During this period nucleocapsid proteins are synthesized to ensure penetration into the nucleus and onset of viral replication. The viral nucleocapsid DNA activates the major immediate early gene (IE). This gene encodes a protein that is the main regulator of virus transcription and activation. During replication, antigens are synthesized and accumulated in the nucleus, viz. the virus grows up. Next the 24 hour early phase ensues. It begins with the synthesis of a new viral DNA, DNA polymerase and enzymes that are necessary for the production of new virions. As a result, the newly replicated DNA is coated with a capsid. Then the late phase begins, namely, the escape of the virus into the cytoplasm of the host cell. Here it is enriched with the secondary coat – envelope. The envelope is synthesized in the endoplasmic reticulum and the Golgi apparatus [13, 14, 26]. Depending on the type of the affected cell, 2 variants of late phase development are possible, but the result in both cases is cell lysis. In the epithelium of mucous membranes – virus exocytosis occurs, while in lymphocytes, monocytes and fibroblasts – latent persistence is found. In the late period, the virus cannot be recognized due to a weak expression of IE gene [26, 27, 28, 29]. The activation of the infectious process can occur with the differentiation of monocytes into macrophages. This occurs during an active infectious process. The viral fragments are recognized by professional antigen-presenting cells, namely dendritic cells, macrophages, B-lymphocytes, CD8 + and CD4 + lymphocytes. Being activated, they release pro-inflammatory cytokines and activate NK and antigen-specific T-helpers. In turn, NK lyses cells in which the MHC class 1 disappeared due to the virus. Pathologies resulting from direct action are described in the section *Clinical manifestations*. CMV also has an indirect effect on the body, causing its immunosuppression via the following mechanisms: 1) preventing the antigen presentation of CD8+ and CD4+ cells, viz., suppressing their cytotoxicity; 2) preventing the expression of MHC 1-2 molecules on the cell surface; 3) viral protein blocking of the cytotoxic action of NK [12]. Due to the suppression of T-lymphocytes and B-lymphocytes functions, the antibody synthesis diminishes (IgM, IgG). This misleading aspect results in diagnosis difficulties. A discordance is observed between the patient's condition and the process activity, especially in cases of AIDS and allogeneic immunosuppressions [30]. This manifests as transplant rejection and superinfection [10].

Clinical manifestations

Clinical manifestations are very variable due to the tropism to the multitude of body tissues, as well as different sensitivity of these tissues to the virus action. This is not uncommonly misleading when making diagnoses, being disguised as other nosologies. The epithelium of the mucous membranes of the respiratory and intestinal tracts is the most sensitive, as well as the epithelium of the bile ducts, neuroglial and hematopoietic cells.

CMV in *immunotolerant humans*, is manifested as CMV mononucleosis or sialoadenitis. CMV mononucleosis begins with general symptoms of intoxication, fever, as well as sore throat and pain in the projection area of the salivary glands. Objectively, swollen cervical and submandibular lymph nodes are determined and enlarged liver by2-3 cm from the costal arch. In sialoadenitis, on the background of subfebrile temperature, the parotid salivary glands are bilaterally impaired.

In *children*, depending on the infection period, CMV can manifest itself in several forms: 1) congenital (intrauterine infection), the symptoms appear in the first 2 weeks of life; 2) perinatal (infection during childbirth or neonatal period); 3) acquired (infection of children from 1 month to 2-5 years) [13, 31]. In the congenital form, 90% of babies lack clear signs of CMVI. This happens when an infected woman gets pregnant. Such children have a high risk of developing complications, such as developmental delay and neurosensory deafness. The frequency of the latter is about 15%, [32, 33]. In the congenital form, only 10% of newborns have obvious symptoms of CMV lesion. Of these, 40–90% have hepatitis, neurological disorders, developmental delay, cerebral palsy, microcephaly, and mental retardation [34, 35, 36]. The cases of sensorineural hearing loss amount to

about 35-65% [37]. This type of CMVI is called "cytomegalic inclusion disease". This type also develops in the case of primary infection of the mother during pregnancy. The aspect of the newborn is "blueberry muffin baby", due to the defeat of the hematopoiesis cells and the development of thrombocytopenic purpura [13]. The CNS impairment occurs, namely, cerebral atrophy, chorioretinitis with optic nerve atrophy, microcephaly, ventriculomegaly and the presence of intracerebral calcificates. At the same time, a little more than 30% of newborns are premature. Different combinations of syndromes are characteristic [38, 39, 40]. Sucking and swallowing disorders are objectively revealed, as well as strabismus, epileptic seizures, paraparesis, plegia and muscular hypotonia is replaced by hypertension. There is an assumption that CMV influences the development of neurological symptoms in Down`s syndrome [41]. In the case of perinatal infection some conditions are characteristic, such as neonatal cholestatic hepatitis, hematological disorders, lymphadenopathy, focal nephritis and pneumonitis (viz., atypical inflammation of lung tissues). In acquired infection, mononucleosis syndrome, acute CMV hepatitis, or prolonged fever syndrome are possible. The outcome of an acute process in any tissue is the formation of calcifications and interstitial fibrosis [42].

In patients with *post-transplant immunosuppression*, any kind of CMV infection has a more severe course with a high incidence of lethal outcome, and usually occurs in the first 120 days after the intervention. The clinical manifestations depend on the type of transplant and the degree of immunosuppression. Bone marrow recipients most often develop interstitial pneumonia, hepatitis, and esophago-gastro-duodenal impairment. In the case of recipients of solid organs, the transplanted organs are mainly affected. The symptoms of the affected organ are associated with fever of unknown origin and typical for CMV changes in the overall blood picture [43]. In liver transplantation, the differential diagnosis of rejection and CMV hepatitis is difficult [10, 43].

In *HIV-infected persons*, the eyeball tissue is usually the first to be affected. Most often the process is bilateral, especially in the absence of ART, the level of CD4 + is<50 cells / $mm^3$and the immune recovery syndrome develops**.** CMV retinitis does not always begin with scotomas, decreased visual acuity, photophobia, loss of visual field and the appearance of "floating flies" before eyes [28, 35]. Sometimes the symptoms may be absent. But when examining the retinal fundus, the "omelette with ketchup" symptom is always detected [35], which are perivascular yellow-white infiltrates with hemorrhages [28, 35]. When there is immune recovery syndrome, vitreitis may develop, that is, the inflammation of the vitreous body. This syndrome usually manifests 1-3 months after the onset of highly active ART. The reduced visual acuity and vision loss, as complications in these cases, result from cataracts, retinal detachment, cystoid macular edema, or damage to the zone 1 of the cerebral cortex [35]. Colitis occurs in 5-10% of HIV-infected people in AIDS stage. Such symptoms as severe weakness, fever, abdominal pain, profuse diarrhea, weight loss, and anorexia are characteristic. Some complications can occur, such as intestinal hemorrhage and perforation of the intestinal walls. CMV-esophagitis is rare, being accompanied by fever, odynophagia, localized retrosternal pains and nausea [28, 35]. To make the diagnosis of CMV pneumonitis, several criteria must be considered: 1) the presence of interstitial infiltrates in the lungs on a radiograph or CT scan; 2) intracellular inclusions in the lung tissues (typical for CMV); 3) the absence of another pathogen that can cause pneumonitis [35]. The damage to the nervous system is clinically manifested as dementia, myeloradiculitis and ventriculo-encephalitis [28, 35]. At the same time, they are obligate in CSF mononuclear pleocytosis and proteinuria [35]. The association with the following symptoms determines the process nature and localization. The appearance of fever, drowsiness and impaired consciousness indicates the development of dementia. Disturbances of consciousness can result in reduced attention and memory, delirium, spatial and/or temporal disorientation. The appearance of progressive paresis of the lower extremities and dysfunction of the pelvic sphincters subsequently indicates myeloradiculitis. In ventriculoencephalitis, in addition to ataxia, nystagmus, progressive delirium and cranial nerves lesions, MRI revealed signal amplification in the periventricular zone [44].

Laboratory diagnosis

Studies are performed on certain groups of patients: 1) women who are pregnant or who are planning their pregnancy, with the history of miscarriages, congenital malformations and stillbirths; 2) pregnant women diagnosed with hepatitis, hepatosplenomegaly, fever of unknown origin, or detected by ultrasound, symptoms of intrauterine infection; 3) patients with immunodeficient conditions (HIV, cancer, treatment with immunosuppressants, hemodialysis, etc.); 4) children with congenital or acquired CMVI symptoms; 5) all patients with sepsis, meningoencephalitis, severe pneumonia, hepatitis, impairment of the digestive tract and eyes [45, 46, 47]; 6) donor and recipient of blood components, organs, tissues and sperm before each donation; 7) history of sexual contact with a seropositive partner.

Methods of laboratory diagnosis

Virusoscopic method. The main morphological feature, active infection – giant cells with intranuclear and intracytoplasmic inclusions, "cytomegaly." This symptom is found only in 50% of cases.

Virological method. Almost any body fluid or tissue can be cultivated. The culture for the virus should consist of a single layer of human embryonic fibroblasts and a double layer of human lung cells. The cultivation duration is up to 6 weeks.

Serological methods. Their essence is to identify viral antigens (Ag) interacting with each other and antibodies (Ab) produced by the body against them. Enzyme-linked immunosorbent assay (ELISA) is the most common and affordable test. It determines the degree of avidity and the

presence of CMV antibodies of classes IgA, IgM and IgG. (Avidity is the stability of Ag-Ab immunocomplexes, which characterizes the antibodies activity). In 5-7 days, after the initial infection Ab IgM appear in the blood and persist for 1-2 months [44]. After 10-14 days of infection, low-avid IgG appear. Within 1–3 months, high-avid IgG appear and grow, and remain in the blood for the entire life [48]. When the process is reactivated, the hyperproduction of IgA is more typical than IgM. The presence of low avid antibodies indicates a primary infection, and a highly avid infection, a reactivation or latent infection. To establish the process activity, ELISA is repeatedly carried out, in order to determine the follow-up changes in the level of Abs [49]. It is to be noted that this method does not always reflect the real state of the patient. As in the immunodeficiency conditions, the immune system is not able to produce a sufficient amount of antibodies, which can be regarded as a low process activity [30]. If a congenital infection is suspected, antibodies are detected in the blood in the first 3 weeks after birth. Otherwise, Ab can be detected after 3 weeks, as a result of natural feeding with infected CMV milk [13]. Immunofluorescence (IF) reactions are used to establish the infectious process activity. IF is based on the detection of fluorescent Abs. Ab labeled with fluorochrome do not lose the ability to connect with the corresponding Ag and thereby cause a blue-violet glow. The presence of antigen luminescence in the nucleus and cytoplasm of cells reveals pp72 and pp65 proteins [49]. Another method of indirect immunofluorescence is applied after 12-24 hours of cultivation in a test tube, centrifuged and stained with monoclonal CMV-specific antibodies. This method is much more sensitive and accelerates the duration of the study [50, 51]. Also, researchers carry out reactions of binding complement and indirect hemagglutination.

M**olecular biological method** is a specific and highly sensitive study, and it is used as the main method of diagnosing CMVI as an opportunistic infection [52, 53]. The viral DNA genome is determined by the polymerase chain reaction (PCR). It is possible to determine the viral load, which is important in determining the follow-up treatment efficacy. Both latent and active infections are detected. The presence of DNA in the blood and urine indicates a high viral activity, i.e. the presence of CMV, while the DNA presence only in saliva, and indicates an infection [54]. The presence of CMV DNA in the amniotic fluid indicates 100% damage to the fetus [53].

Cytological method – its positive result proves the presence of CMVI in 100%. The virus can be detected in all the body secretions, CSF, urine, tissue biopsies. The staining of biomaterial smears and tissue sections is carried out using dyes (hematoxylin-eosin or Romanovsky-Giemsa). Specific cells, cytomegalovirus, containing large intranuclear and cytoplasmic inclusions, similar to the owl's eye, are identified in various shapes and sizes. They contain a breeding virus inside.

Histological examination is the "gold standard" in obstetrics. The placenta study determined its hyperplasia, cytomegaly, thrombosis and focal ischemic infarctions, fibrinoid necrosis of the chorionic villi stroma and damage to the basal decidual cells [51]. But the method has a number of drawbacks: low sensitivity of intravital diagnosis, that is why, a negative result requires the biomaterial test to be repeated daily for 3-5 days. This study is effective only at the infectious process peak.

Treatment

The treatment method depends on the age, the immune system state and the presence of associated conditions. Currently, only antiviral nucleoside drugs and a specific human anti-cytomegalovirus immunoglobulin have shown their antiviral efficacy in evidence-based medicine. The immunoglobulin containing donor IgM CMV provides passive immunity [54].

The indications for the use of immunoglobulin are prevention in and treatment of: pregnant women with acute CMV infection during the initial infection (IgM and IgG are present in the blood); pregnant women with active CMV infection (detection of CMV DNA, IgM in the blood and urine, CMV DNA in the amniotic fluid); newborns and children under 3 years old, with CMV DNA found in the biomaterial in the first 2 weeks of life; premature or hypotrophic newborns and children under 3 years old with intranatal infections; children with active CMV (CMV DNA in blood); patients before transplantation; recipients in the post-transplantation period [55].

Children under 3 years of age are administered 1 mg / kg once every 48 hours (6 times) [55]. Anti-cytomegalovirus immunoglobulin is intravenously administered 150 mg / kg 72 hours before transplantation and in the next 2, 4, 6, 8 weeks after transplantation, and in 12 and 16 weeks, 50-100 mg [8].

The first-line antiviral drugs include ganciclovir and valganciclovir, the second is foscarnet and cidofovir. Ganciclovir is a synthetic analogue of purine and is converted by phosphotransferase (UL 97) and other enzymes in infected host cells to ganciclovir triphosphate. The latter, in turn, is embedded in the synthesized viral DNA. This leads to the termination of CMV replication. This drug has proven to be highly effective against the rest of herpes viruses. Valganciclovir is ether and a prodrug of ganciclovir. Valganciclovir has an advantage: its oral absorption and bioavailability are the same as the intravenous administration of ganciclovir [56]. The disadvantages of these drugs are: prolonged therapy causes the development of resistance, due to the mutation of UL97 [13, 28], and the suppression of hematopoiesis, especially leukocytes [57]. Ganciclovir is administered intravenously 5 mg / kg every 12 hours for 14-21 days, or 6 mg / kg orally per day for 5 days (+ 100-120 days after the transplant). Valganciclovir, 900 mg per day for 14-21 days. Foscarnet- pyrophosphate compound inhibits herpes virus DNA polymerase. It differs from previous drugs, namely, it does not need phosphorylation for its activity. Also, it inhibits HIV retrotranscriptase. Disadvantages: only endove-

nous route of administration, reduced levels of calcium, magnesium, potassium, blood phosphorus and nephrotoxicity. It is administered 60 mg / kg every 8 hours for 2–3 weeks, afterwards 90–120 mg / kg. Cydofovir is a synthetic nucleoside analogue which stops the synthesis of viral DNA by inhibiting DNA polymerase. It is active not only against herpes viruses, but also human papilloma viruses, pox viruses and adenoviruses. Intravenous administration – 5 mg / kg per week for 2 weeks in a row.

Indications for the administration of antiviral drugs are: treatment of CMV retinitis, prevention of CMV intransplant recipients, prevention of CMVI in HIV-infected people (ganciclovir for adults and children only), treatment of CMV colitis, esophagitis.

Prevention

Non-specific prophylaxis includes public awareness of transmission routes, risks of infection, and consequences for children and immunocompromised people.

Pregnant women should avoid contact with CMV infected children. After the birth of the fetus with CMVI, it is necessary to keep a 2 year pause until the next pregnancy. Measures should be taken to identify seronegative pregnant women and their laboratory monitoring.

The Federal Agency of the USA *Department of Health Centers for Disease Control and Prevention* (CDC) was created in 1946 in the state of Georgia, and its role is to ensure the protection of public health by providing information in order to improve health care solutions. It made recommendations on the need to raise awareness of the population, especially women of childbearing age for the specific prevention of CMV. In 2000 the National Medical Academy of the United States of America published an article about the high priority of developing a vaccine against CMV, which has become a big stimulus for vaccine manufacturers.

At present, still there is no officially specific prevention, but active research is underway to develop a vaccine against CMV. But there are several recombinant CMV vaccines undergoing clinical trials. It was found that women vaccinated with recombinant gB were 50% less likely to become infected compared to women who received placebo [58].

There were identified 4 groups of patients in need of a vaccine against CMV: CMV seronegative and CMV seropositive women of childbearing age, seronegative recipients of solid organs and bone marrow obtained from CMV seropositive donors [59].

Conclusions

1. Cytomegalovirus infection is very common and poses a great danger to people with low immunity and children.

2. The infection frequency indicates a low level of social development of the population.

3. Currently, there is an increase in the infection rate all over the world, which is connected both with the improvement of the diagnosis quality and real disease growth.

4. The study of CMVI characteristics is rapidly developing, although some issues concerning diagnosis, treatment and prevention are still open: expensive diagnosis and treatment, lack of vaccine.

5. According to the CDC recommendations, more extensive public information is needed, especially for young women of childbearing age.

6. It is also necessary to pay great attention to CMV study in the training of doctors in the Republic of Moldova.

References

1. Froberg MK. CMV escapes! Ann Clin Lab Sci. 2004;34(2):123-30.
2. Drozdov VN. Evoliutsiia infektsionnykh boleznei k iskhodu XX veka [The evolution of infectious diseases by the end of the twentieth century]. In: Materialy vserossiiskogo soveshchaniya Infektsionnye bolezni cheloveka. Omsk; 2001. p. 3-9. Russian.
3. Ershov FI, Kas'ianov NV. Tsitomegalovirusnaia infektsiia. Sovremennye predstavleniia ob epidemiologii, klinike, diagnostike i terapii [Cytomegalovirus infection. Modern aspects of epidemiology, clinical, diagnosis and therapy]. Infekt Antimikrob Ter. 2002;(4):116-119. Russian.
4. Ozhegov AI, Miakisheva LS. Rasprostranennost' tsitomegalovirusnoi infektsii u detei [Prevalence of cytomegalovirus infections in children]. Ross Pediatr Zh. 1999;(3):16-18. Russian.
5. Parkhomenko YuG, Solnyshkova TG, Tishkevich OA. Morfologicheskie izmeneniia fibroblastov pri tsitomegalovirusnoi infektsii [Morphological changes of fibroblasts during cytomegalovirus infection]. Arkh Patol. 2006;(1):3-8. Russian.
6. Almedia LN, Azevedo RS, Amacu M, Massad E. Cytomegalovirus, seroepidemiology in a urban community of San Paulo, Brazil. Rev Saude Pable. 2001;35(2):124-129.
7. Bruggeman CA. Cytomegalovirus and latency: an overview. Virchows Arch B Cell Pathol Incl Mol Pathol. 1993;64(6):325-333.
8. Velimirovic B, Greco D, Grist C. Infectious diseases in Europe. Copenhagen: WHO; 1984. p. 245.
9. Kisteneva LB. Tsitomegalovirusnaia infektsiia kak problema perinatal'noi patologii: etiologiia, patogenez, diagnostika [Cytomegalovirus infection as a problem of perinatal pathology: etiology, pathogenesis, diagnosis]. Ross Vestnik Perinatol Pediatr. 2003;(4):55-59. Russian.
10. Kushchevoi EV, Kriachok IA, Stepanishina IaA, et al. Tsitomegalovirusnaia infektsiia u gematologicheskikh i onkologicheskikh patsientov [Cytomegalovirus infection in hematology and oncology patients]. Klin Onkol. 2013;(2):102-107. Russian.
11. Gibson CS, Goldwater PN, MacLennan AH, et al. Fetal exposure to herpesviruses may be associated with pregnancy-induced hypertensive disorders and preterm birth in a Caucasian population. BJOG. 2008 Mar;115(4):492-500.
12. Ordzhonikidze NV, Tiutiunina VN. Tsitomegalovirusnaia infektsiia i beremennost' [Cytomegalovirus infection and pregnancy]. Akusherstvo Ginekol. 2002;(3):59-63. Russian.
13. Schleiss MR, Steele RW. Pediatric cytomegalovirus infection treatment & management. Medscape. 2018 Apr 06. [cited 2018 Aug 12]. Available from: https://emedicine.medscape.com/article/963090-treatment
14. Vartanyan RV. Problemy tsitomegalovirusnoi infektsii [Problems of cytomegalovirus infection]. Priroda. 2003;(4)54-9. Russian.
15. Dudgeon JA. Cytomegalovirus infection. Arch Dis Child. 1971;46(249):581-3.
16. Akhter K. Cytomegalovirus (CMV). Medscape. Updated 2018 May 05. [cited 2018 Aug 12]. Available from: https://emedicine.medscape.com/article/215702-overview
17. Watzinger F, Ebner K, Lion T. Detection and monitoring of virus infections by real-time PCR. Mol Aspects Med. 2006 Apr-Jun;27(2-3):254-98. Epub 2006 Feb 14.
18. Guseva LN, Rogova LA, Egorova NIu, et al. Tsitomegalovirusnaia infektsiia: klassifikatsiia i varianty techeniia [Cytomegalovirus infection: classification and course options]. Det Infekt. 2003;(1):57-61. Russian.

19. Volodin NN. Pokazateli smertnosti i rozhdaemosti v Rossiiskoi Federatsii [Indicators of mortality and fertility in Russian Federation]. Pediatriia. 2006;(1):5-8. Russian.
20. Dworsky M, Yow M, Stagno S, Pass RF, Alford C. Cytomegalovirus infection of breast milk and transmission in infancy. Pediatrics. 1983 Sep;72(3):295-9.
21. Bate SL, Dollard SC, Cannon MJ. Cytomegalovirus seroprevalence in the United States: the national health and nutrition examination surveys, 1988-2004. Clin Infect Dis. 2010 Jun 1;50(11):1439-47.
22. Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol. 2010 Jul;20(4):202-13.
23. Adler SP, Starr SE, Plotkin SA, et al. Immunity induced by primary human cytomegalovirus infection protects against secondary infection among women of childbearing age. J Infect Dis. 1995 Jan;171(1):26-32.
24. Centers for Disease Control and Prevention. Guidelines for the Prevention and Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents, 2015. Cytomegalovirus Disease. [cited 2018 Nov 12]. Available from: https://aidsinfo.nih.gov/guidelines/archive/adult-oi-guidelines/
25. Shirobokov VP. Meditsinskaia mikrobiologiia, virusologiia i immunologiia [Medical microbiology, virology and immunology]. Vinnytsia: Nova Kniga; 2015. p. 456-457. Russian.
26. Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. Clin Microbiol Rev. 2009;22(1):76-98.
27. Ho M. Cytomegalovirus: biology and infection. 2nd ed. New York: Springer; 1991. 440 p.
28. He Z, He YS, et al. The human cytomegalovirus UL97 protein is a protein kinase that autophosphorylates on serines and threonines. J Virol.1997 Jan;71(1):405-11.
29. Kisteneva LB, Martynova KA, Khizhniakova TM. Tsitomegalovirusnaia infektsiia u beremennykh. Diagnostika, traktovka rezul'tatov obsledovaniia [Cytomegalovirus infection in pregnant women. Diagnostics, interpretation of examination results]. Vopr Virusol. 2003;(6):4-6. Russian.
30. Dolgikh TI. Strategiia i metodicheskoe obespechenie diagnostiki infektsionnykh zabolevaniI [Strategy and methodological maintenance of infectious diseases diagnostics]. Omsk; 2007. p. 57. Russian.
31. Williamson W, Demmler G, Percy A, et al. Progressive hearing loss in infants with asymptomatic congenital cytomegalovirus infection. Pediatrics. 1992 Dec;90(6):862-6.
32. Foulon I, Naessens A, Foulon W, et al. A 10-year prospective study of sensorineural hearing loss in children with congenital cytomegalovirus infection. J Pediatr. 2008 Jul;153(1):84-8.
33. Bale JF, Miner L, Petheram SJ. Congenital Cytomegalovirus Infection. Curr Treat Options Neurol. 2002 May;4(3):225-230.
34. Dollard SC, Grosse SD, Ross DS. New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection. Rev Med Virol. 2007 Sep-Oct;17(5):355-63.
35. Cheeran MC, Lokensgard JR, Schleiss MR. Neuropathogenesis of congenital cytomegalovirus infection: disease mechanisms and prospects for intervention. Clin Microbiol Rev. 2009 Jan;22(1):99-126.
36. Schlesinger Y, Halle D, Eidelman AI, et al. Urine polymerase chain reaction as a tool for the detection of congenital cytomegalovirus infection. Arch Dis Child Fetal Neonatal Ed. 2003;88(5):371-4.37.
37. Bokovoi AG. Gerpesvirusnye infektsii u detei: diagnostika, klinika, lechenie. Rol' v formirovanii kontingenta chasto boleiushchikh detei: uchebnoe posobie [Herpes virus infections in children: diagnosis, clinic and treatment. The role in the formation of a contingent of frequently ill children]. Moscow: MAKS Press; 2008. p. 144. Russian.
38. Kliegman R., editor. Nelson textbook of pediatrics. 18th ed. Philadelphia: Saunders; 2007. p. 252.
39. Alakaeva IB. Osobennosti gemopoeza vo vnutriutrobnom periode i vliianie na nego vrozhdennykh infektsii [Peculiarities of hematopoiesis in the intrauterine period and the influence of congenital infections on it]. Pediatriia. 2009;(4):122-5. Russian.
40. Cannon MJ, Davis KF. Washing our hands of the congenital cytomegalovirus disease epidemic. BMC Public Health. 2005;5:70.
41. Demidova SA, Semenova EI, Zhdanov VM, et. al. Tsitomegalovirusnaia infektsiia cheloveka [Human cytomegalovirus infection]. Moscow: Meditsina; 1976. p. 167. Russian.
42. Arama V. Actualitati in diaglosticul si terapia infectiilor cu virus citomegalic la pacienti cu transplante de organe [Updates in the diagnosis and therapy of cytomegalovirus infections in organ transplantation patients]. Ro J Infect Dis. 2002;5(2). Romanian.
43. Coll O, Benoist G, Ville Y, Weisman LE, et al.; WAPM Perinatal Infections Working Group. Guidelines on CMV congenital infection. J Perinat Med. 2009;37(5):433-445.
44. Bartlett J, Gallant J, Pham P. Medical management of HIV infection. Durham: Knowledge source solutions; 2009-2010. p. 367-372.
45. Sampedro MA, Martinez LA, Teatino PM, et al. [Diagnosis of congenital infection]. Enferm Infecc Microbiol Clin. 2011;29 Suppl 5:15-20. Spanish.
46. Yinon Y, Farine D, Yudin MH. Screening, diagnosis, and management of cytomegalovirus infection in pregnancy. Obstet Gynecol Surv. 2010;65(11):736-743.
47. Ljungman P, Hakki M, Boeckh M. Cytomegalovirus in hematopoietic stem cell transplant recipients. Hematol Oncol Clin North Am. 2011 Feb;25(1):151-69.
48. Krasnov AV, Kozhevina GI, Kulagina OI, et al. Tsitomegalovirusnaia infektsiia: Metodicheskie rekomendatsii dlia prakticheskikh vrachei, internov i studentov meditsinskikh vuzov [Cytomegalovirus infection: Guidelines for practitioners, interns and medical students]. Kemerovo; 2012. 57 p. Russian.
49. Gleaves CA, Smith TF, Shuster EA, et al. Comparison of standard tube and shell vial cell culture techniques for the detection of cytomegalovirus in clinical specimens. J Clin Microbiol. 1985 Feb;21(2):217-21.
50. Samohin PA. Tsitomegalovirusnaia infektsiia u detei [Cytomegalovirus infection in children]. Moscow: Meditsina; 1987. p. 157. Russian.
51. Shakhgil'dian VI. Diagnostika i lechenie tsitomegalovirusnoi infektsii u beremennykh i novorozhdennykh [Diagnosis and treatment of cytomegalovirus infection in pregnant women and newborns]. Neonatol Nov Mneniia Obuchenie. 2017;(3):70-82. Russian.
52. Razonable RR. Cytomegalovirus infection after liver transplantation: current concepts and challenges. World J Gastroenterol. 2008 Aug 21;14(31):4849-60.
53. Kochkina SS, Sitnikova EP. [Cytomegalovirus infection in children]. Det Infect. 2016;(1):39-44. Russian.
54. Murray PR, Rosenthal KS, Pfaller MA. Medical Microbiology. 8th ed. Philadelphia: Elsevier; 2016. p. 441.
55. Kaplan JE, Masur H, Holmes KK. Guidelines for preventing opportunistic infections among HIV-infected persons – 2002. Recommendations of the US Public Health Service and the Infectious Diseases Society of America. MMWR Recom Rep. 2002 Jun 14;51(RR-8):1-52.
56. Karpov IA, Salavei MU. [Valganciclovir as a highly effective drug for the prevention and treatment of herpes infections]. Med Nov. 2018;(3):49-52. Russian.
57. Fowler KB, Pass RF. Risk factors for congenital cytomegalovirus infection in the offspring of young women: exposure to young children and recent onset of sexual activity. Pediatrics. 2006 Aug;118(2):e286-92.
58. Vasil'ev VV, Volodin NN, Gorshkova DA. Vrozhdennaia tsitomegalovirusnaia infektsiia: Klinicheskie rekomendatsii [Congenital cytomegalovirus infection: Clinical guidelines]. Moscow; 2016. [Cited 2018 Nov 12]. Available from: https://medi.ru/klinicheskie-rekomendatsii/vrozhdennaya-tsitomegalovirusnaya-infektsiya_14344/. Russian.
59. Duarte RF, Lyon S. Novel approaches to CMV after HCT: report from the 27th European Congress of Clinical Microbiology and Infectious Diseases, Vienna, Austria, 22-25 April 2017. Future Sci OA. 2018;4(5):FSO296.

DOI: 10.5281/zenodo.2590028
UDC: 616.89-008.45/.47-02:616-089.168.1

Postoperative cognitive dysfunction: physiopathological aspects and clinical evidence

Ghenadie Severin, MD, Assistant Professor

Valeriu Ghereg Department of Anesthesiology and Reanimathology
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova

Corresponding author: gseverin@mail.ru
Manuscript received December 12, 2018; revised manuscript February 05, 2019

Abstract

Background: Postoperative cognitive dysfunction (POCD) represents a decrease of cognitive abilities (memory, learning, concentration), which develops in the postoperative period after a variable amount of time (days or weeks). Today, the pathogenesis of the POCD development is not fully known. Literature suggests multiple possible mechanisms of POCD development. Certainly, the neuro-inflammatory effect (generated by the surgery itself) from the cortical zones responsible for learning and memory, is one of the phenomena frequently noted in these patients. This article is a narrative synthesis of literature on postoperative cognitive dysfunction – a quite spread phenomenon found in patients during postoperative care. We described suggested theories and the pathophysiological mechanisms involved in the development of this clinical condition. Its incidence according to different types of surgery is presented. We reviewed the available tools for identification and qualitative assessment of postoperative cognitive dysfunction, including biomarkers. Also, we discuss the risk factors for postoperative cognitive dysfunction and their role in clinical decision making process.
Conclusions: Postoperative cognitive dysfunction is a common complication after the surgery. It occurs in frail patients or in individuals presenting general risk factors. It looks like there is a genetic predisposition for the development of postoperative cognitive dysfunction. Patients at risk of postoperative cognitive dysfunction can be identified by neurocognitive testing tools.
Key words: postoperative cognitive dysfunction, risk factors, biochemical markers.

Introduction

Postoperative cognitive dysfunction (POCD) represents a decrease of cognitive abilities (memory, learning, concentration), which develops in the postoperative period after a variable amount of time (days or weeks).

Today, the pathogenesis of the POCD development is not fully known. Literature suggests multiple possible mechanisms of POCD development. Certainly, the neuro-inflammatory effect (generated by the surgery itself) from the cortical zones responsible for learning and memory, is one of the phenomena frequently noted in these patients [1]. Among POCD risk factors we find: age, intraoperative hypoxemia, intensity of pain in the perioperative period, extent and length of surgery, number of surgical interventions, postoperative complications (infectious, respiratory, stroke) etc [2, 3].

POCD causes a decrease in quality of life and enhances 1 year mortality [4]. POCD incidence is reported to be between 24 and 79% (short term) and 57% (long term) [5].

Neuroinflammatory theory

Riedel B. et al. [6] comes with a recent data analysis in order to understand physiopathology of POCD. So, POCD is a well-known syndrome, present in approximately 15% of patients of 60 years and older, characterized by a decrease of cognitive function as a consequence of anesthesia and surgery. Recent data suggests that POCD is mediated by the neuro-inflammatory response that is strongly related to the surgical intervention. Questions arise regarding the casualty of the inflammatory process, endothelial dysfunction and POCD.

Systemic inflammatory response, in combination with endocrinological, metabolic and immunological modifications plays an essential role in recovery and wound healing. Even though, all recent studies show that the inflammatory status is associated with negative perioperative results. Therefore, interventions that modulate the inflammatory response, surgery, anesthesia and drugs may enhance the recovery and reduce complications. Research about wound physiology underline the importance of genetic variability in systemic inflammatory response [7]. Zhu J. et al. [8] published a study where he finds links between appearances of cognitive dysfunction following cardiac ischemia. The study was performed on animal model, where direct implication of the neuroinflammatory step in POCD is shown.

Theory about mediators disbalance

Recent studies tend to prove the relationship between POCD and disbalance of mediators. Cytokines that are activated by various factors during surgery are capable of affecting memory operational system. Interleukin IL-1β is one of the most important mediators that leads to inflammatory response in the brain. IL-1β has local effects that depend on concentration and act on the hippocampus and memory.

Lipopolysaccharides, one of the components of the external membrane of gram negative bacteria are a strong trigger of inflammatory response. Confirmation of this data can be found in publications of Fidalgo A. et al. [9].

Anti-inflammatory cholinergic pathway is a neurohumoral mechanism that plays an important role in inflammatory response suppression. Usage of cholinesterase inhibitors

raises cholinergic transmission, thus, may act as a potential approach for prevention of neuroinflammation. Kalb A. et al. [10] uses an animal model to reach results that state: intraoperative administration of cholinesterase inhibitors leads to a decrease in the pro-inflammatory response and lowers neurodegeneration in the cortex and hippocampus. This combination may represent an instrument in pathogenesis of POCD.

Genetics' theory

Apolipoprotein E (APOE) is a lipoprotein with small molecular weight, synthesized predominantly in the liver. Its role is to control cholesterol metabolism. Three types of APOE exist: E2, E3, E4; being encoded as alleles: e2, e3, e4 according to Gerdes L. et al. [11]. Allele e4 is the one that correlates with atherosclerosis and Alzheimer. People having this allele have difficult rehabilitation after cerebral injury. Tardiff B. et al. [12] performs a study which included 65 patients after cardiac surgery, analyzing plasmatic concentration of APOE, especially allele e$, evaluates preoperative data about neuropsychological tests, age and educational level, and concludes that there is an important association between APOE e4 and POCD at 6 weeks distance after cardiac surgery. In 2007, Olney J. et al. [13] affirms that APOE allele e4 is the factor which predisposes to late postoperative delirium. Controversial results are brought by Abildstrom H. et al. [14] in a study performed on 976 patients of the same age (40 years old) that underwent non-cardiac surgery. The conclusion states that POCD is not associa-ted with APOE allele e4. The same conclusions are reached by McDonagh D. et al. [15] that affirms that patients with APOE genotype do not correlate with POCD in non-car-diac surgery. Mathew J. et al. [16] creates a hypothesis which states that POCD is genetically drived as a result of genetic polymorphism of biological inflammatory regulation, cellular adhesion, coagulation, fat metabolism and vascular reactivity. Authors aim to monitor 37 unique nucleotides associated with cognitive decrease at 6 weeks after the surgery. Genetical variations of C-reactive protein and P-selectine were implied in the cognitive decrease after cardiac surgery. As a consequence of these genetic variations, patients can be placed in risk groups, and it could be useful as a perioperative anti-inflammatory strategy.

If physiopathological mechanisms of POCD are open for new research, then the clinical aspects, through the prism of evidence based medicine, seem to offer a much larger picture.

POCD in cardiac and vascular surgery

First researches made in order to prove POCD were made in cardiac surgery. Cerebral complications are frequent in cardiac surgery. Incidence of POCD in cardiac surgery varies between 30 and 80% during the first postoperative week and around 60% after several months, Rasmussen L. et al. [17]. A recent study performed by Toeg H. et al. [18], confirms the presence of POCD in 38% of cases at discharge and 19% at 3 months after the surgery. Tournay-Jette E. et al. [19] published results that cognitive dysfunction that appears after cardiac surgery has an incidence of 80.7% in elderly population. POCD was documented after different types of surgery. Evered L. et al. [20] performs a study where he compares different types of surgeries, which were followed by POCD. Evered L. includes patients that underwent coronary angiography with sedation, total hip replacement and coronary by-pass under general anesthesia. It was noticed on postoperative day 7, POCD is more frequently seen in patients after coronary by-pass, and after 3 months no differences were found between the study groups. Newman M. et al. [21] concludes that ¾ of patients that underwent coronary by-pass, suffer from neurocognitive dysfunction at discharge, and about 1/3 of patients – at 6 months distance from the surgery. Thus, Newman M. performed another study on patients after 5 years from the surgery to evaluate POCD in long term. POCD incidence at 5 years after the surgery was almost the same as POCD incidence at discharge. POCD at discharge is predictive for its long term presence as well. This justifies the early POCD treatment. A similar study was made by Knipp S. et al. [22] with similar results.

Dijk D. et al. [23] as well studied POCD in patients after coronary by-pass surgery at 5 years distance. Many authors attributed cognitive decline to age modifications rather than a surgical event. This theory was sustained by Selnes O. et al. [24]. Controversial results are brought by Rosengart T. et al. [25] which performed a study on patients that were about to have surgery for coronary by-pass. No differences were found between control and study group. This difference between other studies and the study of Rosengart T. was explained by different evaluation methodologies and statistical analysis. The same affirmations we find in the study of Sweet J. et al. [26]. POCD was not confirmed after coronary by-pass surgeries.

POCD in non-cardiac surgery (minor and major)

Researches from modern medicine evaluated patients with non-cardiac pathologies. Such studies were performed in 1998 by the research group ISPOCD1 which evaluated patients that underwent a major non-cardiac surgery, pro-ving that 26% of patients suffer from POCD at 1 week distance and about 10% – at 3 months distance. Monk T. et al. [2] also proves POCD in patients that underwent major non-cardiac surgery and has a significant impact on 1 year mortality. Newman S. et al. [27] affirms that POCD during the first weeks after cardiac surgery is significant and its percentage increases with age. POCD at 6 months distance is very low and could be occasionally present in particular cases. POCD in major non-cardiac surgery was studied by Dijkstra J. et al. [28]. POCD was found in the first postope-rative week. At 3 months distance, patients had good results, cases of cognitive changes are particular, and do not reflect the surgical factor, but other factors such as depression and age.

POCD in elderly patients (minor and major surgery)

For the first time, POCD in elderly patients was described by Bedford in 1955 [29]. In such a way, Bedford starts a new trend of demonstrating, proving and researching a new branch of pathologies that interferes with surgery and anesthesia. One of the biggest studies is the ISPOCD1, made by Moller et al. [30] which confirms appearance of long term POCD in elderly patients. Major surgery is frequently associated with POCD in elderly patients. POCD after minor surgery is found in publications of Canet J. et al. [31]. POCD incidence in elderly patients after minor surgeries reaches

9.7%. Comparing with previous studies of Canet J. in minor and major surgery, it is stated that incidence of POCD in minor surgery in elderly is lower.

MRI is used in monitoring of Alzheimer starting with pre-symptomatic phases in order to predict earlier the symptomatic phase. Kline R. et al [32], based on MRI data of patients with Alzheimer, fried to demonstrate the hypothesis that surgery might have an impact on the brain structures with progression of dementia postoperatively. The study included elderly patients. The obtained results showed that in patients that underwent surgery, in the first 5-9 months after the surgery (but no later), rates of gray matter atrophy increased in the cortex, hippocampus and lateral ventricle extended, comparing with the control group (non surgical). Neuro-psychological tests applied to these patients elucidated cognitive decrease in patients from the surgical group. Thus, elderly patients that underwent surgery had higher rates of cerebra atrophy and an increased risk of POCD. On the other hand, no correlation has been found between cerebral atrophy and patients that had mild cognitive dysfunction in the immediate postoperative period and with significant results of cognitive dysfunction later.

POCD in middle-aged patients (minor and major surgery)

POCD in patients of middle age in non-cardiac surgery has been studied by Johnson T. et al. [33] in 2002. POCD after non-cardiac surgery is associated with age, on the other hand, patients of middle age have a lower incidence. Thus, the research hypothesis appeared, and the researches analyzed 463 patients at 1 week postoperatively, and found that 19.2% suffer from POCD. At 3 months distance, POCD has decreased to 6.2%. As mentioned before, Abildstrom H. et al. [14] studies POCD in patients of middle age and has stated an incidence of 11.7% of POCD.

POCD in young patients (minor and major surgery)

Monk T. et al. published data about POCD in young patients in 2008 [2]. The study aimed at identifying POCD in non-cardiac surgery, where patients of all ages are included. Authors were able to prove that POCD was found to be equal in young patients, middle age and elderly. Fact which contradicts the ISPOCD1 study. Monk T. also affirms that recovery at 3 and 12 months after the surgery is better in young patients.

POCD in pregnant (minor and major surgery)

Minor and major surgical procedures are associated with cognitive changes: memory loss and lack of concentration. If this is not diagnosed and treated promptly, this pathology may lead to serious consequences. The author supposes that POCD may appear as well after obstetrical interventions such as C-section and natural delivery, influencing the mother or the baby. Obstetrical or neonatal POCD is now fully known. The majority of studies performed focused on the risk factors such as: type of anesthesia, medication, intervention, stress etc. C-sections and obstetrical anesthesia are being used more frequently nowadays, and we can affirm that POCD will be found in this group of patients as well. Thus, maternal and child brain is subject of an increased risk of cognitive decrease with serious consequences. In conclusion, we can cite S. Ghosh [34]: "*Real nature and incidence of POCD is complex and remains to be explored as its existence in obstetrical anesthesia cannot be excluded*".

Predictive and risk factors for POCD

Predictive and risk factors for POCD are not well established, but the recent studies try to clarify this problem. POCD seems to be a multifactorial. These factors can be divided into pre, intra and postoperative.

a) Preoperative factors – are factors that were present before surgery (genetic, APOE allele e4 theory, C-reactive protein and P-selectine).

Demographic factors (age and educational level). Age is one of the most controverted factors, cognitive decline is inevitable with age and it is difficult to draw conclusions on this matter (where exactly we have normal cognitive function and where we have a pathological decline due to hospitalization). Regarding educational level: patients with a higher educational level have a lower incidence of POCD.

Obtained risk factors during life time: general medical conditions, pain-killers, a specific disease (such as hypertension), POCD established before sugery, substance abuse, etc.

b) Intraoperative factors or *stressing factors* are those which start on the day of the surgical intervention. This group contains the surgery itself, anesthesia, used pain-killes, hypoxia and intraoperative hypotension etc.

c) Postoperative factors are those factors that encouraged and maintained POCD: sedation, analgetics in the postoperative period, infections etc [35]), (tab. 1).

Methods of quantification and evaluation of POCD

Psychrometric tests

Appreciation of postoperative cognitive decline was made through a large variety of methods; large number of types of surgical interventions and the big variety of psychological tests make standardization difficult. Cognitive performance of each individual is different, due to educational level, neuronal reserve, factors that influence each patient during examination (tiredness, insomnia, stress etc). The majority of authors agree that cognitive performance of the patient should be compared only with the performance of the same patient.

Ghoneim M. et al. [47] considers that POCD may be diagnosed using only neuro-psychological tests. Neuro-psychological tests have the goal to identify and quantify cognitive abilities. They are designed to evaluate different domains of cognition such as: general intellectual function, memory, attention, concentration, speed of processing and executive function. There are several tests in each domain. Test selection aims to identify even minor changes, it is difficult to achieve high scores at these tests. This kind of tests is used for patients with high intellectual level that have high scores at initial testing. Sometimes it is necessary to use low-score tests for specific patients. Ghoneim M. suggests using several, most popular, tests for POCD appreciation. *"Digit Span"* and *"Wechsler Adult Intelligence Scale – Revised Test"* are oriented to test working memory. *"Digit Symbol Substitution"* characterizes speed of processing of information and working memory. *"Rey Auditory Verbal Learning Test"* has the goal to teach verbally, revoke and reproduce. *"The Stroop*

Table 1

Risk factors for POCD

Reference	Risk factors and comments
	Genetic factors and specific enzymes
Tardiff B et al. 1997 [12]	Apolipoprotein allele E4
Mathew J et al. 2007 [16]	C-reactive and P-selectine
Gaudet J et al. 2010 [36]	MMP 9 (matrix metalloproteinase)
Rasmussen L et al.1999 [37] Moller J et al. 1998 [30] Johnson T et al. 2002 [33] Monk T et al. 2008 [2] Moritz S et al. 2008 [38] Carrascal Y. 2005 [39] Sanders RD et al. 2010 [40]	Advanced age Patients older than 60 have a significant risk of long-term cognitive dysfunctions Increased mortality during the first postoperative year
Monk T et al. 2008 [2] Sanders RD et al. 2010 [40]	Educational level
Johnson T et al. 2002 [33]	Alcohol abuse. Interferes with effects of premedication and could lead to POCD in the immediate postoperative period
Bodolea C. 2010 [35]	POCD present before admission
Monk T et al. 2008 [2] Sanders RD et al. 2010 [40]	Stroke
Bodolea C. 2010 [35]	Arterial hypertension
Monk T et al. 2008 [2]	POCD at discharge is a risk factor for cognitive decrease at 3 months postoperatively
	Surgery
Sanders RD et al. 2010 [40]	Major surgery and history of surgeries in the past
Carrascal Y et al. 2005 [39] Evered L et al. 2011 [20]	Cardiac and valvular surgeries
Olney JW et al. 2000 [13] Culley DJ et al. 2007 [41] Kavanagh T et al. 2012 [42] Sanders RD et al. 2010 [40]	Type of anesthesia
Moller J et al. 1998 [30] Browne S et al. 2003 [43] Sanders RD, et al. 2010 [40] Saricaoglu F et al. [44]	Hypoxia and hypotension during surgery
Sanders RD et al. 2010 [40] Stanley T et al. 2002 [45]	Atrial fibrillation
Leiendecker J et al. 2010 [46]	Migrant micro-embolism during surgical interventions
Sanders RD et al. 2010 [40]	Infections and postoperative complications in the past

Test" evaluates attention, concentration and executory function. *"Grooved Peg Board"* – manual dexterity and psychomotor coordination. "The Trail Making Test" – attention, mental flexibility and motor function. Also, it is recommended to test anxiety and depression, as these factors may lead to lower performance during testing. Anxiety is usually tested with *"State Trait Anxiety Inventory"*. Depression is usually tested with *"The Beck Depression Inventory"* and *"Center for Epidemiological Studies Depression Scale"*.

Carrascal Y. et al. [39] proposes a single test for POCD appreciation. He performs a study, enrolling 132 patients and uses *"Paced Auditory Serial Addition Test"*. Authors conclude that the percentage of POCD after cardiac surgery was equal to results of studies made using several tests. These tests are easy to use, they can be reproduced and they represent a simple and practical method of POCD testing after cardiac surgeries.

A Swedish study made by Jildenstal P. et al. [48] studies the evoked auditory potential during ophthalmological surgeries. Peculiarities of ophthalmologic surgeries limit application of a large spectrum of neuro-psychological tests; nevertheless, authors prove the utility of auditory evoked potential.

How neuro-psychological tests are applied

An important role in POCD appreciation is played by the fact how the tests are applied, the timing, number of evaluations, number of tests used etc. Rasmussen et al. [17] comes with several recommendations regarding the design of such studies. We will discuss these aspects.

Selection and application of tests

a) Objective vs. subjective tests. Neuropsychological tests should detect even small cognitive changes by evaluating several intellectual and personal aspects. One can use MCQ-blank, pencil-paper or various computed applications. Usually these tests require time and effort but can contain subjective complaints from patients. These tests are highly dependent on patients' expectancies, self-respect and should not be implied for POCD evaluation.

b)Variability. Neuropsychological tests should be the same in both testing sessions, under the same circumstances, at the same time of the day. External exciting factors should be minimized, drugs that impede cognitive function should also be discontinued (opioids, hypnotics), also testing should not be performed if patient is in pain.

c) *Basal Performance.* Basal, preoperative, normal levels should be evaluated if patients experience tiredness, anxiety and depression while hospitalized. Rasmussen recommends primary evaluation to be done 1-2 weeks before surgery.

d) Exercising effect. Using the same set of tests favours memorizing. This way, performance is artificially improved. In order to exclude that, it is recommended to use parallel forms of these tests, re-testing with longer intervals of time or using a control group.

e) Intervals between testing. Time intervals between testing depend on specific clinical situations. Tests are applied upon discharge or at the first postoperative control. Demotivation and tiredness may lead to subestimation and lower levels of cognitive performances, than can easily be confounded with demotivation and chronic tiredness. If patients are not tested because of pre-existent cognitive dysfunction, the impossibility of testing may hide patients with cognitive decline and subestimate obtained data.

f) Emotional modifications and anxiety. Depression and anxiety lead to lower cognitive performances. Rasmussen recommends questionnaires that evaluate these variables.

g) Drop-out. Drop-out means losing patients during the study. Patients abandon the study due to lack of interest, lack of time at discharge and even because of POCD itself. It is recommended to report drop-out, this way; these patients may be attributed to POCD.

Rasmussen et al. [17] recommends basic criteria of test selection:

1) *Linguistic and cultural issues.* Most of the tests are written in English. Translating and adapting tests requires careful analysis and word selection according to local culture and using control groups. Patients with low intellectual levels may have difficulties in understanding some words.

2) *Parallel versions.* Parallel versions that are used in order to exclude familiarization with the test may induce a cognitive decline that is improperly calculated by a non-equivalent form of testing.

3) *Sensibility.* Tests with low sensibility, such as "*Wechsler Adult Intelligence Scale*" or "*Wechsler Memory Scale*" are frequently used for POCD evaluation but do not identify fine cognitive dysfunction.

4) *Inferior and superior limit effect.* Neuro-psychological tests that are difficult to achieve a minimal score, or, vice-versa, tests that are very easy in obtaining the highest score lead to subestimation of obtained data.

5) *Time and errors.* It is preferably to use tests that can count the number of errors, and in case of no mistakes – that can take into consideration the total time of testing.

Nevertheless, POCD evaluation requires a set of tests in order to be able to appreciate a larger spectrum of cognitive functions. But it is important to have in mind the time required to perform the testing. Testing should not make the patient tired, because this will lead to incorrect data. Some authors recommend calculation of performances from all tests in a single score. This may cause another difficulty, allowing some researchers to lower or increase the proportion of POCD, Bodolea C. [35].

Biochemical markers

POCD is currently diagnosed with neuropsychological tests, this method being the main assessment tool.

Variability and difficulty in applying neuropsychological tests led researchers to look for POCD-specific biochemical markers. Determination of specific markers will allow the tracking of the evolution and the prevention of this pathology. Thus, Rasmussen et al. [17], analyses the action of neuron-specific enolase (NSE) and S-100b protein, both of which are early markers in cerebral injuries. Obvious correlations have been observed in S-100b plasma levels in abdominal surgery and postoperative delirium patients. There was no correlation between POCD and blood concentrations of NSE and S-100β protein in abdominal surgery. While in cardiac surgery, namely coronary bypass, the authors had a significant increase in both biomarkers at 24 and 48 hours. Except that NSE in the statistical analysis correlates with POCD having a significant increase at 24 hours postoperatively. While the increased plasma concentration of the S-100b protein correlates with the duration of surgery. So NSE seems to be a blood marker for early POCD after coronary bypass.

The role of biomarkers in the DCPO predictor such as the S-100β protein and the anticholinergic active serum (AAS) was evaluated by Plaschke K. et al. [49]. This hypothesis is put by Plaschke on the grounds that AAS has previously been described as a risk factor in the appearance of the delirium to elderly patients. In contrast to the increased intraoperative plasma concentration of S-100β protein, the association of AAS with POCD was not detected. Nitric oxide is a powerful vasodilator. In the central nervous system, nitric oxide functions as a neurotransmitter. The increase in the concentration of nitric oxide and its products, nitrites and nitrates, was observed by Molnar T. et al. [51] in his paper he aims to evaluate the link between PODC and the biomarkers of immuno-endothelial dysfunction, which may be linked to ischemia and neurocognitive changes in pulmonary onco-surgery. Thus, it analyzes a series of biomarkers such as soluble P-selectin, soluble ligand, CD40 as the biomarker of platelet activation, soluble vascular cell adhesion molecule-1, MCP-1 (monocyte chemoattractant protein-1) Interleukins IL-6, IL-8, C-reactive protein and S100B protein. Therefore, the platelet and endothelial-related molecules had a preoperative plasma concentration and 48 hours postoperatively plasma concentration in patients who

developed POCD. Significant increase at 48-hour postoperative was notes for values of MCP-1 and S100B-protein. The authors assume that the endothelium and thrombocytes are activated as a response to the immune system and the presence of tumor agents. Translocation of leukocytes with deterioration of the blood-brain barrier and brain ischemia with the appearance of POCD occurs after the preoperative activation of endothelium and platelets. Following the increase of these preoperative markers, patients in the risk group who may develop POCD could be identified and it is possible to even modify therapeutic course, and why not, avoid surgical intervention in case of risk of the major neurocognitive injury of oncological patients.

In 2010, Gaudet et al. [36] comes with an article which discusses the correlation between the plasma concentration of the marker MMP 9 (matrix metalloproteinase) and POCD in patients that undergo endarterectomy. MMP 9 being a proteolytic enzyme that acts on the progression and destabilization of atheromatous plaques contributes to damage to the blood-brain barrier and contributes to the formation of cerebral edema, resulting in cerebral ischemia. Increased MMP 9 preoperatively correlated with patients who developed POCD. There were no increases in postoperative MMP 9 values. Thus, the authors recommend the use of MMP 9 as a predictor of POCD.

Other evaluation methods

Cerebral oximetry is based on Beer-Lambert's principle, where the concentration of a substance can be measured according to the degree of light absorption. Regardless of the factors that act on the human body in causing cognitive dysfunction, ultimately the brain suffers through hypoxigenation, ischemia, and as a consequence the onset of POCD. The variety of studies, were performed in both cardiac and non-cardiac surgery. Thus, Murkin J. et al. [52] makes a hypothesis that controlling oxygen concentration in the brain during heart surgery by the method of brain oximetry monitoring would have beneficial effects on systemic organs. As a result, Murkin J. asserts that monitoring and modulating intraoperative conduction based on cerebral oximetry values in coronary bypass patients avoids profound brain desaturation and is associated with fewer major organ dysfunction. However, the given method suffers from some limitations, the monitoring of cerebral oximetry does not continue in the ICU (intensive care unit), brain oxyge-nation is performed on a small part of the brain and may be influenced by anatomic particularity or cerebral edema.

Another study by Slater J. et al. [53], in patients to be operated in cardiac surgery, prolonged intra-operative cerebral desaturation has an increased risk of developing POCD and prolongs in-hospital stay.

Casati A. et al. [54] in his study uses brain oximetry monitoring in major abdominal surgery in elderly patients. The results are to show that intraoperative adjustment of cerebral oximetry and avoidance of cerebral desaturation prevents cerebral ischemia in elderly patients, thus preventing the onset of POCD and shortens the patient hospital stay, approaching the discharge period.

Critical analysis of POCD study methodologies in publications

In spite of the fact of the years of study in the unraveling of POCD, many questions remain unclear. In 2013, Uysal S. and Reuch D. [55] confirmed that the difficulty relates to a number of factors, including very different methods of POCD assessment, and variations in surgical management and surgical techniques over time. The sensitivity, specificity, and overall usefulness of neurocognitive research methods are well established, but the application of these methods to cardiac surgery over the past 30 years has often been mistaken.

In 2007, Newman S. et al. [27] comes with a study based on investigations of POCD-related publications in non-cardiac surgery. The authors assert that no study was able to elucidate the possible mechanisms in the occurrence of POCD. Research suffers from major shortcomings in the number of studies in the field and a number of other technological difficulties. This includes the high variability of the type of surgery and anesthesia, the number of patients enrolled in the study, the diversity of recruitment, the variability of neuropsychological tests with different sensitivity, the diversity of statistical analysis, and even the variety of application of the definition used in the classification of study participants as POCD. These differences make it difficult to compare studies with each other.

Rasmussen L. et al. [17] asserts that the low frequency of POCD in some studies is caused by the incorrect methodology chosen to detect cognitive deviations. Using lower threshold tests will not show the presence of POCD correctly. Using easy memorable tests will result in underestimations, and obtaining the same postoperative outcome in comparison with the pre-operative shows certainly that POCD has taken place, which many authors do not take into account.

Conclusions

Postoperative cognitive dysfunction is a common complication after the surgery. It occurs in frail patients or in individuals presenting general risk factors. It looks like there is a genetic predisposition for the development of postoperative cognitive dysfunction. Patients at risk of postoperative cognitive dysfunction can be identified by neurocognitive testing tools.

References

1. Wan Y, Xu J, Ma D, et al. Postoperative impairment of cognitive function in rats: a possible role for citokyne-mediated inflammation in hyppocampus. Anesthesiology. 2007;106(3):436-43.
2. Monk T, Weldon BC, Garvan CW, et al. Predictors of cognitive dysfunction after major noncardiac surgery. Anaesthesiology. 2008;108(1):18-30.
3. Newman M, Mathew JP, Grocott HP, et al. Central nervous system injury associated with cardiac surgery. Lancet. 2006;368(9536):694-703.
4. Phillips-Bute B, Mathew JP, Blumenthal JA, et al. Association of neurocognitive function and quality of life 1 year after coronary artery bypass graft (CABG) surgery. Psychosom Med. 2006;68(3):369-75.
5. Rasmussen L, Siersma V; ISPOCD Group. Postoperative cognitive dysfunction: true deterioration versus random variation. Acta Anesthesiol Scand. 2004;47(9):1137-43.
6. Riedel B, Browne K, Silbert B. Cerebral protection: inflammation, endothelial dysfunction and postoperative cognitive dysfunction. Curr Opin Anesthesiol. 2014;27(1):89-97.
7. Nicholson G, Hall G. Effects of anaesthesia on the inflammatory response to injury. Curr Opin Anesthesiol. 2011;24(4):370-4.

8. Zhu J, Jiang X, Shi E, et al. Sevoflurane preconditioning reverses impairment of hippocampal long-term potentiation induced by myocardial ischaemia-reperfusion injury. Eur J Anaesthesiol. 2009;26(11):961-8.
9. Fidalgo A, Cibelli M, White J, et al. Systemic inflammation enhances surgery-induced cognitive dysfunction in mice. Neurosci Lett. 2011;498(1):63-6.
10. Kalb A, von Haefen C, Sifringer M, et al. Acetylcholinesterase inhibitors reduce neuroinflammation and degeneration in the cortex and hippocampus of a surgery stress rat model. PLoS One. 2013 May;8(5):e62679.
11. Gerdes L, Klausen I, Sihm I, et al. Apolipoprotein E polymorphism in a Danish population compared to findings in 45 other study populations around the world. Genet Epidemiol. 1992;9(3):155-67.
12. Tardiff B, Newman M, Saunders A, et al. Preliminary report of a genetic basis for cognitive decline after cardiac operations. Ann Thorac Surg. 1997;64(3):715-20.
13. Olney J, Farber N, Wozniak D, et al. Environmental agents that have the potential to trigger massive apoptotic neurodegeneration in the developing brain. Environ Health Perspect. 2000;108 Suppl 3:383-8.
14. Abildstrom H, Christiansen M, Siersma V, et al. Apolipoprotein E genotype and cognitive dysfunction after noncardiac surgery. Anesthesiology. 2004;101(4):855-61.
15. McDonagh D, Mathew J, White W, et al. Cognitive function after major noncardiac surgery, apolipoprotein E4 genotype, and biomarkers of brain injury. Anesthesiology. 2010;112(4):852-9.
16. Mathew J, Podgoreanu M, Grocott H, et al. Genetic variants in P-selectin and C-reactive protein influence susceptibility to cognitive decline after cardiac surgery. J Am Coll Cardiol. 2007;49(19):1934-42.
17. Rasmussen L, Moller J. Central nervous system dysfunction after anesthesia in the geriatric patient. Anesthesiol Clin North America. 2000;18(1):59-70.
18. Toeg H, Nathan H, Rubens F, et al. Clinical impact of neurocognitive deficits after cardiac surgery. J Thorac Cardiovasc Surg. 2013;145(6):1545-9.
19. Tournay-Jetté E, Dupuis G, Bherer L, et al. The relationship between cerebral oxygen saturation changes and postoperative cognitive dysfunction in elderly patients after coronary artery bypass graft surgery. J Cardiothorac Vasc Anesth. 2011;25(1):95-104.
20. Evered L, Scott DA, Silbert B, et al. Postoperative cognitive dysfunction is independent of type of surgery and anesthetic. Anasth Analg. 2011;112(5):1179-85.
21. Newman M, Kirchner J, Phillips-Bute B, et al. Longitudinal assessment of neurocognitive function after coronary-artery bypass surgery. N Engl J Med. 2001;344(6):395-402.
22. Knipp S, Matatko N, Wilhelm H, et al. Cognitive outcomes three years after coronary artery bypass surgery: relation to diffusion-weighted magnetic resonance imaging. Ann Thorac Surg. 2008;85(3):872-9.
23. van Dijk D, Moons K, Nathoe H, et al. Cognitive outcomes five years after not undergoing coronary artery bypass graft surgery. Ann Thorac Surg. 2008;85(1):60-4.
24. Selnes O, Grega M, Bailey M, et al. Do management strategies for coronary artery disease influence 6-year cognitive outcomes? Ann Thorac Surg. 2009;88(2):445-54.
25. Rosengart T, Sweet J, Finnin E, et al. Stable cognition after coronary artery bypass grafting: comparisons with percutaneous intervention and normal controls. Ann Thorac Surg. 2006;82(2):597-607.
26. Sweet J, Finnin E, Wolfe P, et al. Absence of cognitive decline one year after coronary bypass surgery: comparison to nonsurgical and healthy controls. Ann Thorac Surg. 2008;85(5):1571-8.
27. Newman S, Stygall J, Hirani S, et al. Postoperative cognitive dysfunction after noncardiac surgery. Anesthesiology. 2007;106(3):572-90.
28. Dijkstra L, Houx P, Jolles J. Cognition after major surgery in the elderly: test performance and complaints. Br J Anaesth. 1999;82(6):867-74.
29. Bedford P. Adverse cerebral effects of anaesthesia on old people. Lancet. 1955;269:259-63.
30. Moller J, Cluitmans L, Rasmussen L, et al. Long-term postoperative cognitive dysfunction in the elderly: ISPOCD1 study. Lancet. 1998;351:857-61.
31. Canet J, Raeder L, Rasmussen L, et al. Cognitive dysfunction after minor surgery in the elderly. Acta Anaesthesiol Scand. 2003;47(10):1204-10.
32. Kline R, Pirraglia E, Cheng H, et al. Surgery and brain atrophy in cognitively normal elderly subjects and subjects diagnosed with mild cognitive impairment. Anesthesiology. 2012;116(3):603-12.
33. Johnson T, Monk T, Rasmussen L, et al. Postoperative cognitive dysfunction in middle-aged patients. Anesthesiology. 2002;96(6):1351-7.
34. Ghosh S. The possibility of postoperative cognitive dysfunction in obstetric anaesthesia following caesarean section. Eur J Anaesthesiol. 2012;29(2):61-3.
35. Bodolea C. Disfuncția neurocognitivă postoperatorie a pacientului vârstnic [Postoperative neurocognitive dysfunction in elderly patients]. Oradea: Primus; 2010. p. 49-82. Romanian.
36. Gaudet J, Yocum G, Lee S, Granat A, et al. MMP-9 levels in elderly patients with cognitive dysfunction after carotid surgery. J Clin Neurosci. 2010;17(4):436-40.
37. Rasmussen L, Steentotf A, Rasmussen H, et al. Benzodiazepines and postoperative cognitive dysfunction in the elderly. Br J Anaesth. 1999;83(4):585-9.
38. Moritz S, Arlt A, Voolkel V, et al. The role of global cerebral hypoperfusion in the development of postoperative cognitive dysfunction. Eur J Anaesthesiol. 2008;25(Suppl 43):23-24.
39. Carrascal Y, Casquero E, Gualis J, et al. Cognitive decline after cardiac surgery: proposal for easy measurement with a new test. Interact Cardio Vasc Thorac Surg. 2005;4(3):216-21.
40. Sanders RD, Maze M. Neuroinflammation and postoperative cognitive dysfunction: can anaesthesia be therapeutic? Eur J Anaesthesiol. 2010;27(1):3-5.
41. Culley D, Xie Z, Crosby G. General anesthetic-induced neurotoxicity: an emerging problem for the young and old? Curr Opin Anaesthesiol. 2007;20(5):408-13.
42. Kavanagh T, Buggy D. Can anaesthetic technique affect postoperative outcome? Curr Opin Anesthesiol. 2012;25(2):185-98.
43. Browne S, Halligan P, Wade D, et al. Postoperative hypoxia is a contributory factor to cognitive impairment after cardiac surgery. J Thorac Cardiovasc Surg. 2003;126(4):1061-4.
44. Saricaoglu F, Celiker V, Basgul E, et al. The effect of hypotensive anaesthesia on cognitive functions and recovery at endoscopic sinus surgery. Eur J Anaesthesiol. 2005;22(2):157-9.
45. Stanley T, Mackensen B, Grocott H, et al. The impact of postoperative atrial fibrillation on neurocognitive outcome after coronary artery bypass graft surgery. Anesth Analg. 2002;94(2);220-95.
46. Leiendecker J, Hocker J, Meybohm P, et al. Postoperative neurocognitive function and microembolus detection in patients undergoing neck dissection:a pilot study. Eur J Anaesthesiol. 2010;27(5):417-24.
47. Ghoneim M, Block R. Clinical, methodological and theoretical issues in the assessment of cognition after anaesthesia and surgery: a review. Eur J Anaesthesiol. 2012;29(9):409-22.
48. Jildenstal P, Hallen J, Rawal N, et al. Effect of auditory evoked potential-guided anaesthesia on consumption of anaesthetics and early postoperative cognitive dysfunction: a randomized controlled trial. Eur J Anaesthesiol. 2011;28(3):213-9.
49. Plaschke K, Hauth S, Jansen C, et al. The influence of preoperative serum anticholinergic activity and other risk factors for the development of postoperative cognitive dysfunction after cardiac surgery. J Thorac Cardiovasc Surg. 2013;145(3):805-11.
50. Clark R, Kochanek P, Obrist W, et al. Cerebrospinal fluid and plasma nitrite and nitrate concentrations after head injury in humans. Crit Care Med. 1996;24(7):1243-51.
51. Molnar T, Jakab L, Palinkas L, et al. Increased levels of baseline biomarkers reflecting platelet and endothelial activation predict early cognitive dysfunction after lung surgery. Eur J Anaesthesiol. 2009;26(8):708-10.
52. Murkin J, Adams S, Novick R. Monitoring brain oxygen saturation during coronary bypass surgery: a randomized, prospective study. Anesth Analg. 2007;104(1):51-8.
53. Slater J, Guarino Th, Stack J, et al. Cerebral oxygen desaturation predicts cognitive decline and longer hospital stay after cardiac surgery. Ann Thorac Surg. 2009;87(1):36-44.
54. Casati A, Fanelli G, Pietropaoli P, et al. Continuous monitoring of cerebral oxygen saturation in elderly patients undergoing major abdominal surgery minimizes brain exposure to potential hypoxia. Anesth Analg. 2005;101(3):740-7.
55. Uysal S, Reuch D. Neurocognitive outcomes of cardiac surgery. J Cardiothorac Vasc Anesth. 2013;27(5):958-71.

Declaration of conflicting interests
Nothing to declare.

GUIDE FOR AUTHORS

The authors are kindly requested to visit our web site www.moldmedjournal.md and strictly follow the directions of the **Publication Ethics and Malpractice Statement.**

The articles must be sent electronically to editor@moldmedjournal.md by the author, responsible for the correspondence, using the **Authorship Statement Form** (www.moldmedjournal/authorship-statement/).

All papers are to be executed in the following manner:

1. The manuscript should be typed in format A4, 1.5-spaced, with 2.0 cm margins, printing type 12 Times New Roman, in Microsoft Word.

2. The original article (presents new and original scientific findings, explain research methodology and provide data) has to be less than 16 pages long and should consist of an Introduction, Material and Methods, Results, Discussion, Conclusions and be followed by not more than 40 references.

3. The review article (provides an overview of a field or subject, synthesize previous research) must not exceed 25 pages and contain not more than 100 references.

4. The title page should include the first and family name of all the authors, their academic degrees, the name of the department and institution from which the paper has arrived, the phone number and e-mail address of the corresponding author.

5. The abstract should be written on the title page and limited from 220 to 240 words. The abstract of original article should have 4 parts: Background, Material and methods, Results, Conclusions. The abstract of review article should have 2 parts: Background and Conclusions. The abstract should end with 3 to 6 key words.

6. The tables and figures must be typed, consecutively numbered and followed by an explanatory text. The figures that have to emphasize a comparison or details are published in color. If colored figures are to be placed, the author must pay an additional fee of €100 per page (1-8 figures on a page).

7. The references are to be listed in order of their appearance in the text, and the appropriate numbers are to be inserted in the text in square brackets in proper places. The references must comply with the general format outlined in the Uniform Requirements for the Manuscripts Submitted to Biomedical Journals developed by the International Committee of Medical Journal Editors (www.icmje.org), chapter IV.A.9. The references in the Cyrillic script should be transliterated into Latin script using the American Library Association and Library of Congress Romanization Tables as follows: А–A, Б–B, В–V, Г–G, Д–D, Е–E, Ё–E, Ж–ZH, З–Z, И–I, Й–I, К–K, Л–L, М–M, Н–N, О–O, П–P, Р–R, С–S, Т–T, У–U, Ф–F, Х–KH, Ц–TS, Ч–CH, Ш–SH, Щ–SHCH, Ъ–", Ы–Y, Ь–', Э–E, Ю–IU, Я–IA. Immediately after the transliteration the translation of the title in English in the square brackets should follow. For example: Ivanov IV, Shchukin NF, Men'shikov VM, Ad"yunktov AM. Transplantatsiia organov i tkanei [Transplantation of organs and tissues]. Vestnik Khirurgii. 2010; 26(6):45-49. Russian.

Submission and Peer Review Process

1. Submitted articles are first put under consideration to decide whether a given article fits into the area of the journal thematic, then articles are sent to reviewers for further approval, usually to leading experts in the field. The names of the authors and reviewers and their affiliation are not shown to each other.

2. For an article to be published, it must have a well-reasoned approval of the two reviewers, which is then considered and further approved by the Editorial Board. If one of two reviews is "negative", the Editorial Board is approving the rejection or the article is sent to another reviewer.

3. If the article requires some modifications, it is returned to the author(s) to be revised, taking into account the reviewer's recommendations. The modified version is then again forwarded to the reviewer, to consider a possibility for the publication of the article.

4. The accepted article is forwarded for scientific editing and proofreading. The final version is adjusted with authors for approving and for correction of possible inexactitudes. However, essential modifications by the author(s) after editing and proofreading are not allowed.

5. In case an article is rejected, the author(s) receive a well-reasoned explanation from the Editorial Board.

Responsibility of the Editorial Board

1. The Editorial Board members are responsible for making decisions on accepting or rejecting the submitted articles. Criteria for publication are the scientific value and originality of materials and their conformity with the scope and thematic range of the journal.

3. The Editorial Board considers only previously unpublished manuscripts, containing the results of original research or detailed thematic review on the medicine, dentistry, pharmacy, social medicine and public health. In case of submitting a previously published article (or sent for publication in any other journal), repeating the pending article for more than 50%, the Editorial Board reserves the right to reject the article from being published in the "The Moldovan Medical Journal".

4. The editors do not impose authors to cite papers published in "The Moldovan Medical Journal" in order to artificially improve scientometric indicators of the journal.

5. The Editorial Board ensures that submitted material will be subject for a peer review by anonymous independent experts.

Responsibility of authors

1. Authors are responsible for the content of the article, confirming the use of only original scientific data. In case of borrowing materials from other researchers (or from an earlier publication of the given authors) the correct reference or citation should be made. Regarding plagiarism and fraudulent data please read chapter 2.3, 2.4 and 4 of the Publication Ethics and Malpractice Statement.

2. Authors guarantee that submitted article has not been previously published in English or any other language nor simultaneously submitted to another journal.

3. Authors should write the article in conformity with the Guide for Authors. Otherwise, the Editorial Board reserves the right to reject the article from consideration for publication.

Responsibility of reviewers

1. Reviewers must present an objective expertise of the manuscript. In case of any conflict of interests (self-interest, personal animosity, financial or scientific conflicts, etc), the reviewers must immediately inform the Editorial Board about it.

2. Reviewers are required to remain confidential and not to disclose information about or from the article under review to an unauthorized person.

Address of the Editorial Office

192, Stefan cel Mare Avenue, Chisinau, MD-2004, the Republic of Moldova
Telephone: +37322205209. Mobile: +37379429274
www.moldmedjournal.md editor@moldmedjournal.md