The Moldovan Medical Journal

The Publication of the Scientific Medical Association of Moldova

Frequency – 4 per year
Category – B+

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ORIGINAL RESEARCH

Changes of autonomic tonus of the heart during induction of general anesthesia with two intravenous anaesthetics

*[1,2]Iuliana Feghiu, MD, Assistant Professor; [1]Sergiu Cobiletchi, MD, Assistant Professor;
[1]Galina Frunza, MD, Assistant Professor, [1]Sergiu Sandru, MD, PhD, Professor, [3]Anatol Scripnic, MD

[1]Valeriu Ghereg Department of Anesthesiology and Intensive Care No 1
[2]Department of Pathophysiology and Clinical Pathophysiology
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova
[3]Institute of Emergency Medicine, Chisinau, the Republic of Moldova

*Corresponding author: i.dimitriu@yahoo.com
Manuscript received July 09, 2019; revised manuscript September 09, 2019

Abstract

Background: Induction of general anesthesia with midazolam or thiopental is often associated with cardiovascular changes.

Material and methods: The study group involved 94 patients. The analysis of heart rate variability and the changes in cardiac vegetative tonus was performed after premedication with fentanyl solution and after induction of general anesthesia with midazolam combined with fentanyl (midazolam group) or thiopental combined with fentanyl (thiopental group).

Results: After administration of fentanyl in doses of 1.0 mkg/kg for premedication there were no significant changes of heart rate variability and vegetative heart tonus in both groups. Administration of midazolam 0.2-0.3 mg/kg combined with fentanyl 1.0 mkg/kg for induction of general anesthesia leads to a significant reduction of heart rate variability. The LFun (marker of sympathetic heart tonus) reduced by 24.2% (69.1 (95%CI 65.9-72.3) vs 52.4 (95%CI 42.9-70.0) (p=0,02), meantime the HFun (marker of parasympathetic cardiac tonus) enhanced by 34,9% (30,9 (95%CI 27.6-34.1) vs 47.5(95% CI 30.4-57.4) (p=0.01). Administration of thiopental 6.0-7.0 mg/kg combined with fentanyl 1.0 mkg/kg for induction of general anesthesia leads to a significant reduction of heart rate variability.

Conclusions: Administration of fentanyl solution in doses 1.0 mkg/kg for premedication is not associated with significant changes of vegetative tonus of the heart. Administration of midazolam in combination with fentanyl for induction of general anesthesia leads to significant decrease of heart rate variability and enhanced parasympathetic cardiac tonus. Induction of general anesthesia with thiopental and fentanyl leads to enhanced sympathetic tonus of the heart and reduced parasympathetic tonus of the heart.

Key words: heart rate variability, sympathetic heart tonus, parasympathetic heart tonus.

Introduction

Midazolam is a hypnotic agent used for sedation as well as for induction of general anesthesia. Frequently, its intravenous administration is associated with blood pressure and heart rate changes. Midazolam acts via gamma-aminobutyric acid (GABA) receptors which have an important role in regulation of vegetative nervous system [1, 2].

Thiopental is an ultra-short acting derivative of barbiturates. Large clinical application of the drug has been accompanied by an enormous increase in the knowledge of the pharmacology, in particular the effects on GABA receptor and GABA-induced effects on nerve cell membranes. Despite the development of new agents for induction o general anesthesia, thiopental still has a firm place in clinical applications. Currently it is mainly used in obstetrics for induction of cesarean sections under general anesthesia. Also, this is preferred agent of induction in neurosurgery [3-6].

Fentanyl is an opioid used in combination with other hypnotic agents for induction of general anesthesia [7].

The sympathetic and parasympathetic influences on the sinus node in the heart are manifested by cyclic changes of the RR interval on the ECG, a phenomenon known as heart rate variability (HRV). HRV is a widely used method to assess changes in vegetative tonus of the heart in different medical fields [8, 9, 10]. Some recent studies have demonstrated the efficacy of HRV analysis for risk assessment of hemodynamic instability during induction of anesthesia in abdominal surgery [11, 12].

Induction of general anesthesia with thiopental or midazolam is associated with changes in blood pressure and heart rhythm. These changes can be attributed to direct effects of the drugs on the heart, changes in arterial blood pressure and activation of baroreceptor mechanisms, peripheral vasodilation (preferential mechanism for barbiturates like thiopental). In the literature there are several studies which analysed the effects of midazolam [1, 13-19] and the effects of thiopental [20, 21, 22] on sympathetic-parasympathetic balance of the heart. There is not a single comparative study

regarding changes in autonomic tonus of the heart during induction of anesthesia with midazolam or thiopental.

This study tested the hypothesis that induction of general anesthesia with thiopental or midazolam is associated with changes in autonomic tonus of the heart. The study hypothesis started from the clinical observation that the combination of midazolam and fentanyl for induction of anesthesia frequently is associated with development of arterial hypotension and sinus bradycardia, while induction with thiopental and fentanyl more often led to arterial hypotension and sinus tachycardia.

Material and methods

This is a prospective randomized study to evaluate the changes of vegetative heart tonus after induction of general anesthesia with two different anesthetic agents: midazolam and thiopental, both of them combined with fentanyl. The protocol of study was approved by the Ethic Committee of the Nicolae Testemitsanu State University of Medicine and Pharmacy, Chişinău.

The study groups involved ASA physical status I-II patients scheduled for elective surgical procedures aged under 60 years (to exclude age-related changes of HRV). We obtained an informed consent from all participants in the study. Patients with diseases that could interfere with vegetative heart tonus (endocrine, neurological, cardiovascular diseases) were excluded from the study. Another exclusion criterion was the presence of more than 20% of artifacts on ECG trace. Another compulsory criterion was the presence of sinus rhythm on ECG in patients enrolled in the study group (fig. 1).

For registration of continuous ECG to provide analysis of HRV in order to find the change of autonomic tonus of

the heart was used a Holter device (Holter TLC 5000, USA). We attached 10 electrodes on the chest and abdomen of the patients and connected them to Holter monitor. Continuous ECG registration was performed within 25-30 minutes after admission of patients to the surgical room. HRV parameters were analyzed at rest (T1), after premedication with fentanyl 1.0 mkg/kg (T2) and after induction of general anesthesia with midazolam 0.2-0.3 mg/kg with fentanyl 1.0 mkg/kg (midazolam group) and thiopental 6.0-7.0 mg/kg with fentanyl 1.0 mkg/kg (thiopental group) (fig. 1). After administration of midazolam or thiopental and development of bradypnea or apnea, the mask ventilation was initiated in order to ensure a frequency of ventilation of 14-16/min and a tidal volume 7.0-8.0 ml/kg, an important requirement for correct registration and analysis of HRV. During induction of general anesthesia, oxygen was delivered to ensure a SpO_2 above 95%.

HRV parameters and changes in sympathetic and parasympathetic vegetative heart tonus were analyzed by Holter computerized system. Parameters of HRV and their significance were interpreted according to the recommendations of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology [13]. Total Power (TP) of HRV represents all vegetative influences on the heart (sympathetic, parasympathetic, influences from chemoreceptors and baroreceptors)(physiological ranges – 3466.0 ± 1018.0 ms^2); spectral power of normalized low frequency power (LFun) (physiological ranges – 54.0 ± 4.0) represents sympathetic and baroreceptor influences on the heart; spectral power of normalized high frequency power (HFun) (physiological ranges – 29.0 ± 3.0) represents parasympathetic influences on the heart; LFun/HFun ratio (physiological ranges – 1.5-2.0) – represents sympathetic-parasympathetic balance of the heart [8, 10].

Statistical analysis of the results was done with the statistical program GraphPad Prism 8 (GraphPad Software, San Diego, California, SUA). For analysis of HRV changes within one group were used paired t-test and repeated measures ANOVA (for values with parametric distribution) and Wilcoxon and Friedman tests (for values with non-parametric distribution). For statistical analyses between groups (thiopental group vs. midazolam group) were used unpaired t-test (for values with parametric distribution) and Mann-Whitney and Kruskal–Wallis tests (for values with non-parametric distribution). Results are presented in form of average and 95% confidence interval (for parametric data) and median with interquartile range (IQR - for non-parametric data). Value of $p<0.05$ was considered statistically significant. The number of patients involved in the study group was determined in order to ensure a study power of 80%.

Results

A total of 94 patients comprising 43 men and 51 women were studied. None of the patients was excluded from the study. Demographic data are shown in Table 1. There were

94 patients
ASA I-II
Sinus rhythm on ECG
T1

Premedication with Fentanyl 1.0 mkg/kg
T2

Thiopental group
n=47
Thiopental 6.0-7.0 mg/kg
with fentanyl 1.0 mkg/kg

Midazolam group
n=47
Midazolam 0.2-0.3 mg/kg
with fentanyl 1.0 mkg/kg

Analysis of HRV and assessment of changes in autonomic tonus of the heart by Holter computerized system
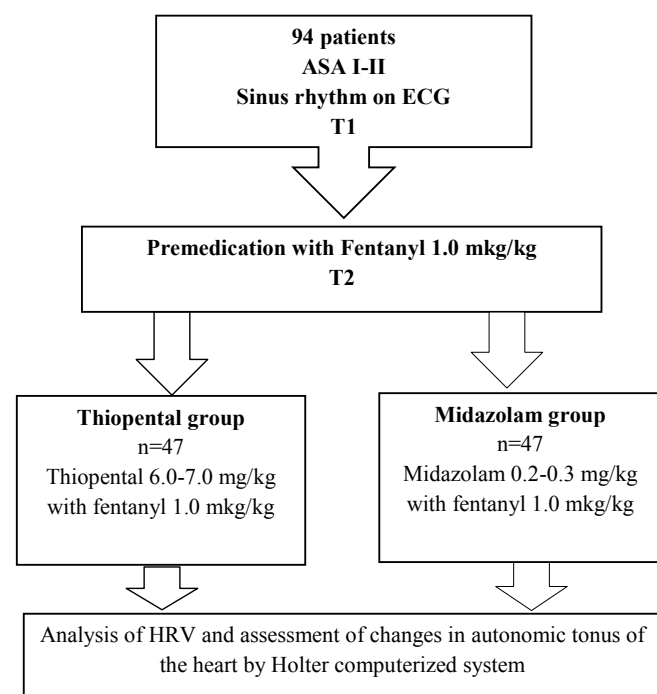
Fig. 1. Flowchart of the study.

no significant differences between groups in terms of demographic data. The distribution of ASA physical status classification and operative procedures was also comparable in two groups (tab. 1 and 2).

Table 1

Demographic data

Parameters	Group		p
	Midazolam	**Thiopental**	
Age in years (mean±SD)	38.0±12.0	35.4±11.2	0.86
BMI in kg/m² (mean±SD)	24.5±3.3	23.9±4.1	0.19
Male/Female	20/27	23/24	NS
ASA I/II	21/26	19/28	NS

SD=Standard deviation, BMI = body mass index; NS=Not significant, ASA=American Society of Anesthesiologists

The baseline values of HRV parameters (TP, LFun, HFun and LFun/HFun) for both groups are presented in the table 3. There was no statistically significant difference between groups. It can be observed that the baseline value of LFun/HFun was 3.1 (95%CI 2.4-3.8) in midazolam group and 2.7 (95% CI 2.1-3.3) in thiopental group, indicating enhanced cardiac sympathetic tonus in the patients of both study groups.

Table 2

Distribution of operative procedures in the study groups

Operative procedures	Midazolam group (n=47)	Thiopental group (n=47)
Laparoscopic cholecystectomy	18	18
Mandible osteosynthesis	9	9
Discectomy	12	8
Rhinoplasty	5	4
Sinusotomy	3	2
Others	-	6

After administration of fentanyl 1.0 mkg/kg for premedication the parameters of HRV didn't change significantly when comparing to baseline values. There were no attested significant differences between groups as well (table 3). The major changes in HRV parameters were attested after administration of midazolam 0.2-0.3 mg/kg or thiopental 6.0-7.0 mg/kg for induction of general anesthesia.

After intravenous administration of midazolam the spectral power of TP decreased by 81.9% (149.3 ms² (IQR 52.0-320.0) vs 829.1 ms² (IQR 438.5-2395.0), (p=0.001). After intravenous administration of thiopental the spectral power of TP decreased by 88.5% (100.4 ms² (IQR 54.7-188.8) vs 869.5 ms² (IQR 512.2-1633.0) (p<0.0001) (fig. 2).

Table 3

HRV parameters in both study groups

HRV parameters		Baseline (T1)	After premedicatio (T2)	After induction (T3)	p
TP (ms²) *	Midazolam group	924.2* (404.2-1913.0)	829.1* (438.5-2395.0)	149.3* (52.0-320.0)	0.0001
	Thiopental group	889.5* (481.7-1585.0)	869.5* (512.2-1633.0)	100.4* (54.7- 188.8)	**<0.0001**
	p	**0.68**	**0.9**	**0.014**	
LFun	Midazolam group	67.7 (62.9-72.5)	69.1 (65.9-72.3)	52.4 (42.9-70.0)	0.02
	Thiopental group	65.5 (60.8-70.1)	65.8 (61.9-69.6)	73.5 (68.4-78.6)	<0.001
	p	**0.49**	**0.18**	**0.03**	
HFun	Midazolam group	27.4 (21.4-37.0)	30.9 (27.6-34.1)	47.5 (30.4-57.4)	**0.01**
	Thiopental group	34.5 (29.8-39.2)	34.2 (30.4-38.1)	24.5 (20.3-28.7)	**<0.001**
	p	**0.5**	**0.18**	**0.01**	
LFun/HFun	Midazolam group	3.1 (2.4-3.8)	2.8 (2.2-3.4)	1.1 (0.6-1.8)	**0.03**
	Thiopental group	2.7 (2.1-3.3)	2.4 (2.0-2.8)	4.4 (3.5-5.2)	**<0.001**
	p	**0.33**	**0.26**	**0.01**	

Note. Statistical analysis was performed with repeated measures ANOVA and Friedman test* (for analysis within the group) and unpaired t-test and Mann-Whitney test* (for analysis between groups). Values are presented as average and 95% confidence interval for values with parametric distribution and median with interquartile range for parameters with non-parametric distribution*.

It is worth mentioning that induction of general anesthesia with thiopental and fentanyl depresses HRV more than induction with midazolam and fentanyl (p=0.014).
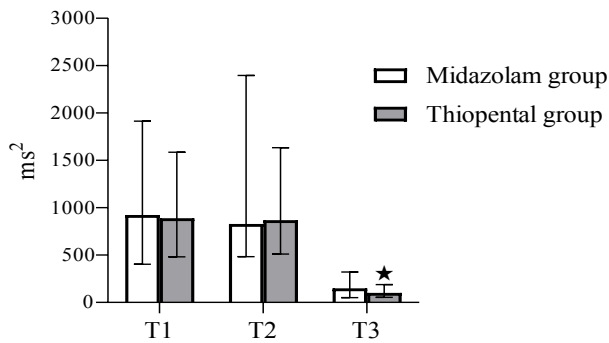
Fig. 2. Changes of spectral power of TP of HRV in both study groups.

(*p<0.05) Values are represented as median with intercuartile range (error bars.)

There were attested significant changes in the spectral power of LFun and HFun after administration of thiopental or midazolam. These changes are different in each group, in such a way emphasizing the different effects of thiopental and midazolam on the cardiac vegetative tonus.

In the midazolam group in T3, LFun decreased by 24.2% (52.4 (95%CI 42.9-70.0) vs 69.1 (95%CI 65.9-72.3), (p=0.02), demonstrating the sympatholytic effect of the drug, while in thiopental group LFun enhanced by 10.5% (73.5 (95%CI 68.4-78.6) vs 65.8 (95%CI 61.9-69.6) (p<0.001), proving sympathomimetic effect of anesthetic agent (fig. 3).
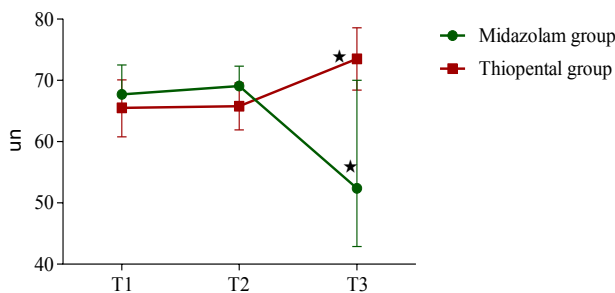
Fig. 3. Changes of spectral power of LFun in both study groups.
(*p<0.05) Error bars represent 95% confidence interval.

Spectral power of HFun (marker of parasympathetic heart tonus) also changed significantly after administration of thiopental or midazolam. Changes of spectral power of HFun after administration of midazolam proved the vagotonic effect of the drug since power of HFun increased by 34.9% (47.5 (95%CI 30.4-57.4) vs. 30.9 (95%CI 27.6-34.1) (p=0.01). In the thiopental group spectral power of HFun has reduced by 28.4% (34.2 (95%CI 30.4-38.1) vs. 24.5 (95%CI 20.3-28.7)(p=0.001). Reduction in power of HFun demonstrated the vagolytic effect of thiopental when given in doses for induction of general anesthesia and combined with fentanyl (fig. 4).
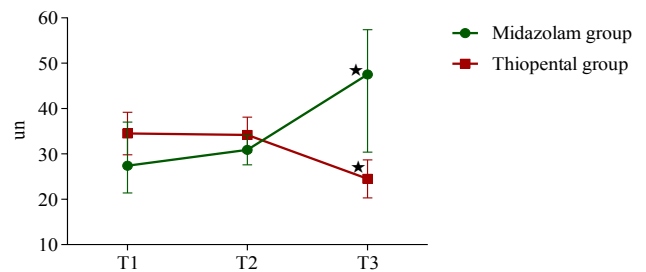
Fig. 4. Changes of spectral power of HFun in both study groups.
(*p<0.05) Error bars represent 95% confidence interval.

Changes of LFun and HFun in both groups lead to changes in LFun/HFun ratio.

After induction of general anesthesia with midazolam and fentanyl the LFun/HFun ratio decreased by 60.7% (1.1 (95%CI 0.6-1.8) vs. 2.8 (95%CI 2.2-3.4)(p=0.03). The value of LFun/HFun ratio after induction was 1.1 which indicated enhanced cardiac parasympathetic tonus in the patients of the study group. In the thiopental group the average LFun/HFun in T3 was 4.4, signaling the predominance of sympathetic tonus of the heart, showing an enhancement by 45.5% (2.4 (95%CI 2.0-2.8) vs. 4.4 (95%CI9 3.5-5.2) (p<0.001) (fig. 5).
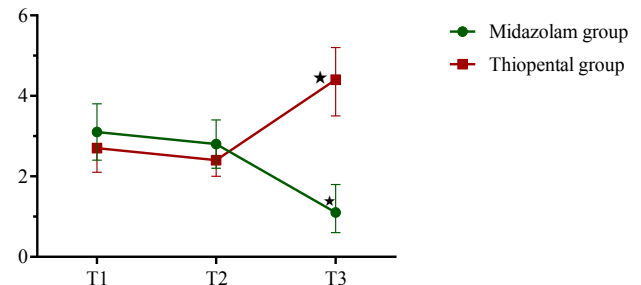
Fig. 5. Changes of LFun/HFun ratio in both study groups.

(*p<0.05) Error bars represent 95% confidence interval.

Discussion

The sinus node of the heart is under permanent control of vegetative nervous system thus controlling the heart rhythm and performing adaptation to different physiological or pathological factors. The changes in heart rhythm and subsequently changes in HRV are triggered by sympathetic and parasympathetic input on the sinus node. HRV is a frequent tool used in medical field for analysis of changes in sympathetic and parasympathetic influences on the heart. Nowadays to analyze HRV is an easy goal since modern Holter devices are equipped with computerized system for analysis of HRV and can appreciate the changes in heart vegetative tonus. It was generally accepted and proved in many clinical researches, that the LFun/HFun ratio represents the sympathetic-parasympathetic balance of the heart, the LFun represents the sympathetic and baroreceptors influences on the heart and the HFun represents the parasympathetic tonus of the heart [9, 10, 13].

ORIGINAL RESEARCH

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Several clinical studies used analysis of HRV to find the effect of midazolam on vegetative regulation of the heart. The fact should be mentioned that in most of these studies midazolam was administered intravenously for sedation [1, 14, 15]. So, it is difficult to compare their results with the results of this study, since the midazolam dose was higher (0.2-0.3 mg/kg) and it was administered in combination with fentanyl (1.0 mkg/kg).

In a recent study Nishiyama T. (2018), demonstrated that administration of midazolam 0.06 mg/kg in combination with 0.5 mg of atropine reduced sympathetic tonus. The final conclusion of the study was that midazolam, but not hydroxyzine premedication, inhibited sympathetic activation at induction of anesthesia with midazolam and thiopental [1].

In another study performed by Tsugayasu R. et al. [14], sedation with midazolam 0.01 mg/kg decreased cardiac sympathetic tonus without significant effect on cardiac parasympathetic tonus. Smith A. et al. showed that premedication with midazolam 2.5 mg in combination with differential doses of fentanyl (50 mkg, 75 mkg, 100 mkg and 150 mkg) didn't change significantly the cardiac vegetative tonus. The final conclusion of this clinical study was that midazolam for sedation in combination with fentanyl didn't change the autonomic balance of the heart and the enhanced cardiac sympathetic tonus in the patients from the study group mostly was triggered by changes in respiratory pattern [15].

Contrary to this, in another clinical research by Dogan I. et al. was proved that sedation with midazolam 0.05 mg/kg for transesophageal echocardiography significantly reduced cardiac sympathetic tonus and significantly increased parasympathetic tonus [16]. The results of this study are similar to our results, even if the dose of midazolam was lower. In our study value of LFun/HFun after induction of general anesthesia with midazolam and fentanyl decreased to 1.1 thus signaling enhanced cardiac parasympathetic tonus. This decrease could be attributed to the effects of midazolam, as premedication with fentanyl didn't significantly change LFun/HFun ratio. Benzodiazepines can influence autonomic neurocardiac regulation, probably through their interaction with the GABAA receptor complex in the brain [2].

Hidaka S. et al. in a prospective clinical research, involving forty ASA physical status I and II patients scheduled for knee surgery investigated the effect of propofol and midazolam on cardiac autonomic nervous system activity during combined spinal-epidural anesthesia [17]. In this clinical study, propofol was more potent than midazolam in causing sympatholytic effect during combined spinal and epidural anesthesia. Our research proved the same sympatholytic effect of midazolam when combined with fentanyl and given in doses for induction of general anesthesia.

In a clinical study involving thirty dental patients, Win N. et al. proved dominant sympathetic effect of midazolam [18]. In this clinical research, midazolam was associated with an increase in LF/HF ratio (2.3±1.1 versus 3.7±1.8). It should be emphasized that the dose of midazolam in this study was 0.075 mg/kg, much lower than in our study.

In a controlled, randomized, double-blinded study by Sherif S. et al. aiming to investigate the effects of intravenous midazolam on HRV, patients received midazolam 0.05 mg/kg. In this clinical research, midazolam administered in sedative doses induced a significant decrease in TP and HF power, reflecting decreased parasympathetic activity. There was a decrease in LF power that did not reach statistical significance [19].

There are several studies which analyzed the effects of thiopental on autonomic tonus of the heart by analysis of HRV according to recommendations of Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology [13]. Tsuchiya S. et al. in a clinical study involving 17 patients scheduled for minor surgical interventions proved the fact that thiopental given in small doses for sedation, significantly reduced parasympathetic tonus of the heart without visible influences on sympathetic tonus of the heart [20]. Another remark of the study was that effect of thiopental on vegetative balance of the heart is in direct relation with the level of sedation. In our study induction with thiopental significantly enhanced sympathetic tonus of the heart and significantly reduced parasympathetic tonus of the heart, but the doses of the drug were higher. In another clinical research by Omerbegovic M. et al. [21] was compared the effect of propofol and thiopental on heart autonomic balance. The study group comprised only patients scheduled for surgery with ASA I-II risk. In this study the effect of propofol on HRV didn't differ significantly from the effect of thiopental, as induction in both groups of study leads to mark reduction of TP of HRV, LFun and HF. So, in this study was confirmed the sympatholytic and vagolytic effect of thiopental. Their results are different from ours, as in our study induction with thiopental and fentanyl reduced significantly HRV and HFun, thiopental having a vagolytic effect. The spectral power of LFun after administration of thiopental enhanced significantly, proving a sympathomimetic effect of the drug.

In a study conducted by Riznyk L. et al. [22], aiming to compare the effects of thiopental and propofol on heart rate variability during fentanyl-based induction of general anesthesia, after administration of fentanyl 3.0 mkg/kg there was a significant reduction in spectral power of LFun, proving the sympatholytic effect of opioid. In our study after premedication with 1.0 mkg/kg fentanyl were not attested significant changes in spectral power of LFun, HFun and LFun/HFun ratio. This may be explained by a lower dose of the drug which we used for premedication. After administration of thiopental in the study by Riznyk L. at al. as well as in this study, was proved the sympathomimetic effect (enhanced power of LFun and LFun/HFun ratio) and vagolytic effect (reduced power of HFun) of thiopental.

This clinical research of HRV analysis used to find changes in sympathetic-parasympathetic tonus of the heart proved its clinical applicability. By analysis of changes in spectral power of TP, LFun, HFun and LFun/HFun ratio

was demonstrated the sympatholytic and vagotonic effect of midazolam and sympathomimetic and vagolytic effect of thiopental. This can be of huge clinical significance when choosing the drugs for induction of general anesthesia in patients with cardiovascular disorders or other diseases which interfere with autonomic regulation of the heart.

Conclusions

1. Induction of general anesthesia with thiopental and fentanyl depresses HRV more than induction with midazolam and fentanyl.

2. Administration of midazolam combined with fentanyl for induction leads to enhanced parasympathetic tonus of the heart (vagotonic effect) and reduces sympathetic tonus of the heart (sympatholytic effect);

3. Administration of thiopental combined with fentanyl for induction leads to enhanced sympathetic tonus of the heart (sympathomimetic effect) and reduces parasympathetic tonus of the heart (vagolytic effect).

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Particularities of gynecological history in patients with primary infertility associated with endometrial dysfunction

Mihaela Burac, MD, PhD Applicant

Department of Gynecology, Obstetrics and Human Reproduction
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova

Corresponding author: mihaelaburac@gmail.com
Manuscript received October 07, 2019; revised manuscript December 02, 2019

Abstract

Background: Despite the positive dynamics of global demography, infertility remains one of the current challenges of contemporary gynecology. The endometrium represents the mirror that reflects the state of the pathological processes that occur in the pelvic organs, and the frequency of morphofunctional disorders of the endometrium in infertility is quite high. The aim of the study was to assess the gynecologic history in primary infertility patients.
Material and methods: The study included 96 patients divided into 2 groups. The study group - 48 patients with primary infertility and the control group – 48 fertile patients. The patients were interrogated according to a questionnaire that included 130 questions.
Results: The evaluation of menstrual function revealed that according to the following criteria: age of menarche, duration of menstruation, study groups were homogeneous. The age of onset of menarche was within the normal range in 97.9% (n = 47) of patients in both groups and averaged 12.77±1.27 years. Patients in the study group had regular menstrual cycle in 70.8% (n = 34) of cases, and those in the control group in 93.8% (n = 45) of cases, c^2 = 8.649; p = 0.003. The duration of the menstrual cycle averaged 35.23 ± 12.54 days in Study group (L_1) versus 28.33 ± 3.09 days in Control group (L_0), p <0.001. The duration of menstruation was between 2 and 7 days in both groups with a mean of 4.35±1.12 in the study group and 4.73±1.12 in the control group, p = 0.1.
Conclusions: Patients in the study group reported more often an irregular menstrual cycle and a prolonged interval between menstrual periods, hypomenorrhea, intermenstrual and postcoital bleeding, algodysmenorrhea, dyspareunia, premenstrual syndrome indicating the existence of endometrial dysfunction at the basis of infertility pathogenesis.
Key words: endometrial disfunction, primary infertility, endometrium.

Introduction

The fertility rate is a fundamental and integral criterion in the socio-economic wellbeing of a country. Despite the positive dynamics of the global demography, infertility remains one of the current challenges of contemporary gynecology [1, 2]. Despite the fact that the etiological factors and the pathogenetic mechanisms of infertility are diverse, the fundamental mechanisms in pregnancy occurrence are represented by the quality of the embryo and the morphofunctional state of the endometrium [3, 4, 5, 6]. For many decades, researchers have shown a special interest for the study of the endometrium, in which complex molecular interactions of biologically active substances take place in order to create optimal conditions for the most important function - implantation of the embrio and pregnancy occurance, but so far it was not possible to disclose its functional activity until the end [7, 8]. It is necessary to note that the first mention about the endometrium, especially its pathology as a cause of infertility is found in the works of Hippocrates [7]. With the development of medicine, subsequent knowledge about the structure and functional activity of the endometrium has been refined and expanded. The endometrium is the mirror that reflects the state of the pathological processes that occur in the female genital organs, and the frequency of the morphofunctional disorders of the endometrium in infertility is quite high [9, 10].

Endometrial dysfunction represents the morphofunctional changes of the endometrium, which can be reversible or irreversible, based on disorders of molecular mechanisms, which subsequently lead to infertility, disturbances in the implantation of the embryo and placenta [5, 10, 11, 12, 13]. Factors that contribute to the development of endometrial dysfunction are chronic inflammatory processes of the endometrium. The most important signs of chronic endometrial inflammatory processes and endometrial dysfunction are disturbances of the reproductive function in women (infertility, miscarriages, missed abortion), disturbance of menstrual function (irregular menstrual cycle, abnormal uterine bleeding), pain syndrome (dysmenorrhea, dyspareunia) and dysregulation of secretory functions [4, 14, 15, 16, 17, 18, 19, 20].

Material and methods

A prospective cohort study was conducted at the Department of Obstetrics, Gynecology and Human Reproduction at the clinical base of Municipal Clinical Hospital No.1, and Maternity No.2, Nicolae Testemitsanu State University of Medicine and Pharmacy. The study included 96 patients divided into 2 groups. The study group (L_1) included 48 patients with the established diagnosis of primary infertility and the control group (L_0) included 48 fertile patients.

The protocol of this study was approved by the Research Ethics Committee of the Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova (no. 79/62 of 26.04.2017). Patients signed informed consent for participation in the research.

The inclusion criteria for the study group were: patients suffering from primary infertility with indications for laparoscopy and hysteroscopy, age of the patient 20 - 40 years, lack of hormone and antibiotic therapy during the last 6 months, lack of intrauterine manipulations in anamnesia, agreement to participate in the research. Inclusion criteria for the control group: patients who have had a live birth in the last 2 years and are not breastfeeding, patients who do not have complicated reproductive gynecological anamnesis (infertility, miscarriage, missed abortion), lack of hormonal and antibiotic therapy in the last 6 months, research participation agreement. The exclusion criteria from the research were: patients with acute genital infection, age < 20 years and > 40 years, patients suffering from congenital uterine malformations, patients who have had previously intrauterine surgical manipulations, atypical endometrial hyperplasia, patients who refused voluntary participation in the research.

The clinical examination consisted of the evaluation of patient's complaints and the anamnesis. Assessment of the average age of menarche, establishment of menstrual function, duration and variations of the menstrual cycle and menstrual flow. Evaluation of the regularity of the menstrual cycle and the presence of such characteristics as: dysmenorrhea, the onset of pain syndrome with menarche, dyspareunia, the presence of pain and their nature during the menstrual cycle. In the study of the anamnestic data, attention was paid to the premorbid background, gynecological and extragenital disorders, reproductive and menstrual function. Were determined the factors that contributed to the onset of the disease. A general physical and gynecological examination was performed in the patients from the examined groups.

Statistical data processing was performed using Microsoft Excel 2016 and SPSS 20. The results are expressed as mean values ± standard deviation for the parametric variables and for the categorical variables as a percentage. The Pearson test was applied for correlation analysis. The values $p<0.05$, were considered statistically significant.

Results

The study included 48 patients in each group who met the inclusion criteria, the study group – patients with the diagnosis of primary infertility and the control group – fertile patients.

According to the age criterion, marital status, living environment, the examined lots were homogeneous. The age of the patients included in the study group was between 22 and 39 years with an average of 29.00 ± 4.58 years and in the control group was between 20 and 35 years with the average of 29.23 ± 4.29 years $p = 0.80$ (fig. 1).
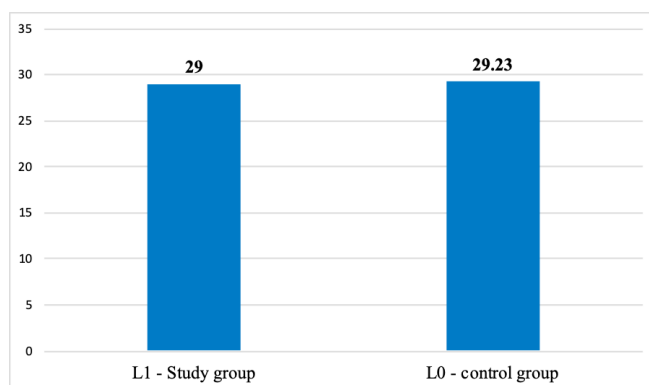
Fig. 1. Distribution of groups according to age criterion (years).

The majority of patients in both groups were from urban area L_1 – 64.6% (n = 31) vs L_0 – 75% (n = 36) (fig. 2).

Each second patient suffering from primary infertility had higher education – 54.2% (n = 26) whereas in the control group only 43.8% (n = 21) of patients and 54.2% (n = 26) of the patients in the study group reported the presence of harmful factors at work, while patients in the control group only – 22.9% (n = 11).

The evaluation of menstrual function in the patients included in the study revealed that according to criteria such
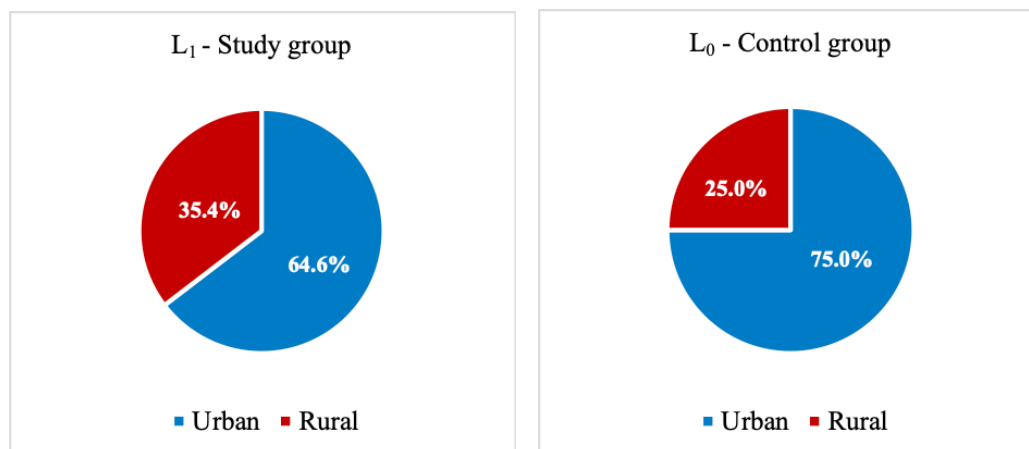
Fig. 2. Distribution of study groups according to the living environment.

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as the age of menarche, the duration of menstruation the study groups were homogeneous. The age of onset of the menarche was within the norm within 97.9% (n = 47) of patients in both groups and constituted on average 12.77±1.27 years. Patients in the study group had a regular menstrual cycle in 70.8% (n = 34) cases, and those in the control group – in 93.8% (n = 45) of cases, $c^2 = 8.649$; p = 0.003. The duration of the menstrual cycle was on average 35.23 ± 12.54 days in L_1 versus 28.33 ± 3.09 days in L_0, p<0.001. The duration of menstruation was between 2 – 7 days in both groups with the average of 4.35 ± 1.12 in the study group and 4.73±1.12 in the control group, p = 0.1.

As a result of the study of the peculiarities of the menstrual cycle, we found that every 5th patient suffering from primary infertility reported hypomenorrhea compared with the fertile patients – 18.8% (n = 9) vs 2.1% (n = 1), $c^2 = 7.839$; p = 0.020, the presence of intermenstrual and postcoital bleeding was reported only by patients in the study group with a frequency of 14.6% (n = 7), $c^2 = 7.551$; p = 0.006 and 4.2% (n = 2) $c^2 = 2.043$; p = 0.15 in the control group. Algosdymenorrhea was more frequent in patients in the study group – 60.4% (n = 29) vs the control group – 35.4% (n = 17), $c^2 = 6.010$; p = 0.014. Each of the 2 patients in the study group reported premenstrual syndrome – 47.9% (n = 23) vs 29.2% (n = 14), $c^2 = 3.562$; p = 0.059, and each 5th patient – dyspareunia 20.8% (n = 10) vs. 4.2% (n = 2), $c^2 = 8.095$; p = 0.014 (table 1).

Table 1

The complaints of the patients included in the study

The evaluated criterion	Study group L_1 %(n)	Control group L_0 %(n)	p
Regular menstrual cycle	70.8 (34)	93.8 (45)	0.003
Hypomenorea	18.8 (9)	2.1 (1)	0.020
Intermenstrual bleeding	14.6 (7)	0	0.006
Postcoital bleeding	4.2 (2)	0	0.15
Algodysmenorrhea	60.4 (29)	35.4 (17)	0.014
Premenstrual syndrome	47.9 (23)	29.2 (14)	0.059
Dyspareunia	20.8 (10)	4.2 (2)	0.014

The analysis of the gynecological pathologies that had an impact throughout the life of the patients included in the study revealed the following: the pathology of the fallopian tubes was found in 68.8% (n = 33) L_1 vs 0% (n = 0) L_0, $c^2 = 50.286$; p < 0.001, ovarian pathology was reported by patients in 52.1% (n = 25) L_1 vs 8.3% (n = 4) L_0, $c^2 = 21.789$; p < 0.001, uterine pathology – 16.7% (n = 8) L_1 vs 2.1% (n = 1) L_0, $c^2 = 6.008$; p = 0.014; of which endometrial polyps in 2.1% (n = 1) vs 0% (n = 0), intramural myoma – 2.1% (n = 1) vs 0% (n = 0), subserous myoma – 14.6 % (n = 7) vs. 2.1% (n = 1), multinodular myoma in 2.1% (n = 1) vs. 0%

(n = 0) (fig. 3). Each 5th patient in the study group had a sexually transmitted disease during her lifetime – 22.9% (n = 11) L_1, vs 6.3% (n = 3) L_0, $c^2 = 5.352$; p = 0.021, of which chlamydiosis – 12.5% (n = 6) vs 2.1% (n = 1), trihomonia – 2.1% (n = 1) vs 0% (n = 0), genital herpes – 2.1% (n = 1) vs 2.1% (n = 1), human papilloma virus (HPV) – 2.1% (n = 1) vs 2.1% (n = 1), mycoplasmosis – 8.3% (n = 4) vs 0% (n = 0), ureaplasmosis – 12.5% (n = 6) vs 0% (n = 0).

In the study group the duration of primary infertility was 4.2% (n = 2) up to 1 year, 10.4% (n = 5) one year, 20.8% (n = 10) - 2 years, 22.9% (n = 11) – 3 years, 6.3% (n = 3) – 4 years, 8.3% (n = 4) – 5 years and 27.1% (n = 13) more than 5 years (fig. 4).
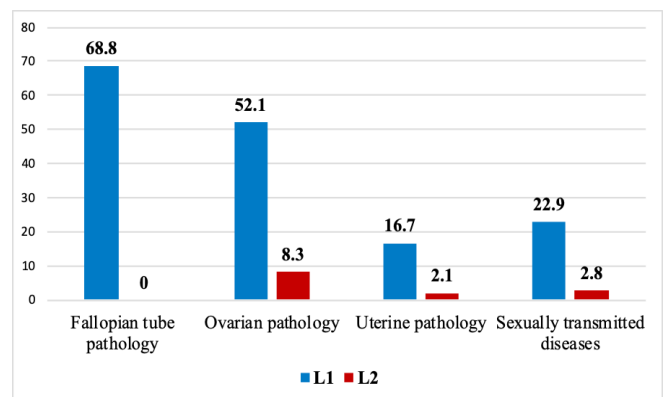
Fig. 3. Structure of gynecological history in patients included in the research (%).
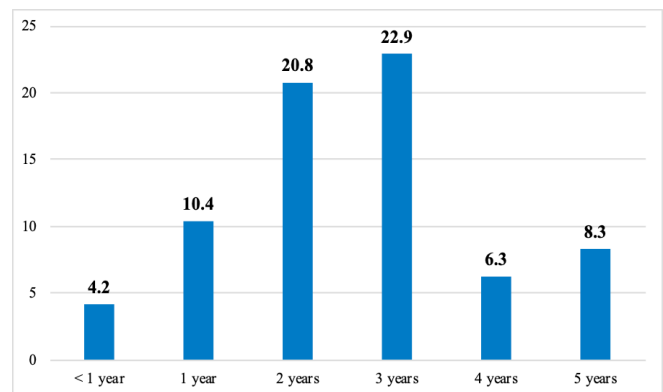
Fig. 4. Duration of primary infertility in patients in the study group (%).

Discussion

The endometrium is a complex, hormone-dependent functional tissue, which undergoes cyclic and structural changes under the influence of sex steroid hormones. Optimal morphofunctional characteristics of the endometrium are the basic elements in the occurrence and development of pregnancy [5, 7]. The pathological processes of the pelvic organs have both a direct and indirect effect on the state of the endometrium. Changes in their structural and functional characteristics determine the development of infertility, spontaneous abortions and implantation defects [14, 21, 22].

In the presented study we evaluated the clinical-anamnestic characteristics in patients with primary infertility in order to determine which conditions most frequently lead to the development of endometrial dysfunction. Currently, an important social factor is the fact that women delay the planning of a pregnancy closer to 30 years, which leads to the accumulation of both somatic and gynecological pathologies [22, 23]. The socio-economic factors of a woman's life such as studies, career, lack of life partner, often become fundamental moments in the process of performing the reproductive function [24]. The results of the study showed that most of the women suffering from infertility and included in the research were between 25 and 34 years of age (73%), of which 41.7% were between the age of 25-30 years and 31.3% of the patients were 30-34 years old, a share of 14.6% occupied the patients included in the age category of 35-40 years.

The assessment of the menstrual function of the patients showed that the age of onset of menstruation and the duration of menstruation in both groups correspond to normal sexual development. Thus, the average values of the studied parameters were not statistically significant and were within the average range. Menstrual function in patients suffering from primary infertility is the mirror of the morphofunctional status of the endometrium and denotes the degree of its impairment by a number of pathological factors mentioned by the patients throughout their life. According to different studies, the main complaints of patients suffering from infertility and endometrial damage are the following menstrual disorders: the presence of hypomenorrhea, oligomenorrhea, intermenstrual bleeding, bleeding or postcoital spotting [9, 10, 25, 26]. These results were also obtained in our study, so patients with primary infertility reported more frequently, compared with fertile patients: hypomenorrhea (18.8%), intermenstrual bleeding (14.6%), postcoital bleeding (4.2%). Another important factor that leads to changes in quality of life and working capacity in infertile patients is the presence of chronic pain syndrome with such manifestations as algodysmenorrhea, dyspareunia, dysuria, premenstrual syndrome, these complaints have also been more frequently reported by patients in the study, compared to the control group. Premenstrual syndrome and algodysmenorrhea have been reported 2 times more frequently by patients suffering from infertility, whereas dyspareunia have been accused 5 times more frequently, which is consistent with other international studies [12, 14].

According to some authors, early sexual onset and lack of knowledge about appropriate contraception methods are responsible for the development of a series of infectious gynecological pathologies that have serious repercussions on women's reproductive health [21]. International studies broadly describe the association of sexually transmitted diseases, pelvic inflammatory disease with the development of endometrial dysfunction in patients with infertility, in particular the pathological and cytopathic action of viral infection (herpesvirus, cytomegalovirus, HPV) on the endometrium [27, 28, 29]. The results of our study indicated a high incidence among patients with primary infertility of the sexually transmitted diseases (22.9%), especially those with silent evolution and with cytopathic effect on the endometrium such as chlamydiosis – 12.5%, genital herpes – 2.1%, HPV – 2.1%, mycoplasmosis – 8.3% and ureoplasmosis – 12.5%. This subsequently led to the high frequency of repeated pelvic inflammatory diseases such as salpingitis (52.1%), salpingoophoritis (12.5%), endometritis (6.3%), cervicitis (33.3%). The results obtained coincide with the data obtained by other researchers [12, 28, 30, 31]. The high frequency of urogenital infections independent of the causal factor ultimately leads to endometrial damage and the development of endometrial dysfunction with infertility, spontaneous abortions, missed abortion, premature births, intrauterine growth restriction of the fetus, fetal death.

Conclusions

Patients suffering from primary infertility more often reported irregular and prolonged menstrual cycle. Also, the patients in the study group reported a series of menstrual cycle disorders such as: hypomenorrhea, intermenstrual and postcoital bleeding, algodysmenorrhea, dyspareunia, premenstrual syndrome, which indicates the existence of endometrial dysfunction based on the pathogenesis of infertility. Gynecological anamnesis was more frequently complicated with the pathology of the fallopian tubes, ovaries and most importantly was complicated by sexually transmitted diseases.

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Eligilibility criteria for video-observed anti-tuberculosis treatment at patients from Chisinau city

Evelina Lesnic, MD, PhD, Associate Professor; **Tatiana Osipov,** MD, Lecturer;
Alina Malic, MD, PhD, Associate Professor

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

Abstract

Background: It is known that the main barriers in the anti-tuberculosis treatment delivery are social, economic, educational and psychological issues. According to the estimations the Republic of Moldova (RM) remains a high risk zone showing an inadequate concern regarding social determinants that represent the risk factors for achieving high treatment outcome. Tuberculosis is concentrated in areas with high density of the population, poor environmental and sanitation conditions: poverty, food insecurity, low living conditions.

Material and methods: A retrospective selective, descriptive study of socioeconomic, epidemiological peculiarities, case-management, diagnosis and microbiological characteristics of 693 patients with tuberculosis registered in Chisinau in 2016 was performed.

Results: Despite the fact that criteria for selection of patients for video-assisted anti-tuberculosis treatment (VOT) were defined, a range of risk factors can endanger treatment performing, such as: deep social economic vulnerability, comorbidities associated or not with psychic impairment, disease related characteristics, such as extensiveness, severity, duration of the tuberculosis evolution, positive microbiological state and multi-drug resistance are conditions which can exclude the ambulatory treatment and VOT. The low treatment outcome shows the limited potential of VOT to improve the epidemiological indices due to the complexity of patient's risk factors.

Conclusions: VOT can be implemented in the management of tuberculosis patients in the actual epidemiological state of the RM, if a complex of patients supporting measures are performed.

Key words: tuberculosis, treatment, outcome.

Introduction

Tuberculosis is one of the 10 causes of death worldwide [1]. The lack of an appropriate and adequate treatment according to the drug resistance profile contributes to the death in a couple of years [2]. The main objectives of the anti-tuberculosis treatment constitute: 1. To cure the patient; 2. To prevent the death from active disease or its late effects; 3. To prevent relapse of tuberculosis; 4. To decrease the risk of the mycobacteria transmission to others; 5. To prevent the development of the acquired drug resistance [1, 2].

According to the World Health Organization guideline "Treatment of tuberculosis" and TB report drug susceptible tuberculosis is treated with the first-line anti-tuberculosis drugs: isoniazid, rifampicin, ethambutol, pirazinamide and streptomycin [1, 2]. Tuberculosis determined by the multidrug resistant strains (MDR-TB) is treated during 18-24 months with 2nd line antituberculosis drugs according to the drug susceptibility test [3]. The standard treatment for MDR-TB consists in injectable antibiotics – aminoglycozides (kanamycin, amikacin or capreomycin) and orally administrated anti-tuberculosis drugs: fluoroquinolones (levofloxacin, moxifloxacin or gatifloxacin), ethionamide, prothionamide, paraaminosalicylic acid and cycloserine) [1, 2]. There are 3 types of the anti-tuberculosis treatment administration options: 1. Community or home-based directly observed treatment (DOT) when the treatment is delivered in the community close to the patient's home or work [1, 2]; 2. DOT administered by specialised healthcare providers such as in the hospitals or specialised services [1, 2]; 3. Video-observed treatment (VOT), based on the principle when the staff involed in its performing can observe the administration of the anti-tuberculosis drugs using electronic devices (personal computer, notebook, smartphone with Android system) through a web camera [3, 4, 5].

The technology required for VOT are broadband Internet and availability of an electronic device connected to a specialised in VOT platform. The option for VOT is real-time communication or recorded video. VOT can replace the DOT when video communication technology is available and the healthcare providers and the patients are well trained. VOT allows to observe adherence to treatment from distance, avoiding the direct contact of the patient with the healthcare worker. VOT is more flexible for patients, achieves a higher level of interaction between patients and medical staff and probably has a lower cost than DOT [6]. There were performed cohort studies in high income countries and no data were found from low and middle income countries which compared the treatment effectiveness of DOT compared with VOT [3, 4]. The studies showed that there is no statistical difference in the treatment completion and mortality among the groups treated through DOT and VOT [2, 3, 4].

In the Republic of Moldova the methodology of the VOT was established by the law no. 153-XVI of 4.07.2008 related to the control and prevention of tuberculosis, recommendations of the National Tuberculosis Control Program for 2016-2020, approved by the decision no. 1160 of 20.10.2016, the objective of the Strategic Program for the Technological Upgrade of the Government (E-Transformare) approved by the decision no. 710 of 10.09.2011 and the National Clinical Protocol "Tuberculosis in adults" 123 approved by the decision no.1081 of 29.12.2017. The regulation established that the responsibility for the initiation of the VOT lies on the pulmonologist specialised in tuberculosis and the primary healthcare worker responsible for the case management in the outpatient settings. In the Republic of Moldova the VOT facilitates the interaction between the healthcare worker and the patient; however, it does not replace the DOT. The including criteria for video-observed treatment (VOT) in the RM are: 1) The patient has an available electronic device (personal computer, notebook, smartphone with android system) and a web camera through which the medical staff involed in its performing can observe the administration of the anti-tuberculosis drugs; 2) The patient is residing in the RM. 3) the patient can administrate independently the anti-tuberculosis treatment [7, 8, 9, 10, 11, 12]. The technologies required for VOT to be available for the patient are: broadband Internet and availability of an electronic device connected to a specialised in VOT platform. The option for VOT according to the actual regulation is the recorded video available to be sent for validation through the VOT platform.

The steps to be performed by the trained in VOT healthcare worker are:

1. Before the initiation of the anti-tuberculosis treatment the patient must be informed by the healthcare worker about the possibility to accomplish it using the video-assistance.

2. To create an account on the site www.vot.tuberculosis.md on E-Sanatate platform on the page "Medici".

3. Before the initiation the VOT the healthcare worker should identify if the patient is eligible according to the including criteria established in the "Eligibility Checklist for Including in VOT".

4. If the patient accomplishes 14 days of 100% treatment compliance the healthcare worker will appreciate him eligible according to the evaluation form "Eligibility Checklist for Including in VOT".

5. After the patient's assessment through the "Eligibility Checklist for Including in VOT" the pulmonologist will decide to include or exclude the patient from VOT.

6. The VOT will be monitored and followed-up according to the recommendations of the National Clinical Protocol No 123 "Tuberculosis in adults" [7, 11].

The trained patient will receive the anti-tuberculosis drugs for 14-30 days confirmed by the signature in the TB01 register. Before the video recording the patient must prepare the drugs on a white paper visible in the webcam and a transparent glass with water in an illuminated place. After the onset of the video recording the patient has to present himself and to enumerate the drugs prepared and the number of the pills. The patient should be placed in front of the webcam and to swallow the drugs one by one with the water prepared in the transparent glass. The patient has to open the mouth and to show the tongue after the swallowing of the pills. At the end of the administration the patient will stop the video recording and will send to validation. The healthcare worker must assess and validate the video recording from 1 to 3 points. The value 1 means the treatment was administrated and the dose was validated. The value 2 means that there is no certainty that the pills were swallowed. The value 3 means that the treatment was not administrated or the dose of a drug was not swallowed. The patient is responsible for the storing the anti-tuberculosis drugs in special conditions such as dry and dark place, far from children.

The regulation establishes excluding from VOT criteria or criteria which cannot allow the patient to be enrolled in VOT. The patient should be treated using the DOT instead of VOT if: a) he refuses to sign the informed consent for VOT; b) the therapeutic regimen includes injectable drugs; c) the patient has no available electronic device (personal computer, notebook, smartphone); d) the electronic device has no Internet connection or the connection has a low speed; d) the patient is unable to take independently the anti-tuberculosis drugs, e) the patient is diagnosed with mental disorders.

There are several criteria which ensure the transfer of the patient from VOT to DOT: a) the patient's requirement; b) the patient fails to transmit for validation the recorded video for at least 2 days; c) the patient does not answer the telephone; d) the hospitalisation in the emergency department; e) imprisonment; f) the patient left the Republic of Moldova for more than 1 month; g) the patient has a low tolerance of the anti-tuberculosis drugs or experiences adverse drug effects; h) the referral pulmonologist decides to stop the VOT.

Before the initiation of the VOT the healthcare worker must register the patient on the site www.vot.tuberculoza.md and complete the electronic file of the health state ("Dosarul electronic de sănătate) with the patient's data about diagnosis and treatment. Special duties are attributed to the nurse specialized in the case management, such as:

1. Supporting the patient in the creating the account on www.vot.tuberculoza.md,

2. To explain what means VOT and its principles;

3. To establish the number of the doses, the frequency of the administration, the modality of the video recording and sending for validation, the steps to be followed in different issues (technical problems, lack of electricity, low Internet speed).

4. To receive and to validate the video files and to confirm the administration of the anti-tuberculosis drugs according to the recommended regimens.

5. To complete the treatment register TB01 after the VOT video files validation.

6. To explain and ensure that the patient could recognize

the clinical signs of the adverse drug reactions and declare them.

However, the main barriers in the anti-tuberculosis treatment delivery are social, economic, educational and psychological issues [7, 9, 13, 14, 15]. According to the estimations the Republic of Moldova (RM) remains a high risk zone showing an inadequate concern regarding social determinants, that represent the risk factors for achieving high treatment outcome. Tuberculosis is concentrated in areas with high density of the population, poor environmental and sanitation conditions: poverty, food insecurity, low living conditions. The most affected groups, being assessed as hard-to-reach groups, are homeless, migrants, individuals living with HIV, drug injected users, alcohol abusers. Accumulated evidence suggested that not only the deficiencies in performing an effective antituberculosis treatment is a problem for the public health care system, but also the lack of intervention to resolve social and economic problems of tuberculosis patients. All factors that diminish the treatment success rate could be assessed as excluding criteria from the VOT. In this paper we evaluated tuberculosis patients diagnosed in Chisinau according to the social, demographic and economic characteristics for identifying target groups for VOT. So, the aim of the study was to assess the including and excluding criteria from VOT in a cohort group of tuberculosis patients from Chisinau city. The objectives were: 1. Assessment of the socioeconomic and epidemiological risk factors of patients with tuberculosis distributed in including and excluding for VOT criteria. 2. Evaluation of the case management, diagnosis, radiological patterns and microbiological characteristics of tuberculosis patients distributed in including and excluding for VOT criteria.

Material and methods

It was performed a retrospective selective, descriptive study targeting social, demographic, economic and epidemiological peculiarities, case-management, diagnosis, radiological aspects and microbiological characteristics of 693 patients registered with tuberculosis in Chisinau in 2016. The electronic system for monitoring and follow-up of tuberculosis cases (SIME TB) was used for the selection. Data were extracted from the statistic templates F089/1-e "Declaration about the patient's established diagnosis of new case/relapse of active tuberculosis and restart of the treatment and its outcomes" and F090/e "Declaration and follow up of multidrug-resistant tuberculosis". The inclusion criteria were: age more than 18 years old, tuberculosis diagnosed by the specialist and signed informed consent. All patients with tuberculosis were investigated and treated according to the National Clinical Protocol 123 "Tuberculosis in Adults" [8]. Statistic analysis was carried out using the quantitative and qualitative research methods [16].

Results and discussion

According to the data obtained from the monitoring and follow-up of the cases during the period of 2016, were registered 693 tuberculosis cases among all residents of Chisinau, which included 581 (84%) patients from the urban sectors and 112 (16%) from rural communes. So, the VOT could be implemented mainly in patients from urban sectors where broadband Internet and electronic devices connected to specialize in VOT platform are more available than in rural regions. While distributing selected patients according to the sex, it was established the statistical predominance of men 474 (68%) compared with women 219 (31%), with a male/female rate 2.1/1 (fig 1).

Repartition of patients into age groups, according to the WHO recommendation identified that the largest subgroups were between 25 and 34 years old, and also between 35 and 44 years old, respectively 173 (25%) and 162 (23%) patients. Less numerous were patients from the subgroups 45-54 years old – 116 (17%), 55-64 years old – 100 (14%), 18-24 years old – 78 (11%) and older than 65 years – 64 (9%) patients. The total number of young patients who were between 18 and 44 years old constituted 413 (60%), which showed that VOT should target young patients (fig. 2).
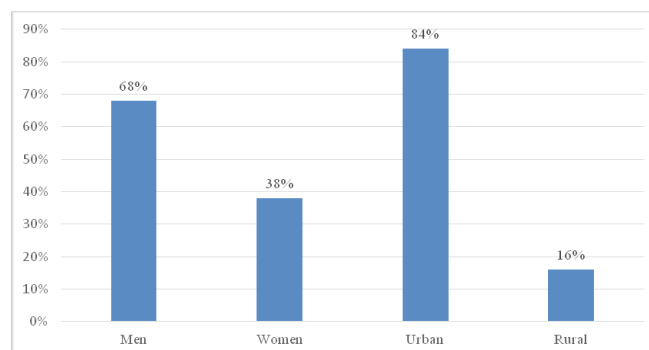
Fig. 1. Distribution of patients by sex and demographic residence (%).
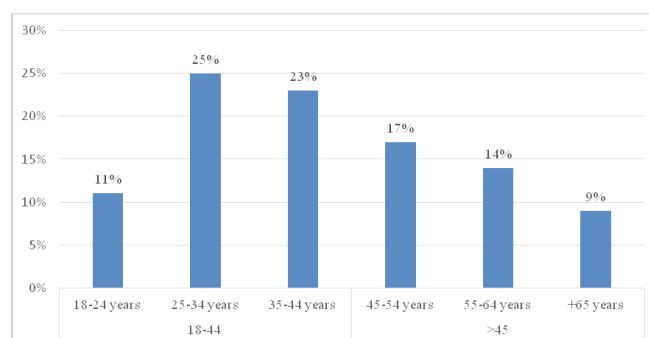
Fig. 2. Distribution of patients by age (%).

When distributing patients, according to the economic status, it was established that the were 158 (23%) employed persons, contributing to the health budget by paying taxes. So, according to the economic segregation of the patients, the financial capacity for supporting the VOT by acquiring electronic devices, such as personal computer, notebook, smartphone connected to a broadband Internet could have only one fourth. 82 (12%) patients were retired. Older than 65 years were 83 (12%) patients, being eligible for VOT, however, they are less likely to use electronic devices connected to a broadband Internet. 61 (9%) patients were

disabled, which have a high risk to be excluded due to the incapacity to take the pills independently. Unemployed patients made up the majority of the group – 377 (54%) cases, which can also be excluded due to the economical incapacity. There were 14 (2%) pupils and students. Most of them should be excluded due to the age criteria and the fact that they form infectious clusters made up preponderantly by children (fig. 3).
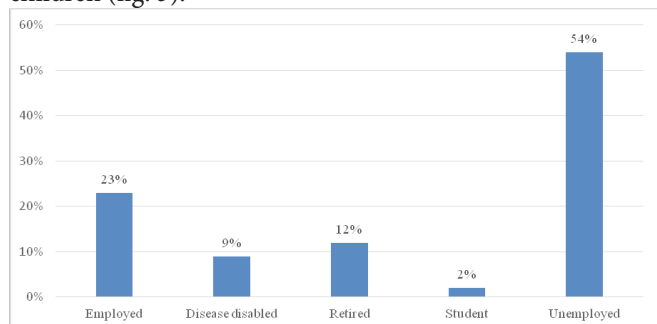
Fig. 3. Distribution of patients in economic subgoups (%).

Assessing the educational level, we established that most of the patients had secondary education – 291 (42%) cases. Technical vocational education had 181 (26%) and bachelor studies – 49 (7%) patients. So, according to the educational level, 521 (75%) could be eligible to perform VOT, considering their intellectual ability to use electronic devices (personal computer, notebook, smartphone). Lack of studies, only primary and incomplete secondary education were established in each fourth patient – 172 (25%) and could not be eligible for VOT (fig. 4).
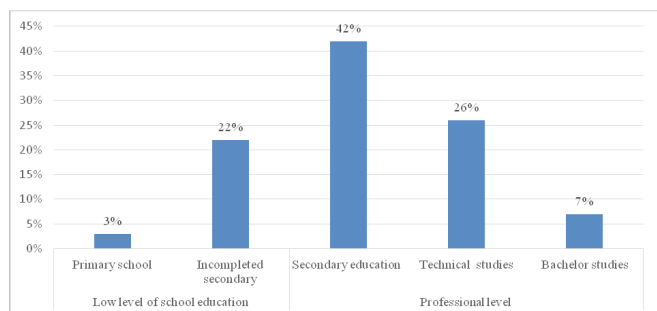
Fig. 4. Distribution according to the last graduate level (%).

The extreme poverty, caused by homelessness or lack of the demographic registration was identified in each fourth patient – 147 (21%). So, certainly every fourth patient will not be eligible for VOT. Migrants were defined persons who left the Republic of Moldova for more than 3 months during the year of the tuberculosis diagnosis. One of excluding criteria for VOT is the situation when the sick person leaves the Republic of Moldova for more than 1 month. The data confirmed that 70 (10%) patients are not eligible for VOT because they could be lost from follow-up due to their absence in the Republic of Moldova. The history of detention during the last year was identified in 38 (5%) cases. This type of patients is not eligible for VOT according to the regulation establishing the conditions for VOT (fig. 5).
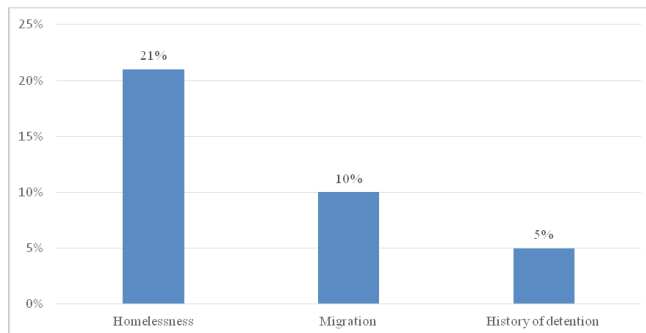
Fig. 5. Excluded from VOT patients.

Close infectious contact with a member of a family who was previously diagnosed with tuberculosis was established in 70 (11%) patients. The ambulatory treatment of the patients from infectious clusters makes the video-assistance a challenge. The VOT of patients with associated diseases raises big issues due to frequent severe adverse drug effects, incapacity to recognize them and to perform independently the treatment. Hospitalization in other departments than those specialized in the treatment of tuberculosis is a criteria which stops VOT and starts the DOT. There were 225 (32%) comorbid patients, which shows that each third case has a high risk to be transferred from VOT to DOT or to be illegible for video-assistance. Among comorbidities predominated HIV-infection – 62 (9%). The co-infection TB-HIV raises the rate of severe and disseminated forms with high risk of death. Those conditions make impossible the treatment in ambulatory conditions and make the patients not eligible for VOT. In a high proportion were diagnosed patients with chronic alcoholism – 59 (8.5%). Drug users were 10 (1.4%) patients. Psychiatric diseases were diagnosed in 12 (5%) patients. Numerous mental disorders were diagnosed in 81 (12%) and constitute certain exclusion criteria from the VOT. Diabetes mellitus was diagnosed in 11 (5%) cases. Due to a high rate of adverse drug effects diabetic patients have a high risk to be excluded from VOT. Immune suppressive conditions such as neoplastic diseases, treatment with corticosteroids and chronic renal failure were diagnosed in 15 (2%) cases (fig. 6). Due to frequent hospitalizations of immune suppressed patients they will be excluded from VOT.
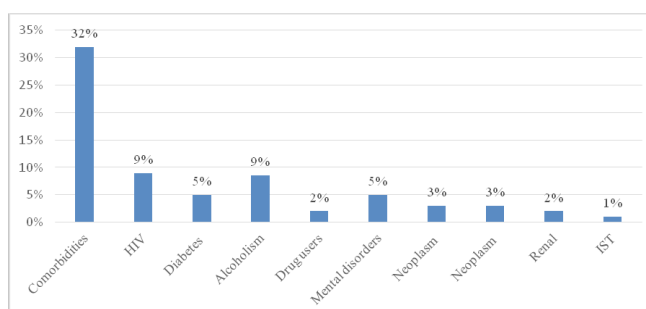
Fig. 6. Distribution according to the risk groups.
Note: IST-immune suppressive treatment.

Studying case-management, it was identified that the general practitioners were involved in the detection of the most of the patients – 299 (43%) and the specialists detected 210 (30%) patients. Screening of the patients from high risk groups performed by the general practitioners detected 82 (12%) cases and through the investigation of the symptomatic cases were detected 217 (31%) cases. Pulmonologists detected 167 (24%) symptomatic patients and 43 (6%) from high risk groups. 43 patients (6%) came directly for hospitalization into a specialized institution and were hospitalized due to the personal requirement. Most of those patients were not admitted for the ambulatory treatment and could not be eligible for VOT.

While distributing patients, according to the registered case type, it was identified that the new cases, never treated cases, predominated – 425 (61%) compared with the relapses – 165 (24%) cases. New cases and relapses are eligible for VOT and their number constituted 590 (85%) with other excluding criteria will not be identified. Patients recovered after a previous "loss to follow-up" made up 69 (10%) and treatment failure – 31 (5%). The total number of the patients previously treated and not allowed for VOT due to the therapeutic incompliance was 100 (15%) cases (fig. 8).
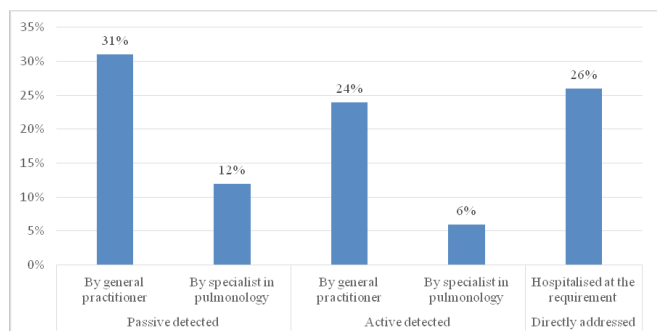
Fig. 7. Distribution according to the medical staff involved in the case detection.
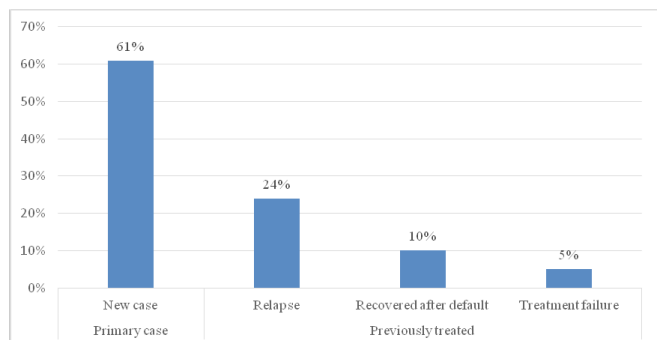
Fig. 8. Distribution according to the case type.

While identifying the clinical, radiological forms of tuberculosis, it was established that pulmonary forms were diagnosed in a higher proportion 656 (94%) cases. Extrapulmonary forms of tuberculosis were diagnosed in 34 (5.4%) patients. Generalised tuberculosis was diagnosed in 3 (0.4%) cases. Severe with extensive destructions pulmonary infiltrative tuberculosis – caseous pneumonia was

diagnosed in 41 (6%) cases. Disseminated tuberculosis and fibro-cavernous tuberculosis were diagnosed in 60 (8%) patients. Severe, disseminated, generalised and chronic forms of tuberculosis can not be treated in ambulatory conditions due to the risk of death and were diagnosed in at least 104 (15%) patients. Extended tuberculosis in both lungs was diagnosed in two thirds of patients – 484 (70%), which can raise difficulties for ambulatory treatment.
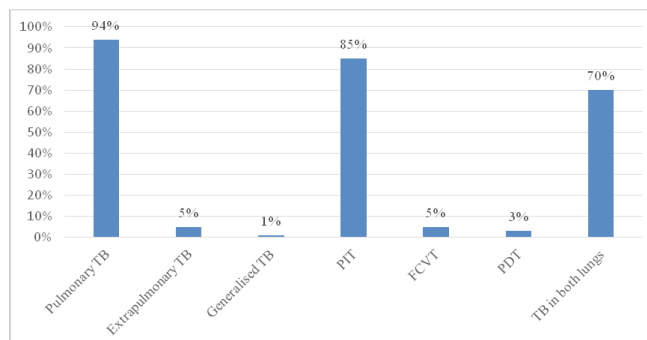
Fig. 9. Distribution according to the clinical radiological forms.

Note: PIT – pulmonary infiltrative tuberculosis, FCVT – fibro-cavernous tuberculosis, PDT – pulmonary disseminated tuberculosis.

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, 200 (29%) patients. Microscopic positive patients are non-eligible for ambulatory treatment due to epidemiological threat, which they expose on the family and social community. A lower proportion of patients were identified to have positive bacteriological results at cultivation on solid Lowenstein-Jensen ether liquid MGIT BACTEC media: 144 (21%) patients. The molecular genetic assay was performed in all cases, but positive results were obtained in 278 (40%) cases, including rifampicine sensitive were 179 (26%) and resistant 99 (14%) cases. Microscopically positive for AFB and cultivation on the conventional media established Mycobacterium tuberculosis (MTB) in 104 (15%) being assessed as non-eligible for the ambulatory treatment. Patients with MDR-TB should be treated compulsory during the intensive phase, for 6 months, in the hospital due to the therapeutic regimen, which includes injectable drugs. So, 116 (17%) of patients were not allowed for VOT for the treatment in ambulatory conditions and VOT during the intensive phase (fig. 10).

The standard treatment for the 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: 2 months with H, R, E, Z, S and 1 month with H, R, E, Z and 5 months with H, R and E. Patients with rifampicin-resistant or MDR-TB were treated with second-line drugs for 18 months or more divided in two phases The regimen com-
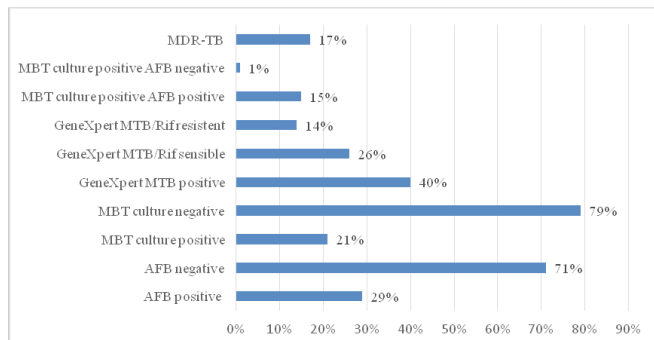
Fig. 10. Distribution according to the microbiological characteristics.

Note: MBT – Mycobacteria tuberculosis, AFB – acid fast bacilli, Rif – rifampicine.

position during the intensive phase lasts 6 months and included 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 of Lfx, PAS, Etho, Cs and Z. The standard treatment for drug susceptible tuberculosis with first-line anti-tuberculosis drugs was used for the treatment of 577 (83%) cases and for MDR-TB with second-line anti-TB drugs were treated 116 (17%), of which 7 (1%) patients with extensive drug resistance (XDR-TB) should be treated in specialized service.

All the patients were managed and treated with the standard treatment for tuberculosis. First-line anti-tuberculosis drugs were used in 577 (83%) patients from urban groups vs. 13 (11.7%) patients from the rural group. Successfully treated were 450 (65%) cases, failed the treatment – 9 (1%), were lost to follow-up –51 (7%) cases and died 81 (12%) patients. 61 (9%) patients were still continuing the treatment and not available data was established in 41 (6%) cases, which are the candidates for lost to follow – up. So, the low therapeutic outcome, which included therapeutic failure, lost to follow-up and patients without available outcome was established in every third case – 182 (26%). Information is exposed in the figure 11.
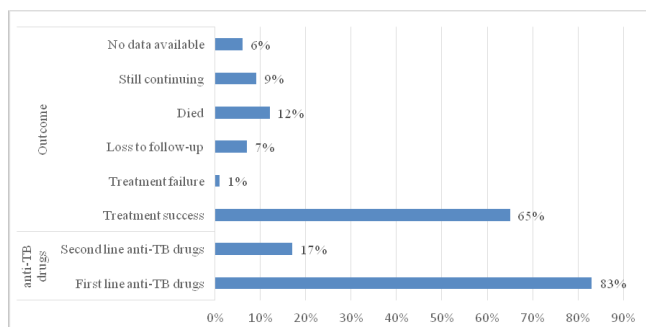
Fig. 11. Treatment outcome of tuberculosis patients.

An important research outcome represents the groups of patients in which the priority interventions for implementation of VOT are most suitable and the groups of patients which the excluding criteria will not allow to start the VOT.

It was established that the risk factors which contribute to the excluding from VOT or the transfer from VOT to DOT were linked with the sociovulnerability: unemployment, low level of the school education, homelessness or lack of the residence visa, harmful habits, migration, present emprisonment or history of imprisonment. Medical biological conditions which contribute to the excluding or lack of eligibility for VOT are: comorbidities, mental disorders and harmful habits with mental impairment. Epidemiological risk factors which arise challenges for the ambulatory treatment were close contact and clusters composed by children. Disease related characteristics which make non-eligible patients for ambulatory treatment are severe, extended, disseminated and chronic evoluated tuberculosis. Every tenth patient could not be allowed for VOT due to the enumerated conditions. One third of the groups were microscopic positive for AFB, which exclude the possibility for the ambulatory treatment and VOT as well. Second-line anti-tuberculosis treatment with injectable drugs in the intensive phase was used for the treatment of 17% of patients which make them non-eligible for the video-assistance. Generally, the treatment outcome did not achieve the 85% of success, as recommended by WHO [1]. The final results were diminihsed by a high proportion of patients, which had a low outcome due to therapeutic incompliance, severe forms of tuberculosis and comorbidities.

The relation between tuberculosis indices and treatment delivery was widely studied [1, 2]. Globally, the epidemic of tuberculosis is much higher in socially vulnerable subpopulations [1, 2]. It can be explained by the complexity of risk factors, which reflects the barriers for accessing the healthcare services and to achieve the treatment completion [3, 4, 5]. In the RM the specialised institutions offer a standard approach, which corresponds to the international recommendation and national regulations [10, 11]. The actual international recommendation imposes the ambulatory treatment of tuberculosis patients and implementation of VOT instead of DOT. Our research established increased rate of socially vulnerable patients (unemployed, homeless, migrants, patients with history of imprisonment) with low degree of school education which can reduce the effectiveness of the VOT implementation. No similar studies assessing the impact of social vulnerability on VOT were identified. Tuberculosis indices are linked with overcrowding, low level of sanitation and infectious clustering, which also endanger the treatment results; however, no studies assessed these conditions. Disease related characteristics, such as extensiveness, severity, duration of the tuberculosis evolution, drug resistance spectrum were not included as conditions with high impact on the treatment outcome in the international papers.

Conclusions

VOT represents a modality for the anti-tuberculosis treatment delivery in high income countries. VOT facilitates the interaction between the healthcare worker and the

patient, however, it does not replace the DOT in tuberculosis treatment.

The including criteria for video-observed treatment (VOT) in the RM are: 1) the patient has an available electronic device 2) the patient is residing in the RM. 3) the patient can administrate independently the anti-tuberculosis treatment.

The informal excluding criteria from VOT were deep social economic vulnerability, associated or not with migration, homelessness, detention and infectious clustering.

Associated diseases, which can reduce the VOT effectiveness are those which reduce the immune resistance (TB-HIV, diabetes mellitus, immune suppressive treatment, neoplastic diseases) and which exclude patients due to psychic impairment (psychiatric disorders, harmful habits such as chronic alcoholism and drug use).

Disease related characteristics, such as extensiveness, severity, duration of the tuberculosis evolution, positive microbiological state and multi-drug resistance are conditions which can exclude the ambulatory treatment and VOT as well.

The low treatment outcome during DOT shows indirectly that VOT will not improve the outcome due to the complexity of patient's risk factors.

VOT can be implemented in the management of tuberculosis patients in actual epidemiological state of the RM, if a complex of patients supporting measures is performed.

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Correlation between body mass index and the results of the treatment of iron deficiency anemia in pregnant women

*Diana Turlacova, MD; **Ianos Coretchi,** MD, PhD, Associate Professor

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

*Corresponding author: diana_turlakova@mail.ru
Manuscript received May 06, 2019; revised manuscript September 10, 2019

Abstract

Background: Iron deficiency anemia (IDA) is a hematologic syndrome characterized by the deregulation of hemoglobin synthesis due to iron deficiency. During pregnancy, there is an increase of about six times of iron. A correlation between body mass index (BMI) and hemoglobin (Hb) in pregnant women with IDA during the treatment with "Sorbifer Durules" (SD) was evaluated.

Material and methods: A retrospective, cohort study. 40 medical cards of pregnant women diagnosed with IDA during the pregnancy and who were treated with SD: one tablet 2 times per day, were studied. The BMI and Hb levels were assessed in each trimester of pregnancy. The dynamic of Hb values was determined: ΔHb_{II-I} – the difference between Hb values of the 2nd and of the 1st trimesters, ΔHb_{III-II} – the difference between Hb values of the 3rd and of the 2nd trimesters. Statistics: Pearson's correlation coefficient.

Results: IDA was diagnosed in 15 out of 40 pregnant women in the 1st trimester of pregnancy (IDA1), in 19 – in the 2nd trimester (IDA2), in 6 – in the 3rd trimester (IDA3). 7 pregnant IDA1 with normal body weight (BMI = 18,5-25 kg/m²) had ΔHb_{II-I}=14,28 g/l, 8 pregnant IDA1 with grade I obesity (BMI=30-35 kg/m²) had ΔHb_{II-I}=26,12 g/l. Pearson correlation coefficient between BMI in the 1st trimester of pregnancy and ΔHb_{II-I} in pregnant IDA1: r=+0,617, p=0,014. The associations between BMI and ΔHb in pregnant IDA2 and IDA3 were negligible.

Conclusions: There is a substantial and significant association between BMI in pregnant women who developed IDA in the 1st trimester and Hb increase during the treatment with SD.

Key words: iron deficiency anemia, pregnancy, body mass index, hemoglobin.

Introduction

There are many diseases nowadays. For example, the International Classification of Diseases (ICD-10) developed by WHO in 1994, lists about 20 000 diseases. There are even more drugs in the world, and their number is increasing every year. It is very difficult for a modern doctor to keep track of innovations on the pharmaceutical market. He must know everything about the medicine: its belonging to a certain pharmacological group, its mechanism of action, take into account the indications and contraindications for its use, possible side effects. The doctor should know the form of release and dosage of this particular medicine.

Paracelsus said, "The dose makes the poison". The dosage is the key factor that determines the drug's effect on the body.

The study of the pharmacokinetic properties of the drugs allows us to determine the optimal route of their administration, which in the future contributes to a rational dosage for its use in medical practice. The information about the pharmacokinetic properties of drugs can clarify the indications and contraindications of their use. So, substances that easily penetrate the hematoplacental barrier should be used with caution during pregnancy. Antimicrobials that are actively excreted by the kidneys or accumulated in the liver are suitable for the treatment of urinary or biliary tract infections, respectively. The pharmacokinetics of drugs creates the basis for a rational search for new drugs with the desired patterns of distribution in the body, with higher activity or a wider spectrum of action [1].

The modification of the pharmacokinetics of drugs occurs due to important physiological changes in the mother's body during pregnancy. It is important to understand the dose-response relationship for optimizing the safe and effective use of drugs, especially in such a vulnerable population as pregnant women. The optimal dosage of drugs during pregnancy should provide maximal therapeutic efficacy, while minimizing the risk of maternal and fetal toxicity [2].

The study of rational pharmacotherapy during pregnancy is significant due to the limited possibilities of clinical research of drugs involving pregnant women, possible fetus complications, side effects of drugs on both the mother and the fetus. The need for drug support for a normal pregnancy is also a relevant issue [3].

According to various sources, 80% of women in Russia, 83% in Brazil, 62% in the USA, take at least one drug during pregnancy [4]. The average number of drugs per 1 pregnant woman is 11 ± 5.3.

The purpose of this study is to provide scientific evidence for the relationship between the pharmacokinetics of drugs and their dosage in pregnant women.

It is important for the practicing physician to know the particularities of dosage of drugs during pregnancy, depend-

ing on the principles of pharmacokinetics. The pharmacokinetics of the drug depends on many factors: genetics, co-existing diseases, physiological changes that the pregnant body undergoes. If these conditions are not taken into account, the risk of incorrect prescription of a drug dose is high. As a result, an insufficient pharmacological effect will occur in case of administering a small dose. Alternatively, in case of a larger dose, there is a danger of the effect of accumulation and an increased risk of side effects of the drug to appear. It is important to find the golden middle when setting the dose.

The features of the pharmacokinetics of drugs during pregnancy

Age, weight, body mass index, gender, race, ethnicity, renal and hepatic functions, genetic polymorphism, concomitant pathology, therapy, smoking, alcohol and nutrition – all these factors can contribute to the variability of the pharmacological response [5]. Pregnancy is also a physiological state of the body in which the pharmacokinetics of drugs changes (fig. 1).
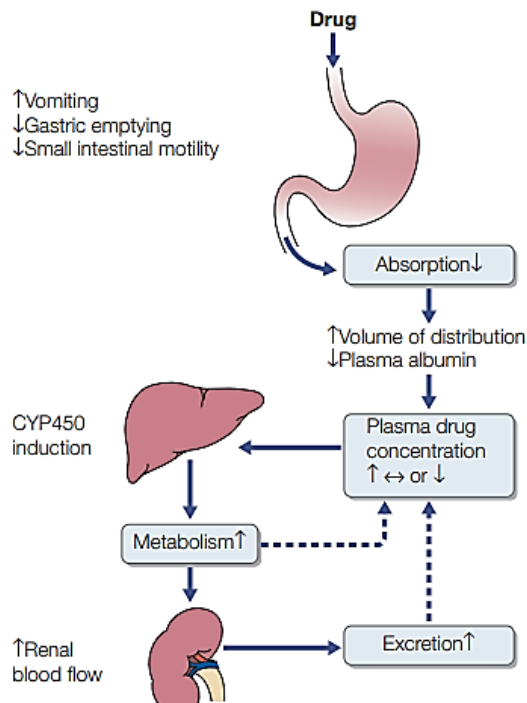
Fig. 1. Pharmacokinetic changes during pregnancy [6].

The absorption of drugs is influenced by many factors, such as: acidity of the stomach, transit time of food, metabolic and transport processes in the intestine. Nausea and vomiting, characteristic for the first trimester of pregnancy, can reduce the amount of drug available for absorption, so it is important to take the drug when nausea is minimal, for example, in the evening [2, 6]. During pregnancy, the production of gastric juice decreases and the secretion of mucus increases, as a result of which gastric pH increases to 5.6, with normal values of 1.5. Such changes can increase the ionization of weak acids (for example, acetylsalicylic acid) and reduce its absorption, but weak bases (for example, caf-

feine) will diffuse better, because they will not be ionized. Slowed intestinal motility and increased cardiac output improve blood circulation in the intestine and increase drug absorption [7] and accelerate its onset of action [6].

The pressure of increased in size uterus on the pelvic veins and the inferior vena cava prevents the outflow of blood from the rectum, which can interfere with absorption during the rectal route of administration. An increase in the body fat during pregnancy is a cause of deregulation of drug absorption in subcutaneous administration. In contrast, airway absorption may increase due to an increase of tidal volume characteristic for pregnancy [2].

The distribution of drugs was also changed. The plasma volume increases by 42%, reaching a total of 3.5 liters, and in parallel there is an increase in the volume of fluid in all parts of the body. Edema, which at least one third of women experience during pregnancy, can add up to 8 liters to the volume of extracellular fluid [6]. An expansion of the extracellular fluid volume will increase the distribution volume for hydrophilic drugs, but will decrease the plasma concentration of the drug. An expansion of the extracellular fluid volume will increase the distribution volume for hydrophilic drugs, but will decrease the plasma concentration of the drug. During pregnancy, the volume of the fat depot increases by about 4 kg, so the distribution volume for lipophilic drugs also increases.

It is known that the amount of plasma proteins changes both during normal pregnancy and in pathological conditions. With a normal pregnancy, albumin concentration decreases on average by about 10% after 20 weeks and by 13% after 32 weeks. The change of albumin's concentration is important in the prescription of drugs such as phenytoin, valproic acid, carbamazepine. Another plasma protein such as α-1-glycoprotein, which is involved in the binding of betamethasone, bupivacaine, lopinavir and lidocaine, is lower by 52% at the end of pregnancy (30–36 weeks of gestation) [8].

A complex biological barrier appears – the placental barrier. Lipophilic compounds pass through it (by diffusion). Ionized polar substances (e.g. Quaternary ammonium salts) cross the placenta poorly. The placenta also has a P-glycoprotein transporter [9]. Glycoprotein P is expressed on the maternal side of the placental membrane of syncytiotrophoblast. It removes xenobiotics and drugs from the circulatory system of the fetus into mother's circulatory system and also prevents the passage of several substrates through the blood-brain barrier to the fetus: calcium channel blockers, statins, macrolides, and some cytostatics [10]. For example, in antiretroviral therapy in a pregnant woman in order to prevent fetal HIV infection, it is extremely important to know that HIV protease inhibitors (for example, saquinavir), being a substrate of glycoprotein P, do not cross the placenta and thus do not protect the newborn [11].

The deposition of drugs during pregnancy in some tissues can lead to side effects. For example, tetracyclines bind to calcium and are deposited in bone tissue, contributing to impaired development of the skeleton of the fetus [12].

The metabolism of drugs by the liver during pregnancy is increased, mainly due to the induction of enzymes, possibly due to an increased level of hormones. Moreover, blood circulation in the liver does not change. This can lead to an increase in the excretion rate of these drugs (eg, theophylline) [6].

The activity of cytochrome P (CYP) isoforms such as CYP3A, CYP2D6, CYP2C9 increases, as a result of this the period of action of the non-metabolized form of the drug decreases and the daily dose of certain drugs should be increased: amlodipine, erythromycin [8]. However, each organism is individual. For example, in clinical practice, in connection with depression, pregnant women often take the antidepressant fluoxetine, which is metabolized by the CYP2D6 isoenzyme, the gene of which has a polymorphism. It was found that "slow CYP2D6 metabolizers" have adverse reactions during treatment with fluoxetine (sedation, cardiotoxicity, arrhythmias, etc.) more often, which is explained by high concentrations of the drug in the blood. Therefore, before prescribing antidepressants to pregnant women, it is necessary to conduct genotyping to identify the carriage of allelic variants of the CYP2D6 gene [11].

However, the activity of CYP1A2 and CYP2C19 decreases, therefore, the daily dose of drugs such as clozapine, theophylline, ondansetron, clopidogrel, omeprazole should be reviewed. It is known that progesterone and pregnanediol, the concentration of which increases during pregnancy, activate sulfation of a number of drugs, and vice versa they block the enzymes of UDP-glucuronyl transferase, which leads to a slowdown in glucuronidation in the second phase of metabolism for a number of drugs (for example, lamotrigine) [11].

Excretion of drugs by the kidney during pregnancy depends on filtration, secretion and reabsorption. During the first trimester, the glomerular filtration rate increases by 50% and continues to grow in the future. Little information about the effect of pregnancy on tubular secretion and drug reabsorption is available. An increase of tubular secretion during pregnancy for digoxin and amoxicillin has been reported. The renal clearance of ampicillin, cefuroxime, ceftazidime, cefradine, cefazolin increases in the second and third trimester, compared to non-pregnant women [2]. In this case, a dose adjustment of the drug is required.

During pregnancy, hepatic blood flow increases, which, in association with decreased binding of drugs to proteins, leads to an increase in clearance and lowered plasma concentrations of drugs [2].

Medicines and the fetus

The problem of evaluating the effect of drugs on the course and outcome of pregnancy is one of the most complex and least studied areas of clinical pharmacology. For most drugs, if they are not intended to treat complications of pregnancy and childbirth, for ethical reasons, special studies of their safety in pregnant women are not carried out. At the same time, most women use drugs of various pharmacological groups (antimicrobial, antianemic, painkillers, anti-

inflammatory, psychotropic, multivitamins, etc.) during the gestational period, however, the benefit / risk ratio of their use during pregnancy has not been established.

The greatest danger poses the teratogenic effects of drugs, which are understood as anatomical malformations, impaired histogenesis with subsequent functional inferiority of the fetal organs and systems. In the early 60s of the twentieth century, more than 1000 children with phocomegaly were born in Europe (congenital absence of upper (proximal) parts of the limbs; in this case, the hands or feet, and sometimes both of them, are connected to the body by means of short stump). That is when the relationship of this developmental malformation with the use of the thalidomide tranquilizer during pregnancy was proven, i.e., the fact of drug teratogenesis was established. Preclinical studies of this drug, performed on several types of rodents, did not reveal its teratogenic properties. In this regard, in the absence of embryotoxic, embryoletal and teratogenic effects of the drug in the experiment still prefer not to recommend its use in humans during pregnancy until confirmation of the complete safety of such a drug after a statistical analysis of the results of controlled clinical trials of its use in pregnant women is performed [13].

Most countries use classifications of risk categories of drugs in pregnancy to indicate the potential risk of drugs to the fetus. The first of them was introduced in Sweden in 1978, and the next was the FDA (Food and Drug Administration) classification (1979), which was most widely used in the world. Based on FDA recommendations, the following categories of drugs are distinguished depending on teratogenicity:

• Category A: drugs in this group are harmless to the fetus throughout the whole pregnancy period (potassium chloride, iron preparations, multivitamins, triiodothyronine);

• Category B: experimental studies did not reveal teratogenic effects, or complications observed in animals were not found in children whose mothers were taking drugs included in this group (insulin, acyl salicylic acid, metronidazole);

• Category C: in animal studies, teratogenic or embryotoxic effects of the drug were detected, control tests were not carried out, or the effect of the drug was not studied (isoniazid, fluoroquinolones, gentamicin, antiparkinsonian drugs, antidepressants);

• Category D: the use of drugs carries a certain risk to the fetus, but the benefits of their use exceed the possible side effects (diazepam, doxycycline, kanamycin, diclofenac);

• Category X: the teratogenic effect of drugs of this group has been proven, their use is contraindicated before and during pregnancy (isotretinoin, carbamazepine, streptomycin) [14].

Material and methods

A retrospective cohort study of 40 cards of pregnant women, which were received at the University Hospital of Primary Care from 2017 to 2018, was conducted.

All women developed iron deficiency anemia (IDA) at a certain stage of pregnancy. IDA was confirmed by a hemoglobin blood test. Blood hemoglobin (Hb) values below 110 g/l. (trimesters I and II), in the trimester II – below 105 g/l and up to 90 g/l indicate IDA I degree, IDA degree II– hemoglobin – 70–89 g/l, IDA degree III – hemoglobin less than 70 g/l, according to WHO [15].

The incidence of pregnant with IDA depends on several factors: age, nationality, socioeconomic status, eating habits, diagnosis criteria [16, 17]. The study examined the following personal data of pregnant women: the age of the pregnant woman, gestational age at the time of registration, gestational age at the time of diagnosis IDA, the number of pregnancy, height, weight (in each trimester), hemoglobin (in each trimester). The study also looked at whether the pregnant woman suffered from co-existing diseases, such as liver, cardiovascular, respiratory, gastrointestinal, endocrine, gynecological, autoimmune diseases. In addition, the intake of medications for IDA was taken into account: the name, the dosage, the frequency of administration, before / after meals, and other medications.

The indicators of the first trimester corresponded to 11-12 weeks of pregnancy, the second – 23-24 weeks, the third - 32-33 weeks, in accordance with the antenatal visits of the pregnant woman to the clinic.

Additional calculations were performed to calculate the body mass index (BMI) of pregnant women in each trimester: BMI = weight (kg) /height (m)^2.

Women were divided into groups according to BMI:

<18.5 kg/m^2 – underweight.
18.5 – 25 kg/m^2 – normal weight.
25 – 30 kg/m^2 – overweight.
30 – 35 kg/m^2 – obesity class I.
35 – 40 kg/m^2 – obesity class II.
> 40 kg/m^2 – obesity class III.

Unfortunately, due to the lack of data, the gestational weight gain was not calculated since the weight prior to the pregnancy was not indicated in the medical cards.

The difference (increase) in hemoglobin between two trimesters was also calculated:

$$\Delta Hb_{m-n} = Hb_m - Hb_n$$

The difference in BMI between trimesters was calculated:

$$\Delta IMT_{m-n} = BMI_m - BMI_n$$

SPSS STATISTICS and MICROSOFT OFFICE EXCEL programs were used for calculations.

Results

The following results were revealed after processing the data.

Pregnant women were grouped into the following age groups (fig. 2).

The pregnancy number among all examined pregnant women is indicated in fig. 3. In the 1st place – women who are pregnant for the second time, in the 2nd place – pregnant women for the first time, in the 3rd place – women expecting a third child.
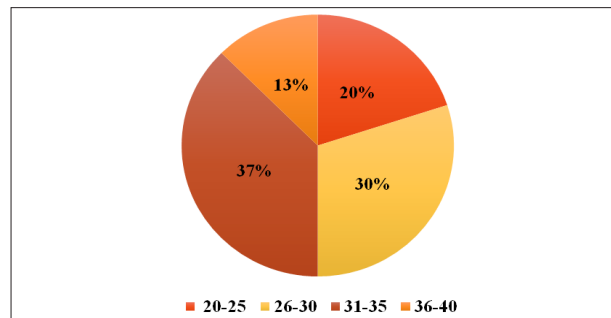
Fig. 2. The age groups of pregnant women.

Most pregnant women are between the ages of 31-35 years, a little fewer are between 26-30 years old, which may be explained by the achievement of a certain financial stability and favorable conditions for the birth of children by this period of life.
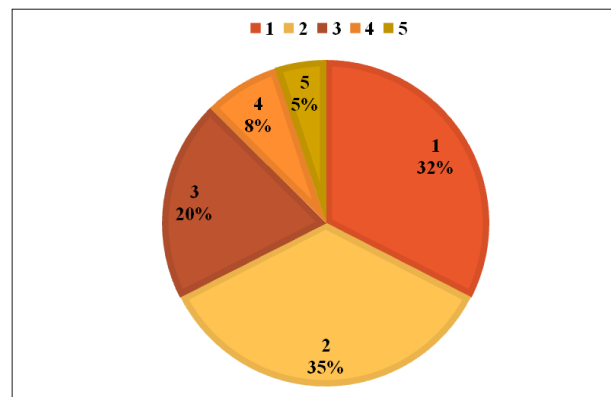
Fig. 3. Pregnancy number.

Two hundred and eighty-six women planning a pregnancy took part in a study conducted in Chisinau in 2010. The results of the study were summarized after childbirth. In conclusion, the author points out the following risk factors for the development of IDA in pregnant women: 1) meat consumption less than 1 time per week, 2) use of iron preparations less than 60 days during pregnancy, 3) low level of education, 4) three and more born children [18]. Note that 1/3 of the pregnant women in our study have already become mothers of many children. Why is IDA so common in pregnant women?

Iron deficiency (ID) is the most common malnutrition in the world [19] and the most common cause of anemia in pregnant women (up to 75%) [20]. According to the WHO, ID can be found in 3.6 billion people, among whom 2 billion people, that is, more than 30% of the world's population, suffer from anemia. The highest need for iron is observed in children in their first years of life (about 1 mg per day), which is associated with high rates of development; in the puberty, especially in girls in connection with the onset of menstruation (about 2 mg / day); in women of childbear-

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ing age with monthly menstrual loss of iron (about 2.5 mg / day), in pregnant women (in the first trimester, 0.8 mg / day, in the second trimester – 4.0-5.0 mg / day, in the third trimester – up to 6.3 mg / day) due to active growth and fetal formation [21]. In general, uncomplicated pregnancy and childbirth are accompanied by a loss of 650 mg of iron [22]. It takes at least 2-3 years to restore the reserves of iron spent during pregnancy, childbirth and lactation [23]. Iron reserves do not have time to replenish with repeated pregnancy, in the presence of additional risk factors. ID in pregnant women is dangerous both for maternal health: decreased performance, general weakness, gestosis, premature detachment of a normally located placenta [24], and for the fetus: the risk of premature birth, the birth of a low birth weight child and even inhibition of the postnatal physical and neuropsychic development of the child are increased [25, 26].

It is important to understand that it is impossible to cure the patient IDA only with products rich in iron, since in them iron is mainly in the trivalent form (Fe^{3+}). But this does not mean that pregnant women should not be recommended to enrich their menu with food containing such an important trace element [27]. It is necessary to pay attention not so much to the amount of iron in the product as to the form in which it is presented. Iron is most effectively absorbed from products of animal origin, in which it is contained in the form of a heme, identical to that which is part of hemoglobin [28, 29]. The heme iron is absorbed by intestinal enterocytes unchanged. Gem is found in beef tongue, liver, rabbit, turkey, chicken, beef, fish [29]. Plant products: beans, pumpkin and sesame seeds, whole grains, thyme, parsley, field salad, contain non-heme iron, which is absorbed much worse, as it is presented in the form of Fe^{3+} and Fe^{2+}. Non-heme iron can be captured by cells of the intestinal mucosa only in the form of Fe^{2+} [28]. The intake of a large number of apples, pomegranates, carrots, beets, buckwheat, recommended earlier in the USSR, is not justified from the point of view of the limited absorption of iron from them [30].

It is impossible to eliminate IDA only by means of a diet, since the absorption of Fe from food is no more than 2.5 mg per day, while it is absorbed 15–20 times more from drugs [30].

Iron deficiency anemia (IDA) is a hematological syndrome characterized by impaired hemoglobin synthesis due to iron deficiency [31, 32] and, as a result, a decrease in the number of circulating red blood cells per unit blood volume is below normal for a given age and gender. IDA is hypochromic microcytic anemia, which is an independent nosological unit [33], but as a rule, IDA is associated with some disease or condition of the body that causes absolute iron deficiency. This gave some scientists reason to believe that IDA is always secondary, there is no idiopathic form of this disease [22].

Further in our study, concomitant diseases of pregnant women were identified (fig.4.). Gynecological pathology is a uterine fibroid, an ovarian cyst; autoimmune – vulgar

psoriasis, autoimmune thyroiditis; endocrine – autoimmune thyroiditis, hyperthyroidism, hypothyroidism, type I diabetes mellitus; diseases of the respiratory system – bronchial asthma; cardiovascular diseases – varicose veins of the lower extremities, arterial hypertension of pregnant women, sinus tachycardia, WPW syndrome, hemorrhoids; liver disease – hepatitis B.
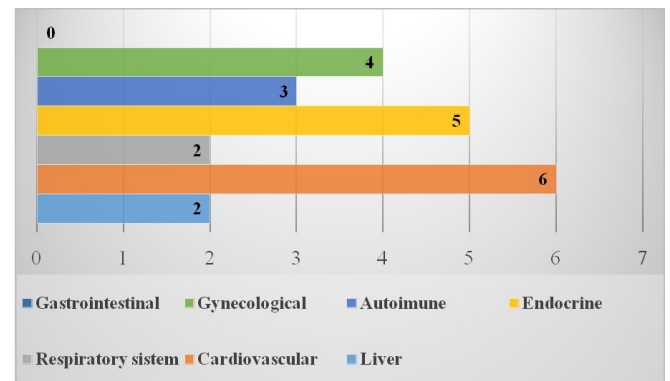
Fig. 4. Concomitant diseases during pregnancy.

As a result of counting, 19 out of 40 women suffered from concomitant pathology, which proves to us a high incidence of women of childbearing age with chronic diseases, which is probably one of the reasons for the development of IDA.

Pregnant women have been gaining weight for 9 months and BMI in each trimester has changed accordingly (fig. 5).
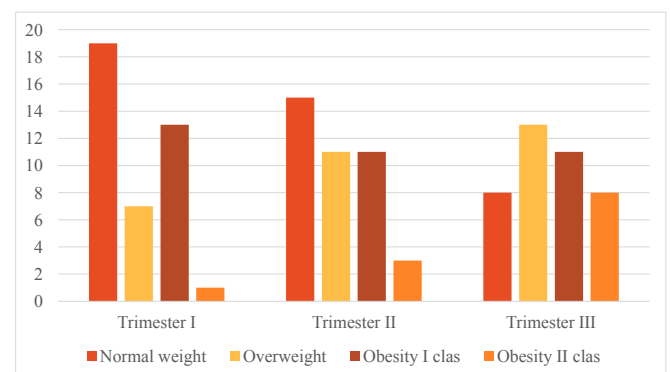
Fig. 5. BMI dynamics in three trimesters of pregnancy.

Thirty-two women out of 40 in the last trimester of pregnancy had a body weight greater than normal, but nevertheless if we compare the body weight parameters of pregnant women with the presence of IDA (fig. 6), we can draw interesting conclusions: during treatment with iron preparations, the best effect was observed in overweight women.

A prospective, cohort study of 100 pregnant women with different BMIs was conducted in Moscow, and doctors concluded that the higher the BMI, the less likely the development of anemia in the third trimester [34].

Iron absorption increases to 30-60% of the total amount in the diet in women with obesity during pregnancy, since the iron depot is exhausted and there is an increase in the

rate of erythropoiesis, as well as a slowdown of intestinal motility, which is characteristic of the second half of pregnancy, which lengthens the absorption period.

It is generally accepted that there are three possible causes that can lead to ID and anemia in obesity: 1) nutritional deficiency of iron, 2) an increase in the volume of circulating blood due to the intensive development of adipose tissue and, as a consequence, an increased need for iron, 3) the development of a chronic systemic inflammatory process in obesity [35]. The most likely cause of IDA in pregnant women is iron deficiency due to increased body need.

WHO (2016) recommends oral supplements with a content of 30-60 mg of elemental iron daily for pregnant women with normal hemoglobin levels to prevent IDA [15]. The equivalent of 60 mg of elemental iron is 300 mg of iron sulfate, 180 mg of iron fumarate or 500 mg of iron gluconate.

Pregnant women with IDA are prescribed 120 mg of iron per day until the hemoglobin reaches 105–110 g / l [36, 37]. After normalizing hemoglobin levels, the pregnant woman continues to take iron as usual (60 mg per day). Weekly supplements taken for at least 12 weeks increase the iron content in the body, as can be judged by the increase in hemoglobin and serum ferritin. The daily use of iron preparations should be continued until the end of pregnancy, as well as the first 6 months of breastfeeding [27].

Iron-containing drugs are recommended to be taken 30-40 minutes before meals, with 100 ml of water or juice. The medicine should not be washed down with tea, coffee, milk or taken with food, as they reduce the absorption of iron [27]. The tannin contained in tea negatively affects the absorption of iron from food [22].

It should be noted that iron medications in the intestinal lumen interact not only with food components, but also with drugs (oxalates, tannins, antacids, tetracyclines, chloramphenicol, penicillins), which complicates the absorption of iron [38, 39].

In the treatment of IDA, the study revealed:

1. Family doctors prescribed 100% of pregnant women an iron-containing drug according to WHO recommendations: Sulfate Fe^{2+} + Vitamin C in a proportion: 320 mg + 60 mg (Sorbifer Durules) 1 tablet 2 times per day; and issued free to insured pregnant women in accordance with the current Order No. 729/230A, issued on June 11, 2018 by the Ministry of Health, Labor and Social Protection of the Republic of Moldova and the National Health Insurance Company [40].

2. The reception of "Sorbifer Durules" was appointed before meals to all women, taken with a glass of water. However, after the appearance of nausea and discomfort in the epigastrium, the reception was postponed for after the meal to 11 pregnant women.

3. At the same time, other multivitamin and polymineral drugs were prescribed, such as Ojestan (folic acid, iodine, omega-3 fatty acids, vitamins E and D3), Prenatal (vitamins A, C, D3, E, B1, B2, B6, B12, B9, PP, iron, zinc, calcium).

Why was Sorbifer Durules preferred?

Firstly, the drug has an inherent delayed release, which is provided by the special Durules technology, when the active substance is contained in a biologically indifferent plastic matrix of a spongy structure. Iron is first released from the surface layer of the system, and then gradually from deeper layers. The empty carrier is destroyed and removed from the body. At the same time, the gastrointestinal mucosa is slightly irritated, due to the lower concentration of iron during its delayed release. The release of the active substance occurs regardless of the pH of the gastrointestinal tract [22].

Secondly, side effects when taking iron medication vary in degrees inherent in almost every drug and are manifested primarily by symptoms of gastrointestinal discomfort. These include a tendency to constipation or diarrhea, a change in the color of feces (black), nausea, heaviness in the epigastric region, and a metallic taste in the mouth [22, 41]. In retard forms of Fe^{2+} and Fe^{3+} medication, side effects are minimal.

Thirdly, in the work of P. A. Vorobyov [42] it is indicated that prices for iron-containing drugs can vary 10-15 times, and therefore patients and doctors are concerned about the ratio of cost and the resulting positive effect of the therapy. As an example, an analysis of several iron-containing drugs is carried out, on the basis of which the author concludes that the preparation "Sulfate Fe + Vitamin C" (Sorbifer Durules) has the lowest cost of ferrous iron, therefore, this medication is the most economically feasible in terms of "cost-efficiency".

The change in the number of pregnant women with IDA by trimester of pregnancy is indicated in fig. 6.
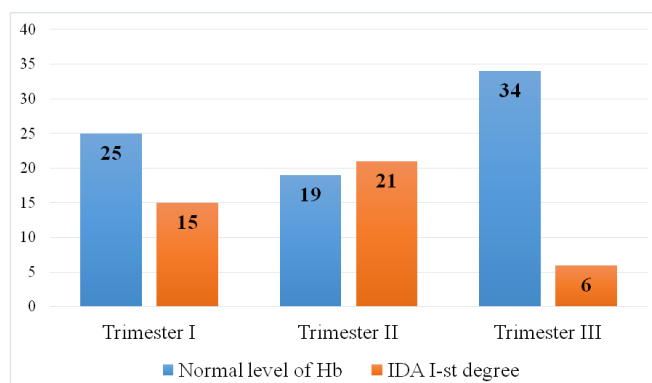
Fig. 6. The dynamics of IDA by trimester of pregnancy.

The largest number of pregnant IDA was observed in the second trimester, it includes women whose drug effect of Sorbifer Durules has not yet increased hemoglobin to normal levels in women with IDA detected in the first trimester, as well as newly discovered cases of IDA. In the second trimester, the stage of organ formation is very active in the growing fetus, as a result of which iron is used more intensively than in the first and hemoglobin decreases in direct proportion to the decrease in the mother's serum iron.

Table 1

Correlation of BMI_1 with ΔHb_{2-1} and ΔHb_{3-}

Para-meter	Me-dium	Standard deviations	Pearson correlation (r)	Bilateral signifi-cance (p)	N
BMI_1	27.58	5.26			15
ΔHb_{2-1}	20.6	9.14	+0.617	0.014	15
ΔHb_{3-2}	3.86	7.53	+0.027	0.925	15

Note: we studied 15 cases of pregnant women who developed IDA in the first trimester and were prescribed treatment with iron-containing drugs.

Table 2

Correlation between ΔBMI_{2-1} and ΔHb_{2-1}

Para-meter	Me-dium	Standard devia-tions	Pearson correlation (r)	Bilateral signifi-cance (p)	N
ΔBMI_{2-1}	1.59	0.66			15
ΔHb_{2-1}	20.6	9.14	-0.59	0.836	15

Table 3

Correlation between ΔBMI_{3-2} and ΔHb_{3-2}

Para-meter	Me-dium	Standard devia-tions	Pearson correla-tion (r)	Bilateral signifi-cance (p)	N
ΔBMI_{3-2}	1.7	0.91			15
ΔHb_{3-2}	3.86	7.53	-0.318	0.247	15

Pearson correlation (r) is a two-dimensional measurement of the bond strength between two variables. If an increase in the values of one variable corresponds to an increase in the values of another variable, then the relationship is called direct (positive +); if an increase in the values of one variable corresponds to a decrease in the values of another variable, then the relationship is inverse (negative -). To assess the strength of the correlation the Cheddock table is used (tab. 4). The lower the probability (p-level value), the higher the statistical significance of the result. The result is considered statistically significant if the p-level does not exceed 0.05. That is, if p <0.05, the correlation is significant, if p> 0.05, then the correlation is negligible.

We conclude that there is a positive (+), noticeable (r = 0.617) statistically significant relationship p = 0.014, p <0.05 between BMI_1 and the increase in hemoglobin from the first to the second trimester of pregnancy (ΔHb_{2-1}) while taking the drug "Sorbifer Durules" (tab. 1).

Further attempts to find a connection between ΔIMT_{2-1} and ΔHb_{2-1}, between ΔIMT_{3-2} and ΔHb_{3-2} were unsuccessful, since the bilateral significance in the first case is r = 0.836, and in the second case r = 0.247 and both of them have p > 0.05, therefore, there is low significance (tab. 2, 3).

Moreover, among 15 women with IDA in the first trimester of pregnancy, in pregnant women with normal weight (n = 7), the increase in hemoglobin over 3 months of treatment with Sorbifer Durules averaged 14.28 g/l, and in pregnant women with class I of obesity (n = 8) – 26.12 g/l, that is, almost 2 times more.

These results are comparable with other sources. Women with normal and low BMI have a longer duration of anemia, even during treatment with Sorbifer Durules, as well as a slower increase in hemoglobin when taking iron supplements, compared with overweight women [43].

Women, especially those with normal and low BMI, can be recommended to increase the dosage of "Sorbifer Durules": 2 tablets – 2 times a day, which may contribute to an increase in Hb levels to the corresponding trimester of pregnancy values.

The absorption of iron medication can be accelerated by the simultaneous administration of succinic, ascorbic, pyruvic, citric acids, as well as fructose, sorbitol, methionine and cysteine. Calcium, vitamins C, B12, gastric acid, pepsin and copper contribute to the absorption of iron, especially if they come from animal sources [44].

Conclusions

1. The physiological changes that develop during pregnancy have a significant effect on the pharmacokinetics of drugs, which is reflected in the need to make appropriate amendments in the dosage regimen.

2. With obesity during pregnancy, iron absorption increases to 30-60% of the total amount in the diet, since the iron depot is exhausted and there is an increase in the rate of erythropoiesis, as well as a slowdown of intestinal motility, which lengthens the absorption period.

3. There is a positive, noticeable, statistically significant relationship between the BMI of pregnant women in whom IDA developed in the first trimester of pregnancy and the growth of hemoglobin during treatment with "Sorbifer Durules".

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Modern methods in treatment of deep caries

***Valentina Nicolaiciuc,** MD, PhD, Associate Professor; **Shiran Yed,** MD

Department of Odontology, Paradontology and Oral Pathology
Nicolae Testemitsanu State University Medicine and Pharmacy, Chisinau, the Republic of Moldova

*Corresponding author: vnicolaiciuc@inbox.ru
Manuscript received May 20, 2019; revised manuscript November 14, 2019

Abstract

Background: Deep carious lesions cause pulpal inflammation, if not managed, they may result in pulp necrosis and involvement of the periradicular tissues, with possible pain requiring, endodontic treatment or extraction.
Material and methods: This study included 35 patients with deep dental caries, 14 males and 21 females with the age range 25-37. The patients are divided into 2 groups. First group – with deep caries treated by capping material "Trioxident" based on mineral trioxide aggregate (MTA), and second group – with deep caries treated using calcium hydroxide.
Results: In our work for the treatment of deep caries, we used the preparations: "Trioxident" based on MTA and "Ultra Blend Plus" based on calcium hydroxide. Applications by material "Ultra Blend Plus" for 3-6 weeks on dentin surface show good results because sterile environment, significant alkaline reaction and calcification of the dentinal tubules. In this regard, it should be noted that the material "Trioxident" does not have porosity in the formed "dentin bridge" and is free from this disadvantage. The use of medical pads with MTA and calcium hydroxide with direct and indirect pulp capping methods did not reveal negative results (complicated caries).
Conclusions: Theoretical data is important for setting correct diagnosis and most suitable protocol of treatment. The described in the article materials – "Trioxident" and "Ultra Blend Plus" provide anti-inflammatory, analgesic and plastic effect. They stimulate the formation of a new secondary dentin layer. The first material – "Trioxident" (based on MTA), in our opinion, is more preferable because it has a number of useful advantages.
Key words: deep caries, treatment, trioxide mineral aggregate, calcium hydroxide.

Introduction

Dental caries is the most common disease. In economically developed countries, its prevalence among the population reaches 95-98%. According to the data of the World Health Organization (WHO), there is a sharp increase in the incidence of caries among the population of developing countries.

Caries is one of the problems of dentistry, important in theoretical and practical terms. A progressive lesion of the hard tissues of the tooth, such as pulpitis and periodontitis, is the cause of severe humidification – periostitis, phlegmon, osteomyelitis, mediostenitis and septic conditions [1]. In order to prevent these complications, it is necessary to try to keep the tooth living. The use of medical pads based on calcium hydroxide and the mineral trioxide aggregate (MTA) can stop the development of the carious process.

Medical pads should have the following properties:
♦ Stimulate the reparative functions of the pulp of the tooth;
♦ Have a bactericidal and anti-inflammatory effect;
♦ Have an analgesic effect;
♦ Do not irritate the pulp of the tooth;
♦ Have good adhesion;
♦ To be plastic;
♦ Have pressure resistance after hardening.

The treatment of acute deep caries has the following features. The acute course of the disease leads to a more rapid spread of the pathological process (the entire thickness of the dentin is affected).

The protective layers of transparent and secondary dentin do not have time to form. The carious cavity is separated from the pulp only with a thin layer of partially or fully demineralized dentin. In the pulp, the first manifestations of its focal inflammation are usually noted. Such clinical features of acute deep caries lead to the fact that during its treatment, in addition to the preparation and filling of carious cavity, additional measures are necessary to prevent pulp inflammation. It is necessary to stimulat pulp plastic aimed at remineralization of softened dentin. All this determines some features of the preparation, sterilization of the cavity and the need for additional use of medications for the treatment of acute deep caries [2].

While preparation of the carious cavity, the softened dentin from the bottom of the cavity must be removed carefully – with light movements with an excavator, and not with bur. In this case, you need to be very careful not to accidentally open the tooth cavity. The supra-pulp dentin layer is thinned and demineralized. Partially demineralized dentin can be left at the bottom of the cavity. Medicaments during treatment remineralize it. Antiseptic treatment of the cavity is carried out in warm solutions (36-37 °C): 0.02% furatsilin solution, microcid, 0.5% ethonium solution, 0.05% chlorhexidine solution, 4% betadine solution. Dry the carious cavity by stream of warm air and sterile cotton balls.

For drug treatment of acute deep caries, you can use a large number of antibacterial therapeutic pastes – suppressing microflora and odontotropic (plastic stimulating) – stimulating the deposition of pulp of secondary dentin.

Today, pastes containing calcium hydroxide are the most effective. This calcium compound creates an alkaline environment due to the high pH value (12.2), which, together with calcium ions, has an anti-inflammatory effect on the pulp and remineralizes the demineralized supra-pulp dentine. The high alkaline reaction of calcium hydroxide neutralizes the acid reaction that occurs with inflammation. These drugs have antibacterial effects. With direct capping of the pulp, calcium hydroxide causes surface coagulation of its proteins and stimulates the formation of a protective barrier from secondary dentin (dentinal bridge). Widespread compositions with calcium hydroxide based on acrylic resins (chemical and light polymerization), glass-ionomer cements, Life (Kerr), Calcimol LC.

Treatment pads are applied to the bottom of the carious cavity with a thin layer of 1-1.5 mm. Do not cover the paste with calcium hydroxide isolation pad of phosphate cement, because it contains phosphoric acid, which neutralizes the alkaline reaction of calcium hydroxide and the paste loses its effectiveness.

Acute deep caries is usually treated in two visits. In the first visit, a medical curative lining is applied and the carious cavity is closed with a temporary filling for 7-14 days. In the second visit, in the absence of patient complaints, a temporary seal is removed. Then carry out inspection of the cavity and electroodontomerty (6-10 µA). If necessary, carry out intraoral targeted radiography. Then, an isolating pad and a permanent filling are applied. If necessary, a temporary filling in the cavity can be saved for several months, i.e. extend the duration of the medical curative paste.

Modern filling materials allow departing from the classical rules for the preparation of the carious cavity in the treatment of caries.

You can carry out a necrotomy of the hard tissues of the tooth with hand tools (excavator, enamel knife). This is an Atraumatic Restorative Treatment (ART) technique [2]. Subsequently, the cavity is treated with an adhesive system and sealed with glass ionomer cement or composite. Modern filling materials release fluorine continuously and intensively, which provides anti-carious effect. In the absence of complaints and signs of inflammation, the seal may be left for a long time. After this time, the temporary seal is replaced with a stronger permanent seal.

The appearance of new filling materials leads to a change in the traditional methods of preparation and treatment of caries [1-5]. Flowable composite materials and compomers: "Filtek Flow (3M)", "Dyract Flow" ("Dentsplay"), "Revolution" ("Kerr") – they can be easily inserted into carious cavities and there is no polarization stress during polymerization. They firmly connect with the hard tissues of the teeth when filling. The organic matrix specific for resin composites ("Dyract Flow") and filler (reactive silicone glass) provides a significant and long-lasting fluoride release, which provides a pronounced anti-carious effect. "Dyract Flow" is characterized by minimal irritating effect on the pulp. These materials can reduce the amount of intact tooth tissue removed. When using them, there is no need to form box-shaped carious cavities [6, 7].

Purpose of the study. To evaluate the success result of deep acute caries treatment using calcium hydroxide and MTA.

Material and methods

This study included 35 patients with deep dental caries, 14 males and 21 females with the age range 25–37.
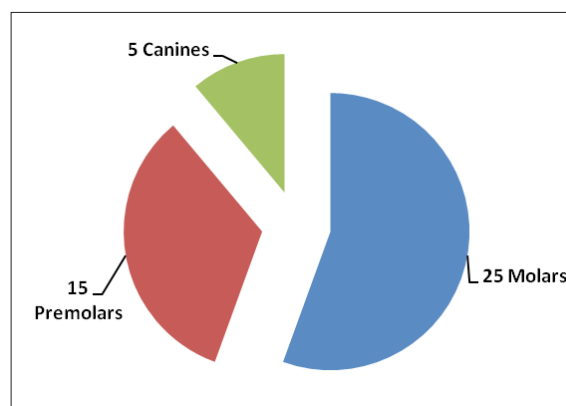
Fig. 1. Total were treated 45 teeth.
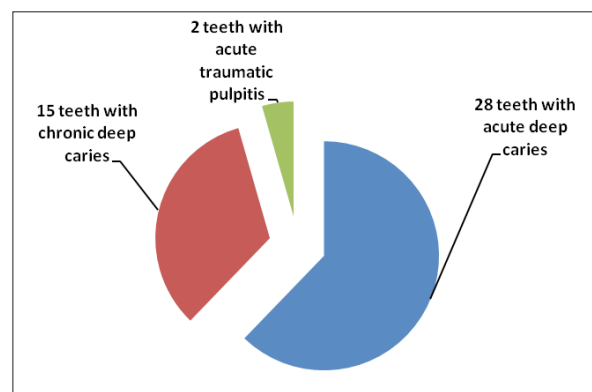
Fig. 2. Teeth distribution by disease.

The patients were divided into 2 groups. First group – with deep caries treated by capping material based on MTA "Trioxident", and second group – with deep caries treated using calcium hydroxide (fig. 1, 2).

Mineral Trioxide Aggregated material "Trioxident"

In 2002, Loma Linda University (USA) developed and currently successfully uses the new Mineral Trioxide Aggregated (MTA) material. This material is truly revolutionary and not replaceable in modern dentistry (fig. 3).

The chemical composition of MTA includes oxides SiO_2, K_2O, Al_2O_3, Na_2O, Fe_2O_3, SO_3, CaO_2, Bi_2O_3, MgO, as well as insoluble precipitate CaO, KSO_4, $NaSO_4$ and crystalline silica. MTA powder consists of small hydrophilic particles; when mixed with water, it first goes into a gel form and then hardens within 10-15 minutes. Final crystallization occurs in a day. Moreover, to complete the treatment procedure, it is not necessary to wait for the complete solidification of MTA [6].

Now MTA is used for retrograde filling, for filling upper apex part of canal with unfinished root formation, for sealing perforations of root canal, for treatment-isolating pulp covering.

Advantages of MTA

1. Water based chemistry, so requires moisture for setting,
2. Excellent biocompatibility,
3. Normal healing response without inflammation,
4. Least toxic of all the filling materials,
5. Reasonably radiopaque,
6. Bacteriostatic in nature,
7. Resistance to marginal leakage.

Disadvantages of MTA

1. Difficult to manipulate,
2. Long setting time (3-4 hours),
3. Costly.

The main indications for the use of MTA are:

➢ Direct pulp capping,
➢ Closure of perforations in the bifurcation area,
➢ Closure of lateral root perforation,
➢ Closure of root resorption,
➢ Pulpotomy,
➢ Apexification in the teeth with an unformed root apex,
➢ Retrograde filling after resection of the apex of the root.

A wide range of applications of the material makes it possible to save even "hopeless" teeth. The material is biologically fully compatible with natural tissues, hardens in a humid environment, providing reliable sealing.

The material has excellent sealing properties, has pronounced antibacterial properties, prevents the migration of microorganisms, stimulates the healing process and osteosynthesis. The material has excellent edge adaptation. Immediately after mixing, MTA has a pronounced alkaline reaction (pH = 12). In terms of X-ray contrast, MTA is comparable to gutta-percha – higher than that of dentin and bone tissue, which makes it possible to distinguish it well in X-rays. The original color of MTA is light gray-brown. Over time, it was proposed for aesthetic reasons to produce white powder for the frontal group of teeth.

Method of application

On a sterile glass for mixing, one measured spoon of powder or the contents of one sachet of MTA and one drop of distilled water are applied.

1. Using a spatula, the powder and distilled water are mixed for 30 seconds to achieve a homogeneous consistency similar to wet sand.
2. Using a suitable sterile instrument or gutta-percha pin, the paste with MTA is applied to the desired area and condenses it.
3. Excess water that appears on the surface is removed using a cotton ball or paper pin.
4. Ultrasound can be used to get the best result.

Based on MTA was created material "Trioxident". Main components of "Trioxident" material are calcium, silicon and aluminum oxides. Standard powder to distilled water ratio is 3:1. Obtained dough keeps its plasticity for 10-15 minutes at temperature 18-23ºC and 50±10% humidity due to plasticizer introduced into powder.

The material begins to harden during 4 hours. Total hardening time in root canal is 24 hours.

The material comprises copper-calcium hydroxide, i.e. active bacteriostatic additive.

Material hardening process comprises 3 stages:

1. First, calcium oxide contacts with water. As a result, calcium hydroxide is obtained providing high pH level (12, 8).
2. Then calcium exudes from solution in amorphous state covering particles of radiopaque filler (bismuth oxide) and uniting all the components into bonded mass. Calcium hydroxide particles compress.
3. Then calcium silicate obtained increasing mechanical strength of the cement.

Calcium hydroxide prevents resorption of supra pulpar dentine bone tissue and stimulates dentinal bridge formation in case of pulp covering.

"Trioxident" material features good bactericidal effect, high biocompatibility, low solubility, high mechanical strength. The material possesses high biocompatability, low solubility and high mechanical durability, and also provides impermeability of bacteria.

By means of set of instruments with various form cannulas and plastic nozzles (applicators) it is possible to be dosed easily without special efforts [8, 9].

Fig. 3. Material "Trioxident" based on MTA.

Calcium-containing "Ultra Blend Plus" material

"Ultra-Blend Plus" is a unique photopolymer pad containing calcium hydroxide and calcium hydroxyapatite based on urethane dimethacrylate. Ultra-Blend plus has a high filler load to minimize shrinkage and a higher viscosity to facilitate ease of placement. It can be easily contoured with a high- or low-speed hand piece and is available in dentin and opaque white shades (fig. 4).

Compared to other liners, "Ultra-Blend plus" liner is highly filled for minimal shrinkage and superior calcium release. It has been proven the best light-cured material for pulp capping.

The material prevents pulp from toxic effect of permanent filling. The material features high stability and low solubility, thermoisolating effect and chemical affinity with polymeric materials providing high adhesion with composites, unlimited manipulation time and short hardening time. The controlled short time of the material hardening at non-limited working time creates additional conveniences in its usage.

Material advantages:
- Bioactive liner and pulp-capping material,
- Superior calcium release,
- Controlled, precise syringe delivery,
- No mixing necessary,
- Will not dissolve over time,
- Light cured when clinician is ready,
- Radiopaque,
- Highly filled.

Fig. 4. Calcium-containing material "Ultra Blend Plus".

Treatment methods in acute deep caries

Indirect pulp capping is a complex therapeutic method that provides disinfection of the wound surface, closure of the dentinal tubules, protection of pulp from physical and chemical agents and prevention of inflammation with the use of anti-inflammatory drugs and stimulation mechanisms of neo-dentinogenesis [10].

Indications for indirect capping method
1. Acute deep caries.
2. Absence of severe concomitant chronic or acute illness before or during the treatment.
3. No changes on X-rays in the apex of the root.
4. Lack of allergic reactions to drugs used.
5. The tooth is not subject to prosthetics.
6. Electro excitability of pulp should be 2-10 mkA.
7. Supra pulpal dentin in consistency and color should be similar to normal (unaffected), dentin.

Contraindications
1. Reduction of pulp electro excitability more than 25-35 mkA.
2. Radiographic changes in the periapical area of the tooth.
3. The tooth is subject to prosthetics.
4. Allergic reactions to drugs.
5. Acute diffuse pulpits.
6. Different forms of chronic pulpits.

Treatment is carried out in one or two visits

Indirect capping method in one visit
1. Antiseptic preparation of oral cavity and washing the carious cavity with a stream of warm water.
2. Anesthesia.
3. The tooth is isolated by cofferdam or by sterile cotton rolls.
4. Mechanical manipulations of the cavity with the principles and stages of preparation. Carious cavity is prepared with sterile sharp spherical burs.
5. Medicamental preparation of carious cavity. Cleaning of caries cavity must be done with not irritant antiseptic

solutions, low concentration [11, 12]. We recommend the following drug:
   1. 0.1-10% solution of Dimexidum.
   2. 0.06-0.3% solution of Chlorhexidine.
   3. 1% solution of Iodinol.
   4. 4% solution of Betadine.

6. Degrease and drying of the cavity is carried out with sterile cotton rolls and a jet of warm air. Alcohol and ether are not applied because they are irritant.

7. Using of curative pads (layer): "Trioxident" or "Ultra Blend Plus".

8. Permanent filling of carious cavity with glass-ionomer cements or light cure materials.

Indirect capping method in two visits

The first visit
1. Antiseptic preparation of oral cavity and washing the carious cavity with a streem of warm water.
2. Anesthesia;
3. The tooth is isolated by cofferdam or by sterile cotton rolls.
4. The surface of the affected and the two adjacent teeth is treated with 2% iodine solution, 1% chlorhexidine or other antiseptics.
5. We must prepare thoroughly the caries cavity. This operation should be carried out in a professional manner, with a clear representation of topographic relation – "carious cavity – the cavity of a tooth" [13, 14].
6. Carious cavity must be disclosed maximally to remove all infected tissue. Special attention during the preparation should be paid to the state of supra-pulpal dentin at the bottom of the cavity, success of treatment often depends on this. Softened carious dentin is removed carefully with a sharp bur [15, 16].
7. Medicamentous preparation of dentinal wound of caries cavity. Cleaning of caries cavity must be done with not irritant antiseptic solutions of low concentration. We recommend the following drug:
- 0.1-10% solution of Dimexidum.
- 0.06-0,3% solution of Chlorhexidine.
- 1% solution of Iodinol.
- 4% solution of Betadine.
8. Degrease and drying of dentinal wound is carried out with sterile cotton rolls and a jet of warm air. Alcohol and ether are not applied because they are irritant;
9. Then apply curative base (layer): "Trioxident" or "Ultra Blend Plus". Curative base is applied with a thin layer (0.5 mm) at the bottom of dentinal wound [17].
10. Temporary filling for 1-2 weeks: Cavidur, Zinc-Eugenat, Dentin paste.

The second visit
1. In the absence of patient's complaints and if electro excitability of the pulp is 6-10 mkA, there is an indication for the second visit.
2. The tooth is isolated by cofferdam or by sterile cotton rolls.

3. Antiseptic treatment of the operating field by the 3% solutions hydrogen peroxide and 4% solution of Betadine.

4. Remove the temporal filling from the caries cavity.

5. Impose a paste based on calcium hydroxide or MTA. Its alkaline reaction stimulates the production of secondary dentin. Cover indirect – with "Trioxident" or "Ultra Blend Plus".

6. Place a permanent filling, using glass-ionomer cements or light cure materials.

Direct pulp capping. Removal of altered dentin from deeper cavities can lead to accidental opening of the pulp chamber and imposes direct pulp capping. We used this method in two cases on young teeth with accidental opening of the pulp chamber.

Indications

1. In accidental opening on healthy pulp teeth.

2. At young people with good capacity of pulp reaction.

3. At healthy patients with good immunological reaction.

4. On teeth with good hygiene.

Contraindications

1. The size of pulp chamber opening orifice bigger than 1 mm^2.

2. When the time of pulp tissue exposure is longer than 2-3 hours.

3. Teeth selected for prosthetic therapy.

Vital pulp therapy is indicated in some cases. By placing MTA over the exposed area often allows healing and preservation of vital pulp without further treatment. Rinse the cavity with sodium hypochlorite to disinfect the area. Mix the MTA with enough sterile water to give it a putty consistency. Apply it over the exposed pulp and remove the excess. Blot the area dry with a cotton pellet and restore the cavity with an amalgam or composite filling material.

Results and discussion

In our work for the treatment of deep caries, we used the preparations: "Trioxident" based on MTA and "Ultra Blend Plus" based on calcium hydroxide (Ca(OH)$_2$). The results of our studies allowed us to compile a table of comparative characteristics of both drugs (tab. 1).

Applications by material "Ultra Blend Plus" based on Ca (OH) 2 for 3-6 weeks on dentin surface show good results because of sterile environment, significant alkaline reaction and calcification of the dentinal tubules. The access of bacteria and products of their life activity along the dentinal tubules to the pulp is completely stopped, which prevents its subsequent infection. It should be noted that in the works of E. Joffe data appeared on the inferiority of the structure of the resulting "dentin bridge" when using drugs based on calcium hidroxide. This is due to the porosity of substitution dentin. Bacteria can enter the pulp through them and cause inflammation [18].

In this regard, it should be noted that the material "Trioxident" (based on MTA) does not have porosity in the formed "dentin bridge" and is free from this disadvantage.

Table 1

Comparative characteristics Ultra Blend Plus and Trioxident

Property	"Ultra Blend Plus" based on (Ca(OH)$_2$)	"Trioxident" based on MTA
Hard tissue formation	Not much	Root and induction
Calcific bridge	Not continuous slow	Continuous with dentin fast
Biocompatibility	Low	High
Degree of inflammation	High	Low
Sets	Not hard	Hard
pH	High	High
Solubility	Partially dissolve	Less soluble
Permeability	Permeable to fluids	Non permeable
Resorbtion	Rate varies with density	Non resorbable

The use of medical pads with MTA and calcium hydroxide with direct and indirect pulp capping methods did not reveal negative results (complicated caries).

We give one clinical case as an example.

Clinical case

Basic information. Patient X, age 35, female.

Subjective examination

➢ **Complaint.** Pain that was triggered by hot\cold stimuli that disappeared immediately after their removal.

➢ **History of the disease (Anamnesis Morbi).** The disease started about two years ago, but the patient didn't visit the clinic.

➢ **History of the patient's life (Anamnesis Vita).** Absence of hereditary, congenital and systemic diseases. The patient smokes up to 10 cigarettes per day. Alcohol and narcotics consumption was denied. No allergic history.

Objective examination of the patient

Extra oral:

• Inspection: The high part of the lower 1/3 of the face is stable, no asymmetry of the face. Normal level of mouth opening. No deviation during mandibular movements.

• Palpation: Trigger points are painless. Regional lymph nodes didn't reveal any pathology.

Intra oral:

Dental formula:

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38
					R	C		AB	AB		T.P		A	A	A	A

• **Inspection:** Labial mucosa, gums, cheeks, hard and soft palate have pale pink color without pathological changes. Tongue has normal size without deposits. Pathological abrasion of teeth No 31-41. Tooth No 43 nas deep wide caries cavity with dark dentin.

• **Probing:** The bottom of the cavity is painful, without pulp communication.

• **Thermal test:** Positive, when water and air was used, the patient felt pain that disappeared after removal of the stimuli.

• **Percaution:** Horizontal and vertical are painless.

• **EOD:** 10 MkA.

Primary diagnosis: Deep dental caries in tooth No 43.

Radiographic examination

Intense dark line which indicates a caries lesion of hard tissue. Between caries tissue and pulp remains only a small, dense and healthy dentin that can indicate about chronic deep caries of tooth No 43. No pulpal communication. Periapical pathological changes are absent in the affected tooth (fig. 5).

Differential diagnosis
• Acute deep caries.
• Acute focal pulpitis.
• Medium caries.

Final Diagnosis

Chronic deep caries of tooth No 43

Treatment
▪ Rinsing the mouth with antiseptic "Mouth wash solution" (sodium benzonate, menthol, thymol, coloring and flavor).
▪ Infiltrative anesthesia Septanest sol. with adrenalin 4% – 1.7 ml.
▪ Isolation of the treated tooth from saliva by using "Rubberdam".
▪ Preparation of caries cavity tooth No 43.
▪ Antiseptic cleaning with a Chlorhexidine solution of 0.05%, and drying of the cavity with varm air jet.

Fig. 5. Deep dental caries in tooth No 43.

▪ Application of medical pads on the cavity floor based on MTA "Trioxident" and thin layer of SDR.
▪ Etching with 37% phosphoric acid for 20 sec, washing 20 sec; drying with air jet.
▪ Bonding application "All bond universal" Bisco.
▪ Application of protective base by using SDR.
▪ Restoration of the cavity with photopolymer material "G-aenial" tooth No 43.
▪ Finishing and polishing of the restoration by using burs, polishing discs and polishing paste.

A total of 45 teeth were cured
▪ 28 teeth with acute deep caries.
▪ 15 teeth with chronic deep caries.
▪ 2 teeth with acute traumatic pulpitis.

Out of 28 teeth with acute deep caries 18 teeth were

Fig. 6. Tooth No 43 during caries cavity opening.

Fig. 7. Tooth No 43 after cavity enlargement and necrotomy.

Fig. 8. Tooth cavity after "Trioxident" MTA application.

Fig. 9. Application of etching.

Fig. 10. Tooth No 43 during finishing.

Fig. 11. Tooth No 43 during polishing.

treated using capping material "Trioxident" and 10 teeth were treated using capping material "Ultra Blend Plus". Out of 15 teeth with chronic deep caries 8 teeth were treated using capping material "Trioxident" and 7 teeth – using capping material "Ultra Blend Plus". Two teeth with acute traumatic pulpitis were treated using "Trioxident".
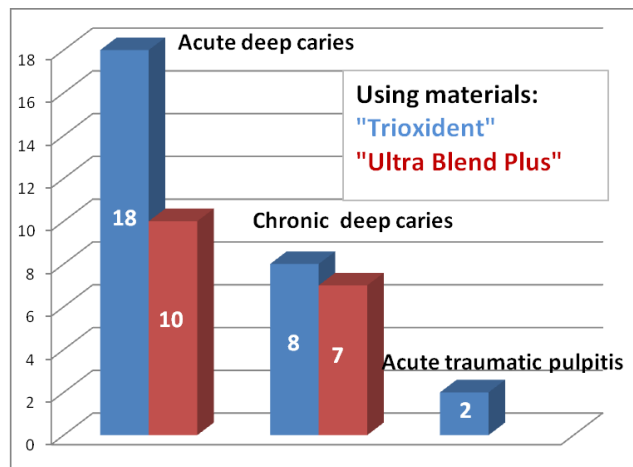
Fig. 12. Distribution of using capping materials.
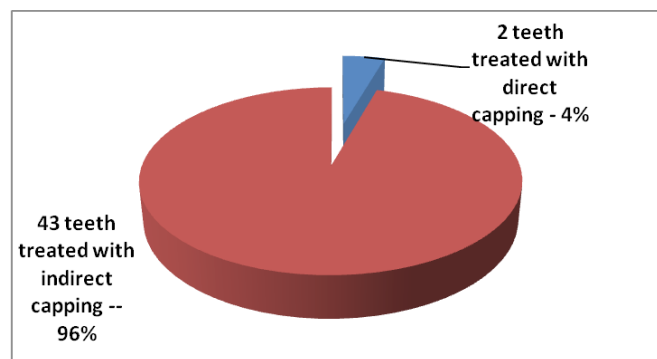
Fig. 13. Number of teeth treated with indirect and direct capping.

Teeth with chronic deep caries were treated with indirect capping in one visit, and with acute deep caries in two visits. Teeth with acute traumatic pulpitis were treated using method of direct capping in three visits.

Conclusions

1. Studying the theoretical data is important in order to give the correct diagnosis and to treat the disease with the most suitable protocol treatment.

2. Therapeutic filling materials containing calcium hydroxide and MTA provide anti-inflammatory. analgesic and plastic stimulating effect on the pulp of the tooth. Overlaying them on carious dentin causes sclerosis of the dentinal tubules and stimulates the formation of secondary dentin, which makes it possible to use them as therapeutic linings in the treatment of deep caries.

3. Testing of "Trioxident" (based on MTA) and "Ultra Blend Plus" (based on Calcium Hydroxide) demonstrated that the former has the following advantages:

➢ Higher success rate in direct pulp capping.

➢ Maintains long-term tooth vitality.

➢ Less toxic and has less pulpal inflammation.

➢ Has more predictable hard tissue barrier formation.

4. In the case of indirect capping both materials, "Trioxident" (MTA) and "Ultra Blend Plus" (Calcium Hydroxide) have positive effects.

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ORIGINAL RESEARCH

The bacterial strains isolated from trophic ulcers and their persistence factors

Greta Balan, MD, MPH, PhD, Associate Professor

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

*Corresponding author: greta.balan@usmf.md
Manuscript received October 07, 2018; revised manuscript November 29, 2019

Abstract

Background: Recently, a particular attention has been drawn to the study of the microbial persistence properties and their correlation with the rate of elimination from the source of infection, as well as the prognosis of the disease progression.

Material and methods: There were examined 44 samples taken from patients with trophic ulcers. The bacteriological examination, as well as tests on determining both the persistence factors and the antibiotic susceptibility of the isolated strains were carried out according to the current method.

Results: There were determined 80 isolated bacterial strains. Two and more strains were isolated in over half of these cases (52.3%). The most commonly involved strains were the genus *Staphylococcus*, followed by *Enterobacter spp.*, *Pseudomonas spp., Candida spp.,* and enterococci. Both gram-negative and gram-positive species exhibited a high-level antimicrobial resistance. The study of the persistence factors revealed that the strains isolated in mixed culture showed a higher rate of virulence (1.0-1.5 times higher) compared to isolates in pure culture.

Conclusions: The main bacterial strains isolated from trophic ulcers are the genus *Staphylococcus* and the *Enterobacteriaceae* family. Isolated strains showed higher level of antimicrobial resistance and multiple persistence factors. The study results proved that treatment of trophic ulcers is still a major problem, requiring rational monitoring and management strategies.

Key words: trophic ulcers, microbial spectrum, antibiotic resistance, persistence factors.

Introduction

In recent years there has been a qualitative change of some microbial strains involved in the infectious disease pathology, which tend to increase the incidence of mixed infections caused by potentially virulent gram-negative and gram-positive bacteria and characterized by a marked clinical polymorphism due to a simultaneous exposure of several etiological agents, each of which exhibiting a range of pathogenicity factors [1].

Microorganisms, involved in mixed infections, commonly present antibiotic resistance and a number of pathogenicity factors, such as lecithinase, haemolytic, antilizozyme, DNA-staining, and adherent activity, etc. [2].

Long-term persistence of bacteria within the host organism is due to multiple factors that inactivate the antimicrobial mechanisms of the immune system. Thus, it is highly recommended to study the persistence properties of the microorganisms in purulent infections, since these are responsible for the elimination rate from the site of inflammation, as well as for the prognosis of the disease. It is well known that the bacterial persistent potential is dependent upon the length of pathogenic harboring within the macro-organism, whereas its suppression via drugs may weaken this infectious potential [3-7].

The studies, conducted across different countries, have revealed a range of species isolated from trophic ulcers and their antimicrobial resistance, as well as the incidence of multidrug resistance (MDR) cases, strains of methicillin-resistant *Staphylococcus* spp. (MRS) and extended-spectrum beta-lactamases (ESBL), thus, suggesting that administra-

tion of empirical antimicrobial therapy might increase the rate of a treatment failure [8-10].

Treatment of trophic ulcer is a challenging task for clinicians and remains a current and relevant issue [11].

As regarding to the aforementioned, the purpose of the study was to determine the spectrum of bacteria isolated from trophic ulcers, to study the antibiotic susceptibility of the bacterial strains and to determine their hemolytic, lecithinase, anti-lysozyme, and anti-complementary properties, as well as to prove their diagnostic importance in detection of the bacterial targets in order to select the appropriate drug therapy.

Material and methods

Studies were conducted on 80 microbial strains isolated from trophic ulcers. The microbiological investigations, as well as the persistence factors and antibiotic sensitivity assessment of the isolated strains were performed within the microbiological laboratory of the National Agency for Public Health. The bacterial strains were isolated and detected according to the methodology described in "Basic laboratory procedures in clinical bacteriology". Antibiotic susceptibility was tested by Vitek 2 compact and Kirby-Bauer disk diffusion method. Antibiotic susceptibility testing and data interpretation have been standardized, in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for current antibiotic assessment [12]. Antibiotic disk diffusion testing included: cefoxitin (30 mg), erythromycin (15 mg), clindamycin (2 mg), gentamicin (10 mg), cefepime (30 mg), ceftazidime (10 mg) , me-

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ropenem (10 mg), aztreonam (30 mg), ciprofloxacin (5 mg), linezolid (10 mg), tetracycline (30 mg), amikacin (30 mg), chloramphenicol (30 mg), rifampicin (5 mg), ampicillin (10 mg). Strains that showed resistance to three or more antibiotic groups were considered polyresistant [13].

The persistence factors were determined in the most common isolates from trophic ulcers. The lecithinase activity was assayed on the egg yolk salt agar, the hemolytic activity on a blood agar plate and the anti-lysozyme and anti-complementary activity we determined according to the method described by Bukharin O. et al. [14-16].

Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC-25922) and *Pseudomonas aeruginosa* (ATCC-27853) reference strains were used for quality control. Statistical data analysis was carried out via EpiInfo 2000.

Ethical Issues

The strains used in this study were obtained from the routine analysis of clinical specimens. Sample collection did not involve direct contact with the patient, thus no consent was required. The study was conducted and approved by the Ethics Committee no. 65 / 12.04.2017 of Nicolae Testemitanu State University of Medicine and Pharmacy from the Republic of Moldova.

Results

The bacteriological study was conducted on 44 samples collected from patients with trophic ulcers. A single bacterial strain was isolated in 36.3% of cases, two and more species – in 52.3% and no strains – in 11.4% of cases.

A total of 80 bacterial species were isolated and identified. The most common strains, isolated from trophic ulcers, were the *Staphylococcus* (predominantly *S. aureus*), then enterobacteria (*Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., *Serratia* spp., *Escherichia* spp.), non-fermenting bacilli *Pseudomonas spp.,* levuriform fungi of *Candida* type and enterococci. (fig. 1).
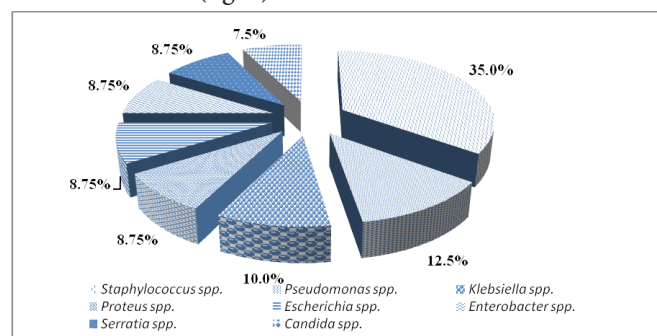
Fig. 1. The etiological spectrum of microorganisms isolated from trophic ulcers.

Among the infections caused by a single strain, the most common was found *Staphylococcus aureus* (43.75%), along with the other isolates such as *Pseudomonas aeruginosa* (18.75%), *Staphylococcus haemolyticus* (12.5%), *Proteus mirabilis* (12.5%), *Klebsiella pneumoniae* and *Enterobacter gergoviae* (6.25%).

S. aureus was determined in 82.6% of mixed infections, whereas 30.4% of cases were associated with *Klebsiella* and

Pseudomonas species. Two-strain associations were recorded in 52.2% of cases, three-strain in 13.1%, 4 and 5 species were found in 13.1% and 4.3% of cases, respectively.

Staphylococcus spp. strains showed a marked sensitivity to vancomycin (96.4%) and only 1 strain showed intermediate resistance to vancomycin, tetracycline (89.3%) and linezolid (82.1%). Of the 28 tested staphylococcus strains, 13 (46.4%) were methicillin-resistant (MRS). MRS strains were more sensitive to vancomycin (100%), tetracycline (84.6%) and linezolid (76.9%), followed by chloramphenicol (79.2%), whereas a reduced sensitivity was recorded to erythromycin (27.5%) and ciprofloxacin (17.3%). Moreover, the obtained data highlighted a number of strains with multiple antibiotic resistance and only 3 (10.7%) of the 28 strains were sensitive to all the tested antibiotics.

Carbapenems were found to be the most effective antibacterial drugs (86.1%) in treatment of enterobacterial infections; however, the bacteria exhibited a marked resistance to aminoglycosides (> 70%), fluorquinolones and cephalosporins (> 80%).

Furthermore, this study detected 15 extended-spectrum beta-lactamase strains (BLSE), which showed susceptibility to meropenem (86.6%), followed by amikacin (60.0%), gentamicin (53.3%), ceftazidime (26.6%) and ciprofloxacin (13.3%).

The bacterial strains of *Pseudomonas* genus presented susceptibility rates to aminoglycosides (100%) and monobactam drugs (90%) and resistance to fluoroquinolones (100%), carbapenems (90%) and cephalosporins (80%).

Levuriform fungi of the genus *Candida* isolated from trophic ulcers made up 7.5%. In the present study, 66.7% of *Candida* spp. were susceptible to fluconazole, 100% to amphotericin B, 83.3% and 50.0% to voriconazole and itraconazole, respectively.

In the next step of our study, we determined the levels of expression for some persistence factors found in the most common bacterial strains, isolated from trophic ulcers.

The study of the persistence factors of bacteria isolated from trophic ulcers showed a higher- level expression in strains isolated from mixed infections (1.0-1.5 times) compared to those isolated in pure culture.

Lecithinase was among the studied persistence factors. This enzyme destroys lecithin and releases the receptors with which microorganisms interact [16]. Of the total 26 *S. aureus* strains, 24 (92.3%) showed lecithinase activity and 2 (7.6%) strains were inactive.

Hemolysin was determined as another persistent factor, leading to development of chronic infectious disease. Hemolytic activity was detected in 38 (47.5%) bacterial strains isolated from trophic ulcers. A higher level of expression was also recorded in strains isolated from the mixed infections, compared to those isolated in pure culture.

Lysozyme is a carbohydrase that selectively breaks down the glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine and is a component of the peptidoglycan cell wall. If the peptidoglycan network is destroyed, the osmotic pressure between the inside and the outside of the cell is no longer balanced and thus is being destroyed [17]. Therefore, an important strategy of the bacteria is to protect themselves against this enzyme, aiming to survive longer

in the host organism. Of 26 strains of *S. aureus*, 14 (53.8%) strains showed antilysozyme activity and 12 (46.2%) were inactive. The antilysozyme activity was assessed by lysozyme titres in the medium, which revealed that out of 14 Staphylococcus aureus strains, 5 (35.7%) strains showed a lysozyme concentration greater than 10 µg/ml, 6 (42.9%) – a concentration of 5-10 µg/ml and 3 (21.4%) – 5 µg/ml.

The antilysozyme activity of *Enterobacteriaceae* strains was also assessed, showing that of 36 strains, 24 (66.7%) strains exhibited an antilysozyme activity, of which 6 (25.0%) in concentration greater than 10 µg/ml, 8 (33.3%) – in concentration from 5-10 µg / ml, 10 (41.7%) – in concentration of 5 µg/ml and 12 (33.3%) strains did not present any activity (p <0.05).

Another important factor that provides persistence for the microorganisms within the infection site is the ability of the bacterial cells to inactivate the complement system of the macroorganism [16] . The study of the anti-complement activity of the strains isolated in pure culture showed that 62.5% of the strains inactivate the complement and 37.5% of the strains did not present anti-complement activity.

Of the 26 Staphylococcus aureus strains, 24 strains (92.3%) exhibited complementary activity, of which 7 (29.2%) strains inactivated the complement at a concentration of 5 CH50/ml, 3 (12.5%) – at concentration from 5 – 15 CH50/ml and 35 (58.3%) in a concentration greater than 15 CH50/ml. Only two strains did not exhibit anti-complement activity (7.7%).

Anticomplementary activity is a common feature among the bacteria of the *Enterobacteriaceae* family. 34 (94.4%) strains of enterobacteria out of 36 isolates from trophic ulcers showed anti-complementary activity. 1 (2.9%) strain inactivated the complement at a concentration of 5 CH50/ml, 6 (17.6%) at a concentration from 5-15 CH50/ml and 27 (79.4%) strains at a concentration greater than 15 CH50/ml.

The data study of the anti-complementary activities in monocultures compared to isolated cultures in associations (co-culture isolates) showed that the latter are often related to medium and high anti-complementary activity (p<0.05).

Conclusions

1. The study of the spectrum of microorganisms isolated from trophic ulcers has revealed the significant role of strains belonging to the genus *Staphylococcus*, followed by enterobacteria, *Pseudomonas* spp., levuriform fungi of the genus *Candida* and streptococci. *Staphylococcus aureus* strain was predominantly isolated in both pure and mixed cultures.

2. Both gram-positive and gram-negative strains isolated from trophic ulcers showed a marked resistance to the antimicrobial drugs tested.

3. The study of the persistence factors confirmed that the strains isolated from trophic ulcers exhibit a range of properties to inactivate the natural resistance factors of the macroorganism.

4. It is essential to understand the pathogenic persistence factors, since it might provide effective targeted therapies for controlling the microbial growth in trophic ulcers.

5. The study results have proved that treatment of trophic ulcers is still a major medical concern, requiring current management strategies.

Conflict of Interest Declaration

There are no known conflicts of interest and financial or non-financial support associated with this publication.

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Survival predictive models in severe trauma patients' transportation within Moldovan medical system

Oleg Arnaut, MD, PhD, Associate Professor

Department of Human Physiology and Biophysics
Valeriu Ghereg Department of Anesthesiology and Intensive Care No 1
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova

Corresponding author: oleg.arnaut@usmf.md
Manuscript received September 09, 2019; revised manuscript December 02, 2019

Abstract

Background: Trauma remains an unresolved medical problem and its severity often requires the transfer of patients to specialized trauma institutions (centers). The elaboration of the predictive models represents an effective tool for improving the prognosis of the transported patients by optimizing the management of the trauma and/or improving the national interhospital transfer system. The survival probability predictive models in severe trauma were proposed in this pilot research.

Material and methods: Data were collected from 39 patients with severe trauma (NISS > 15) transported to the Emergency Medicine Institute (EMI), Chisinau, the Republic of Moldova, from district hospitals. These data were statistically processed using multivariate logistic regression where NISS, MPMoIII, age and biological gender were considered as covariates.

Results: There were developed three predictive models: based on the estimation of anatomical lesions (NISS), based on physiologic parameters estimation and conditions during/immediately after hospital admission (MPMoIII) and their combination (NISS + MPMoIII). The last of these showed significance only after the resampling, the characteristics of the model being superior (the coefficient of determination over 0.8, the sensitivity and the specificity over 80%) compared to the first two taken separately. Age and biological gender were insignificant and were not included in the equations.

Conclusions: Developed models are perspective (especially a combined one) in predicting survival rate of severe trauma patients transported to EMI from district hospitals. At the same time, taking into account the particularities and limitations related to the pilot study, the models can be recommended for use in clinical practice after validation procedure only.

Key words: severe trauma, predictive models, interhospital transportation.

Introduction

Trauma remains an unresolved medical problem. According to data from the literature, traumatic lesions occupy the third place in the overall structure of the lethality and are the first cause of death in the category of patients between the ages of 1 and 44 years [1]. The mentioned trends are also characteristic for the Republic of Moldova. According to the data of the National Management Center of the National Agency of Public Health for the period 2008-2017, traumas are placed on the fourth place, constituting 8.1% (36889 cases) of all the death cases registered after the diseases of the circulatory system (61%, 226195 of cases), tumors (15.8%, 58518 cases) and diseases of the digestive system (10%, 36889 cases). The analysis of the lethality structure by age shows that in the first year of life the traumas are placed second (30.3%) after the diseases of the respiratory system (57.9%). Subsequently, as the age progresses, the rate of deaths caused by trauma increases and reaches maximum values at 18 years (81.3%), after which it is decreasing, predominating until the age of 45 years (27.2%) compared to other causes of death, continuing to decrease to zero at old age [2].

Often, patients with trauma are admitted to a medical institution and subsequently, for different reasons, require transfer to the trauma center, sometimes being in a serious or critical condition during transfer. On the one hand, transporting patients from one institution to another represents an increased risk for complications and even death. On the other hand, the transfer of patients to specialized institutions has benefic effects for patients. But, unfortunately, currently for patients with severe trauma are not unanimously accepted criteria for the need, the right time, and the mode of transport between two medical institutions [3, 4, 5].

One of the criteria to determine the tactics for transferring to a specialized institution is to determine the severity of the injuries and the prognosis of the patient's condition. These are crucial for trauma management. Currently, two approaches need to be considered in order to mark patients at high risk of complications, including death. The first is the use of terms such as "severe trauma", "major trauma" and "polytrauma". The analysis of the number of records / documents in the Web of Science database in 2016 highlighted 24441, 19471 and 2813 records for these notions, respectively. The terms "severe trauma" and "major trauma" are very close, interchangeable, but the criteria are not well established, the critical value of ISS (Injury Severity Score) or NISS (New Injury Severity Score) varies in different studies at the level of 16-17 points [6, 7, 8]. Polytrauma is one of the most complicated and unexplored categories of trauma, being a restricted notion compared to severe trauma or major trauma. According to the Berlin definition, polytrauma is

defined as lesion of at least two regions of the body, assessed by AIS (Abbreviated Injury Scale) with score ≥ 3 and presence of at least one of the 5 physiological parameters (systolic pressure ≤ 90 mmHg, GCS ≤ 8, acidosis, coagulopathy and age ≥70 years [9]. This approach has as a disadvantage – the lack of the possibility of individualizing the management of a patient with traumatic lesions arising from the particularities of their evolution, the circumstances of the trauma, etc. As a result, the most severe patients within each group cannot be identified and there are no indications of the probability of survival/death, of developing complications, which of the parameters/variables are effective in determining the treatment results, which of the examined factors would have the greatest influence, which of the patients requires admission in Intensive Care Unit or how rational it is to benefit from a procedure, etc.

Another approach – the use of traumatic scores (NISS, ISS, MPMoIII, ASCOT, TRISS etc.) as well as the development of predictive models, which represent effective tools for solving the mentioned disadvantages. Thus, the predictive models have a potential for improving the prognosis of the transported patients by optimizing the management and/or by improving the interhospital transfer system in the Republic of Moldova [3].

In the pilot research, three predictive models have been proposed and analyzed for estimating the survival probability of patients with severe trauma, transferred from the district hospitals to the EMI through the AVIASAN service.

Material and methods

Analyzing the observation data of the patients admitted to the EMI for 2012, a retrospective pilot study was performed. The study included 39 severely traumatized patients transferred through the AVIASAN service from district hospitals to EMI by the reanimatologic team. The criteria used for severe trauma was the NISS score greater than 15 points [10].

The research project was approved by the ethics committee of the Nicolae Testemitsanu State University of Medicine and Pharmacy.

The elaboration of the predictive models was carried out by the logistic regression analysis, taking into consideration the recommendations for the multivariate analysis. The minimum number of respondents was estimated by the ratio 1:10 (for each covariate included in the model at least 10 respondents) [11].

In addition to NISS, for the determination of patient status, the MPMoIII (Mortality Probability Admission Model) score was used [12]. The age and gender of the respondents were also taken into account. Specifically, these four skills were considered as effective maintenance variables in the predictive models of survival rate for transferred severe trauma patients. Considering the relatively small number of respondents for the mixed model, the resampling was performed by bootstrapping.

Results

Age of the studied group varied from 20 to 74 years (Median 45, interquartile range 32), from which 30 were males evaluated with NISS 16-66 (Median 48, interquartile range10), MPMoIII variated between 17.1 and 91.2 (Median 73.8, interquartile range19) upon admission to IMSP IMU.

Totally, three models were developed: a model based on anatomical lesions (NISS), another model based on the physiological parameters and some patient parameters during/immediately after admission to the hospital (MPMoIII) and the third, mixed one (NISS + MPMoIII), results being adjusted to the age and gender only in the case of the NISS score. Age and biological gender were insignificant and were not included in the final equations.

Model based on the estimation of anatomical lesions (NISS)

For the NISS-based model, the following hypotheses were formulated: *The null hypothesis* - the covariates included in the model cannot predict the probability of survival in severely traumatized transported patients better than a model that is based only on constant. *Alternative hypothesis* – at least a variable can predict the probability of survival in patients with severe trauma better than a model that is based only on constant.

The model presented the following characteristics. *Omnibus Test of Model Coefficients* ($\chi 2$ (df=1) = 23.05 p<0.001). The test was a significant one, which allowed us to reject the null hypothesis and to analyze further, which of the studied covariates is relevant for predicting survival rate in severe trauma. The coefficient of determination, *Nagelkerke R Square*, was estimated at 0.641 (64.1%), which tells us that the variables included in the model (NISS) determine about 2/3 of the dispersion of the examined variable (probability of occurrence of an event). *The Hosmer-Lemeshow test*, ana-

Fig. 1. The ROC curve of the predictive model for the probability of survival in patients transported with severe trauma. SPSS 22 Output.

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Table 1

Variables in the equation

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
NISS	-.369	.128	8.255	1	.004	.692	.538	.889
Constant	18.983	6.405	8.783	1	.003			

Note: Constant – the value of the equation constant; B – the coefficients B; S.E. – standard errors; Wald – Wald statistic; df – degrees of freedom; Sig. – significance threshold; Exp (B) – values for odds ratio; 95% C.I. for EXP (B) – confidence interval for odds ratio.

lyzing the model in terms of the ability to predict positive and negative results, presents the result as insignificant ($\chi2$ (df = 6) = .332, p = 0.999), which tells us about the increased fidelity of the obtained results. The classification table highlighted a *sensitivity* of 96.4% (27 cases out of 28), the *specificity* being 63.6% (7 out of 11 cases), the average validation appreciated at the level of 87.2%.

The surface under the ROC curve, for the proposed model, was 0.912, with 95% confidence interval (0.819, 1.000) and with a significant difference from the value 0.5 (p <0.001) (fig. 1). Thus, the logistic regression classified the model as significantly better model than the random model.

The model includes the constant (B = 18.983) and the NISS values (B = - 369) (tab. 1). NISS is a predictor for survival of patients with severe trauma, OR (odds ratio) = .692 (95% CI, .538, .889). If the NISS value increases by one point, the probability of survival will decrease by approximately 30% (tab. 1). Age and biological gender showed no significance. The analysis of the classification graph (fig. 2) did not reveal possibilities for improving the specificity.

The model based on the estimation of physiological parameters and indicators during/immediately after hospital admission (MPMoIII)

The following hypotheses were formulated. *The null hypothesis* – the covariates included in the model cannot predict the probability of survival in transported severely traumatized patients better than a model that is based only on constant. *Alternative hypothesis* – at least a variable can predict the probability of survival in patients with severe

trauma better than a model that is based only on constant.

The model presented the following features. After performing the *Omnibus Test of Model Coefficients* ($\chi2$ (df = 1) = 17.094 p <0.001) The null hypothesis was rejected. The coefficient of determination, *Nagelkerke R Square* = 0.51 (51%) was reduced from the model based on anatomical lesions. The fidelity of the results was confirmed by performing the *Hosmer-Lemeshow test*, ($\chi2$ (df = 7) = 3.338, p = 0.847). The classification table shows a *sensitivity* of 89.3% (25 cases out of 28), the *specificity* being 72% (8 out of 11 cases), the average validation appreciated at the level of 84.6%.

The surface under the ROC curve, for the proposed model, was 0.878, with 95% confidence interval (0.773, 0.983) and with a significant difference from the value 0.5 (p <0.001) (fig. 3). Thus, the logistic regression classified the model developed as significantly better model than the random model.

The model includes the constant (B = 14.385) and the values of MPMoIII (B= -.178) (tab. 2). MPMoIII is a predictor with OR = .837 (CI95% .735, .954), that means that if the value of MPMoIII increases by one point, the probability of survival decreases to almost 16% (tab. 2). Age and biological gender are the components of the score and were included in the model.

Fig. 2. **The classification chart for the NISS model (N – non-survived, S – survived).**

Fig. 3. **The ROC curve of the predictive model for the probability of survival in transported patients with severe trauma. SPSS 22 Output.**

Table 2

Variables in the equation

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
MPMoIII	-.178	.067	7.144	1	.008	.837	.735	.954
Constant	14.385	5.272	7.445	1	.006			

Note: Constant – the value of the equation constant; B – the coefficients B; S.E. – standard errors, Wald – Wald statistic; df – degrees of freedom; Sig. – significance threshold; Exp (B) – values for odds ratio; 95% C.I. for EXP (B) – confidence interval for odds ratio.

The analysis of the classification graph (fig. 4) highlights the possibility of improving the specificity (increasing of the cut-off to .62 instead 0.5), but due to the fact that the results are not stable (standard error of MPMoIII coefficient had more than 30% of B), there is a chance of not highlighting the survivors (*overfitting*).

Fig. 4. The classification chart for the model MPMoIII (N – non-survived, S – survived).

The mixed model (NISS + MPMoIII)

For the mixed model, the following hypotheses were formulated. *The null hypothesis* – the covariates included in the model cannot predict the probability of survival in transported severely traumatized patients better than a model that is based only on a single constant. *Alternative hypothesis* – at least a variable can predict the probability of survival in patients with severe trauma better than a model that is based only on a single constant.

The Omnibus Test of Model Coefficients ($\chi 2$ (df = 1) = 32.023 p <0.001), being a significant one, allowed us to reject the null hypothesis and to analyze further, which of the studied covariates is relevant. The coefficient of determination, *Nagelkerke R Square* = 0.805 (80.5%), explaining 4/5 of the dispersion of the examined variable (probability of occurrence for an event), reached the optimal level for the prognostic models. The *Hosmer-Lemeshow test*, analyzing the model in terms of the ability to predict positive and negative results, presents the result as insignificant $\chi 2$ (df = 8) = 2.037, p = 0.980 and increased fidelity to the obtained results. The classification table shows a sensitivity of 96.4% (27 out of 28 cases), the specificity being 81.8% (9 out of 11 cases), the average validation appreciated at 94.5%. As with the coefficients of determination, the optimum values were reached.

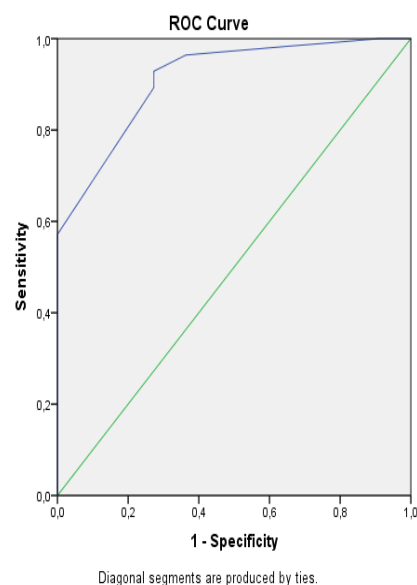The surface under the ROC curve, for the proposed model, constituted 0.977, with 95% confidence interval

Fig. 5. The ROC curve of the predictive model for the probability of survival in transported patients with severe trauma. SPSS 22 Output.

Table 3

Variables in the equation

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
MPMoIII	-.331	.202	2.686	1	.101	.718	.484	1.067
NISS	-.400	.175	5.247	1	.022	.670	.476	.944
Constant	46.233	22.835	4.099	1	.043			

Note: Constant – the value of the equation constant; B – the coefficients B; S.E. – standard errors, Wald – Wald statistic; df – degrees of freedom; Sig. – significance threshold; Exp (B) – values for odds ratio; 95% C.I. for EXP (B) – confidence interval for odds ratio.

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Fig. 6. Chart of classification for the model MMMIII and NISS (N – non-survived, S – survived).

(0.938, 1.000) and with a significant difference from the value 0.5 (p <0.001) (fig. 5). Thus, the logistic regression classified the model as significantly better model than the random model.

The model includes the constant (B = 46.233), the values of MPMoIII (B = -.331) and NISS (B = -.400). The adjustment of NISS to MPMoIII improved the characteristics of the model (the coefficient of determination, sensitivity and specificity). At the same time, NISS represents an efficient covariate, OR = 0.670 (CI95% .476, .944), MPMoIII being insignificant. OR = .718 (CI95% .484, 1.067) (tab. 3). The resampling by bootstrapping showed the significance of parameter MPMoIII (tab. 4). The analysis of the classification graph (fig. 6) does not reveal possibilities of improving the specificity/sensitivity.

Thus, this model is one of perspective, combining an anatomical score with a physiological one, in order to predict the survival in patients with severe trauma transported to the specialized institution.

Table 4

Resampling by bootstrap (997 samples)

	B	Bias	Std. Error	Sig.	95% C.I. for B	
					Lower	Upper
MPMoIII	-.331	.202	2.686	.004	-40.230	-.166
NISS	-.400	.175	5.247	.005	-47.976	-.202
Constant	46.233	22.835	4.099	.002	29.144	5836.065

Note: Constant – the value of the equation constant; B – the coefficients B; Std. Error – standard errors;
Sig. – significance threshold; 95% C.I. B – confidence interval for the coefficients.

Discussion and conclusions

In our research, three predictive models have been developed for the survival rate of severely traumatized patients transferred from district hospitals to the EMI. The model that includes NISS, an anatomical score, has been proven as relevant, the effect of anatomical lesions for this category of patients being estimated quantitatively by the coefficient of determination, the OR and the coefficient for the logistic regression equation. The coefficient of determination constituted .641 (64.1%), compared to .41 (41%) estimated for traumatized hospitalized directly in the EMI. Thus, it can confirm that interhospital transfer of patients increases the effect of the anatomical lesions on the treatment results (outcomes).

The MPMoIII model also predicted the survival rate of a patient with severe trauma. The coefficient of determination constituted .51 (51%), being close to the NISS effect and confirms the idea that the physiological parameters, as well as some indicators of the patient's condition at the admission to the hospital have a prospective predictive potential. The combination of NISS and MPMoIII in the mixed model increased the coefficient of determination over .80 (80%), a practically ideal value, the sensitivity and specificity also being over 80%. But, the significance for MPMoIII was obtained only by resampling.

Thus, all these models are perspective models (especially the combined model) for predicting survival in severely traumatized patients transported to the EMI from district hospitals.

On the one hand, the proposed models can't be recommended for use in daily practice due to limitations related to

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the particularities of the pilot study. The most important of them – a small number of respondents was analyzed, which cannot ensure a high level of accuracy of the coefficients in the logistic regression equation (for example in the NISS covariate mixed model it had a coefficient B = -0.400 and a standard error = .175). On the other hand, the models have a potential to be improved by supplementing with efficient variables.

The implementation procedure can't be initiated without obtaining an accuracy of the coefficients (narrow confidence intervals) and validation of the elaborated models, both obtained in studies with higher level of evidence.

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The influence of respiratory biofeedback training on the breathing pattern and anxiety

Andrei Ganenco, MD, Assistant Professor

Department of Human Physiology and Biophysics
Nicolae Testemitsanu State Medical and Pharmaceutical University, Chisinau, the Republic of Moldova

Corresponding author: andrei.ganenco@usmf.md
Manuscript received September 05, 2019; revised manuscript November 25, 2019

Abstract

Background: The purpose of the respiratory biofeedback method is to change the dysfunctional respiratory pattern to the normal one, and to decrease the patient's general anxiety, as biofeedback training can influence the parameters of the respiratory pattern and the level of anxiety.

Material and methods: 12 subjects (3 men and 9 women), mean age 21.9 ± 1.1, with high level of trait anxiety, were selected for recording the respiratory pattern and respiratory biofeedback (RBF). Respiratory minute volume (MV), tidal volume (TV), duration of inspiration (Ti), duration of respiratory cycle (Tt), respiratory drive (TV/Ti) and ratio of inspiration (Ti/Tt) were measured. Breathing was recorded under the following conditions: resting breathing, paced voluntary hyperventilation, the recovery period after hyperventilation, voluntary apnea and recovery period after voluntary apnea, anticipatory stress. Respiratory biofeedback consisted of 12 sessions of abdominal, deep, 10 breaths/min, visually guided by the route on the computer screen.

Results: After biofeedback, trait anxiety scores decreased in 11 subjects. TV, TV/Ti and MV after biofeedback have been decreased in all phases of research. Tt during the rest and hyperventilation periods did not change, but it was extended in all subsequent phases. RBF did not substantially change the Ti and Ti/Tt in all recording phases.

Conclusions: RBF had a greater impact on volume parameters (TV, TV/Ti, MV) and little or no impact on time parameters.

Key words: respiratory biofeedback, state and trait anxiety, breathing pattern

Introduction

Non-drug methods of prophylaxis and treatment have become increasingly popular lately. One of the effective methods is the biofeedback method. Biofeedback is the process of displaying through the applied psychophysiological feedback of involuntary physiological processes, usually through electronic tools and learning to voluntarily influence those processes. Biofeedback is also a therapeutic tool for facilitating the learning of self-regulation of autonomous functions for improving health. Due to advances in technology and increasing interest in alternative therapies, biofeedback remains in the attention of researchers on possible applications of the method in medicine [1].

Respiratory biofeedback (RBF) has proven to be a method with positive clinical and experimental results. The effectiveness of this method is due to the fact that the respiratory function in the human body has two regulation contours –- the involuntary, automatic, based on maintaining partial pressure of CO_2 in the blood, and the voluntary, behavioral one, based on the involvement of the upper floors of the central nervous system in directing motor activity of the respiratory muscles. The respiratory biofeedback method has been shown to be effective in the prophylaxis and treatment of cardiovascular, pulmonary and neuropsychiatric disorders. As a result of extensive research, the decisive role of this method has been proven in reducing the negative effects of stress on the human body, reducing anxiety and improving the quality of life of patients [2].

The respiratory biofeedback method is based on per-forming voluntary directed respiratory movements with the purpose of changing (reeducating) the dysfunctional respiratory pattern into a normal physiological pattern. The practice of respiratory biofeedback method includes manual and instrumental methods. The most effective ones have been proved to be the instrumental methods that involve the patient's use of technical devices that provide the patient's feedback with the result of his voluntary action on the respiratory pattern by sound or visual signals [3].

Through extensive research it has been shown that the dysfunctional respiratory pattern is characterized by modifications of some of its parameters [4, 5, 6]. This disturbed pattern becomes the source of the disturbing symptoms for the patient increasing the general anxiety of the patient. The purpose of the respiratory biofeedback method is to change (adjust) the dysfunctional respiratory pattern to the normal one. This change leads to the decrease of the patient's general anxiety, the disappearance of the unpleasant symptoms and the change for the better of the quality of life [7, 8, 9].

Multiple researches in respiratory biofeedback, however, have very few references to the influence of biofeedback training on the parameters of the respiratory pattern and their connection with the level of anxiety in healthy people. This is the purpose of the present work.

Material and methods

The study included 63 subjects (24 men and 39 women), aged from 19 to 25 (mean age 22.3 ± 1.1 years). The volunteers did not have a psychiatric, neurological or pulmonary

disorder. All subjects presented written informed consent and the study was approved by the Research Ethics Committee of Nicolae Testemitsanu State University of Medicine and Pharmacy. Subsequently, after performing the Spielberger test, subjects with trait anxiety score greater than 41, 12 subjects (3 men and 9 women), mean age 21.9±1.1, were selected for recording the respiratory pattern and biofeedback.

Respiratory pattern recording was performed on the subject in the lying position, using the inductance plethysmography method (VISURESP, RBI Instrumentation, Meylan, France). Variations of respiratory volumes have been calculated after calibration, performed with a known air volume.

Respiratory minute volume (MV), tidal volume (TV), duration of inspiration (Ti), duration of respiratory cycle (Tt), respiratory drive (TV/Ti) and ratio of inspiration (Ti/Tt) were measured.

Breathing was recorded under the following conditions:
a) Resting breathing, 3 minutes (RB).
b) Paced voluntary hyperventilation (guided by metronome, 10 breaths / min), 3 minutes (HV).
c) The recovery period after hyperventilation (posthyperventilation, PHV), 3 minutes and more until the complete restoration of end-tidal CO_2 concentration (EtCO2) to the values in RB. For the calculation, however, the first 3 minutes of PHV were taken.
d) Voluntary apnea and recovery period after voluntary apnea (PAV), 3 minutes and more until the complete restoration of EtCO2 values to the values in RB. For the calculation, the first 3 minutes of the PAV were taken.
e) Anticipatory stress period (AS), 3 minutes, the subject was persuaded that he is currently stimulated by low intensity electric currents.

All respiratory data was stored on a laptop. The room temperature was maintained at 20 ± 1° C.

The level of anxiety of each subject was determined using Spielberger's State-Trait Anxiety Inventory (STAI) [10]. The instrument comprises two scales, one for measuring trait anxiety level and one for measuring state anxiety level. Each scale has 20 statements and the levels of anxiety for the subjects are indicated by the rating score from 20 to 80. The personal anxiety score evaluates how people generally feel, while the state anxiety score evaluates how people feel "right now" in different situations. The trait score is generally stable, while the state score changes depending on the situation. Scores higher than 44 indicate high trait anxiety, and scores lower than 43 reflect normal or low trait anxiety in women (in women the scores are generally higher). Scores higher than 41 indicate high anxiety, and scores lower than 40 reflect normal or low anxiety in men [11]. In this study, subjects were asked to assess their anxiety level using STAI prior to the start of physiological recordings.

Subjects were selected for biofeedback on the basis that their trait anxiety score is greater than 44, according to Spielberger's State Anxiety Inventory (STAI).

Respiratory biofeedback treatment consisted of 12 ses-

sions of abdominal, deep, visually guided by the route on the computer screen, with the frequency set of 10 breaths per minute and the maximum possible volume.

The breathing pattern was recorded once again after biofeedback, in the same conditions as before RBF.

All statistical analyses were performed with SPSS 10.0. Comparisons of all respiratory parameters before RBF and after RBF were analyzed using the t-test.

Results

The values of anxiety are shown in table 1. The scores of personal anxiety ranged from 46 to 61, the mean value being 52.7 ± 3.2. The scores of the state anxiety ranged from 21 to 43, the average being 29.3 ± 2.4. After biofeedback, trait anxiety scores decreased in 11 subjects and remained the same for one person, ranging from 29 to 52, mean 44.9 ± 2.7. The changes in the state anxiety scores had a variable character, the values increased in 4 people, decreased in 4 people and remained the same in 4 people, ranging from 17 to 38, the mean value 30.8 ± 3.2.

Table 1

The trait and state anxiety scores before and after RBF, 12 subjects. Scores are presented as mean ± SD.

	Before RBF	After RBF
Trait anxiety	52.7 ±3.2	44.9 ± 2.7*
State anxiety	29.3 ± 2.4	30.8 ± 3.2

* – indicate statistical difference p≤0.05.

The tidal volume after biofeedback decreases in all phases of research (fig. 1). After the BFR there is a decrease in the TV from 0.69 l to 0.57 l (p≤0.05). This decrease continues in all phases of the recording. The TV increases obviously during HV, mainly during the period before biofeedback (2.32 l vs. 2.10 l). During the recovery after HV, TV returns
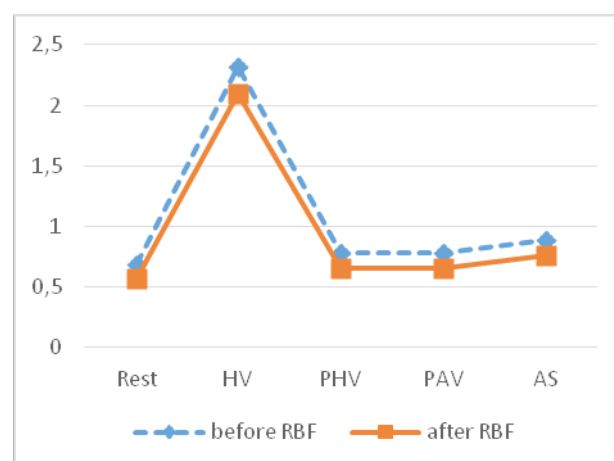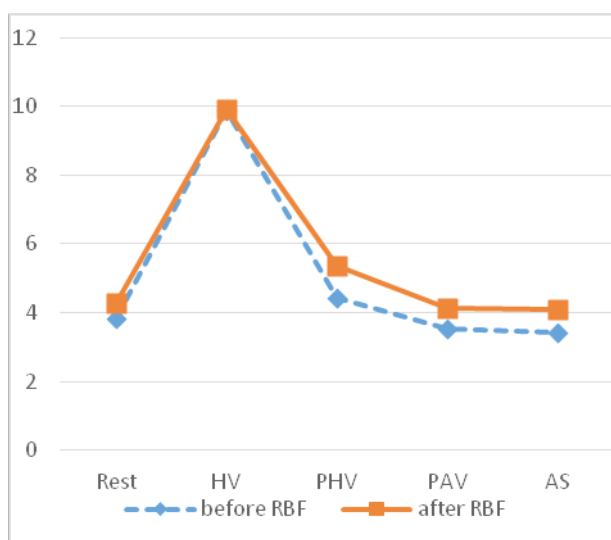

Fig. 1. Tidal volume (TV, l), recorded before and after RBF, in different conditions of recording. * – indicates statistical difference, p ≤0.05.

to the values before the HV, but does not do it completely, showing higher values, especially before RBF (0.78 l and 0.65 l). Approximately the same values manifest during the recovery period after voluntary apnea (0.78 l and 0.65 l). In the recovery period after the anticipatory stress, the values of the TV are higher than in the resting breathing (0.89 l and 0.76 l).

The duration of the respiratory cycle (fig. 2) during the rest and hyperventilation periods was not changed by the RBF (3.82 s and 4.27 s in the resting breathing, 9.89 s and 9.91 s in the hyperventilation). In contrast, RBF extended the duration of the respiratory cycle in all subsequent phases: during the recovery period after hyperventilation – from 4.41 s to 5.37 s, during the recovery period after voluntary apnea – from 3.52 s to 4.13 s, during anticipatory stress period – from 3.43 s to 4.08 s.

Fig. 2. Duration of respiratory cycle (Tt, s), recorded before and after RBF, in different conditions of recording. * – indicates statistical difference, p ≤0.05.

RBF did not substantially change the duration of inspiration at any of the recording phases (fig. 3). Ti values were 1.25 s before RBF and 1.31 s after RBF during normal breathing; 4.35 s and 4.36 s respectively in the period of hyperventilation; very little increased during the recovery period after hyperventilation, from 1.24 s to 1.34 s respectively; also slightly increased during the recovery period after voluntary apnea, from 1.15 s to 1.27 s; and during the period of anticipatory stress the values are close, 1.16 s and 1.22 s respectively.

The ratio of inspiration did not change substantially in all phases of registration (fig. 4). During the rest period, Ti/Tt decreased after the BFR from 0.33 to 0.31, during the hyperventilation period they were the same (0.44), it decreased again in the recovery period after hyperventilation from 0.28 to 0.25, during the recovery period after voluntary apnea from 0.33 to 0.31 and during the anticipatory stress period from 0.34 to 0.3.

Changes in the respiratory drive had the same route as the changes in tidal volume. Thus, Vi/Ti had a value of 0.55
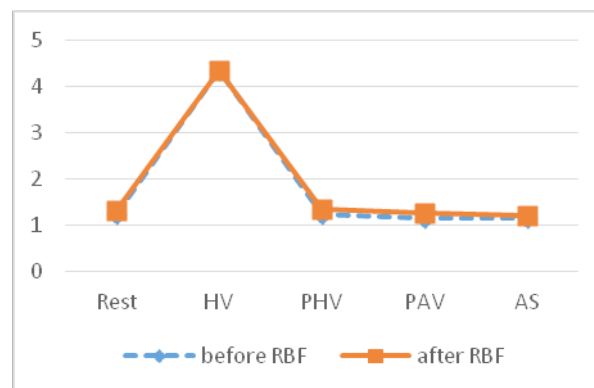
Fig. 3. Duration of inspiration (Ti, s), recorded before and after RBF, in different conditions of recording.
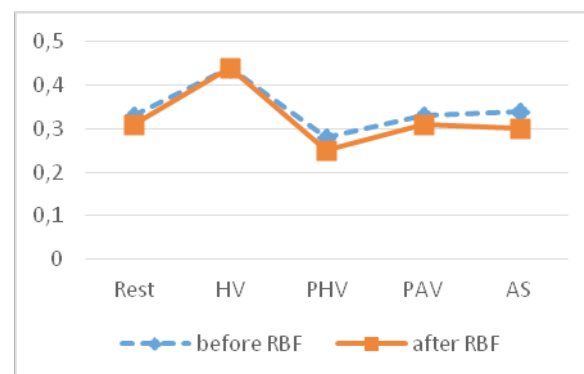
Fig 4. Ratio of inspiration (Ti/Tt), recorded before and after RBF, in different conditions of recording.

l/s in the rest period before the RBF and 0.44 l/s in the same period after the RBF. During the hyperventilation period the respiratory drive was 0.53 l/s before RBF and 0.48 l/s after RBF. The values of the respiratory drive manifested in the same way in other phases – 0.63 l/s and 0.48 l/s in the recovery period after hyperventilation, 0.68 l/s and 0.51 l/s in the recovery period after voluntary apnea, 0.77 l/s and 0.63 l/s during the anticipatory stress period.

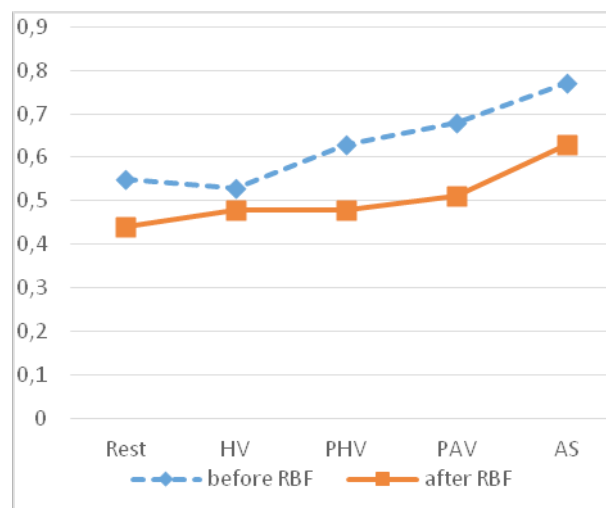Changes in respiratory minute-volume after RBF were

Fig. 5. Respiratory drive (TV/Ti, l/s), recorded before and after RBF, in different conditions of recording. * – indicates statistical difference, p ≤0.05.

similar to changes in tidal volume and respiratory drive (fig. 6). Thus, MV decreased after RBF during the resting breathing from 10.78 l/min to 8.05 l/min; during hyperventilation – from 14.05 l/min to 12.72 l/min; in the posthyperventilation period – from 10.65 l/min to 7.25 l/min; during the period after voluntary apnea – from 13.31 l/min to 9.47 l/min; during the anticipatory stress period – from 15.6 l/min to 11.22 l/min.
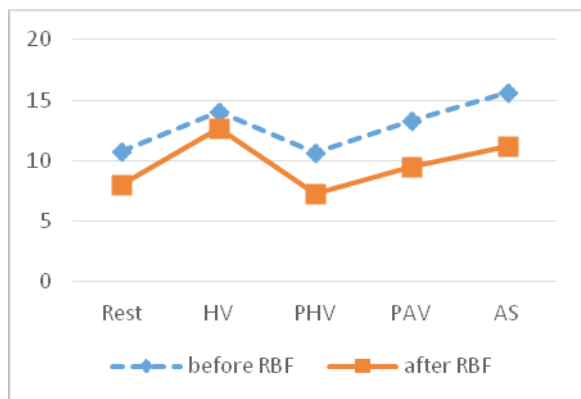

Fig. 6. Respiratory minute volume (MV, l/min), recorded before and after RBF, in different conditions of recording. * – indicates statistical difference, p ≤0.05.

Discussion

It is known that the respiratory function is regulated in the human body by two mechanisms (contours) of regulation: the metabolic one – by the partial pressure of CO_2 and O_2 gases in the blood and the behavioral one – by the activity of the suprapontine superior centers of the central nervous system. This suprapontine, behavioral regulation of breathing, and respectively of the respiratory pattern is influenced by changes in the emotions experienced by the human being, such as fear, anxiety, joy, sadness, etc. [6, 11, 12]. It is remarkable that both components of the regulation – metabolic and behavioral are preserved in the structure of the respiratory pattern [11].

Data obtained in this study have shown that by modeling certain situations induced by voluntary changes of breath (hyperventilation and voluntary apnea, modeling of anticipatory stress), sublime changes of the respiratory pattern can be evidenced with a possible functional diagnosis of the pathological conditions of the central nervous system.

At the same time, the results of the research open up the perspectives of the implementation of the training through respiratory biofeedback as an effective method of prophylaxis and treatment of the suprapontine disorders of the central nervous system.

Conclusions

1. Respiratory biofeedback training reduced the level of the trait anxiety.

2. Respiratory biofeedback had a greater impact on volume indices (VT, Vt / Ti, MVR) and little or no impact on time indices

3. This impact was especially accentuated during the transition periods from the functional tests to the stationary period: namely, in the periods after voluntary hyperventilation, after voluntary apnea and anticipatory stress.

4. We consider that the data obtained in this research using the functional tests of hyperventilation and voluntary apnea, as well as anticipatory stress, will be useful in explaining the clinical phenomena in the patients with hyperventilation syndrome / dysfunctional respiratory syndrome and / or anxiety disorders.

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REVIEW ARTICLES

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Tissue engineering of heart valves – challenges and opportunities

*1Tatiana Malcova, MD, PhD Applicant; 1Tatiana Balutel, MD, Undegraduate Student;
2Anatol Ciubotaru, MD, PhD, Professor; 1Viorel Nacu, MD, PhD, Professor

1Laboratory of Tissue Engineering and Cell Cultures, Department of Topographic Anatomy and Operative Surgery
2Department of Surgery No 4, Nicolae Testemitsanu State University of Medicine and Pharmacy
Chisinau, the Republic of Moldova

*Corresponding author: malcovatatiana92@mail.ru
Manuscript received October 21, 2019; revised manuscript December 02, 2019

Abstract

Background: Heart valve disease is a clinically serious condition. The replacement of damaged valves practiced since the 1950's is the ultimate treatment for end-stage heart failure caused by severe valve dysfunction. The choice of adequate prosthesis is challenging. Unfortunately, the treatment options available today do not satisfy completely physicians and scientists' needs. Mechanical valves require long-term anticoagulation therapy because of poor hemocompatibility. Biological substitutes have better hemodynamics, but need replacement in ~ 10 years due to calcification and degeneration. In order to overcome the shortcomings of current treatment options many researches are motivated to fabricate a functional, living heart valve replacement by tissue engineering.
Conclusions: Tissue engineering is a promising approach that may lead novel constructs that will satisfy the need and overcome the limitations of current valve prosthetics. Scaffolds, fabricated from synthetic or biological materials, do not require donor tissue, but have struggled to recreate the macro- and micro valve anatomy and mechanical properties of native valve. Decellularized cardiovascular grafts have the opportunity to improve patients care by reducing the risk of sensitization to donor antigens, calcify and stenosis and providing with a good graft that will grow (especially important in children). In this way the emotional and financial drain on the patient and family of enduring multiple surgeries may be significantly minimized. The choice of decellularization method can be rational if mechanism of action is contemplated and clearly understood.
Key words: tissue engineering of heart valve, decellularized scaffolds, hybrid starter matrices.

Introduction

Valvular heart diseases remain a serious clinical condition, a major health problem and one of the main causes of morbidity and mortality worldwide. Even if the prevalence and incidence of valvulopathies increase with age, it represents an important problem for pediatric patients too (1% -2% of all live birth are affected by congenital heart diseases, the most common of which affects the heart valves) [1].

Etiology of heart valve diseases is various [2]**, including:**

a. Congenital defects (bicuspid aortic valve, tetralogy of Fallot, congenital pulmonary valve insufficiency, pulmonary artery hypoplasia).

b. Inflammatory/immunological disorders (rheumatic fever, syphilis, antiphospholipid syndrome, angiosarcoma of the aorta or pulmonary artery angiosarcoma).

c. Heritable disorders of connective tissue (Marfan syndrome).

d. Endocardial disorders with valvular involvement.

e. Diseases and disorders of other organs (such as, chronic renal failure).

f. Aging (valve calcification).

g. Post interventional valvular diseases.

For better understanding of valve condition and produced pathophysiological disturbances it is necessary to know the role of each heart valve.

The heart consists of four chambers: two atria and two ventricles, and four flap-like membranous structures, namely valves. Valves determine the direction of blood flow from the atria to the ventricles and from the ventricles to the great vessels.

The valves located between the atria and ventricles, or atrioventricular valves, are:
- ♦ Tricuspid valve, between the right atrium and right ventricle.
- ♦ Mitral valve, between the left atrium and the left ventricle.

The valves located between the ventricles and great arteries, or the semilunar valves, are:
- ♦ Pulmonary valve, between the right ventricle and pulmonary artery.
- ♦ Aortic valve, between the left ventricle and aorta.

As it is known, the cardiac cycle consists of two phases: diastole phase and systole phase. During the diastole phase, the atrioventricular valves are opened and semilunar valves are closed, and during the systole phase, the atrioventricular

valves are closed and semilunar valves are opened, passive openings and closings being determined by a transvalvular pressure gradient. When the valve is damaged or diseased, it does not open or close properly and the blood flow becomes disrupted [3].

Valvular heart diseases can be broadly characterized by the following pathological disorders:

♦ Stenosis: the valve opening becomes restricted and the blood flow out is prevented. In order to move blood, the heart needs to contract with increased force;

♦ Regurgitation: the valve does not fully close, causing the blood flowing back instead of forward flow through the valve;

♦ And heart valves can have both malfunctions at the same time [4, 5].

The contemporary medicine offers a few strategies for the treatment of heart valve diseases: special medications that help to control the symptoms and to avoid further valve damage (diuretics, anti-arrythmic medications, vasodilators, etc.), valve repair, and valve replacement.

Since the first mitral valve repair in 1923 and the first successful prosthetic valve replacement in 1960 described by Starr and Edwards, surgery for valvular diseases has advanced significantly [6].

Due to better long-term results of valve repair and lower morbidity and mortality this procedure is used in preference when possible [7]. However, when heart valve repair is not possible, open-heart surgery with removing of damaged valve and implantation of an artificial one in its place is recommended. During the last decades more than 80 models of prostheses have been developed, however, none of them corresponds completely to the criteria of an "ideal" product, described in cardiovascular surgical literature, such as [8-10]:

➢ Non-thrombogenicity,
➢ Excellent hemodynamics,
➢ Availability in a range of sizes,
➢ Excellent handling characteristics,
➢ Long-term valve function,
➢ Low-to-moderate price,
➢ Low infections potential,
➢ Potential for growth (in particular in pediatric patients).

Mechanical and biological valve substitutes used currently have struggled to recreate the macro- and micro-valve anatomy and mechanical properties of native valve [11]. As a result, their long-term performance is associated with major limitations. Thus, none of them may be considered "ideal" solution.

Mechanical valve substitutes: general characteristic

Three types of mechanical valvular prosthesis are available now: ball valves, disc valves, or monoleaflet valves, and bileaflet valves.

Even if mechanical valves remain the most structurally durable replacements, they have poor hemocompatibility because of their non-physiological surfaces and flow abnormalities. As a result, life-long anticoagulation therapy is necessary for prevention of thromboembolic complications. At the same time, anticoagulation therapy can cause serious spontaneous bleeding and embolism [12]. In addition, mechanical valve substitutes are noisy and susceptible to infection [13, 14].

Biological valve substitutes: general characteristic

By application in practice of biological heart valve replacements the hazards of anticoagulation treatment were avoided.

Different types of bioprosthetic valves are described, such as autografts, xenografts (for example, porcine aortic valves or bovine pericardial valves) and homografts, or allografts (valves taken from human donors) [8, 15].

In 1967 Donald Ross [16] described a new procedure for the treatment of aortic valvular disorders. It involves replacement of patient diseased aortic valve with his own pulmonary valve and then installation of a mechanical or bioprosthetic valve in the hemodynamically weaker pulmonary position. The procedure is associated with a significant surgical risk and risk of postoperative complications, transforming the patient with one pathological valve into a patient with two diseased valves.

Even if cryopreserved, donor valves are closest to the natural valve, have low thrombogenicity, superior hemodynamic performance and resistance to infection. Their main disadvantages are limited availability and failure to regenerate and grow *in vivo*. Moreover, the recipient can become sensibilized to the donor Major Histocompatibility Complex (MHC) antigens, which are present in endothelial cells linking the luminal surface (MHC I) and smooth muscle cells in the media of the arterial wall (MHC II) [17]. Also, when compared to mechanical valves, the structural degeneration of bioprosthetics due to inflammatory/immune response and calcification occurs earlier (in about 10-20 years).

None of currently available biological substitutes shows any potential to grow, regenerate and develop *in vivo*. All these characteristics are important especially in the treatment of pediatric patients [18].

Even the progress in the field of development of new types of valve replacements is undoubted, tissue engineering is the unique approach that may propose a promising strategy to overcome the limitations mentioned above and to provide the surgeons with alternative suitable substitutes, which are able to grow and remodel as the age of the patients advances [19, 20].

Material and methods

Articles containing the keywords "Valvular diseases", "Heart valve replacement", "Tissue Engineering of Heart Valves", "Polymeric starter matrices", "Decellularization", "Decellularized scaffolds", "Biological/Polymeric starter matricies" were selected from PubMed and SpringerLink databases.

The following filters were used: articles published since January 2008 in English. After a preliminary analysis the bibliography of the identified articles has been studied also

in order to find other relevant articles on this topic. Subsequently, information was systematized highlighting the main aspects of contemporary vision on advantages and disadvantages of existing heart valve replacements, scaffolds used in fabrication of a tissue engineered heart valve, improving the procedures of scaffolds development, main characteristics of new valvular prostheses.

Discussion

Being motivated by the lack of adequate replacements pediatric surgeons were the first who introduced the concept of tissue engineering of heart valve [12] and, perhaps, Grim *et al* were the first who presented an example of a tissue-engineered heart valve at the University of Vienna in 1990's. They demonstrated the possibility of including and growing of endothelium on glutaraldehyde-fixed bovine pericardium [21]. Between February 1986 and February 1992, 144 patients received 149 bovine pericardial valve bioprostheses. Even short-term results were satisfactory, long-term results were as follows – 10 patients required reoperation because of valvular dysfunction (valvular stenosis – 7, valvular regurgitation – 2, paravalvular leakage - 1), defect bioprosthesis being removed 34 to 81 months after implantation [22].

The advancement in the field of heart valve tissue engineering since the first published study till today is undoubted. Future development of TEHV needs elaboration of appropriate starter matrices that are able to support cell growth and cell-to-cell interaction with tissue formation. Apart from standard requirements for general tissue-engineered scaffold, like biodegradability, biocompatibility and non-immunogenicity, scaffolds used for tissue engineered heart valve (TEHV) should correspond to several other important criteria [4, 23-26]:

- ➤ Non-thrombogenicity.
- ➤ Mechanically resistance.
- ➤ Growth with patient.
- ➤ Anatomically-shaped.
- ➤ Non-obstruction.
- ➤ Ability to close promptly and completely.

According to these criteria, three main types of starter vehicles are applied in TEHV:

- ♦ Polymeric (synthetic) bioresorbable starter matrices (such as polyglatin, polyglicolic acid, polylactic acid etc.),
- ♦ Decellularized allogeneic starter matrices,
- ♦ Biological / Polymeric hybrid starter matrices [4].

A. Characteristics of Polymeric Scaffold

The concept of use of polymeric starter matrices in tissue engineering is simple – the cells of a particular phenotype seeded on a porous material are expected to generate the tissue growth and organ formation as the scaffold degenerates (important, the degeneration rate of the scaffold should be controllable and proportional to the rate of tissue formation). Except being biocompatible and biodegradable, the vehicles used should match the mechanical properties of

the native tissue, exhibit a cell-favourable surface chemistry and to be at least 90% porous (interconnected pore network is essential for cell growth, nutrient supply and removal of metabolic waste products) [27].

The first models of synthetic biodegradable scaffolds were constructed from aliphatic polyester like polyglatic (in 1995), polyglicolic acid (PGA, in 1996), polylactic acid (PLA, in 1998) and copolymer of PGA and PLA (PGLA, in 1997) [25, 26, 28, 29]. Because these materials demonstrated to be too stiff, new more compliant scaffolds, like polyhydroxyalkanoate (PHAs, in 2000) and poly-4-hydroxybutyrate (P4HB, in 2000) have been investigated [30] to create trileaflet heart-valve conduits. Combination of aliphatic polyesters and PHAs, as alternative composite polymers, has demonstrated promising results in TEHV [31].

As conclusion, the use of polymeric starter matricies has been already broadly demonstrated for cardiovascular tissue-engineering [12] with good results at short-term follow-up. Unfortunately, the mid- to long-term results are not clear yet.

B. Characteristics of decellularized starter matrices

It has been supposed that by decellularization of cryopreserved cardiovascular grafts and removal of donor cells and cell membrane associated MHC I/MHC II proteins the immunogenic potential may be reduced. The main challenge remains elaboration of an appropriate processing method.

According to the definition, decellularization is the process of removing cellular (including nuclear) material from the extracellular matrix (ECM) with its' preservation. Unaltered extracellular matrix and proteins play an important role in promoting tissue regeneration and repair and serve as a native scaffold for cell migration growth and differentiation [32, 33].

The first clinical implantation in pediatric patients of decellularized homografts engineered with autologous endothelial progenitor cells for pulmonary valve replacement was performed in 2002 (since 2005 only non-seeded decellularized allografts have been implanted). The first clinical application in humans of decellularized aortic homografts for aortic valve replacement was performed in February 2008 in Chisinau, the Republic of Moldova [15].

There are different methods used for tissue decellularization, such as [34]:

a. Chemical agents:
- ♦ Acids and bases.
- ♦ Hypotonic and hypertonic solutions.
- ♦ Detergents: ionic – sodium dodecyl sulphate (SDS), sodium deoxycholate (SDC), N-Lauroylsarconsinate (NLS); non-ionic – Triton X-100, Tween-20; and – zwitterionic detergents.
- ♦ Other solvents – alcohols, acetone, tributyl phosphate (TBP).

Complete removal of residual chemicals from ECM after decellularization is obligatory, because even low residual concentration may influence negatively on ECM-scaffold properties [35].

b. <u>Biological agents</u> [34]:
♦ Enzymes: nucleases – DNase and RNase; trypsin; collagenase; lipase; dispase, etc.
♦ Non-enzymatic agents: chelating agents – ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA).

c. <u>Physical and miscellaneous agents</u> [36-38]:
♦ Temperature (freeze-thaw processing).
♦ Force and pressure: mechanical abrasion.
♦ Non-thermal irreversible electroporation.

Because of a variety of techniques, in the context of heart valve decellularization the following criteria were elaborated [17, 31, 32]:

➢ It should be stringent enough to ensure completely cellular material removal (DNA, mitochondria, membrane lipids, cytosolic proteins) in order to avoid any adverse cellular immune response post-implantation.
➢ It should be gentle enough to preserve the biomechanical strength and structural properties of the remaining ECM, because the conduits and leaflets are under extreme environmental demands.
➢ It should preserve potential for recellularization.
➢ It should reduce of immunogenicity and thrombogenicity.

Broadly speaking, the choice of the method of processing is of key importance in decellularization strategy.

The most often employed decellularization combinations for cardiovascular tissue

It's very important to understand the effects of the decellularization technology on the properties of donor heart valve.

a. <u>Biological agents</u> [32, 39, 40]
♦ *Nucleases (DNase/RNase)* cleave nucleic and sequences into shorter segments, expediting their removal from the ECM.

♦ **Trypsin** (a serine protease) cleaves proteins hydrolytically and is used to digest cellular proteins in the decellularization process. Because the structural proteins of ECM have limited resistance to trypsin cleavage, visible histological damage to the ECM is often determined. As conclusion, even it is known that tyrosine cleaves proteins at the arginine or the lysine amino acid residue on the carboxyl side, except when followed by proline; it is capable of degrading the extracellular matrix and cannot be considered a "perfect" strategy for decellularization of cardiac tissue.

♦ **Trypsin+ EDTA**, most often employed enzyme-based combination. Intracellular proteases released as the cells are being trypsinized are inactivated by EDTA. In this way degradation of extracellular matrix by proteases can be avoided, but, unfortunately, all the proteolytic activity of the intracellular proteases cannot be inhibited by it.

Thus, biomechanical integrity of ECM could be adversely affected due to aggressive effect of biological agents.

b. <u>Chemical agents</u>
Detergents have a hydrophilic head and hydrophobic tail, and by reducing the surface tension of the local environment they can penetrate the extracellular matrix and cell membranes [41]. They are classified into three main categories based on the property of the hydrophilic head group: non-ionic, ionic, and zwitterionic.

Detergents are very effective agents because they are able to solubilize cell membrane, lyse cells, and dissociate DNA.

Characteristics of ionic detergents
Ionic detergents contain a head group with a net charge that can be either negative (anionic) or positive (cationic). Ionic detergents can disrupt protein-protein interactions along with lipid-lipid and lipid-protein interactions, and they may denature proteins [42].

Anionic detergents (SDS, SDC) are stronger solubilizing agents than non-ionic detergents and are often used in valve decellularization for cells and DNA removing from ECM [32].

♦ **SDS** (Sodium-dodecyl-sulphate) is a good candidate detergent due to its known ability to denaturate proteins [42], but also SDS has the potential to reduce the biomechanical strength of obtained cell-free scaffold, predisposing the allograft to anevrysm formation once *in vivo*, and to increase the immunogenic potential of the allograft due to denaturation of the extracellular matrix proteins [8]. In addition, complete SDS removal from the tissue is difficult and residual detergents can adversely affect cell adhesion and repopulation [35].

So, SDS seems to be effective for removing cell residues from tissue compared to other detergents, but it is also disruptive to ECM [43].

♦ **SDC** (Sodium Deoxycholate) is an ionic detergent (even it tends to act more like a non-ionic detergent, because of its polar properties it is classified as ionic one) that is useful for disrupting and dissociating many types of protein interactions [44].

♦ **NLS** (N-Lauroyl Sarconsinate) is an effective solubilizer that permits a complete decellularization, additionally it possesses bactericidal properties [45]. In conjunction with a recombinant endonuclease it has been successfully utilized to decellularize pulmonary artery patch grafts [46, 47].

Characteristics of non-ionic detergents
Non-ionic detergents contain unchanged hydrophilic head groups and are suited for breaking lipid-lipid and lipid-protein interactions [42]. Even if **Triton X-100** has proven effective at cell and DNA removal from thicker tissues where enzymatic and osmotic methods are insufficient and appears to be more effective for tissue delipidation than ionic detergents [35, 48], it has demonstrated to lack sufficient strength to decellularize cardiovascular tissue in some hands.

Characteristics of zwitterionic detergents
Zwitteronic detergents offer combined properties of ionic and non-ionic detergents. They do not possess a net charge like non-ionic detergents, but are able to break protein-protein interactions like ionic detergents [42]. For example, **CHAPS** (3-(cholamidopropyl)dimethylammonio)-

1-propansulfonate) is effective for decellularization of thinner tissues and is less effective for cell removal from thicker tissues [49].

To summarize, there are many different detergents that can be used in decellularization protocols, but it is critical to understand how different detergents with distinct chemical properties effect ECM scaffolds in the process of decellularization [42].

c. Osmotic gradient, or osmotic shock, can be used to lyse cells, but it is not efficient at removing the hydrophobic cell membranes and remnants. Thus, it cannot be recommended as the sole decellularization technique [50, 51], but if used in combination with detergents or enzymatic-based methods as an initial step, the required enzyme concentrations and/or exposure time may be reduced [52, 53].

The methodical evaluation of the effect of different agents on the ECM scaffold can be performed by applying the following criteria (safety and effectiveness assessments) [4, 17]:

1. DNA content: < 50 ng ds DNA/mg ECM (dry weight) or < 200 bp DNA fragment lengths.

2. Histological and immunohistochemical assessments:

2.1 Hematoxylin and Eosin (H&E) and 4,6-diamidino-2-phenylindole (DAPI) assess for cellularity and inflammation (lack of visual nuclear material).

2.2 Movat's Pentachrome assesses for extracellular matrix structure.

2.3 Alizarin Reds assesses for the presence of calcification.

2.4 Factor VIII assesses for the presence of endothelia cells.

2.5 Alpha smooth muscle actin assesses for myofibroblasts and smooth muscle cells.

2.6 TUNEL assesses for apoptotic cells and Hsp 27 assesses for this chaperonin protein specifically expressed during the manufacture of collagen Types I and III.

3. Residual assessment:

3.1 Enzyme Residuals may be assessed by ELISA, mass spectroscopy or zymography.

3.2 Detergent Residuals can be assessed by radiolabeling the detergent and conducting a time course experiment or performing a colorimetric assay.

4. Biomechanical assessments:

4.1 Uniaxial tensile.

4.2 Ball burst testing (assesses the biaxial strength of the conduit).

4.3 Fluid mediated burst.

4.4 Hydrodynamic assessment

4.5 Durability testing.

4.6 *In vivo*, durability and functional assessments usually performed in the female juvenile sheep model (according to ANSI/ISO/AAMI 5840 "Cardiovascular Valve Prostheses").

To summarize, decellularization of the tissue to produce extracellular matrix (ECM) scaffold is a complex process that is not standardized even for a specific anatomic source tissue, furthermore it is highly desirable to preserve the complex composition and three-dimensional ultrastruc-ture of the ECM. But it is recognized that all methods of tissue decellularization result in some degree of disruption of the architecture with potential loss of surface structure and composition, that may subsequently impact the host response (such as chronic inflammation, fibrotic encapsulation, and scar tissue formation or a constructive remodeling response with the formation of site-specific functional tissue) [34, 42, 54].

Numerous protocols with applying different agents for decellularization are reported. However, no references exist on how each one may affect the properties of the final ECM scaffold.

C. Characteristics of biological/polymeric starter matricies

The engineered construct with single material and single technique can hardly mimic the whole structure, properties, and function of native valve tissue [5]. Biological/polymeric composite materials are complex structures and have recently been introduced as a further strategy in tissue-engineering. These hybrids may be used for production of heart valves, e. g. fabricated from decellularized porcine aortic valve and enhanced with bioresorbable polymer. Assessments of a novel hybrid heart valve (tensile tests, suture retention strength, pulse duplicator system used for functional testing of the valve under physiological systemic load conditions) demonstrated its feasibility for an application in tissue engineering [12, 55].

Conclusions

Fabricating of a living valve that can grow and functionally integrate to patients' cardiovascular system is the ultimate goal. Heart valve tissue-engineering is a field already almost 20 years old and has advanced considerably since the first published study that galvanized the research. Tissue engineering is a promising approach that may lead novel constructs that will satisfy the need and overcome the limitations of current valve prosthetics.

Some more common and traditional techniques have been improved, including using biopolymers and decellularization. Scaffolds, fabricated from synthetic or biological materials, do not require donor tissue, but have struggled to recreate the macro- and micro valve anatomy and mechanical properties of native valve.

Decellularized cardiovascular grafts have the opportunity to improve patients care by reducing the risk of sensitization to donor antigens, calcification and stenosis and providing with a good graft that will grow (especially important in children). In this way the emotional and financial drain on the patient enduring multiple surgeries may be significantly minimized. Decellularization process typically involves exposure to different agents (chemical, biological, physical ones) that unavoidably cause disruption of the associated ECM. Although some of decellularized valve technology showed promising results, the critical weakness of obtained decellularized TEHV is a somewhat unpredictable rapid graft failure because of immune response and incom-

plete recellularization. As conclusion, the choice of decellularization method can be rational if mechanism of action is contemplated and clearly understood. In addressing to challenges associated with the TEHV, researches must achieve the following goals:

♦ Improvement of decellularization technique.
♦ Preservation of valve biomechanical properties (equal with valve functional safety).
♦ Achieving of the entire valve recellularization *in vivo.*

To summarize, many challenges have been encountered in the pursuit of a TEHV and, probably, it may take another 20 years before many complex challenges are finally solved.

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Haemostatic system changes during pregnancy and puerperium

Liliana Profire, MD, PhD, Associate Professor

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

Corresponding author: liliana.profire@usmf.md
Manuscript received September 09, 2019; revised manuscript December 02, 2019

Abstract

Background: The activity in the hemostasis system is determined by two opposite processes that function simultaneously – blood clotting (fibrin clot formation) and fibrinolysis (the process of fibrin clot breakage). A normal balance between the processes of coagulation and fibrinolysis produces neither coagulation nor lysis, and vice versa, the imbalance of these processes is potentially dangerous in the development of coagulopathic or lytic events. Both systems (coagulation and fibrinolysis) undergo substantial changes in the physiological pregnancy, changes in an increase of coagulation factors concomitant with a decrease of anticoagulants and suppression in the fibrinolysis system. The predominance of prothrombotic activity gives the pregnancy a hypercoagulable status, with an increased risk of intravascular thrombi formation and thromboembolic complications (e.g. venous thrombosis, DIC syndrome). On the other hand, physiological hypercoagulation during pregnancy contributes to preventing the loss of blood during the immediate postpartum period by providing hemostasis in placental wounds and birth pathways.

Conclusions: The hemostasis system in pregnant women is marked by an increase in coagulation at each stage (from endothelium to circulatory factors of coagulation) which presumes the risk of thrombo-embolic complications, and the inhibition in the fibrinolytic system prevents peripartum bleeding.

Key words: haemostatic system, pregnancy, puerperium.

I. Changes in the coagulation system in pregnant women

The blood coagulation involves the interaction of the vascular endothelium, platelets, plasmatic coagulation factors and consists of two stages: primary hemostasis (involves the vascular wall, vascular endothelium and platelets) and secondary hemostasis (activation of the coagulation cascade). The vascular endothelium is the component of the vascular wall with an important hemostatic function at local and systemic level through the secretion of substances with various actions (e.g. procoagulant, anticoagulant, fibrinolytic, antifibrinolytic, etc.), the molecular interactions of which favour coagulation in the subsequent stages and fibrinolysis.

Endothelial cells secret:
- Prothrombotic factors (tissue factor, von Willebrand (fvW) factor, plasminogen activator inhibitor (PAI 1 and PAI 2), platelet activator factor (PAF), endothelins, fibronectin, collagen);
- Antithrombotic factors: protein S, protein C, thrombomodulin, tissue factor pathway inhibitor (TFPI), heparan, antithrombin III, tissue plasminogen activator, urokinase, nitric oxide (NO), prostacyclin (PGI_2);
- Vasodilating factors: prostacyclin (PGI_2), NO;
- Vasoconstrictor factors: endothelins (ET-1, ET-2, ET-3).

The role of vasoactive substances (thromboxane (TX) and PGI_2)) in maintaining hemostasis is their mutual annihilating actions on thrombocyte functions (TX and PGI_2 counteract each other's actions to maintain homeostasis with respect to platelet function). PGI_2 inhibits platelet aggregation and TX is a strong platelet aggregator and contributes to the activation of other platelets. In addition, PGI_2 is a potent vasodilator and contributes to increased blood flow in the uthero-placental complex and TX has a vasoconstrictor action. Endothelial dysfunction is accompanied by a reduction in PGI_2 synthesis, and the platelet aggregation-increasing TX effects and induction of vasoconstriction are determinant [1-3].

An important source of TX and PGI_2 during pregnancy are placenta, placental vessel endothelium, umbilical cord and uterus; ductus arteriosus; derivatives of placenta – amnion, chorion and decidua. The healthy placenta produces an almost equal amount of TX and PGI_2, that is why their biological action on vascular tone, platelet aggregation and uterine contractility is balanced. It should be mentioned that in physiological pregnancy the concentration of both substances increases – PGI_2 (middle of pregnancy) and TX (towards the end of pregnancy). TX is primarily produced by trophoblast and stroma tissues, and PGI_2 is the primary product of endothelial cells of placental vessels and in smaller amounts of trophoblast (fig. 1). The increased serum concentration of TX derivatives and β-thromboglobulin (increased in the wall of the spiral arteries and in the intervillous space of the placenta) in the third trimester of pregnancy is further evidence of platelet activation.

In comparison with the physiological pregnancy in preeclampsia TX production increases and PGI_2 production decreases, so that the balance of the biological actions of these substances tend to favour TX actions. Decreased PGI_2 is also found in complicated pregnancies with intrauterine fetal development delay.

Another component of the coagulation system is platelets. Generally the number of platelets does not change during normal pregnancy (tab. 1), but it varies considerably within the normal baseline ($50-70 \times 10^9$ /L) (tab. 1). Gesta-

tional thrombocytopenia is defined as a reduction in platelets below 150×10^9/L determined in 5-12% of healthy pregnant women. Gestational thrombocytopenia occurs at an advanced gestational age (3rd trimester), rarely enough to have an impact on postpartum bleeding, and is secondary to the dilution effect in pregnancy when the plasmatic volume increases (at the end of the second trimester of pregnancy); reduction of platelets survival in pregnancy; increase in platelets breakage, etc. Restoration of normal platelets number occurs within the first 2 months after childbirth [4-6].

A peculiarity of the coagulopathic changes is related to platelet functions. The platelet membrane is composed of glycoproteins, phospholipids and cholesterol. Some glycoproteins act as membrane receptors (the most important complexes Ib-IX and IIb-IIIa). Transmembrane complex Ib-IX – role of thrombin receptor and von Willebrand factor in platelet adhesiveness. Complex IIb-IIIa – receptor for fibrinogen (essential in platelet aggregation) for Ca^{2+}, von Willebrand factor.
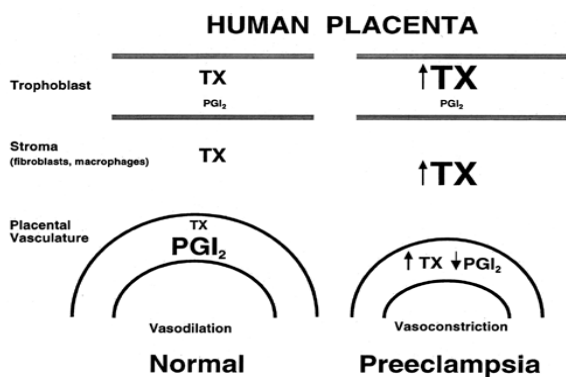
Fig. 1. Secretion distribution of vasoactive substances (PGI2, TX) by placenta in normotensive and with preeclampsia pregnant women.

Hemostatic functions of platelets:

1. Adhesion – the property of adhesion, their attachment to subendothelial structures (collagen, microfibrils, basal membrane). The adhesion process requires the presence of von Willebrand factor that binds the platelets to the subendothelial structures.

2. Platelet activation – follows thrombocyte adhesion and consists in synthesis and release of thrombocyte factors and some active substances (histamine, serotonin, adrenaline, immune complexes, PAF, arachidonic acid, TXA2, thrombin, etc.). All the released substances are designed to attract other platelets to the source, to activate them and determine platelet aggregation and promote coagulation.

3. Aggregation – represents the property of platelets to join together to form conglomerates.

Increased adhesion, activation and aggregation of platelets is marked in complicated pregnancy induced by hypertension, preeclampsia, placental abruption, HELLP-syndrome, prolonged retention of the dead fetus in utero, massive intra- and postpartum bleeding. Changes in adhesion, activation and aggregation functions of platelets are described differently in normotensive pregnant women [7, 8].

Von Willebrand factor as well as platelets is a component of coagulation that occurs at the primary hemostasis stage. FvW is the vascular (endothelial) component of the coagulation system and has a mediator function in the platelet adhesion process to endothelial injured structures. In addition, it is the transporting protein of VIII factor. The fvW level increases progressively during the physiological pregnancy (tab. 1). A parallel increase of fVIII is marked out. The increase in fvW far exceeds its normal level from a normal pregnancy in obstetric complications, the appearance and development of which are attributed to endothelial dysfunction (e.g. from preeclampsia) [9].

Essential changes in the blood coagulation system in pregnant women are determined by an increase of coagulation factors and a concomitant decrease of inhibitors of blood coagulation (anticoagulants). Factors with direct contribution to blood hypercoagulability (with the exception of XI and XIII factors) during pregnancy are: fI (fibrinogen); fVII (proconvertin); fVIII (antihemophilic A factor circulating in plasma associated with von Willebrand factor); fX (Stuart-Prower factor); fXII (Hageman factor); fvW (von Willebrand) (tab.1). The IX (antihemophilic B factor or Christmas factor), II (prothrombin), and V (proaccelerin) factors increase insignificantly or remain unchanged (tab. 1) [2, 6, 9, 10]. The high serum concentration of coagulation factors is reflected on the diagnostic parameters of blood coagulation during pregnancy, marked by shortening of coagulation time; shortening of PT and aPTT (sometimes below lower reference limits); increase of thrombo-elastographic parameters such as maximum clot firmness (MCF) and maximum amplitude (MA) [4, 11-14].

The serum level of fibrinogen in pregnancy at term constitutes (4–6 g/l) (tab.1), a double exceeding as against its reference values outside the pregnancy (2-4 g/L). Thus, the fibrinogen level considered normal for non-pregnant status – 2 g/l, in postpartum bleeding (hypo-atonic) may indicate the loss of a significant amount of blood [1, 15-17].

Increased formation of thrombin-antithrombin complexes (TAT), prothrombin fragments 1 and 2 in the second and third trimester of pregnancy is another indicator of hypercoagulability in pregnant women. The TAT complex is formed as a result of the interaction of these factors; it is a non-active complex, the formation of which is followed by the loss of activity of both components, thrombin and antithrombin. The presence of serum complexes TAT is a marker of increased thrombin formation and their progressive increase may indicate a possible depletion of antithrombin. Prothrombin fragments 1+2 (FP1+2), as well as TAT complexes, are indicators of thrombin formation. FP1+2 fragments are generated during prothrombin transformation into thrombin (tab. 1) [3, 18-20].

With the evolution of pregnancy the fibrinopeptid A concentration increases. Fibrinopeptid A is a marker of fibrin formation. Under the proteolytic action of thrombin the fibrinogen is cleaved into fibrinopeptids A, B and fibrin monomers. The serum level of fibrinopeptid A is maximal in the last trimester of pregnancy, the increase of which in

plasma indicates increased formation of thrombin and fibrin generation in physiological pregnancy (tab. 1).

Table 1

Changes of hemostasis system in pregnancy

Physiological components of blood	Out of pregnancy	In pregnancy
Platelets	$150-350 \times 10^9$	Unchanged
Von Willebrand (fvW) factor	100%	Increases
Fibrinogen (fI)	2.0-4.5 g/l	4.0-6.5 g/l
F II (prothrombin)	75-125%	100-125%
F V (proaccelerin)	75-125%	100-150%
F VII (proconvertin)	75-125%	150-250%
F VIII (antihemophilic A factor)	75-150%	200-500%
F IX (antihemophilic *B* factor or Christmas factor)	75-125%	100-150%
F X (Stuart-Prower factor)	75-125%	150-250%
F XI, antihemophilic globulin C (Rosenthal factor)		Does not change
F XII (Hageman factor)	75-125%	100-200%
F XIII (fibrin stabilizing factor)	75-125%	35-75%
Prekallikrein		Does not change
Kininogen with high molecular weight		Does not change
Protein S	100%	Decreases($\downarrow$)
Protein C	100%	Does not change/ increases resistance to protein C
Antithrombin III	80-130%	Does not change
Heparin cofactor II		Increases
Complexes TAT		Increase
Fragment 1 of prothrombin		Increases
Fragment 2 of prothrombin		Increases
Fibrinopeptid A		Increases
Complexes PAP		Increase
Tissue plasminogen activator(t-PA)	1.6-13µg/l	3.3-9.2 µg/l ($\downarrow$)
TAFI		Increases
Inhibitors of plasminogen activator (PAI-1, PAI-2)	100%	Increase
Thrombomodulin		Increases
Fibrin degradation products (FDP)		Increase
D-dimers	<0.5 mg/l	0.13-1.7 mg/l

There are a number of factors with inhibitory action on blood coagulation preventing in such a way the formation of thrombi. Some of the important physiological inhibitors are antithrombin, protein C, protein S, heparin cofactor II, tissue factor pathway inhibitor or coagulation extrinsic pathway inhibitor (TFPI). Hypercoagulability during pregnancy is favoured/supported by changes of anticoagulants marked by a significant reduction (40-50% decrease in protein S) or neutrality (e.g. antithrombin, protein C, which remain unchanged). Antithrombin III – powerful inhibitor of blood coagulation by inactivating most enzymes: thrombin (f II), fIX (antihemophilic B or Christmas factor), fX (Stuart-Prower), f XI, fXII (Hageman factor), and of VIIa complex factor and tissue factor.

Protein S is an anticoagulant that works in association with protein C. The decrease of protein S during pregnancy reduces the amount of thrombomodulin-thrombin-protein C and S complexes formation, thus diminishing their inhibitory action on V and VIII factors with increased procoagulant activity of the blood. In pregnancy the level of thrombomodulin increases [2, 21-23].

Out of pregnancy the interaction of the mentioned anticoagulants works differently. At the endothelial cells level thrombomodulin binds thrombin. The formed complex activates the protein C which in the presence of proteins S and Ca^{2+} leads to a decrease in blood coagulation through activation of factors V and VIII.

One of the important mediators of blood coagulation is the tissue factor (TF). In pregnancy a substantial increase in tissue factor concentration in decidua, myometrium, placenta, fetal membranes (especially in amnion) and amniotic fluid is determined. Inhibition of TF activation is accomplished by the tissue factor pathway inhibitor (TFPI) which has anticoagulant impact, the placenta being an important source of TFPI production and release in the maternal circulation. Thus, the concentration of TFPI increases significantly and progressively in the physiological pregnancy, the maximal level of which is reached in the pregnancy at term, with a dramatic decrease close to the non-pregnant level in the first postpartum day [1, 20, 21, 24].

II. Fibrinolysis and fibrinolytic activity in pregnancy

Fibrinolysis is a mechanism of prevention of thrombosis, consisting of a set of reactions that lead to fibrin degradation with a repermeabilization of the injured vessels and resumption of blood circulation. Plasmin is the enzymatic key of fibrinolysis that plays the role of thrombus lysis. The plasmin is formed from the activated plasminogen (in circulation the plasminogen is inactive). The activation of plasminogen (under the influence of fibrinolysis activators) is accompanied by its transformation into plasmin, and the plasmin acting on fibrin degrades it into fragments until final products are formed (fig. 2).

Plasmin is inactivated by antiplasmins (inhibitors of fibrinolysis with action on plasmin) – α_2-antiplasmin and α_2–macroglobulin (fig. 2), with the formation of plasmin-antiplasmin complexes (PAP), the concentration of which increases in the physiological pregnancy. The increased PAP complexes concentration during pregnancy is also due to the increase in antiplasmins. Plasmin in the formed complexes (PAP) loses the ability to destroy the fibrin.

Fibrin degradation product (FDP) increases progressively in pregnancy through increased formation of fibrin and secondary enhancement of fibrinolysis. Excessive production of FDP (e.g. in obstetrical complications that involve coagulation system activation, preeclampsia, abrup-

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tion placentae, DIC syndrome, etc.) has an anticoagulation impact and inhibits blood coagulation [5, 25].

A more specific indicator for the fibrinolysis process than FDP is D-dimers. The level of D-dimers begins to gradually increase from the 1st trimester of pregnancy, so that at the end of pregnancy its levels may be 3-4 times higher than the initial level. Significant increase in D-dimers is determined in pregnant women with complications of pregnancy (e.g. DIC syndrome, thrombosis of deep veins, thromboembolic complications in pregnancy, etc.) (tab. 1). Excess concentration of D-dimers in such complications indicates activation of fibrinolysis preceded by the activation of coagulation cascade with excessive fibrin formation.

D-dimers and PAP complexes are markers of the fibrinolysis process. Thus, hemostasis markers change during pregnancy, reflecting the increase in thrombin generation (fragment 1 and 2 of prothrombin, TAT complexes, fibrinopeptid A) on the one hand, and increase of fibrinolysis (D-dimers, PAP complexes) on the other hand [21, 26-28].

Similar to the coagulation system, the fibrinolytic process is the result of the interaction of activators and inhibitors. At the action of fibrinolitic activators the endovascular deposits of fibrin are removed, but the inhibitors prevent premature lysis of the hemostatic thrombus that could lead to a prolonged bleeding. The fibrinolysis can be activated in two ways – extrinsic and intrinsic [10, 11, 23, 24].

The activators of extrinsic fibrinolysis are tissue plasminogen activator (t – PA); U – PA urokinase and activators of bacterial origin (streptokinase, staphylokinase, proteolytic enzymes, e.g. trypsin). The activation of intrinsic fibrinolysis is triggered by f XII (Hageman), kallikrein. The final effects of the activators of both pathways of fibrinolysis are unanimous, to transform the plasminogen into plasmin.

The fibrinolysis inhibitors act upon the enzymes (plasminogen and plasmin) involved in the most important fibrinolysis reactions: 1. The inhibitors of the tissue activator of plasminogen (t-PA) impede in such a way the transformation of plasminogen into plasmin; 2. Inhibitors with direct action on the plasmin (the effect of stopping the thrombus lysis) (fig. 2).

The fibrinolysis inhibitors to neutralize the tissue activator of plasminogen are collectively called PAI (plazminogen activator inhibitors): PAI-1 – produced by endothelial cells, platelets, hepatocytes and stimulated by proinflammatory cytokines; PAI-2 – is met exclusively in pregnant women, produced by the cells of chorionic villosities, progressively increases in pregnancy, returning to normal values 6 weeks after childbirth; PAI-3 – a powerful inhibitor of protein C.

Both substances, PAI -1 and PAI-2, can inhibit the activators of the intrinsec pathway of fibrinolysis (t-PA and U-PA).

The inhibitors with direct action on the plasmin are α_2–antiplazmin, α_2 macroglobulin, α_1 antitrypsin. The fibrinolysis inhibitor activated by the thrombin TAFI. TAFI modifies fibrin filaments, inhibiting the binding of plasminogen to the fibrin network and implicitly its activation to plasmin;

The activity of the fibrinolysis system is opposed to the activity of coagulation system, marked by the decrease in activators and increase in inhibitors, with subsequent suppression in the fibrinolytic system in pregnancy.

Changes in fibrinolysis activators during pregnancy:
- Plasminogen increases but its fibrinolytic activity decreases;
- Decrease in tissue plasminogen activator (t-PA) due to the increase of the levels of inhibitors of plasminogen activators during pregnancy – PAI-1 and PAI-2.

Changes in coagulation inhibitors in pregnancy:
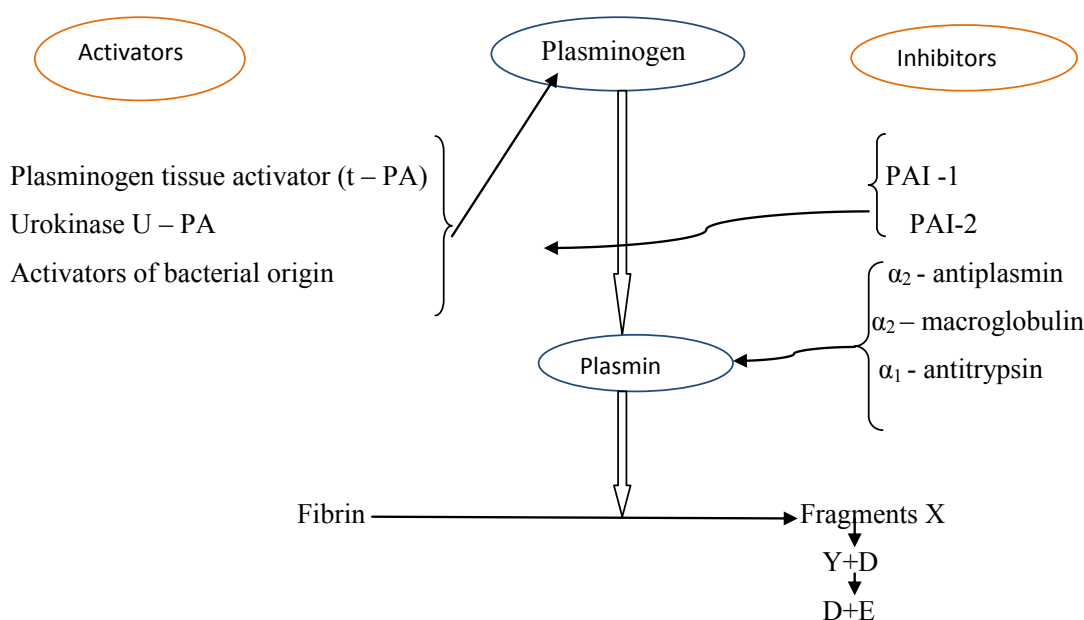- Increase in inhibitors of plasminogen activator – PAI-

Fig. 2. Fibrinolysis and regulation of activity in fibrinolytic system.

L. Profire. Moldovan Medical Journal. December 2019;62(4):56-60

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1 and PAI-2. In particular, the inhibition of fibrinolysis in pregnancy is due to the increase of inhibitor of type 2 (PAI-2) of the plasminogen activator.

♦ Significant increase of inhibitors with direct action on plasmin – α_2 antiplazmin; α_2 – macroglobulin, α_1-antitrypsin.

♦ Increase of the fibrinolysis inhibitor, activated by the thrombin – TAFI. TAFI reaches maximal values up to 35-39 SA with a rapid decrease in 24 hours after childbirth.

Conclusions

The hemostasis system in pregnant women is marked by an increase in coagulation at each stage (from endothelium to circulatory factors of coagulation) which presumes the risk of thrombo-embolic complications, and the inhibition in the fibrinolytic system prevents peripartum bleeding.

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Bone marrow-derived mononuclear cells therapy for ischemic stroke

*¹**Petru Butucel,** MD, Undergraduate Student; ¹**Viorel Nacu,** MD, PhD, Professor;
²**Vitalie Lisnic,** MD, PhD, Professor

¹Laboratory of Tissue Engineering and Cells Culture, ²Department of Neurology No 1
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova

*Corresponding author: petrubutucel@yandex.ru
Manuscript received September 21, 2019; revised manuscript November 24, 2019

Abstract

Background: Nowadays, the cerebrovascular event is the second cause of death and the third cause of disability worldwide. In the last few decades, stem cell-based approaches are widely analyzed as a potential treatment for this disease. One of these types of cells are bone marrow-derived mononuclear cells (BMMNCs).
In this review, we analyzed 9 completed clinical trials with the use of BMMNCs in patients with ischemic stroke, which we found in the clinicaltrials. gov and PubMed databases, using the keywords "stroke" and "bone marrow mononuclear cells". Our goal was to analyze the safety and efficiency of this therapeutic approach, as well as the optimal therapeutic time window, transplantation route and cell dose used.
The best stroke phase to apply this therapy is the subacute stage. Higher numbers of CD34+ cells, derived from BMMNCs were correlated with a trend toward a better outcome. All the clinical trials support the idea that BMMNCs transplantation is a safe therapy.
Conclusions: In conclusion the author points out that the autologous transplantation of BMMNCs is harmless and not associated with severe complications. Although some clinical studies stated a better outcome in patients treated with BMMNCs, further clinical trials are needed to establish their therapeutic efficiency.
Key words: ischemic stroke, bone marrow mononuclear cells, transplantation, treatment.

Introduction

The cerebrovascular event (stroke) is a medical condition in which the blood flow to the brain is diminished due to arterial ischemia or arterial rupture. Usually this results in severe brain damage, which includes neuronal death, microvasculature disturbances, local inflammation and acid-base imbalance. Stroke is the second cause of death and the third cause of disability worldwide. About 87% of strokes are ischemic, the rest being hemorrhagic. Disability affects 75% of stroke survivors enough to decrease their employability [1]. There were many efforts to elaborate a pharmaceutical medication that would reduce the severity of stroke and support intensive therapy. These led to some achievements, for example the production and use of tissue Plasminogen Activator (tPA), which can be administered in ischemic stroke patients and contribute to degradation of blood clots. Unfortunately, the time window for application of this therapy is a serious limitation, so than it cannot be administered to patients who have suffered an ischemic stroke for more than 4.5 hours after onset. As a result, very few patients benefit of tPA therapy; a study that reviewed records from the National Inpatient Sample from the U.S.A. has shown that from 2005 to 2011, overall 3.8% of patients received tPA, although with the number growing each year [2].

Another important therapy that has evolved in recent years is the mechanical thrombectomy. It implies the use of cerebral clot extracting devices in acute large-vessel occlusion, which results in vascular recanalization. However, this treatment also has some limitations: it is indicated for patients with acute ischemic stroke due to a large artery occlusion in the anterior circulation, who can be treated within 24 hours of the time last known to be well. According to some clinical studies, only 9-10 % of ischemic stroke patients can qualify for mechanical thrombectomy [3-8].

In the last few decades, stem cell therapy is being regarded as a promising therapeutic approach for stroke patients. There are several cell types that could be transplanted in the post stroke patient and have the potential to improve the outcome: bone marrow-derived mononuclear cells (BMMNCs), bone marrow mesenchymal stem cells (BMSCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs) and multilineage-differentiating stress-enduring (Muse) cells, to name just a few.

BMMNCs are a group of cells which contain lymphoid cells, myeloid cells, hematopoietic and mesenchymal stem cells. Preclinical studies have shown an efficiency of treatment with such cell types, by means of different mechanisms of actions, such as neurogenesis, angiogenesis, arteriogenesis and modulation of inflammation [9, 10, 11]. BMMNC autologous transplantation has some remarkable advantages over transplantation of other cell types. These cells can be rapidly prepared for transplantation within hours after harvest; there is no need for *in vitro* expansion in a culture medium, there is no risk of immune reaction associated with their transplant and there are no ethical issues regarding such a therapeutic approach.

In this review we have analyzed 9 completed clinical trials with BMMNC autologous transplantation as a treatment for ischemic stroke patients. The aim of this review is to analyze the safety and efficiency of this therapeutic approach, as well as the optimal therapeutic time window, transplantation route, cell dose and to discuss the correlation between these variables and patient outcomes. Secondly, we analyze and discuss the correlation between BMMNC transplantation and the levels of some relevant blood markers, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF) and β-nerve growth factor (β-NGF) and the relation between these levels and such biological processes as neurogenesis, arteriogenesis, angiogenesis and inflammation.

Material and methods

We have analyzed the clinical trials regarding BMMNC therapy for ischemic stroke with published results, which we found in the databases Pubmed and Clinicaltrials.gov. As a selection filters, we have used the keywords: "stroke" and "bone marrow mononuclear cells", and selected just articles in the English language. After processing the materials according to the search criteria, we found 12 finished clinical

trials with the use of BMMNCs in order to treat ischemic stroke (excluding case-report studies). The final bibliography of this review included 9 clinical studies, which were considered to be representative and sufficient to describe the overall situation of cerebrovascular event therapy with BMMNC autologous transplantation, including the safety and clinical efficiency of this treatment method.

Clinical studies

The 9 clinical trials analyzed relate to the use of BMMNCs in order to treat ischemic stroke. Their importance consists, firstly, in confirming the BMMNC autologous transplant safety for stroke survivors and lack of association with severe complication. Secondly, some of these trials have also showed that this method of treatment could improve the patient's outcome. Nowadays, it became clear that for the proper understanding of the correlation between BMMNC transplantation and the patient's health condition after the treatment, much more clinical studies are needed.

Valeria Battistella et al. study [12] included 6 patients who had suffered ischemic stroke 59-82 before they received BMMNC intra-arterial transplantation, aged between 24 and 65 years, in their study. The mean quantity of infused

Table 1

Completed clinical studies concerning BMMNC transplantation in ischemic stroke

Study reference	Route of administration	Patient's age (years)	Time period of administration after stroke onset	Number of BMMNCs transplanted	Period of follow-up	Patients treated for ischemic stroke/ Total of patients treated with BMMNCs
Valeria Battistella et al. 2010 [13]	IA	24 - 65	Day 59 - 82	3.058×10^8	180 days	6/6
Sean I. Savitz MD et al. 2011 [14]	IV	55.6 ± 15	24 - 72h	8 patients: 10^7/ kg 1 patient: 7×10^6 / kg 1 patient: 8.5×10^6/ kg	6 months	10/10
Francisco Moniche et al. 2012 [15]	IA	66.9 ± 13.9	Day 5 - 9	1.59×10^8	6 months	10/10
Maurício A. G. Friedrich et al. 2012 [16]	IA	30 - 78	Day 3 - 7	22.08×10^7	6 months	20/20
Alok Sharma et al. 2014 [17]	IC	27 - 79	4 - 144 months	10^6/ kg	6-54 months	14/24
Kameshwar Prasad et al. 2014 [18]	IV	50.7 ± 11.6	7 - 30 days	280.75×10^6	1 year	58/58
Akihiko Taguchi et al. 2015 [19]	IV	57 - 75	7 - 10 days	6 patients: 2.5×10^8 6 patients: 3.4×10^8	6 months	12/12
Azza Abass Ghali et al. 2016 [20]	IA	46 - 66	12 – 32 Days (mean = 22 days)	10^6	12 months	21/21
Ashu Bhasin et al. 2016 [21]	IV	Group I: 48.6 ± 7.1 Group II: 48.1 ± 9.1	3 months – 1.5 years	10^6/ kg	12 months	10/10

IV – intravenous, IA - intra-arterial, IC - intrathecal.

* Information about the group that has received BMMNCs infusion is undisclosed.

cells was 3.058×10^8 (range between 1×10^8 and 5×10^8). Also, the authors have investigated the distribution of BMMCs labeled with ^{99m}Tc 2 and 24 h after transplantation and observed that the infused cells were localized in the brain, although at 24 h, cell homing could only be visualized in the brains of two patients. 2 patients suffered seizures approximately 200 days after the cell infusion and were placed under an extended follow-up. At the 180 day of follow-up all patients had improved NIHSS (National Institute of Health Stroke Scale) scores in comparison with the pre-transplantation values (range – 1 to 8 points). This study confirms that BMMNC autologous transplantation is safe for ischemic stroke patients and can lead to an improvement in patient outcomes, but the absence of a control group should be pointed out as a study limitation.

Sean I. Savitz et al. [13] have included 10 patients with acute ischemic stroke in their open-label prospective study. Within 24-72 hours after the stroke onset, the BMMNCs were infused intravenously. 8 patients received approximately 10^7 cells/kg, one patient received 7×10^6 cells/kg and the other one revived at 8.5×10^6 cells/kg. Two patients had infarct expansion between enrollment and harvest and subsequently underwent hemicraniectomy. One patient died on the 40th day after enrollment in the experiment due to a pulmonary embolism related to the stroke and the patient's request to discontinue medical therapy. The Median NIHSS score was 13 before harvest of the BMMNCs, 8 – on 7 day after BMMCS infusion, and 3 – 6 months after BMMCS infusion. At 6 months, all surviving patients had shifted down by at least 1 point on the mRS (modified Rankin Scale) compared to day 7. 7 out of 10 patients achieved a BI (Barthel Index) ≥ 90. Also when comparing with the historical controls, the majority of the BMMNC treated patients were within the 95% confidence interval (CI) range or showed a better outcome at 90 days on the mRS scale. This study confirms that BMMNC transplantation is a safe treatment for ischemic stroke patients and may lead to a better outcome, but the lack of a control group should be noted as a limitation.

Francisco Moniche et al. [14] have completed a single-blinded (outcomes assessor) controlled Phase I/II study. They included 20 ischemic stroke patients, from which 10 formed a BMMNC treated group, and 10 formed the control group. The mean NIHSS score was 15.6 in the BMMNC treated group and 15.0 in the control group ($P=0.82$). Autologous transplantation was done 5 to 9 days after stroke onset. BMMNCs were injected in the M1 segment of the infarct-related MCA (medial cerebral artery) at low pressure. A mean of 1.59×10^8 cells were transplanted in the BMMNC treated group, from which a mean of 3.38×10^6 were CD34+ cells. 2 patients from this group had an isolated partial seizure (at 3 months). In both cases an antiepileptic drug was administered and there were no recurrent seizures. There were no statistically significant differences in the neurological function at 180 days of follow-up. At 6 months, a greater insignificant proportion of BM-MNC-treated patients had mRC modified Rankin Scale scores of ≤2 (20% versus

0%, $P=0.47$). There was a trend towards a better outcome when higher numbers of CD34+ cells were injected, especially in the BI Barthel Index at 1 month after transplantation ($P=0.09$). Higher significance levels of β-nerve growth factor (β-NGF) appeared in BM-MNC-treated patients than in control subjects: after 8 days β-NGF levels were 12.8±2.7 in BMMNC treated group versus 3.9±2.5 I control group ($P=0.029$). This study shows that BMMNC autologous transplant is safe for ischemic stroke patients, and confirms that BMMNC infusion is associated with an elevated level of β-NGF in the blood.

Maurício A. G. Friedrich et al. [15] included 20 patients with moderate to severe acute middle cerebral artery infarcts in their study. The mean baseline NIHSS score was 17 ± 5.6 (median 15.5; range 9–28). The mean time from stroke onset to treatment was 6 ± 1.8 days (range 3–10) and the mean BMMNCs in the infused solution was 22.08×10^7 cells (range 5.1×10^7-60×10^7). There were no serious adverse effects related to the experimental procedure. 2 patients died during the follow-up. One of them was discharged in a good condition but suffered an acute myocardial infarct 43 days after treatment. The other patient has undergone a hemicraniectomy 2 days after intra-arterial infusion and responded well to this procedure. However, he died 61 days after the IA ABMMC infusion from infectious complications related to an elective cranioplasty. A significant reduction of NIHSS score between the pretreatment period and 180 days after transplant was observed ($p<0.001$). 6 patients (30%) achieved satisfactory clinical improvement in functional recovery at 90 days. A total of 8 patients (40%) achieved a mRS ≤2 at 90 days. This study confirms that intra-arterial BMMNC transplantation is safe and can lead to a better clinical outcome for ischemic stroke patients. The main limitation is the absence of a control group.

Alok Sharma et al. [16] have included 24 patients in their study, 14 of which had suffered an ischemic stroke, and 10 who had suffered a hemorrhagic stroke. Between 24h and 48h before cell harvesting, patients were infused with granulocyte colony stimulating factor. Patients were infused with a quantity of $10^6 \times$ kg of body weight of BMMNCs, intrathecal, in the L4-L5 lumbar space. The authors have concluded that out of 24 patients 12 have shown improvements in ambulation, 10 in hand functions, 6 in standing balance, 9 in walking balance, and 10 patients in functional status. Also, it was observed that patients aged less than 60 years showed a high improvement percentage compared with older patients. Also, the percentage of improvement was higher in patients whose stroke episode happened less than 2 years prior, as compared to patients whose stroke episode happened more than 2 years prior to the study. Out of 24 patients, 9 had affected higher mental functions. 2 out of these 9 patients showed an improvement in higher mental functions after BMMNC transplantation and neurorehabilitation. Patients were followed-up for a minimum of 6 months to a maximum of 4.5 years. None of the patients had any major adverse events. This study confirms that BMMNC transplantation using the intrathecal

route is safe and has the potential to lead to a better outcome, but it should be pointed out that 14 out of 24 patients have suffered the ischemic stroke, the remainder having suffered a hemorrhagic stroke. The main limitation of the study is the lack of a control group.

Kameshwar Prasad et al. [17] have conducted a phase II, multicenter, parallel group, and randomized trial with a blinded outcome assessment that included 120 patients that had suffered from ischemic stroke. In the marrow mononuclear stem cells (BMSCs) treated group, 58 patients were intravenously infused with BMSCs (initially there were 60 patients, but 2 missed because of withdrawal and logistical difficulties). Other 60 patients formed the control group. The mean number of BMSCs infused was 280.75×10^6 cells. The transplantation took place between 7 and 30 days after the stroke onset (median of 18.5 days). 5 (8.4%) out of 59 patients in the BMSC group and 5 (8.3%) out of 60 in the control group died before day 180. Three more patients died at day 195, day 206, and day 221 in the BMSC group. No significant differences in the NIHSS score and changes in infarct volume at day 90 and day 180 were observed between the BMSCs and the control group. The BI score on day 90 and day 180 of the both groups was also similar. Analysis adjusted for infarct volume, baseline NIHSS, and baseline BI did not change the results. Scores of mRS in the control group versus the BMSC group at day 180 showed no difference. No relationship was observed between cell dose and outcomes. This study confirms that BMSC transplant is safe for ischemic stroke patients but does not present any improvements in outcomes correlated with such a therapeutic approach.

Akihiko Taguchi et al. [18] have conducted a phase1/2a clinical trial and included 12 patients that have suffered an ischemic stroke of embolic etiology in their study. Patients were aged between 57 and 75 years old (mean age=67.4 ± 5.4 years). Mean NIHSS scores were 16.6 ± 4.7 and 16.3 ± 3.3 on admission and day 7 after stroke, respectively. The BMMNC transplantation took place on day 7-10 after stroke. A group of 6 patients were intravenously infused with a mean number of $2.5 \pm 0.5 \times 10^8$ cells, and another group of 6 patients were infused with a mean number of $3.4 \pm 1.3 \times 10^8$ cells. Patients were followed up 6 months after treatment, and serious adverse effects were observed in two patients. One of them experienced aspiration pneumonia and sepsis 3 months after cell therapy. An independent data monitoring committee concluded that cell transplantation had no association with the occurrence of aspiration pneumonia and sepsis. The other patient experienced a recurrent stroke. The independent data monitoring committee concluded that the association between cell transplantation and the recurrent stroke in this patient was unclear. Mean NIHSS scores on day 7 after stroke and day 30 after cell transplantation were 16.3 ± 3.3 and 11.6 ± 4.8, respectively. Mean improvement in NIHSS score was 4.8 ± 4.6 ($P < 0.01$, 95% CI). Although there were no statistically significant differences between the low-dose and high-dose groups, administration of the higher dose of BMMNCs consistently showed a trend to-

wards an improved neurological recovery. Also, comparing patients who received cell therapy with historical controls, a trend favoring improvement was observed in the group treated with bone marrow mononuclear cells. Significant differences were observed between the two groups in NIHSS scores at the time of discharge ($p < 0.05$) and change of the NIHSS score between day 7 after onset of stroke and discharge ($p < 0.05$). This study confirms that BMMNC autologous transplantation is safe for ischemic stroke patients and has the potential to enhance neurological improvements. The main limitation of this study is the absence of a control group.

Azza Abass Ghali et al. [19] included 39 patients with sub-acute cerebral infarct in their study. The patients had suffered stroke from 1 week up to 3 months before they were included in the study. At that time, their National Institutes of Health Stroke Scale (NIHSS) scores were between 4 and 20. 21 patients were in the group treated with BMMNCs transplant, and 18 patients were in the control group. Three days before the procedure, patients received a daily subcutaneous injection of granulocyte colony stimulating factor (Pegfilgrastim). The BMMNCs treated group received a quantity of approximatively 1×10^6 BMMNCs, by infusion in the ipsilateral carotid artery. The time period of BMMNCs administration after stroke onset was between 12 and 32 days, with a mean of 22 days. At the beginning of this study, there were no significance and differences between both groups in NIHSS ($p = 0.364$), modified Rankin Scale (mRS) ($p = 0.452$), Barthel index (BI) ($p = 0.84$) scores were not significant and different in both groups. At the fourth month of the follow-up, a significant improvement in NIHSS within each group was observed, but without statistically significant comparisons ($p = 0.376$). After 12 months of follow-up both groups showed significant improvement in mRS and BI but aslo without statistical significance on comparison, with p = 0.290 for mRS and $p = 0.745$ for BI, respectively. The language deficit, which was evaluated via the Arabic version of the Comprehensive Aphasia Test, was also insignificant in both groups initially ($p = 0.513$); at the end of follow-up there was a marked improvement in both groups, but again without any statistical significance on comparison ($p = 0.691$). There were no severe complications during the treatment and follow-up which could be associated with the BMMNC autologous transplantation. This study confirms that such treatment is safe for ischemic stroke patients, but does not prove any improvement in outcomes associated with BMMNC transplantation.

Ashu Bhasin et al. [20] have carried out a randomized placebo-controlled clinical trial. 20 patients that have suffered an ischemic stroke and 20 age-matched healthy controls were included in this study. 20 patients were randomized and formed 2 groups, with 10 patients in each of them. One group was treated with BMMNC autologous transplantation and the other group with infused placebo. The subjects were diagnosed with ischemic stroke from 3 months up to 1.5 years before being included in the study. The BMMNC treated group received 10^6 BMMNC/kg. After 2 months,

there were no statistically significant differences between BMMNC treated group and the control group, according to modified Barthel index (mBI) (p=0.31) and Fugl Meyer (FM) scale for upper limb (p=0.25). Modified Ashworth scale (MAS) and the Medical Research Council (MRC) for muscle strength were statistically insignificant between the 2 groups (p>0.05). Also, the vascular endothelial growth factor (VEGF) and the brain-derived neurotrophic factor (BDNF) levels were found to be more elevated in BMMNC treated group compared to the control group, but without statistically significant differences (VEGF: 442.1 vs. 400.3 pg/ml, p = 0.67; BDNF: 21.3 vs. 19.5 ng/ml). There were no severe complications during the treatment or follow-up. This study confirms that BMMNC treatment is inoffensive for ischemic stroke patients.

Discussion

Therapeutic time window

There are reasons to consider the optimal therapeutic time window for BMMNCs autologous transplantation to be the subacute stage of the ischemic stroke, although there are some studies that suggest that this treatment could be effective even during the chronic stage [12]. One of the reasons to administrate BMMNCs in an optimal therapeutic time window is that these cells could support the endogenous neurogenesis, especially during its peak after stroke. In rodent stroke models, neural stem cells in the poststroke brain, in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus were observed, all of them capable of differentiating into new neurons. Between 7 and 10 days after stroke, there seems to be an increase in mitotic activity whithin the SVZ, then a decrease during weeks 3-5 is observed, and thereafter it continues at lower levels over the course of the following year [21, 22]. Other studies have pointed out that administration of BMMNCs in rodents between 2 and 14 days after stroke lead to significant positive effects [23].

A histopathological study conducted by Nakayama D et al. [24] has shown that the peak in endogenous neurogenesis in stroke patients occurs on the fourth day and 10-24 days after stroke. Temporal profiles of 2 markers in poststroke cortex: nestin- and musashi-1-positive cells were provided. Also, according to these temporal profiles, day 17 after stroke onset is the last day in which the levels of both of these markers were elevated at the same time, although the level of Musashi-1-positive cells were found to be raised up to 24 days after stroke.

In the first 24-72 hours after stroke, patients are usually neurologically unstable. In the sudy conducted by Sean I. Savitz MD et al. [13], the patients were treated with BMMNCs within 24-72 h after stroke. 2 out of 10 patients had infarct expansion between enrollment and harvest, and required hemicraniectomy after transplantation. In the study conducted by Maurício A. G. Friedrich and colleagues [15] it was also reported that a patient developed hemorrhagic transformation of his infarct before the BMMNCs transplantation (before day 3 poststroke), and a hemicraniectomy was performed 2 days after the IA infusion of BMMNCs.

In the Francisco Moniche study [14] the patients have been treated with BMMNCs between 5 and 9 days after stroke. Although no correlation between the functional status and the amount of transplanted BM-MNCs was detected, there was a trend towards a better outcome when higher numbers of CD34+ cells were injected, especially in the Barthel Index BI at 1 month after transplantation (r=0.57, P=0.09). Also, higher significance levels of β-nerve growth factor appeared in BM-MNC-treated patients than in control subjects; after 8 days these were 12.8±2.7 versus 3.9±2.5, respectively (p=0.029).

In the study conducted by Maurício A. G. Friedrich [15] the patients were treated with BMMNCs within 3 to 7 days from stroke onset, and satisfactory clinical improvement occurred in 6/20 (30%) patients at 90 days. 8 out of 20 patients (40%) showed a good clinical outcome.

In the study conducted by Akihiko Taguchi [18] patients have been treated with BMMNCs within 7-10 days after stroke. Although there were no statistically significant changes on NIHSS, iB (BI) and mRS between the patients that were treated with BMMNCs IV and the control group that was not, when comparing patients who received cell therapy with historical controls, a trend favoring improvement was observed in the group treated with bone marrow mononuclear cells. Also, the author has pointed out that analysis of cerebral blood flow and metabolism in patients after autologous BMMNC transplantation showed a trend favoring an increase in rCBF (regional cerebral blood flow) and $rCMRO_2$ (regional cerebral metabolic rate of oxygen).

In the study conducted by Valeria Battistella and colleagues [12], NIHSS scores were improved (range – 1 to 8 points) during follow-up in all patients, although they received intra-arterial BMMNCs 59–82 days after stroke. Even so, it should be noted that the patients from this study had a lower NIHSS score when they were included in this study (range between 4 and 13), comparing to other clinical studies [14, 15, 18].

In the study conducted by Kameshwar Prasad and colleagues [17], the time window for BMMNCs transplantation after stroke onset was 18.5 days (median), in the study conducted by Azza Abass Ghali [19] – the time period of 12 to 32 days, with a mean of 22 days poststroke onset, and in the clinical study conducted by Ashu Bhasin and colleagues [20] – 3 months up to 1.5 years after stroke onset. This time period could be a reason for which they did not point out any beneficial effects in stroke treatment.

Optimal cell transplantation route

An optimal cell delivery route should bypass the peripheral filtering organs, provide a maximal possible cell grafting and confirm a maximal safety for the patient. There were 3 types of transplantation routes used in these 9 clinical trials (Fig.1). In 4 studies, the route of choice was the intravenous route, in other 4 studies – the intra-arterial route, and only one study used the intrathecal route.
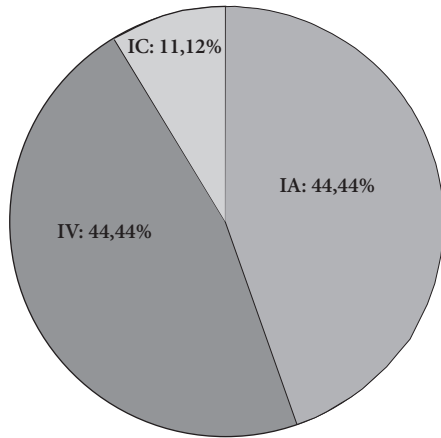
Fig. 1. Diagram showing different BMMNCs delivery routes.
IV – intravenous, IA – intra-arterial, IC – intrathecal

There are some concerns about safety regarding the intravenous and intra-arterial delivery routes, namely microemboli formation and development of microstrokes. On the other hand, the intrathecal route may result in most grafted cells, but it is also the most invasive one.

All the clinical trials have confirmed the safety for their chosen delivery route. There were no serious adverse reactions during the treatment or follow-up in all 9 studies linked to any of the chosen delivery routes. BMMNCs have a smaller size, comparing with other stem cells, for example mesenchymal stem cells (MSC), and a preclinical study has shown that infusion of BMMCs resulted in a 30-fold pulmonary passage increase as compared to a single MSC bolus [25]. Also, their smaller size decreases the risk of emboli formation in the blood. In studies that have chosen the intra-arterial delivery route, the infusions were performed using a microcatheter, which is considered to preserve the anterograde blood flow, and therefore to avoid the of microstrokes [26].

Unfortunately, only one study [12] has analyzed the biodistribution of the labeled BMMNCs. It has been concluded that at 2h after transplantation, the ^{99m}Tc-labeled cells were present in the brains of all patients, and the activity of the isotope was 0.6–5.1% of the activity in the whole body. At 24h, the cells were seen to be in the brain in only 2 out of 6 patients. Also, the author has mentioned that the absence of labeled cells in the brain of the remaining patients could be due to the decay of the radioactivity compound below the levels of detection and/or to the decrease in the number of cells at the lesion site. It is not possible to compare these transplantation routes and to conclude which one is more efficient, as the BMMNCs were administered in different time windows after stroke and the number of studies is too small. However, some observations could be made concerning a potential superior efficiency of the intrathecal route over the intravenous route. In the study conducted by Alok Sharma and colleagues [16] the patients were treated in the chronic phase (4-144 after onset) with a mean number of 10^6/kg BMMNCs via intrathecal route, and in the study conducted by Ashu Bhasin and colleagues [20] the

patients were treated similarly in the chronic phase (3-18 months after onset) with 10^6/kg BMMNCs. The first study has revealed that patients had a better outcome, as 38% have improved their functional independence measure (FIM) score, 50% improved in their ambulation, 42% in hand functions, 38% in walking balance and 25% in standing balance. By contrast, the second study did not find any significant improvement in patient's outcome, which can lead to the opinion that at least in the chronic phase the intrathecal route is more efficient. The major limitations here are that the study which used the intrathecal route is uncontrolled and the studies did not use the same clinical outcome measures. Another observation is that 3 out of 4 studies in which the intra-arterial delivery route was used have shown some encouraging results. The study conducted by Francisco Moniche et al. showed a trend towards a better outcome when higher numbers of CD34+ cells were injected [14], in the study conducted by Maurício A. G. Friedrich et al. [15] 40% of patients have shown a good clinical outcome, and in the study conducted by Valeria Battistella et al. [12] improved NIHSS scores during follow-up in all patients have been observed. Some of the limitations here are that the last 2 studies are uncontrolled, and in the study conducted by Valeria Battistella et al. the patients had a lower initial NIHSS comparing to other studies [14, 15, 18].

Cell dose

The range of the number of BMMNCs infused varies between 10^6 cells to 10^7 / kg cells (fig. 2, tab. 2). Each quantity has proven to be safe for autologous transplantation in poststroke patients. The number of cells to be infused was selected either by extrapolating the dose from rodents to humans based on their weight or brain size or was based on other clinical trials with cell transplantation.
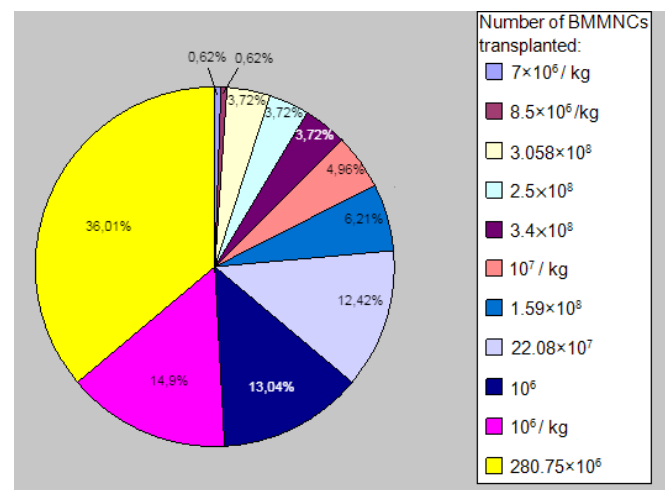

Fig. 2. Chart summarizing the percentage of patients that received certain doses of Bone Marrow-derived Mononuclear Cells (BMMNCs).

It is not possible to make an objective correlation of cell doses with a change in functional outcome as there are other variables that have a marked influence on it, for example

A comparison of the different BMMNCs transplantating doses in clinical studies

Authors and year of study	Transplantation dose (cells)	Time period of administration after stroke onset	Route of administration	Improvement in outcome
Valeria Battistella et al. 2010 [13]	$1 \times 10^8 - 5 \times 10^8$ (mean of 3.058×10^8)	Day 59 - 82	IA	+
Sean I. Savitz MD et al. 2011 [14]	7×10^6 / kg - 10×10^6 / kg	24 – 72 h	IV	++
Francisco Moniche et al. 2012 [15]	1.59×10^8	Day 5 - 9	IA	-*
Maurício A. G. Friedrich, et al. 2012 [16]	22.08×10^7	Day 3 - 7	IA	+
Alok Sharma et al. 2014 [17]	10^6 / kg	4 - 144 months	IC	+
Kameshwar Prasad et al. 2014 [18]	280.75×10^6	Day 7 - 30 (median of 18.5 days)	IV	-
Akihiko Taguchi et al. 2015 [19]	6 patients: 2.5×10^8 6 patients: 3.4×10^8	Day 7 - 10	IV	++
Azza Abass Ghali et al. 2016 [20]	10^6	12 - 32 days	IA	-
Ashu Bhasin et al. 2016 [21]	10^6 / kg	3 months - 1.5 year	IV	-

IV – Intravenous; IA – intra-arterial; IC – intrathecal;
"-" – no significant difference in patients outcome;
"+" – an improvement in patients' outcome but no control group in study;
"++" – an improvement in comparison with historical controls;
* – there were no significant differences in neurological function during follow-up, but a positive correlation trend between the number of CD34+ cells injected and Barthel Index was found (r=0.56, P=0.09).

the time window of administration, the route of administration and patient heterogenicity. It should be pointed out that one study [19] has tested 2 different dosages for 2 groups of 6 patients, one of which has received 2.5×10^8 BMMNCs, and the other one – 3.4×10^8 BMMNCs. The author has concluded that administration of the higher dose of BMMNCs consistently showed a trend towards enhanced neurologic recovery, although without statistically significant differences between groups.

Mechanisms of action

The protective mechanisms of action of BMMNCs are thought to be: stimulation of arteriogenesis and angiogenesis, modulation of local and systemic inflammation and secretion of neurotrophic factors.

Arteriogenesis and angiogenesis

After cerebral ischemia, especially after obstruction of the medial cerebral artery (MCA), there is usually a substantial injury of the neural tissue supplied by the artery. Nevertheless, a part of this tissue could be saved, as there are leptomeningeal collateral vessels from the anterior cerebral artery (ACA) and the posterior cerebral artery (PCA), which appears to allow for perfusion of some brain tissues to persist [27]. However, the arteriogenesis is relatively slow and self-limiting and cannot compensate sufficiently for MCA obstruction [28]. Thus, stimulation of arteriogenesis could be an important strategy in the treatment of ischemic stroke. BMMNCs contain endothelial progenitor

cells, which have been reported to contribute to revascularization of ischemic tissues [9]. In a preclinical study, Wang et al. reported that transplanted BMMNCs can differentiate into smooth muscle cells (SMCs) and endothelial cells (ECs) after permanent MCA obstruction in rats [29]. The differentiated cells exhibit an increased arteriogenesis (especially for leptomeningeal anastomoses) and angiogenesis by direct incorporation in collateral vessel walls. Other studies, as that conducted by Youshi Fujita et al. [30] did not find any evidence of direct structural incorporation of BMMNCs into ECs. Instead, donor BMMNCs with morphological features of pericytes were observed in the vessel walls. Another study has shown that BMMNC treatment induced an increase in vascular endothelial growth factor (VEGF) and Ser1177 phosphorylated endothelial nitric oxide synthase (eNOs) levels and resulted in an enhanced cerebral blood flow (CBF) in the acute phase [30]. Although the exact mechanism is not known, these preclinical studies show that BMMNCs promote arteriogenesis and angiogenesis through upregulation of eNOs, increasing of VEGF level in the blood, stimulation of endogenous EC proliferation and stimulating the direct differentiation into ECs and pericytes. The VEGF is a key mediator of arteriogenesis and angiogenesis. VEGF has been shown to increase vascular permeability and the proliferation of vascular endothelial cells and to inhibit endothelial cell apoptosis [31]. Unfortunately, there are few clinical trials that have evaluated the level of

VEGF after BMMNCs transplantation. In the clinical study conducted by Akihiko Taguchi et al. [18] a nonquantitative SPECT imaging was performed in a 48h window before cell transplantation, and at 1 and 6 months after cell transplantation the rCBF, rCMRO$_2$ and OEF were measured with a PET imaging. The author has pointed out that the analysis of cerebral blood flow and metabolism in patients after autologous BMMNC transplantation showed a trend favoring an increase rCBF in contralateral hemisphere and an increase in rCMRO$_2$ in both hemispheres. In parallel with the increase of rCBF, a decrease in OEF was observed in contralateral hemisphere. Although, it is important to point out that in 6 out of 12 patients these measures could not be obtained at either 1 or 6 months after treatment because of restlessness of the patient or maintenance/replacement of the PET machine. This study did not show any significant change in vascular endothelial growth factor (VEGF) after BMMNCs infusion. The clinical study conducted by Ashu Bhasin et al. [20] has shown the serum VEGF at baseline was higher in severely affected patients than in moderately affected patients (316.1 vs. 257.4 pg/ml), which remained high at 2 months predicting a good functional recovery. The study has also shown that at 2 months after BMMNCs transplantation, the patients treated with autotransplant had a higher level of VEGF than the control group (mean 453.5 ± 89.1 vs. 408.4 ± 93.3 pg/ml, 95% CI 13.3-6.7, p = 0.96), although without a statistical difference. The author made the conclusion that in chronic strokes (without classification into stroke subtype and volume), VEGF might have been increased already at acute onset in severely affected patients it stimulates angiogenesis and provides neuroprotection.

Modulation of inflammation

The brain responds to ischemic injury with an acute and prolonged inflammatory process, which tends to give rise to cytotoxic damage to the surviving neurons, neural glia and endothelial cells in the peri-infarct area [32]. Some studies have shown the BMMNC infusion can suppress inflammation. The study conducted by Francisco Moniche et al. [33] has shown that there is a negative correlation between the levels of matrix metalloproteinase-2 (MMP-2) at day 4 after transplantation and the number of CD34+ cells injected ($r = -0.667$, $p = 0.071$). Also, lower levels of MMP-2 at day 4 were correlated with lower neurological deficit (NIHSS at day 30) ($r = 0.775$, $p = 0.041$). MMP2 induce shedding of cytokines and growth factors and may contribute to the creation of a chemotactic gradient and subsequent immune cell recruitment to sites of vascular injury [34]. Another study conducted by Francisco Moniche et al. [14] revealed a positive correlation trend between the number of CD34+ cells injected and the BI ($r=0.56$, $P= 0.09$). On the other hand, a strong correlation was detected between serum levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) at day 90 after transplantation and the total number of BM-MNCs injected ($r = 0.929$, $p = 0.001$) and BM-MNC per kilogram injected ($r = 0.929$, $p = 0.003$). GM-CSF functions as a cytokine which stimulates stem cells to produce granulocytes and monocytes, thus promoting inflammation.

Secretion of neurotrophic factors and enhancing the neurogenesis

As stated before, the NSCs residing in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus are capable of producing new neurons in adult brains. Moreover, it is known that NSCs develop in the poststroke brain [35].

A histopathological study conducted by Nakayama D et al. has analyzed poststroke cerebral cortices in autoptic human brains and has confirmed that the NCSs are found in the human poststroke cortex [24]. Also, this study has shown that there is a peak in endogenous neurogenesis in stroke patients at the fourth day and 10-24 days after stroke. During this time period, it is absolutely essential to sustain the neurogenesis with neurotrophic factors. The study conducted by Francisco Moniche et al. [14] has shown that higher significance levels of β-nerve growth factor (β-NGF) appeared during the first week in BMMNC-treated patients than in control subjects: β-NGF levels after 4 days were 10.3±3.1 versus 8.5±2.9 ($P=0.68$) and after 8 days were 12.8±2.7 versus 3.9±2.5 ($P=0.029$). β-NGF is involved primarily in the growth, as well as the maintenance, proliferation, and survival of neurons. The study conducted by Akihiko Taguchi et al. [18] has shown an increase in brain-derived neurotrophic factor (BDNF) after infusion of 3.4 × 10^8 BMMNCs (2.721.7 ± 2.052.4 pg/mL at the baseline vs 4.319.0 ± 5.002.8 pg/mL 1 day after transplant) but without any statistically significant changes. Another study has also analyzed the level of BDNF but did not find any statistically significant improvement within 8 weeks between the group treated with BMMNCs and the control group (mean 32.8 ± 9.2 vs. 27.3 ± 9.1 ng/ml).

Conclusions

BMMNC autologous transplant is a safe therapy for patients that have suffered ischemic stroke without any severe complications associated. There are reasons to consider the subacute stage of the stroke to be the optimal therapeutic time window for this method of treatment. Although some clinical studies stated a better outcome in patients treated with BMMNC, further clinical trials are needed to establish their therapeutic efficiency.

Competing interests

The author declares no conflict of interests regarding publication of this paper.

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Direct-acting antivirals: a new strategy in the treatment of hepatitis C virus infection in patients with cirrhosis

Mariana Avricenco, MD, PhD Applicant

Department of Infectious, Tropical Diseases and Medical Parasitology
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chişinău, the Republic of Moldova

Corresponding author: avricencomariana@gmail.com
Manuscript received November 18, 2019; revised manuscript December 02, 2019

Abstract

Background: Hepatitis C virus (HCV) infection has a significant worldwide impact. Patients with hepatic cirrhosis with HCV have an annual risk of decompensation of 3-5%, a risk of developing hepatocellular carcinoma between 1.4-6.9% and a risk of mortality of 2% / year. Therefore, the treatment of chronic HCV infection is a priority for patients with severe hepatic fibrosis and cirrhosis. The emergence and approval of direct-acting antivirals (DAA) in recent years have revolutionized antiviral therapy, especially for patients with liver cirrhosis. Following numerous studies it has been found that, this treatment is well tolerated by these patients. The combination of DAA from different groups has a potent enhancing effect, and the sustained viral response (SVR) rate reaches up to 85-98% in patients with liver cirrhosis. In general, the chance of performing SVR with DAA in patients with compensated cirrhosis (Child-Pugh A) is comparable to non-cirrhotic patients. However, there is a risk for decompensation and acute liver failure during and after treatment. Patients with decompensated liver cirrhosis and advanced liver fibrosis may have greater benefit from antiviral therapy after liver transplantation.

Conclusions: The data obtained from the analyzed studies suggest that DAA antiviral therapy prevents the progressive evolution of the disease towards hepatocellular carcinoma or decompensation. At the same time, a correct therapeutic approach and a permanent monitoring of these patients can improve the quality of life, significantly prolonging the years of life.

Key words: direct-acting antivirals, cirrhosis, hepatocellular carcinoma, hepatitis C virus.

Introduction

Hepatitis C virus (HCV) infection is a disease with a significant global impact. According to the World Health Organization (WHO) data, 71 million people worldwide are infected with HCV [1]. About 700,000 people die annually from HCV complications, including cirrhosis, hepatocellular carcinoma (HCC), liver failure. In Western European countries, approximately 5 million people are infected with HCV, 40% of whom are in the stage of liver cirrhosis and 30% are candidates for liver transplant [1, 2].

In the Republic of Moldova the prevalence of HCV infection in the general population was estimated at 4.5-5.0%, with the prevalence of genotype (GT) 1b – 98% [3-5]. According to the cumulative data, at the end of 2016 in the Republic of Moldova, there were 15.400 people infected with HCV [6, 7]. At the same time, in the last years, there is an increase in the prevalence of cirrhosis through HCV from 21.4 (2005) to 52.8 (2014) cases / 100.000 inhabitants [5].

Due to the fact that most cases (about 80%) are asymptomatic, the actual incidence of HCV infection is much higher.

After HCV infection, the rate of chronicization is 55-85%, and the rate of development of cirrhosis after 20 years after infection is 15-30% for infected persons after the age of 40 [2, 8]. The evolution of the disease is not linear; the progression of fibrosis is accelerated after the age of 50, regardless of the infection [9, 10]. Patients with cirrhosis and HCV have an annual risk of decompensation of 3-5%, a risk of developing HCC between 1.4-6.9% and a risk of mortality of 2% / year [11-13].

In the context of the exposed data and the fact that the majority of patients take non-clinical forms, we can conclude that, from an epidemiological point of view, HCV is a problem, which has a negative impact on public health. Thus, the objectives proposed by WHO, included in the strategy for Global Health 2015-2030, are to increase the percentage of people tested for hepatitis C from 20% to 90% and those treated from 7% to 80% [1].

Direct-acting antiviral (DAA) treatment

Until hepatitis C was identified as an agent of non-A non-B hepatitis, Interferon (IFN) – alpha contributed to the normalization of transaminases and to the improvement of liver histology in some patients. Over time, the sustained virus response rate (VRR) increased from 5-20% in interferon monotherapy, to 40-50% in the combination of IFN and ribavirin (RBV) [14].

Due to the limited efficacy and secondary extensive side effects of standard pegylated alpha – IFN (PEG) and RBV antiviral combination therapy, new antiviral drugs were needed.

The opportunity to administer direct-acting antiviral drugs (DAAs) is a substantial advantage in the treatment of chronic HCV infection, having the possibility of oral administration, short duration of treatment, high sustained viral response (SVR), decreased liver stiffness, improved liver function, and minimal side effects [15, 16]. The combi-

nation of DAA from different groups has an enhanced potentiation effect, and the SVR rate reaches up to 85-98% in patients with cirrhosis [17, 18, 19].

The data obtained from the analyzed studies suggest that such treatments can extend the life span of the cirrhotic patients, preventing the progressive evolution of the disease towards HCC or decompensation. Thus, patients with an advanced degree of fibrosis and an increased risk of liver complications, as well as those with severe extrahepatic manifestations will have priority over immediate treatment, using the most advantageous therapeutic options.

A correct therapeutic approach and a permanent monitoring of these patients can improve the quality of life, significantly prolonging the life years.

Although there are still barriers that prevent the complete eradication of HCV infection, mutual international efforts to overcome them determine optimism regarding the future of treatment for this disease.

Treatment with DAA in cirrhosis with HCV infection: objectives, response to treatment, monitoring, adverse events

Liver cirrhosis represents the final evolutionary stage of any liver disease, being the consequence of destroying liver cells and reducing the ability of liver tissue to regenerate. The rate of chronicization and progression to cirrhosis is correlated with the age of infection (greater than 40-50 years), male sex, presence of HBV / HIV coinfection, alcohol consumption, severity of liver fibrosis, presence of steatosis [20-22]. For people with chronic infection, the risk of cirrhosis is between 15 and 30% for a period of 20 years [9].

At the same time, a diagnosis of compensated cirrhosis is associated with a 4.7 times higher risk of death compared to the general population, and decompensated cirrhosis is associated with a 9.7 times higher risk [22, 23].

The emergence and approval of DAA in recent years have revolutionized antiviral therapy, especially for patients with cirrhosis. Following numerous studies, it has been found that this treatment is well tolerated by patients with advanced liver disease [24, 25]. The current therapeutic possibilities have the advantage of being highly effective, and the main purpose of DAA therapy is to eradicate the infection as early as possible and to prevent the evolution of the disease in order not to reach the advanced stages of the disease.

Before making the decision in favor of a particular treatment regimen with DAA, several factors that may influence this therapy should be considered. First, the HCV genotype must be determined. Most DAA regimes are available and active against GT1. Second, previous antiviral therapies should be considered. Patients with relapse or unresponsiveness after treatment with PEG-INT and RBV still have high chances of viral eradication. However, previous treatments followed by DAA may be associated with resistance, which may influence the outcome of therapy with other DAA regimens [24, 25]. Here, resistance analysis is recommended to select an effective DAA combination. Also, the interaction between the drugs administered in the asso-

ciated diseases and those of the antiviral therapy with DAA should be checked.

Advantages of DAA administration in patients with liver cirrhosis:
  – Possibility of oral administration.
  – Short duration of treatment.
  – High SVR and minimal adverse reactions [18, 26, 27, 28].
  – Decreased hepatic stiffness (fibrosis) in patients with SVR [15, 29].
  – Improvement of liver function [30, 31].

Before initiating antiviral therapy, patients with liver cirrhosis should be examined in order to assess: presence / absence of esophageal varices, HCC and signs of hepatic decompensation (hepatic encephalopathy, ascites, etc.). In general, the chance of performing SVR with DAA in patients with compensated cirrhosis (Child-Pugh A) is comparable to non-cirrhotic patients. However, there is a risk of decompensation and acute liver failure during and after treatment [25]. Therefore, patients with advanced and decompensated cirrhosis should be treated and monitored in experienced centers, and the possibility of liver transplantation should be evaluated.

Patients with decompensated liver cirrhosis and advanced liver fibrosis may have greater benefit from antiviral therapy after liver transplantation [19, 32].

The combination of sofosbuvir (SOF) / daclatasvir (DCV) with / without RBV and SOF / ledipasvir (LDV) with / without RBV clearly influences hepatocytolysis syndrome in patients with hepatic cirrhosis, the transaminase profile being significantly improved at the end of treatment (88-95% of patients had normal values), recording the biochemical response [27, 33]. On the other hand, the combination of 2 DAA and RBV in patients with compensated liver cirrhosis showed a higher efficacy (SVR 96%), compared to the schemes without RBV (SVR 88%)[33, 34].

RVS rates are decreased (82-87%) in patients with decompensated cirrhosis, especially in those with platelets <75000 [17, 27]. Studies have shown that the effectiveness of DAA therapy decreases with the degree of decompensation of cirrhosis. Thus, the SOLAR-2 study evaluated the use of SOF / LDV and RBV in 329 patients with decompensated cirrhosis for 12 and 24 weeks. RVS rates at 12 weeks ranged from 87% to 96% for Child Pugh B patients and 72-85% for Child Pugh C patients (genotype 1) [30]. Similar data were obtained in the ALLY-1 study, patients being treated with SOF / DCV and RBV: the 12-week RVS rate was 96% in Child Pugh B patients and 56% in Child Pugh C patients [32, 35].

FDA (Food and Drug Administration) recommends 12 weeks of RBV treatment in naive patients with compensated / subcompensated cirrhosis [36]. The European Association for the Study of Liver Disease (EASL) recommends 24 weeks without RBV in patients with decompensated cirrhosis or those with pre / post liver transplant and 12 weeks with RBV in patients with compensated cirrhosis [37].

Afdhal N. et al. reported in a batch of 50 patients with

cirrhosis and HCV genotype 1 and 4 (60% Child Pugh B stage) in treatment with SOF and RBV, in 89% of patients a rapid viral response (RVR) was obtained) at week 4 of treatment and 97% at week 8 [18]. Out of a total of 108 patients with cirrhosis Child Pugh B genotype 1 and 4 treated with SOF / LDV and RBV, SVR was achieved in 89% of those who received 12 weeks of treatment [38]. It is remarkable that these rates of SVR are comparable to those for compensated cirrhosis or even non-cirrhotic patients. DAA treatment in patients with cirrhosis improves liver function by about 40% [2, 8, 31].

The combination paritaprevir (PTV) / ritonavir (RTV) / ombitasvir (OBV) plus dasabuvir (DVR) (3D regimen) was approved by the FDA in December 2014 for the treatment of HCV GT1 infection. The use of a 12-week PTV / OBV regimen stimulated with RTV with RBV (without DVR) in the treatment of HCV GT4 infection is studied in studies PEARL-1, AGATE-1 and AGATE-2. PEARL-1 is a study of 91 naïve patients with cirrhosis, where all patients had SVR [39]. The AGATE-1 and AGATE-2 studies added the results of the PEARL-1 study by including patients with cirrhosis. All participants in the AGATE-1 study had cirrhosis, where 97% SVR rates were reported (59/61) [40]. The AGATE-2 study investigated patients with and without cirrhosis. In these cohorts, SVR rates of 97% (30/31) and 94% (94/100) were obtained. Extending treatment duration to 24 weeks did not increase SVR rate in patients with cirrhosis [41].

The OPTIMIST-2100 Phase III study had patients with HCV GT1 cirrhosis who were treated with SOF / simeprevir (SMV) for 12 weeks. RVS rates made up 83% (86/103) [26].

In June 2016, the FDA approved the first pangenotypic regimen – SOF / velpatasvir (VEL), which introduced a new era of DAA therapy. This combination simplifies the management of HCV infection treatment, because the need to determine the genotype before initiating antiviral therapy disappears. ASTRAL-1-5 studies have confirmed the pangenotypic efficacy of SOF / VEL, as well as the efficacy of this regimen in HIV co-infection and in decompensated liver disease [42-44]. SOF / VEL with / without RBV has been shown to be an effective pangenotypic therapeutic option including in cirrhosis with HCV.

The American Association for the Study of Liver Diseases (AASLD) and EASL recommend the administration of DAA regimens containing SOF with one of the following preparations: LDV, VEL, DCV in combination with RBV in patients with decompensated cirrhosis [2, 45].

The EASL recommends monitoring with abdominal ultrasound and alpha-fetoprotein (AFP) every 6 months, for early detection of HCC, for all patients with FibroScan> 9.5 kPa (Metavir ≥ F3) [2]. The EACS (European AIDS Clinical Society) recommends surveillance only for cirrhotic patients, and FibroScan> 12.5 kPa is considered to indicate cirrhosis [46]. The occurrence of esophageal varices after SVR is rare, if varicose veins were not present at pre-treatment endoscopy. Endoscopic control for varicose veins is recommended every 2 years after SVR in all patients with cirrhosis [26]. According to the Baveno VI statement, patients with

compensated cirrhosis can avoid endoscopy provided they have platelets> 150,000 and FibroScan <20 kPa [47].

Invasive assessment of hepatic gradients of venous pressure before and after antiviral treatment showed a partial regression and normalization in most patients with portal hypertension who had SVR [48].

Although, it has been shown that an SVR for antiviral treatment with DAA induces regression of liver cirrhosis and reduces the risk of mortality in cirrhotic patients, however, a significant risk for HCC development, cholangiocarcinoma and hepatic decompensation is still present, and long-term surveillance is mandatory. The results of the studies showed that, in these patients, the risk is significantly reduced compared to those who failed the treatment [49-52].

Adverse events of DAA therapy

There are few studies describing the adverse events (AE) associated with DAA therapy in patients with liver cirrhosis. A study aimed at AE research included 102 patients (74% cirrhosis) with chronic HCV infection who underwent DAA therapy for 12 or 24 weeks. All patients received SVR. About 90% of patients reported at least one AE associated with current treatment. The most common AEs reported were: fatigue (43%), headache (42%), neuropsychiatric symptoms (30%) and nausea (26%). Neuropsychiatric symptoms were more frequent in patients with previous antiviral treatment experience compared to naive patients [28].

Current guideline recommendations support the use of SOF-based DAA regimens in combination with LDV, VEL or DCV, with or without RBV, for the treatment of HCV infection in patients with cirrhosis. NS3 / 4 protease inhibitors (Telaprevir, Boceprevir and Simeprevir) are not recommended in cirrhosis because of their potential to aggravate liver disease. Apart from SOF that is mainly excreted by the kidneys, most DAAs are metabolized by the liver with bile excretion as a major pathway. Therefore, in patients with severe renal impairment (glomerular filtration rate <30 ml / min), the administration of SOF is contraindicated and treatment of HCV infection should be postponed until after transplantation. At the same time, data from some studies suggest that SOF therapy can be used safely and effectively in those with chronic kidney disease in stages 4 and 5, although patients with compensated liver disease were included in the studies [53, 54].

Most EAs are related to the administration of RBV, so dose adjustment is needed. RBV-induced anemia may be moderate / severe, requiring dose adjustment or withdrawal of therapy with this preparation. In patients with decompensated cirrhosis, it is suggested to administer RBV with an initial dose of 600 mg / day and increased depending on the tolerability of the patients.

It has been noted that, most commonly, adverse reactions to RBV manifest in patients with a higher degree of cirrhosis [16, 33, 55]. Studies in such patients have shown that RBV cancellation or dose reduction during treatment does not significantly influence the virological response to treatment [30, 33, 56]. However, patients with hepatic cirrhosis

require hospitalization at the initiation of antiviral therapy, mainly due to complications caused by the disease.

The effectiveness of the PTV / RTV / OBV plus DVR combination is similar compared to LDV / SOF. However, the 3D regimen has two main disadvantages: the greater number of pills administered per day and the potential drug interactions [45]. The most common adverse events encountered during approval studies were: sleep disorders, nausea and pruritus. Increases of bilirubin to more than three times the upper limit of normal value were more frequently seen in patients with cirrhosis (9.7%). Relevant increases of ALAT were also noted. An Israel multicenter cohort study reported 7 patients who received PTV / RTV / OBV plus DVR and developed decompensation within 1 to 8 weeks of starting therapy, and one patient died [57]. Due to these possible hepatotoxic effects and worsening of liver function in patients with advanced disease, this treatment cannot be recommended for patients with decompensated cirrhosis[58]. Patients with compensated cirrhosis who receive this regimen should monitor the clinical picture and symptoms of hepatic decompensation, being subjected to hepatic laboratory tests at the beginning and at least every 4 weeks during therapy [45].

Hepatic fibrosis

Hepatic fibrosis, following chronic infection, is the most important factor related to HCV morbidity and mortality.

Hepatic fibrosis is a prognostic marker for the evolution of HCV infection. Thus, three types of fibrosis progression can be identified: the rapid progressive type (develops cirrhosis in less than 20 years), the intermediate type (develops cirrhosis between 20-50 years), the slow progressive type (without evolution towards cirrhosis or very slow evolution in more than 50 years) [59].

The researchers identified several factors that influence the regression or evolution of fibrosis. There were no significant associations with the patient's sex, age, race / ethnicity, other medical conditions or complications of cirrhosis [60, 61]. However, diabetes and esophageal varicose veins have been associated with a lower likelihood of fibrosis improvement [29].

A meta-analysis of 111 studies revealed that fibrosis progression was nonlinear, with an estimated risk of cirrhosis of 16% and 41% after 20 and 30 years of infection respectively [61]. Other studies have also shown nonlinear development, with major acceleration of fibrosis progression after age 50 [62].

When advanced fibrosis develops (stage F3 after the METAVIR scale), the risk of progression to cirrhosis is approximately 10% per year.

Studies were conducted with monitoring of the fibrosis degree in cirrhosis with HCV after treatment with DAA obtained SVR. Thus, in a study in which 65 people with cirrhosis were evaluated after DAA treatment, 55% showed improvement and 45% of the fibrosis remained unchanged. It was found that the average time to improvement was 2.5-3.0 years from the time of initiation of therapy, indicating that those with less severe hepatic injury have a faster improvement [15].

HCV infection has increased the interest for the study of the cellular and molecular mechanism of hepatic fibrosis, with a view to identifying effective therapeutic, etiological, pathogenic and antifibrotic means.

Hepatocellular carcinoma

Patients who develop liver cirrhosis prior to initiation of antiviral therapy should be maintained in the HCC surveillance program because the risk of malignant development is high, even if elimination of SVR infection is achieved.

The appearance in the liver cirrhosis of regenerative nodules or hyperplastic nodules is the main alarm signal, because following genetic changes that occur during repetitive cell proliferation, these nodules change into dysplastic nodules, a process that leads to liver damage [63].

Numerous studies have been carried out over the years, targeting patients who have developed CHC following antiviral therapies [28, 37-39].

In the study that included 103 patients with a history of HCC in a previously treated (surgical) history with a complete response (absence of characteristic nodules), they received DAA treatment. Patients pretreated with IFN in the background were not included in the study. After a monitoring of approximately 6 months, 18 patients had recurrence of HCC with the development of the characteristic intrahepatic tumor nodules [64].

On the other hand, another study conducted on 819 patients evaluated the risk of developing HCC after DAA treatment compared to patients treated with IFN. It was found that rates of HCC development did not differ between patients treated with DAA and those receiving IFN. At the same time, all patients who developed HCC were in the stage of liver cirrhosis [65].

Data from some studies have shown that patients with cirrhosis and HCC have SVR rates (74%) lower than patients with cirrhosis, but without HCC (91%) [66, 67].

There was also a correlation between HCC and high levels of alpha-fetoprotein, platelet count ≤110 x109 / l, advanced fibrosis (F4), adverse effects on more common antiviral therapy, RVS rate in comparison with lower DAA with those who do not develop HCC (87.3 vs 95.5%) [68].

Until recently, the category of patients with HCC associated with viral hepatitis C had a lower survival rate than those with HCC of other etiology, due to the lack of effective treatment for the underlying hepatitis C virus infection, but the new interferon-free therapies significantly improved these figures.

Treatment with DAA in patients with chronic HCV hepatitis does not increase the risk of developing HCC, so this treatment is considered a method of preventing the progression of the disease to cirrhosis and HCC.

Conclusions

1. Interferon-free treatment simplified therapeutic behavior and exponentially reduced adverse effects.

2. New generations of drugs (DAAs), which provide high SVR, are the best and reasonable option including for patients with advanced liver cirrhosis.

3. Based on the results presented in different clinical studies, it is recommended to initiate DAA therapy earlier in order to ensure a faster decrease in liver stiffness after treatment.

4. The choice of antiviral treatment regimen and its duration is individualized according to the degree of fibrosis, genotype, concomitant diseases and adverse effects that may occur.

5. In patients with hepatic cirrhosis, DAA therapy has been shown to be the most effective prevention for the development of hepatocellular carcinoma.

6. After obtaining SVR, patients with liver cirrhosis, however, present a significant risk for developing hepatic decompensation, so long-term surveillance is mandatory.

7. The risk of HCC and mortality is significantly reduced, but not completely eliminated in cirrhotic patients who have obtained SVR, as opposed to untreated patients and patients who do not get SVR, especially in the presence of other causes of hepatic impairment: metabolic syndrome, consumption of alcohol and co-infection with HBV / HIV.

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