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Welcome to the Moldovan Medical Journal!

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

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

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ORIGINAL RESEARCHES

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Micromolecular inhibitors of superoxide radicals

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Abstract

Background: Currently, there is a growing interest in new copper (Cu^{2+}) heterocyclic coordination compounds (CC), isothiosemicarbazide derivates, which demonstrated multiple beneficial properties, but their effect on reactions with free radicals such as the superoxide radical has not been investigated.
Material and methods: The action of new micromolecular complexes of copper (Cu^{2+}) chloride and bromide with methyl n- (prop-2-en-1-yl) -2- (pyridin-2-ylmethylidene) hydrazine carbimidothioate on capturing activity of the superoxide radical was determined by the spectrophotometric method *in vitro* experiments.
Results: It was established that the micromolecular complexes of copper (II) chloride and bromide with methyl n-(prop-2-en-1-yl)-2-(pyridin-2-ylmethylidene) hydrazine carbimidothioate have been found to possess strong superoxide radical inhibitor properties when interacting with a superoxide radical. In addition to this, the IC_{50} of the studied compounds depends on the nature of the acid-ligand in the internal sphere of the complex and increases in the following sequence: Cl^-–Br^-.
Conclusions: The established property of mentioned compounds is new, because their use as micromolecular inhibitors of superoxide radicals has not been described so far. The synthesized CC expand the arsenal of superoxide radical inhibitors with high biological activity. Their possible significance for the development of new treatment strategies for diseases associated with the overproduction of superoxide radicals is discussed.
Key words: superoxide radical inhibitors, coordination compounds, isothiosemicarbazide derivates.

Cite this article
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Introduction

In the pathogenesis of acute and chronic degenerative diseases (the most common diseases) an important role is attributed to reactive oxygen and nitrogen species (ROS/RNS), in particular, the superoxide radical $O_2^{\cdot-}$, which from a biological point of view, can be generated from two major sources: mitochondrial respiratory chain and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase – an enzymatic complex found in the plasma membrane, as well as in the membranes of phagosomes of nucleated polymorphic leukocytes of the blood to destroy microorganisms [1-3].

The superoxide radical $O_2^{\cdot-}$ – a product of the mitochondrial respiratory chain is also a crucial component of the immune system defense. Due to the high reactivity, superoxide radicals $O_2^{\cdot-}$ through their potential to oxidize nucleic acids, proteins, lipids or carbohydrates are responsible for multiple harmful actions on the body, such as inflammation, cancer, cardiovascular disease, hypertension, ischemia / reperfusion, diabetes, neurodegenerative diseases (Alzheimer's and Parkinson's disease), rheumatoid arthritis, alcohol-induced liver disease, ulcerative colitis, senescence and atherosclerosis. The free radical scavengers presence in biological systems from a wide variety of sources endogenous and / or exogenous, limits the harmful effects of oxigen radical species (ORS), allowing the body to fight efficiently in various pathological situations, limiting the lesions and not allowing their spread [4-7].

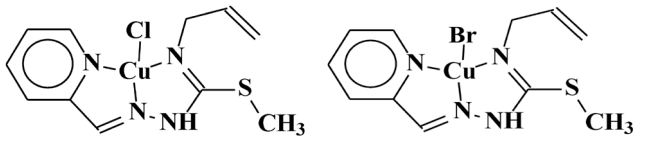
Therefore, the therapeutic inhibition of superoxide radicals is a new contribution, because the compounds with antiradical activity show a strong curative effect, thus preventing multiple harmful actions on the body.

Respectively, one of the priority directions of modern applied chemistry is the synthesis of new compounds, which capture and neutralize ORS, thus preventing the development of cell and tissue damage, including various pathologies caused by exacerbation of free radicals.

The aim of the study is to elucidate the effects of new copper (Cu^{2+}) heterocyclic coordination compounds (CC), isothiosemicarbazide derivates, on oxidation processes with free radicals, such as superoxide radical $O_2^{\cdot-}$, which can be used to prevent and treat many multifactorial acute and chronic diseases.

Material and methods

At the State University of Moldova, in the Laboratory of Advanced Materials in the Biopharmaceutical and Technical Field was synthesized a number of new copper coordination compounds, from the class of isothiosemicarbazides, in particular, the coordinating compounds of copper (2+) - dichloro- [methyl-N-(prop-2-en-1-yl)-2-(pyridin-2-ylmethylidene)-hydrazinecarbimidothioate] copper (**compound I**) and dibromo-[methyl-N-(prop-2-en-1-yl)-2-(pyridin-2-ylmethylidene) hydrazinecarbimiothioate] copper (**compound II**) of the general formula:



I II

The synthesis of compounds I and II, their structure, physicochemical, antimicrobial and anticancer properties have been described [8-10].

Quantitative measurement of free superoxide radicals is difficult due to their exceptional reactivity and short half-life. The most commonly used in biological and chemical systems are classical methods, which are based on the generation of superoxide radicals by the phenazine metosulfate / nicotinamide adenine dinucleotide reduced system (PMS / NADH) by oxidation of NADH, and superoxide radicals reduce the tetrazolium salt – Nitro Blue Tetrazolium (NBT) in blue-purple formazane. Briefly, the capturing activity of the superoxide radical was determined by the spectrophotometric method, described in the literature [11, 12] with some modifications.

At first, the working dilutions of tested compounds in dimethylsulfoxide (DMSO) solution so that the final concentrations are equal to 0.1; 1.0; 10.0; and 100 μmol /L were prepared. Next, 20 μl of each working dilution of tested compounds was poured into the wells of the 96-well microplate and 180 μl of reagent mixture containing 0.02 M phosphate buffer (pH 7.4), 0.1 mM NADH and 0.09 mM nitro-blue tetrazolium (NBT) was added. Each dilution was poured in duplicate. The control samples (in duplicate) were mounted in the same way as the test samples, but the dilutions of the tested compounds were replaced with an equivalent volume of solution containing 0.02 M phosphate buffer (pH 7.4). The microplate was shaken for 10-15 s and the absorbance (A) was measured at 560 nm [A_0]. Then, in all wells, 20 μl of 8.0 μM solution of phenazine metosulphate (PMS) was added, and all the sample was shaken for 10-15 s and was incubated for 5 minutes at room temperature, after which the absorbance was measured again [A_1] at 560 nm. The percentage of *superoxide radicals scavenging activity* was calculated according to the formula:

Superoxide radical scavenging activity (%) = [(A_0 –A_1)/A_0 x 100];

where: A_0 is the absorbance of the control; and A_1 is the absorbance of the test compounds or of the standard and / or reference substances.

As a standard for determining the s*uperoxide radical scavenging activity* – quercetin (3,3',4,5,6-pentahydroxyflavone), which is a natural flavonol, was used [13].

Of all the chemical compounds described in the literature which contain the 4-allyl-S-alkylisothiosemicarbazide moiety and which inhibit superoxide radicals ($O_2^{\cdot-}$) the highest antiradical effect was obtained in the case of compound bis (m_2-acetate-o)-bis {[N-prop-2-en-1-yl-N'- (pyridin-2-ylmethylidene) carbamo-hydrazonothioate] copper} dihydrate (prototype and structural analogue) [14] with the formula:

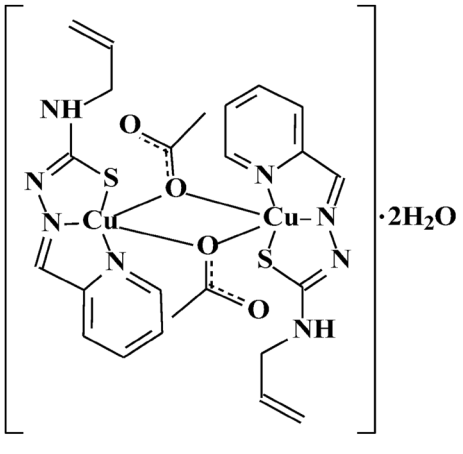


Fig. 1. Chemical structure of the compound bis (m_2-acetate-o)-bis {[N-prop-2-en-1-yl-N'-(pyridin-2-ylmethylidene) carbamo-hydrazonothioate] copper} dehydrate (structural prototype) [14]

Results and discussion

The obtained experimental data presented in tab. 1 demonstrated that the compounds I and II manifest high antiradical activity in the IC_{50} range equal to 0.05-0.06 μmol/L which is 6-7.0 times higher than the antiradical activity of the prototype and the structural analogue [compound bis (m_2-acetate-o)-bis {[N-prop-2-en-1-yl-N'- (pyridin-2-ylmethylidene) carbamo-hydrazonothioate] copper} dihydrate] [14], which manifests the highest antiradical effect among all biometal complexes with ligands from the thiosemicarbazonic class, described in the literature.

In addition to this, as can be seen from the presented

data, the IC_{50} of the studied compounds depends on the nature of the acid-ligand in the internal sphere of the complex and increases in the following sequence: Cl-–Br-.

Table 1. Superoxide radical scavenging activity of tested compounds compared to prototype and structural analogue

Tested compound	IC_{50}, μmol/L
Quercetin (3,3',4,5,6-pentahydroxyflavone) [1]	61.86±2.5
Bis (μ_2-acetate-O)-bis {[N-prop-2-en-1-yl-N'- (pyridin-2-ylmethylidene) carbamo-hydrazonothioate] copper} dehydrate (prototype structural analogue) [1]	0.35±0.07
Dichloro- [methyl-N- (prop-2-en-1-yl) -2- (pyridin-2-ylmethylidene) –hydrazine-carbimidothioate] copper (**compound I**)	0.06±0.01
Dibromo - [methyl-N- (prop-2-en-1- yl) -2- (pyridin-2-ylmethylidene) hydrazinecarbimidothioate] copper (**compound II**)	0.05±0.01

The established property of above mentioned compounds I and II is new, because their use as micromolecular inhibitors of superoxide radicals has not been described yet.

Comparative analysis of compounds I and II with the prototype and the structural analogue demonstrates that they differ in that the monodeprotonated salicylidene moiety of azomethine is eventually replaced by the neutral picolinic moiety, due to which the six-atom metallocycle in the structural analogue composition changed to the five-atom metallocycle and the coordination nodes formed by isothiosemicarbazone are changed from O, N, N to N, N, N. In the compounds claimed in addition, in the internal sphere of declared compounds nitrate-ion and water are replaced by chlorine and bromine ions. Due to these changes in the internal sphere of compounds I and II, a new combination has already known chemical bonds.

Detected properties of dichloro- [methyl-N-(prop-2-en-1-yl)-2-(pyridin-2-ylmethylidene)-hydrazincarbimidothioate] copper and dibromo- [methyl-N-(prop-2-en- 1-yl)-2-(pyridin-2-ylmethylidene) hydrazine carbimidothioate] copper are of interest to medicine in terms of expanding the arsenal of highly efficient synthetic inhibitors of superoxide radicals.

After antiradical activity tested compounds are over 1030-1237 times more effective than quercetin, used as a standard for determining the activity of superoxide radical inhibition and 6-7 times more effective than the most active synthetic antiradical inhibitor described in the literature [Bis (m_2-acetate-O)-bis {[N-prop-2-en-1-yl-N'-(pyridin-2-ylmethylidene) carbamo-hydrazonothioate] copper dihydrate (prototype, structural analogue)].

The high toxicity of the superoxide anion is due to its ability to inactivate enzymes containing critical iron and sulfur groups in a wide variety of metabolic pathways, thus releasing free iron into the cell, which can undergo the Fenton reaction and generate highly reactive hydroxyl, as well as other free radicals that can damage important molecules, such as DNA, proteins, lipids and carbohydrates [15].

The human body is under constant attack by these radicals formed as a result of normal metabolic activity; therefore, there are a number of defense mechanisms against free radicals (FR), including antioxidant enzymes and non-enzymatic compounds. The involvement of FR in the etiology and progression of several acute and chronic clinical disorders has generated a growing interest in the search for substances that can eliminate FR and increase the antioxidant defense system.

There are several antioxidant tests to evaluate the antioxidant potential of different substances. For this purpose are widely used various variants of the ABTS radical cation discoloration test ($ABTS^+$) [16]. This method is based on the spectrophotometric determination of the discoloration rate of the blue-green chromophore $ABTS^+$ [(2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)] when an antioxidant is added. $ABTS^+$ is added to ABTS and discolors it. $ABTS^+$ is a stable radical that has not been found in the biological systems and the human body, at the same time, the main impediment is the inability to determine / evaluate and quantitatively separate the activity of inhibition of the radical superoxide (O_2^-) is explained by the fact that the indicated method is non-specific, able to determine / evaluate the summary annihilation activity of various reactive oxygen and nitrogen species (ROS/RNS), such as the superoxide radical ($O_2^{\cdot-}$), hydroperoxyl radical (HO_2), hydroxyl radical, hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), lipid peroxyl radical (LOO^-) etc., but the rate of uptake of each of these radicals cannot be appreciated, because it depends on several factors that are difficult to take into account. Note that $ABTS^+$ radicals can be annihilated by both hydrogen and electron transfer, and the dominant reaction varies depending on antioxidant, solvent and pH, complicating the comparison of results in different systems.

Thus, ABTS is an indirect method based on the reduction of various persistent radicals, which does not allow to appreciate exactly the inhibitory activity of the superoxide radical (O_2^-).

The shortcomings of the $ABTS^+$ cationic radical method and its limitations are reflected in a number of publications and synthesis articles [17-21].

Thiosemicarbazones continue to attract the interest of researchers as potential anticancer drugs. Moreover, in recent decades, thiosemicarbazones have found wide application as effective remedies for a variety of diseases, including tuberculosis, viral infections, malaria and a number of multifactorial diseases. Regarding malignancies, the class of α-N-heterocyclic thiosemicarbazones should be considered as drugs that influence various biological pathways in a complex mode of action and with multiple cellular signaling targets [22]. Note the particularly high selectivity that normal cells have, which manifests itself on a stronger redox balance of them [23].

The antitumor activity of these compounds could relate to the disturbance of the cancer cells homeostasis, in par-

ticular the interruption of pivotal pathways for signaling cancer cells [24].

It should be noted, moreover, that highly concentrated oxygen ventilation in patients with low SpO_2 levels can produce an extremely large number of harmful superoxide free radicals, which occurs as in extremely severe patients with SARS-Cov-2 infection. In this aspect micromolecular inhibitors of superoxide radicals could counteract the negative effects of oxidative stress and inflammation; they could improve the severity of the disease and treatment outcomes, especially in patients with underlying complications of COVID-19. Further investigations are needed in this direction line to provide effective strategies for treating and preventing the complications of this terrible disease of the third millennium [25-27].

Therefore, the therapeutic inhibition of the superoxide radicals is a new contribution, because the micromolecular compounds with antiradical activity show a strong curative effect, thus preventing multiple harmful actions on the body.

Further studies have to confirm the therapeutic utility of this bioactive micromolecular compound under investigation.

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Authors' contribution
LA and VP conducted/performed the laboratory work, biochemical investigation; AG conceptualized the idea; IS, VG VM and VM designed the research, reviewed statistics and interpreted the data, wrote the first draft; VG revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Ethics approval and consent to participate
The research protocol was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (protocol No 81 of 19.09.2020). No animals or human subjects were used in this work.

Conflict of Interests
The authors declare that there is no conflict of interests.

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Maternal morbidity of adolescent pregnant women

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Abstract

Background: In Mexico, it is estimated that the adolescent population represents 29% of the population of childbearing age. The present study aimed to analyze the obstetric results of 3310 adolescent pregnant women attended in a third level hospital.
Material and methods: All records of pregnant women aged 19 years or less up to the date of admission were analyzed at the *Mónica Pretelini Saenz* Maternal Perinatal Hospital during the period from January 2018 to June 2020, with the following variables: age, pregnancy, resolution obstetric, severe preeclampsia, preeclampsia and gestational hypertension.
Results: A total of 13874 pregnant women were attended, of which 3310 (24%) patients were adolescents. The overall frequency of obstetric complications was 21%, including obstetric hemorrhage (13%) and hypertensive disorders of pregnancy (8%). Regarding postpartum obstetric hemorrhage events, classified according to the Advanced Trauma Life Support shock scale, they were categorized as Grade 1 – 338 cases, Grade II – 76 cases, Grade III – 11 cases and Grade IV – 1 case. Hypertensive disorders of pregnancy highlight preeclampsia as the most frequent with a total of 97 cases, followed by 89 cases of severe preeclampsia, 58 cases of gestational hypertension, 14 cases of chronic hypertension and 3 cases of chronic hypertension with preeclampsia.
Conclusions: The main complications found in the Mexican pregnant adolescent population were obstetric hemorrhage, which was more frequent in the population aged 15 to 19 years, and hypertensive disorders, which occurred more frequently in the population aged 9 to 14 years.
Key words: adolescence, maternal morbidity, preeclampsia.

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Introduction

According to the World Health Organization (WHO), an average of 16 million adolescents, between 15 and 19 years of age, account for 11% of annual births worldwide. The WHO defines adolescence as the period of human development between childhood and adulthood (early adolescence: 10-14 years and late adolescence: 15-19 years) [1].

In Mexico, it is estimated that the adolescent population represents just over 18% of the total population and 29% of the population of childbearing age; the main biological problem of adolescent pregnancy is the lack of maturity for gestation. A determining factor of reproductive health in adolescents is reflected in the first trimester, when there is the importance of transgenerational events that make it possible to reduce the main diseases that affect pregnancy, such as diabetes, hypertension, cancer, preeclampsia, prematurity, intrauterine growth restriction, etc. [2].

Complications during pregnancy and childbirth are the second leading cause of mortality worldwide in this population. In this regard, the WHO reports that 830 women die daily from these complications and that 95% of them occur in developing countries [3]; in Latin America, almost half of the female population has had at least one delivery before the age of 20 years [4].

In the same line, the National Institute of Statistics, Geography and Informatics (INEGI), in 2018 published the data informing that in Mexico 17.5% of births are to teenage mothers; and teenage pregnancy continues to be one of the main contributors to maternal and infant mortality. Complications of pregnancy and childbirth are the leading cause of death among girls aged 10-19 years worldwide [5, 6].

Adolescent mothers (aged 10-19 years) face higher risks of eclampsia, puerperal endometritis, and systemic infections than women aged 20-24 years [4]. Besides, about 3.9 million unsafe abortions occur among girls aged 13 to 19 years each year, contributing to maternal mortality and long-lasting health problems [7]. Also, the emotional, psychological, and social needs of pregnant adolescents may be greater than those of other women.

Early childbearing can increase risks for newborns as well as for young mothers. In low- and middle-income

countries, babies born to mothers younger than 19 years face increased risks of low birth weight, preterm delivery, and serious neonatal conditions [4]. In some settings, rapid repeated pregnancy is common for young mothers, presenting additional risks to both mother and child [8].

Some research has concluded that these adverse events are due to age, and even to biological immaturity that may predispose very young mothers to subclinical infections; to an increase in the production of prostaglandins, with a consequent increase in the incidence of preterm delivery and fetal growth restriction [9, 10]. The present study had the purpose of analyzing the obstetric outcomes of adolescent pregnant women attended in a third level hospital in Toluca, Mexico.

Material and methods

This was a retrospective and descriptive study of patients admitted to the *Mónica Pretelini Sáenz* Maternal Perinatal Hospital (MPSMPH), Health Institute of the State of Mexico (HISM), Toluca, Mexico.

All the records of pregnant women aged 19 years from January 2018 to June 2020 were analyzed with the following variables: age, gestation, obstetric resolution, severe preeclampsia, preeclampsia, and gestational hypertension.

The present study followed the ethical principles of human research stipulated in the Declaration of Helsinki [11] and was considered risk-free since only information from the hospital databases was collected. The ethics committee MPSMPH, approved the protocol (code: 2021-03-721).

The descriptive analysis was performed according to the nature of the variables: continuous variables were expressed as means and medians with standard deviation, while categorical variables were summarized as frequencies and percentages. Also, a measur were used for continuous variables. All statistical analyses were performed using the SPS® program, version 23.0.

Results

During the time of this survey, 13874 pregnant women were attended in the MPSMPH, of which 3310 patients were adolescents (24%). The median age was 17 years (range 9 to 19 years), 42.5% of the patients were between 17 and 18 years old, however, 908 patients were younger than 15 years old and there was a record of 4 patients aged 9 years. 59% (1952) resolved the pregnancy vaginally, 33% (1081) through the abdominal route, and 8% (277) underwent uterine evacuation.

The overall frequency of obstetric complications was 21%, including obstetric hemorrhage (13%) and hypertensive disorders of pregnancy (8%); but there were no maternal deaths. The diagnosis of hypertensive disorders of pregnancy was made according to the time of onset of clinical data, gestational age of onset, and biochemical alterations. The most frequent diagnosis was preeclampsia with a total of 97 cases, followed by preeclampsia with a severity of 89 cases, gestational hypertension – 58 cases, chronic hypertension with 14 cases, and chronic hypertension with added preeclampsia with 3 cases. It should be noted that there were no cases of eclampsia, as well as admissions to the Obstetric Intensive Care Unit (O-ICU) with a total of 256 cases.

As for postpartum obstetric hemorrhage events, classified according to the Advanced Trauma Life Support shock scale, they were categorized only according to the estimated amount of loss: Grade I with a total of 338 cases, Grade II with 76 cases, Grade III with 11 cases, and Grade IV with only 1 case that was sent to the O-ICU.

Discussion

Compared to other Latin American countries, the timing of sexual debut in Mexico is later [12]. However, 15% of our population were between 9 and 16 years old; thus, it can be affirmed that there are indicative data that the sexual life beginning of the Mexican population is moving to early ages. The results of this study may be useful, for example, to estimate at what age it is convenient to vaccinate young people against human papillomavirus or to know when it is the right time to include information on family planning methods.

It was documented that the resolution of pregnancy was 59% vaginally, which is consistent with the findings of Amaya J. [9] and Fory JL. [10], where 33% of pregnancies were resolved by cesarean section; disagreeing with a study conducted in Nepal by Maharjan M. et al. [13], where they studied 2050 pregnant adolescents and reported a cesarean section rate of 11.9%, well below our results; the variation could be because this study was conducted in a rural hospital. According to the Nepal Demographic Health Survey in 2016, the total cesarean section in adolescents constituted 24%; adolescent pregnancy is higher in South Asian countries due to the common practice of early marriage and the social expectation of having a child soon after marriage.

Garcia-Salgado A. et al. in 2019 [14] mentioned that adolescent pregnancy carries a five times higher risk of maternal mortality compared to adult pregnant women; however, at the MPSMPH adolescent maternal deaths in the period from January 2018 to June 2020 were not confirmed; attributing it to the interdisciplinary management of this unit. The results of the present study show that adolescent pregnancy is associated with complications due mainly to obstetric hemorrhage and hypertensive disorders of pregnancy, these findings are consistent with those reported in the literature consulted [10, 14-18].

Finally, eight percent of the pregnant adolescents presented hypertensive diseases; most frequently preeclampsia (97) and preeclampsia with severity (89); studies show that adolescents of younger ages (< 15 years) seem to be the most prone to suffer from this complication [14, 17].

Although no statistically significant differences were observed in the present study concerning the frequency of threatened abortion, urinary tract infections, gestational diabetes, and ectopic pregnancies, a higher frequency of obstetric hemorrhage episodes were detected, representing 13% of pregnant adolescents, in agreement with studies conducted in populations, such as Argentina, Guatemala, India, Kenya, Pakistan and Zambia [17] and international, multi-

center registries from countries with limited resources [19].

Conclusions

The main complications encountered in Mexican pregnant adolescent population were obstetric hemorrhage, which was more frequent in the population aged 15-19 years and hypertensive disorders, which occurred more frequently in the population aged 9-14 years.

Current health programs have privileged the care of children and the elderly, leaving the adolescent population uncovered.

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Authors' contribution

CAPC conceptualized the project and designed the research, collected the data and drafted the manuscript; JAH interpreted the data and drafted the manuscript; DAAL interpreted the data, drafted the manuscript and revised the manuscript critically; LPG collected the data; RAMO revised the manuscript critically; HMZ made statistical analysis, drafted the manuscript and revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of *Mónica Pretelini Sáenz* Maternal-Perinatal Hospital, Toluca, Mexico (the protocol code: 2021-03-721).

Conflict of interests

No competing interests were disclosed.

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Chondrocytes isolation from hyaline cartilage by continuous monitoring method

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Abstract

Background: Articular cartilage has poor regenerative capacities. Numerous cartilage repair techniques are known, including implantation of autologous chondrocytes.
Material and methods: From 18 rabbits pieces of cartilage were harvested from femoral condyle. Minced cartilage was treated with 0.25% trypsin-EDTA. In the 1st group (n=9) the cartilage was digested with 0.6% collagenase in 15 ml tubes by shaking in incubator at 37°C, 5%CO_2. In the 2nd group (n=9) digestion was performed in 25cm^2 cell culture flasks placed on the lateral side, monitoring the process under a microscope after 120 minutes. The isolated cells were cultured to a 80-90% confluence. The chondrocytes were identified using histochemical staining after culturing for 16 days in overconfluence.
Results: Chondrocytes isolation in the 1st group lasted a fixed 360 minutes, in the 2nd group – 140±10 minutes. In the 1st group were isolated $9.2x10^4$±$3.1x10^4$ chondrocytes with a viability of 85.36±16.41%, but in the 2nd group – $1.6x10^5$±$3.4x10^4$ chondrocytes with a viability of 98.09±3.85%. The mean period of cell culture in the 1st group was 15±2 days, in the 2nd group – 11±3 days. In first passage of the 1st group were obtained – $1.2x10^6$ ±$4.3x10^5$ chondrocytes and in the 2nd group – $2.92x10^6$ ±$3.6x10^5$ chondrocytes. The secreted extracellular matrix by chondrocytes was stained specifically for cartilaginous tissue.
Conclusions: The method used for chondrocytes isolation has a direct impact on the number of isolated cells, their viability, but also upon the culture period and the number of cells obtained during the first passage.
Key words: cartilage, chondrocytes isolation, continuous monitoring.

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Introduction

The cartilaginous articular tissue has a poor regenerative capacity [1-4], as a result, the lesion of this tissue can have serious consequences upon the diarthrodial joints, especially the large ones [3, 5-7]. It is known that the number of patients with articular cartilage lesions increases about for a million cases per year [7]. Several nonsurgical and surgical methods (microfractures, allo- or autologous osteochondral tissue transplantation, etc.) have been used as a strategy to repair, or to stimulate the reparation processes of articular cartilage defects, resulting in formation of fibrocartilaginous tissue with reduced mechanical properties and faster wearing compared to hyaline cartilage [5, 8]. The method for treating cartilage defects is autologous chondrocyte transplantation technique, which currently represents the "Golden Standard" in the regeneration of articular cartilage [9, 10]. The method is used particularly in treatment of articular cartilage defects in the knee joint. It consists in harvesting pieces of hyaline cartilage from non-weight bearing articular surface of the femur, and release of chondrocytes from it by enzymatic digestion, their culture *in vitro* and subsequent transplantation in an articular defect, using a three-dimensional collagen matrix [11,13,14], or covering the defect with a periosteal flap and subperiosteal inoculation of chondrocytes [2, 6, 7, 13, 15]. Being a dynamic process, consisting of many stages, isolation of chondrocytes from articular hyaline cartilage is one of the essential steps of the treatment method. This involves one or more types of enzymes for enzymatic digestion process of cartilage to release the chondrocytes. Their speed depends directly on the enzyme concentration [4, 16-19]. Different periods of time are recommended for enzymatic digestion of cartilage to obtain a sufficient number of cells needed for culture, from 3 to 24 hours, depending on used enzyme combination [11, 16, 19]. Isolation of a large number of chondrocytes allows to reduce the period for cell culture and the number of cell passages in order to obtain a sufficient number of cells for transplantation in a short period of time. Continuous monitoring of chondrocyte isolation process from hyaline articular cartilage has reduced the time period for chondrocyte isolation and obtaining of a larger number of cells for culture. This is very important, because chondrocytes de-

grade in cells with a fibroblastic phenotype called chondrocyte dedifferentiation. As a result, the quality of synthesized extracellular matrix decreases, becoming fibrocartilaginous [1, 20, 21].

Material and methods

In the study were used 18 domestic rabbits 5±1 months old, 8 females and 10 males, with an average weight of 3.4±0.6 kg. To perform the experiments the following solutions and culture media were prepared:

1. Chondrocyte culture media: DMEM culture media (Sigma, UK) – 500 ml; fetal bovine serum (VBS) (Lonza, Belgium) – 55 ml; fibroblast growth factor (FGF) (Prospec, USA) – 5.5 μg; transforming growth factor β-2 (TGFβ-2) (Prospec, USA) – 550 ng / ml; vitamin C – 13.75 mg; 200 mM ultraglutamine (Lonza, Belgium) – 5.5 ml; penicillin – 55000 U; streptomycin – 55 mg; amphotericin B – 137.5 μg.

2. Collagenase solution 0.6%: by dissolution of 600 mg collagenase from Clostridium histolyticum (Sigma, UK) in 100 ml DMEM culture media (Sigma, UK).

3. Tripsin-EDTA solution 0.25%: Hank's Balanced Salt Solution (HBSS) without calcium and magnesium, with phenol red (Gibco, UK) – 100 ml; EDTA – 37.2 mg (Sigma, Germany); Trypsin from porcine pancreas (Sigma, USA); 0.1 N NaOH solution.

All solutions were prepared in sterile conditions under a laminar air flow hood and sterilized by filtering at 0.22 μm (Sofra, China).

Isolation and culture of chondrocytes

Under general anesthesia (with 5 mg/kg xylazine, 35 mg/kg ketamine and 2 mg/kg diazepam), under sterile conditions, from the non-weight bearing surface of the femoral condyle at the knee joint, pieces of articular cartilage were taken and placed in a 15 ml test tube filled with preheated chondrocyte culture media. The wound was sutured and the animals were isolated in vivarium. The harvested cartilage, under sterile conditions in laminar flow hood (LN 090, Nuve) was washed with warm PBS (HiMedia, India) 3 times. Then the cartilage was minced with a scalpel to fragments of 1-2 mm^3 and placed in a sterile 15 ml tube with 5 ml of 0.25% trypsin-EDTA solution and shaked for 10 minutes in a orbital thermo-shaker (ES-20, Biosan) at 150 rpm, 37°C. The tube was centrifuged for 3 minutes at 50 x *g*. After supernatant removal, the cartilage pieces were washed with warm PBS and centrifuged again (fig. 1).

The cartilage pieces were resuspended in 2 ml of 0.6% collagenase solution [16], with subsequent separation into 2 groups. In the 1st group (n = 9) the cartilage pieces with collagenase were transferred to 15 ml tubes, and in the 2nd group (n = 9) they were introduced in a 25 cm^2 cell culture flasks (Nunc, Denmark) positioned on the lateral side (fig. 2). With the slightly opened cap, the vessels with pieces of cartilage and collagenase were introduced into an incubator (HealForce, Start Cell) at 37°C, 5% CO_2 on a rocker shaker (MR-1, Biosan) and shaked at 20-25 oscillations per minute. In the 1st group, the cartilage pieces were subjected to an enzymatic digestion with 0.6% collagenase for 6 hours [16].

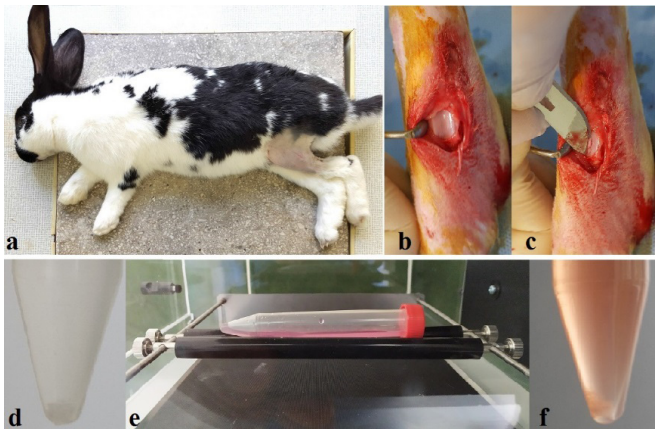

Fig. 1. Articular cartilage harvesting and trypsinization: (a) animal preoperative management, (b) knee joint opening, (c) cartilage harvesting from the non-weight bearing surface of the femoral condyles, (d) cartilage after mincing and washing, (e) treatment with 0.25% trypsin-EDTA, (f) articular cartilage after trypsinization

In the 2nd group, the process was monitored under microscope after 120 minutes of enzymatic digestion every 10-20 minutes, with the interruption of the process when chondrocytes were spread in large number (occupying 50-60% of field of view), even if the cartilage pieces were incompletely digested. Collagenase digestion was stopped by diluting the solution with 10 ml of preheated PBS. To separate the cells from the undigested cartilage, the solution was filtered through a 70 μm pore nylon filter (Sigma, UK) and centrifuged at 170 x *g* for 10 minutes. After supernatant removal, the chondrocytes were washed with 10 ml of chondrocyte culture media and centrifuged repeatedly. After centrifugation, the chondrocytes were resuspended in 5 ml of chondrocyte culture media, the cells number and viability were measured with the hemocytometer by trypan blue exclusion method (1, 2, 3). Chondrocytes were seeded in 25 cm^2 cell culture flasks (Thermo Fisher, Sweden) at different densities ranging from $2x10^3$ to $8x10^3$ living cells per cm^2 and incubated at 37°C, 5% CO_2, changing half of the culture media every 2-3 days. At 70-80% chondrocytes confluence, the cells were detached from the cell culture surface with 0.25% trypsin-EDTA, counted and used by destination: transplantaion, cytotoxicity tests for cryopreservation.

No of cells/ml = Average No of cells in *n* squares x Dilution fraction x 10^4 (1);

Total No of cells = No of cells/ml x Volume (2);

% living cells = (No of living cells x 100%)/Total No of cells (3);

Chondrocytes identification

From one passage $5x10^5$ chondrocytes were taken and placed in two 25 cm^2 cell culture flasks in equal number. The cells were cultured in overconfluence for 16 days, with complete changing of culture media every 2 days. After culture in overconfluence the specific cartilaginous extracellular matrix secreted by chondrocytes was identified by

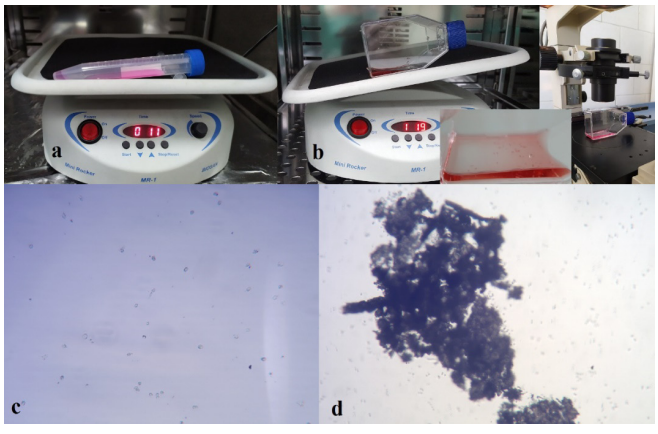

Fig. 2. Enzymatic digestion with 0.6% collagenase on a rocker shaker in the incubator: (a) in a 15 ml tube for 360 minutes and (b) cartilage enzymatic digestion in a cell culture flask positioned on the lateral side with the possibility of monitoring under a microscope of chondrocytes releasing process, (c) the isolated chondrocytes (x60) and (d) the chondrocytes released from cartilage (x40)

specific staining with Safranin O and Toluidine blue with Fast Green.

As a control group in chondrocytes identification process were used the 3rd passage of primary rabbit bone marrow mesenchymal stem cells (MSC) which were stored at -85°C [22]. The $5x10^5$ MSCs were thawed and suspended in 10 ml culture medium consisting of DMEM/F-12 Ham (Sigma, UK) with 10% FBS (Lonza, Belgium) and antibiotics with antimycotic solution. After pipetting, in two 25 cm^2 cell culture flasks were introduced by 5 ml of cell suspension. MSCs were cultured parallely with chondrocytes in incubator at 37°C, 5% CO_2 completely changing the culture media every 1 to 2 days [23].

The staining for chondrocytes identification with Safranin O was realised after removing of cell culture media from the cell culture flask and cells washings with PBS. In cell culture flask were added 5 ml of 0.1% glutaraldehyde in PBS for 20 min at room temperature. After 3 consecutive washings with PBS, 3 ml of 0.001% Fast Green were added for 5 min and 1% acetic acid for 10 seconds. The cells were washed repeatedly with PBS and 3 ml of 0.1% Safranin O solution were added for 15 minutes. After repeated washings, 2 ml of PBS were added for microscopic examination. For Toluidine blue and Fast Green staining, after removing of cell culture media, 3 ml of 0.4% Toluidine blue were added for 10 minutes. The cells were washed gently 3 times for 30 seconds with dH2O followed by addition of 3 ml of 0.02% Fast Green. The cells were washed again 2 times with dH_2O and other 2 ml of dH_2O were added for microscopic examination [24].

Results

As a result of this new method implementation, the number of isolated chondrocytes has increased significantly. The time period of cartilage exposure to collagenase digestion in the 1st group was 360 minutes, whereas in the 2nd group this period varied, having an average of 140±10 minutes. As a result, in the 1st group were isolated $9.2x10^4±3.1x10^4$ chondrocytes, while in the 2nd group, using continuous monitoring, were isolated almost 2 times more cells – $1.6x10^5±3.4x10^4$ chondrocytes (p = 0.005) (fig. 3).

The duration of enzyme exposure, also influenced the viability of the isolated cells, not just their number. After cells counting with hemocytometer by exclusion with trypan blue, in the 1st group was obtained a cell viability of 85.36%±16.41%, and in the 2nd group – 98.09±3.85% (p = 0.081). An uncritical difference, but in case of isolation of a small number of cells, a low viability may have negative effects on the cellular culture potential. Since the number of viable cells cultured in the first passage was about $7.5x10^4±2.1x10^4$ and in the 2nd group – $1.6x10^5±3.5x10^4$ (p <0.0001), the time for chondrocyte culture including adhesion to the cell culture surface, multiplication, formation of cellular colonies with a tendency to confluence and reaching a confluence of 70-80% was different in both groups. In the 1st group the culture period of the first passage was 17±2 days, but in the 2nd group – 11±3 days (p <0.0001) (fig. 4). As

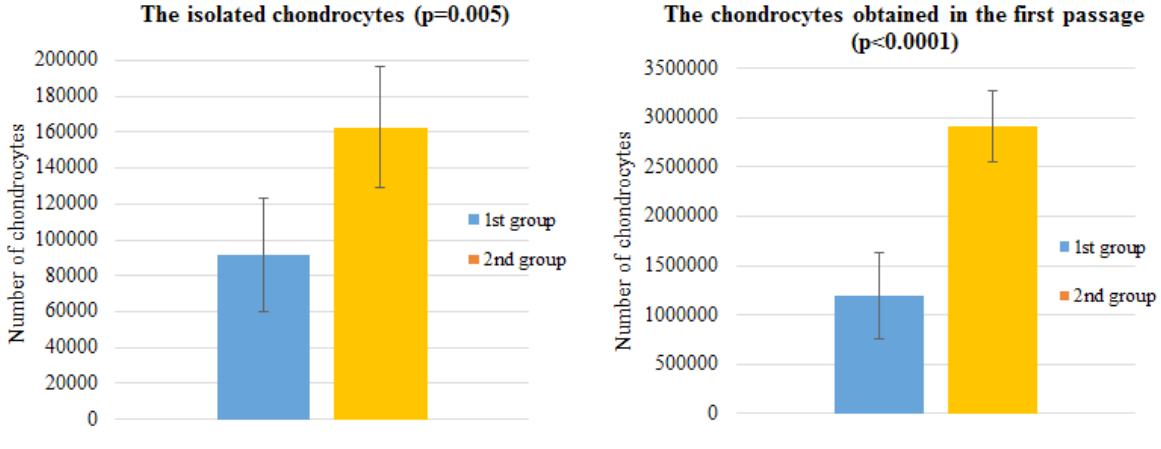

Fig. 3. Comparative presentation of isolated chondrocytes and the number of total chondrocytes obtained in the first passage

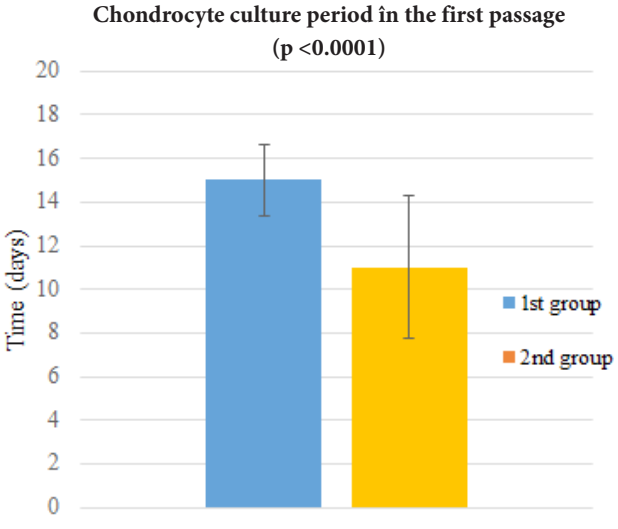

Fig. 4. Chondrocyte confluency during the first passage

a result, the number of cells obtained in first passage by both groups was different. In the 1st group were obtained $1.2x10^6 \pm 4.3x10^5$ chondrocytes, and in the 2nd group – $2.92x10^6 \pm 3.6x10^5$ cells (p <0.0001), with a 100% viability in both groups.

The histochemical staining techniques of the extracellular matrix secreted by chondrocytes during 16 days of culture in overconfluence, allowed to stain the secreted matrix in red-orange with Safranin O, while in the control group (MSC) the staining was absent or poorly expressed. The same is characteristic for Toluidine blue and Fast green staining, where the secreted by chondrocytes extracellular matrix was stained blue-purple, and the staining of extracellular matrix also was absent. This indicates that the isolated cells from hyaline articular cartilage secrete cartilage-specific extracellular matrix and are chondrocytes, but no difference was observed between the stainings of both chondrocytes groups (fig. 5).

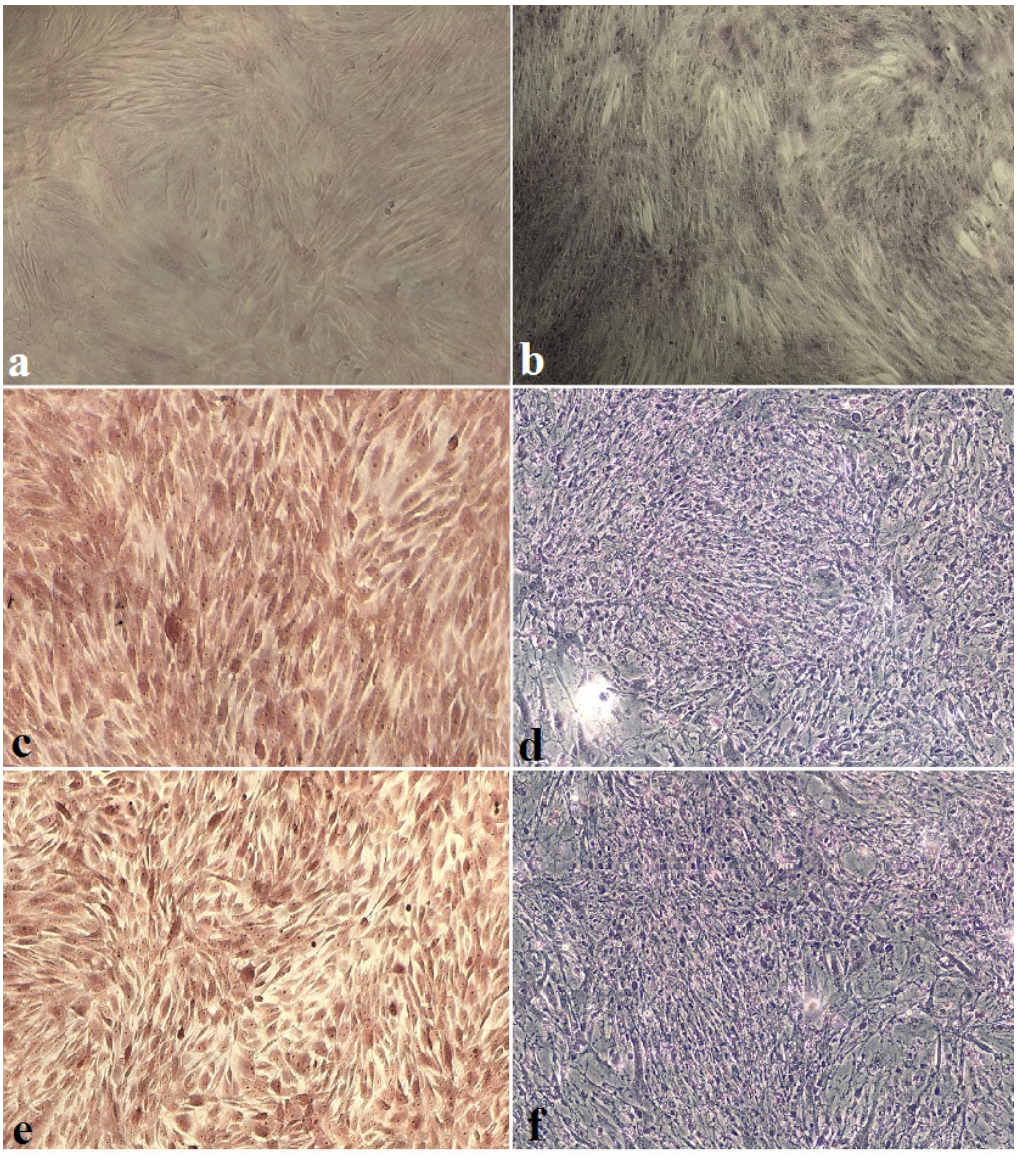

Fig. 5. Cartilage specific staining with Safranin O and Toluidine blue with Fast green (x100): Mesenchymal stem cells – control group (a, b); the 1st group chondrocytes (c, d), the 2nd group chondrocytes (e, f)

Discussion

Chondrocytes isolation and culture is an important step for the autologous chondrocyte transplantation process for articular cartilage repair [2, 6, 7, 9, 11-14]. Chondrocytes can also be used *in vitro* to test the effects of different substances [25], implants, grafts designed for treatment of cartilage defects or other intra-articular lesions leading to osteoarthritis of the joints [17, 26].

The surgical procedure for autologous chondrocyte transplantation has several weak parts, related to both the surgical and the laboratory component. Namely, the laboratory stage ensures the treatment procedure with a sufficient number of chondrocytes, but, in addition to the high risk of contamination and infection of isolated chondrocytes, a negative factor in the culture process to obtain a sufficient number of cells for transplantation is the fibroblastic degradation of chondrocytes, also called chondrocyte dedifferentiation [1, 15, 19]. This dedifferentiation is characterized by changing of chondrocyte shape from round to spindle-like, characteristic for fibroblast, decreased expression of GAGs, COL2A1 and ACAN genes, enhancing the expression of COL1A1 genes. As a result, reducing the synthesis of glycosaminoglycans, type II collagen and aggrecan, but stimulating of type I collagen [1, 19-21], which intensifies during cultivation, starting with the second passage [19] and becomes evident after 5 consecutive passages [20]. As a result, reducing the time period for chondrocytes culture in passages is mandatory because it reduces not only the risk of chondrocyte cultures infection, but also the degree of their degradation [27]. To obtain a sufficient number of normal chondrocytes for transplantation, in the literature are described methods of chondrocyte redifferentiation, such as chondrocytes culture on non-adherent surfaces [17], culture on the three-dimensional matrices with their subsequent transplantation [20], culture in condition with a low oxygen pressure [28], mechanical stimulation [29], and utilisation of chondrocyte differentiation factors [30] which we used. Continuous monitoring of chondrocyte releasing process by enzymatic digestion of articular cartilage is aimed to obtain a large number of chondrocytes cultured in a reduced number of passages to obtain the required amount of cells. When a small number of viable chondrocytes is obtained and they are seeded at a density less than 3-3.5x10^3 chondrocytes/cm^2, is observed formation of isolated, nonconfluent chondrocyte colonies as in 7 cases from the 1st group (fig. 6), or stagnation of cellular multiplication. Once the number of cells is sufficient to form uniform cell colonies on a cell culture surface, the number of obtained cells per passage will be higher. The isolated growth of cell colonies dictated by a small number of seeded cells results in occupation of a reduced part of the cell culture surface and, as a result the number of cells obtained per passage is smaller.

Another effective method for chondrocytes obtaining without involvement of enzymatic digestion of articular cartilage is the explant method [14, 23]. Since after isolation of chondrocytes from articular cartilage by continuous monitoring permanently remain undigested pieces of cartilage, it is reasonable to combine both methods, which consists in initial release by enzymatic digestion of a large number of condocytes with following utilisation of undigested cartilage as an explant (fig. 7). Also, undigested cartilage pieces

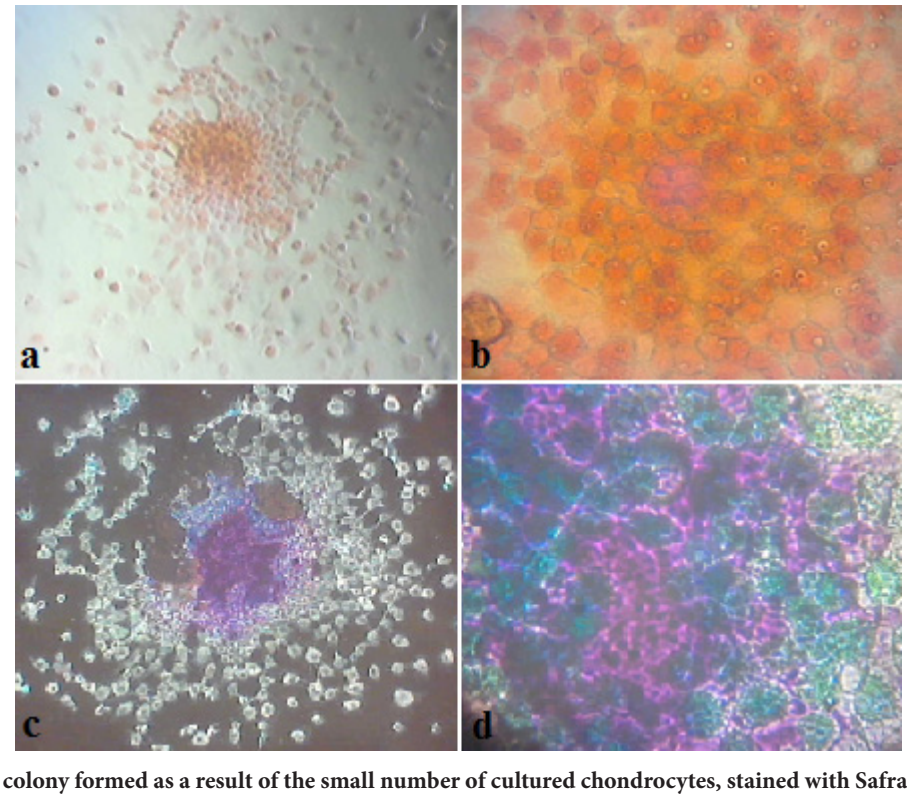

Fig. 6. Isolated cell colony formed as a result of the small number of cultured chondrocytes, stained with Safranin O (a) (x100), (b) (x150) and Toluidine blue with Fast green (c) (x100), (d) (x150)

may undergo a repeated enzymatic digestion with a fresh dose of collagenase solution.

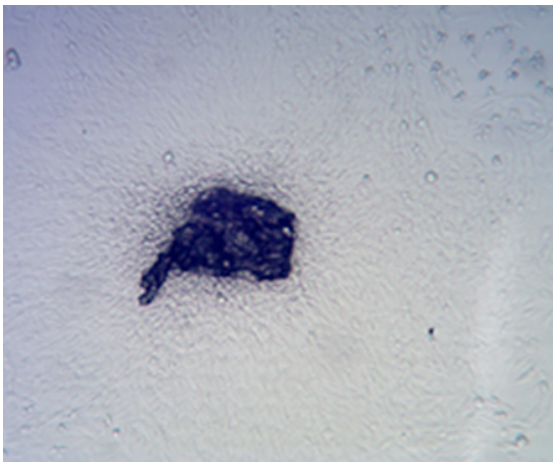

Fig. 7. Chondrocyte colony formation around an explant (x60)

Identification of isolated cells is a mandatory part of cellular isolation and culture process not only for chondrocytes but and for other types of cells [22, 23]. Histochemical methods, such as Safranin O and Toluidine blue with Fast green stainings used for histological analysis of cartilage, are qualitative methods and were sufficient to identify the presence of glycosaminoglycans and proteoglycans specific to cartilaginous tissue in chondrocytes cultured in overconfluence.

Conclusions

The short period of enzymatic exposure of cartilage during chondrocyte isolation directly influences not only the number but also the viability of isolated cells. Microscopic monitoring of the enzymatic digestion process allowed to isolate approximately 2 times more viable cells ($p < 0.0001$) in a shorter period of time, and in the first passage the time period for chondrocyte culture was almost one week shorter ($p < 0.0001$).

Isolation of a large number of viable chondrocytes directly influenced in the first passage duration for cell culture and the number of obtained cells in the first passage. Although there is a segnificant difference between the quantity of isolated chondrocytes in both methods, comparative histochemical examination did not reveal any differences related to the secretion of specific extracellular cartilaginous matrix after culture in overconfluence of the second celular passage.

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Authors' contributions
VC conducted literature review, obtained raw data and wrote the manuscript; LV monitored the experiment and critically revised the manuscript; MJ interpreted the data and drafted the manuscript; VN conceptualized the idea, designed the research and monitored the experiment. All the authors approved the final version of the manuscript.

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Ethics approval and consent to participate
The project was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 31, 14.12.2016).

Conflict of Interests
No competing interests were disclosed.

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Aspects of frailty syndrome, nutritional status and comorbidities in the elderly

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Abstract

Background: Frailty syndrome is one of the most important multifactorial medical syndromes, which is characterized by a decrease in functionality of many systems and organs.
Material and methods: In order to establish the nutritional determinants that contribute to the onset of frailty syndrome, a study was performed on a group of 50 patients, aged ≥65 years with chronic pathologies and geriatric syndromes. All participants were examined according to clinical features (history, clinical examination), Mini Nutritional Assessment and of the Complex Geriatric Assessment, which included: the data of the frailty tools, age category, Vulnerable Elders Survey, Charlson Comorbidity Index, autonomy – Activity Daily Living, Instrumental Activity Daily Living, Tinetti scale, psychoaffective status – by memory test Mini-Mental State Examination and the Geriatric Scale of Depression in the context of nutrition in the elderly. A clustered analysis (*k*-means method) of nutritional status showed that the most relevant indicators that separated the clusters were: age category, gender, clinical scale of frailty, comorbidities and polymedication.
Results: Frailty through the multidimensional aspects that it meets has an increased prevalence among the elderly with an unfavorable prognosis. Following the proposed study, it was revealed that insufficient nutrition and comorbidities can lead to the weakening of the institutionalized age. The results obtained by evaluating the bio-psycho-social aspects characterize the profile of the institutionalized elderly and can be used as a basis for the development of effective strategies aimed at reducing physical, cognitive and social frailty.
Conclusions: The comparative evaluation between both groups of elderly people by gender, showed a normal nutritional status with a higher share in women in the group of 75-84 years, compared to older men, and malnutrition was practically manifested equally in both groups in the study (men/women).
Key words: nutritional status, frailty, comorbidities, elderly.

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Introduction

The concept of frailty is increasingly attracting the interest of specialists, being widely used in scientific research and medical practice. According to consensus of international experts in 2013, frailty is characterized by a decrease of functionality in several systems and organs, accompanied by increased vulnerability to stressors, falls, frequent hospitalizations, long-term care and increased mortality [1-3]. A major problem is the fragile lonely and institutionalized elderly persons [4-6].

Frailty, comorbidity and disability are three interdependent clinical entities, which have only recently been shown to be distinct, and their multidimensional assessment should insist on a geriatric clinical decision. Recent research has shown that there is a constant category of frail people with no comorbidities or disabilities [2, 7]. In the context of new changes resulting from the need to formulate certain approaches to solving the problems of the elderly, there is a need for new scientific research in the field of geriatrics, so one of the most relevant, but at the same time little understood, is the frailty syndrome one of the most important multifactorial medical symptoms.

Frailty often precedes disability, while disability and comorbidity can contribute to the development of frailty [8]. In addition, increased attention was paid to the subtypes of frailty: social, functional, nutritional and cognitive [9, 10]. At the same time, the role of nutrition as a means of delaying frailty in the elderly is well established.

Insufficient dietary intake is often associated with multiple conditions, such as: increased risk of chronic diseases and osteoporotic fractures, impaired immune responses, frailty. Assessment of nutritional status and diagnosis of malnutrition requires a variety of nutritional screening tools and laboratory biochemical markers [10].

Currently, the literature does not fully elucidate the nutritional determinants that would contribute to the emergence of frailty syndrome and its impact on public health. Identifying and integrating these factors would help phy-

sicians and geriatric care teams in developing prevention and treatment strategies for the vulnerable population. Numerous recent studies have highlighted data on the prevalence of frailty and various factors that most often influence its appearance and development. The presence of frailty is considered a predictor of negative prognosis and high rates of morbidity and mortality. Given the increasing incidence of different types of frailty among the elderly population in many countries and the unfavorable prognosis of frail patients, there is a need for a number of clinical trials to implement measures for primary and secondary prevention and effective methods of preventing and treating frailty and, in particular, nutritional frailty.

The purpose of the study was to assess aspects of frailty syndrome, nutritional status and comorbidities according to the gender of the elderly.

Material and methods

The epidemiological study was part of the Institutional *Project 20.80009.8007.25 Frailty: diagnosis and prevention in relation to the medico-psycho-social problems of the vulnerable elderly*, with a positive opinion of the Ethics Committee with No 51 of 16.06.2020, which included 50 elderly people between 65 and 93 years old, institutionalized at the Republican Asylum for the Disabled and Retired.

The inclusion criteria were: the elderly ≥65 years with chronic somatic pathologies and geriatric syndromes, and the exclusion criteria: the elderly with various forms of dementia and oncology. The study was conducted in November-December 2020. Members were enrolled in research only after signing the informed consent to participate in the study.

All participants were examined according to clinical features (history, clinical examination) and the Complex Geriatric Assessment, which included: the data of the frailty phenotype and the frailty index – Fried's criteria, Gröningen Frailty Index (GFI) [11, 12], nutritional score – Mini Nutritional Assesment (MNA) [13], sarcopenia – SARC-F (A Simple Questionnaire to Rapidly Diagnose Sarcopenia) [14], age category [15], VES-13 (Vulnerable Elders Survey) [16], SPPB (The Short Physical Performance Battery) [12], Charlson Comorbidity Index [12], physical status by assessment of autonomy – ADL (Activity Daily Living), IADL (Instrumental Activity Daily Living) [17] and gait /balanceTinetti scale [18], MMSE (Mini-Mental State Examination) [19], the geriatric scale of depression in the context of nutrition in the elderly [13].

The data obtained from the programed investigations were analyzed by methods of variational, correlational and cluster analysis in the STATISTICA 6.0 software package.

Results and discussion

The study was performed on a group of 50 people, aged between 65-93 years, the average being 78 years. Females (80.64%) versus males (19.36%) predominated.

A multidisciplinary approach of understanding the determinants of frailty is the key to success in geriatric populations. The phenomenon of clinical frailty includes 3 fundamental aspects of evaluation: standardized geriatrics, clinical examination and the social side. Currently, the literature emphasizes the bio-psycho-social model of frailty, which includes areas such as: cognitive and mood disorders, functional deficiencies, malnutrition or lack of social support [9].

The results of this study presented data of the social subtype of the elderly placed in the nursing home, where according to the jobs they performed – workers predominated (58%), followed by intellectuals (30%) and peasants (12%), by the level of education, they were distributed as follows: secondary and higher – 68%, primary school – 20% and without education – 12%.

According to the international code of diseases [20], the morbidity structure was established in the Republican Asylum for the Disabled and Retired in the evaluated period, from the number of concomitant diseases of the elderly, cardiovascular pathology prevailed – 74% of cases, followed by vision disorders – 66% and hearing impairment – 66 %, neurological pathology – 64%, osteoarticular – 42%, digestive – 28%, diabetic – 18% and pulmonary – 6%.

After examining the nutritional indicator score (MNA): 38% had normal nutritional status, 40% – risk of malnutrition and 22% were malnourished.

In the specialized works of the last years, the researchers reported the complexity of the frailty syndrome in the elderly population, mentioning the importance of Complex Geriatric evaluation through certain grids, in terms of establishing subtypes of frailty: functional – ADL, IADL, GFI, SPPB, VES-13, SARC-F, gait and balance – Tinetti, cognitive – MMSE and bio-psycho-social (Charlson Comorbidity Index) [12, 21, 22].

Vermeulen J. et al. [8] noted that frail people from a multidimensional perspective of impairment are susceptible to a higher risk of functional frailty determined by the ADL score, and researchers Batko-Szwaczka A. et al. [23], Bekić S. et al. [24], Montero-Odasso MM. et al. [25], highlighted the phenotype of frailty through the prism of physical and mental determinants in the risk of frailty.

Pearson's correlation analysis of geriatric scores established high positive correlations between the Charlson Comorbidity Index – Gröningen Frailty Index ($r = 0.56^*$), Activity Daily Living – Instrumental Activity Daily Living ($r = 0.61^*$), Activity Daily Living – gait and balance Tinetti ($r = 0.62^*$), Instrumental Activity Daily Living – gait and balance Tinetti ($r = 0.68^*$), Gröningen Frailty Index – A Simple Questionnaire to Rapidly Diagnose Sarcopenia ($r = 0.69^*$), The Short Physical Performance Battery – gait and balance Tinetti score ($r = 0.62^*$) and maximum correlation between Gröningen Frailty Index – Geriatric Depression Scale ($r = 0.78^*$) ($p \leq 0.05$), results confirmed in other specialized works [8]. The high degree of correlation between the scores indicates that there is a pronounced positive dependence between the levels of their expression. In the case of ADL and IADL scores, this dependence is natural, be-

cause both scores show the degree of functionality of the elderly, so the higher the value of the ADL score, the higher the value of the IADL. The Charlson Comorbidity Index correlation with Gröningen Frailty Index demonstrates that, the Charlson Comorbidity Index being a marker of chronic comorbidities, Gröningen Frailty Index is sensitive to the process of weakening the vulnerable elderly (fig. 1, 2).

The analysis of the statistical results of a study in 2011 on a group of 81 patients showed that frailty correlates with age and moderate cognitive impairment, and another study conducted on a group of 185 participants showed that nutrition is negatively correlated with cognitive frailty [26, 27].

In our work, the correlational analysis of Mini Nutritional Assesment and Mini-Mental State Examination established high negative correlations between Mini Nutritional Assesment – Geriatric Depression Scale (r = -0.78*), Mini Nutritional Assesment – Gröningen Frailty Index (r = -0.73*), Mini Nutritional Assesment – Charlson Comorbidity Index (r = -0.54*), Mini-Mental State Examination – Gröningen Frailty Index (r = -0.56*) (p≤0.05), which shows that at a high nutritional value, there is the risk of developing functional, cognitive, psychological frailty and chronic comorbidities (fig. 3, 4).

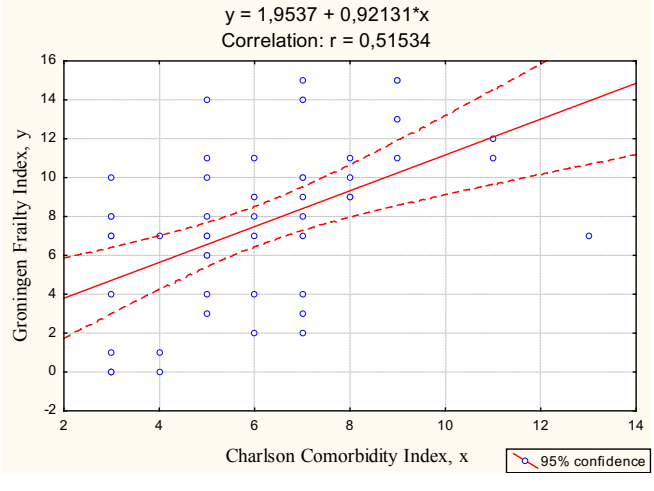

Fig. 1. Correlation and linear regression between the Charlson Comorbidity Index and the Gröningen Frailty Index

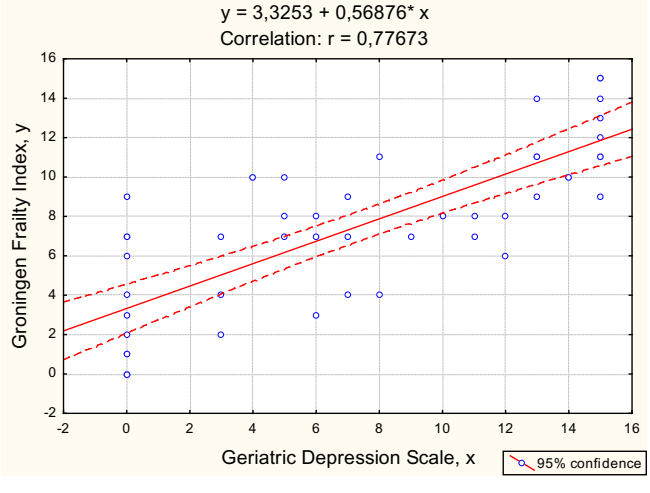

Fig. 2. Correlation and linear regression between Gröningen Frailty Index and Geriatric Depression Scale

Depression is the leading cause of mental suffering as we age and affects morbidity and geriatric patients. In the case of this study, e.g. Mini Nutritional Assesment – Geriatric Depression Scale or Mini Nutritional Assesment – Gröningen Frailty Index at a high nutritional value, decreases the risk of developing depression and frailty of the vulnerable elderly.

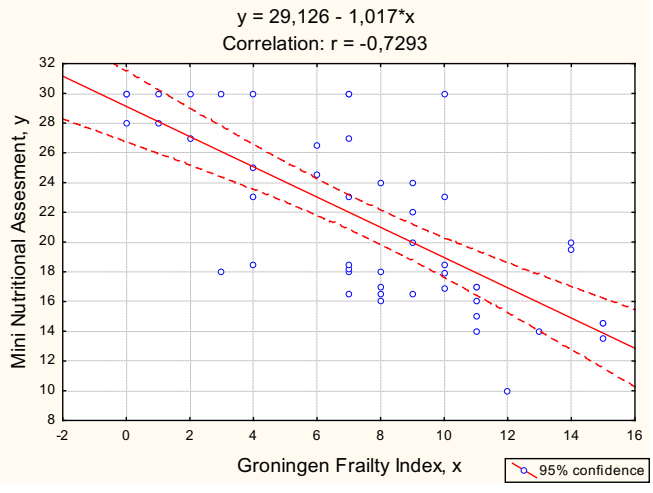

Fig. 3. Linear correlation and regression between Mini Nutritional Assesment and Gröningen Frailty Index

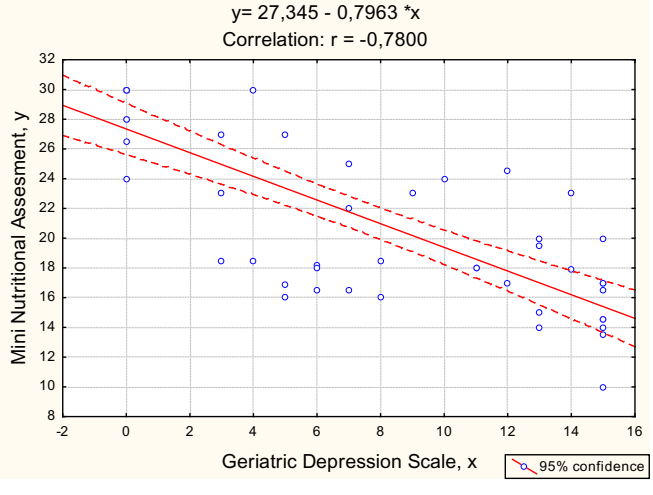

Fig. 4. Correlation and linear regression between index MNA and Geriatric Depression Scale

Based on the correlational analysis, the regression analysis was performed, which indicates not only the degree of dependence (0.00… 1.0) and the orientation between factors (+/-), but also the mathematical equation of the obtained correlations, which has a predictive importance in medical practice. Taking into account the determining role of the frailty and nutritional risk of the vulnerable elderly, it was established as an independent factor in the regression analysis. Thus, for the correlations Gröningen Frailty Index – Geriatric depression scale (r = 0.78*), the Comorbidity

Table 1. Clustered analysis of nutritional status (MNA) by age, gender, clinical scale of frailty, comorbidities and drugs

Women, n=31				Men, n=19			
Cluster	**Frequent indices in clusters**			**Cluster**	**Frequent indices in clusters**		
1, n=14	MNA ≥ 24 MNA (17-23,5) MNA ≤ 17		57.14% 28.57% 14.28%	1, n=6	MNA (17-23.5) MNA ≤ 17		83.33% 16.66%
	75-84 years		92.85%		65-74 years		100%
	Frail Prefrail Robust		50% 28.57% 21.14%		Robust Frail		16.66% 83.33%
	No comorbidities	1	14.28%		No comorbidities	1	16.66%
		3	21.42%			4	33.33%
		4	28.57%			5	33.33%
		5	28.57%			6	16.66%
	No drugs	4	57.14%		No drugs	4	16.66%
		5	14.28%			5	50.0%
2, n=11	MNA ≥24 MNA (17-23,5) MNA ≤17		27.27% 36.36% 36.36%	2, n=7	MNA (17-23.5) MNA ≤ 17		71.42% 28.57%
	65-74 years		100%		75-84 years		85.71%
	Frail		81.81%		Frail		100%
	No comorbidities	3	18.18%		No comorbidities	3	28.57%
		4	18.18%			5	71.43%
		5	45.45%				
		6	18.18%				
	No drugs	3	27.27%		No drugs	4	14.28%
		5	27.27%			5	71.42%
3, n=6	MNA ≥24 MNA (17-23,5) MNA ≤17		33.33% 33.33% 33.33%	3, n=6	MNA ≥24		100%
	85-93 years		100%		65-74 years 75-84 years 85-93 years		33.33% 33.33% 33.33%
	Prefrail Frail		16.66% 83,33%		Robust Prefrail		66.66% 33.33%
	No comorbidities	4	16.16%		No comorbidities	1	50%
		5	16.16%			4	16.66%
		6	33.33%			6	33.33%
	No drugs	4	50.0%		No drugs	3	16.66
		5	33.33%			4	16.66%
						5	16.66%

Index Charlson – Gröningen Frailty Index (r = 0.56*), Mini Nutritional Assesment – Gröningen Frailty Index (r = -0.73*), Mini Nutritional Assesment – Geriatric Depression Scale (r = -0.78*) (p≤0.05), the regression equations are: y = 1.9537 + 0.92131 * x, y = 3.3253 + 0.56876 * x, y = 29.126 - 1.017 * x, y = 27.345 - 0.7963 * x (Fig. 1-4).

As a result of these analyses, high positive and negative dependencies of the Gröningen Frailty index and Mini Nutritional Assesment, as well as the mathematical equations of dependencies were established, which is of predictive importance in medical practice.

Cluster analysis is a useful method for identifying pro-

files associated with multifactorial aspects. The authors Fried L. et al. [2] and Rockwood K. et al. [28], who are the pillars of the concept of frailty, highlighted through this method the main aspects of the frailty phenotype model and the frailty index model.

In the present study, the most relevant indicators were used, which separated the clusters, such as: age category, frailty subtype, MNA nutritional score, number of chronic polypathologies and daily polymedications administered, which can be easily applied in trials clinics by nutritionists and clinicians [29]. At the same time, the aim was to elucidate the frequency of relevant clinical manifestations that would serve as markers of the evolution of frailty depending on nutritional status and sex.

The *k*-means cluster analysis divided the elderly into 2 sublots according to gender (female / male) with 21 different parameters, which included nutritional data, frailty, the presence of comorbidities and the number of drugs administered per day (tab. 1).

The cluster analysis by the centroid method of *k*-means established that the groups of elderly (female), separated into 3 clusters, differed according to the level and variability of the researched parameters. The elderly in cluster I were of the age category 75-84 years, who presented 3 subtypes of frailty with moderate prevalence of comorbidities, the daily administration of 4 drugs and the nutritional status not being affected. Cluster II was composed entirely of young elderly persons of the 65-74 age group, but more vulnerable in terms of frailty and nutritional status (risk of malnutrition – malnourished), associated with the highest number of chronic polypathologies (No 5), but with reduced drug use. Cluster 3 consisted mainly of old elderly people (85-93 years), with an equal frequency of normal nutritional status – risk of malnutrition – malnutrition, the most vulnerable in terms of vulnerability, with the highest number of chronic diseases (No 6) and the administration of the average number of daily drugs.

Regarding the age groups (male), they were distributed practically equally in number of participants, but with a frequency of different indices in clusters. Cluster I was composed of elderly people aged 65-74 years with the highest risk of malnutrition – 83.33%, the highest share of frail, with the largest variety of comorbidities. Cluster II was noted for the highest frequency of frail people in the 75-84 age category with increased use of daily medication, and cluster III was noted for having the best indicator of nutritional status, being robust according to the frailty scale and using as a frequency the lowest number of prescribed medications.

Due to the comparative assessment between both groups of elderly (women / men), it can be mentioned that the normal nutritional status was found with a higher share in frail elderly in the category 75-84 years being associated with a high spectrum of comorbidities and lower comparative polymedicine with the same parameters in older men. The nutritional risk was found more frequently in frail elderly men in the young category of elderly (65-74 years) being associated with a wide range of comorbidities, and malnutrition was practically manifested equally in both groups from the study.

Conclusions

1. Frailty through its multidimensional aspects, has an increased prevalence among the elderly with an unfavorable prognosis.

2. Following a multilateral research, it was highlighted that insufficient nutrition and comorbidities can lead to the weakening of the institutionalized elderly through bio-psycho-social aspects.

3. According to the cluster analysis (*k*-means method), the most relevant indicators that separated the clusters were: age category, sex, clinical scale of frailty, comorbidities and drugs.

4. The results obtained characterize the profile of institutionalized elderly and can be used as a basis for the development of effective strategies aimed at reducing physical, cognitive and social frailty.

5. The comparative evaluation between both groups of elderly people by gender, showed a normal nutritional status with a higher share in women in the group of 75-84 years, compared to older men, and malnutrition was practically manifested equally in both groups in the study (men/women).

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FLV conceptualized the study, designed the research, collected and interpreted the data, drafted the first manuscript; GS conducted the laboratory work and revised the manuscript critically; AP collected data and revised the manuscript critically; AN conducted the management work and revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Ethics approval and consent to participate
The research was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (protocol No 51 of June 16, 2020).

Conflict of Interests
The authors have no conflict of interests to declare.

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In vivo experimental study of the arterial supply of the rabbit posterior limb

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Abstract

Background: The use of bone graft has been a successful step in the treatment of a large number of diseases of the osteoarticular system. But a massive bone defect remains a dilemma for modern reconstructive surgery. Present methods used have a high level of morbidity and complication. Literature indicates the absence of an optimal solution in massive bone defects healing. The aim of this study: to perform an *in vivo* preliminary study of vascularization of the hind limb in the rabbit model, for obtaining a graft able for further inclusion in the host blood circulation, without immunosuppression by decellularization.
Material and methods: The study was performed on the 12 laboratory rabbits. After euthanasia of the rabbit, the femoral and tibiofibular bone was collected without soft tissue, only with the vascular pedicle, and keeping the passage through the vessels. In the abdominal aorta was injected contrast material, with the subsequent preparation of the arterial vessels, succeeded by anatomical, morphological, radiography, and microangiography study of this vascularized bone segment.
Results: The principal nutrient artery of the rabbit femur springs from the lateral circumflex femoral artery. The optimal segment for vascularized allografting (the rabbit model) was determined the upper third of the femur with the up to the level of the internal iliac artery. So, it could be used as a bone graft for further conservation and decellularization.
Conclusions: The vascularized allogeneic bone without immunosuppression would be a perfect alternative in the treatment of the massive bone defects.
Key words: vascularized bone grafts, bone allograft surgical revascularization, angiography.

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Introduction

Reconstruction of the large bone defects, caused by severe trauma, infection, tumor resection, or congenital deformity, is an actual clinical problem and the approach challenges. They were dictated by a multitude of clinical factors, including the defect size, patient comorbidities, soft tissue condition, and the risk of infection present in the defect. Current reconstructive options include the use of cryopreserved allografts, bone transport, prosthetic replacement, membrane-induced osteogenesis, re-implantation of autoclaved tumor bone and, vascularized bone autografts, all with a significant incidence of complications and failure. Unfortunately, almost all of these substitutes have been only used to refill small cavitary defects in the clinic. A multidisciplinary approach that involves orthopedic, plastic, and vascular surgeons are essential to optimize success rates [1-3].

The large bone defects, called critical-sized defects (CSDs), describe a bone void that will not spontaneously fill, without intervention. That means CSDs would not heal despite simple stabilization and require further intervention in addition to fixation. This type of defect is difficult to characterize because diagnosis is subjective. Whereas, the critical defect varies based on patient age, overall health, and the size of the lack of bone. The significant defects that are less than 4 to 6 cm are classified as small-scale defects, and those that are larger than 4 to 6 cm are considered large-scale defects. In adult patients, a critical bone defect generally has circumferential loss of 50% or a length of 2 cm. The anatomic location and the condition of the surrounding soft tissue are the two contributing factors that dictate the healing potential of a defect. Healing potential varies based on the anatomic location of the defect [3-5].

The healing of bone fractures is a well-orchestrated physiological complex process involving interactions between different cells and signals to form new mineralized tissue, to replace and repair bone tissue without scar formation. Blood vessels serve as a basic template, around which bone development takes place and also brings together the critical elements for bone homeostasis into the osteogenic microenvironment, including minerals, growth factors, and osteogenic progenitor cells [2, 3, 4, 6].

The healthy growth and development of a bone are exclusively linked to its vascular and, in particular, its

arterial supply. Well, it is generally agreed that there are three sources of blood supply in long bones. They are the nutrient artery, epiphyseal-metaphyseal arteries, and periosteal arteries. Johnson R. concluded that the nutrient artery was the essential source, by interfering with two of the three sources of blood supply of the tibia in dogs. The nutrient artery is capable of maintaining the viability of the entire shaft and of supporting the repair of bone defects, and that the periosteal source was the least important [7, 8]. An interesting question may be raised. From where does the remaining intact blood supply come to these three segments after ligation of the nutrient artery? The quantitative observation in this study that the nutrient artery contributes at least 71 percent of the total blood supply of the shaft agrees very well with their comments. Very effective communication between the Bone Marrow vascular system and external circulation must exist, a fact that has been exploited in emergency medicine for many years. Initially developed for battlefield administration of fluids and analgesics, the use of direct intraosseous infusion is now widely utilized in emergency medicine when peripheral venous access is difficult. Here it is shown that murine long bones are supplied with approximately 16 nutrient arteries and a central sinus with two exit sites. Trans-cortical vessels (TCV) require the presence of narrow canals in the cortical bone that is then lined by endothelial cells, in all types of murine long bones investigated. These TCVs can be either arterioles or venules and effectively transport blood and thereby also neutrophils. TCVs can be either arterial or venous and directly connect the periosteum to BM [9, 10].

The "diamond concept," being a conceptual framework for a successful bone repair response, gives equal importance to mechanical stability and the biological environment. Moreover, adequate bone vascularity and the physiological state of the host are thought to be essential within this framework of fracture repair. Overall, the "diamond concept" refers to the availability of osteoinductive mediators, osteogenic cells, an osteoconductive matrix (scaffold), optimum mechanical environment, adequate vascularity, and addressing any existing comorbidities of the host [11].

The bone graft is commonly used in reconstructive surgery, a complicated surgical procedure that replaces missing bone. The bone grafts can be autografts, allografts (cadaveric bone usually obtained from a bone bank), or synthetic (often made of hydroxyapatite or other naturally occurring and biocompatible substances) with similar mechanical properties to bone. Vascularized autografts – the gold standard between grafts is limited primarily to the fibula and iliac crest for significant skeletal defects. Each site of autologous bone graft has its advantages, but also has the disadvantages like donor site morbidity, the limited volume available, no structural capability, and occasional unsatisfactory biologic activity have led to increased use of allografts. For CSDs, there are two primary options to consider, in the actual clinical approach: induced membrane technique and distraction osteogenesis. Choosing between these two techniques should be based on the associated soft tissue injury, the local vascularity, and the possibility of residual infection. Transplantation of living vascularized allogeneic bone should have the potential to combine the same biological benefits as vascularized autografts with the mechanical advantages provided by the size- and shape-matched allogeneic bone segments [12-14]. The significant benefits of vascularized bone grafts are more rapid and complete incorporation of the graft, which provides immediate structural support. The orthobiologic ability of the transplanted (living) bone to form new bone and the addition of new blood supply to the recipient is the crucial element of the "diamond concept." Free vascularized bone grafts allow living bone tissue to be transplanted to replace a bone defect. The use of vascularized bone grafts requires microvascular dissection and attachment to a recipient site artery and vein [15-17].

Therefore, the data of the literature presents suggestions of the need to increase vascularized bone allograft, which remains at the stage of preclinical studies on the need for long-term post-interventional immunosuppression, which is irrational in the case of bone transplantation. Current studies are investigating alternative options to maintain the viability of the allograft by practicing drug-induced and surgical neoangiogenesis with the short-term immunosuppression [18, 19].

The vascularization of the rabbit femur, the closest segment to the required demand of *in-vivo* study, has been earlier researched [10, 20].

In this literature context, the aim of *in-vivo* research for the first stage is to study the vascularization of the posterior limb – femur and the tibiofibular segment in the rabbit model and establish the most suitable portion of bone with the vessel. This bone portion must be able to serve as a vascularized allograft, for the future microsurgical inclusion in the host circuit. The success after a bone vascularized allograft procedure is ensured by keeping the passage of nutrient artery and microcirculation of this graft.

Material and methods

This study is a part of the doctoral program of the Doctoral School of Medical Sciences. It was approved by the Research Ethics Committee, dated 21.05.2018, and performed during 2019-2020, in the Tissue Engineering and Cells Cultures Laboratory, of *Nicolae Testemitanu* State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova.

The first stage of the present study was performed in the animal model (New Zealand White Rabbit) on their bodies. The study was carried out on twenty adult white rabbits of about four months old and weighing about three kilograms. The animals' bodies were taken from the abattoir, mandatory that has the sanitary-veterinary certificate for euthanasia. Following dissection, the individual bones were stripped of all extra-osseous soft tissue, only with the vascular pedicle and including periosteum to permit clear visualization of

intraosseous vessels alone, and to avoid confusion arising from the superimposition of vessels in soft tissues. This procedure kept the passage through the vessels and didn't remove periosteal arterial twigs from the cortex since. In the abdominal aorta was injected contrast material, with the subsequent preparation of the arterial vessels, succeeded by anatomical, radiography, histology, and microangiography study of this vascularized bone segment. Thus, was determined the vascularized bone segment that could be used as a bone graft for further conservation and decellularization.

The macroscopic study of the vascularization of the posterior limb in the rabbit model

The research was performed on the vascularization of the posterior limb in the carcasses of laboratory animals, New Zealand White rabbit. We injected polymerizing material (Protacryl – M) in the abdominal aorta, and the *inferior vena cava* (fig. 1), with the subsequent preparation of the limb's vessel (fig. 2, 3).

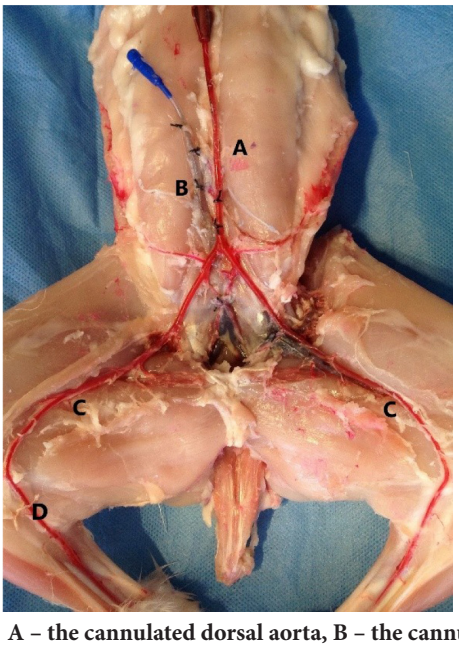


Fig. 1. A – the cannulated dorsal aorta, B – the cannulated *inferior vena cava,* C – the femoral artery, D – the saphenous artery

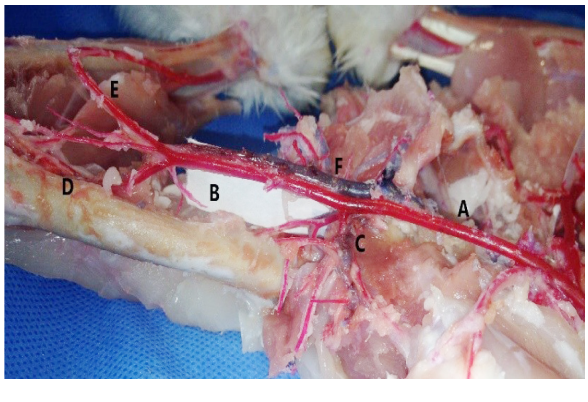


Fig. 2. A – the common iliac left artery, B– the femoral artery, C – the lateral circumflex femoral artery, D – *Arteria suprema genu,* E – the saphenous artery, F – the femoral intercondylar vein

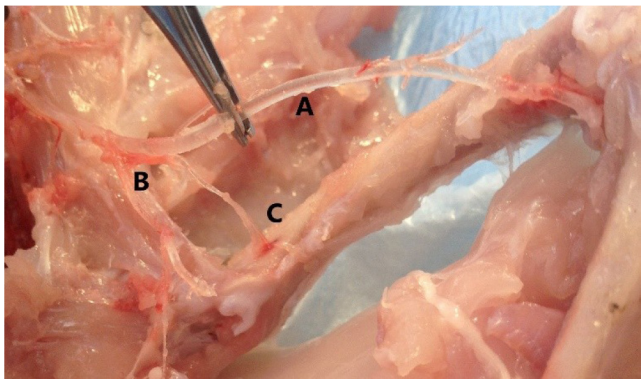


Fig. 3. A – the femoral artery, B – the lateral circumflex femoral artery, C – the principal nutrient artery of the rabbit femur

The angiographic study of vascularization by introducing the contrast substance into the abdominal aorta.

The contrast substance-Urografin® 30% was injected in the abdominal aorta under pressure. The radiological examination was performed in the standard way (fig. 4).

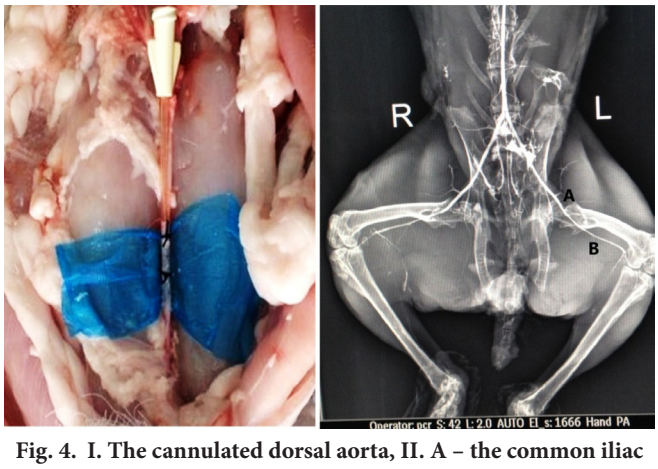
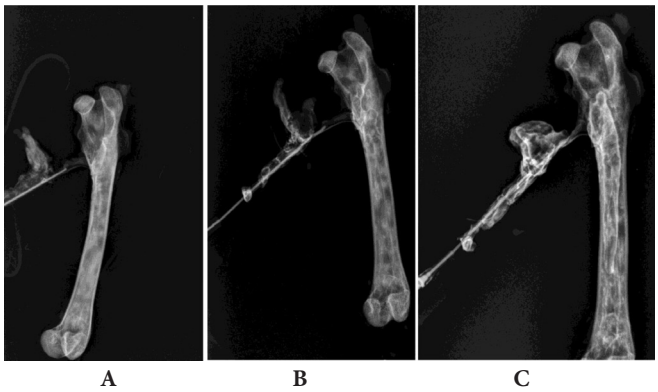


Fig. 4. I. The cannulated dorsal aorta, II. A – the common iliac artery, B – the femoral artery

The microangiography study of the vascularized bone segments (femur)

Through the cannulated internal iliac artery, the



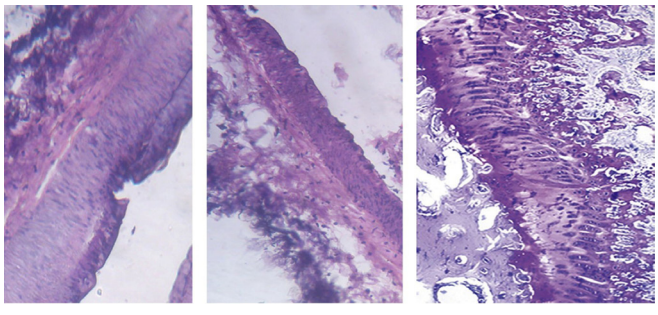
A B C

Fig. 5. A – the radiological examination at 1 min, B – the radiological examination at 3 min, C – the radiological examination at 3 min

Urografin® 30% was introduced under pressure. The radiological examination was performed at 1 min, 3 min and 5 minutes after the start of the introduction of the contrast substance (fig. 5).

The morphological study of the dorsal aorta, the internal iliac artery, and the vascularized bone segments (femur)

Histological sections of the dorsal aorta (fig. 6 A), the internal iliac artery (fig. 6 B), and the proximal femur (fig. 6 C) of rabbits, were stained with hematoxylin-eosin showing.



A B C

Fig. 6. A – the histological sections of the dorsal aorta, B – the histological sections of the internal iliac artery, C – the histological sections of the proximal femur

Results

The macroscopic study of the vascularization of the posterior limb in the rabbit model

The arterial supply of femur in a rabbit model begins at the *dorsal aorta* then divides into two *common iliac arteries* and a median *caudal artery* to the tail (fig. 7). Each common iliac artery gives a small *ilio-lumbar artery* that supplies the dorsal body wall. The *common iliac artery* divides into the *internal iliac artery* which supplies the organs of the pelvis and *external iliac artery* that supplies the hind limb. The external iliac terminates as a *femoral artery* in each hind limb. It has branches the – *lateral and medial circumflex femoral* which passes around on the hip joint to terminate as a contribution to the trochanteric anastomosis. From it, anterior cervical arteries descend into the femoral neck. Another branch of the same artery concerned in the nutrition of the femur is the *artery of the trochanteric fossa*. In one specimen, it sprang directly from the femoral, distal to the lateral circumflex femoral artery. It passes downwards for 2 cm before disappearing in the nutrient foramen situated on the medial surface of the shaft just below the lesser trochanter. So, *the principal nutrient artery* of the rabbit femur springs from the *lateral circumflex femoral artery.* In the distal part from the *femoral artery* begins the *arteria suprema genu.* Then the femoral artery continues as the *popliteal artery*, giving origin to the *supracondylar (genicular) arteries*, which pass outwards, supplying fine nutrient twigs to the posterior face of the inferior metaphysis, and the condyles. They join the medial and lateral condylar loops. From the popliteal begins the *anterior tibial artery*, a sizeable middle genicular artery arises, which pierces the joint capsule, passes above the point of crossing of the cruciate ligament, and sinks into a foramen in the anterior wall of the intercondylar notch. The principal *nutrient artery of the tibia,* derived from the *anterior tibial artery* descends on the posterior surface of the bone before reaching the nutrient canal situated 5 mm above the level of the tibiofibular synostosis. The second principal nutrient artery given off by the anterior tibial at the synostosis sinks into the bone anteriorly just below the level of fusion. The shaft of the fibular portion of the bone has no nutrient artery of its own. The *saphenous artery* divides into the *medial and lateral plantar arteries.*

Venous drainage

A single *vena comitans* accompanies each artery. A simple circulus venosus is formed on the superficial surface of each condyle. The femoral intercondylar vein joins the tibial intercondylar veins to drain into the anterior tibial vein. The veins of the tibiofibular segment show those general features which have been described for the femur. The following individual points are to be noted. A vein issues from the fibular border of the shaft below the synostosis and drains into the peroneal vein, on the posterior surface of the tibia, two and occasionally three large veins issue from the bone. Veins from the intercondylar ridge join those draining the inferior femoral epiphysis and pass ultimately into the anterior tibial vein.

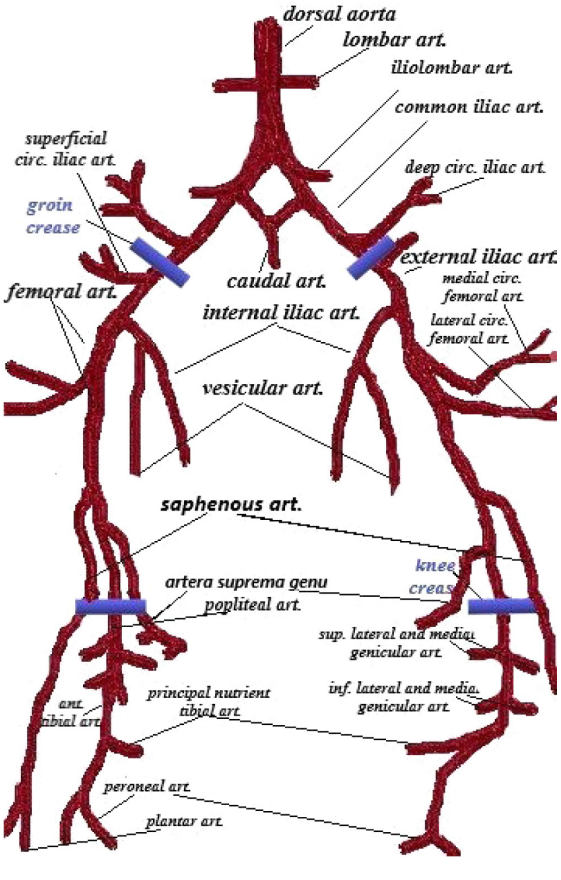


Fig. 7. The sketchy representation of the arterial supply of the posterior limb in the rabbit model.

The angiographic study of the vascularization in the hind limb in the rabbit model

The contrast substance (Urografin® 30%) was introduced into the abdominal aorta under pressure. The gross arterial supply of the hindlimb was determined, and the visualization of the anatomical correlation with the femoral bone after a standard radiological examinatio**The microangiography study of the vascularized bone segments (femur) in the rabbit model**

The internal vascularization of bone as revealed by radiography is presented at 1 min, 3 min and 5 minutes after the start of the introduction of the contrast substance (Urografin® 30%). The rate of blood flow through the principal nutrient artery of the femur indicates that it contributes at the 50-80 percent of the surface to the femur (depending on the epiphysial, metaphyseal or diaphyseal region). The epiphyseal and metaphyseal arteries, and the periosteal arteries also have a significant contribution to blood arterial flow.

The morphological study

The histologic examination was done on the dorsal aorta, the internal iliac artery, and the vascularized bone segments (proximal femur). It demonstrates that the content of the extracellular matrix of the vessel is optimal up to the level of the internal iliac artery, for a next microsurgical inclusion in the host blood circulation. The grafts were stored at -84.4^{0}C to be subsequently decellularized by the combined method. The next *in vivo* step will involve the orthotopic inclusion of the decellularized graft in the host circuit (fig. 8).

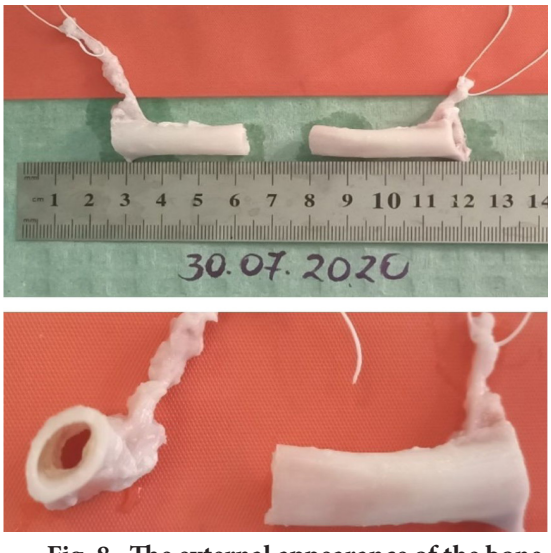


Fig. 8. The external appearance of the bone vascularized grafts

Discussion

Bone regeneration is a well-orchestrated physiological process. It is sufficient when the vascularization of the segment remains optimal. Performed by the intramedullary arteries, periosteal vessels, and the arteria nutricia, artery nutrition plays an essential role in strengthening throughout the whole bone consolidation process [21, 22]. The success after bone vascularized allografting is ensured by keeping the circulation on nutrient artery and microcirculation of blood. The microsurgical anastomosis of the allograft pedicle would admit the creation of optimal conditions for sufficient revascularization. That ended in consolidation, resistance, and increased rigidity of the segment [14, 23-25]. Thus, vascularized bone grafting is considered superior to the non-vascularized one. Its task is not only to structurally replace the defect but also to biologically engage in the formation of new bone. Because the bone allografts not only replace the missing bone but also help the reconstruction of the lost bone by acting as a scaffold for osteoconduction and as a source of osteogenic and osteoinductive molecules for bone formation. To increase bone healing it is essential to provide a suitable vascularization and excellent mechanical stability [26]. The interaction of immunocompetent blood cells with the graft's vascular endothelium is the first stage in the cascade that induces ischemia, reperfusion disorder, and rejection. Stopping the microcirculation through the allograft is considered the principal cause of graft failure [27, 28]. Immunosuppression marks the progress in contemporary transplantology. It was shown that vascularized bone grafts are superior, especially for the reconstruction of large defects, because they retain the property of osteogenesis. But rest the morbidity of the donor site, and the source of autografts is limited. In this context, the research of vascularized bone allografts is imperative [29-32]. The dilemma required by vascularized bone allografts means the necessity of an after-graft immunosuppression and immunomodulation. Grafting of limbs, joints, bone tissue is not similar to the transplantation of vital organs, such as the heart, liver, which require long-term immunosuppression to omit systemic complications. And in the case of musculoskeletal tissue, the need for medication is 2-3 times higher than in the case of organ transplantation. On a large scale, long-term immunosuppression doesn't argue, and the risk induced by organic toxicity, malignancy, or other complications, does not justify it in these vitally uncritical situations [26, 31, 33].

The vascularized living allogeneic bone, without the need of the immunosuppression, would be a perfect alternative in the treatment of the massive bone defects. In other words, the research into vascularized bone allografts to omit their immunogenic potential by decellularization has increasing importance. Current decellularization methods of the vessel preserve vascular stiffness [34]. In this context, the *in vivo* experiment of including in the host blood circuit the orthotopically decellularized vascularized bone allograft will permit the description of both the vascular and the bony aspects.

Conclusions

The orthobiologic ability of the transplanted (living) bone to form new bone and the addition of new blood supply to the recipient is the crucial element of the "diamond concept". The significant benefits of vascularized bone grafts

are more rapid and complete incorporation of the graft, which provides immediate structural support.

The principal nutrient artery of the rabbit femur springs from the lateral circumflex femoral artery. Graft, able for the subsequent inclusion in the host blood circulation, without immunosuppression by decellularization, is the proximal third of the femur with the vascular pedicle to the internal iliac artery.

The further evaluation of the decellularization methods of the tissue, organs by preserving vascular stiffness, represents the reason to continue research in this field.

Transplantation of native allogeneic vascularized bone can be a potential perfect solution, only if significant and unjustified risks of long-term immunosuppression can be avoided.

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Authors' contribution
PE and SA designed the study, collected, processed, and interpreted the data and drafted the manuscript; GV and NV designed the trial and revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Ethics approval and consent to participate
The research was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (protocol No 70/75 of 21.05.2018).

Conflict of Interests
Nothing to disclose.

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Cerebrovascular disease associated with Parkinson's disease in Moldovan cohort study

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Abstract

Background: Parkinson's disease (PD) is frequently associated with brain vascular lesions (BVLs), which may influence the severity of the disease.
Material and methods: BVLs on MRI were determined in 78.4% of 111 consecutive PD patients (mean age 64.87 ± 7.69 y.o.; disease duration 50.21 ± 38.61 mo.; 48 women (43.2%), 63 men (56.8%)).
Results: White matter lesions were present in 73 patients (p.) (65.77%): 61p. (54.95%) – deep white matter, 46p. (41.44%) – periventricular white matter, and 41p. (36.94%) – both locations. Lacunas were determined in 19p. (17.12%), cerebral fissures deepening – 52p. (46.85) %), perivascular spaces dilation – 34p. (30.63%), ventricular system dilation – 29p. (26.13%). Patients with and without BVLs had similar ages, ages at PD onset and disease duration. They had insignificantly higher Beck (7.26 ± 5.62 vs 6.86 ± 4.34), PDQ39 (Parkinson's Disease Questionnaire) (59.71 ± 20.38 vs 51.94 ± 27.69) and NMS (Non-Motor Symptoms) (75.06 ± 45.21 vs 71.67 ± 26.35) scores; and lower MoCA (Montreal Cognitive Assessment) scores (21.92 ± 4.25 vs 22.38 ± 4.57). QRISK3 scores (19.68 ± 16.16 vs 12.90 ± 6.58) and levodopa equivalent daily dose (639.98 ± 223.05 vs. 439.69 ± 404.87) were significantly higher in patients with BVLs.
Conclusions: Brain vascular lesions were common in our PD patients, and were associated with higher QRISK3 scores and higher levodopa equivalent daily dose, suggesting more disease severity.
Key words: Parkinson's disease, brain vascular lesions

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Introduction

Neurodegenerative disorders, such as Parkinson's disease (PD), more frequently occur in the context of other chronic conditions associated with aging. PD and cerebrovascular disease (CVD), both are common in aged populations. PD, as well as brain vascular changes in strategic regions, may display: tremor, rigidity, slowness, gait disturbance, postural instability, urinary incontinence, mood disorders, and cognitive impairment [1]. Cognitive impairment and dementia are recognized features of PD, but these symptoms may relate to comorbid cerebrovascular disease too [2]. Parkinson's disease (PD) is frequently associated with white matter hyperintensities and other brain vascular lesions (BVLs). Studies suggest that the latter may influence the severity of the disease. White matter hyperintensities may be a contributing factor for cognitive impairment [3], as well as to increased motor severity and gait impairment [4].

There are conflicting literature data on PD and CVD association: (1) no clear relationship between PD and CVD [5]; (2) protective effect of PD from CVD [6]; (3) increased risk of CVD in PD [7].

Earlier studies provided no clear relationship between PD and CVD [5].

Protective effect of PD from CVD may be due to the decreased amount of smoking among PD patients. PD patients seem to experience more of their CVD as TIA than stroke: they have increased access to neurological care, so TIAs are more readily recognized and treated and are therefore less likely to lead to stroke [8]. Dopamine deficiency in PD could ameliorate ischemic damage. It has been found that dopamine depletion with either lesions of the substantia nigra lessen ischemic damage [9].

Increased risk of CVD in PD may be due to the shared pathogeneses between the two diseases or to PD-related effects. Patients with PD should be more aware of the risk of CVD despite having fewer traditional vascular risk factors. In a meta-analysis of four clinical case–control studies, PD was more associated with CVD (OR: 2.89, 95% CI: 1.36–6.13); and in three postmortem cohort studies PD patients were at higher risk of CVD during the follow-up period (HR: 1.84, 95% CI: 1.34–2.54)[7].

Material and methods

These are preliminary data of a cohort study of Moldovan patients with incident Parkinson's disease. Diagnosis of PD was based on widely acknowledged criteria [10]. Structured

interview on complaints, medical history and family history of cardiovascular, neurological and psychiatric diseases and drug history was applied; general neurological and medical examinations were conducted. Severity of parkinsonism and disability were assessed by the Modified Unified Parkinson's Disease Rating Scale (MDS-UPDRS) [11]. Cognitive impairment was quantified by Montreal Cognitive Assessement (MoCA) score [12], depression – by Beck score [13], non-motor symptoms – by Non-Motor Symptoms score [14], quality of life – by PDQ39 (Parkinson's Disease Questionnaire) score [15].

All brain MRI (magnetic resonance imaging) examinations were performed using a 3.0 T MRI scanner.

MRI Analysis of cerebrovascular disease markers included: lacunas, white matter lesions / hyperintensities, perivascular spaces dilation / enlarged perivascular spices, cerebral fissures deepening, ventricular system dilation.

Lacunas were defined as round or ovoid cerebrospinal fluid-filled cavities in the basal ganglia or white matter, usually 3–15 mm, with low signal on T1WI and DWI, and high signal on T2WI [16]. Periventricular white matter lesions and deep white matter lesions were graded using the Fazekas scale [17], perivascular spaces dilation were defined as punctate hyperintensities on T2WI in the basal ganglia, usually <3 mm in diameter [18].

Patients were sub-classified at baseline into two groups according to presence of any of mentioned above cerebrovascular disease as: (1) PD patients with brain vascular lesions; and (2) PD patients without brain vascular lesions.

The data analysis was performed via statistical program Stat-Direct, using descriptive, variation, and correlational analysis. Student's t tests or Mann-Whitney tests were used as appropriate. P values less than 0.05 were considered statistically significant.

BVLs on MRI were determined in 78.4% of 111 consecutive PD patients, (mean age 64.87 ± 7.69 y.o.; disease duration 50.21 ± 38.61 mo.; 48 women (43.2%), 63 men (56.8%)).

Results and discussion

The study included 111 consecutive PD patients. The mean age in the cohort was 64.87 ± 7.69 years old and disease duration of 50.21 ± 38.61 months. In 88 (78.4%) of our PD patients was determined the presence of brain vascular lesions. White matter lesions were present in 73 patients (65.77%): 61 of them having (54.95%) deep white matter lesions, 46 (41.44%) – periventricular white matter lesions, and 41 patients (36.94%) – a combination of periventricular and deep white matter lesions. Lacunas were determined in 19 patients (17.12%), cerebral fissures deepening – in 52 patients (46.85%), perivascular spaces dilation – in 34 patients (30.63%) and ventricular system dilation – in 29 patients (26.13%).

In Ma X. et al. [19] study, lacunas were found in 9.3% of patients with PD, periventricular white matter hyperintensities – in 89.7%, deep white matter hyperintensities – in 81.3%, enlarged perivascular spaces – in 85%, and cerebral microbleeds – in 2.8%. In their study PD patients, showed higher periventricular white matter hyperintensities and deep white matter hyperintensities scores compared with normal controls. Advanced PD patients, versus early PD group exhibited greater periventricular white matter hyperintensities (P = 0.041), deep white matter hyperintensities (P = 0.046), and total cerebral small vessel disease score (P = 0.044) than the early PD group. In Ma X. et al. [19] study, higher Hoehn&Yahr stage was independently correlated with increased total cerebral small vessel disease score (OR = 2.667, 95% CI 1.154–2.266) and periventricular white matter hyperintensities score (OR = 2.237, 95% CI 1.084–1.696).

In this study, patients with and without brain vascular lesions had similar ages (65.43 ± 7.64 vs 61.01 ± 7.64), similar ages at PD onset (60.95 ± 8.09 vs 56.01 ± 8.59) and similar disease duration (49.98 ± 36.76 vs 60.01 ± 52.31).

Examined PD patients with brain vascular lesions had insignificantly higher Beck score (7.26 ± 5.62 vs 6.86 ± 4.34). PDQ39 (Parkinson's Disease Questionnaire) score (59.71 ± 20.38 vs 51.94 ± 27.69) and NMS (Non-Motor Symptoms) score (75.06 ± 45.21 vs 71.67 ± 26.35) were slightly higher in patients with brain vascular lesions. MoCA (Montreal Cognitive Assessment) scores (21.92 ± 4.25 vs 22.38 ± 4.57) were lower in patients with brain vascular lesions compared to the control.

Forbes E. et al. [20], noted the influence of depression and other non-motor signs on cognitive performance of PD patients; they showed that depression scores ($\beta = -0.034$, $P < 0.001$), along with: Higher Body Mass Index (BMI) ($\beta = -0.009$, $P = 0.039$), anxiety scores ($\beta = -0.005$, $P < 0.001$), Epworth Sleepiness scores ($\beta = -0.017$, $P = 0.003$), and REM Sleep Behavior Disorder Screening scores ($\beta = -0.037$, $P < 0.001$) were associated with faster rates of MoCA decline [20]. Shibata et al. suggested a relationship between cognitive decline and increased cerebral small vessel disease score [19]. Increasing age and reduced MoCA scores were associated with increased small vessels disease burden. Logistic regression analyses of their study demonstrated that periventricular white matter hyperintensities, enlarged perivascular spaces in the basal ganglia, and atrophy were predictors of cognitive impairment in PD.

Liu H. et al. [21] meta-analysis had the aim to review systematically and to identify the relationship between the severity and location of white matter hyperintensities (WMHs) and the degree of cognitive decline in patients with PD. PD demented patients had a significantly higher burden of white matter hyperintensities (SMD = 0.8, 95% CI: 0.44 to 1.71, $p < 0.0001$), especially deep white matter hyperintensities (SMD = 0.54, 95%CI: 0.36 to 0.73, $p < 0.00001$) and periventricular hyperintensities (SMD = 0.70, 95% CI: 0.36 to 1.04, $p < 0.0001$), than PD-normal cognition patients, regardless of the adjustment of age. Liu H. et al. [21] concluded that WMHs might be imaging markers for cognitive impairment in PD dementia but not in PD-mild cognitive impairment, regardless of age, vascular risk factors, or race.

In the present study QRISK3 scores (19.68 ± 16.16 vs 12.90 ± 6.58) were significantly higher in patients with brain vascular lesions.

High vascular risk is related to impaired scores for: cognition, bradykinesia, axial, postural-instability-gait-disorder and freezing-of-gait score. Furthermore, high vascular risk was identified as a potential predictor of both mild cognitive impairment and dementia in PD. It was found that the presence of more than 2 vascular risk factors was associated with worse UPDRS 3 motor scores (beta coefficient 4.05, 95% confidence interval 1.48, 6.61, p = .002) and with cognitive impairment (ordinal odds ratio 2.24, 95% confidence interval 1.34, 3.74, p = .002). Presence of white matter leukoaraiosis (but not lacunas) was associated with impaired cognition (p = .006) and postural instability gait difficulty (p = .010), [22].

Similarly, in this study, levodopa equivalent daily doses (639.98 ± 223.05 vs 439.69 ± 404.87) were significantly higher in patients with brain vascular lesions – as an indicator for more severe disease in this group.

Cerebrovascular disease may play a critical role in patients with PD. The total cerebral small vessel disease score is a potential neuroimaging marker for monitoring the progression of PD, as the Hoehn&Yahr stage is independently correlated with the total cerebral small vessel disease score [19]. In Vesely B. et al. [3] review of PD patients with mild cognitive impairment and dementia they had significantly more white matter lesions than the group without mild cognitive impairment and dementia. There was significant relationship between increasing total white matter lesions volume and worse performance on executive function, memory and language. Patients with vascular parkinsonism and dopaminergic denervation have more severe frontal lobe dysfunctions than patients with PD. According to Stojkovic T. et al. [22] results, motor scores were significantly higher in cognitively impaired patients, and only axial score discriminant between mild cognitive impairment and dementia. Whole brain white matter volume was associated with PD dementia, freezing of gait and attention deficits. Additionally, age and bradykinesia scores were independently associated with PD-mild cognitive impairment and age, axial score and whole brain white matter lesions volume with PD-dementia.

Conclusions

In in the present study cerebrovascular disease was common in MRI evaluated Parkinson's disease patients. Brain vascular lesions were more prevalent in patients with a higher QRISK3 score. PD patients with cerebrovascular comorbidity had higher levodopa equivalent daily dose, suggesting more PD severity.

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LR conceptualized the idea, conducted literature review, wrote the manuscript, revised and finalized the text.

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Sonographic approach to the tumours of retroperitoneal space

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Abstract

Background: Timely diagnosis of primary retroperitoneal tumours is one of the current challenges of clinical oncology. This is due to the rarity, polymorphism and diagnostic difficulties of primitive retroperitoneal tumours.
Material and methods: The study is cross-sectional, prospective and retrospective. The study group is represented by 118 patients with abdominal and retroperitoneal space tumours. Using the receiver operating characteristic (ROC) analysis curve and calculating the average quality of the diagnostic model, the informativeness of ultrasonography in the diagnosis of primary retroperitoneal tumours (PRT) was appreciated.
Results: For tumour localization, the ultrasonography (USG) as a diagnostic model demonstrated an appropriate use criteria (AUC) of 0.641 (95% CI 0.541, 0.740, p <0.001), and the mean quality of the diagnostic model was 0.54. Following the statistical analysis, was found a partial correlation between the size of the tumour and the dimensions estimated at USG of 0.540 (95% CI 0.295, 0.737, p <0.001), which represents a high positive correlation. To determine the uni- or multicentric character of the tumour, the USG demonstrated an integrative value of sensitivity and specificity of 0.644 (95% CI 0.415, 0.873, p <0.001. In assessing the proximity ratio of retroperitoneal tumours, the highest AUC was recorded in the assessment of the ratio of tumour to pancreas – 0.838 (95% CI 0.705.0.971, p <0.001) and kidney – 0.861 (95% CI 0.699, 1.024, p <0.001).
Conclusions: Ultrasonography is a fairly informative imaging diagnostic method in the diagnosis of retroperitoneal tumours. The characteristics of the tumours obtained after the ultrasound examination provide indirect information about the malignant or benign nature of the primitive tumour, which allows the assessment of the next stages of diagnosis and treatment.
Key words: ultrasonography, retroperitoneal tumours.

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Introduction

Primary retroperitoneal tumours (PRTs) are an extremely heterogeneous group of tumours of mesenchymal, neuroectodermal or vestigial origin and occur in the retroperitoneum. PRTs can be benign or malignant, the most common being retroperitoneal sarcoma. Approximately 70–80% of primary retroperitoneal soft tissue tumours are malignant; however, they represent only 0.1–0.2% of all malignancies [1, 2]. Retroperitoneal sarcomas (RPS) are rare tumours of mesenchymal origin. The incidence of these tumours is difficult to determine, being 0.31 per 100000 people per year. About 53–56% of patients are women, and the average age at diagnosis is 59–61 years old [3, 4].

Timely diagnosis of PRTs is a challenge for clinicians due to the rarity of this pathology and the difficulty of diagnosing these tumours. The difficulties of diagnosis are due to the peculiarities of the late clinical manifestation of retroperitoneal tumours, which are exaggeratedly large at the time of diagnosis.

The use of ultrasonography with Doppler technique significantly improves the early differential diagnosis of non-organ tumours of the peritoneal cavity and retroperitoneal space.

Data on the semiology of PRTs are not sufficiently systematized. This is due to the low incidence and lack of mainstream clinical trials to systematize the data on this topic. Definition of the diagnostic criteria for the malignant or benign type of tumours requires further research.

The aim of the study was to evaluate the informativeness of ultrasonography as a method of imaging diagnosis in primitive retroperitoneal tumours.

Material and methods

This is a complex study, prospective and retrospective structural analysis of clinical, imaging, morpho-pathological and immunohistochemical data of 118 patients with tumours of peritoneal cavity and retroperitoneal space investigated and treated at the Institute of Oncology of the Republic of Moldova, 2015-2020.

To determine the informativeness of the investigation method used, the representative study group was calculated in the EpiInfo 7.2.2.6 Program, "StatCalc-Sample Size and

Power" section for cross-sectional study based on the following parameters:

- 95.0% confidence interval for significance of results,
- Statistical power – 80.0%,
- Frequency 0.01-0.2% is on average up to 1.0%.

Ultrasonography of the abdominal cavity and retroperitoneal space was performed in 2 D, colour and spectral Doppler to assess the status of the tumour against the main blood vessels (abdominal aorta, renal arteries, inferior vena cava, portal system, etc.) or to determine tumour vascularization. If the tumour was in the small pelvis, the sonographic examination was performed with a full bladder.

The final diagnosis was established by morphological or immunohistochemical examination of the removed tumour or bioptate taken by diagnostic laparotomy or by ultrasound guided biopsy. The statistical processing of the data obtained in the study was performed following the unanimously accepted principles.

Descriptive statistics were used for both categorical and nominal parameters, represented by absolute and relative frequencies, supplemented by 95% confidence intervals, and for continuous, mean, median, standard deviation and percentile parameters (25% and 75%).

The evaluation of ultrasonography as the diagnostic method used in the study, as well as of the imaging semiology, was performed by the receiver operating characteristics (ROC) analyses curve and by determining the average quality of the diagnostic model.

In addition, some statistical tests were performed for the independent groups, the procedures being selected according to the level of measurement of the studied parameters, some particularities of the studied data and the distribution of continuous data. Thus, the $\chi 2$ test with continuity corrections, the Fisher test and the Mann Whitney test were used.

The obtained data was processed using IBM / PC, using the statistical processing software "Statistical Package for the Social Sciences" SPSS 17 for Windows 10.0.5 (SPSS, Chicago, IL, USA) and GraphPad PRISM® 5.0 for Windows 5.0 (GraphPad Software, Inc.).

Results

Although the initial study group consisted of 118 patients, the status of primary retroperitoneal tumour was confirmed morphopathologically and immunohistochemically in only 84 (71.18%) of them, 34 (28.81%) of the patients had retroperitoneal metastases or organic tumours. Imaging evaluation of all patients included in the study was performed by ultrasonography of peritoneal cavity and retroperitoneal space, and pelvic cavity using the contrast CT.

Among the patients with a confirmed diagnosis of primary retroperitoneal tumour, 36 (42.9%) were men and 54 (57.1%) were women, the mean age was 57 years (σ = 12.0), Me = 59.

Although, in most cases, the PRTs are malignant, they metastasize quite rarely, but they have a high recurrence rate. Thus, among the patients with PRTs included in the

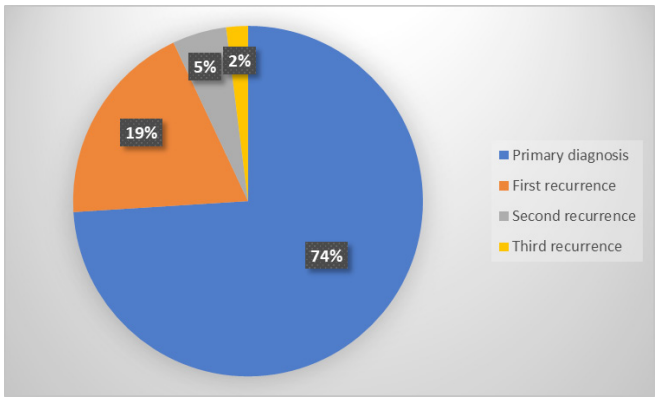

Fig. 1. The recurrence rate of PRTs

Following the statistical analysis, was found a partial correlation between tumour size and ultrasonographically estimated dimensions – 0.540 (95% CI 0.295, 0.737, p <0.001), which is a high positive correlation. The appropriate use criteria (AUC) of ultrasonography was calculated to determine the retroperitoneal location of the tumours is 0.641 (95% CI 0.541, 0.740, p <0.001), the overall model quality being 0.54. The edge characteristics of PRTs sonographic determined can suggest the benign or malignant type of the tumour ($\chi 2$ = 9843, df = 1, p <0.001). Thus, tumours located in the retroperitoneal space, with irregular edges have a higher probability of malignancy. A significant association was also identified between the consistency of the tumour (solid, cystic, mixed) and the benign or malignant type of the tumour ($\chi 2$ = 7526, df = 2, p = 0.023). Therefore, solid or mixed-textured tumours can be considered malignant. To determine the unicentric or multicentric type of the tumour, the ultrasonography demonstrated an integrative value of sensitivity and specificity of 0.644 (95% CI 0.415, 0.873, p <0.001).

In order to assess the treatment tactics, it is essential to determine the proximity of the tumour to the neighbouring organs. In the research group the most affected organs by retroperitoneal tumours were: the colon – in 15 cases (12.7%), the small intestine – 22 cases (18.64), the main blood vessels – 23 cases (19.49%), the pancreas – 17 cases (14.40%), the kidney – 9 cases (7.62%), the adrenal gland – 9 cases (7.62%), the spleen – 7 cases (5.93%),the stomach – 3 cases (2.54%), the bladder – 3 cases 2.54%). Ultrasonography of the abdominal cavity and retroperitoneal space used as a diagnostic test in determining the invasion of adjacent organs, demonstrated an integrative value of sensitivity and specificity as follows: involvement of the colon in the tumor process – AUC 0.767 (95% CI 0.611, 0.922, p <0.001), the involvement of the small intestine in the process calculated AUC was 0.795 (95% CI 0.672, 0.917, p <0.001), AUC calculation for determining the invasion of the pancreas by the tumour was 0.838 (95% CI 0.705, 0.971, p <0.001), for kidney invasion – 0.861 (95% CI 0.699, 1.024, p <0.001), spleen – 0.567 (95% CI 0.326, 0.808, p <0.001), main blood vessels (aorta, inferior vena cava, superior mesenteric artery and vein) – 0.674 (95% CI 0.532, 0.816, p <0.001) (tab. 1).

Table 1. The informativeness of ultrasonography in PRTs diagnosis

		Ultrasonography	
		AUC	Overall model quality
The localization of the tumour		0.641 (IC 95% 0.541, 0.740, p < 0.001).	0.54
The unicentric/multicentric type of tumour		0.644 (IC 95% 0.415, 0.873, p < 0.001).	0.42
The edge characteristics		0.720 (IC 95% 0.601, 0.838, p < 0.001).	0.49
Relations between tumours and adjacent organs	Colon	0.767 (IC 95% 0.611, 0.922, p < 0.001).	0.61
	Small intestine	0.795 (IC 95% 0.672, 0.917, p < 0.001).	0.67
	Pancreas	0.838 (IC 95% 0.705, 0.971, p < 0.001).	0.71
	Kidney	0.861 (IC 95% 0.699, 1.024, p < 0.001).	0.70
	Spleen	0.567 (IC 95% 0.326, 0.808, p < 0.001).	0.33
	Adrenal gland	0.593 (IC 95% 0.377, 0.808, p < 0.001).	0.38
	Blood vessels	0.674 (IC 95% 0.532, 0.816, p < 0.001).	0.53

All patients involved in this study underwent curative or diagnostic laparotomy. Although benign tumours were found, excision biopsy was performed in 12 patients (35.3% (95% CI 20.9, 52.0)) and excision of the tumour in 17 patients (34.0% (95% CI 22.1, 47.7)). Diagnostic laparotomy was performed in 15 patients (44.3% (95% CI 20.9, 52.0)) with histopathologically confirmed malignancies, and in 27 cases (54.0% (95% CI 40.3, 67.3)) the tumour was excised.

Although retroperitoneal cysts are in most cases benign, which was demonstrated in this study, all 6 cysts identified were benign, complete excision of the cysts was successful in only 2 cases (4.0% (95% CI 0.8, 12.2)), in 4 cases that constituted (11% (95% CI 20.9, 52.0)) the partial excision of the cysts was performed.

Non-Hodgkin's lymphomas with primary involvement of the retroperitoneal lymph nodes were confirmed in 7 cases: in 3 cases by excisional biopsy (8.8% (95% CI 4.1, 25.6)), and in 4 cases, when the disease was manifested by the presence of a tumour, solitary or lymph node conglomerate, the tumour was completely removed (8.0% (95% CI 2.8, 17.9)).

Discussion

PRTs are histologically heterogeneous benign and malignant neoplasms, being categorized on the basis of a single principle, the anatomical space where they develop – the retroperitoneal space [5].

Presented symptoms are often not specific and dependent on the anatomical site involved. The retroperitoneal sarcoma (RPS) usually grows as a mass, causing compression symptoms on other organs and a sense of abdominal discomfort, especially when it reaches a considerable volume. More frequently, RPS are incidental findings at the imaging tests performed for other reasons. Some of the most frequent symptoms are abdominal pain and discomfort, back pain, bowel obstruction, urinary and gynaecological symptoms. When the mass becomes bulky, it can be palpated externally [6-8].

A correct evaluation of the diagnostic images is paramount to stage the disease, establish the best therapeutic pathway and evaluate the surgical resectability.

Ultrasonography is an imaging diagnostic method that allows the evaluation of retroperitoneal tumours by providing information in an acceptable volume to assess subsequent diagnostic and treatment tactics. Due to the fact that the PRT becomes quite large at the time of diagnosis, the average tumour size of the patients in the study being 17.3 cm (± 10.5 cm), the assessment of tumour size is difficult. In assessing the retroperitoneal or intraperitoneal localization as well as in determining the unicentric or multicenter tuple of the tumour, ultrasonography demonstrated integrative values of credible sensitivity and specificity, 0.641 and 0.644, respectively. Determining the margins and tumour texture by assessing tumour echogenicity at ultrasound examination plays a key role in determining the tactics and volume of surgical treatment applied. The surgical treatment applied can be palliative or radical. If curative surgery is expected, an assessment of the tumour's proximity to adjacent organs is essential. PRT being in 80% malignant cases, most often the tumour invades the adjacent organs. Ultrasonography, as a diagnostic method used in assessing organs invaded by the neoplastic process, has demonstrated integrative values of sensitivity and specificity of high veracity (the highest AUC being used to determine the invasion of the colon, small intestine, pancreas and kidney). An advantage of ultrasonography over other imaging methods used in the diagnosis of PRT is that it is available in all medical institutions, it is a harmless method that can be applied to all categories of patients and it is an inexpensive method that meets practically all the conditions for a screening investigation method.

Conclusions

Ultrasonography is an imaging diagnostic method that can be easily used in the diagnosis of PRT as a preoperative diagnostic step. It is an imaging method that provides information in acceptable volume for making a decision to approach a patient with a retroperitoneal tumour, but does not provide enough information to plan surgery.

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Authors' contributions

VS interpreted the data and performed the analytical part of the work, drafted the first manuscript; VT and NG conceptualized the project, designed the research and revised the manuscript critically.

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The authors are independent and take responsibility for the integrity of the data and accuracy of the data analysis.

Ethics approval and consent to participate

The research project was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 84, 20.06.2017).

Conflict of Interests

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REVIEW ARTICLES

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Could human amniotic membrane be a source for acupoint thread embedding therapy?

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Abstract

Background: Peripheral neuropathy usually leads to a major cause of motor disability, but the functional restoration after treatment continues to show modest results. Acupoint thread-embedding therapy is a subtype of acupuncture treatment in which biodegradable threads are inserted into skin, subcutaneous tissue or muscles at specific points for long stimulation. Different biodegradable materials have been developed and widely used. Human amniotic membrane is rich in collagen, extracellular matrix proteins and growth factors. The avascular, low immunogenic, anti-inflammatory, antibacterial, anti-fibrotic and non-tumorigenic properties of amniotic membrane make it valuable in medical applications and its use has no ethical problems. Elasticity, stiffness and other biomechanical properties also make it possible to use the amniotic membrane for various medical purposes. AM is almost always considered as discarded substance, it satisfies most of the criteria of an ideal biological tissue and shows almost zero rejection phenomenon.
Conclusions: The human amniotic membrane, the cellular compounds and extracellular matrix have a lot of benefic proprieties that are or could be used in treatment of many human diseases. Its biological and biomechanical properties are promising in the manufacture and use of filaments in acupoint thread embedding therapy.
Key words: peripheral neuropathy, acupoint thread embedding therapy, amniotic membrane.

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Introduction

Peripheral neuropathy is a damage or dysfunction of one or more peripheral nerves, which usually leads to numbness, tingling, muscle weakness, and pain in the affected area [1]. Globally, it is estimated that 2-3% of the population suffers from peripheral neuropathy, and the prevalence increases with age [2]. A study estimated that the prevalence of peripheral neuropathy in family medicine is 8% in people from 55 years old [3]. Peripheral nerve trauma remains a major cause of motor disability, at the same time functional restoration after treatment continues to show modest results [4].

In 1960s, in China appeared a treatment method by implanting absorbable materials (e.g. catgut) substituting for filiform needles into the acupoints, which realized long-time needle retaining and also avoided the danger of filiform needle retaining. This method was then termed acupoint thread-embedding therapy (ATET) [5]. ATET is an invasive treatment which can prolong point stimulation, reduces the frequencies of pain and psychological fear of patients [4, 6]. Different biodegradable materials have been developed and widely used. They are divided into natural (e.g., catgut) and synthetic types (e.g., polyglycolic acid, polylactic acid etc.) according to material sources. Both have advantages and disadvantages [6]. The ideal embedding materials are required to be safe, non-toxic, biocompatible, and to have excellent swelling and biodegradation behaviors. ATET can be a promising treatment method of peripheral nerve disorders [4].

The amniotic membrane (AM) is a biological material of the human placenta, constituting the inner wall of the fetal membranes [7]. It surrounds the embryo/fetus and delimits the amniotic cavity, which is filled by amniotic fluid [8]. Fetal membranes are composed of two layers: an outer layer (chorion), which contacts maternal cells and an inner layer (amniotic membrane) [9].

AM is a gift of nature which not only protects the fetus inside the womb but also has several medicinal proper-

ties. It serves as a natural barricade to protect the fetus from bacterial infection and trauma [10]. The fetal membranes facilitate the exchange of gas, nutrients, and waste, serving as a barrier to protect fetus from the maternal immune system and synthesizing certain hormones and enzymes that are critical during pregnancy and parturition [11-14]. AM is not just a simple avascular structure; it has multiple metabolic functions, such as the transport of water and soluble materials and the production of bioactive factors, including vasoactive peptides, growth factors and cytokines [15, 16].

Although AM is almost always considered as discarded substance, it satisfies most of the criteria of an ideal biological dressing and shows almost zero rejection phenomenon [17]. It is one of the thickest membranes in the human body and can withstand current cryopreservation techniques [15, 18]. The translucent, avascular, low immunogenic, anti-inflammatory, anti-scarring, and wound healing properties of AM allow this material function beyond its role *in vivo* and assume a wide range of applications in regenerative medicine [19, 20].

The earliest known clinical applications of amnion go back to the beginning of the previous century. Until the beginning of the seventies only a few reports can be found. The use of amnion has become firmly established in addition to other new methods [21].

The literature search was performed using the search terms "peripheral neuropathy", "acupoint thread embedding therapy", "amniotic membrane" and were selected from databases, such as PubMed, Hinari, Springer, Elsevier and Science Direct. The material was selected based on the studies published until 13/01/ 2021, which aimed to elucidate the structure and properties of human amniotic membrane. After processing the data obtained from databases according to the search criteria, we found 107 articles related to human amniotic membrane properties and structure. The articles used were written in English and Romanian. The final bibliography included 97 relevant sources that were considered representative materials on this topic and sufficient to formulate the main ideas of this text. The information systematized the main aspects of human amniotic membrane structure and properties. The articles which do not correspond to this article goal were excluded.

Amniotic membrane structure

AM thickness varies from 0.02 mm to 0.05 mm and consists of three main histological layers: the epithelial layer, the thick basement membrane and the avascular mesenchymal tissue or stroma [18]. The AM contains no blood vessels or nerves; instead, the nutrients it requires are supplied directly by diffusion out of the amniotic fluid and/or from the underlining decidua. Two cell types, extracellular matrix proteins, and growth factors are placed in the mentioned layers [19, 22].

Epithelium is a monolayer of metabolically active cuboidal cells with microvilli present on its apical surface which are in direct contact with amniotic fluid [18, 20]. These cells have a large irregular nucleus with a large homogeneous nucleolus and many intracytoplasmic organelles and pinocytic vesicles [23]. The first epithelial layer is composed of collagen I, II, and V and expresses some of the crucial epidermal markers, such as glycoprotein CA125 and oxytocin receptors and are also positive for antigen CD44 and desmin [24, 25]. Erythropoietin and its receptors are expressed in human amniotic epithelial cells. Erythropoietin, whose functions are still unknown in the AM, stimulates the differentiation, proliferation and survival of erythroid precursors and its production is regulated by the concentration of oxygen in the blood [22, 26].

Ogawa et al. (2003) have reported that erythropoietin production in human amniotic epithelial cells is stimulated by progesterone but is not stimulated by hypoxia or 17β-estradiol [26].

The basement membrane is made up by reticular fibers. It is made up of type IV, V and VII collagen in addition to fibronectin and laminin. Although thin, it is one of the thickest basement membranes found in the human body and can withstand cryopreservation [18, 27]. The basement membrane contains large amounts of proteoglycans that are rich in heparan sulphate and that serve as a permeable barrier to amniotic macromolecules and several molecules with a structural function enabling the maintenance of membrane integrity. These molecules are actin, α-actinin, spectrin, ezrin, several cytokeratins, vimentin, desmoplakin and laminin [28-30].

The stroma of AM can be subdivided further into a compact layer, a fibroblast layer, and an outer spongy layer [18]. The collagens of the compact layer are secreted by mesenchymal cells situated in the fibroblast layer. Interstitial collagens (types I and III) predominate and form parallel bundles that maintain the mechanical integrity of AM. Collagens type V and VI form filamentous connections between interstitial collagens and the epithelial basement membrane. The spongy layer of the stromal matrix sits adjacent to the chorionic membrane. Its abundant content of proteoglycans and glycoproteins produces a spongy appearance in histologic preparations, and it contains a nonfibrillar meshwork of mostly type III collagen [31]. Closely connected to the chorionic membrane, the spongy layer consists of wavy bundles of reticulum bathed in mucin; hence, AM is easily separated from the chorion by means of blunt dissection [19].

Amnion-derived cells

Cells of AM have pluripotent properties and, for this reason, are an attractive source for transplantation [24]. Pluripotent stem cells are self-renewing cells, capable for differentiating into all 3 germ layers of the developing embryo: ectoderm, mesoderm, and endoderm. The amniotic membrane includes amniotic mesenchymal cells (AMCs) and amniotic epithelial cells (AECs) which are responsible for the production of extracellular matrix (ECM), different cytokines and growth factors [19]. These cells have several properties which make them as an appropriate cell source for stem cell therapy. AMCs exhibited plastic adherence and fibroblastic morphology, while AECs displayed a cobblestone epithelial phenotype [32, 33]. One of the most abundant proteins found in AM derived cells is laminin, which

plays a key role in differentiation, cell shape and migration, and tissue regeneration [24, 34]. Several studies have documented that amniotic cells express various surface markers associated with embryonic stem cells, e.g. stage-specific embryonic antigen 3 and 4 (SSEA-3 and –4), TRA-1-60 and TRA-1-81 [19]. Epithelial and mesenchymal amniotic cells also express various stem cell markers, such as octamer-binding transcription factor 4 (OCT-4), hepatocyte nuclear factor 3β (HNF-3β), nanog and nestin [32, 35, 36].

The mesenchimal stem cells (MSCs) derived from amniotic membrane have been reported as a better new prospective field of regenerative medicine compared with other MSCs sources, because of the easiness of their acquisition, reduced donor damage, multipotency, low immune response, acceptable ethical issue [24, 37]. The AMCs are positively expressed for the mesenchymal specific markers including CD44, CD73, CD29, CD105 and CD90 and do not express the hematopoietic markers and human leukocyte antigen including CD34, CD45, CD11b, CD19, HLA-A, HLA-B and DR antigens [38]. AMCs also secrete some anti-inflammatory cytokines, such as PGE2, IDO, HGF and TGF-b [39]. AMCs promote angiogenesis through secretion of some angiogenic factors, such as angiogenin, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [40]. RT-PCR analysis showed that AMSCs expressed genes, such as Oct-3/4, zinc finger protein 42 (zfp42 or Rex-1), stem cell factor protein (SCF), neural cell adhesion molecule (NCAM), nestin (NES), bone morphogenetic protein 4 (BMP-4), GATA binding protein 4 (GATA-4), and hepatocyte nuclear factor 4α (HNF-4α) even in high passages [32, 38]. AMCs shared similar phenotypic characteristics with the ones derived from adult sources. AMCs exhibited a higher proliferation rate compared to MSCs derived from adult sources and a multilineage differentiation potential into cells derived from the three germ layers [32, 33, 37].

AECs have some properties that make them a precious candidate to be considered as a source of pluripotent stem cells [19]. The epithelial cell population could be exclusively isolated from the amnions of term human placentae by specific enzymatic digestion [41]. It has been shown that cultured AECs secrete various morphogens and growth factors, such as epidermal growth factor, Noggin, Activin [42], platelet-derived growth factor, vascular endothelial growth factor, angiogenin, transforming growth factor-beta-2 (TGF-β2), and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) [43].

AECs express surface markers commonly found in embryonic stem cells, such as SSEA-3, SSEA-4, TRA1–60, TRA-1–81 and pluripotent stem cell-specific transcription factors like Oct-4 and Nanog, fibroblast growth factor 4 (FGF4), Rex-1, cryptic protein (CFC-1), and prominin 1 (PROM-1) [33, 44, 45]. AECs express the non-polymorphic, non-classical human leukocyte antigen (HLA-G) which is involved in the induction of immune tolerance. Therefore, the risk of rejection or immune reaction will be reduced upon transplantation of AECs [46, 47]. AECs express secretory leukocyte proteinase inhibitor (SLPI) and elafin which have anti-inflammatory properties. In addition, they both are a part of amnion's innate immune system which prevents infection. AECs also produce β -defensins that are anti-microbial peptides [48, 49].

Tehrani et al. reported for the first time that the AM cells contain cathelicidin LL-37 as a polypeptide involved in innate antibacterial property which can be augmented in the cells after exposure to inflammatory signal IL-1β [50]. Another merit of AECs is the number of cells derived from a single source of tissue. The average yield is reported to be more than 100 million cells per amnion [35].

AECs and AMCs possess unique characteristics which make them an excellent candidate for clinical applications. These cells exhibit low immunogenicity that protects them against the host immune system [51]. Although AECs decrease angiogenesis, AMCs are able to improve angiogenesis via expression of some angiogenic components, such as interlukin-6 (IL-6), growth related oncogene (GRO), monocyte chemoattractant protein-1 (MCP1), C-X-C Motif Chemokine Ligand 8 (CXCL8), intracellular adhesion molecule (ICAM) and migration inhibitory factor [40].

There is no evidence of tumorigenic behaviors of amniotic-derived stem cells. Furthermore, the AM has the merits of decreasing loss of protein, electrolytes, and fluids, reducing the risk of infection, minimizing pain, speeding up the process of wound healing and appropriate handling properties [52].

The potential of human amniotic membrane-derived stem cells for the treatment of hepatic disorders has also been examined. The capacity of these cells to differentiate into liver cells was evaluated, using periodic acid-Schiff staining on amniotic membrane cells in order to evaluate glycogen storage. The positive staining demonstrated that the amniotic membrane cells were able to carry on important physiological function of hepatocytes such as glycogen storage [53].

Takashima et al. have found that several hepatic genes are expressed in AECs, namely: albumin, α1- antitrypsin, cytokeratin-18, glutamine synthetase, carbamoyl phosphate synthetase I, phosphoenolpyruvate carboxykinase, cytochrome P450 2D6 and 3A4 (genes involved in drug metabolism). Hepatocyte nuclear factor 3γ (HNF-3γ), one of the isoforms of transcription factor HNF-3 and CCAAT-enhancerbinding protein-α (C/EBP-α) are genes involved in the regulation of the process of transcription in hepatocytes and have been identified in amniotic cells. The HNF-3γ gene induces the expression of albumin, α-fetoprotein, α1-antitrypsin and transthyretin. On the other hand, the C/EBP-α gene controls glycogen storage and the gene expression of albumin, α-fetoprotein, transthyretin, and tyrosine aminotransferase [36].

The capacity of human AECs to differentiate into type II pneumocytes has also been explored in several studies. One of the studies that evaluate the effect of human AECs in lung injury induced lung inflammation and fibrosis in a mouse model with bleomycin, treating the mice with a human AECs transplant. After the transplant, human AECs were

able to differentiate into phenotypic alveolar epithelium and secrete surfactant protein. The application of human AECs also helped fight lung fibrosis, with a reduction of lung collagen, and inflammatory and fibrotic cytokines [54].

The amniotic cells exhibit some specific cardiac transcription factors, namely cardiacspecific transcription factor GATA4, cardiac-specific genes, such as myosin light chain (MLC)-2a, MLC-2v, cTnI and cTnT, α-subunits of the cardiac-specific L-type calcium channel (α1c) and the transient outward potassium channel [24]. Co-culture experiments have confirmed that amniotic cells have the ability to integrate into cardiac tissue and to differentiate into heart cells. *In vivo* studies have also demonstrated cardiomyocyte differentiation after the injection of amniotic cells into scar tissue post-myocardial infarction [55].

Toda et al. have shown that induction *in vitro* with BMP-2 leads to expression of collagen II and aggrecan. Amniotic cells were implanted into non-cartilage tissue in an animal model with BMP-2 or were implanted with a collagen scaffold into defects generated in rat bone. As a result, morphological changes with the deposition of collagen type II were observed [24].

Wei et al. verified the expression of insulin mRNA in cultivated AECs and the normalization of blood glucose values for several weeks after the implantation of amniotic cells into streptozotocin-induced diabetic mice [56].

Studies evaluating the effect of ASCs in new therapies have been geared toward their utility in several neurological disorders including Parkinson's disease, stroke, traumatic brain injury, and spinal cord injury [57]. The expression of acetylcholine, catecholamines, dopamine, neurotrophic factors, activin and noggin has been found in epithelial and amniotic basement membrane cells [42, 58]. Neural grafts with amniotic epithelial cells in animal models of Parkinson's disease result in the synthesis and release of catecholamine and neurotrophic factors, such as nerve growth factor, neurotrophin-3 and brain-derived neurotrophic factor [24, 59].

In another experiment, human AECs were transplanted into a hemorrhagic stroke model in rats, improving motor skills, and reducing cerebral edema, with survival of transplanted cells in the lateral ventricular wall at 4 weeks [60].

Biological properties of amniotic membrane

Human AM has proven to be an outstanding scaffold for tissue engineering owing to its ability to allow the transport of water and the presence of growth factors such as the epithelial growth factor [61]. Human AM has advantageous characteristics including promotion of epithelization, anti-inflammatory effects, anti-bacterial properties [62], anti-fibrotic properties [19], low immunogenicity as well as immunomodulatory properties [62, 63], anti-angiogenic and non-tumorigenic properties [63].

Basement membrane of AM is composed of collagen type IV, V and VII which facilitates the growth of epithelial cells [18]. Human AM promotes epithelization by excreting EGF, IL-8, insulin-like growth factor 1 (IGF-1), PDGF bFGF, HGF, TGF-b, and other factors that support epithelization and differentiation of different cells [64-66]. Molecules of human AM extracellular matrix, such as fibronectin, laminin-1, laminin-5, collagen type-I, III, IV, V, and VII, also promote cell adhesion and migration [27, 67].

Although the immunogenicity of the AM is controversial, in general, it is believed that the AM possesses low immunogenicity because AECs do not express HLA-A,-B,-D and-DR antigens on the cell surface, but express HLA-G [68]. Presence of interferon – and other immunologic factors has also been observed in the amniotic membrane. It seems that amniotic membrane may induce immunologic reactions in the presence of viable epithelial cells [69]. Human AECs and human AMSCs express low to moderate levels of major histocompatibility complex class I (MHC1) molecules – human leukocyte antigen (HLA), including antigens Ia (HLA-A, B, C) and Ib (HLA-G, E). Moreover, they do not express (or express only very low levels of) HLA II class molecules (HLA-DP, -DQ, -DR) and costimulatory molecules (CD80, CD86) on the cell surface. These properties of hAM decrease the possibility of transplant rejection, which is an important advantage when choosing materials for use in regenerative medicine [51, 70]. In addition, it is generally thought that the immunogenicity of cryopreserved AM tissue is less than that of fresh AM tissues and that cryopreserved cells are expected to be nonviable. This approach guides some researchers to use cryopreserved AM instead of fresh AM [51, 63].

Tissue engineered constructs often provoke an inflammatory reaction known as a foreign body reaction upon implantation. These implanted materials can be degradable or non-degradable. While inflammation can be good in some instances to trigger the healing of an injury, it can also lead to implant failure [71]. There are several reports of the AM reducing inflammation. The AM stromal matrix markedly suppresses the expression of the potent pro-inflammatory cytokines, IL-1α and IL-1β [72]. Matrix metalloproteases (MMPs) are expressed by infiltrating polymorphonuclear cells and macrophages. Natural inhibitors of MMPs have been found in the AM [73, 74]. Hyaluronic acid is a high-molecular-weight glycosaminoglycan that exists in large quantities in the AM and acts as a ligand for CD44, which is expressed on inflammatory cells and plays an important role in adhesion of inflammatory cells, including lymphocytes, to the AM stroma [75]. It has been proved that IL-10 can suppress the effect of pro-inflammatory cytokine IL-6 and tumor necrosis factor-alpha [76]. The IL 10 also suppresses the production of IL-8, a pro-inflammatory chemokine which attracts migration of neutrophils [77]. AM also contains inter-α-trypsin inhibitor which also possesses anti-inflammatory action [78]. AM can also suppress the staffing of inflammatory cell, such as polymorphonuclear cells, CD3 cells, CD4 cells, T cells and CD11b cells to the wounded site thus decreasing inflammation [56, 75].

AM contains natural antimicrobial molecules which are component of innate immune system thus act as safeguard against Gram-negative and Gram-positive bacteria, viral and fungal infection [49, 79]. The innate immune system has evolved to eliminate microorganisms upon entry into

the tissues, creating antigens necessary to produce an adaptive immune response. AECs also have the ability to produce βdefensins [49]. The β-defensins are an important group of antimicrobial peptides which resist microbial colonization, expressed in AECs. Beta 3-defensin is the most prevalent defensin of AECs [80]. Some other important antimicrobial components expressed in AM are low molecular mass elastase inhibitor, secretory leukocyte proteinase inhibitor and elafin [81]. In addition to their anti-inflammatory properties, elafin and secretory leukocyte proteinase inhibitor have antimicrobial actions and act as components of the innate immune system to protect related surfaces from infection [48].

Kim et al. showed that histones H2A and H2B, which possess antimicrobial and endotoxin-neutralizing activity, were localized in the cytoplasm and on the extracellular surface of human AECs [82].

Foreign body reactions evoke stimulation of giant cells and macrophages that produce cytokines and attract fibroblasts, leading to fibrosis. These fibroblasts are activated by the transforming growth factor (TGF)β [71]. The AM downregulates TGF-β and its receptor expression by fibroblasts and in doing so, reduce the risk of fibrosis [83]. Human AM reduces the risk of scarring and adhesion due to secretion of TIMP-1, -2, -3, and -4, which reduce proteases activity on the site of application [74].

Angiogenesis is the formation of new blood vessel from pre-existing one. Some specific compounds have been detected in AM which can prevent angiogenesis. Anti-angiogenic chemicals identified in both epithelial and mesenchymal cells of AM are thrombospondin-1, endostatin and all four types of tissue inhibitors of metalloproteases (TIMP-1, 2, 3 and 4) [73]. Thrombospondin-1 is a potent anti-angiogenic chemical produced by only 20% of mesenchymal cells, whereas endostatin is a powerful anti-angiogenic and endothelial cell growth inhibitor. MMP-1, MMP-2, MMP-3, MMP-4, IL-1 receptor antagonist, collagen XVIII and IL-10 are some of the proteins found in AM and that might have anti-angiogenic activity [73]. Pigment epithelium-derived factor (PEDF) expressed in the AM plays a major role in eliciting the anti-angiogenic activity of AM [84].

Another critical part of safety evaluation is to identify whether human AESCs have an effect on the tumor generation and promotion. In previous studies, the non-tumorigenicity of AECs has been confirmed in many species, including in humans [85-88]. Human AECs and human AMSCs or their conditioned medium (culture medium, which was in contact with hAM or human AM-derived cells during culture) are capable of inducing apoptosis in several cells lines (HeLa cervical cancer cells, MDA-MB231 breast cancer cells, hepatocarcinoma cancer cells HepG2, Hep3B2.1-8, HuH7) [89, 90].

Magatti M. et al. [91] have shown that human AMSCs induce the cell cycle arrest of hematopoietic and nonhematopoietic cancer cells in co-culture by inhibition of positive regulators of the cell cycle (cyclins, cyclin-dependent kinases, mini-chromosome maintenance complex, proliferating cell nuclear antigen) and upregulation of cell cycle inhibitors (cyclin G2, CDK inhibitor 1A, CDK inhibitor N2B). Additionally, Cullin-1 (mediator of ubiquitination and degradation of several proteins, including p21) and RB-1-like protein (p107) are downregulated and retinoblastoma protein (pRB) is upregulated. Consequently, this leads to cell cycle arrest of cancer cells in the G0/G1 phase and prevention of cell cycle progression to S phase.

Mechanical properties of amniotic membrane

Thickness of normal amniotic membrane lies between 0.02 and 0.05 millimeters which includes around 6–8 layers of cells. An average surface area of this membrane is about 1600 square centimeters [92]. The elasticity, stiffness and other biomechanical properties of the ECM depend on the variation in its ingredients, such as collagen, proteoglycan, and elastin [93]. The orientation of the collagen fibrils in the ECM is responsible for the tensile strength, whereas the elastic deformation is related to the presence of elastin fibers [62], laminin, hyaluronic acid, and glycosaminoglycan [94]. Some research has shown the amniotic membrane shear modulus to be between 100 and 400 Pa, with the difference in measurements related to the state of the human AM used [95]. Decellularized human AM presents higher shear modulus than native one, because the denudation process dehydrates the membrane and thus decreases its thickness. It has also been shown that the elastic modulus decreases with increasing human AM thickness [95, 96]. Moreover, the reported values of elastic modulus differ between human AM from distal (135 Pa) or proximal parts (62 Pa) in the placenta [95], which could be attributed to the thickness of the membrane, the tissue composition, and to a time-dependent viscoelastic property of creep of the human AM (tab. 1). This viscoelastic property is related to the increase in the amniotic fluid volume and fetal development during gestation [62].

Table 1. Mechanical properties (intact AM)[97]

Amniotic membrane features	Contributing factors	References
• Direct tensile mechanical properties	Placental: Force before rupture: 1.2 ± 0.2 N Strain at break: 19% ± 3% N Peripheral: Force before rupture: 0.68 ± 0.08 N Strain at break: 16% ± 1% N	Litwiniuk et al., 2017
• Young's modulus	2.29–3.6 MPa	Niknejad et al., 2008
• Tensile strength	5.475 ± 0.135 MPa	Cai et al., 2015;
• Elastic modulus	4.048 ± 1.702 MPa	Ramesh et al., 2017.

Conclusions

The human amniotic membrane, the cellular compounds and extracellular matrix have a lot of benefic proprieties that are/or could be used in treatment of many human diseases.

AM satisfies most of the criteria as an ideal biological tissue and shows almost zero rejection phenomenon.

Taking into consideration the strength, biological properties and grown factors of amniotic membrane it may be a source for embedding therapy treatment.

Biomechanical and good biodegradable properties of human amniotic membrane make it possible to be used for various medical purposes including the manufacture of threads for acupoint embedding therapy and for treatment of peripheral nerve disorders.

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Sevoflurane anesthesia: impact on postoperative cognitive dysfunction

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Abstract

Background: Sevoflurane is the inhalational anesthetic agent that is used widely in operating room. It is currently the most commonly used inhalation anesthetic in operating rooms. A series of studies on animal and human model detected the association of intraoperative use of sevoflurane and postoperative cognitive dysfunction (POCD) manifestation. On the other hand other studies demonstrate the same POCD associated with intravenous agents. Relevant multicentric trials got the reasons to suspect other key factors in developing postoperative cognitive dysfunction.
Conclusions: The intra-anesthetic use of sevoflurane has been associated for a long time with the higher incidence of POCD. The mechanism was not identified, and the theory of neuroinflammation remained the main key of pathophysiological reaction that leads to cognitive dysfunction. Recent multicentre trial gives reliable information that the use of intravenous anesthetic agents is associated with the same POCD. Neuroinflammation remains to be the mediator of cognitive disorders, and apparently IL-6 keeps a major role in them. Future studies are needed to be conducted to identify the role of anesthetic agents in determining the neuroinflammation.
Key words: sevoflurane, propofol, outcome, postoperative cognitive dysfunction.

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Introduction

Inhalational anesthetic agents have a long lasting history, from the beginning of general anesthesia and the discovery of ether in 1866, and our days where we can use a large variety of inhalational agents: desflurane, sevoflurane, isoflurane and others, to provide a safe general anesthesia. Despite large use of them, worldwide there are still many questions remain to be clarified concerning the use of inhalation anesthesia and its effects on the recovery of the patients. Sevoflurane is one of the most widely used anesthetics for the induction and maintenance of general anesthesia. Despite the large use, however, its anesthetic mechanism remains unclear like for the rest of the agents. Sevoflurane reportedly causes amnesia, analgesia, coma, and quiescence, primarily by inhibiting N-methil-D-aspartat (NMDA) receptors. In addition, *in vivo* studies have suggested that gamma-aminobutyric acid (GABA) type A (GABAA) receptors, nicotinic acetylcholine receptors, and voltage-gated sodium channels are potential targets for sevoflurane related to its hypnotic effects.

The clinical use of the sevoflurane extended in countries from Eastern Europe as well, for the last 10 years. It is currently the most commonly used inhalation anesthetic in operating rooms. It has been demonstrated excellent respiratory tolerance in different clinical cases, as well as the hemodynamic stability in cardiopulmonary bypass, providing a safe anesthetic process for different categories of surgery from one-day surgery to complex long-lasting surgery.

Material and methods

There was made a scientific review of the latest articles from PubMed database that were published between 2016-2021, concerning sevoflurane and its effects. The selection criteria were: prospective studies, guidelines, trials and meta-analyses. Key words used in search: "sevoflurane", "propofol", "outcome", "postoperative cognitive dysfunction" (POCD). The articles contain a detailed analysis and synthesis of the recommendations, concerning the use of the sevoflurane and cognitive disfunction after its use. There were selected articles, taking into consideration their title, and selecting abstracts. In the process of searching by title using key words, there have been found 686 results. After selecting the period of years 2016-2021, 352 results were found.

Results

One of the most frequent postanesthetic complications, especially in elderly patients, after the use of the sevoflurane is POCD, which is a multifactorial, neurodegenerative condition, mechanisms of which remain unclear. Many studies and repeated clinical trials have shown that after exposure to sevoflurane-inhalation anesthesia, humans and animals

experience varying degrees of cognitive dysfunction [1, 2]. Different hypotheses like neuroinflammation, changes in neurotransmitters, a decrease in brain-derived neurotrophic factor (BDNF), mitochondrial oxidative stress, and changes in Aβ concentrations regarding the pathogenesis of sevoflurane-induced POCD, are described by researchers. Still these mechanisms are not completely independent and interact with each other, finally resulting in POCD. In recent years, increasing attention has been paid to the relationship between sevoflurane-induced POCD and neuroinflammation, changes in neurotransmitters, and BDNF reduction [3]. Alalawi R. and Yasmeen N. in 2018 showed that the incidence of POCD was at least twice as high in individuals older than 60 years as compared to younger age groups [4]. The high incidence of POCD in older patients may be related to specific susceptibility factors. Aging itself is a risk factor for cardiovascular, respiratory, renal, and neurodegenerative diseases. Immune responses also decrease with age. Also, the pharmacokinetics and pharmacodynamics of older patients are considerably altered compared to younger patients. Sevoflurane-based anesthesia in older patients results in a lower minimum alveolar concentration of gas and an increased cumulative effect of it, with gradual degeneration of various organ functions [4]. Hence, sevoflurane may remain in the blood for longer periods in older than in younger patients after anesthesia [5]. The fragile balance between neuroinflammation and neuronal functioning in older patients is easily interrupted upon pathological invasion [6, 7]. In 2015 Qiao Y. et al., have found elevated plasma concentrations of S-100β protein, TNF-α, and IL-6 in patients receiving sevoflurane anesthesia [7]. Other animal study in 2020, headed by Yang L. et al., determined that sevoflurane has been found to induce increased inflammation and apoptosis of hippocampal neurons in older rats [6]. Sevoflurane-induced anesthesia disturbs the balance between neuronal functioning and neuroinflammation, especially in older patients, increasing the incidence of POCD. To reduce the incidence of sevoflurane-induced POCD the optimal management of pre-existing diseases and maintenance of optimum functioning of the nouronal homeosthasis may be an effective way. Another randomized controlled trial of sevoflurane-induced anesthesia in laparoscopic cholecystectomy, showed aggravated POCD compared to propofol group of patients after laparoscopic cholecystectomy [8]. On the other hand, some studies have confirmed that sevoflurane exerts neuroprotective effects through specific pathways, in a rat model of focal cerebral ischemia [9]. Recently in 2020, Lu G. et al., presented the results of an animal model study where sevoflurane pre-treatment exerted a neuroprotective effect by reducing signaling activity and activating autophagy [10]. Also, sevoflurane post-treatment can regulate mir-203 expression to attenuate cerebral ischemia – reperfusion-induced neuroinflammation by targeting MyD88 [11].

Neuroinflammation is one of the multifactorial determinants, particularly in the hippocampus, that has been shown to play an important role in POCD. Block et al., in 2007, then Lu B. et al., in 2020 showed in their studies that the activation of microglia may play the most important role in the development of POCD, as inflammation of microglia is recognized as the main source of pro-inflammatory cytokines and chemokines in the central nervous system (CNS) [12, 13]. Another study headed by Su W. et al., in 2020 is concerning neuroinflammation and microglial activation trigger that amplify a complex cascade of reactions, including immune response activation, microcirculatory changes, increased hippocampal oxidative stress, and increased blood-brain barrier permeability [14]. Other studies that researched the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α determined that they were statistically significantly increased in the brains of rats exposed to sevoflurane in a number of studies [8].

Zhu X. et al., in their study from 2021 showed the correlation between the property of sevoflurane to be highly and lightly diffused across the cell membrane without binding to specific receptors on the cell membrane and to direct stimulation of NF-κB signaling. Sevoflurane has been shown to increase intracellular Ca2+ by activating GABA receptors, inducing mitochondrial damage, and increasing the levels of intracellular reactive oxygen species (ROS) [15]. A sevoflurane-induced increase in intracellular Ca2+ may activate NF-κB signaling and lead to increased concentrations of pro-inflammatory cytokines. IL-17A, a novel cytokine, increases statistically significantly in the hippocampus of sevoflurane-induced aged rats and can promote the binding of Act1 (an activator of NF-κB) and IL-17R to induce activation of the NF-κB signaling pathway [15].

Neuroinflammation is a critical neuropathological process for postoperative cognitive dysfunction. This finding is mostly based on results from animal studies. It has been shown that surgery on peripheral tissues causes systemic inflammation that then induces neuroinflammation in rodents [16, 17]. Consistent with this finding, serum interleukin-6 concentrations were increased after surgery in patients with or without delayed neurocognitive recovery, but the interleukin-6 concentrations were higher in patients with delayed neurocognitive recovery than that in patients without delayed neurocognitive recovery, providing initial evidence that heightened inflammation may be a pathologic process for delayed neurocognitive recovery in humans. There were no changes in the serum interleukin-1β, interleukin-10, and tumor necrosis factor-α within 24 h after the surgery. These results suggest that only selected cytokines, like interleukin-6 in this surgical population, are induced after surgery. Interestingly, the serum concentrations of vascular endothelial growth factor, intercellular adhesion molecule, transforming growth factor-β1, C3α, and advanced glycation end products were first decreased and then recovered after surgery. The first four factors can modulate immune functions. Advanced glycation end products are an oxidative stress marker. The reasons for this pattern of change are not known but may indicate a decreased immune function at the end of surgery, which recovers with time after surgery [18].

A multicenter, randomized trial on intravenous versus volatile anesthetic effects on postoperative cognition in elderly patients undergoing laparoscopic abdominal surgery, published in 2021, showed that patients with laparoscopic abdominal surgery under propofol-based anesthesia had a delayed neurocognitive recovery incidence similar to that of patients under sevoflurane-based anesthesia. This recent study determines clinicians to take into consideration that anesthetic choice may not be a decisive factor for delayed neurocognitive recovery [8]. That study has identified the fact that a high concentration in serum interleukin-6 is an independent risk factor for delayed neurocognitive recovery, and this is a marker that should be taken into consideration regarding patients with risk to develop POCD. This finding provides clinical evidence for the role of inflammation in delayed neurocognitive recovery.

In the sevoflurane model studies, calcium influx and increased exposure to ROS can lead to the activation of the NLRP3 inflammasome, which may influence the neurological outcome in postoperative period (fig. 1).

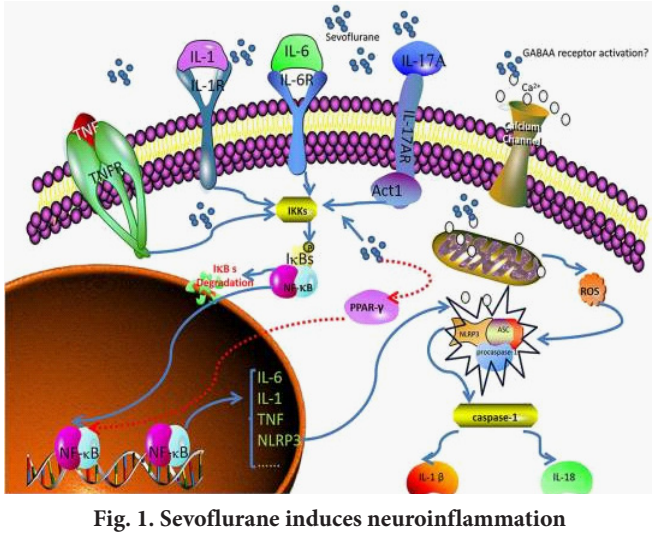

Fig. 1. Sevoflurane induces neuroinflammation and leads to POCD [8]

The damage of mitochondria and exposure to mitochondrial contents, such as ROS, are essential for the assembly of the NLRP3 inflammasome. Also is required calcium influx for optimal activation of the NLRP3 inflammasome [8].

Dong P. et al. in their study in 2018, confirmed as well that sevoflurane could contribute to inducing the neuroinflammation mediated by microglia by downregulating hippocampal PPAR-γ, exacerbating cognitive dysfunction [19]. The theory of neuroinflammation, has more power to demonstrate the incidence of POCD after sevoflurane model anesthesia since peroxisome proliferator-activated receptor-γ (PPAR-γ) is a ligand-inducible transcription factor of the nuclear hormone receptor family and is expressed in several cell types in the brain, including microglia, astrocytes, and neurons. The Nod-like receptor protein 3 (NLRP3) inflammasome is determinant to the immune response, and includes NLRP3, apoptosis-associated speck-like protein containing a caspase-recruitment domain, and procaspase-1.

Discussion

In recent years, many studies provided information considering the relationship between sevoflurane-induced POCD and neuroinflammation, changes in neurotransmitters, and BDNF reduction.

Postoperative cognitive disfunction is a multifactorial, neurodegenerative condition mechanisms of which remain unclear due to many studies conducted for the last 5 years. Several clinical trials, as well as many animal studies have shown that after sevoflurane-inhalation anesthesia, humans and animals experience different degrees of cognitive disfunction. Hypotheses regarding the pathogenesis of sevoflurane-induced POCD, including neuroinflammation, changes in neurotransmitters, decrease in BDNF, mitochondrial oxidative stress, and changes in Aβ concentrations, couldn't give a complete answer to the question of correlation between sevoflurane anesthesia and POCD. The answer is more difficult to get as long mechanisms are not completely independent and they interact with each other.

The importance of patient-related factors, such as age, cognitive status before the surgery, may also affect the incidence of POCD, being related or not to the choice of the anesthetic agent, shows a recent multicenter randomized trial [20]. Related studies that confirm the correlation between sevoflurane anesthesia and neuroinflammation, exacerbating that way cognitive dysfunction, recent randomized trials show the same cognitive dysfunction being relative to intravenous anesthesia agents. Neuroinflammation still remains the key factor of POCD and its correlation or not with the selection of anesthetic agent was not determined.

Regarding the limitation of studies, it was determined by the American Society of Anesthesiology that the physical status classification, mini-mental status examination scores, and duration of hospitalization were predictors for delayed neurocognitive recovery. Regardless of the type of anesthetic agent, a high blood interleukin-6 concentration in postoperative period is a risk factor for developing delayed neurocognitive recovery. In fact, interleukin-6 concentration in the blood at this time is the only independent risk factor for delayed neurocognitive recovery as determined by multiple studies [21, 22]. However, its concentration as a risk factor for delayed neurocognitive recovery has not been reported.

The role of intravenous and volatile anesthetics in postoperative cognitive dysfunction in humans remains unclear. Therefore, future research should be aimed at further elucidating the pathogenesis of induced POCD and developing an effective combination therapy to minimize the neuroinflammation and protective strategies for minimizing POCD, and the length of stay in hospitals.

Conclusions

The intra-anesthetic use of sevoflurane has been associated for a long time with the higher incidence of POCD. The mechanism was not identified, and the theory of neuroinflammation remained the main key of pathophysiological reaction that leads to cognitive dysfunction. Recent multi-

centric trial gives reliable information that the use of intravenous anesthetic agents is associated with the same POCD. Neuroinflammation remains to be the mediator of cognitive disorders, and apparently IL-6 keeps a major role in them. Future studies are needed to be conducted to identify the role of anesthetic agents in determining the neuroinflammation.

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Author's contribution
VR conceptualized the idea, conducted literature review, collected the data, interpreted the data, and wrote the manuscript.

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Ethics approval and consent to participate
No approval was required for this study.

Conflict of Interests
The author has no conflict of interests to declare.

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