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

The Moldovan Medical Journal

The Publication of the Scientific Medical Association of Moldova

Frequency – 4 per year
Category – B+

Vol. 64, No 2
June 2021

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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.

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Index for subscription – 32130

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Publisher

"Revista Curier Medical"
192, Stefan cel Mare Avenue
Chisinau, the Republic of Moldova

Printing House Tipografia Sirius
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https://doi.org/10.52418/moldovan-med-j.64-2.21.01
UDC: 616.831-005.4-053.2

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Ischemic stroke in children depending on risk factors

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Manuscript received June 18, 2020; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: Ischemic stroke (IS) in children is a major neuropediatric emergency. The incidence of stroke in children is from 2 to 13 for 100000 children. IS in perinatal period occurs in 1 for 2300 – 5000 live births.
Material and methods: In 2010 – 2019 in the Republic of Moldova was carried out a retrospective as well as prospective study on a cohort of 458 children diagnosed with stroke. Were studied possible risk factors related to IS. Out of 458 children, 284 children with IS were selected and diagnosed during the reference period.
Results: IS was determined in 284 cases with the diagnosis of stroke (62%, 95CI 59.73-64.27). Among the most common risk factors for the development of neonatal IS are pathologies of amniotic membranes in 113 cases (39.8%, 95CI 36.9-42.7), pathologies of amniotic fluid with meconium in 135 cases (47.5%, 95CI 44.54-50.46), and history of urgent caesarean section in 132 cases (46.5%, 95CI 43.54-49.46). Among the etiological causes of IS in the studied children were: congenital heart anomalies in 52 cases (18.3%, 95CI 16.01-20.59), neonatal encephalopathy in 27 cases (9.5%, 95CI 7.76-11.24), genetic syndromes in 18 cases (6.3%, 95CI 4.85-7.75), sickle cell disease – 5 (1.8%, 95CI 1.06–2.54), MELAS syndrome – 4 (1.4%, 95CI 0.7-2.1).
Conclusions: IS risk factors are an important problem in clinical research. Most often, there is not a single risk factor responsible for the development of IS in children.
Key words*:* stroke, ischemic, children, risk factors.

Cite this article
Sprincean M, Hadjiu S, Calcii C, Lupusor N, Revenco N, Groppa S. Ischemic stroke in children depending on risk factors. *Mold Med J.* 2021;64(2):5-11. https://doi.org/10.52418/moldovan-med-j.64-2.21.01.

Introduction

Stroke is a rare pathology in children and adolescents, often underestimated. The incidence of ischemic stroke in pediatric patients according to several studies is from 2 to 13 of 100000 children, from 1 to 5 of 100000 children for hemorrhagic stroke and 0.67 of 100000 children for sinus venous thrombosis [1]. Ischemic strouke (IS) most often occurs in the prenatal period and in the first 28 days with a frequency of 1:4000 live newborns. IS can also occur during the fetal period, from the 14th week of pregnancy to birth. Perinatal IS is caused by an ischemic lesion that occurs from the 20th week of pregnancy to 28 days after the birth. Data from the literature show that perinatal IS occurs in 1 of 2300 to 5000 live newborns, with an estimated mortality rate of 3.49 for 100000 annually [2].

Studying the risk of IS occurring in children in aspects of risk factors is a currently important issue for contemporary neurology both nationally as well as internationally and is an object of extremely valuable scientific studies. There is currently a large number of children in the Republic of Moldova who have had IS, and actual incidence of this pathology is not known.

The risk factors of IS in children and adolescents are different from those of adults. Scientific research revealed that IS in children is often the result of a simultaneously acting several risk factors. Thus, IS etiology in childhood is multifactorial. Were determined five main groups of stroke in children, such as: (1) diseases of the blood, i. e., Shonlein-Henoch disease, aplastic anemia, hemophilia, hemoglobinopathies, leukemia, von Hippel-Lindau syndrome; (2) various types of thrombocytopenies, coagulopathies and vasculopathies; (3) congenital heart anomalies; (4) congeni-

tal disorders of metabolism; (5) vasculitis, e. g., rheumatic vasculitis, primary cerebral vasculitis, Moyamoya disease, Takayasu's disease, Behcet's disease, etc. [3]. Genetic diseases are considered to be significant risk factors in more than half of stroke cases. Among genetic diseases at risk of developing of IS in children we should note tuberous sclerosis, fibromuscular dysplasia, Moyamoya disease, MELAS syndrome, hereditary connective tissue dysplasia, sickle-cell disease, hereditary hemorrhagic teleangiectasia, i.e. Osler-Weber-Rendu syndrome, hyperhomocysteinemia, homocysteineuria, Fabry disease, cerebrotendineous xanthomatosis etc. [4].

The aim: Studying the risk of stroke in children in aspects of risk factors based on analysis of statistical data and pathologies in newborns and children of pediatric age, with the aim of improving early diagnosis.

Material and methods

For the investigation we studied the possible risk factors for the development of IS in 458 children with stroke from the Republic of Moldova during the years 2010 – 2019. 284 children with IS diagnosed during the given period were included in the target group. The etiological diagnosis of IS included obtaining the historical data, i.e., prenatal history, diseases of mother, course of pregnancy, perinatal and postnatal history, neurological status and general somatic status, the results of neurological investigations, i.e., ultrasound visualization of nervous system and electroencephalography, and neurological imaging methods, i.e., magnetic resonance imaging and cerebral computed tomography.

Results

A retro- and prospective study of a cohort of 458 children from the Republic of Moldova who suffered stroke in the period from 2010 to 2019 was carried out. Clinical diagnostic and imaging methods have allowed the detection of stroke in children from the earliest stages of intrauterine development. The age of the children included in the study ranged from that of newborns to 18 years. 284 children with IS were selected, and was performed the analysis of etiology and predictive risk factors for the development of the disease.

The results of the study and statistical analysis of a cohort of 458 children with pediatric stroke revealed the following results of gender distribution, namely, in the review predominate the male patients, i.e., 272 cases (59.4%, 95CI 57.11-61.69), compared to the female patients, i.e., 186 cases (40.6%, 95CI 38.31-42.89) (fig. 1).

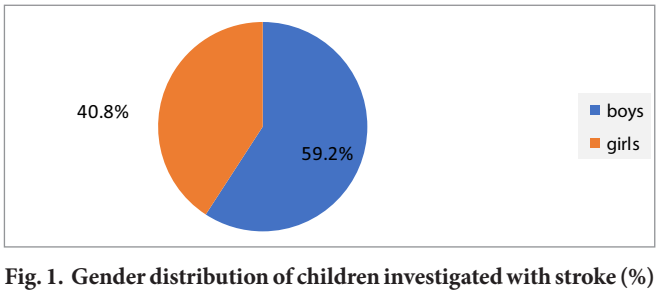

Fig. 1. Gender distribution of children investigated with stroke (%)

According to the obtained results, the structure of stroke in investigated children is as follows: SI in 284 cases (62%, 95CI 59.73-64.27), hemorrhagic stroke (HS) in 144 cases (31.4%, 95CI 29.23-33.35) and mixed stroke in 30 cases (6.6%, 95CI 5.44-776) (fig. 2).

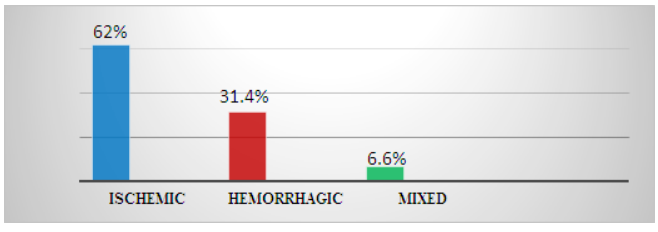

Fig. 2. Types of stroke in investigated children (%)

According to the results of the study, out of the total number of 284 children with IS, in 206 cases IS developed during the neonatal period (72.5%, 95CI 69.85-75.15), in 12 cases they suffered IS during the fetal period (4.2%, 95CI 3.01-5.39) and in 66 cases children suffered IS in early childhood and adolescence (23.2.0%, 95CI 20.69-25.71) (fig. 3).

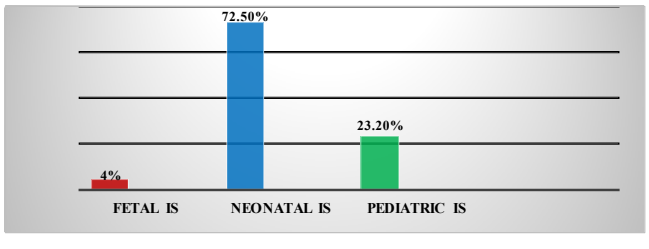

Fig. 3. Distribution of the patients according to the age of onset of IS

Data on the variables of neonatal IS showed the following results: pathologies of fetal membranes were in 113 cases (39.8%, 95CI 36.9-42.7), meconial amniotic fluid in 135 cases (47.5%, 95CI 44.54-50.46), early urgent caesarean section in 132 cases (46.5%, 95CI 43.54-49.46), placental pathologies in 104 cases (36.6%, 95CI 33.74-39.46), umbilical cord pathologies in 122 cases (43.0%, 95CI 40.06-45.94), and some other factors are shown in figure 4.

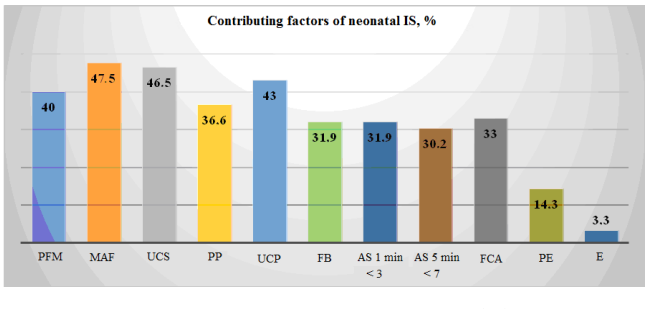

Fig. 4. Risk factors of neonatal IS (%)

Note. Pathology of fetal membranes, PFM; meconium in amniotic fluid, MAF; urgent cesarean section, UCS; placental pathology, PP; umbilical cord pathology, UCP; first birth, FB; Apgar score (1 min) < 3, AS 1 min < 3; Apgar score (5 min) < 7, AS 5 min < 7; fetal cardiac arrhythmia, FCA; pre-eclampsia, PE; eclampsia, E.

Analysis of obstetric history shows the presence of an unsatisfactory pregnancy development in about half of

cases. One of the risk factors in the development of perinatal pathologies is the maternal age, especially up to 18 years and that which exceeds 30 years. The age of mothers at birth of premature babies included in the study was from 17 years to 45 years (average 25.4±5.2). The total number of underage mothers was 11 (3.9%, 95CI 2.76-5.04), that of mothers over 30 years of age was 135 (47.5%, 95CI 44.54-50.46), 18 of whom were over 40 years of age (6.3%, 95CI 4.85-7.75). The vast majority of children in the study group came from rural areas, i.e., 187 children (65.8%, 95CI 62.99-68.61), while 97 children were urban (34.2%, 95CI 31.39-37.01). Analyzing the data of the obstetric history in the basic group we found that 103 of pregnant women (36.3%, 95CI 33.45-39.15) had miscarriages or stopping evolution of pregnancies. Pathological evolution of pregnancy is characterized by various complications arising from the first and second trimesters of pregnancy.

Among the significant risk factors for perinatal IS should be noted pathologies of the placenta, amniotic membranes, umbilical cord, meconium in amniotic fluid, eclampsia, urgent cesarean section, pre-eclampsia, first birth, low Apgar score, and oligoamnios. Thus, the most common complication of the antenatal period was the imminence of premature birth in 138 cases (48.6%, 95CI 45.63-51.57), pre-eclampsia in 42 cases (14.8%, 95CI 12.69-16.91) and eclampsia in 10 cases (3.5%, 95CI 2.41-4.59) (fig. 5).

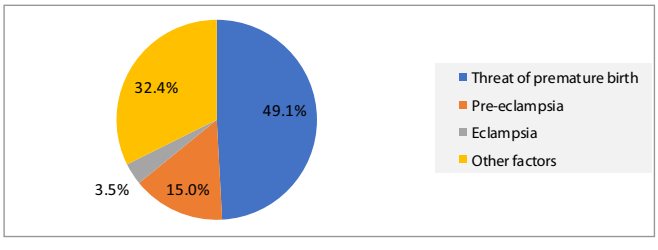

Fig. 5. Factors for perinatal IS

Among the etiological causes of IS in the studied children we should note congenital heart anomalies in 52 cases (18.3%, 95CI 16.01-20.59), systemic diseases in 45 cases (15.8%, 95CI 13.63-17.97), neonatal encephalopathy in 27 cases (9.5%, 95CI 7.76-11.24), genetic syndromes in 18 cases (6.3%, 95CI 4.85-7.75), sickle cell disease in 5 cases (1.8%, 95CI 1.06-2.54), MELAS syndrome in 4 cases (1.4%, 95CI 0.7-2.1), metabolic diseases in 8 cases (2.8%, 95CI 1.82-3.78) and also other pathologies which are presented in tab. 1.

Table 1. Etiology of IS in children

Etiology of IS	Abs. No.	%, 95CI
Congenital heart anomalies	52	18.3%, 95CI 13.21-17.99
Systemic diseases	45	15.8%, 95CI 13.63-17.97
Neonatal encephalopathies	27	9.5%, 95CI 7.76-11.24
Genetic syndromes	18	6.3%, 95CI 4.85-7.75
Sickle cell disease	5	1.8%, 95CI 1.06-2.54
MELAS syndrome	4	1.4%, 95CI 0.7-2.1
Metabolic diseases	8	2.8%, 95CI 1.82-3.78
Cerebral vasculitis	5	1.8%, 95CI 1.06-2.54
Infections	15	11%, 95CI 3.97-6.63
Moyamoya syndrome	4	1.4%, 95CI 0.7-2.1
Cerebral vascular anomalies	9	3.2%, 95CI 2.16-4.24
Coagulopathies	8	2.8%, 95CI 1.82-3.78
Posr varicella angiopathy	3	1.1%, 95CI 0.49-1.71
Oncological factors	5	1.8%, 95CI 1.06-2.54
Trauma	6	2.1%, 95CI 1.25-2.95
Non determined etiology	34	12.0%, 95CI 10.07-13.93

Discussion

The characteristics of variables of IS in children are very different from those of adults. Stroke risk factors are an important problem in clinical research. The diversity of risk factors creates a heterogeneous patient population. In addition, studies focused on pediatric stroke etiology are relevant for ischemic stroke, not for hemorrhagic one. Some studies have improperly combined ischemic and hemorrhagic strokes for risk analysis. More than half of children with stroke develop disabilities at an early and pre-school age. Repeated strokes are observed in 20% of patients [5]. Some authors note common risk factors for ischemic stroke in children, such as congenital heart defects, homocysteine metabolism disorders and thrombophilic disorders, as well as upper respiratory tract infections, mild head trauma etc. [6].

Genetic diseases which are risk factors for IS in children. Homocystinuria may cause IS and should be suspected in the presence of Marfanoid phenotype and mental retardation associated with the dislocation of the lens and occasionally *pectus excavatum*. Homocystinuria is a rare hereditary condition affecting amino acids metabolism, namely methionine. This autosomal recessive disorder is characterized by abnormal storage of homocysteine and its metabolites methionine, and S-adenosyl derivatives in blood and urine. Although homocystinuria is usually associated with ischemic stroke, the sudden occurrence of stroke as a result of homocystinuria is very rare in infancy. Increasing of thickness of carotid plaques was associated with high levels of homocysteine and with lowering the level of vitamin B12 and with following increasing risk of stroke. This association between homocystinuria and vascular complications was reported for the first time in 1976 [2] and since then, several studies have confirmed this association [7]. Nutritional deficiencies of folic acid or vitamin B12 can also cause hyperhomocystinemia, which leads to stroke.

MELAS syndrome or mitochondrial myopathy, encephalopathy, lactic acidosis and stroke is a multisystem and progressive neurodegenerative disorder. Cases of MELAS syndrome may occur sporadically or as hereditary transmission on a maternal line with a variable expressiveness of clinical manifestations. Patients with MELAS syndrome may have the following symptoms such as: mitochondrial encephalopathy, lactic acidosis and stroke events, but also with other manifestations such as headache, seizures, cognitive and verbal disorders, sensory neural deafness, muscle weakness and mental retardation.

Hereditary dysplasias of connective tissue are considered to be significant risk factors in about 10% of cases [8].

Genetic background of ischemic and hemorrhagic accident is often polygenic or multifactorial. It can be determined in some cases by a particular single gene disease, especially in children and young adults. Apart from the mentioned risk factors, many types of dysplasia of connective tissue can cause stroke. Hereditary dysplasias of connective tissue (HDCT) represent a group of hereditary single gene pathology determined by mutations in genes responsible for collagen synthesis and metabolism. HDCT may be characterized by severe manifestations, are relatively common and sufficiently understood at the molecular level to provide useful paradigms for a number of associated diseases [8].

Of the most prevalent HDCT should be noted Ehlers-Danlos syndrome, Marfan syndrome, osteogenesis imperfecta, spondyloepiphyseal dysplasia congenita, achondrogenesis, Stickler syndrome, hereditary angiopathy, Alport syndrome, benign family hematuria, etc. These are caused by mutations in collagen and the extracellular matrix genes. For example, mutations in the COL4A1 gene are considered to be the cause of small vessels anomalies in adults presenting ischemic stroke or intracerebral hemorrhage [9].

Cerebrotendineous xanthomatosis is a hereditary disorder, caused by mutations of the CYP27A1 gene, characterized by abnormal storage of lipids in many parts of the organism [9]. In this disorder in the organism of the patients certain lipids such as cholesterol cannot effectively decompose so these fats form fatty yellow nodules called xanthomas, which accumulate in the body, especially in the brain and in tendons. Symptoms may include diarrhea, cataracts and progressive neurological problems, such as seizures, movement disorders, stroke, dysarthria, sensitivity disorders, peripheral neuropathy, hallucinations and depression. Other symptoms may include fragile bones that are prone to fractures and an increased risk of developing cardiac or pulmonary impairment due to the accumulation of lipids [9].

Fibromuscular dysplasia (FMD) is a hereditary condition that causes cell growth of the arterial walls. Extracellular growth leads to narrowing the arteries and causing reduction of blood flow. It can also cause aneurysms and dissections in the carotid arteries with the development of a hemorrhagic stroke.

Genetically determined pathologies are increasingly

Table 2. Genetic diseases which are risk factors for IS in children [9]

Diagnosis/Pathology	Description	Genetic testing	Treatment
Homocystinuria.	Deficiency of cystathionine beta synthase, autosomal recessive inheritance.	Increasing the serum level of homocysteine.	Supplement of B6, B12 vitamins, folic acid, betaine. Diet with methionine exclusion and supplement of cysteine, C vitamin.
MELAS syndrome, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes.	Maternal inherited mitochondrial dysfunction manifested with lactic acidosis, seizures and IS episodes.	Increasing the serum level of pyruvic acid and lactate, biopsy of skeletal muscles.	Coenzyme Q10, idebenone, L-arginine, supplement of carnitine.
Ehlers-Danlos syndrome.	*COL3A1* mutation in gene encoding procollagen type III. Clinical features are: thin lips, hyperelasticity of skin, increased softness of ligaments, articular hypermobility, vascular fragility which can lead to intrauterine rupture, IS, aortal dissection, tendon ruptures.	Clinical examination, biochemical analyses, molecular genetic analysis of *COL3A1*.	Beta blockers, surgery of dilated aorta.
Fibromuscular dysplasia.	Autosomal dominant inheritance, non-inflammatory non-atherosclerotic vessels pathology. Symptoms: focal arteriopathies, stenotic in children with IS. Can lead to vascular occlusion, dissections or aneurisms.	Clinical history, CT/MRI/angiography, diagnosis of pathological tissues of renal or temporal arteries.	Little studied. Screening of multi- systemic vascular complications.
Marfan syndrome.	Autosomal dominant inheritance, *FBN1* mutation in gene encoding fibrillin 1. Skeletal symptoms are: high waist, long limbs, arachnodactyly, deformities of the chest and spine, etc. Cardiovascular manifestations are: aorta dissection, aorta aneurism, prolapse of mitral valve. Eye manifestations are: flattened cornea, myopia, crystalline subluxation. Neurovascular symptoms are from spontaneous intracerebral artery dissections to cardioembolization or aorta dissections.	Clinical examination, criteria of diagnosis. Molecular genetic analysis of *FBN1*.	Beta blockers, ACE inhibitors, surgery or conservative treatment of aortic aneurism.
Pseudoxanthoma elasticum.	Autosomal recessive inheritance, *ABCC6* mutations, disorder to the assembly of elastic fibres. Cerebral vascular symptoms are: IS, intracerebral aneurisms.	Skin biopsy, sequencing of *ABCC6*.	Treatment in process of researches. Management of risk factors.

recognized as a cause of IS in children. Among the most common genetic diseases at risk of IS in children are: homocystinuria, MELAS syndrome, Ehlers-Danlos syndrome, Marfan syndrome, fibromuscular dysplasia, pseudoxantoma elasticum etc. The list continues to expand, including mutations *COL4A1*, *ACTA2* and pericentrin (*MOPD2*) and syndromes, such as Alagille and PHACE. The most studied syndrome is Moyamoya disease (MMD), which is characterized by a progressive, usually bilateral stenosis or occlusion of intracranial internal carotid arteries, which involves the anterior and medial cerebral arteries. The cause of MMD disease is mutations in the *RNF213* and other mutations of *BRCC3* / *MTCP1* and *GUCY1A3* [10]. Based on the study data presented, in 6.3% of studied children (95CI 4.85-7.75) had various genetic diseases, including homocystinuria; metabolic disorders occurred in 2.8% (95CI 1.82-3.78) of studied children. In 3.2% (95CI 2.16-4.24) of the children surveyed in the current study were cerebral vascular anomalies, and 1.4% (95CI ̲0.7-2.1) of children had Moyamoya syndrome.

Genetic arteriopathy caused by a deficiency of adenosine deaminease 2 (*ADA2*) has been reported with clinical characteristics that included intermittent fever, lacunar stroke from early childhood and acute onset eruptions; histopathological changes included compromised endothelial integrity, endothelial cellular activation and inflammation [11, 12].

Hereditary coagulopathies and thrombophilia. One or more prothrombotic conditions were identified in 20 to 50% of children who had stroke [13]. The main mutations associated with prothrombotic states are described in factor V Leiden, prothrombin G20210A, methylentetrahydrofolate reductase (MTHFR; C677T and A1298C), protein C, protein S, antithrombin and lipoprotein (a) [9]. Most stroke experts consider coagulopathy to be a potential risk factor for a stroke that usually works in combination with other factors, rather than being an independent causal mechanism. Thus, it is reasonable to look for more common prothrombotic conditions in patients with another identified stroke risk factor and in patients with a history of ischemic or thrombotic stroke; in this case, oral contraceptives may be discontinued in adolescents. If homocysteine is found to be high, specific diet or supplementation with folate, vitamin B6 or vitamin B12 can be administered and, in general, patients with a prothrombotic tendency should be consulted by a hematologist [9]. In 2.8% (95CI 1.82-3.78) of the children who had IS were diagnosed hereditary coagulopathies and thrombophilia.

Acquired prothrombotic disorders secondary to protein C and S deficiencies may occur in children with renal pathology and liver disease, including nephrotic syndrome with the loss of coagulation factors. Protein C deficiency was also reported in children taking valproate. The iron deficiency was reported in children with IS and venous thrombosis, with no other apparent etiology.

Heart diseases are the most common cause of stroke in infancy, representing up to one third of all strokes. In children after heart surgery or with catheter, almost 50% of all cases of stroke occur within 72 hours. Prolonged cyanotic episodes provoke polycythemia and anemia, both increasing the risk of tromboembolic stroke. Embolic clots may occur in children with cardiomyopathies, rheumatic and cardiac diseases, artificial heart valves or valvular vegetations in endocarditis. Foramen ovale may occur in more than 35% of patients aged from one to 29 years, and this opening can serve as a prerequisite for venous embolic events in which it is necessary to move embolus from the right to the left side of the heart [2].

Moyamoya disease is a rare, progressive, occlusive disease of cerebral arteries, with a special involvement of circle of Willis and the arteries that vascularized it [10]. The affection can cause a transient ischemic attack or stroke with deterioration of brain functions and cause cognitive and developmental delay. Moyamoya disease most commonly affects children, being associated with the following clinical signs: headache, weakness, numbness or paralysis in the face, arm or leg, usually on one side of the body, visual disturbances, aphasia, developmental delay, involuntary movements, and cognitive decline. These symptoms can be triggered by physical exercise, crying, coughing, tension or fever.

Sickle cell anemia (SCA) is a very frequent cause of pediatric stroke, which occurs in 285 cases to 100000 children affected [7]. The stroke may occur earlier than the age of 18 months, but in most children the disease manifests after the age of five years. IS is more prevalent at the younger age. The stroke may occur in the absence of pain or aplastic crisis. Two-thirds of the children with SCA have had previous strokes, but without the treatment they will have a recurrence. Children with *sickle cell disease* make up another important group of patients at high risk of arteriopathies and stroke. Prior to using the modern primary prevention strategies, up to 11% of children with heart disease had a clinical stroke by the age of 20. In 1992 it was found out that transcranial Doppler ultrasonography (TCD) proved to be effective in identifying patients with sickle cell disease at high risk of stroke, and at present primary prevention is possible using chronic red cell transfusions in patients with sickle cell disease and increased cerebral blood flow on TCD. This approach decreased the prevalence of stroke by about 1% [14]. Based on the data obtained in this study, 1.8% (95CI 1.06-2.54) of the children who had IS have had sickle cell disease.

IS in *metabolic disorders* are rare, but important for children. Energy depletion leads to ischemic lesions in mitochondrial disorders. In TCA cycle disorders, toxic deposits lead to the destruction of brain tissue. For this reason, IS in metabolic disorders do not occur in a certain vascular territory; so, e. g., in MELAS syndrome stroke occurs mainly in the occipital area. Other metabolic problems, such as Fabry disease, lead to focal arteriopathy [9]. MELAS syndrome was found in 1.4% (95CI ̲0.7-2.1) of children included in the present study.

Infection of the upper respiratory airways causes local inflammation of the vascular wall with the development

of cerebral arteriopathy and increased prothrombotic potential. Arteriopathy resulting from a traumatic factor and increased physical activity causes a stroke in 17% to 33% of cases [9]. In present study, 11% (95CI 3.97-6.63) of the children had infections.

Primary thrombophilia caused by mutations in the genes of the haemostatic system is a risk factor for ischemic stroke in 10 – 50% of cases in patients under 18 years of age in the European population [15]. Studies of polymorphisms in the genes methylenetetrahydrofolate reductase (MTHFR), Leiden factor, prothrombin and fibrinogen showed their significant role in the development of stroke in children and adults [16]. However, there are no studies that consider a complex of 11 prothrombotic genes, taking into account all parts of the hemostatic system, i. e., vascular system, platelets and plasma.

Mutations in the MTHFR and MTRR regulate homocysteine metabolism, the excess of which is achieved by endothelial impairment and stimulating prothrombotic reactions [17]. An excess of homocysteine in adults has a systemic harmful effect on vascular endothelium, with the accumulation of low and very low density lipoproteins in the vascular wall, causing atherogenesis, and acts as vascular and clotting risk factors of ischemic stroke [17]. The role of homocysteine metabolism disorders in the development of ischemic stroke has not been adequately studied in children, which requires further researches.

About half of children with a stroke have a known predisposing condition, but in some of them the stroke is unexpected, such as in primary cerebrovascular disease, associated with congenital heart abnormalities, or in the presence of modifiable risk factors, such as hypertension associated with sickle cell disease. Genetic predisposition, trauma, infections and nutritional deficiencies appear to be important, although case control studies will be necessary to demonstrate causality. Appropriate screening for modifiable risk factors may prevent recurrence in some patients. In the long term, an understanding of multiple etiologies of childhood cerebral vascular disease and ischemic stroke can allow development of primary prevention for respective age group and, possibly, for adults [16].

With advances in neuroimaging, arteriopathy appears to be the predominant basic mechanism causing 53% cases of stroke. Furthermore, it is the most important predictor of recurrence, highlighting its role as a target for treatment to prevent the secondary stroke [16].

The most common arteriopathy established in IS is a unilateral intracranial acquired arteriopathy associated with basal IS, characteristically involving the junction of the distal internal carotid artery, proximal middle cerebral artery (MCA) and proximal anterior cerebral artery (ACA). This condition was originally conceived as a transient cerebral arteriopathy (TCA) and characterized by its duration, unilateral localization and the absence of long-term progress [18]. By traditional methods of initial imaging, TCA cannot be differentiated from progressive arteriopathy, such as Moyamoya disease (MMD) or vasculitis that presents unilaterally, with a different duration and prognosis. Few clinical and radiological parameters can predict the evolution of unilateral intracranial arteriopathy in childhood. Patients with progressive arteriopathy have been found to have more often arterial occlusion, ACA involvement and abnormal collateral vessels, and that a predominantly cortical localization is associated with poor functional outcome. The differentiation between TCA and progressive arteriopathy may require further radiological assessment, such as magnetic resonance angiography (MRA) and conventional angiography, and generally the aggravation of the process after 6 months or bilateral involvement suggests a different arteriopathy than TCA [5].

Studies by Rivkin MJ, Bernard TJ, Dowling MM, Amlie-Lefond C. have shown that IS etiology in children is multifactorial and the risk factors are numerous and complement each other. IS are characterized by multiple signs and symptoms, most often subtle, make it difficult the early diagnosing. The patient investigation should be carried out by a multidisciplinary team, i. e., geneticist, neurologist, rheumatologist, nephrologist, etc. [19].

Conclusions

Risk factors and causes of IS in children are heterogeneous and in many cases remain idiopathic. Investigations of variables and causes of cerebral ischemia in children can direct rational research and therapeutic strategies of IS in children. The comprehensive approach to the patient will ensure the certain diagnosis, which has a defining role for the decision of the treatment tactics and to determine the subsequent evolution of the disease. In high-risk families it is necessary to carry out genetic counseling and family planning in order to reduce the rate of morbidity, mortality and improve the quality of life of patients and their relatives.

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MS – designed the research, did statistics and interpreted the data, drafted the manuscript; SH – conducted/performed the laboratory work; CC – interpreted the data; NL – collected the data; NR – conceptualized the project and designed the research; SG – conducted the laboratory work, revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

Funding
It was provided by *Nicolae Testemitanu* State University of Medicine and Pharmacy and Mother and Child Institute. The trial was the authors' initiative. 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 was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (protocol No 69 of March 21, 2017).

Conflict of Interests
There is no known conflict of interests and financial or non-financial support associated with this publication.

https://doi.org/10.52418/moldovan-med-j.64-2.21.02
UDC: 616.853.9-053.2

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Refractory status epilepticus – a major problem for the practitioners

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Manuscript received June 22, 2020; revised manuscript March 18, 2021; published online April 28, 2021

Abstract

Introduction: Status epilepticus (SE) is a life-threatening neurological emergency requiring immediate medical intervention and is associated with high mortality and morbidity. The aim of this research was evaluation of clinical and etiological profile of refractory status epilepticus (RSE) among children aged between 1 month and 18 years.
Material and methods: The study was done between January 1, 2017 and December 24, 2019. All children with the age limits mentioned above, who presented convulsive SE, subsequently with development in refractory status epileptic (RSE), were included in the study. Patients were investigated and evaluated according to a standard protocol. Subsequently, the characteristics of children with RSE and those without an evolution in RSE were compared.
Results: 55 children, out of whom 32 boys with SE were enrolled in the study, of which 20 children (36%) developed RSE. Central nervous system (CNS) infections were the most common causes of SE and development of RSE (51% in SE and 53% in RSE, $p > 0.05$). Noncompliance of antiepileptic medication served as the second cause for evolution of RSE. The overall mortality rate was 10.9%, the chances of death in RSE (20%) being higher than in SE (5.7%). The unfavorable prognosis was seven times higher in children with RSE, compared to children who developed SE.
Conclusions: In the management of CNS infections, pediatricians should be aware of the high risk of developing RSE. In addition, the possibility of developing RSE should be considered and promptly managed in an intensive care unit in order to reduce the risk of mortality and morbidity of this severe neurological condition.
Key words: refractory status epilepticus, childhood epilepsy, CNS infection.

Cite this article
Calcii C, Hadjiu S, Sprincean M, Feghiu L, Lupusor N, Revenco N, Groppa S. Refractory status epilepticus – a major problem for practitioners. *Mold Med J.* 2021;64(2):12-15. https://doi.org/10.52418/moldovan-med-j.64-2.21.02.

Introduction

Status epilepticus (SE) is a life-threatening neurological emergency requiring immediate medical intervention and is associated with high mortality and morbidity. The incidence of SE in children is reported to be of 10-40 children per 100000 people. SE is the most common pediatric neurological emergency [1, 2], being a condition resulting from the loss of the mechanisms responsible for ending of convulsive access or by initiating mechanisms that cause an abnormal convulsive response (after Time 1) [3, 4]. SE is a condition that can lead to long-term consequences (after Time 2), including neuronal death, neuronal injury, alterations of neural networks depending on the type and duration of the attacks [4]. The practical approach suggests that any seizure or series of seizures, lasting more than five minutes, could be considered as SE, most children requiring pharmacotherapy for control of seizures [3, 4]. Recently, Tinka et al. proposed a new definition for focal SE: a duration of 10 minutes of seizures (without the return of consciousness between seizures) and 60 minutes for possible long-term consequences [4]. At the same time, and experimental data support the idea that prolonged seizures cause neuronal damage, therefore the drug intervention is considered critical in such cases [5, 6].

In children, the incidence is higher compared to that in adults; however, the mortality rate of adult patients is higher, around 20%, and in children under 10 years of age, it can be up to 2.6% [7, 8]. Early diagnosis and prompt treatment significantly reduce mortality and are key steps in SE management [9]. After initial supportive treatment (ABC via intravenous [I/V] access), seizure control with Lorazepam I/V is recommended as a first-line treatment [10, 11]. Alternatives to Lorazepam include intravenous administration of Midazolam or Diazepam. If I/V access

is not achieved, Midazolam may be administered orally, intranasally or intramuscularly. Diazepam may also be administered rectally [12, 13]. First-line drugs control the seizures and they are obtained in 80% of children in the first 30 minutes [14]. If the patients continue to have seizures, additional treatment should be given rapidly [15]. Most experts recommend fosphenytoin as a second-line therapy, although there are supporters in favor of the use of other alternative antiepileptic drugs (AED), such as phenobarbital, valproic acid or levetiracetam [16]. While most of the causes of SE are due to epilepsy, in case of SE in children primary causes can be considered atypical febrile seizures, neuroinfections, cerebral hypoxia and innate errors of metabolism [17].

Refractory epileptic status (RSE) is a more severe variant of SE. Currently, the accepted definition of RSE is the persistence of seizures despite the administration of two adequate anticonvulsants in acceptable doses and is estimated in approximately 10-40% of patients with SE. RSE has been shown to be associated with a higher mortality rate and more long-term neurological consequences. Based on the fact that RSE is a major emergency and on the fact that there are no studies on RSE in the Republic of Moldova, this research was carried out with the aim of improving knowledge on the etiology and evolution of RSE among children with SE, to prevent unfavorable prognosis, including mortality.

Material and methods

This study is a part of a larger research, carried out within the project "Integration of epileptogenic mechanisms in order of creating a network of multimodal diagnosis and treatment of epilepsy". The study is retrospective and descriptive (preliminary data attributed to the project). The group of patients included in the study was selected from children admitted to the Departments of Neurology of the Institute of Mother and Child Health Care during the years 2017-2019. SE was defined as a continuous seizure lasting more than five minutes and/or multiple seizures between which the state of consciousness was not regained within at least 30 minutes. The age of the patients included in the study ranged from one month to 18 years. Newborns and children with undocumented SE were excluded from the research. The medical records were reviewed to make an analysis of the data, type of seizures (focal versus generalized), data on epilepsy, as a precursor disease to the installation of SE, analyzed antiepileptic drags (AED) used daily by patients, serum levels of AED at the time of admission (therapeutic or sub-therapeutic), neuroimaging examinations performed, EEG data, possible etiological causes, mortality rate. Continuous data is presented as a median interval; some is presented as a percentage. A *p* value of less than 0.05 was considered significant.

RSE was defined as SE, in which seizures persisted despite the administration of two adequate anticonvulsants at acceptable doses. Unfavorable prognosis included death of the patient, persistent vegetative state or severe disability. In subjects with previously diagnosed epilepsy were collected data about the type of seizures, duration of disease, especially drawing attention to non-compliance and / or other changes in the dosage of drugs. The standard management of SE consisted of two doses of Diazepam, followed sequentially by intravenous phenytoin and intravenous phenobarbital. In severe cases, Propofol infusion was done. After seizure control, neuroimaging and EEG examinations were performed. In the case, if the child had fever, the lumbar puncture was performed (Glasgow Scale > 7 points). The children were monitored daily with appropriate examinations. All data were analyzed using Epi Info software. Different characteristics and results obtained in children with SE and children with RSE were compared.

Results

Of the 55 children (32 boys) with SE, the evolution of SE to RSE was recorded in 20 children (36%). The average age of patients was 6.5 years. There have been documented 6 (10.9%) cases of children who developed a recurrent SE. At the time of admission, the number of children with pre-existing seizures who did not receive routine AED was 9% (5 cases), and 32 children (58.1%) received two or more AED daily. The results of serum AED were evaluated in 77.3% of children, of whom 51.6% children had subtherapeutic levels of AED.

Diazepam was the most common medication given as emergency therapy, used both in children with pre-existing seizures and in those with "*de novo*" SE (62.5% vs 51.1%; p >0.05). The second-line anticonvulsant therapy was phenytoin (45.2% vs 51.1%; p >0.05). Phenobarbital infusion was used in 7.3% of children with pre-existing seizures and in none of the children with "de novo" SE. In 36.3% of cases, endotracheal intubation was required, mainly in the children with RSE (28.5% vs. 60%, p >0.05).

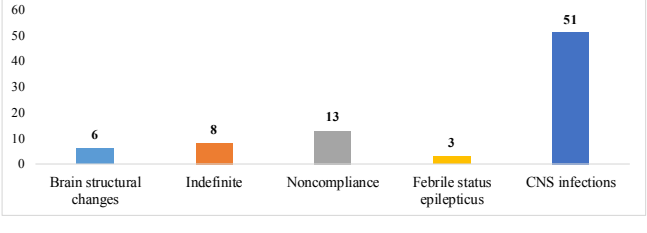

Fig. 1. Etiological factors for convulsive SE in children (n=35), (%)

The gender and type of seizures did not differ significantly between these 2 groups (tab. 1). Electroencephalography (EEG) was performed in 82% of all children admitted with SE. Of these, 72% showed changes on the EEG route. Among children with RSE, EEG was performed in 98% of cases, an abnormal route of EEG was recorded in 85% of cases.

Being admitted to the hospital, children were analyzed for possible causes for the onset of SE. Thus, CNS infection was considered an etiological cause for both SE and RSE (51% in SE and 53% in RSE, respectively) (fig. 2).

Another cause for SE and RSE development was non-compliance with doses and regimes of AED administration, with no statistical difference between groups (p > 0.05).

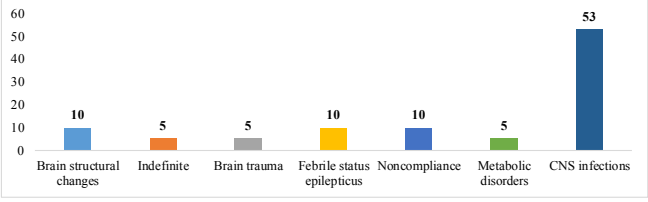

Fig. 2. Etiological factors recorded in RSE (n=20), (%)

The proportion of patients with pre-existing epilepsy, the duration of the disease before SE development and the etiology of epilepsy (genetic / structural) were not significantly different between the two groups (p > 0.05). Most patients (85%) developed generalized seizures. The risk of evolution in RSE was not significantly different for febrile children (PR =1.2; 95% CI : 0.34–3.9; p >0.5), children with pre-existing epilepsy (PR = 0.7; 95% CI : 0.18–2.7, p > 0.6), children with developmental disorders (PR = 1.25; 95% CI: 0.29–5.4; p > 0.8), focal seizures (PR = 5.12; 95% CI: 49–53.2, p > 0.17), (tab. 1).

Table1. General characteristics of children with SE and RSE

Characteristics	SE (n=35), %	RSE (n=20), %
Median age (years)	7.5	5.5
Sex boys girls	18 (32.7%) 17 (30.9)	14 (70%) 6 (30%)
Mean weight (kg)	42	34
Fever association	57.1	70
Pre-existent epilepsy (%)	27.2	25
Intubation (%)	28.5	60
EEG performed on admission (%)	82	98
Pathologic EEG course	68.9	54.7
Neuroimagistic exam at admission (%)	62.6	85.2
Pathological neuroimagistic exam (%)	43.4	62.6
Disorders of children's neurodevelopment (%)	38.1	75
State of shock at admission to the hospital	42.8	25

Six children (10.9 %) died, CNS infection being the most common cause of death (80.2%). The death rates in children with RSE (20%) were higher than those with SE (5.7%). The unfavorable prognosis was seven times higher in children with RSE, compared to children who developed SE (PR= 7.0; 95% CI:1.6–22.3).

Table 2. The outcome of SE

Description	SE (n=35),%	RSE (n=20), %
Death	2 (5.7)	4 (20)
Persistent vegetative state	1 (2.8)	1 (5)
Persistent disability	2 (5.7)	5 (25)
Moderate disability	10 (28.5)	2 (10)
Good reabilitation	18 (51.4)	4 (20)

Discussion

In this observational study based on the hospitalization of 55 children with convulsive SE (including 20 with RSE) at the Hospital of Mother and Child Health Care, we found out that CNS infections were the most common etiological cause in both groups of patients. Most studies in developing countries report CNS infections as the most common etiology of SE. Among pediatric studies, CNS infections are also the most common RSE etiologies [18].

The unfavorable prognosis was seven times higher in children with RSE, compared to children who developed SE. The proportion of generalized seizures varies from 63% to 96% in pediatric studies, similar to our conclusions (85%) [19].

About 1/4 of the subjects in the study had a previously established diagnosis of epilepsy, the conclusion corresponds to similar studies on RSE (16–29%) [20]. In the same way, we were able to perform the EEG exam only in 82% of patients with SE and in 98% of RSE. Neuroimaging was performed in 62.6% of patients with SE and 85.2% with RSE, with pathological changes in 43.4% of patients with SE and 62.6 with RSE, data correlate with previous studies [20].

Non-compliance with AED dosing regimens was an important cause of SE in this study (13%), subtherapeutic levels constituting 51.6%, similar to previous reports in adults (20–27%) [21]. A meta-analysis of paediatric RSE reported a mortality rate of 16%; more recent studies report rates up to 3.7% [22]. Studies in the adult population report mortality rates of 5–35% in RSE [23]. Our study reported a mortality rate of 10.9%, predominantly in the case of RSE.

Conclusions

1. The high proportion of RSE in patients with CNS infections, high rate of mortality in children with RSE and high rates of remote unfavorable prognosis are the highlights in the management of these cases.
2. Early identification of RSE in intensive care unit and emergency care service could reduce mortality in this group of children.
3. Since most patients with RSE have various CNS infections as an etiologic cause, antibacterial treatment should be initiated from the very first minutes of RSE.
4. During the management of children with CNS infections, treating physicians should be aware of the high risk of developing of RSE, and this risk should be managed in an intensive care unit in order to reduce mortality and morbidity due to this severe neurological condition.
5. In addition to infections, another important etiologic cause of RSE was noncompliance with the AED; a situation that could be avoided by improving physician-patient compliance.

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CC drafted the first manuscript; SH conducted/performed the laboratory work; MS interpreted the data, LP collected the data; FL collected the data; NR conceptualized the project and designed the research; SG conducted the laboratory work, revised the manuscript critically. All the authors approved the final version of the manuscript.

Funding
It was provided by *Nicolae Testemitanu* State University of Medicine and Pharmacy and Mother and Child Institute. The trial was the authors' initiative. 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
No approval was required for this study.

Conflict of Interests
There is no known conflict of interests and financial or non-financial support associated with this publication.

https://doi.org/10.52418/moldovan-med-j.64-2.21.03
UDC: 616.891.6+616.895.4]:616.853-055.2

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Anxiety and depressive disorders associated with epilepsy in women of reproductive age

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Manuscript received August 01, 2020; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: Mental disorders in people with epilepsy are an old problem that remains crucial nowadays. The specialized literature has reported increasingly significant values of anxiety and depressive disorders associated with epilepsy in women during their child bearing age. The prevalence of depression comorbidity related to epilepsy has accounted for 55% of cases, many of which remain undiagnosed. Anxiety exceeds the frequency of depressive cases by 10-15%, which tends to be milder, however showing a chronic evolution. It is important to find out the correlation between the occurrences of mental disorder in women with epilepsy from the Republic of Moldova. The purpose of the study was to assess the depressive and anxiety symptoms associated with epilepsy in women of reproductive age.

Material and methods: A retrospective cohort study was conducted on a group of 128 women with epilepsy. Patients were divided into 2 groups, with anxiety and depression respectively. Hospital and Anxiety Depression Scale (HADS) was applied for estimating anxiety and depression, being considered a priority compared to Hamilton scales, the Beck Inventory, the Zung scale.

Results: It has been proved that the disease duration and seizure recurrence are directly proportional to prevalence of depression and anxiety cases. The type of seizure remains a controversial topic.

Conclusions: Anxiety and depression in epilepsy patients show a heterogeneous nature due to clinical pleiomorphism. The HAD scale is effective for the early diagnosis of anxiety and depression in epilepsy women.

Key words: epilepsy in women, epileptic seizures, depression, anxiety.

Cite this article

Duca V. Anxiety and depressive disorders associated with epilepsy in women of reproductive age. *Mold Med J.* 2021;64(2):16-20. https://doi.org/10.52418/moldovan-med-j.64-2.21.03.

Introduction

Epilepsy is a chronic neurological disorder, and the epileptic seizure (ES) is its pathognomonic sign. The disease chronicity and severity lead to mental comorbidities, particularly in women [1]. Psychiatric disorders, such as depression and anxiety, are considered more commonly encountered in women with epilepsy, thus being a challenging issue in the management of epilepsy in this population group [2, 3].

Anxiety is caused by subjective feelings, such as worry and physiological responses like tachycardia, hypercortisolemia, and avoidance behaviors. The characteristic signs of anxiety include a diffuse, unpleasant and vague sense of fear or anxiety, accompanied by vegetative symptoms like headaches, sweating, palpitations, tachycardia, gastric disorders, etc. It consists of two components viz. physiological and psychological ones. It might eventually affect thinking, perception and learning, thus it might potentially distort the perceptions and decrease the power of concentration, associative memory and evocation [4].

Depression is defined as a mental health disorder characterized by a series of symptoms resulting in a lack of positive emotional well-being, such as loss of interest and pleasure in previously rewarding or enjoyable activities and experiences, mood swings and a range of associated emotional, cognitive, physical and behavioral problems. The diagnosis of Major Depression is based on both the severity and persistence of symptoms, as well as on the level of functional and social disabilities, which refer to psychiatry [4, 5].

The Depression and Anxiety Screening is a mandatory for all chronic diseases, especially in epilepsy, thus becoming a medical task for neurologists and epileptologists [6].

Epidemiological data have revealed a significantly prevailing rate of depression and anxiety disorders accounting for 50-65% of cases [7], although these indices vary depending on the research data and the development level of the country. The disease duration and severity influence the onset of these disorders. It has been proved that the prevailing 55% of depressive disorders in epilepsy is related to drug-resistant epilepsy, 20-30% to recurrent seizures and even a 6-7% incidence was found in seizure-free periods [7].

However, it should be underlined that most mental dis-

orders remain undiagnosed [8]. On the one hand, the coexistence of depression and anxiety in epilepsy will worsen the epileptogenic brain process by deepening the brain epileptization, thus increasing the ES recurrence [8]. On the other hand, the development of mental disorders beyond depression and anxiety is likely to result in serious mental illnesses and suicide attempts [8, 9]. Early diagnosis of mental disorders associated with epilepsy first provides possibility to identify the risk factors for the onset of these serious disorders and then optimizes antiepileptic and psychiatric treatment.

The importance of risk factors for developing depression and anxiety disorders related to epilepsy comorbidity is obvious and is under current research studies. The conclusions and recommendations of the field-related specialists are quite controversial. Some authors state that disease chronicity, drug resistance and family history are the key risk factors in developing depression and anxiety disorders, thus disregarding the significance of epileptic seizure type [10]. However, most authors suggest that the main risk factors include the ES type, particularly in focal seizures, ES recurrence, ES duration, the disease duration and hereditary tendency. This study was aimed to assess the risk factors for developing depression and anxiety in epilepsy patients and namely, type of seizure, its frequency, and disease duration.

The depressive episodes, as well as anxiety disorders including panic attacks or different types of phobias and obsessive signs, might exhibit preictal, ictal, postictal and interictal manifestations. Depression and interictal anxiety are considered the most common types of mental disorders in epilepsy [11]. However, the comparative prevalence is not known yet. The interictal period is more helpful in researching mental disorders related to epilepsy.

Therefore, comorbidity of epilepsy-related depressive disorders shows a pleomorphic nature and includes signs, such as anhedonia, irritability, dysphoria, emotional lability followed by crying spells, anxiety, no energy, pain, insomnia, frustration, difficulty of concentrating, etc.

Anxiety more commonly occurs during the interictal period, being characterized by anhedonia, anxiety, phobia, fatigue, turbulence, nervousness, muscle tension, paresthesias, etc. Anxiety and potential panic attacks are memorized by patients, particularly in focal ES, thus becoming the risk factors or triggers for epileptic seizures.

Common symptoms of anxiety and depression related to epilepsy include anhedonia, depressive mood, sense of uselessness and guilt associated with neurovegetative signs. Neurologists should be mandatorily including these signs within the epilepsy patient's screening follow-up. The psychic signs are assessed via the well-known scaled scoring described within specialized literature, such as the Hamilton scale, the Beck's Depression and Anxiety Inventory, the Zung Self-rating Depression Scale and Hospital Anxiety and Depression Scale [12].

A quantitative and qualitative informative algorithm is particularly important for accurate assessment of depression and anxiety symptoms associated with epilepsy in women, thus the Hospital Anxiety and Depression-Anxiety (HAD – A) and Hospital Anxiety and Depression-Depression (HAD – D) scales were selected for screening [13]. The HAD scale includes the common signs of these 2 mental disorders, being easily applicable since no somatic signs are necessary, as well as quite applicable and effective in conducting research studies in the Republic of Moldova.

Material and methods

The retrospective cohort analytical study was carried out in women of reproductive age (aged 15 - 49 years old) and included a sample of 128 women with epilepsy. Patients were enrolled via a primary outpatient consultation and a dynamic patient's assessment, being admitted to hospital with an increasingly recurrent ES and serial ES that show risk of developing into Status Epilepticus. The characteristics of the patients are displayed in table 1.

Table 1. General characteristics of patients

Parameters	Average or number	SD or %
Age 15 – 49 years	30.2	1.72
Education	42.7	39.6
Higher	45	35.2%
Secondary	76	59.4%
Primary	7	5.5%
Marital status	32	37.4
Married	36	28.3%
single/unmarried	88	69.3%
separate/divorced	3	2.4%
Widowed	1	0.8%
Occupation	32	29.9
Employed	74	57.9%
Unemployed	18	14.1%
Students	24	18.8%
Pupils	12	9.4%
Type of ES. Total	130	122.9
Generalized	155 ES	121%
Focal	227 ES	177.3%
not defined	8 ES	6.25%
ES incidence over the past year. Total	1282	2646
1/365 days	12w	9.4%
1/180days	11w	8.6%
1/ 125days	16w	12.5%
1/90days	27w	21.1%
1/60days	24w	24%
1/30days	41w	32%
1/7days	37w	22.7%
1/1day	21w	16.4%
free ES	15w	11.7%
Disease duration (years)	3.5	3.6
0 years	21w	16.4%
1 year	5w	3.9%
2 years	16w	12.5%
3years	27w	21.1%
5years	11w	8.6%
10 years	47w	36.7%

Table 2. A and D distribution depending on ES type

Item	Total	Focal ES	Generalized ES	Focal ES	Generalized ES
Anxiety	**79w – 1.7%**	**61w – .2%**	**18w – 2.7%**	**19w–32%**	**22w – 3.6%**
I feel tense or wound up (points)	170p	132p	38p		
I get a sort of frightened feeling as if something awful is about to happen	164	126	38		
Worrying thoughts go through my mind	171	129	42		
I can't sit at ease and feel relaxed	129	99	30		
I get a sort of frightened like "butterflies" in the stomach	120	96	24		
I feel restless as if I have to be on the move	119	93	26		
I get sudden feelings of panic	143	105	38		
Total	1016 points	780 points	236 points		
Depression	41w – 32%			**19w – 32%**	**22w – 53.6%**
I still enjoy the things I used to enjoy				35p	54p
I can laugh and see the funny side of things				28	30
I feel cheerful				43	48
I feel as if I am slowed down				37	50
I have lost interest in my appearance				17	44
I look forward with enjoyment to things				42	36
I can enjoy a good book or TV program				43	50
Total				245 points	312 points

Table 3. Distribution of HAD-A and HAD-D test response

Anxiety	WWFS		WWGS		Depression
7 groups of questions	**A 61w**	**D19w**	**A 18w**	**D**	**7 groups of questions**
0	0	5	0	0	0
1	15	0	6	2	1
2	18	6	4	2	2
3	27	6	8	16	3
0	3	1	0	0	0
1	9	4	4	10	1
2	30	4	8	4	2
3	18	5	6	4	3
0	6	0	0	0	0
1	6	3	4	8	1
2	27	8	4	6	2
3	21	8	10	8	3
0	0	2	2	2	0
1	15	7	8	2	1
2	24	2	4	8	2
3	18	8	6	10	3
0	15	2	6	2	0
1	18	2	8	6	1
2	18	5	2	6	2
3	9	10	2	8	3
0	9	2	2	2	0
1	27	2	10	2	1
2	15	7	4	12	2
3	9	8	2	6	3
0	9	0	2	0	0
1	18	4	8	4	1
2	27	6	0	8	2
3	9	9	8	10	3
Total score					
8 – 10 p	18w/29,5%	3w/15.7%	8w/44.4%	4w/18.2%	Borderline levels A/D
11 – 14p	24w/39.3%	8w/42.1%	6w/33.3%	10w/45.5%	Medium levels A/D
15 – 21p	19w/31.1%	8w/42.1%	4w/22%	8w/36.3%	Higher leves A/D

Note: WWFS – women with focal seizures, WWGS – women with generalized seizures.

The ES diagnosis was made, based on its definition and ES diagnostic criteria settled by the International League Against Epilepsy (ILAE) [14]. The 1981 ILAE Classification of the epilepsy [15] was used in diagnosing the ES type.

According to the purpose of the research, depression and anxiety disorders were selected as factors being the most commonly associated with epilepsy, particularly in women. The assessment of depression and anxiety was based on the Hospital Anxiety and Depression Scale (HADS), which was considered sufficiently relevant. This method was designed in 1983 and includes 14 questions, 7 of which refer to A: HAD – A, and 7 are characteristic of D: HAD – D [16]. The assessment score was divided into 3 categories in both HAD – A and HAD – D scales, thus: I.> 7 – shows no A or D signs; II. 8-10 p – A / D milder form; III. 11-14 p – moderate form; IV. 15-21p – severe form. The A and D signs were separately assessed. The specialized literature shows that the sensitivity for HAD-A is 93.7% and for HAD-D is 84.6%, whereas specificity makes up 72.6% for HAD-A and 90.3% for HAD-D [16].

Patients with atypical signs and severe anxiety and depressive disorders with past medical history of psychiatric monitoring were excluded from the study.

HAD was administered by a resident-neurologist and specialist-neurologist based on the patient's informed consent. Patients were informed to select responses spontaneously, based on their sensations and experiences.

The statistical analysis was performed via SPSS, the data being represented by the mean and the standard deviation values, which were applied to possible parameters.

Results

The ES, depression and anxiety characteristics were examined in 128 WWE, aged 15-49 years, the mean age was 30.2 ± 11 years. The study inclusion criteria were as following: all types of ES associated with depression and anxiety symptoms, except for severe and atypical forms of mental disorders. 79 (61.7%) patients showed signs of anxiety and 41 (32%) patients – depressive signs. 61 (77.2%) patients with anxiety suffered from simple and complex focal seizures (FS) and 18 (22.7%) – primary and secondary generalized seizures (GS), showing predominantly significant values compared to patients with depression. Signs of depression were found in 41 patients, whereas 19 patients (32%) exhibited FS and 22 (53.6%) – GS. Depending on the severity of the mental disorders, women with FS showed a more pronounced anxiety and made up 39.3% of medium severe cases (HAD-A scored 11-14p) and 31.1% of severe cases (HAD-A score 15-21p) compared to women with GS. The group of GS patients showed anxiety disorders in 33.3% of moderate to severe cases and in 22.1% of severe cases, depending on the HAD-A score.

The higher incidence of depressive symptoms was recorded in GS women – 53.6% of cases vs FS – 32% of patients. Moreover, depending on the disease severity, moderate-to-severe signs of depression were recorded via HAD-D score of 11-14 that were slightly more pronounced in GS cases, and accounted for 45.5% vs. 42.1% of FS cases, respectively. However, signs of severe depression were determined in the group of patients with FS – 42.15% vs 36.3% in women with GS. The anxiety and depression signs, as well as the quantified response were distributed according to the type of epileptic seizure that is shown in tables 2 and 3.

The obtained study results of the correlation between the frequency of epileptic seizures with anxiety and depression, revealed a directly proportional relation between these parameters in 93.7% of cases. Thus, the HAD-A and HAD-D score showed high levels of 15-21 in recurrent ES cases, ranging from 1ES / day, 1ES / week to 1ES/month, in both types of mental disorders. The recurrent ES cases ranging from 1ES / month and 1ES/year, the HADS score had a mean value of 11-14 p. respectively, without revealing any prevalence in 41 patients with depression or 79 patients suffering from anxiety. The 8-10 HADS score registered patients with 1 / year or less than 1 / year ES recurrence, as well as seizure-free patients for over 1 year.

The disease duration over 5 years, as well as over 10 years determined an increasing tendency of depression signs with 15-21 HAD-D score in 65% vs anxiety HAD-A in 53% of cases. Anxiety was determined in 57% of cases, predominantly found in the group of patients with 0-2 years of disease duration with a score of 15-21 HAD-A vs depression – found in 18% of cases.

Discussion

The results of this study showed a significant correlation between epilepsy in women and anxiety and depression that was determined in the dynamics of epileptic disease. The HADS scaled score proved to be sufficiently relevant to assess the prevalence of anxiety and depression disorders, depending on the type and recurrence of seizures, as well as on the disease duration. The research results confirmed that the disease chronicity has a greater impact on depression vs anxiety, being a major risk factor for developing mental disorders in epilepsy [17]. This study revealed about 90% incidence rate of anxiety and depression in the group of women of reproductive age, suffering from epilepsy for over 1 to 10 years. However, some depression and anxiety disorders were likely to be caused by several endogenous and constitutive factors, such as hereditary tendency, antiepileptic medication, non-diagnosed early mental symptoms, etc.

As regarding the ES type, it has been proven that it had a less impact on the development of mental disorders compared to the ES recurrence and disease duration. However, focal ES has been mostly associated with anxiety, whereas generalized ES referred to different types of depressive disorders.

Women with high depression and anxiety severity levels established via HADS score are recommended to be further checked by psychiatrist and undergo a more complex psychometric scale assessment in order to prevent serious mental disorders and initiate an early psychiatric treatment [18].

Conclusions

The analysis of the depression and anxiety symptoms associated with epilepsy in women of reproductive age revealed the following:

1. Depression and anxiety comorbidity in epilepsy exhibit a heterogeneous nature, determined by the polymorphism of the clinical signs.
2. The HADS scale was sufficiently relevant for screening anxiety and depression disorders associated with epilepsy in women aged 15-49 years.
3. The disease duration and the recurrent epileptic seizures are major risk factors for developing depression and anxiety in epilepsy women.
4. Correlation between anxiety and depression and the ES type is quite controversial, thus requiring further studies by using instrumental investigations, such as global neuroimaging and electroencephalography studies, as well as the analysis of antiepileptic drug therapy.
5. The anxiety and depression disorders in women with epilepsy might be potentially diagnosed earlier if applying the screening HADS scale assessment in primary and dynamic neurological examination of all patients with epilepsy, in order to identify early signs of mental disorders, prevent suicide attempts, improve the quality of life and manage proper treatment.

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

Funding
This study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy. The trial was the author's initiative. The author is independent and takes responsibility for the integrity of the data and accuracy of the data analysis.

Ethics approval and consent to participate
The study was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Report No 71 of 17.06. 2016). An informed consent from all participants in the study was obtained.

Conflict of Interests.
There is no known conflict of interests to declare.

https://doi.org/10.52418/moldovan-med-j.64-2.21.04
UDC: 616.24-001-092.9

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Indirect lung injury predictive model in experimental trauma

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Manuscript received January 31, 2021; revised manuscript April 02, 2021; published online April 28, 2021

Abstract

Background: Trauma remains a medical-social problem, still having high lethality rate. Indirect lung injury (ILI) occurs in trauma due to systemic neutrophils activation and proteases release into primarily intact tissues. There are no data in the literature regarding ILI predictive models in trauma.
Material and methods: In the experimental study (19 traumatized male rabbits), the proteases, antiproteases and the pulmonary morphological changes, assessed according to the SAMCRS score (Semiquantitative Reflected Qualitative Changes Assessment Scale) were followed. There were used two statistical instruments – correlational analysis and multivariate linear regression.
Results: Initially, a correlational analysis between the values of the SAMCRS score and the proteases/ anti proteases was performed. The null hypothesis was rejected (F = 7.017, p = .002). The correlation coefficient of the predicted results and the real values of $SAMCRS_{lungs}$ was .854, the determination coefficient being .626. The final model included the following parameters: constant (B = 9.427; 95% CI 7.341, 11.513; p <.001); $\alpha 2\text{-macroglobulin}_0$ (B = -4.053; 95% Cl -6.350, -1.757; p = .002); $AEAMP_0$ (B = .002; 95% CI .000, .004; p = .075); $AEAMP_{24}$ (B = -. 006; 95% CI -.010, -.002; p = .003); $AECG_2$ (B = .081; 95% CI .040, .122; p = .001); AEE_0 (B = -. 026; 95% CI -.040, -.011; p = .002).
Conclusions: In this research, a predictive model for indirect lung injury in experimental trauma was developed, the predictors being some elements of the proteases/antiproteases system. This, in turn, allows the hypotheses emission regarding the pathophysiology, prophylaxis and treatment of ILI.
Key words: trauma, indirect lung injury, predictive model.

Cite this article
Arnaut O, Grabovschi I, Sandru S, Rojnoveanu G, Baltaga R. Indirect lung injury predictive model in experimental trauma. *Mold Med J.* 2021;64(2): 21-25. https://doi.org/10.52418/moldovan-med-j.64-2.21.04.

Introduction

The epidemiological data in the Republic of Moldova regarding the trauma showed alarming numbers, traumatic injuries being the first cause of death in the age group of 1-44 years [1]. The principal causes of "traumatic death" are severe trauma and polytrauma. They are characterized by a series of systemic mechanisms activation with pro-/anticoagulant, pro-/antiinflammatory, endocrine, nervous and immune systems enrolment in order to restore/maintain the homeostasis [2].

Thus, in conditions of traumatic injuries, it's important to consider both, the lesion severity and the host response. Under normal conditions, the aseptic inflammation, generated by trauma, remains local. Polytrauma or severe trauma amplifies the process and Systemic Inflammatory Response Syndrome (SIRS) occurs. As a result, the immune cells, forming inflammasomes, are activated via cytokines and chemokines, vascular permeability being increased via expression of adhesins in the surrounding endothelium. This, in turn, allows the immunocompetent cell accumulation besides injured tissues in healthy, normal tissues with following degranulation at this site. Consequently, some aggressive agents, as reactive oxygen species (ROS) and proteases, determine the lesions in the tissues that are far from the primary traumatic lesions. As named "indirect" injuries, decreasing the functional reserves with organs failure (sometimes multiple organ failure (MOF)) appearance are an unresolved problem in critical care patients management [3].

Literature has described "indirect" injuries in different organs: central nervous system/brain and spinal column – disruption of the blood-brain and blood-spinal barriers, heart – acute coronary syndrome, liver – acute hepatic injuries, kidney – acute kidney injuries, endothelium of systemic vessels – disseminated intravascular coagulation etc. [4-7]. Indirect lung injury (ILI or indirect ARDS (Acute Respiratory Distress Syndrome)) represents the most common type of "indirect" injuries, explanation being neutrophils rapid accumulation (minutes, hours) in the interstitial space and bronchoalveolar fluid of the lungs after the trigger factors influence. This can be explained by some pulmonary microcirculatory bed particularities, neutrophils redistribution before trigger factor actioning, their passage through alveolocapillary barrier and late apoptosis. Compared with

other organs where cells can concentrate in the postcapillary venules, in the lungs, they will cumulate in the capillaries themselves that are connected in a short segment network. This will increase about 50 times the capillary walls exposure period to neutrophils compared to other body areas [8]. Existing therapies, especially synthetic antiproteases administration, did not show efficiency in order to decrease the mortality rate – data from randomized clinical trial [9] and meta-analysis from 2017 [10].

Taking into account the information above ILI needs additional researches. The study aim was indirect post-traumatic lung injury predictive model elaboration for hypotheses emission regarding the pathophysiological mechanisms, prophylaxis and potential therapies of ILI.

Material and methods

In the experimental study were used 19 severely traumatized rabbits according to the method described above [11]. The proteases, antiproteases and the pulmonary morphological changes, assessed according to the Semiquantitative Reflected Qualitative Changes Assessment Scale (SAMCRS), were analyzed.

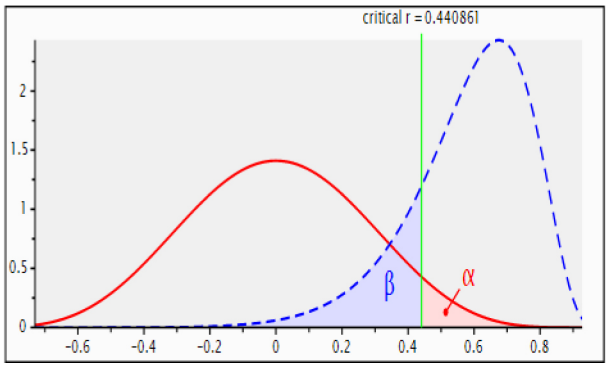
Protease/antiprotease system components were used as biomarkers/predictors of "indirect" lesions and lung functional state at 24 hours after trauma. From collected and frozen samples, later, Elastase (AEE), Cathepsins G (AECG), D (AECD), L (AECL), H (AECH), Trypsin (AET), Adenosinedesaminase (AEADA) and Adenilatdesaminase (AEAMP) enzymatic activity, the same as ά2-macroglobulin and ά1-antitrypsin was measured before, at 2, 5 and 24 hours after the trauma, using spectrophotometry method.

Collected tissue samples analysis was used as an instrument for "indirect" lesion quantification. Initially, the collected samples followed the hematoxylin eosin coloration technique: fixation, washing, dehydration, waxing, sectioning, etalation, dewaxing, hydration, coloration and mounting. Morphological pieces examination was performed using artificial light optical microscope ("Micros", Austria) using objectives needed for an optimal amplification (x100 or x200 each time) of the studied structures. Histological samples were evaluated from 0 to 3 based on SAMCRS as follows: 0 – no any notable changes, 1 – weak changes, 2 – moderate changes, 3 – excessive changes. SIRS characteristics where analyzed for every tissue. Interstitial edema, venous congestion, interstitial granulocyte infiltration, hemorrhagic impregnation, lung hemosiderosis were attested. $SAMCRS_{lungs}$ score was appreciated by summing all the intensities of the listed above changes observed in the lungs [11].

There were used two statistical instruments – correlational analysis (Spearman ρ test) with effect size estimation and multivariate linear regression. Initially, by building a histogram (extremes identification) and by distribution analysis (Shaporo test) of the measured biochemical and histological parameters, they were identified and where needed, normalized (by logarithmic function), the data was prepared for identification of the potential biomarkers/predictors of the "indirect" lung injury. Using the Spearman ρ test there were identified the associations ($p<.05$) or tendencies to associate ($p<.1$) of the protease/antiprotease system components with SAMCRS for ILI. At the same time, there were analyzed the associations between different proteases/antiproteases system components in order to identify the potential sources for multicollinearity as an obstacle for predictive models' elaboration.

Minimal sample size was estimated by using version 3.1.9 GPower software [12]. Left side of the figure shows the distribution plot estimating correlation coefficient critical value. On the right side are listed the parameters needed for sample size estimation (fig. 1). Calculated minimal number of the statistical units was 15 (power 0.8, $\alpha=0.05$, expected ρ value being 0.6, using unilateral hypotheses).

Having 19 statistical units, we can consider a sufficient research power. Finally, was elaborated a predictive model for "indirect" lung injury in the experimental severe trauma. The applied statistical method to elaborate the models able to predict the "indirect" lesion severity was the linear regression (backward method) used according to the standards recommended by the literature sources [13].



Exact – Correlation: Bivariate normal model

Options:	exact distribution		
Analysis:	A priori: Compute required sample size		
Input:	Tail(s)	=	One
	Correlation ρ H1	=	0.6
	α err prob	=	0.05
	Power (1−β err prob)	=	0.80
	Correlation ρ H0	=	0
Output:	Lower critical r	=	0.4408608
	Upper critical r	=	0.4408608
	Total sample size	=	15
	Actual power	=	0.8058718

Fig. 1. Distribution with critical r (left) and sample size estimation (right)

Table 1. Linear regression coefficients and collinearity analysis for $SAMCRS_{lungs}$ values prediction in experimental severe trauma

	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B		Collinearity Statistics	
	B	Std. Error	Beta			Lower Bound	Upper Bound	Tolerance	VIF
(Constant)	9.427	.966		9.763	.000	7.341	11.513		
α_2-macroglobulin$_0$	-4.053	1.063	-.847	-3.813	.002	-6.350	-1.757	.421	2.373
AEAMP$_0$	.002	.001	.430	1.937	.075	.000	.004	.423	2.366
AEAMP$_{24}$	-.006	.002	-1.353	-3.569	.003	-.010	-.002	.145	6.905
AECG$_2$	.081	.019	1.089	4.306	.001	.040	.122	.325	3.076
AEE$_0$	-.026	.007	-.698	-3.840	.002	-.040	-.011	.630	1.588

Note: Std. Error –standard error for B coefficient, t – t test, Sig. – significance, VIF – variance inflation factor, α_2-macroglobulin$_0$ - α_2-macroglobulin enzymatic activity before trauma, AEAMP$_0$ - Adenosinedesaminase before trauma, AEAMP$_{24}$ – Adenosinedesaminase measured at 24 hours after trauma, AECG$_2$ – Cathepsin G enzymatic activity measured at 2 hours after trauma, AEE$_0$ – Elastase enzymatic activity measured before the trauma

Results

Correlation analysis showed the following correlations (p<.05) or tendencies for correlations (p<.1). $SAMCRS_{lungs}$ was associated with AET_0 (r=-.343, p=.075, effect size .12), AET_2 (r=.466, p=.022, effect size .22), AET_{24} (r=-.358, p=.066, effect size .13), α_2-macroglobuline$_2$ (r=-.401, p=.044, effect size .16), $AEAMP_{24}$ (r=.311, p=.097, effect size .01), $AECG_2$ (=.590, p=.004, effect size .35), $AECG_{24}$ (r=-.317, p=.093, effect size .10), $AECL_2$ (r=.441, p=.029, effect size .20), AEE_0 (r=-.479, p=.019, effect size .23), AEE_{24} (r=-.342, p=.076, effect size .17). These potential biomarkers were used for ILI prediction. Also, because of possible predisposition for ILI, were considered the initial values, before the trauma.

Predictive model for $SAMCRS_{lungs}$ was elaborated using backward technique. The final model correlation coefficient between predicted histological modifications and real $SAMCRS_{lungs}$ value was .854, determination coefficient equal to .626, the sum of squares being 17896 from 24526 possible. The null hypothesis (there are no parameters with predictive potential for $SAMCRS_{lungs}$ values at 24 hours after experimental trauma) was rejected (F=7.017, p=.002).

The final model included the following parameters (tab. 1):

- Constant (B=9.427; 95%CI 7.341, 11.513; p<.001);
- α_2-macroglobulin$_0$ (B=-4.053; 95%CI -6.350, -1.757; p=.002);
- AEAMP$_0$ (B=.002; 95%CI .000, .004; p=.075);
- AEAMP$_{24}$ (B=-.006; 95%CI -.010, -.002; p=.003);
- AECG$_2$ (B=.081; 95%CI .040, .122; p=.001);
- AEE$_0$ (B=-.026; 95%CI -.040, -.011; p=.002).

Other potential biomarkers like AET_0, AET_2, AET_{24}, $AECG_{24}$, $AECL_2$, AEE_{24} the same as for their value before the trauma were not significative, thus, they weren't included in the final model of "indirect" lung lesions prediction. The

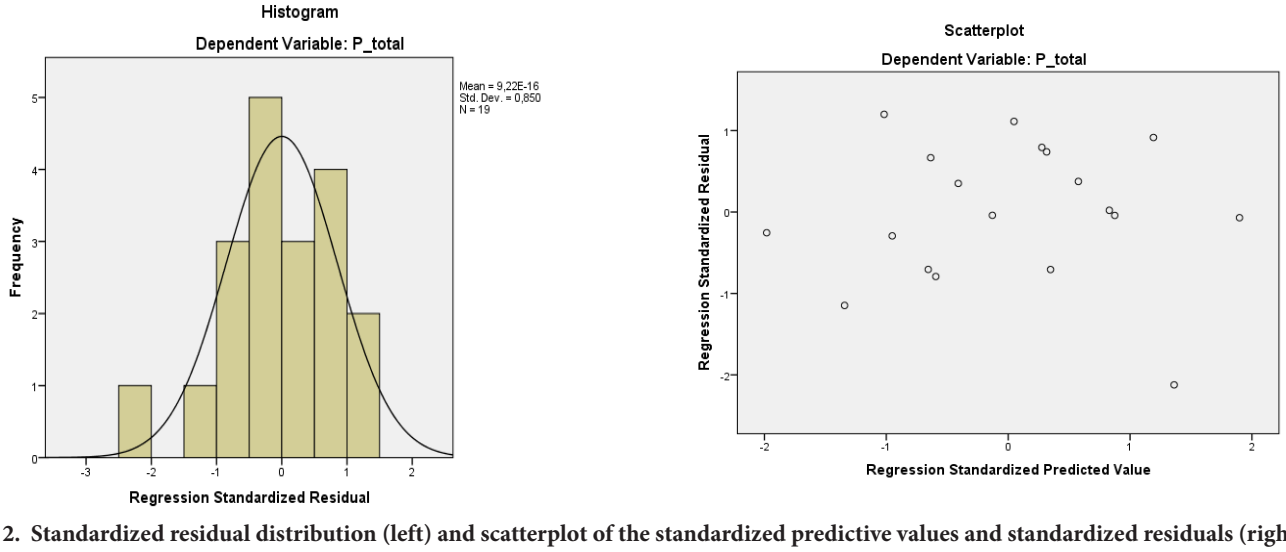


Fig. 2. Standardized residual distribution (left) and scatterplot of the standardized predictive values and standardized residuals (right)

obtained model has the following mathematical expression (Equation 1):

$$SAMCRS_{lungs} \text{ at 24 hours} = 9.427 - \alpha_2\text{-macroglobulin}_0 * 4.053 + AEAMP_0 * .002 - AEAMP_{24} * .006 + AECG_2 * .081 - AEE_0 * .026 \text{ (Equation 1)}$$

As the analysis of collinearity showed, the quality of the prediction is not affected by the potential strong correlations between the parameters included in the model (Tolerance and VIF being more than 0.1 and less than 10 respectively). From a quantitative point of view, it has been demonstrated by standardizing the coefficients that the effects of $AEAMP_{24}$ on $SAMCRS_{lungs}$ are the most significant (Beta=-1.353), followed by $AECG_2$ (Beta=1.089), α_2-$macroglobulin_0$ (Beta=-.847), AEE_0 (Beta=-.698) and $AEAMP_0$ (Beta=.430).

In addition, the developed model also met the necessary conditions for residual linear regression. Their analysis demonstrated an almost normal distribution and lack of associations between standardized predictive values and standardized residuals (fig. 2). All these together allow us to consider the model as a suitable one.

Considering that the model was developed on a relatively small number of participants, which increases the risk of model instability, especially since the latter included five biomarkers in addition to the constant, resampling was performed by bootstrapping (tab. 2). The model has shown its stability, $AECG_2$, $AEAMP_0$ and $\alpha2$-$macroglobulin_0$ being potential biomarkers for distant lung damage. The effects of $AEAMP_{24}$ and AEE_0, even if significant and stable, require verification in subsequent studies.

Discussion

In severe trauma or polytrauma through cytokine storm immunocompetent cells are activated, infiltrate intact tissues and produces "indirect" lesions [14]. Actual research had the aim to probe the proteases/antiproteases components, deposited in neutrophils and other immunocompetent cells, as predictors for posttraumatic ILI by histological modification modeling. Obtained information, in perspective, will complete the knowledge in this field.

In general, elaborated model showed acceptable characteristics with no multicollinearity, no residuals problem and stability, according to linear regression procedure [13]. The model included both, proteases and antiproteases, each of them having protective or destructive potential. The concept of the antiproteases protective effects and the proteases destructive effects in our research is supported by the signs in front of the regression coefficients of $\alpha2$-$macroglobulin_0$, $AEAMP_0$ and $AECG_2$. α2-macroglobulin (macromolecular antiprotease) is a plasma glycoprotein best known for its ability to inhibit a broad spectrum of serine, threonine, and metalloproteases as well as inflammatory cytokines [15]. Cathepsin G activates coagulation, having immunostimulatory and antimicrobial effects or it can increase vascular permeability promoting edema. Also, it increases metalloproteinase activity with further vascular matrix destruction [16-18]. Because $AEAMP_{24}$ and AEE_0 are proteases, the negative signs in front of the regression coefficients can suggest some suspect results. Possibly, this fact can be explained by the need to complete the model (1/3 of the dispersion is not explained, the constant being significant), their adjustment to the potential effective variables will reverse their sign or will exclude them from the final model. Other possible variants – the proteases are balanced by antiproteases before the trauma or they have protective effects in case of pulmonary lesions. The model took into account the predictor's value before the trauma (α_2-$macroglobulin_0$, $AEAMP_0$, AEE_0). There are some opinions that it can show a predisposition for ILI in conditions of severe trauma.

At the same time, the elaborated model has some limitations. First, the model needs to be improved by adding some effective parameters/variables up to .80 (80%) value of the determination coefficient to remove one of the research's imperfections, namely that about one third of the $SAMCRS_{lungs}$ at 24 hours after trauma dispersion remains unexplained. Second, the activated neutrophils ROS releases besides proteases, that were not investigated and, probably, could improve the model [3]. Third, the confidence intervals range needs precision. Fourth, the research is experimental one, model being male rabbits – the argument to validate or adapt the model for human being. At the same time, similar research in clinical practice could be performed only by changing the design.

Table 2. Resampling by bootstrapping. $SAMCRS_{lungs}$ predictive model at 24 hours after trauma

	B	Bias	Std. Error	Sig. (2-tailed)	95% Confidence Interval	
					Lower	Upper
(Constant)	9.427	.047	1.369	.001	6.683	12.193
$AECG_2$	.081	-.003	.026	.009	.022	.129
$AEAMP_0$	.002	3.004E-05	.001	.079	.000	.005
$AEAMP_{24}$	-.006	.000	.002	.016	-.011	-.002
AEE_0	-.026	.000	.008	.011	-.039	-.009
α_2-$macroglobulin_0$	-4.053	-.166	1.514	.039	-7.364	-1.264

Note: Std. Error – standard error for B coefficient, Sig. – significance, $AECG_2$ – Cathepsin G enzymatic activity measured at 2 hours after trauma, $AEAMP_0$ – Adenosinedesaminase before trauma, $AEAMP_{24}$ – Adenosinedesaminase measured at 24 hours after trauma, AEE_0 – Elastase enzymatic activity measured before the trauma, α_2-$macroglobulin_0$ – α_2-macroglobulin enzymatic activity before trauma.

Conclusions

In the current research, a predictive model for indirect lung injury in experimental trauma was developed. This, in turn, allows the hypotheses emission regarding the pathophysiology, prophylaxis and treatment of post-traumatic ILI. The model needs validation/adaptation in clinical research.

Three predictors are represented by the proteases/antiproteases system components before the trauma, two proteases ($AEAMP_0$, AEE_0) and antiprotease α_2-$macroglobulin_0$. This can suggest a predisposition of some individuals for developing a post-traumatic ILI.

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Funding
This study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy. The trial was the authors' initiative. 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 46, 16.12.2016).

Conflict of Interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.64-2.21.05
UDC: 616.127-005.8-089.844

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Myocardial remodeling in NSTEMI patients with intermediate and low cardiovascular risk exposed to delayed revascularization

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Manuscript received March 05, 2021; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: Nowadays, the impact of the delayed myocardial revascularization (DMR) (>72h) in patients with myocardium infarction without ST-segment elevation (NSTEMI) having either intermediate or low cardiovascular risk (ILCR) on quality of post-infarction myocardial remodeling is not well established. Aim of the study: The comparative evaluation of cardiac functional recovery of NSTEMI patients undergoing either revascularization <72h or DMR (72h–30 days) in a follow-up of 6 months.
Material and methods: The study was realized in 2 homogenic series of NSTEMI patients with ILCR exposed to revascularization: <72h (control) or to DMR (72h–30 days). The echocardiographic and physical test indices were registered at the 2nd day since revascularization and after 6 months.
Results: The increasing ratio of ejection fraction was significantly higher in patients with DMR compared to control (5.24% vs 1.73%). Likewise, the contractility ability of left ventricle improved better, proven by systolic volume diminution, lower value of akinetic areas, and less patients with class III of heart failure according to New York Heart Association (4 vs 29%). More than that, DMR was associated with higher physical endurance.
Conclusions: NSTEMI patients with ILCR exposed to delayed myocardial revascularization (72h–30 days) had a better post-infarction recovery after 6 months according to dynamics of echocardiographic and physical tolerance indices in comparison with patients revascularized <72h.
Key words: myocardial infarction, delayed revascularization, myocardial remodeling, echocardiographic indices.

Cite this article
Surev A, Cioban L, Ivanov M, Popovici I, Cobet V, Popovici M. Myocardial remodeling in NSTEMI patients with intermediate and low cardiovascular risk exposed to delayed revascularization. *Mold Med J.* 2021;64(2):26-32. https://doi.org/10.52418/moldovan-med-j.64-2.21.05.

Introduction

The post-infarction remodeling of the myocardium is a complex phenomenon that activates the main systems of homeostasis, in the acute phase of myocardial necrosis and is marked by morpho-functional and metabolic changes meant to ensure the adaptation of the heart's contractility by reducing the number of sarcomeres. The quality of the post-infarction remodeling, defined by structural and geometrical changes in the first 4-6 months from the onset of the myocardial infarction (MI) will lately dictate the clinical and functional evolution of the patient as well as the major advanced cardiovascular events (MACE) rate. It obviously depends on a series of factors, but one of the most important is the necrosis area, that usually is widespread in the case of MI with elevated ST segment (STEMI) due to a total or subtotal occlusion (>90%) of a large caliber epicardial artery, associated with a conclusive inflammatory response.

MI without ST-segment elevation (NSTEMI), from the physiological point of view, remains an intriguing question and a challenge for most cardiologists due to the similar in-hospital mortality percent (5-7%) and even higher at one year distance, versus STEMI, despite its specificity of evolution in the limit of one coronary artery with a total occlusion just in ⅓ of cases but with a better distal collateralization than STEMI. In the rest, when the coronary occlusion is <50%, pathogenetically important, is attributed the role of microcirculatory coronary dysfunction, where the subendocardial coronary arteriola (<200 mkm) manifests a prothrombotic status, increased by the lasting spasm due to endothelial dysfunction.

In STEMI, the revascularization time of the "culprit" artery is a decisive factor for the prognosis of evolution post MI, the optimal accepted window is between 6 and 12 hours from the onset of the myocardial necrosis.

For NSTEMI, the European Society of Cardiology (ESC) guidelines recommendations regarding the timing of the invasive treatment are estimated through the 2 scores: Global Registry of Acute Coronary Events (GRACE) score and Thrombolysis In Myocardial Infarction (TIMI) score. In patients with high and very high cardiovascular risk, primary reperfusion is recommended at the distance of 2 and 24h respectively from the MI onset [1]. In patients with moderate or low cardiovascular risk (ILCR), the ESC guidelines recommend applying the invasive strategy in the first 72h from the establishment of NSTEMI diagnostic, and in case of the impossibility to perform the invasive treatment lo-

cally, the patient should be transferred to a medical center with interventional cardiology [2].

Our experience reveals the existence in the Republic of Moldova of a large number of patients eligible to this recommendation, mainly due to the newly installed social restrictions because of COVID-19 pandemic, that postpones the myocardial reperfusion even to 30 days.

It is important to emphasize, in this context, the impact of the delayed angioplasty on the prognosis of post MI evolution at distance in contiguity with the estimation of the character of the morpho-functional remodeling of the myocardium, functional class of heart failure according to New York Heart Association (NYHA) as well as on MACE rate.

Aim of the study: the comparative assessment of the effects of reperfusion <72 h and delayed reperfusion (72h–30 days), on echocardiographic parameters and physical endurance in NSTEMI patients at the 6 months distance.

Material and methods

The study was realized on two groups of NSTEMI patients with GRACE score <140 (intermediate and low cardiovascular risk), created depending on the angioplasty time:

- Group 1, control series (126 patients) revascularized <72 h;
- Group 2 (126 patients) revascularized in the time between 72h–30 days.

Both groups were considered homogeneous regarding the age, gender, the onset of comorbidities symptoms (hypertension, diabetes mellitus) and other cardiac risk factors (tab. 1).

Patients included in the study were investigated in the Institute of Cardiology and in the polyvalent hospital NOVAMED. The NSTEMI diagnostic was established on the base of the clinical, electrocardiographic, echocardiographic and enzymatic criteria: (i) the onset of symptoms of acute myocardial ischemia lasting more than 20 minutes, (ii) ST-segment depression more than 1.0 mm and/or T-wave changes in more than 2 leads on ECG and/or evidence of loss of viable myocardium or new regional wall motion abnormality and (iii) increase of cardiac biomarkers (CK-MB and cardiac Troponins TnT).

GRACE score was calculated via the standard formula, taking into consideration the age, heart rate (HR), systolic blood pressure (SBP), creatinine, ST-segment deviation on ECG, abnormal cardiac enzymes and history of cardiac arrest.

Exclusion criteria were:

- High and very high cardiovascular risk;
- History of myocardial infarction;
- Dilated cardiomyopathy;
- Post-inflammatory cardiomyopathy;
- Hypertrophic cardiomyopathy;
- Coronary stenosis that caused the infarct < 50% and FFR (Fractional Flow Reserve) > 0.80;
- The presence of stenosis > 75% on other vases than the one causing the MI;
- History of CABG (coronary artery bypass surgery) or PCI (Percutaneous Coronary Intervention);
- Significant valve disease (III-IV degree of insufficiency, moderate or severe stenosis);
- Diffuse LV (left ventricle) hypokinesis on ECHO (echocardiography);
- Severe renal failure.

The following examinations were done at admission and at a 6 months interval post MI:

Registration of ECG in 12 leads at rest, using the electrocardiographic device "CARDIOLINE AR1200adv" (Cardioline S.p.A, Italy) repeated three times: at admission, the day after revascularization and at a 6 months distance.

Transthoracic echocardiography (ECHO), realized using

Table 1. The clinical feature of NSTEMI patients on admission

Indices		General group (N = 252)	Group 1 (N = 126)	Group 2 (N = 126)	p
Age		59.940±0.639	59.675±0.919	60.206±0.891	>0.05
Gender:	Male	208 (82.5%)	104 (82.5%)	104 (82.5%)	>0.05
	Female	44 (17.5%)	22 (17.5%)	22 (17.5%)	
Resident:	Urban	149 (59.1%)	79 (62.7%)	70 (55.6%)	>0.05
	Rural	103 (40.9%)	47 (37.3%)	56 (44.4%)	
Occupation:	Sleep	10 (4.0%)	3 (2.4%)	7 (5.6%)	>0.05
	Physical effort	90 (35.7%)	44 (34.9%)	46 (36.5%)	
	Intellectual work	26 (10.3%)	14 (11.1%)	12 (9.5%)	
	Retired	126 (50.0%)	65 (51.6%)	61 (48.4%)	
Marital status:	Single	6 (2.4%)	5 (4.0%)	1 (0.8%)	>0.05
	Married	209 (82.9%)	103 (81.7%)	106 (84.1%)	
	Divorced	7 (2.8%)	3 (2.4%)	4 (3.2%)	
	Widower	30 (11.9%)	15 (11.9%)	15 (11.9%)	
Type of admission:	Emergency room	181 (71.8%)	124 (98.4%)	57 (45.2%)	<0.001
	Programmed	71 (28.2%)	2 (1.6%)	69 (54.8%)	
Main symptom:	Pain	234 (92.9%)	121 (96.0%)	113 (89.7%)	<0.05
	Dyspnea	17 (6.7%)	4 (3.2%)	13 (10.3%)	
	Palpitations	1 (0.4%)	1 (0.8%)	0 (0%)	

Presenting of symptoms:	At rest	107 (42.5%)	77 (61.1%)	30 (23.8%)
	Minor physical effort	101 (40.1%)	32 (25.4%)	69 (54.8%)
	Moderate physical effort	37 (14.7%)	12 (9.5%)	25 (19.8%)
	Major physical effort	7 (2.8%)	5 (4.0%)	2 (1.6%)
				<0.001
Onset of symptoms:	Sudden	168 (66.7%)	88 (69.8%)	80 (63.5%)
	Slow	84 (33.3%)	38 (30.2%)	46 (36.5%)
				>0.05
History of angina pectoris:	Yes	79 (31.3%)	35 (27.8%)	44 (34.9%)
	No	173 (68.7%)	91 (72.2%)	82 (65.1%)
				>0.05
Angina's functional class in history:	I	0 (0%)	0 (0%)	0 (0%)
	II	14 (17.7%)	6 (17.1%)	8 (18.2%)
	III	51 (64.6%)	24 (68.6%)	27 (61.4%)
	IV	0 (0%)	0 (0%)	0 (0%)
	Unstable angina	14 (17.7%)	5 (14.3%)	9 (20.5%)
				>0.05
Nitrate administration:	No	230 (91.3%)	114 (90.5%)	116 (92.1%)
	Yes	22 (8.7%)	12 (9.5%)	10 (7.9%)
				>0.05
Risk factors				
Hypertension HTA:	absent	76 (30.2%)	49 (38.9%)	27 (21.4%)
	Gr. I	3 (1.2%)	2 (1.6%)	1 (0.8%)
	Gr. II	87 (34.5%)	37 (29.4%)	50 (39.7%)
	Gr. III	86 (34.1%)	38 (30.2%)	48 (38.1%)
				<0.05
Obese or overweight:	No	106 (42.1%)	46 (36.5%)	60 (47.6%)
	Yes	146 (57.9%)	80 (63.5%)	66 (52.4%)
				>0.05
Diabetes Mellitus:	No	193 (76.6%)	99 (78.6%)	94 (74.6%)
	Yes	59 (23.4%)	27 (21.4%)	32 (25.4%)
				>0.05
Dyslipidemia:	No	136 (54.0%)	73 (57.9%)	63 (50.0%)
	Yes	116 (46.0%)	53 (42.1%)	63 (50.0%)
				>0.05
Family history:	Present	44 (17.5%)	26 (20.6%)	18 (14.3%)
	Absent	208 (82.5%)	100 (79.4%)	108 (85.7%)
				>0.05
Physical activity:	<30 min/day	143 (56.7%)	75 (59.5%)	68 (54.0%)
	>30 min/day	109 (43.3%)	51 (40.5%)	58 (46.0%)
				>0.05
Smoking:	No	175 (69.4%)	84 (66.7%)	91 (72.2%)
	Yes	77 (30.6%)	42 (33.3%)	35 (27.8%)
				>0.05
Alcohol intake:	No	214 (84.9%)	100 (79.4%)	114 (90.5%)
	Regular	32 (12.7%)	20 (15.9%)	12 (9.5%)
	Exaggerated	6 (2.4%)	6 (4.8%)	0 (0%)
				<0.05
Coffee consumption:	No	206 (81.7%)	100 (79.4%)	106 (84.1%)
	Yes	46 (18.3%)	26 (20.6%)	20 (15.9%)
				>0.05
Physical examination				
Hight		170.806±0.281	170.810±0.412	170.802±0.385	>0.05
Weight		77.579±0.618	78.786±0.895	76.373±0.843	>0.05
Body weight index		26.537±0.173	26.940±0.261	26.133±0.222	<0.05
Systolic blood pressure		134.786±1.310	134.460±1.909	135.111±1.803	>0.05
Diastolic blood pressure		81.710±0.751	80.952±1.069	82.468±1.056	>0.05
Heart rate		75.087±0.809	76.984±1.109	73.190±1.158	<0.05

the device "PHILIPS Hd11 Xe" (Koninklijke Philips N.V., Holland) in M, B and Doppler mode with a 2.0-2.5 mHz transducer. The functional and geometrical parameters regarding structural and functional remodeling were estimated.

The stress test was performed using the cycloergometric device (CEM) "CARDIOLINE cube stress" (Cardioline S.p.A, Italy) through the continuous method, pedaling speed was 60 rotations per minute, starting from 25 Wt, and every step was taking 2 minutes. ECG, HR and SBP were constantly monitored. At each step the load was increased by 25 Wt.

The criteria for stopping the test were:

- Reaching the target criteria, 85% from the maximal HR (220 – age in years);
- A strong angina seizure;
- Severe dyspnea;
- Ischemic changes on ECG;
- Set of rhythm abnormalities (frequent extrasystole, atrial fibrillation, etc.);
- Lowering BP less the initial one or elevation more than 230/130 mm Hg;
- Claudication – ischemic pain in the inferior extremities;

The test was considered positive in the case when the elevation or depression of ST segment > 1 mm at 0.08 sec from the j point was registered, compared with the ECG at rest.

The coronarography was conducted in the catheterization laboratories in the Institute of Cardiology and in the

Table 2. Main echocardiographic parameters from NSTEMI patients on the 2nd day after revascula

Parameters	Group 1	Group 2	p
LV diastolic diameter (mm)	53.556±0.475	52.437±0.507	>0.05
LV diastolic volume (ml)	148.341±2.653	144.016±2.240	>0.05
LV systolic diameter (mm)	36.762±0.579	35.683±0.550	>0.05
LV systolic volume (ml)	75.294±2.055	70.627±1.773	>0.05
Interventricular septal wall (mm)	11.500±0.154	11.849±0.150	>0.05
LV posterior wall (mm)	10.274±0.090	10.389±0.128	>0.05
Ejection fraction (EF) (%)	47.063±0.785	48.754±0.748	>0.05
Normokinetic pattern No	112 (88.9%)	108 (85.7%)	>0.05
Normokinetic pattern Yes	14 (11.1%)	18 (14.3%)	>0.05
Relaxation disorders Yes	118 (93.7%)	120 (95.2%)	>0.05
Relaxation disorders No	118 (93.7%)	120 (95.2%)	>0.05

polyvalent hospital NOVAMED, using the device General Electric INNOVA and Siemens "Artis One".

Depending on the degree of stenosis, the coronary lesions were divided in (1) insignificant lesions (25%); moderate (25-50%); moderate-severe (51-75%); severe (76-90%); critical (91-99%) and total occlusion (100%). In the case of severe, critical and total occlusion lesions feasibility of collateralization was estimated.

After revascularization and stenting the "culprit" artery, on the final flow was applied the TIMI system score and myocardial "blush" (MBG – Myocardial Blush Grade).

Optical Coherence Tomography (OCT) was realized with the "Saint Jude Medical" device, model "ILUMIEN" just in the case of those patients whose morphologic pattern couldn't be appreciated through coronary angiography.

Fractional flow reserve (FFR*)* was estimated with the "Saint Jude Medical" device, model "ILUMIEN" in those patients who were suspected to have abnormality in coronary microcirculation, as a result of a moderate coronary stenosis diagnosed during coronary angiography.

Laboratory investigations have included a large specter of circulating markers regarding: irreversible cellular lesions, dyslipidemia, endothelial dysfunction, systemic inflammation, hyperglycemia, hemostasis, etc.

The angioplasty was realized, using 2 types of stents: bare metal stent (BMS) and drug eluting stent (DES). The diameter of the used stents was in the range from 2.25 mm to 5 mm, while the length between 8 mm and 48 mm.

For the statistical processing of the numerical material, were used the accepted biostatistical maneuvers: t-Student index (comparison of averages in 2 groups), ANOVA (comparison of averages in 3 groups), method of variational analysis, correlation, $\chi 2$ index (comparison of nonparametric variables), Pearson correlation coefficient, U-Fischer criterion. Statistically significant in all methods of analysis was considered the value $p < 0.05$.

Results

The echocardiographic data from NSTEMI patients estimated on the 2nd day after angioplasty are similar in both groups (without significant discrepancy) and prove the imminent morphofunctional pattern of failed heart (tab. 2).

The ejection fraction was average in both groups, below 49%, that associated with elevated left ventricular end diastolic volume (LVEDV) that exceeded on average 144 ml. Interventricular septal wall (IVSW) and left ventricular posterior wall (LVPW) thickness, that are considered an important index in appreciation of myocardial hypertrophy degree, registered values above 11 mm and 10 mm respectively for both groups.

Except for the rate of severe stenosis and total occlusions in the second group *versus* the first group, data obtained during coronarography are similar (tab. 3).

Table 3. Coronarography data of the NSTEMI patients

Index		Group 1	Group 2	p
Approach:	Radial	108 (85.7%)	119 (94.4%)	
	Femoral	13 (10.3%)	6 (4.8%)	>0.05
	Brachial	5 (4.0%)	1 (0.8%)	
Atherosclerotic lesions:				
	1 coronary	32 (25.4%)	30 (23.8%)	>0.05
	2 coronaries	22 (17.5%)	23 (18.3%)	
	3 coronaries	72 (57.1%)	73 (57.9%)	
"Culprit" artery:	LM	1 (0.8%)	0 (0%)	>0.05
	LAD I	43 (34.1%)	25 (19.8%)	
	LAD II	30 (23.8%)	39 (31.0%)	
	LAD III	2 (1.6%)	2 (1.6%)	
	DIA	1 (0.8%)	3 (2.4%)	
	aCX I	4 (3.2%)	0 (0%)	
	aCX II	8 (6.3%)	15 (11.9%)	
	aCX III	3 (2.4%)	0 (0%)	
	OM	1 (0.8%)	3 (2.4%)	
	RCA I	10 (7.9%)	10 (7.9%)	
	RCA II	15 (11.9%)	16 (12.7%)	
	RCA III	8 (6.3%)	13 (10.3%)	
Lesion degree of the target segment:	25-50%	1 (0.8%)	1 (0.8%)	<0.01
	50-75%	3 (2.4%)	1 (0.8%)	
	75-90%	7 (5.6%)	16 (12.7%)	
	90-99%	38 (30.2%)	58 (46.0%)	
	Total occlusion	77 (61.1%)	50 (39.7%)	
Distal recharge:	No	43 (39.8%)	28 (28.3%)	>0.05
	Yes	65 (60.2%)	71 (71.7%)	
Recharge type:				
Intrasystemic collateral		19 (25.0%)	21 (24.7%)	>0.05
Extrasystemic collateral		22 (28.9%)	14 (16.5%)	
Intra-extrasystemic collateral		9 (11.8%)	11 (12.9%)	
Anterograde *via* thrombus		37 (48.7%)	43 (50.6%)	

Angiographic aspect of: Atherosclerotic plaque		60 (47.6%)	87 (69.0%)	<0.01
Thrombotic masses		66 (52.4%)	39 (31.0%)	
Lesion length:	0-15 mm	33 (26.2%)	39 (31.0%)	>0.05
	16-25 mm	71 (56.3%)	70 (55.6%)	
	26-40 mm	17 (13.5%)	15 (11.9%)	
	>40 mm	5 (4.0%)	2 (1.6%)	
Calcification:	0	72 (57.1%)	65 (51.6%)	>0.05
	1	36 (28.6%)	51 (40.5%)	
	2	12 (9.5%)	5 (4.0%)	
	3	6 (4.8%)	5 (4.0%)	
Tortuosity:	0	48 (38.1%)	43 (34.1%)	>0.05
	1	58 (46.0%)	55 (43.7%)	
	2	19 (15.1%)	28 (22.2%)	
	3	1 (0.8%)	0 (0%)	
Bifurcation:	No	105 (83.3%)	98 (77.8%)	>0.05
	Yes	21 (16.7%)	28 (22.2%)	

One of the notable points for estimating the quality of post-myocardial infarction remodeling is the severity of heart failure. It is remarkable that a higher rate of patients with NYHA II in group 2 compared to group 1 at a 6 months interval after angioplasty (66.7 vs 52.4%) while the rate of NYHA III, on the contrary is significantly lower (4 vs 29%).

This observation indicates a better functional heart recovery in NSTEMI patients treated with postponed revascularization, at 72 h. In this aspect, the dynamics of echocardiographic indices attested at a distance of 6 months after revascularization is also intelligible (tab. 4).

It is important to mention a more significant increase of EF in group 2 *versus* group 1 (5.24% vs 1.73%) that is also associated with a notable LV contractility recovery visible through a 5.56% decrease of LV end systolic volume meanwhile it increased by 1.05% in the control group. A similar dynamic was observed regarding LV systolic diameter.

Table 4. Relative deviations (%) of echocardiographic parameters among a period of 6 months

Parameters	Group 1			Group 2			P
	M	m	p	M	m	p	
Left atrium diameter	+0.484	0.191	<0.05	-0.341	0.136	<0.05	<0.01
LV diastolic diameter	+1.097	0.210	<0.001	+1.103	0.434	<0.05	>0.05
LVEDV	+2.742	1.028	<0.01	+0.944	1.128	>0.05	>0.05
LV systolic diameter	+0.508	0.302	>0.05	-1.960	0.243	<0.001	<0.001
LV end systolic volume	+1.048	1.080	>0.05	-5.556	0.615	<0.001	<0.001
IVSW	-0.121	0.09	>0.05	-0.159	0.05	<0.01	>0.05
LVPW	-0.085	0.06	>0.05	+0.071	0.05	>0.05	>0.05
EF	+1.734	0.58	<0.01	+5.238	0.36	<0.001	<0.001
Right ventricle diameter	-0.177	0.18	>0.05	-0.683	0.23	<0.01	>0.05
Right atrium	+0.637	0.24	<0.01	-0.175	0.19	>0.05	<0.01
Pulmonary systolic arterial pressure	-7.113	0.65	<0.001	-9.873	0.48	<0.001	<0.01
hypokinetic areas	+1.135	0.12	<0.001	+1.397	0.12	<0.001	>0.05
akinetic areas	-0.095	0.1	>0.05	-0.159	0.07	<0.05	<0.05
dyskinetic areas	-0.325	0.09	<0.001	-0.04	0.04	>0.05	<0.01
areas involved in the aneurysm	-0.294	0.08	<0.001	-0.048	0.05	>0.05	<0.01

Table 5. The results of the stress test at NSTEMI patients 6 months after revascularization

Parameter		General group	Group 1	Group 2	p
Initial systolic blood pressure (SBP)		124.627±0.735	124.202±1.120	125.032±0.962	>0.05
Initial diastolic blood pressure (DBP)		70.656±0.591	71.639±0.889	69.720±0.777	>0.05
Initial heart rate (HR), 1/min		67.545±0.402	68.151±0.613	66.968±0.520	>0.05
Max intensity effort, Wt		105.430±1.710	99.370±2.487	111.200±2.244	<0.001
Total time of physical effort, min		8.898±0.159	8.319±0.234	9.448±0.205	<0.001
Max SBP at effort, mm Hg		187.705±1.120	186.261±1.708	189.080±1.457	>0.05
Max DBP at effort, mm Hg		101.631±0.771	100.504±1.073	102.704±1.100	>0.05
Max HR, 1/min		134.377±1.379	131.773±2.187	136.856±1.686	>0.05
Reached target HR, 1/min	No	50 (20.5%)	37 (31.1%)	13 (10.4%)	<0.001
	Yes	194 (79.5%)	82 (68.9%)	112 (89.6%)	
Stress test conclusion:	Positive	8 (3.3%)	7 (5.9%)	1 (0.8%)	<0.001
	Negative	192 (78.7%)	80 (67.2%)	112 (89.6%)	
	Inconclusive	43 (17.6%)	31 (26.1%)	12 (9.6%)	
	Dubious	1 (0.4%)	1 (0.8%)	0 (0%)	
Effort tolerance:	Low	30 (12.3%)	23 (19.3%)	7 (5.6%)	<0.01
	Medium	119 (48.8%)	58 (48.7%)	61 (48.8%)	
	High	95 (38.9%)	38 (31.9%)	57 (45.6%)	
	Very high	0 (0%)	0 (0%)	0 (0%)	
	Very low	0 (0%)	0 (0%)	0 (0%)	

From a pathophysiologic point of view, the decrease of right ventricle (RV) dimension in association with reduction of pulmonary pressure index in both groups is important.

Regarding the dynamics of the kinetic changes of the heart in the post-infarction remodeling, the phenomenon of diminution of dyskinetic areas, akinetic areas and risk areas for aneurysm in both groups are important, that subsequently influences the EF. On the other hand, the sum of hypokinetic areas increased to an incremental value similar in both groups.

The positive heart changes developed during the post-infarctional period are followed by an improved tolerance to physical effort (tab. 5).

It is worth noting that the intensity of the maximum effort made by patients in group 2 was considerably higher compared to group 1: 111.200±2.244 vs 99.370±2.487 Wt ($p<0.001$). The total effort time also became significantly longer, with an average delay of 1.1 min: 9.448±0.205 vs 8.319±0.234 min ($p<0.001$). Was documented as well, a larger number of patients that reached the aimed HR: 89.6% vs 68.9%.

The cumulative benefit of physical endurance in patients at the end of 6-month period after revascularization of NSTEMI, was manifested with high physical tolerance at a much higher rate in the case of delayed angioplasty compared to angioplasty in the first 72 hours: 45.6% vs 31.9%.

Summarizing, delayed revascularization in NSTEMI patients with intermediate and low cardiovascular risk, applied at a 72 h- 30 day distance, proved at 6 months a morphofunctional heart remodeling comparable to the pattern of imminent revascularization applied in the first 72 hours, more than that, some changes are even more conclusive: (1) increase of EF, (2) decrease of LVSV and LVSD, (3) the rate of NYHA II patients, (4) maximal exercise intensity, total physical effort time, and rate of patients with high tolerance to physical effort.

Discussion

The crucial difference in addressing invasive treatment of NSTEMI patients with intermediate and low cardiovascular risk calculated by GRACE score (<140) was determined by the revascularization time: <72h and between 72h and 30 days (delayed revascularization). Results obtained at a distance of 6 months, on the restoration of ECHO indices and tolerance to physical effort, as a reflection of the heart's ability of a post-infarction remodeling, proved to be better in the case of postponed revascularization. This phenomenon reveals new perspectives in the application of angioplasty in this type of patients and may basically serve as an indispensable maneuver of time variable.

Several plausible hypotheses of exegesis and explanation of these benefits exist.

First of all, patients with NSTEMI had an intermediate and low cardiovascular risk according to GRACE score and the coronary stenoses >75% didn't exist. Likewise, the collateral system in the ischemic myocardium zone was quite well developed (the phenomenon of distal coronary artery recharge on coronary angiography was present in 65.7% of patients). Revascularization applied during the first 72 hours coincides with the peak of the inflammatory response, cytokine expression, blood cell infiltration, and due to this, the maximum activity of oxidative stress. Thus, the expected benefit of revascularization is compromised by the detrimental action of these factors. It is well known that the maximum expression of neutrophils in the area of myocardial necrosis occurs within 24-48 hours, while the expression of proinflammatory macrophages (M_1) is found at maximum proportion in the period 48-72 hours [3].

Second of all, delayed revascularization occurs during the period of natural restoration of the intrinsic antioxidant potential, after the ischemic impact, and the power of the oxygen paradox inherent to revascularization is thus depreciated, especially after 72 hours when the expression of anti-inflammatory macrophages (M_2) increases [4, 5].

Third of all, the traumatic impact of delayed revascularization occurs due to a longer and more consistent metabolic remodeling, its main elements being the expression of the growth transformation factor (TGF-β) induced by anti-inflammatory cytokines, which increases the intracellular expression of "heat-shock" proteins, recognized as factors in ensuring the resistance of cells to the action of various endogenous and exogenous lesions [6, 7].

Another important thing, in the patients of group 2 the ratio of atherosclerotic/thrombotic lesions of the "culprit" artery was higher than 1. In the group with revascularization <72 hours, on the contrary, this ratio was <1. Therefore, the prevalence of atherosclerotic paternal coronary lesion is a precondition for a better post-procedural evolution of the myocardial remodeling process compared to the predilection of thrombotic paternal. The latter has an increased risk of post-procedural thromboembolism and the phenomenon of 'low-reflow' or 'non-reflow' during reperfusion, inclusive in patients with MI and elevated ST segment [8, 9]. Deep embolization affects the subendocardial microcirculation and may in the context of «low-reflow» and «non-reflow» phenomena trigger the development of the sidereal and hibernated myocardium. This factor may possibly have conditioned higher LVESV values in patients with NSTEMI treated by revascularization <72 hours at a distance of 6 months.

Altogether, these arrangements may have the significance of beneficial preconditions for the cumulative impact of delayed myocardial revascularization in NSTEMI patients on post-infarction morphofunctional remodeling and clinical evolution.

Another element that should be noticed is that among the factors that determined the benefit of delayed revascularization is the presence of a lower rate of patients with total occlusion of the coronary artery in group of NSTEMI patients exposed to DMR: 39.7% vs. 48.7%. In the VERDICT trial, the rate of NSTEMI patients, in which the coronary angiography examination identified total occlusion, reached 26.6% [10].

Among the caliber trials in which delayed revascularization was applied, the ISAR-COOL study should be mentioned, which excelled in an average angioplasty time of 86 hours and showed benefits similar to our research.

In the ICTUS study, the analysis of the death rate of NSTEMI patients at a distance of 10 years, did not prove conclusive benefits for revascularization <72 hours [11].

Finally, the analysis of the obtained results appeals to some conceptually and practically significant hypotheses:

• Delayed revascularization of patients with intermediate and low risk NSTEMI caused by total coronary occlusion would have a similar benefit to revascularization <72 hours, regarding post-infarction morphofunctional remodeling of the myocardium.

• In case of delayed revascularization of NSTEMI patients with intermediate and low cardiovascular risk, caused by stenosis <50%, the superiority of efficacy over revascularization <72 hours is plausible. In this context, the prompt and accurate diagnosis by use of instrumental markers (e.g. Magnetic resonance imaging) is important. Likewise, it is opportune the use of veritable circulatory markers of coronary microcirculation disorder responsible for subendocardial infarction, especially when cardiac troponins (TnT and TnI) dynamics and ECG outcomes are uncertain. Our experience based on applying of multi-marker strategy validates the predictive value of the markers which refer to the inflammatory response, oxidative stress, endothelial dysfunction and prothrombotic status.

Conclusions

1. In our study, delayed myocardial revascularization (72 hours - 30 days) applied for treatment of NSTEMI patients with intermediate and low cardiovascular risk, had proved the superior benefits in concern to clinical-functional evolution at a 6 months follow-up period compared to revascularization <72 hours.

2. The most important traits of delayed myocardial revascularization benefit are: (i) greater increase of EF, (ii) decrease in both LV end systolic volume and LV systolic diameter, (iii) increase in the number of patients with NYHA II and the reduction of NYHA III cases, as well as physical endurance boosting, manifested by double more patients, who fulfilled maximal physical endeavor.

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Authors' contributions

AS described the material and methods; LC presented introduction. IM wrote the abstract; IP exposed results; VC depicted discussion and corrected the text of the manuscript; MP initiated the idea of this research and revised the manuscript. All the authors revised and approved the final version of the manuscript.

Funding

This study was supported by Institute of Cardiology and Nicolae Testemitanu State University of Medicine and Pharmacy. The trial was the authors' initiative. 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 study was approved by the Research Ethics Committee of the Institute of Cardiology, protocol No 04 of March 03, 2020. The informed consent was received from every patient.

Conflict of Interests

No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.64-2.21.06
UDC: 616.65-002.2-079.4

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Possible differential diagnosis of various chronic nonbacterial prostatites

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Manuscript received November 06, 2020; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: The purpose of the study was to diagnose possible chronic nonbacterial prostatitis (CNP) and chronic pelvic pain syndrome (CPPS) among patients, as well as differentiate between the inflammatory (category IIIA) or non-inflammatory (category IIIB) types in selecting and optimizing differential drug treatment of this category of patients.
Material and methods: The study was conducted on 43 patients diagnosed with CNP/CPPS. The control group included 10 healthy men. Both the production of nitric oxides (NO) by phagocytes, as well as prostate secretion and ejaculate were determined according to the procedure described by Metelyskaya B.A., which was modified by Gudumac V, et al.
Results: There was a 39.0% ($p < 0.05$) decrease in NO production by induced NO-synthase (iNOS), determined in the blood of 11 patients (from the main group – 2) with CNP/CPPS and a 115% ($p < 0.05$) increase was determined in 32 patients (from the main group 1) if compared to the same indices in the control group. The prostatic secretion and ejaculate showed a higher macrophage iNOS activity by 80% ($p < 0.05$) and 75% ($p < 0.05$) if compared to the same parameters from the control group. The iNOS activity in prostatic fluid and split-ejaculate fractions from the main group – 2 did not differ from that of the control group.
Conclusions: The assessment of NO production, prostate secretion and ejaculate allows to somewhat establish the main diagnosis of CNP and category III types (A – inflammatory and B – non-inflammatory prostatitis), which will significantly contribute to the optimization and selection of an appropriate differential treatment based on the drug action mechanisms.
Key words: chronic nonbacterial prostatitis, nitric oxide, chronic painful pelvic syndrome, drugs.

Cite this article
Colta A. Possible differential diagnosis of various chronic nonbacterial prostatites. *Mold Med J.* 2021;64(2):33-36. https://doi.org/10.52418/moldovan-med-j.64-2.21.06.

Introduction

Chronic prostatitis is one of the most common and most difficult to diagnose andro-urological disease that is referred to polyetiological disorders. About 5-10% of chronic prostatitis cases prove to have a bacterial origin. The other 90-95% of cases are classified as "chronic nonbacterial prostatitis" unless the laboratory findings detect a bacterial cause (origin) [1]. Currently, it is also named as "chronic painful pelvic syndrome" [2].

According to the classification of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), chronic nonbacterial prostatitis / chronic pelvic pain syndrome (CNP/CPPS) is a category III prostatitis. The US National Institutes of Health classified and highlighted two types of chronic nonbacterial prostatitis / chronic pelvic pain syndrome (CNP/CPPS): inflammatory (IIIa) and non-inflammatory (IIIb) types [3].

The purpose of the study is to diagnose possible CNP/CPPS among patients, as well as differentiate between the inflammatory (category IIIA) or non-inflammatory (category IIIB) types in selecting and optimizing differential drug treatment of this category of patients.

Material and methods

The study was carried on 53 men, of which 43 patients were diagnosed with CNP/CPPS, aged between 27 to 70 years (the mean age – 48.5 years), whereas the disease duration ranged from 5 to 14 years. The control group included 10 healthy men.

The diagnosis was established based on clinical data, anamnesis, digital rectal examination (DRE) and the Meares-Stamey four-glass test. In order to assess the symptoms of chronic prostatitis and its impact on patient quality of life, there were used the USA National Institutes of Health and the International Prostate Symptom Score (IPSS) systems, as well as the urine cytology, the urethral swab via the polymerase chain reaction, and the blood test on prostate-specific antigen; if required – urodynamic examination, cystoscopy, etc.

The serum nitric oxides (NO) content in phagocytic leukocytes, prostatic fluid and ejaculate were assayed by Griess reaction with diazotized sulphanilic acid (NO is a short-term molecule, which converts rapidly into nitrites, being determined via this reaction). The leukocyte suspension, separated from the blood, the prostate secretion

and ejaculate fraction were mixed with the substrate to obtain NO (to arginine) in saline solution, being incubated at 37°C over 24 hours; afterwards the Griess – Ilosvay (N-naphthylethylenediamine with sulphanilic acid) reagent was added to the studied sample. Over 15 minutes after pink discoloration, the photometry with a wavelength of 540 nm was performed according to the procedure described by Metelyskaya B.A., being modified by Gudumac V.S. et al. [4-6].

The data analysis was performed via statistical software SPSS-10 IBM Statistics for Windows, version 20 Microsoft Excel 2010, by using descriptive, variation, and correlational assessment methods. The quantitative parameters were determined by the mean value and the standard error value, whereas the t-Student criterion was used to estimate the statistical differences between the means of the two groups. The p-values below 0.05 (P <0.05) were considered statistically significant.

Results and discussion

The assessment of the obtained results and the level of NO production by serum phagocytic leukocytes in prostate secretion and ejaculate showed that 32 patients with CNP/CPPS (baseline group 1) had an increase of serum NO production by 115% (p <0.05), an increase of NO – macrophage synthase activity by 80% (p <0.05) in prostate secretion and by 75% (p <0.05) in ejaculate, compared to similar indices of patients from the control group (healthy men) (tab. 1).

Table 1. iNOS-induced NO production in blood, prostate secretion and spermoplasm of patients with chronic pelvic pain syndrome (M±m, n = 43 patients)

Study groups, n – patients	NO production		
	Blood (μM/L)	Prostate secretion (μM/gram of protein)	Spermoplasm (μM/gram of protein)
Baseline group – 1 (n=32 patients)	56.97 ± 0.94 **	7.58 ± 0.32 **	7.49 ± 0.42 **
Baseline group – 2 (n=11 patients)	16.1 ± 0.54*	3.8 ± 0.82	3.9 ± 0.88
Control group (n=10 healthy men)	26.40 ± 0.25	4.21 ± 0.54	4.28 ± 1.2

Note: * – the value significance compared to the control group (p <0.05); ** – the significant value differences of the patients from the baseline study group (1 and 2) (p <0.05)

The iNOS-induced blood NO production decreased by 39.0% (p <0.05) in 11 patients (from baseline group-2) with CNP / CPPS, compared to the same parameter in the control study group (tab.1), whereas the iNOS activity in the prostate secretion and split ejaculate fraction for the same condition (CNP/CPPS) did not differ compared to the control group.

Therefore, a 3.5-fold increase (p <0.05) of NO production by NOS macrophage in the blood, in the prostate secretion and ejaculate in patients from the baseline group 1 (n = 32), compared to the 11 patients from the baseline group 2, shows the presence of an inflammatory response in the prostate of most patients with CNP / CPPS IIIA, and the non-inflammatory type of CNP/CPPS IIIB in 11 patients (from baseline group 2).

The assessment of the patients' overall condition determined the intensity of the pain syndrome in patients with CNP/CPPS IIIA (inflammatory CPPS type, n = 32) based on NIH-CPSI scale, which made up 10.84 ± 1.28 points, the urinary incontinence scored 9.64 ± 1,15 points, and the quality of life index was 11.00 ± 0.91 points. Patients with CPPS IIIB (non-inflammatory CPPS type, n = 11) had the following indices: 10.9 ± 1.1; 9.3 ± 1.0 and 10.9 ± 0.99, respectively. According to the IPSS scale, the urinary symptoms index was 13.44 ± 3.91 for CPPS IIIA (32 patients), and 14.1 ± 3.2 for IIIB (11 patients).

Based on the recent scientific research results [6-11], it has been established that chronic nonbacterial prostatitis refers to diseases that develop on the underlying disorders of proteolytic processes in the blood and prostate [8-13]. The mutual action of proteases and their inhibitors maintains the homeostasis within the body, whereas the successive complex and multi-component reactions are categorized as universal non-specific response to inflammation.

Shangichev A.V. et al. [11] stated that the disruption of the bioregulatory mechanisms of the body's main proteolytic systems, namely the kallikrein-kinin, is a major causative factor of CNP/CPPS [11]. The kallikrein-kinin system (SKK) plays a critical role in the pathophysiology of hyperalgesia and inflammatory diseases, as well as in the regulation of proteolytic cascade systems of blood plasma, kininogenesis, hemocoagulation, fibrinolysis, complement and renin-angiotensin system, which provide adaptation and protection of the body, particularly under stress condition. Kallikrein is a multifunctional proteinase that controls various biological processes, including converting the protein precursor kininogen into bradykinin that is a pain and inflammatory mediator [14-16]. Following a study analysis regarding the body's proteolotic systems that might induce an inflammatory response in the prostate, as well as on the markers of inflammation in blood and prostate secretion in various types of CNP/CPPS, Chernogubova I. A. [8] concluded that the inflammatory CNP / CPPS type, besides its subjective-objective inflammatory signs, such as pain and leukocytosis of prostate secretion, might be confirmed based on the status of proteolytic processes in prostate secretion, thus attesting an active inflammatory response in the prostate, being clinically manifested via the chronic pain syndrome. However, other particularities were found in patients with non-inflammatory types of CNP/CPPS. The lack of laboratory evidences of an active inflammatory response provides reason to assume that prostate inflammation initially "triggers" the pain syndrome occurrence, which although the inflammatory response subsides, the pain syndrome already persists due to some other mechanisms, including proteolytic activation of the blood. Thus, the non-inflammatory CNP/CPPS type, though clinically being a predominantly local

prostate disease, should be referred as a disorder associated with systemic pathogenetic mechanisms. According to the author, the metabolic pathways during an inflammatory response in chronic nonbacterial prostatitis are impaired due to the underlying imbalance in the proteinase-inhibitor system, uncontrolled proteolytic activity in the prostate and a weakened body's natural resistance, being the major causative factor of CNP/ CPPS.

Therefore, a significant interest to the pathogenesis and diagnosis of CNP/CPPS is shown to determining the superoxide dismutase activity (SOD) and catalase (CT) in the blood, prostatic secretion and ejaculate, as well as the NO production and its level in this category of patients, which might serve as biochemical markers in the development of an inflammatory response, being used to monitor the treatment effectiveness in patients with CNP/CPPS III.

The present research findings reported NO as a biochemical marker of inflammatory prostatitis in CNP / CPPS IIIA, by increasing the NO production in blood, in prostate secretion and ejaculate fraction. The high NO activity is likely to lead to the accumulation of superoxide and peroxynitrite in blood, prostatic secretion and ejaculate in case of CNP/CPPS IIIA. Excessive NO production in CNP/ CPPS is likely to increase the vessel wall permeability and thus leading to the impairment of the hematoprostatic and hematotesticular barrier gradient [17-19].

It is well known that the treatment of chronic prostatitis (CP) is an extremely challenging task. It depends on the patient's category and symptoms. Most patients present obstructive infravesical phenomena, sometimes being associated with irritative ones. There are several complex treatment approaches, which inevitably act on the etiology and pathogenesis of the disease. The use of new biologically active substances seem to offer great perspectives due to their complex immunostimulatory and antioxidant action. Recently, there have appeared a wide range of such drug classes (cytomedins and polypeptides, showing a systemic delivery and oriented effect (prostatotrope)). This treatment has its own peculiarities, depending on the patient's age and overall condition (immunity and mental status), presence and types of clinical manifestations, evolutionary peculiarities, stages of CP and the level of the adjacent organ involvement.

However, the obtained study results will contribute to an appropriate treatment selection, based on the drug action mechanisms and on the types of the underlying condition. Currently, these treatment schemes are subjected to considerable updating, including entomotherapy by using specific preparations (adenoprosine, imupurine, etc.) that exhibit anti-inflammatory, immunomodulatory and antioxidant properties [20-25].

Conclusions

The NO production and its level in blood, prostatic secretion and ejaculate allows establishing the basic diagnosis of CNP , as well as determining category III (A- inflammatory and B- non-inflammatory types) prostatitis with its own characteristics, thus significantly optimizing the treatment due to an appropriate and differentiated selection of drugs depending on their action mechanism for patients with CNP/CPPS.

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Author's contribution
AC conceptualized the idea, conducted literature review, wrote the manuscript, revised and finalized the text.

Funding
The trial was the author's initiative. The author is independent and takes 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 65, 12.04.2017).

Conflict of Interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.64-2.21.07
UDC: 616.611-002-053.2

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The role of cell signaling molecules in the pathogenesis of glomerulonephritis in children

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Manuscript received August 01, 2020; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: Cytokines are functional class of tiny proteins and glycoprotein and fundamentally they are monomers that function as soluble mediators in an autocrine or paracrine manner. Cytokines are produced by a number of cell types, predominantly leukocytes, and their targets implicate both immune and non-immune cells.
Material and methods: This study was performed on 75 children with glomerulonephritis (GN), aged from 2 up to 17 years. There were 20 children with steroid-sensitive nephrotic syndrome (SSNS), 15 children with steroid-resistant nephrotic syndrome (SRNS), 20 children with chronic glomerulonephritis (CGN) nephrotic form and 20 children with CGN mixed form. This study was performed on patients experiencing disease relapse and clinical remission. The control group consisted of 20 healthy children.
Results: The results of this study demonstrated increased levels of cell signaling molecules (IL-8, TNF-α, MCP-1, MIP-1α) in the urine during clinical manifestations, valuable result due to their major role in the immunopathogenic mechanism of proteinuria in nephrotic syndrome.
Conclusions: Determination of urinary concentrations of cellular signaling molecules may be useful as a predictive non-invasive method for estimating disease activity, monitoring disease progression, differentiating steroid-sensitive nephrotic syndrome from steroid-resistant nephrotic syndrome, and assessing the effectiveness of treatment in children with different variants of GN.
Key words: cytokine, chemokine, nephrotic syndrome, glomerulonephritis.

Cite this article
Ciuntu A. The role of cell signaling molecules in the pathogenesis of glomerulonephritis in children. *Mold Med J.* 2021;64(2):37-41. https://doi.org/10.52418/moldovan-med-j.64-2.21.07.

Introduction

Nephrotic syndrome (NS) is one of the most commonly encountered evidence of glomerular damage in children. It is characterized by a prevalence of approximately 16 cases per 100.000 children aged under 16 [1]. The reported annual incidence in children varies between 1.2 and 3.5 per 100.000 per year in Western Europe [2-4], 4.7 per 100.000 per year worldwide [5] and up to 6.5 per 100.000 per year in Japan [6]. NS is characterized by the triad of heavy proteinuria, hypoalbuminemia (≤2.5 g/ dL), and generalized edema. The pathophysiological mechanisms of idiopathic nephrotic syndrome (INS) support an underlying role for the immune system [7, 8]. This hypothesis relies on the fact that the main treatment is based on corticosteroids and studies show different inflammatory profiles in patients with INS [9].

Idiopathic NS has been considered "a disorder of T-cell function" mediated by a circulating factor that alters podocyte function resulting in massive proteinuria for the last four decades.

The implicated role of circulatory factors released from T-cells has long been postulated in the pathophysiology of NS; however, a single presumptive factor has not been defined yet [10].

Cytokines are functional class of tiny proteins and glycoprotein (molecular weights of approx. 8–80 kDa) and fundamentally they are monomers that function as soluble mediators in an autocrine or paracrine manner. Cytokines are produced by a number of cell types, predominantly leukocytes, and their targets implicate both immune and non-immune cells [11]. Also known as CXCL8, IL-8 is one of the most widely studied chemokines and is a critical inflammatory mediator. The main role of IL-8 in inflammation is in the recruitment of neutrophils, although it is also responsible for the chemotactic migration and activation of monocytes, lymphocytes, basophils, and eosinophils at sites of inflammation [12].

Tumor necrosis factor (TNF), a pleiotrophic cytokine, that is produced primarily by immune cells, such as macrophages, dendritic cells, and T lymphocytes and is implicated in immune regulation [13]. TNF exerts its biological responses via interaction with two cell surface receptors: TNFR1 and TNFR2. (TNFRs) [14]. TNF is implicated

in many systemic inflammatory diseases as well as kidney diseases [15]. TNF-α affects the expression of nephrin and podocyte cytoskeletal rearrangement [16].

Macrophage inflammatory protein (MIP)-1α/CCL3 is an inflammatory chemokine produced by cells during infection or inflammation. It belongs to the CC chemokine family, which displays potent chemotactic properties. Their findings suggested that the MIP-1 protein was composed of two peptides. Partial sequencing revealed two proteins: MIP-1α and MIP-1β. MIP-1α/CCL3 is crucial for the recruitment of macrophages and T lymphocytes from the circulation to sites of infection or injury; thus, it orchestrates acute and chronic inflammatory host responses [17].

Monocyte chemoattractant protein-1 (CCL2/MCP-1) is a chemokine that mediates renal interstitial inflammation, tubular atrophy, and interstitial fibrosis by recruiting monocytes-macrophages into renal interstitium [18]. Within the glomeruli there is MCP-1 overexpression in both crescent GN and nephrotic conditions [19].

There are no good large-scale empirical evidence and complex studies regarding changes in cell signaling molecules in various clinically evolving variants of glomerulonephritis in children.

The aim of the study was to evaluate urinary concentration of cellular signaling molecules in children with glomerulonephritis with different clinical-evolutionary stages of the disease.

Material and methods

The prospective study was conducted in *Nicolae Testemitanu* State University of Medicine and Pharmacy, Department of Pediatrics, Biochemistry Laboratory and Institute of Mother and Child, Nephrology Unit.

It is based on biological samples collected according to the principles of contemporary research, approved by the Ethics Committee of Research of *Nicolae Testemitanu* State University of Medicine and Pharmacy (favorable review of 13.05.2015, official record No 55).

This study was performed on 75 children with glomerulonephritis, aged from 2 up to 17 years. There were 20 children with steroid-sensitive nephrotic syndrome (SSNS), 15 children with steroid-resistant nephrotic syndrome (SRNS), 20 children with chronic glomerulonephritis (CGN) nephrotic form and 20 children with CGN mixed form. At the first presentation, all patients received oral prednisone at dose of 2 mg/kg/day for 6-8 weeks. Diagnostic criteria for NS were based on the KDIGO Clinical Practice Guidelines for Glomerulonephritis [20]. This study was performed on patients experiencing disease relapse and during clinical remission. Inclusion criteria were: children aged 2-17 years, with primary nephrotic syndrome, CGN nephrotic and mixed form, endogenous creatinine clearance> 60 ml / min / 1.73m^2, and exclusion criteria: patients with congenital NS, secondary NS, endogenous creatinine clearance <60 ml / min / 1.73m^2, cardiovascular, hepatic, metabolic comorbidities, acute injury of the internal organs at the time of study initiation. The control group consisted of 20 healthy, sex- and age-matched subjects.

Quantification of cytokines was performed by sandwich ELISA method. To assess the significant difference of performed indices were used statistical methods which appreciated arithmetic average size [X], average squared deviation, error of median arithmetic average size [± m]. It has also been used nonparametric statistical test "Mann-Whitney U" and the selected level of statistical significance was p <0.05 (StatsDirect statistical software, version 1.9.5, 2001).

Results

The mean value of age in NS (6.52±0.66 years old), CGN nephrotic form (8.9±0.67 years old) and CGN mixt form (10.1±0.99 years old). In the onset of the disease, clinical features were determined by anasarca - (60.0 ± 9.65%), edema - (40.0 ± 7.9%), rare urination - (48. 75 ± 8.7%), headache - (22.5 ± 5.92%), vomiting - (15.0 ± 4.84%), abdominal pain syndrome - (8.75 ± 3.7%), pain in the lumbar region - (10.0 ± 3.95%). Evaluation of blood biochemical markers showed hypoproteinemia and hyperlipidemia in all subgroups of patients with NS (tab. 1).

Table 1. Laboratory findings of patients with idiopathic nephrotic syndrome according to the therapeutic response to steroids

Biochemical parameters	SSNS	SRNS
Total protein, g/l	53.3±1.27	51.13±1.49
Albumine,%	35.72±3.56	20.76±2.63
Urea, mmol/l	6.84±0.80	5.12±0.48
Creatinine, mmol/l	0.064±0.006	0.054±0.037
Total lipids, g/l	9.20±1.13	12.56±1.49
Cholesterol, mmol/l	7.43±0.34	10.05±0.95
β-lipoproteins, u.c.	95.07±3.64	107.23±6.88
Protrombin, %	92.95±1.096	92.50±1.71
Fibrinogen, g/l	4.59±0.38	6.69±1.88
24-Hour Urine Collection- proteine, g/l	3.79±0.66	4.55±0.99

In table 2 are reflected results of the evaluation of urinary interleukins in patients with different forms of GN by estimating the level of IL-8 and TNF-α.

Thus, in the onset period, in patients with SRNS we found an increased level of IL-8 in urine that exceeded 9.5 times, and 4.7 times in SSNS compared to the control values. During remission, the level of IL-8 in the urine remained increased in all clinical variants of GN, compared to the control group.

Maintenance of high levels of IL-8 during remission in our research could be explained by decreased urinary clearance or slow degradation of this cytokine and persistence of the pathological process in the kidneys.

We obtained an increased TNF-α in the urine samples of all patients. The level of urinary TNF-α was more increased in the SRNS in the onset period, compared to the SSNS.

Table 2. Urinary interleukin levels in children with glomerulonephritis (pg / mM creatinine)

Study groups	IL-8		TNF-α	
	relapse	remission	relapse	remission
SSNS	130.7±14.28*** 465.1%	86.0±8.94*** 306.04%, p_1<0.05	95.8±9.85*** 287.7%	59.9±7.37** 179.9%, p_1<0.05
SRNS	267.3±21.87*** 951.2%, p_2<0.001	-	135.2±7.83*** 406.0%, p_2<0.01	82.3±10.34 247.1%, p_1<0.01
CGN nephrotic form	291.0±25.73*** 1035.6%	102.8±5.47*** 365.8%, p_1<0.001	62.3±5.72*** 187.1%	22.1±0.78 66.4%, p_1<0.001
Control group	28.1±3.71		33.3±2.91	

Note: statistically significant difference compared to control group values: * P <0.05; ** P <0.01; *** P <0.001.

p_1 - authenticity compared with the same index registered under relapse. p_2 - authenticity in comparison between SSNS and SRNS.

Thus, the level of urinary TNF-α increased 4.1 times in the SRNS and 2.9 times in the SSNS, compared to the control group, and during the remission the level of TNF-α in the urine, although decreased slightly, remains at increased values in these patients groups.

In CGN nephrotic form, the relapse period, there were also increased values of TNF-α that exceeded almost 2 times the control level. During the remission period, this parameter decreases, being by 33.6% lower than the values of the control group, but without statistical relevance.

We purpose to elucidate the disturbances in the cellular signaling system, so we evaluated the dynamics of urinary chemokines in patients with GN at different clinical-evolutionary stages of the disease (Table 3). The obtained results indicate a statistically authentic increase in the level of urinary MCP-1 in all groups of patients, compared to the control group, the highest values being recorded in children with CGN nephrotic and mixed form (Table 3).

The concentration of urinary MCP-1 in the groups of patients with SSNS and SRNS, during the clinical manifestations and remission period exceeded 2.0 – 2.4 times the values of the control group. It should be noted that the group of patients with SRNS showed higher mean values of MCP-1 in the urine compared to those in the group with SSNS. In the groups of patients with CGN nephrotic and mixed form during exacerbation, the concentration of MCP-1 in the urine increased 3.3 times and 8.5 times, respectively, compared to the control group. During remission in groups of patients with CGN, the concentration of urinary MCP-1 remains increased, which indicates the persistence of the chronic renal damage and the lack of a complete immunobiochemical recovery.

We found significantly higher levels of urinary MIP-1α in all groups of patients during the period of relapse compared to the period of remission. The level of urinary MIP-1α increased 2.7 times in the group of patients with SRNS, compared to SSNS during the clinical manifestations. In the group of patients with CGN nephrotic form, in the relapse period, the concentration of MIP-1α in urine exceeded 4.9 times the level of this index recorded in patients with SSNS and 1.8 times in patients with SRNS.

Discussion

Our study revealed the clinical-diagnostic importance of assessing the concentration of cell signaling molecules in urine for the early diagnosis of renal impairment in children with GN, noting a significant increase in the concentration in their urinary samples. Furthermore, we obtained signifi-

Table 3. Urine chemokine levels of MCP-1 and MIP-1α in children with glomerulonephritis

Study groups	MCP-1(pg/mM creatinine)		MIP-1α (ng/mM creatinine)	
	Relapse	Remission	Relapse	Remission
SSNS	96.99±7.63*** 190.4%	112.13±6.88** 220.2%	0.48±0.08 p_3<0.001	0.16±0.017 p_1<0.05
SRNS	108.02±8.78** 212.1%	121.88±7.26** 239.3%	1.28±0.09 p_2<0.01; p_3<0.01	0.30±0.080 p_1<0.01
CGN nephrotic form	170.32±12.01*** 334.4%	93.21±8.43** 183.01%, p_1<0.01	2.36±0.21	0.50±0.059 p_1<0.001
CGN mixt form	430.76±49.73 845.8%	122.49±15.97* 240.5%, p_1<0.01	7.58±2.72	-
Control group	50.93±3.79		0.0	

Note: statistically significant difference in relation to the values of the control group: * p <0.05; ** p <0.01; *** p <0.001. p_1 – authenticity in comparison with the respective index registered in relapse; p_2 – authenticity when comparing SSNS with SRNS; p_3 – authenticity when comparing CGN nephrotic form with SSNS and SRNS.

cantly increased levels of IL-8 in the urine in all groups of patients during the relapse period, compared to the remission period.

Previous studies had reported the relation between CXCL8/IL8 and disease activity and linked high urinary CXCL8/IL8 levels to proteinurea [21].

According to the study [22] urinary CXCL8/IL8 levels were significantly higher in SRNS than SSNS patients during activity and remission with no significant difference on comparing minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) patients.

According to Souto M. et al. study, they found similar results, increased levels of urinary IL8/CXCL8 in relapsed steroid resistant children when compared to steroid sensitive patients in remission, with a positive correlation with urinary protein levels. These findings suggest that the renal release of the chemokine IL8/CXCL8 might be associated with changes in glomerular permeability [23].

IL-8 was shown to induce changes in the permeability of the glomerular basement membrane (GBM) via decreasing the synthesis of heparan sulfate proteoglycans, which eventually induced proteinuria in rats [24]. The study of Al-Eisa AA et al. detected increased urinary levels of IL-1β, IL-6 and IL-8 in INS patients during relapse which disappeared, except IL-8, during remission of the disease. These findings support the assumption of the important role of these cytokines in the immune process during a relapse [25].

A pediatric study used the TNF pathway in the recurrence of focal segmental glomerulosclerosis (FSGS) and showed improvement in proteinuria after TNF antibodies therapy [26].

During the precocious phases of inflammation, TNF-α may play as a key medium to activate the downstream factors expression, including γ-IFN, IL-1, IL-2, IL-4 and IL-8 and thus start a cascade reaction, which then promotes the nephrotic syndrome return by affecting glucocorticoid response [27].

The study of Besbas N. et al. suggests that increased urinary excretion of MCP-1 in the patients with FSGS is most likely due to enhanced production of MCP-1 in kidney, presumably induced by excessive exposure to plasma proteins filtered from the damaged glomeruli. They also showed that urinary MCP-1 levels correlated with the degree of proteinuria in FSGS patients [18].

Vianna and co-workers found higher urinary levels of MCP-1/CCL2 in patients with chronic kidney disease (CKD) due to FSGS than in cases of congenital uropathies [28]. Additionally, patients with glomerular disease had higher MCP-1 as compared with non-glomerular disease patients [28]. More recently, Matsumoto Y. et al. showed that urinary level of MCP-1/CCL2 was significantly higher in steroid-resistant INS than in steroid-sensitive patients, supporting the idea that urinary MCP-1/CCL2 might contribute to the recruitment of macrophages into glomeruli [29].

Alzawa T. et al. in their study revealed that urinary concentrations of MCP-1 is a significant positive correlation with the degree of occult blood in urine and a significant inverse correlation with the estimated glomerular filtration rate. Furthermore, the urinary CCL2/MCP-1 concentration was significantly correlated with histological chronicity indices in patients with lupus nephritis and IgA nephropathy, supporting the hypothesis that the measurement of this chemokine may be useful as a noninvasive method for predicting the disease activity of glomerulonephritis in children [30].

The study of Ikezumi Y. et al. reported that macrophages play an important role in the pathogenesis of SRNS, showing a significant increase in glomerular macrophage accumulation in biopsies from children with SRNS compared with those with SSNS. These results suggest that MCP-1 could be involved in the mechanism for steroid-resistance via recruitment of monocytes/macrophages into kidney tissue [31].

According to the study of Furuichi K. et al. urinary levels of MIP-1α / CCL3 in patients with crescentric glomerulonephritis correlate with the percentage of cell crescents and the number of CD68-positive infiltration cells and CCR1- and CCR5-positive cells in glomeruli. Increased expression of MIP-1α, MIP-1ß and MCP-1 in glomeruli with cellular and fibrocellular crescents suggests that these chemokines may be involved in crescent progression additionally with macrophage recruitment. MIP-1α can specifically promote and generate glomerular infiltration with macrophages in the acute process leading to the formation of glomerular crescents [32].

Conclusions

Quantifying of urinary concentrations of cellular signaling molecules may be useful as a predictive non-invasive method for estimating disease activity, monitoring disease progression, differentiating steroid-sensitive nephrotic syndrome from steroid- resistant nephrotic syndrome, and assessing the effectiveness of treatment in children with different variants of glomerulonephritis.

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Author's contribution
AC conceptualized the idea, conducted literature review, wrote the manuscript, revised and finalized the text.

Funding
The study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy and Institute of Mother and Child. The author is independent and takes responsibility for the integrity of the data and accuracy of the data analysis.

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 55 of May 13, 2015).

Conflict of Interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.64-2.21.08
UDC: 616-089.843:611.018(478)

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A survey of public knowledge and attitude towards tissue, cell donation and transplantation in the Republic of Moldova

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Manuscript received July 27, 2020; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: Numerous studies focusing on public attitude towards organ donation have been performed at the international level, but very few highlight public attitude towards tissue and cell donation and transplantation.
Material and methods: We conducted a survey among a representative sample of the general adult population (N=427). The questionnaire was developed on the basis of previous study and included reuse of some items from the earlier survey to facilitate historical comparison. The analysis of the data was carried out using SPSS, 2011 and focused on descriptive statistics.
Results: The vast majority of our respondents (81.0%) agreed with the tissue and cell donation and only every 10th respondent did not accept this. The fewer respondents (68.1%) would agree to have tissues and cells transplanted from other people compared to those who were willing to donate tissues and cells (81.0%). Finally, most of the respondents (85.0%) expressed interest in receiving more information, and were very similar to those in receiving more information on organ donation and transplantation (88.0%)
Conclusions: The study revealed the respondents' positive attitude towards tissue and cell donation and transplantation and demonstrated a remarkable growth in the public positive attitude towards donation. Furthermore, as the vast majority of our respondents wanted to receive more information on these issues, it seems to be a clear opportunity to develop educational and promotional strategies to improve awareness and enhance donation rates in our country.
Key words: donation, transplantation, tissue, cell, public attitude, public knowledge.

Cite this article
Timbalari T, Lozan O, Codreanu I, Nacu V. A survey of public knowledge and attitude towards tissue, cell donation and transplantation in the Republic of Moldova. *Mold Med J.* 2021;64(2):42-48. https://doi.org/10.52418/moldovan-med-j.64-2.21.08.

Introduction

Discussions on how best to increase tissue and cell stocks often focus on the issue of donor motivation: in particular, how people can best be encouraged to donate different types of human material [1-3]. It is essential to refer to the Convention on Human Rights and Biomedicine, which, in Article 21, clearly states that "*The human body and its parts shall not, as such, give rise to financial gain*" [4].

A well-known fact is that all donation and transplantation programmes are dependent upon the willingness and voluntary commitment to donate organs, tissues and cells from donors to continue their activity [5-9]. So, it is important that public confidence is maintained by standards of good practice [1, 10].

According to the WHO successful donation and transplantation programs should include at least appropriate public awareness strategies, which promote not only organ donation but also tissue and cell donation [2, 10]. One of these programs is in Spain, named as the Spanish Model of Donation and Transplantation, and today it is recommended as the gold standard worldwide, prioritizing the development of logistics starting with the identification of the donor until the removal of organs, tissues and cells [11, 12].

Numerous studies focusing on public attitude towards organ donation have been performed at the international level, but very few surveys highlight public attitude towards tissue and cell donation and transplantation [13-16].

The purpose of the survey in the Republic of Moldova among a representative sample of the general adult population was to assess the knowledge and attitude towards tissue and cell donation and transplantation to identify the factors that may increase the willingness to donate.

Material and methods

The survey was performed to determine the public knowledge and attitude towards tissue and cell donation and transplantation during the five consecutive working days by the initially trained staff. A total of 450 participants from adult population were randomly selected to complete anonymously the specially developed questionnaire, consisting

of 15 questions. A total of 427 valid questionnaires were obtained. The questionnaire was grouped into compartments according to the information collected. The respondents were asked to carefully choose the answer option considered the closest from their personal point of view. In order to facilitate processing of the results, as well as to avoid the indecision of the respondents, we preferred to eliminate the intermediate or neutral values from the assessment scale. The study protocol was approved by the Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy, Chisinau (No 2, 27.10.2016).

The self-administered questionnaire was voluntary completed by 246 (57.6%) women and 181 (42.4%) men. Depending on age, the respondents were distributed as follows: 156 (36.5%) persons were aged within 18 – 34 years, 145 (34.0%) persons – within 35 – 54 years, 126 (29.5%) persons – within 55 – 82 years. The average age of the respondents was 42.38 ± 0.7 years. The average age was statistically significantly higher in men – 44.75 ± 1.1 years, compared with women – 40.64 ± 1.0 years ($p<0.01$).

Statistical processing of the material was based on the special files elaborated where the primary data were coded – the results of the questionnaires. The analysis of the data was computerized using the software "Statistical Package for the Social Science" version 20.0 for Windows (SPSS, Inc., Chicago, IL, 2011). A multivariate analysis using logistic regression was carried out to find the social determinants of supporting donation and transplantation and information available to them. The results are based on descriptive and inferential statistics.

Results

The general adult population. The study population included statistically significant more women under the age of 35 and more men aged within 35 – 54 years ($p<0.05$).

Depending on the educational level, 131 (30.7%) respondents had university education, 114 (26.7%) respondents had high school or college education, 103 (24.1%) respondents had general or vocational education and 79 (18.5%) respondents had incomplete secondary education (fig. 1).

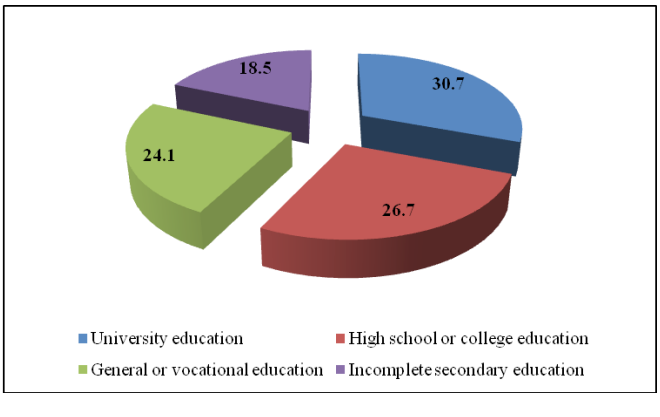

Fig. 1. Distribution of the respondents by their educational level (%)

In accordance with the occupational level, our respondents were distributed as follows: 163 (38.2%) persons were registered as employees, 30 (7.0%) persons were employed as private entrepreneurs, 80 (18.7%) persons were temporarily unemployed and 154 (36.1%) persons did not work (tab. 1).

Table 1. Distribution of the respondents by gender and occupation

Gender	Occupation							
	Employee		Unemployed		Temporarily unemployed		Private entrepreneur	
	abs.	%	abs.	%	abs.	%	abs.	%
Female	97	39.4	88	35.8	46	18.7	15	6.1
Male	66	36.5	66	36.5	34	18.8	15	8.3
Total	163	38.2	154	36.1	80	18.7	30	7.0

More than half of the respondents surveyed – 237 (55.5%) – came from urban areas and 190 (44.5%) respondents came from rural areas.

The survey showed that the respondents from the general adult population were presented by urban women aged 18-34, with university education and were registered as employees.

Attitude towards tissues and cells donation and transplantation. The respondents' attitude towards tissue and cell donation and transplantation has been analysed. We found that the vast majority of our respondents – 346 (81.0%) agreed with tissue and cell donation and only 40 (9.4%) respondents did not accept it, 36 (8.4%) respondents did not know and 5 (1.2%) respondents did not respond (fig. 2). Statistically significant differences in those opinions depending on gender were not found ($p>0.05$).

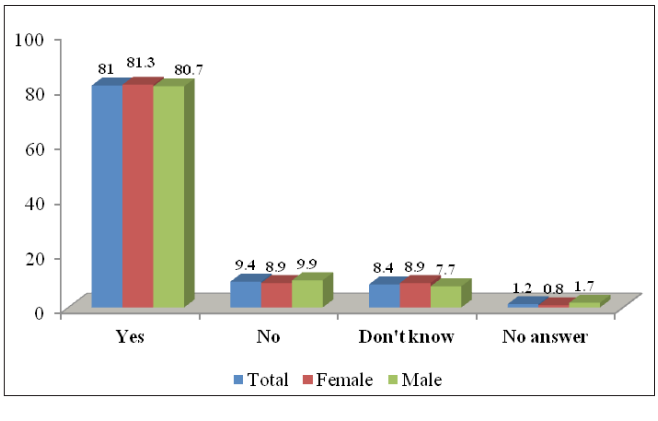

Fig. 2. Willingness to donate own tissues and cells (%)

We correlated this with the age and residential environment of our respondents and found that the statistically significant differences in those opinions were also not revealed ($p>0.05$) (fig. 3, 4).

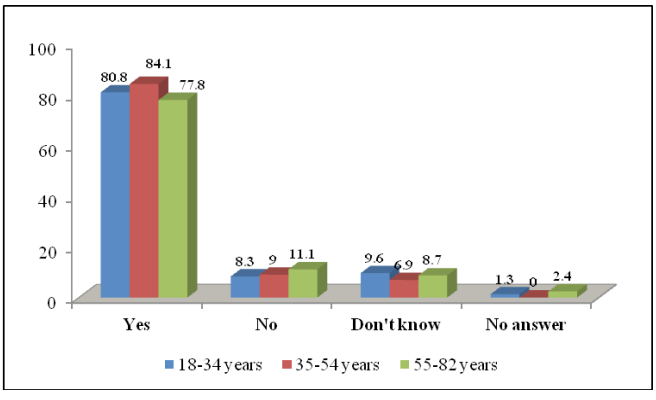

Fig. 3. Willingness to donate own tissues and cells by age (%)

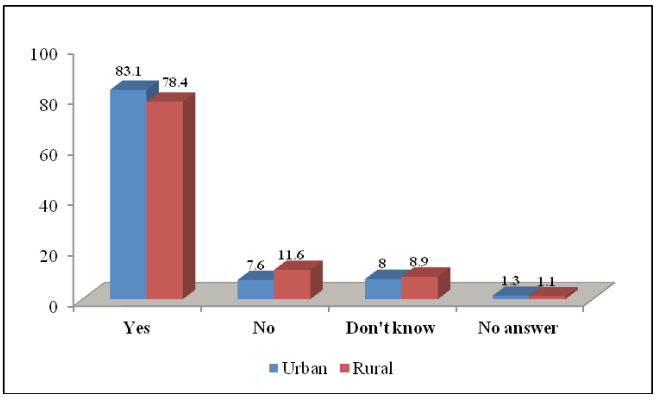

Fig. 4. Willingness to donate own tissues and cells by residential environment (%)

The correlation with the educational level of our respondents found that the respondents with university education agreed with tissue and cell donation, statistically more frequently compared to the respondents with incomplete secondary education (87.0% and 67.1%, respectively; p<0.001), and the respondents with high school or college education agreed with donation, statistically more frequently compared to the respondents with incomplete secondary education (85.1% and 67.1%, respectively; p<0.01). At the same time, the respondents with incomplete secondary education, statistically more frequently compared to the respondents with university education did not agree with donation (12.7% and 6.1%, respectively; p<0.05). From those undecided, the respondents with incomplete secondary education, statistically more frequently compared to the respondents with university education (17.7% and 6.9%, respectively; p<0.05), with high school or college education (17.7% and 5.3%, respectively; p<0.01) and with general or vocational education (17.7% and 6.8%, respectively; p<0.05) answered that they did not know (fig. 5).

The estimation of frequency of this opinion depending on occupational level also found statistically significant differences: the private entrepreneurs agreed with tissue and cell donation statistically more frequently compared to the employees (93.3% and 82.2%, respectively; p<0.05), the respondents who were temporarily unemployed (93.3% and 71.3%, respectively; p <0.01) and the unemployed respondents (93.3% and 82.5%, respectively; p<0.05) (fig. 6).

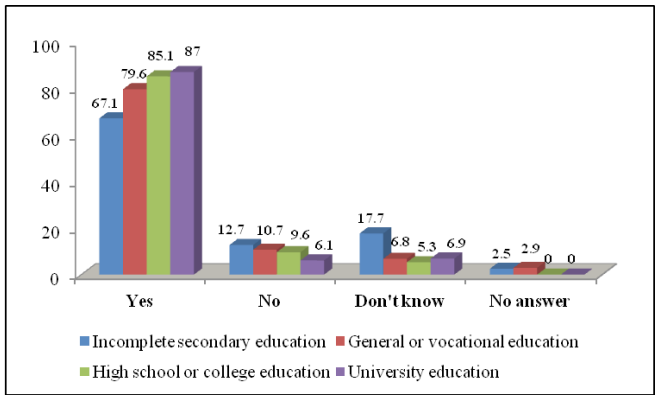

Fig. 5. Willingness to donate own tissues and cells by educational level (%)

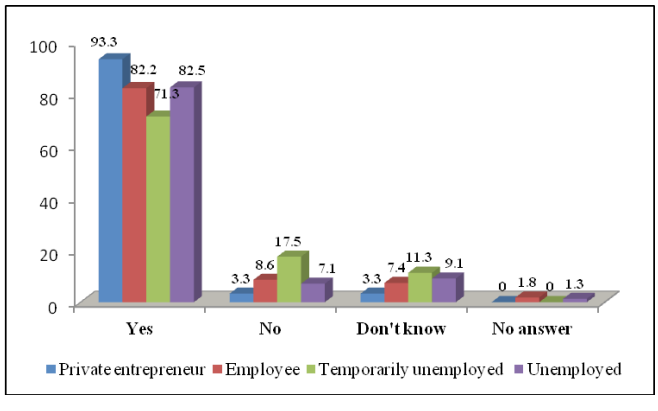

Fig. 6. Willingness to donate own tissues and cells by occupation (%)

Meanwhile, more temporarily unemployed respondents did not agree with donation than private entrepreneurs (17.5% and 3.3%, respectively; p<0.01) and the unemployed respondents (17.5% and 7.1 %, respectively; p<0.05). From those undecided, more temporarily unemployed respondents did not agree with donation than private entrepreneurs (11.3% and 3.3%, respectively; p <0.01) and employees (11.3% and 7.4%, respectively p <0.01).

The survey data analysis showed that in case if the life of a family member could be saved by tissue or cell transplant, 349 (81.7%) respondents would agree to donate these tissues or cells during life, provided that their lives would not be endangered, 40 (9.4%) respondents would not agree to donate, 33 (7.7%) respondents did not know and 5 (1.2%) respondents did not respond. Depending on gender, the results shown above were similar (p>0.05) (fig. 7).

Our study revealed that the respondents' opinion that a close relative can become a deceased donor was generally positive: 109 (25.5%) respondents had a very good opinion, 170 (39.8%) respondents had a good opinion, 24 (5.6%) respondents had a neutral opinion, 24 (5.6%) respondents had a bad opinion, 11 (2.6%) respondents had a very bad opinion, 72 (16.9%) respondents did not know and 17 (4.0%) respondents did not answer. These opinions were statistically similar by gender (p>0.05). So, the significant majority of the respondents (65.3%) had a very good and good opinion in case a close relative became a deceased donor.

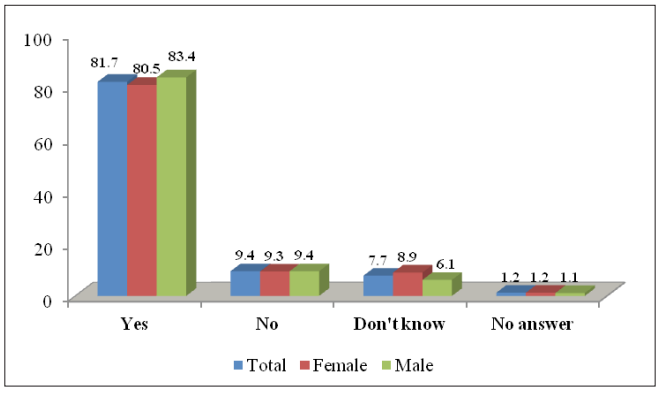

Fig. 7. Willingness to donate own tissues and cells to a family member (%)

As a result of the research it was established that the significant majority of our respondents 299 (70.0%) would accept post-mortem sampling of their healthy tissues to be transplanted to others, 69 (16.2%) respondents would not accept post-mortem sampling, 51 (11.9%) respondents did not know and 8 (1.9%) respondents did not respond (fig. 8). Differences in the frequencies of these results by gender were not statistically significant (p>0.05).

Our study demonstrated that if necessary, the significant majority of the respondents 291 (68.1%) would agree to receive tissues and cells from other people, 61 (14.3%) respondents would not accept this, 69 (16.2%) respondents did not know and 6 (1.4%) respondents did not answer (fig. 9). These results were similar by gender (p>0.05).

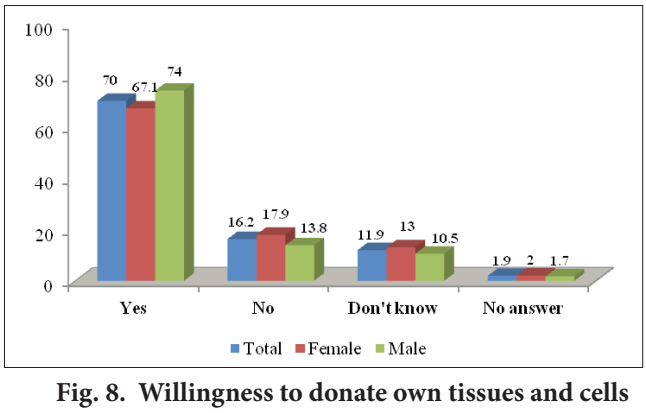

Fig. 8. Willingness to donate own tissues and cells upon death (%)

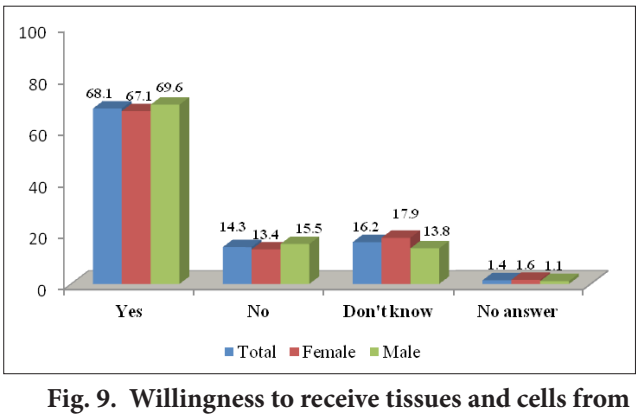

Fig. 9. Willingness to receive tissues and cells from other people (%)

Knowledge in the field of tissue and cell donation and transplantation. The evaluation of the quality and quantity of information on tissue and cell donation and transplantation found that 157 (36.8%) respondents considered they had sufficient information, 183 (42.9%) respondents considered this information insufficient, 71 (16.6%) respondents did not know, 16 (3.7%) respondents did not answer (fig. 10).

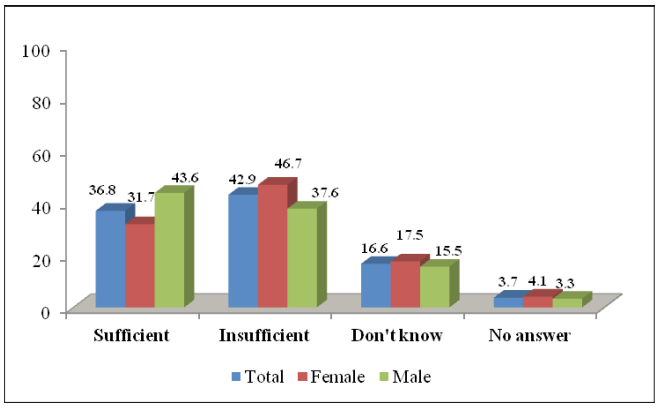

Fig. 10. Volume of information on tissue and cell donation and transplantation (%)

Significant differences in the frequency of our respondents' opinion were found depending on the educational level and the residential environment.

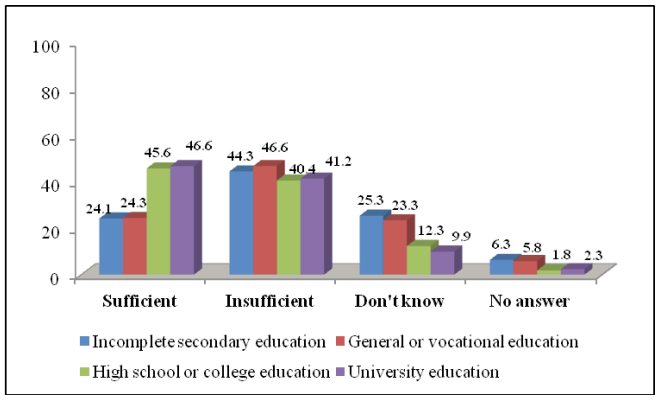

Fig. 11. Volume of information on tissue and cell donation and transplantation by educational level (%)

Our respondents with high school or college education considered this information to be sufficient, statistically more frequently compared to the respondents with incomplete secondary education (45.6% and 24.1%, respectively; p<0.01) and with general or vocational education (45.6% and 24.3%, respectively; p<0.001) (fig. 11). The respondents with university education also considered this information to be sufficient, statistically more frequently compared to the respondents with incomplete secondary education (46.6% and 24.1%, respectively; p<0.001) and with general or vocational education (46.6% and 24.3%, respectively; p <0.001).

Depending on the residential environment, 102 (43.0%)

urban respondents considered they had sufficient information, 98 (41.3%) urban respondents considered this information insufficient, 30 (12.7%) did not know and 7 (3.0%) did not answer. Respectively, 55 (28.9%) rural respondents considered they had sufficient information, 85 (44.8%) rural respondents considered this information insufficient, 41 (21.6%) did not know and 9 (4.7%) did not answer (fig. 12).

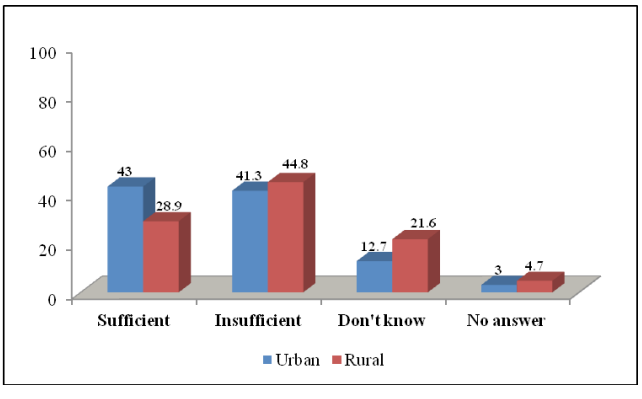

Fig. 12. Volume of information on tissue and cell donation and transplantation by residential environment (%)

Our respondents from urban areas mentioned that this information is sufficient, statistically more frequently compared to the respondents from rural areas (43.0% and 28.9%, respectively; p<0.01), but the respondents from rural areas mentioned that they did not know, statistically more frequently compared to the respondents from urban areas (21.6% and 12.7%, respectively; p<0.05).

It is important to mention that the vast majority of our respondents – 363 (85.0%) wanted to receive more information on tissue and cell donation and transplantation. For such information claimed 87.2% of respondents up to 34 years old, 89.0% of respondents aged 35 -54 years and 77.8% – aged 55 years or above (p<0.05) (fig. 13).

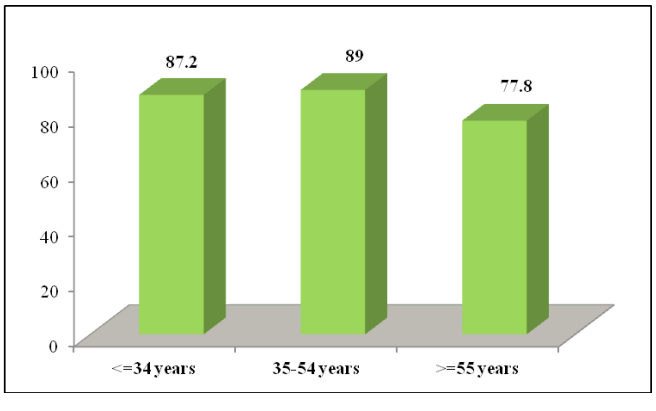

Fig. 13. Interested in having more information on tissue and cell donation and transplantation by age (%)

The correlation with the educational level exposed the fact that the respondents with high school or college education requested the information on tissue and cell donation and transplantation, statistically more frequently compared to the respondents with incomplete secondary education (92.1% and 81.0%, respectively; p<0.05) and with general or vocational education (92.1% and 75.7%, respectively; p<0.01), but the respondents with university education requested such information, statistically more frequently compared to the respondents with general or vocational education (88.5% and 75.7%, respectively; p<0.05 (fig. 14).

Therefore, the opinion of our respondents on the quality and quantity of information on tissue and cell donation and transplantation was similar in terms of age, but depended on gender, educational level and residential environment. Request for more information depended on age and educational level.

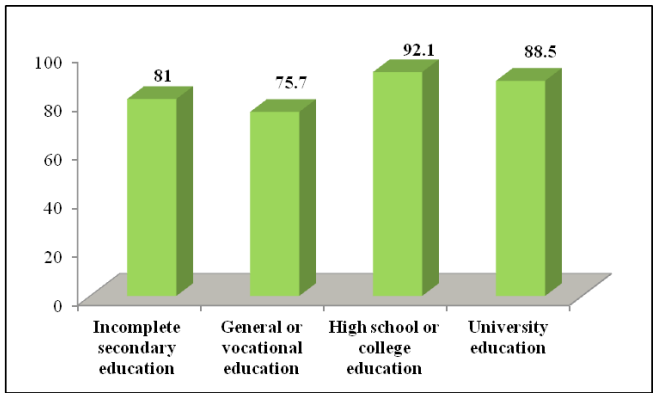

Fig. 14. Interested in having more information on tissue and cell donation and transplantation by educational level (%)

The evaluation of the respondents' opinion on the need to obtain permission from relatives to procure tissues from the deceased person found that about 2/5 – 170 (39.8%) of respondents believed that such permission should always be obtained, 104 (24.4 %) respondents believed that the permission should be obtained when the deceased's opinion is unknown, 99 (23.2%) respondents did not know, 35 (8.2%) respondents believed that the permission should not be obtained in any case and 19 (4.4%) respondents did not respond (fig. 15).

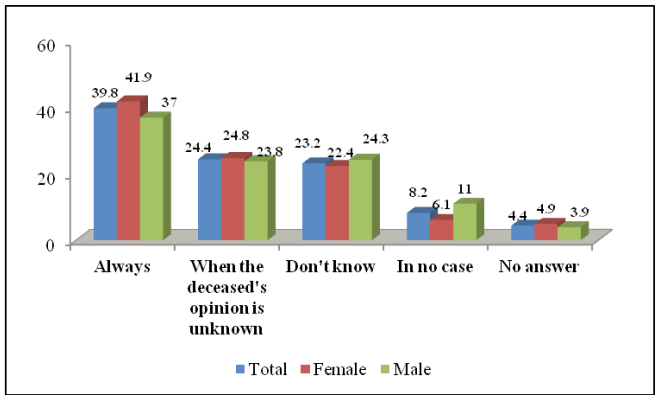

Fig. 15. Attitude towards obtaining permission from relatives for tissue procurement (%)

Discussion

The survey data analysis revealed that the vast majority of our respondents (81.0%) agreed with the tissue and cell donation and only every 10th respondent did not accept this.

Over 4 years, after conducting the first public survey on perception of donation and transplantation in 2014 [17, 18], when the agreement for donation was expressed by only 65.4% of respondents and every 5th respondent did not accept donation, the extension of education and information actions in this field obviously sensitized the population regarding the noble gesture of donation. Our study confirmed that agreement for organ donation as well as for tissue and cell donation depended on the educational level and occupation: the higher the level of education, the higher the level of acceptance of organ, tissue and cell donation; the private entrepreneurs accepted it statistically more frequently compared to employees, temporally unemployed and unemployed respondents [18].

Therefore, in our research we established that the information actions, such as organizing campaigns to promote altruistic donation programs, the annual celebrating of the European Organ and Tissue Donation Day, ensuring citizens' access to sources of medical information, managed to highlight the importance of this medical and social resource and led to a change in the public attitude towards this field.

The results of our study are similar to international ones, which showed that while 90% of people support organ and tissue donation, only about half donate their organs and tissues [19, 20]. For example, in 2012, of all actual donors in the United States authorized through the state donor registry, 40% became organ donors, 48% ophthalmic tissue donors, and 45% donors of other tissues [19]. These numbers continued to grow and in 2018 there were 50% organ donors, 61% ophthalmic tissue donors and 56% donors of other tissues [20].

In Denmark Nordfalk F. et al. found that the vast majority of the respondents (91.9%) are positive or very positive towards organ donation, 85.8% accept the idea of using their body after death, 85.0% are willing to donate their own organs, 82.1% are willing to donate their tissues and only 2.3% believe that too much has been done to promote organ donation [15]. Attitude towards donating an organ or tissue is very similar to the attitude towards receiving an organ (87.4%) or a tissue (88.6%). The fewer of respondents said that they would also be willing to donate tissues (66.2%) and organs (64.7%) of their relatives. Our study revealed that in our country, conversely, fewer respondents (68.1%) would agree to have tissues and cells transplanted from other people compared to those who were willing to donate tissues and cells (81.0%).

The results of our study showed that the vast majority of respondents (85.0%) expressed interest in receiving more information on tissue and cell donation and transplantation, and were very similar to those in receiving more information on organ donation and transplantation (88.0%) [18]. Moreover, the younger, statistically more frequently compared to older respondents, requested information on donation and transplantation. This fact was also confirmed by studying the attitude and knowledge towards donation and transplantation of 14-19 year old students from four urban high schools, when approximately 75% of respondents were willing to receive more information on this subject [21, 22].

Conclusions

1. Our study revealed the respondents' positive attitude towards tissue and cell donation and transplantation. It was found that the vast majority of our respondents generally agreed with tissue and cell donation, as well as would agree to donate their tissues and cells during their lifetime if the life of a family member could be saved by a tissue and cell transplant, provided that their life would not be endangered. We demonstrated a remarkable growth in the public positive attitude towards donation.

2. The survey data analysis showed that the agreement for tissue and cell donation depended on the educational level and occupation. Our respondents with university education and high school or college education accepted donation, statistically more frequently compared to the respondents with incomplete secondary education. The private entrepreneurs accepted donation, statistically more frequently compared to employees, temporarily unemployed and unemployed respondents. No significant differences in the frequency of this opinion according to gender, age and residential environment were found.

3. More than two-thirds of our respondents would agree to have tissues and cells transplanted from other people and would accept post-mortem self-sampling of healthy tissues to be transplanted to others.

4. Only 36.8% of our respondents considered sufficient information on tissue and cell donation and transplantation. Respondents' views on the quality and quantity of this information were similar in terms of age, but depended on gender, educational level and residential environment. So, knowledge determines attitude, which in turn influences donor motivation.

5. Most of our respondents wanted to receive more information on tissue and cell donation and transplantation. The request for information on this issue was similar depending on gender, but depended on age and educational level. This parameter was statistically more frequently met in the respondents up to 54 years old, compared to the respondents of 55 years or above, in the respondents with high school or college education and university education, compared to the respondents with incomplete secondary education and general or vocational education. It seems to be a clear opportunity to develop educational and promotional strategies to enhance donation rates. The successful training programs could significantly increase the number of donors and the transplant rate in the Republic of Moldova.

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Authors' contribution

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Funding

The study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy and Transplant Agency. The survey was a part of the authors' PhD programme. The authors are independent and take responsibility for the integrity of the data and accuracy of the data analysis.

Ethical approval and consent to participate. The study was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (protocol No 2 of 27.10.2016).

Conflict of Interests. No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.64-2.21.09
UDC: 616.314.18-073.756.8

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The dental pulp chamber evaluation by using cone-beam computed tomography

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Manuscript received December 21, 2020; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: Cone-beam computed tomographic (CBCT) imaging is a valuable tool in dental practice. It is widely used in endodontic treatment for the root canal morphology examination. Therefore, the purpose of this study was to use CBCT to calculate the volume of the pulp chamber at different tooth groups.
Material and methods: This study conforms to protocols approved and in accordance with the ethics committee's requirements, informed consent was obtained from each patient. Morphologic measurements of 120 maxillary and 120 mandibular molars (from 40 patients, aged 18–45 years) were included in this study. CBCT images were taken using a Kodak 9500 (Dental Systems, Carestream Health) operated at 90 kVp with a voxel size of 300 mm and a field of view of 90-50 mm. All scans were taken following the manufacturer's recommendation protocol. According to the examination requirements, C-shaped roots, single-rooted molars, crowned teeth, and teeth with caries and/or restorations violating the pulp chamber were excluded. All measurements were taken on the coronal plane view.
Results: In the present study, we used CBCT imaging to gather information regarding pulp chamber volume. With the scanned 3-dimensional images, we were able to clinically determine the pulp chamber parameters using a standardized and defined spatial approach.
Conclusions: The data we collected here serve as a proof of principle for the analysis of dental landmarks before collecting stem cells. In this particular study, existing CBCT scans were used to provide useful information that can be used as a guide to determine the volume of the pulp chamber.
Key words: stem cells, cone-beam computed tomographic imaging, pulp chamber.

Cite this article
Samson S, Nacu V. The dental pulp chamber evaluation by using cone-beam computed tomography. *Mold Med J.* 2021;64(2):49-52. https://doi.org/10.52418/moldovan-med-j.64-2.21.09.

Introduction

Cone-beam computed tomography (CBCT) has become an indispensable method in dental practice. It is widely used in endodontic treatment to examine the morphology of root canals. CBCT can measure and calculate the volume of the pulp chamber, can provide more accurate information to predict the possible number of cells in the dental pulp.

Therefore, the purpose of this study was to use for the evaluation of the volume of the pulp chamber in different teeth groups.

Regenerative medicine based on the methodology of cell therapy and tissue engineering is a developing multidisciplinary field that involves biology, medicine and genetic manipulation. This type of therapy aims to maintain, restore or increase the function of tissues and organs, thus helping to treat human diseases of varying severity, from chronic to life-threatening situations [1]. In diseases that evolve with impaired tissue or organ function, stem cell research promises to provide us with a pathway to regenerative therapy. However, the application of this research in the treatment of human diseases is not possible without a thorough knowledge of the biological properties of all types of stem cells. In the context of a certain disease, it must first be decided which is the most appropriate type of stem cell: the embryonic one or adult type [2]. It is also necessary to identify a source of stem cells that is accessible and in large numbers and does not generate ethical concerns [3].

Possibly, the development of stem cells in dentistry will over time make a bigger revolution than implants. Metal alloys, composites and even titanium implants are not permanent solutions. Instead, stem cell technology will generate tissues that are compatible with the patients.

Stem cells are the cells that form the basis of the formation of all tissues and organs and are characterized by the ability to self-replicate and the ability to differentiate. Thus, from the stem cells in the pulp of temporary and permanent teeth, the substrate necessary for tooth formation can be generated. Teeth are an easily accessible source of harvesting postnatal stem cells from various tissues, including dental pulp, periodontal ligament, dental follicle, apical papilla of developing teeth [4].

Considerable progress has been made in recent years in understanding stem cell biology and new methods have been developed to obtain them and direct them to areas affected by the disease. The teeth have a complex structural composition that ensures both hardness and durability.

However, this structure is vulnerable to trauma and bacterial infections [5]. When the tooth is damaged, but still in a condition that can be treated, regeneration of parts of the tooth structure can prevent or delay the loss of the entire tooth. This is of major importance, because the loss of teeth affects not only the basic functions of the stomatognathic apparatus, but also the quality of life [6]. The regenerative response of teeth to structural deterioration and degeneration is diverse because teeth are complex structures. Of all the dental structures, only the enamel is incapable of regeneration in its original structure, while the remaining tissues possess this quality, to varying degrees, depending on several factors [7]. Dental pulp plays an important role in tooth regeneration by participating in a process called restorative dentinogenesis. When the pulp tissue is exposed to a loss of dentin, direct styling therapy allows the pulp to form a new layer of dentin [8]. It has been observed that the use of various compounds, such as calcium hydroxide and mineral aggregate trioxide, promotes the activity of dentinogenesis [9]. Cells that remain in the healthy part of the pulp migrate to the affected part, proliferate due to growth factors released around the dentin matrix, and attach to the necrotic layer to form osteodentin [10]. Later, cells attached to osteodentin differentiate into odontoblasts to produce tubular dentin, thus forming repair dentin. This early mineralized tissue preserves the integrity of the pulp and serves as a protective barrier. When the tooth is still damaged, dental regeneration becomes difficult because it requires a healthy pulp [11]. Thus, larger traumas or advanced caries are treated by endodontic therapy, in which the entire pulp is cleaned and replaced with a canalicular filling material. However, live pulp is essential for maintaining homeostasis and tooth longevity [12]. An ideal form of therapy could be regeneration approaches in which the necrotic pulp is removed and replaced with regenerating pulpal tissues to revitalize the teeth [13]. In particular, regenerative pulp therapy would reconstitute normal continuous tissue at the pulp-dentin boundary by regulating tissue-specific processes of reparative dentinogenesis [14]. Two types of regeneration of the dental pulp can be considered depending on the clinical situation: partial *in situ* regeneration of the pulp or *de novo* synthesis of the pulp for its total replacement. Tissue engineering and regeneration of dental pulpal tissue remains a difficult task. A regenerated pulpal tissue must be functional and competent: it should be vascularized, contain cells similar to those of the natural pulp, be able to give birth to new odontoblasts, produce new dentin and be re-innervated. The first step for tissue engineering is to isolate cells with the correct phenotypes and propagate them in suitable culture media [15].

Material and methods

Initially to calculate the number of stem cells in the pulp chamber, we determined the volume of the pulp chamber. In this study, 120 upper molars and 120 lower molars were examined in 40 mature patients: 20 women and 20 men, aged between 18 and 45 years. The following study groups were formed: women aged 18-30 years, women aged 31-45, men aged 18-30, men aged 31-45. CBCT images were made using the Kodak 9500 (Dental Systems, Carestream Health) operated at 90 kVp, with a vox size of 300 μm and a field of view of 90x150 mm. All scans were performed using the manufacturer's recommended protocol.

In accordance with the requirements of the Research Ethics Committee, the informed consent was obtained from each patient (protocol No 25 of 31.01.2013).

Criteria for excluding subjects in the study: according to the examination requirements, teeth with C-shaped roots, single-root molars, decayed teeth or large restorations were excluded.

Criteria for inclusion of the subjects in the study: intact teeth without fillings with two and three roots were included.

All measurements were performed in the coronal plane. The appropriate selection of the section was made as follows. Initially the coronal plane of the molar was aligned according to the axial and sagittal point of view of the tooth. It was adjusted so that the coronal view represented a straight longitudinal section of the tooth from the cusp tip to the fork.

Once the coronal plane was identified from the axial point of view, 5 lines were placed at 5 different marks on the tooth (fig. 1, 2): L1: the beginning of the pulp chamber, L2: the floor of the pulp chamber, L3: the first point of separation between the roots (fork) and L4: the last point of separation between the roots (at the complete separation of the root), L5: the tip of the root.

All anatomical landmarks were approximated to plotted points, which were identified based on axial visualization. The slight difference that could have occurred in determining the points was negligible and did not affect the measurement. The volume of the pulp chamber was calculated according to the formula: $V = h * L * l$, where $h = A + B + C + D$. Direct measurements were taken between the 5 lines and the following distances were calculated as follows.

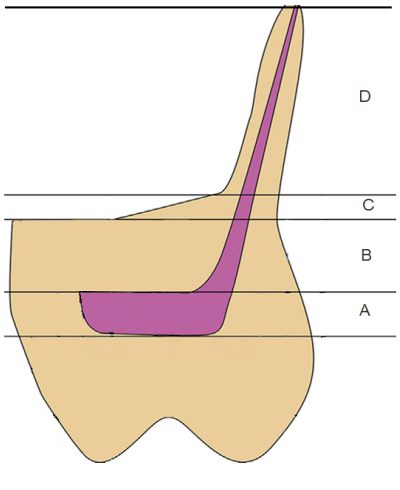

Fig. 1. Lines used as a benchmark for determining the volume of the pulp chamber

A – the beginning of the pulp chamber to the floor of the pulp chamber, B – the floor of the pulp chamber up to the first point of separation between the roots (fork), C – the first point of separation between the roots (the fork) until the last point of separation between the roots (at the complete separation of the root), D – the last point of separation between the roots (at the complete separation of the root) to the top of the root.

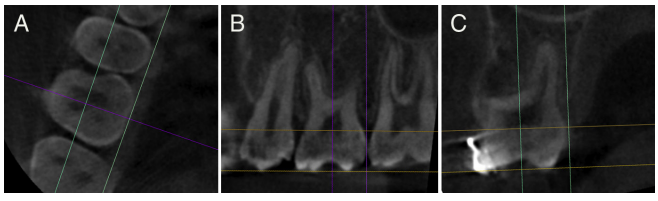

Fig. 2. CBCT images that identify the optimal view for measuring the volume of the pulp chamber of the wisdom tooth

A – axial view, B – sagittal vision, C – the green line of the sagittal plane passes along the longitudinal axis of the tooth. The yellow line of the axial plane passes perpendicular to the line of the sagittal plane and is parallel to the occlusal surface.

Results

We find that initially the indices from the experimental groups were identical, alternating, so that in the end the volume of the pulp chamber increased from the upper molar 3 to the upper molar 1, at the lower molars it inversely decreased from the lower molar 3 to the lower molar 1 (fig. 3). Currently, computed tomography offers us the possibility to automatically calculate on digital images (tomograms) the areas we are interested in. The data obtained show a direct correlation between the height, length, width of the pulp chamber and its volume.

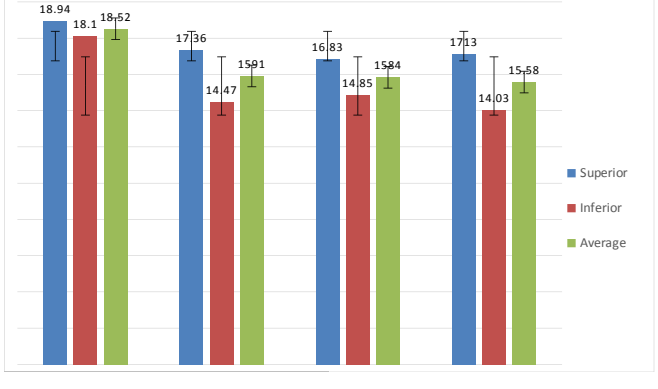

Fig. 3. The volume of the pulp chamber in the upper and lower molars in women and men

Discussion

This study investigated the relationship between the age and the volume of the pulp chamber of the upper and lower molars.

Sex and age are two major variants of estimating the volume of the pulp chamber, which was demonstrated in the present study. CBCT is an important tool for measuring the volume of the pulp chamber. In the present study, we used CBCT to gather information regarding pulp chamber landmarks relevant for volume determination.

Performing measurements of three-dimensional scanned images, allows determining the parameters of the pulp chamber using a defined standard of spatial approach. This study highlights the potential presence at different depths in the fork area and their identification using CBCT [16].

Selecting the optimal section for measuring the parameters was the critical part of this study. The coronal plan was used for the best view of the entire crown and the fork area. Adjustment of CBCT images from axial and sagittal vision allowed us to select the optimal coronal section to measure the parameters of the pulp chamber landmarks [17].

Using axial visualization as a guide allows us to accurately locate the desired parameters and prevented the overlapping of anatomical parameters in different planes. Such standardization would ensure the reproducibility of measurements [18].

In this study, we present 2 types of pulp chamber volume measurements at permanent molars: (1) measurements from the dental cusp tip, as in previous reports and (2) measurements from the central dental fossa. As access preparation is often initiated in the center of the occlusal surface, the central fossa appears to be a more appropriate reference point instead of the cusp tip used in previous studies. In addition, many variables can affect cusp height, such as location and size [19]. In previous studies, the bifurcation is always mentioned as a point where the tooth structure ends, and the roots separate. However, the fork is an area that results from the separation of two or more roots [10]. In this study, using axial vision, it was noted that the point of separation of the roots cannot always happen at the same level along the entire fork area. There were different bifurcation depths more common in the upper jaw (69%) than the lower jaw (34%), indicating that the fork is an area and that cannot be mentioned as a point [20]. Alignment of the scans in the center of the tooth can lead to overlapping anatomical landmarks and failure to identify the most coronal point where the roots separate. In this study, we identified the area of bifurcation at 2 different levels guided by axial vision. This allowed us to identify the different depth levels of the bifurcation area. Despite the wide variation in tooth length, the height of the crown appears to be very similar between all molars (8-9 mm from the central fossa and 10-11 mm from the tip of the tip). The distance from the central fossa to the bifurcation at the maxillary molars (8.78-0.79 mm) was very similar to that of the mandibular molars (8.53-0.65 mm). The variation is probably caused by the discrepancy in the cusp height between the molars of the upper and lower jaw. The height of the pulp chamber had the highest value for the maxillary molars (38.2%) and (44.4%) for the mandibular ones. These findings were similar to previous reports. Such a large variation is probably caused by the ongoing dentin deposition, which reduces the height of the pulp chamber. It can be interpreted that the calcification process appears on the ceiling of the pulp chamber as a protection mechanism against external stimuli. It has been reported by several authors that the reduction in pulp chamber height is the result of dentin deposition on the floor rather than on the ceiling [21].

It should be noted that such measurements and their differences cannot determine the location where the calcification process begins. Further investigations are needed to determine the effect of caries and restorations on the height of the pulp chamber relative to the central fossa [22].

In the present study, we focused only on whole teeth, without restorations or endodontic treatments to allow an adequate identification of the studied parameters.

It seems that CBCT images, with those previously used methods, prove to be a useful and accurate tool to determine the parameters of the pulp chamber and which would allow an approximate assessment of the number of cells contained in the dental pulp.

Conclusions

1. The size of the pulp chambers according to gender showed that for three molars, the volume of the pulp chamber is 17.2 ± mm³ in women's and 17.88 ± mm³ in men's, the difference not being significant.

2. The determination of the volume of the pulp chamber, by non-invasive method, makes it possible to predict approximately the number of nucleated cells that can be obtained from a tooth until its extraction, thus excluding teeth with a lower cell potential.

3. It is necessary to assess the concordance between the volume of the dental chamber and the specific cell content in it and the concordance between the gender, the location of the tooth and the age of the donor.

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Authors' contribution
SS designed the study, collected, processed, and interpreted the data and drafted the manuscript. NV designed the research and revised the manuscript critically. Both authors revised and approved the final version of the manuscript.

Funding
This study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy, and the research project "Nanoarchitecture in base of GaN and tridimensional matrix from biologic material for application in microfluids and tissue engineering" - 20.80009.5007.20, offered by the National Agency for Research and Development of the Government of the Republic of Moldova. 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 was approved by the Research Ethics Committee of *Nicolae Teste*miţanu State University of Medicine and Pharmacy (protocol No 70/75 of 21.05.2018).

Conflict of Interests
Nothing to disclose.

https://doi.org/10.52418/moldovan-med-j.64-2.21.10
UDC: 616-001.17:614.88:612.8

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Experimental substantiation of hyperthermic exogenous and endogenous factors prompt neutralization in burn injuries

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Manuscript received March 23, 2021; revised manuscript April 16, 2021; published online April 28, 2021

Abstract

Background: High mortality and disability of patients with critical and supercritical burns, long-term treatment, unsatisfactory aesthetic and functional results lead to the search for ways to provide assistance aimed at counteracting the formation of a mass of necrotic tissue, which is crucial for life or death of the victim.
Material and methods: The experimental study was performed on 60 sexually mature Wistar rats, which were on a regular diet and weighed 150-160 g. The experimental animals were divided into the main and control groups and were used to simulate burns with boiling water of IIb degree.
Results: The traumatic effect of hyperthermic exogenous and endogenous damage factors of the animals in the main group were immediately neutralized by a gauze napkin soaked in water at a temperature of 18-20 ˚C immediately after the simulation of burns, the duration of which became the criterion for dividing them into subgroups. In animals of subgroup 1 the time of application of a wet wipe to the burn area was 1 min., in the 2nd subgroup it was 5 min., 3rd subgroup – 10 min., 4th subgroup – 15 min., 5th subgroup – 20 min. The application napkin was changed when it was heated to 34 °C. Such applications were not performed to the animals of the control group.
Conclusions: The conclusion was made on the necessity of prompt neutralization of traumatic action of hyperthermic exogenous and endogenous damage factors as the main elements of burn wound depth. For a broader understanding of action mechanisms of the suggested technology of self-help and mutual first aid as well as the nature of the impact of neutralization directly on the tissues, it is advisable to supplement the research with morphological methods.
Key words: hyperthermic factors, burns, neutralization, first aid, necrotic tissues.

Cite this article
Chornopyshchuk R. Experimental substantiation of hyperthermic exogenous and endogenous factors prompt neutralization in burn injuries. *Mold Med J.* 2021;64(2):53-57. https://doi.org/10.52418/moldovan-med-j.64-2.21.10.

Introduction

Burns are one of the most fearsome and widespread forms of injury, which is characterized by prolonged, expensive treatment with not always positive outcomes and a high risk of various complications at different stages of the wound process [1].

The government and health care system of Ukraine today cannot afford the significant financial costs for treating patients with burns as opposed to countries with higher welfare levels. For comparison, in Germany, a total of € 270000 are spent per patient with burns per year, in the UK it is around € 63157, given significant costs of treating patients in intensive care, long and expensive rehabilitation and a high risk of disability [2].

Therefore, the pathogenetic substantiation of knowledge on the provision of self-help, mutual assistance and first aid at the site of injury at the prehospital and hospital-based levels by prompt neutralization of exogenous and endogenous factors of injury with ordinary water at room temperature is so relevant [3].

Objective: experimental study of effective prompt neutralization of the traumatic effects of exogenous and endogenous factors of burn injuries.

Material and methods

The experimental study was performed on 60 sexually mature Wistar rats, which were on a normal diet, weighing 150-160 g. The study followed the international rules and principles of "The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, 18.03.1986), directives of the Council of the European Economic Association for the protection of vertebrate animals (Strasbourg, 24.11.1986), Directive 2010/63/EU of European Parliament and Council on the protections of animals used for scientific purposes, "General ethical guidelines for experiments on animals" (Kyiv, 2011).

Experimental animals were divided into the main and control groups. Simulation of IIb degree burn injuries with boiling water with an area of 27 cm^2 was performed on the rats of both groups according to the method suggested by Pfurtscheller et al. [4]. Prompt neutralization of the trau-

matic action of hyperthermic exogenous and endogenous damage factors of animals in the main group was carried out with a gauze napkin soaked in water at a temperature of 18-20 °C immediately after the simulation of burns. The duration of traumatic action became a criterion for division of animals into subgroups. In animals of subgroup 1 the time of application of a wet wipe to the burn area was 1 min, in the 2nd subgroup it was 5 min, 3rd subgroup – 10 min., 4th subgroup – 15 min, 5th subgroup – 20 min. The application napkin was changed when it was heated to 34 °C. Such applications were not performed to the animals of the control group.

The studies were performed on the 1st, 3rd, 5th, 7th, 14th day and included assessment of the appearance of animals and wounds, determination of temperature in the center of the injured area with infrared non-contact thermometer ThermoFlash LX-26 (SN: 103300706647) compared with that in a homogeneous intact area, analysis of the wound defect area by a planimetric method using mobile application "LesionMeter" [5, 6]. On days 5, 14 blood was taken from the tail vein with leukocyte count [7].

Statistical processing of the obtained results was performed using Microsoft Excel 2016 software and "STATISTICA 5.5" (owned by of National Pirogov Memorial Medical University, Vinnytsya, Ukraine, license No AXXR910A374605FA) with determination of the arithmetic mean and its error (M ± m), Student's criterion (t) and reliability index (p). Differences at $p \leq 0.05$ (95.5%) were considered reliable [8].

Results and discussion

On the first day the formation of gray burn necrosis in the injured area with islets of shading was determined in animals of all groups. Size of the islets increased in proportion to the decrease in neutralization time, mostly in the control group (fig. 1).

Fig. 1. Wound appearance of the animal in the control group on the 1st day of experimental modeling, the formation of burn necrosis

From day 3 the necrotic eschar of the studied animals, which was particularly dense in the rats of the control group, was firmly fixed to the adjacent tissues. In addition, forced position of the body in the animals of the control group and main group 1 with curved torso towards the injury, bowed head and cautious behavior when moving, which suggests significant pain at the site of injury, was determined.

Fig. 2. The characteristic position of the experimental animal of the control group on the 3rd day of observation

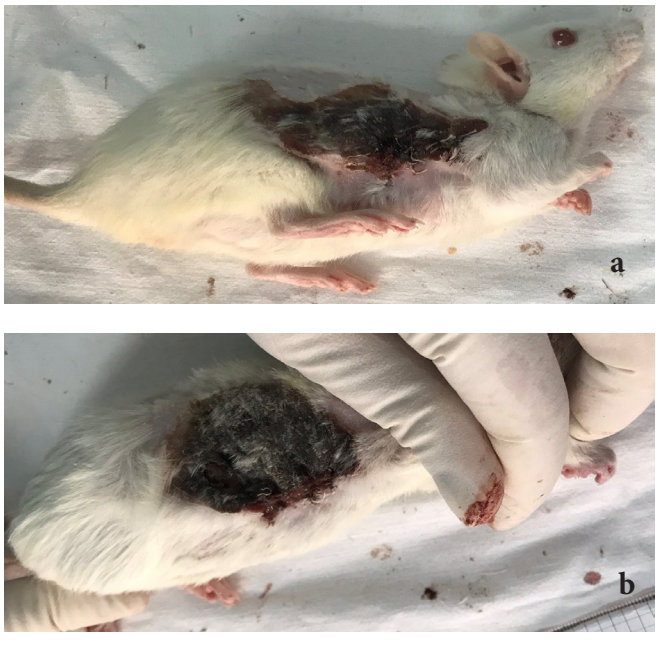

Fig. 3. Wound appearance of animals in the main-5 (a) and control (b) groups on the 7th day of experimental modeling, the wound is covered by necrotic eschar

On day 14 partial exfoliation of the necrotic eschar with signs of epithelialization of the wound surface was observed in animals of the main group, the manifestations of which were especially noticeable in groups 3, 4, 5. The wound defect of animals of the control group remained covered with necrotic eschar (fig. 4).

Complete healing of the wound defect in animals of the main group was determined on days 19-20, while in the control group minor residual wounds with signs of epithelialization were noticed.

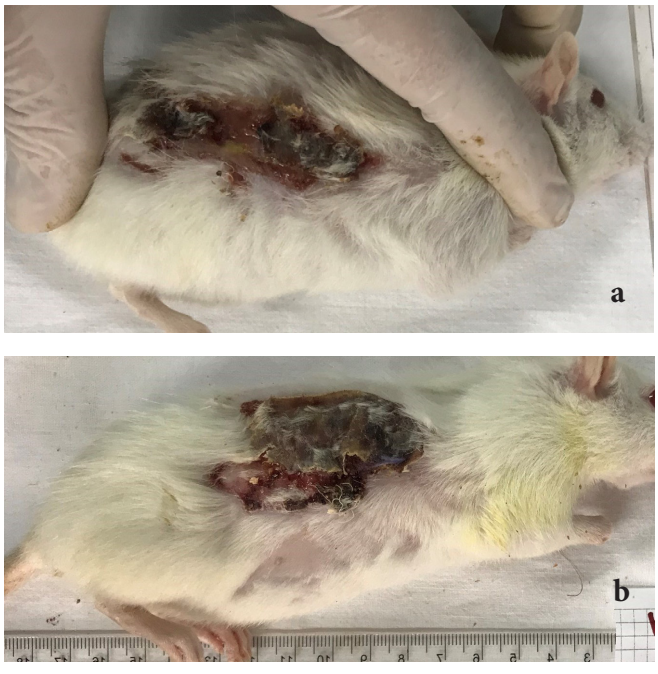

Fig. 4. Wound appearance of animals in the main-5 (a) and control (b) groups on the 14th day of experimental modeling, signs of necrotic eschar exfoliation and marginal epithelialization of wounds of animals in the main group

The skin surface temperature of the rats before the experiment was 37.3±0.4 ˚C. After application of the heated liquid to the skin surface, a sharp increase in temperature in this area to 52.8±1.7 ˚C was determined. The change of wet wipes for neutralization in the animals of the main group was performed after the temperature in the burn area increased over 38.5 ˚C. After completion of neutralization in the appropriate time for the animals of main groups 1, 2, 3, 4, the temperature of the injured area differed from that on a symmetrical intact surface with an average difference of 1.8±0.3 ˚C for 30 minutes. Along with this, in the main groups 1, 2, 3, 4 there was a repeated increase in the temperature of the injured area compared to the intact one. And only in the main group 5 after 20 minutes of application of a wet wipe, the heating of the latter was not determined, and the temperature of the injured area corresponded to that on a healthy body surface. 1 hour after burn modeling, the difference in temperatures of the compared areas in animals of all groups was not reliably detected, which persisted throughout the subsequent observation period. The results of determining the area of the wound defect are shown in table 1.

Thus, on the 1st day after modeling the area of wound defect in animals of the main group 1 was 25.89 ± 0.39 cm^2, group 2 – 24.85 ± 0.26 cm^2, group 3 – 24.50 ± 0.33 cm^2, group 4 – 23.93 ± 0.25 cm^2, group 5 – 22.46 ± 0.27 cm^2; this index was higher in the control group and amounted to 26.14 ± 0.32 cm^2. This difference between the main groups 4, 5 and the control group was reliable. At a later date, these patterns were similar maintaining a positive tendency to reduce the size of the wound defect in animals the temperature factor of which was neutralized. On day 14 these indices constituted 14.94 ± 0.31 cm^2, 13.37 ± 0.28 cm^2, 13.06 ± 0.35 cm^2, 13.04 ± 0.21 cm^2, 12.66 ± 0.19 cm^2, 16.08 ± 0.27 cm^2 in animals of the main and control groups, respectively.

The leukocyte count analysis revealed general patterns for animals of all studied groups: increase in the relative number of eosinophils, monocytes, rod-shaped neutrophils with a decrease in the population of segmental leukocytes and lymphocytes (tab. 2). Difference between the level of rod-shaped neutrophils on days 5 and 14 in the control (15.3 ± 1.1%; 9.5 ± 0.7%;) and main groups (6.8 ± 0.7 %, 6.0 ± 0.7 %; 5.3 ± 0.5 %, 3.9 ± 0.7 %; 4.5 ± 0.9 %, 5.4 ± 0.8 %; 6.0 ± 0.8 %, 4.8 ± 0.6 %; 4.9 ± 0.7 %, 5.2 ± 0.4 % in 1, 2, 3, 4, 5, respectively) was statistically significant, which indicates a more pronounced systemic inflammatory reaction in animals without neutralization of traumatic agent. Statistical differences in indices of a relative level of lymphocytes on the 5th day in animals of the control group (64.2 ± 2.2%) to 71.2 ± 1.7%, 73.0 ± 1.5%, 73.4 ± 2.1%, 70.9 ± 0.9%, 70.8 ± 1.1% in the main groups 1, 2, 3, 4, 5, respectively, confirms the intensity of immunity of the latter.

Thus, the need for prehospital care for patients with burns is not in doubt [9]. It seemed as though the problem was solved and clear step-by-step guidelines for burn care of various genesis and severity were developed [10]. A number of experiments on animals and observations on humans were carried out where the mechanisms of their action were carefully studied and pathogenetically substantiated [11].

Table 1. Dynamics of change in the size of burn injury in the studied rats (cm^2)

Observation group	Terms of observation				
	Day 1	Day 3	Day 5	Day 7	Day 14
Main group-1	25.89±0.39	25.92±0,33	23.68±0.35*	19.01±0.29	14.94±0.31*
Main group-2	24.85±0.26*	25.17±0.51*	22.21±0.39*	18.37±0.40*	13.37±0.28*
Main group-3	24.50±0.33*	24.09±0.46*	21.78±0.37*	18.51±0.38*	13.06±0.35*
Main group-4	23.93±0.25*	23.32±0.39*	21.53±0.45*	18.35±0.25*	13.04±0.21*
Main group-5	22.46±0.27*	21.90±0.45*	20.01±0.33*	17.52±0.31*	12.66±0.19*
Control group	26.14±0.32	26.75±0.41	25.31±0.39	19.66±0.22	16.08±0.27

Note: *$p \leq 0.05$ – index of statistical reliability in comparison with the control group.

Table 2. Dynamics of leukocyte count in rats with burns

Hematological parameters	Observation group	Terms of observation		
		Intact	Day 5	Day 14
Eosinophils, %	Main group-1	1.8±0.5	4.0±0.5	3.3±0.4
	Main group-2		4.2±0.3	3.7±0.5
	Main group-3		3.7±0.5	3.5±0.2
	Main group-4		3.9±0.6	3.2±0.4
	Main group-5		3.5±0.4	3.6±0.3
	Control group		4.5±0.7	3.9±0.5
Rod-shaped neutrophils, %	Main group-1	2.9±0.7	6.8±0.7*	6.0±0.7*
	Main group-2		5.3±0.5*	3.9±0.7*
	Main group-3		4.5±0.9*	5.4±0.8*
	Main group-4		6.0±0.8*	4.8±0.6*
	Main group-5		4.9±0.7*	5.2±0.4*
	Control group		15.3±1.1	9.5±0.7
Segmented neutrophil, %	Main group-1	16.8±2.1	9.3±0.8	12.7±0.6
	Main group-2		8.4±0.5	13.3±0.8
	Main group-3		9.5±0.6	12.9±0.7
	Main group-4		10.1±1.0	14.4±0.9*
	Main group-5		11.5±0.8*	13.8±0.7*
	Control group		8.1±0.9	11.6±0.6
Lymphocytes, %	Main group-1	72.8±3.5	71.2±1.7*	71.0±1.3
	Main group-2		73.0±1.5*	70.3±1.9
	Main group-3		73.4±2.1*	69.0±1.6
	Main group-4		70.9±0.9*	70.1±1.6
	Main group-5		70.8±1.1*	69.2±1.8
	Control group		64.2±2.2	68.6±2.5
Monocytes, %	Main group-1	3.7±0.9	8.7±0.6	6.8±0.4
	Main group-2		9.1±0.8	7.3±0.5
	Main group-3		8.9±0.7	7.9±0.6
	Main group-4		9.1±0.6	8.5±0.3
	Main group-5		9.3±0.7	8.2±0.4
	Control group		7.9±0.9	6.1±0.6

Note: *p ≤ 0.05 – index of statistical reliability in comparison with the control group.

However, numerous fundamental differences in the results of these studies indicate the opposite. One of the main differences is the terminological discrepancy, which in essence leads to a misunderstanding of one of the most important elements of first aid – "cooling". Existing studies have clearly established the negative effects of low temperatures (ice and water at <10 °C) on local tissues, causing vasoconstriction with additional tissue damage in the burn area, disproving previous theories of rapid aggressive wound cooling [12]. Moreover, it has been proven that even topical use of low temperature agents can cause a number of systemic disorders in the body characteristic of general hypothermia [13]. Therefore, the most effective and safe water temperature to date is set in the range of 15-22 °C, and in some cases even 37 °C in order to improve microcirculatory perfusion [14]. In view of this, in our opinion, in contrast to the term "cooling" it would be appropriate to use the term "neutralization", which more clearly reflects the mechanisms and principles of this process.

Another controversial element is the time of onset and duration of local neutralizing action on the injured area. If the opinion of researchers mostly coincides with the need for the earliest possible neutralization of the traumatic agent, the terms suggested by various scientists are fundamentally different [15]. Such discrepancies led to a similar study, which clearly confirmed positive effect of prompt neutralization of traumatic effects of exogenous and endogenous factors of thermal damage on the wound process, wound healing and reducing the activity of general inflammatory reaction on the example of experimental burns on animals. Moreover, the results of research clearly confirmed the interrelation between the duration of care provided and the

nature of further course of the pathological process. Thus, it was established that 20 minutes can be considered the optimal effective duration of prompt neutralization under the conditions of experimental modeling of burns with boiling water in rats. However, it is necessary to keep in mind the numerous anatomical and physiological features of the course of processes in these animals, which are substantially different from human ones. With this in mind, the results obtained should by no means be extrapolated to the human body, which requires further clinical study.

Conclusions

Results of the obtained experimental studies allowed to objectively confirm the importance and necessity of prompt neutralization of the traumatic effect of hyperthermic exogenous and endogenous damage factors as the main elements of burn wound depth formation. This was confirmed not only by the data of visual analysis of the wound area and the dynamics of changes in planimetric parameters, which indicated a positive effect of neutralizing the traumatic agent, but also the results of hematological analysis. Thus, with the exception of monocytes, normal indices of eosinophils, rod-shaped and segmental neutrophils and lymphocytes were better restored in the blood of animals of the main groups, which indicated a rapid attenuation of the inflammatory process and a reduction in burn depth.

For a broader understanding of action mechanisms of the suggested technology of self-help, mutual assistance and first aid and the nature of neutralization impact directly on the tissues it will be appropriate to supplement the research with morphological methods.

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Author's contributions
RC conceptualized the idea, conducted literature review, wrote the manuscript, revised and finalized the text.

Funding
The study was supported by National Pirogov Memorial Medical University, Vinnytsya, Ukraine. The trial was the author's initiative. The author is independent and takes 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 National Pirogov Memorial Medical University, Vinnytsya, Ukraine (protocol No 2, 18.03.2021).

Conflict of Interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.64-2.21.11
UDC: 611.132.2

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Some morphological aspects of myocardial bridges

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Manuscript received February 02, 2021; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: Myocardial bridges are variants of the intramyocardial position of the coronary arteries. In the specialty literature, hot topics in cardiovascular field are myocardial infarction and non-obstructive coronary artery disease with frequent connection with myocardial bridges.
Material and methods: The morphological study was based on the analysis of 200 human hearts and fragments of coronary arteries. The retrospective study was focused on the analysis of 6168 coronary angiography reports, to identify patients with myocardial bridges, their preferred location, the degree of systolic stenosis, the association between myocardial bridges and proximal to bridge and under the bridge coronary atherosclerosis.
Results: The complete myocardial bridges were described in 62% of the analyzed hearts and only in 5.3% of the total number of studied coronarographies. In the majority of cases, the complete myocardial bridges covered the anterior interventricular branch. The degree of subpontine arterial systolic stenosis varied within 10-95%. The comparative study did not determine any correlations between the degree of subpontine vascular compression and the degree of the left ventricular myocardial hypertrophy. In 32% of cases were described proximal to bridge atherosclerotic plaques and only in one case (0.5%) – distal to bridge atherosclerotic plaques, located immediately under the bridge.
Conclusions: The research findings underline the differences in anatomical and angiographic incidence of myocardial bridges, and the inability of all bridges to reduce the lumen of under bridged artery. Current study emphasizes attention to the topography of bridges, the correlation with ventricular hypertrophy and coronary atherosclerosis.
Key words: myocardial bridge, myocardial ischemia, myocardial hypertrophy, coronary atherosclerosis.

Cite this article
Tasnic M, Catereniuc I. Some morphological aspects of myocardial bridges. *Mold Med J.* 2021;64(2):58-64. https://doi.org/10.52418/moldovan-med-j.64-2.21.11.

Introduction

Heart pathology takes the leading place among the diseases, with the highest rate of morbidity and mortality worldwide.

The certain anomalies and anatomical variants of the heart arteries in a certain circumstance, can cause acute and chronic coronary events, myocardial ischemia during physical effort or after it [1]. Myocardial bridges are variants of the intramyocardial position of the coronary arteries [2].

In the specialty literature, it currently becomes a forgotten cause of hot topics in cardiovascular field like myocardial infarction and non-obstructive coronary artery disease (MINOCA) [3] with frequent connection between myocardial bridges and other acute cardiac events, such as: chest pain, malignant arrhythmias, complete atrioventricular block, sudden cardiac death [4-7].

In muscular bridges there is an temporary coronary luminal narrowing. If a patient has a endothelial injury, acute myocardial infarction may occur. Smoking history and nicotine could have damaged the endothelial structure in the bridged segment [5] .

The incidence of myocardial bridges, in the analyzed hearts, as result of necropsies, reaches up to 80-86% [4, 6]. Not all myocardial bridges are able to cause heart ischemia, so the bridges that were determined by angiography are detected only in 0.5-33% of all analyzed coronary angiographies [5], and 3.5–38.5% in coronary CT angiocardiographies [6, 8].

The mechanisms of the myocardial bridges' occurrence are not yet clear, as well as the morphological and functional features of the bridges, which can affect coronary circulation.

The need for an in-depth morphological and clinical study of the problem of morphoclinical correlations of large coronary arteries and their intramural trajectory is dictated by the widespread use of numerous procedures for assessing the subepicardial coronary vessels by minimally invasive methods.

The aim of the current study is to investigate the morphological macroscopic and angiographic aspects of myocardial bridges.

Material and methods

The prospective-morphological study was based on the analysis of 200 human hearts and fragments of coronary arteries applying the thin anatomical dissection and morphometry. The morphological and topographic aspects of the myocardial bridges were analyzed. The retrospective study was focused on the analysis of 6168 coronary angiography reports, performed in patients suspected with coronary ischemic pathology, in order to identify patients with myocardial bridges with and without association with atherosclerotic heart pathology.

The current morphological study investigated the anatomical and angiographic incidence of the variants of the intramural trajectory, the location, the dimensions of the myocardial bridges, the association of the variants of the intramural trajectory of the heart arteries.

In the retrospective study of coronary angiography of patients with myocardial bridges there were evaluated: the preferred location of the myocardial bridges, the degree of systolic stenosis, the association between myocardial bridges and proximal to bridge and under-bridged coronary atherosclerosis. The obtained data were statistically processed.

Results and discussion

The complete myocardial bridge was defined when a portion of the subepicardial coronary artery, on one or more portions of its path, enters the myocardium with its subsequent reappearance, under the epicardium.

The complete myocardial bridges were described in 62% of the analyzed hearts.

In morphological aspect myocardial bridges were classified according the following criteria: width (wide/narrow); thickness (thin/thick); the vessel involved (arterial/venous / arteriovenous); origin of muscle bundles (atrial/ventricular); histoarchitectonics of the myocardial bridge (muscle/ muscular and connective).

The thickness of the complete myocardial bridges was 2-5 mm – 62%, in 27% of cases the thickness of the bridges was up to 2 mm. Myocardial bridges with a thickness of 6-9 mm are described in 15% of cases, and very thick myocardial bridges with a thickness of more than 10 mm – in 3% of cases. The thick myocardial bridges, up to 1 cm, were found exclusively on the trajectory of the middle third of the anterior interventricular branch (fig. 1). Some isolated cases of localization of the intramural segment of the artery have been determined in the immediate vicinity of the left ventricular cavity (fig. 2).

Complete myocardial bridges, usually related to the ventricular muscles, only occasionally were determined as formations similar to the origin and insertion of muscle bundles in the atrial myocardium.

In the majority of cases the complete myocardial bridges cover the anterior interventricular branch, succeeded by the diagonal branches, first marginal branch and the posterior interventricular branch. In only one case the complete myocardial bridge was detected on the path of the main trunk of the right coronary artery and its branches. On the trajectory of the left circumflex branch were found only muscle loops with an atrial or marginal ventricular origin and insertion, while along the atrial vessels complete myocardial bridges were not identified (fig. 4).

The performed study shows that, most commonly complete myocardial bridges cover the distal portion of the proximal third and also the proximal and middle portions of the middle third of the anterior interventricular branch

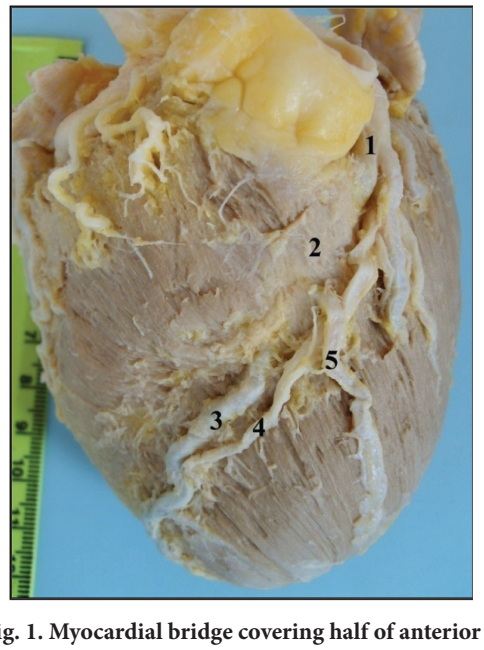

Fig. 1. Myocardial bridge covering half of anterior interventricular branch
1 – proximal to bridge arterial segment; 2 – complete myocardial bridge; 3 – distal to bridge arterial segment; 4 – vena cordis magna; 5 – second diagonal branch.

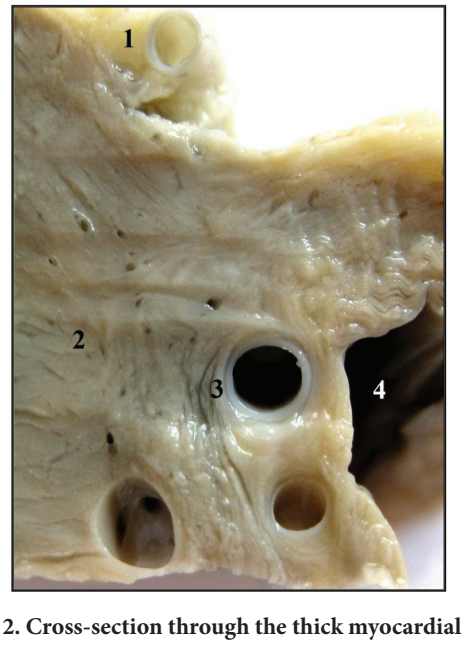

Fig. 2. Cross-section through the thick myocardial bridge covering middle portion of the anterior interventricular branch
1 – first diagonal branch; 2 – septal myocardium; 3 – under the bridged arterial part; 4 – the cavity of left ventricle.

(86% of cases with complete myocardial bridge on the anterior interventricular branch). Rarely, in 32% and 26% of cases complete myocardial bridge covers the distal and proximal third of the LAD as well.

The widest (up to 70 mm) and thickest (up to 8-10 mm) of complete myocardial bridges were detected along the anterior interventricular branch. Only in 4% of cases were found subtotal complete myocardial bridges, covering the anterior interventricular branch over a distance exceeding 75% of the total vessel length.

In 75% of the studied hearts, several complete myocardial bridges were located in the same heart.

The most frequent association is between the complete myocardial bridge on the trajectory of the anterior interventricular branch and the complete myocardial bridges located on the first marginal branch. Such associations were detected in 21% of the investigated cases. Two myocardial bridges were detected in 33% of cases. In 18% of cases 3 myocardial bridges were located in the same heart, and in 3% of cases 4 and more bridges were determined in one heart. The maximum number of the complete myocardial bridges detected per one organ is 7.

The analysis of 6168 reports of diagnostic coronary angiography performed on people with suspected severe coronary atherosclerotic pathology, myocardial bridges were detected in 331 of people, constituting 5.3% of the total number of cases.

A significant discrepancy between the morphological and angiographic frequency of screening for the myocardial bridge is explained only by the inability of all myocardial bridges to reduce the lumen of the artery during systole for various reasons.

Based on the study, it was found that the number of myocardial bridges reported by interventional cardiologists during this period varied from year to year, due to a lack of information. In many cases, the mention of myocardial bridges detected in coronary angiography was considered by some specialists as harmless anatomical variants, which do not require to be mentioned in medical documentation.

The study group was underestimated in patients with myocardial bridges and coronary arteries without severe coronary lesions and in patients with severe coronary lesions. In this way, in the category of patients without moderate and severe coronary artery disease, myocardial bridges were detected in 56% of men and 43% of women, unlike the group of patients with severe coronary heart disease – in 82% of cases patients with severe coronary atherosclerosis and myocardial bridges are men and only 17% – women. This correlation is within the natural ratio of the distribution of atherosclerotic heart diseases by gender, therefore there is no significant difference in the prevalence of myocardial bridges by gender.

The angiographic identification of myocardial bridges during angiographies is possible only by the direct pontine effect on the underlying vessel – systolic compression, in other words – the squeezing effect, "milking" of the blood column under the bridge (fig. 3).

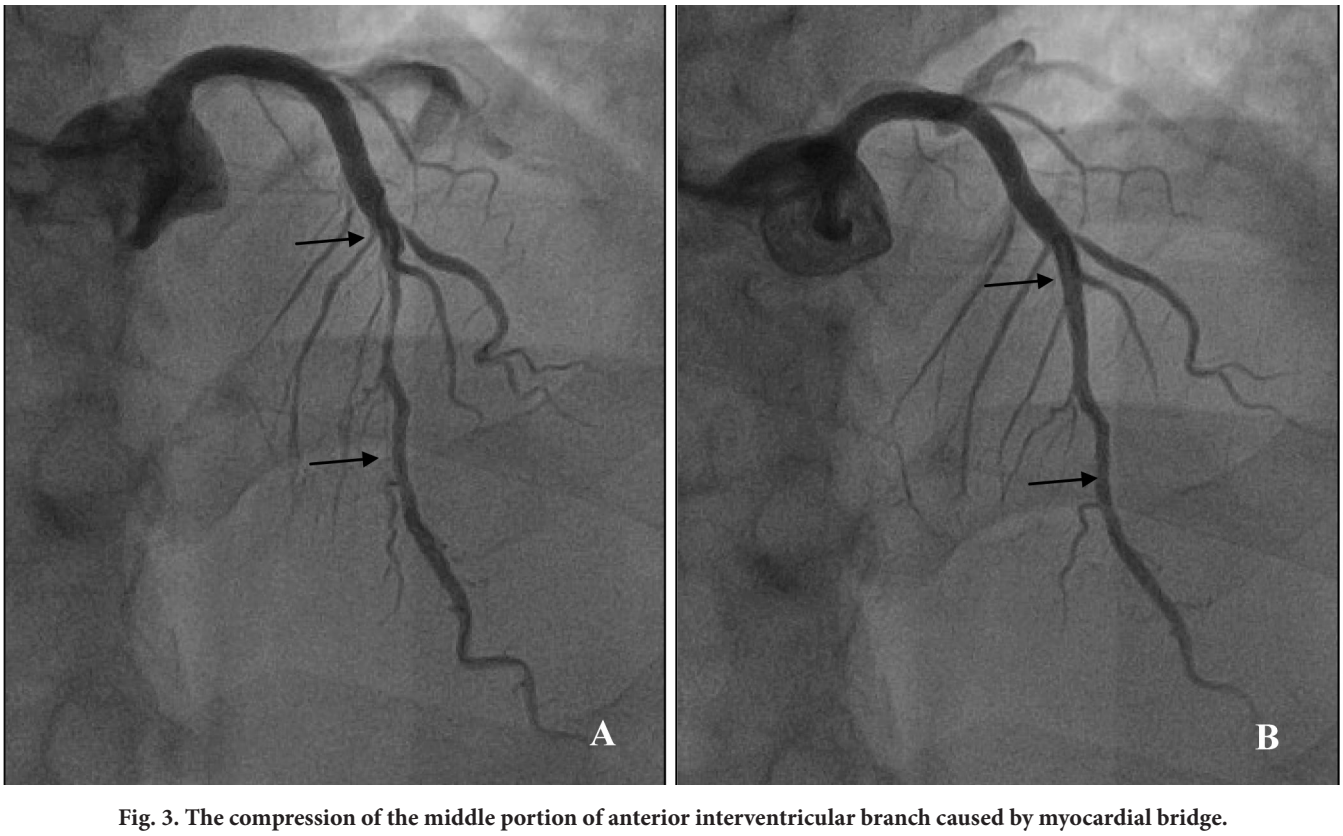

Fig. 3. The compression of the middle portion of anterior interventricular branch caused by myocardial bridge. Images from coronarography

A – during systole of the heart, B – during diastole of the heart

The degree of systolic stenosis is directly proportional to the compression force of the pontine myocardium and inversely proportional to the resistance of the vascular wall, the diameter of the vessel lumen, and the intracoronary pressure.

In the case of bridges that cause the reduction of the vascular lumen up to 50%, the intramural portion of the vessel, at the time of maximum systole, was homogeneously stenotic, having uniform vascular contours. In the case of subtotal systolic compression, the subpontine vascular segment had the appearance of a "sawfish", with the alternation of narrow vascular portions and wider ones.

The non-uniformity of the systolic stenosis of the artery can be caused by the arrangement of the pontine myocardial bundles under the different angles and / or the variation of the areas of anti-systolic resistance of the vascular wall and the tissue structures in the subpontine perivascular space.

Arterial stenosis caused by myocardial bridge is not always limited to systole, but also persists in diastole; can be a part of the diastole or it can be permanent.

In the longitudinal section plane through the muscle-artery complex, the angiographic view in maximal systole takes shape of a "trough".

Often, during the routine coronary examination, the middle and distal portion of the LAD do not show obvious systolic stenoses, but have a "trough" like deformity, which would correspond to the vascular deformation caused by the involvement of the artery under the myocardial bridge, but which, in this case, is systolic inactive. In such cases, it may be appropriate to perform tests to induce coronary spasm (with acetylcholine) and to reduce intracoronary pressure (with nitroglycerin) to detect the activity of myocardial bridges, or to demonstrate the possibility of inducing the vascular spasm in the subpontine portion.

Out of the 331 cases of patients detected with myocardial bridges, in 97% of cases they were located along anterior interventricular branch, and in 3.6% of cases – on other vessels: right coronary artery, circumflex artery, first diagonal branch, marginal branches, posterolateral branch (fig. 4).

Bridges located in the same plane on anterior interventricular branch and diagonal branches were described, being covered by the same "myocardial flap". Similar bridges were found on the trajectory of diagonal branch, intermediate branch and marginal branch, with a shape of myocardial strips located in the same plane, like if they were covered by the same "myocardial flap".

In 65% of cases, the bridges were located in the middle third of the anterior interventricular branch, and in 27% – the myocardial bridges were covering the distal third of the artery. In 4.23% of cases, the bridges located on the LAD were detected as extended, covering 2 segments at the same time.

The degree of subpontine arterial systolic stenosis varies within 10-95%. From the total number of described myocardial bridges, in 50% of cases they were causing insignificant systolic compression of the artery, reducing the lumen of the vessel up to 50% of the initial value (visually appreciated) and only in 16% of cases the degree of compression exceeded 75%.

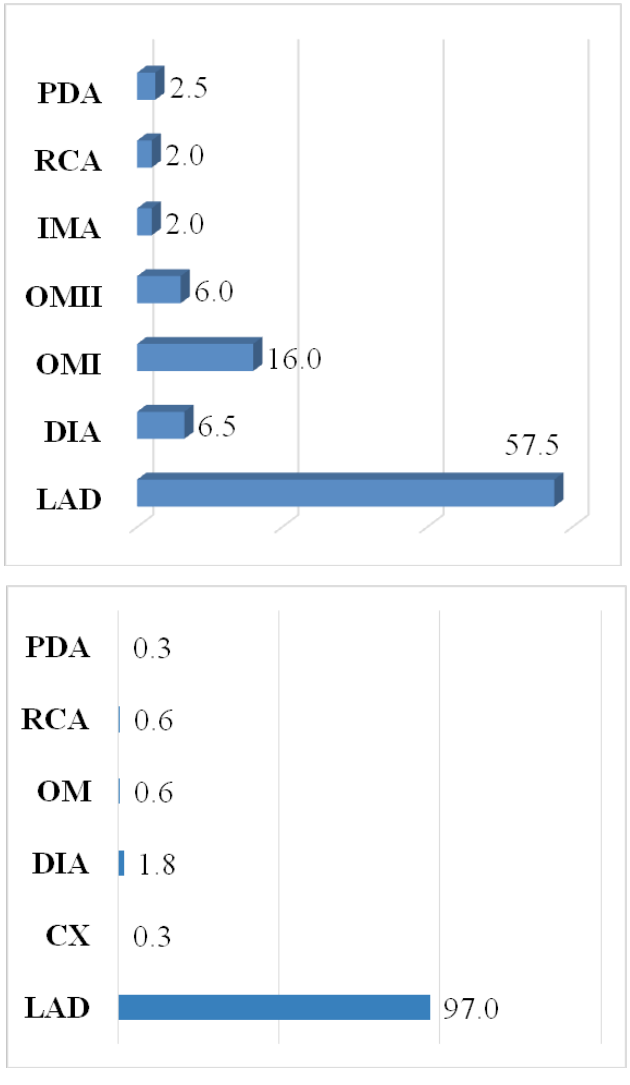

Fig. 4. The topography of myocardial bridges discovered during morphological examination of human hearts and anatomical pieces of coronary arteries (the diagram above), myocardial bridges discovered during coronarographies (the diagram below)
PDA – posterodiagonal branch (posterior interventricular branch); RCA- right coronary artery; OM – obtuse marginal branch; DIA – diagonal branch; CX – circumflex artery; LAD –anterior descendent branch.

Another widely discussed aspect in the literature was analyzed as well – the interdependence of the degree of systolic stenosis of the anterior interventricular branch and the degree of myocardial bridge hypertrophy. The comparative study did not determine any correlation between the degree of subpontine vascular compression and the degree of left ventricular myocardial hypertrophy in the main study group.

Consecutively, has also been investigated the correlation between myocardial bridges and proximal to bridge coronary atherosclerotic lesions. From the total examined reports, in 32% of cases were described proximal to bridge

atherosclerotic plaques located at various distances proximal to the myocardial bridge; and only in one case (0.5%) – distal to bridge; atherosclerotic plaques located immediately under the bridge. Coronary atherosclerosis was not detected in intramural part of the vessel.

The correlation between the degree of dynamic subpontine coronary stenosis and the degree of proximal to bridge atherosclerosis was also not determined.

Discussion

The morphological and clinical study is based on statistically significant number of the research objects, which made possible to formulate statistically true conclusions for the morphological and functional aspects of myocardial bridges.

The obtained morphological data is related with the data reported in multiple studies dedicated to myocardial bridges and the particular location of bridges on the trajectory of the anterior interventricular branch [8, 9].

The discrepancies between the anatomical and angiographic (clinical) incidence are dictated by the inability of the bridges to compress the arteries during the systole. The ability of the myocardial bridge to reduce the lumen of the artery during cardiac contraction is determined by the thickness of the myocardial bridge, width, histological composition, the caliber of the vessel covered by the bridge, the orientation of the myocardium around the bridge [10, 11]. All the mentioned features were the basis for the elaboration of the own morphological classification of the myocardial bridges.

The detection of the myocardial bridges in all branches of the coronary arteries shows that there are no arteries predisposed to intramural localization but their formation is determined in ontogenesis and would correspond to incomplete myocardial resorption around the arteries of the heart [6].

A special attention gets the fact of the association of myocardial bridges on the same vessel or in the same heart, which is detected in 75% of cases by the possibility of amplifying the proischemic effect [9, 10].

Although, in most cases, were described thin myocardial bridges, in the rare cases – 3%, were found thick myocardial bridges of about 10 mm, with a deep localization of the anterior interventricular branch, in the thickness of the interventricular septum. The incidence coincides with the angiographic incidence of myocardial bridges within 5%, suggesting that only thick bridges could reduce the lumen of the vessel.

An angiographic incidence of 5% versus 62% of morphological one suggests that thick myocardial bridges can be considered as coronary arteries anomalies, as they cause visible narrowing on angiograms of the intramural coronary segment. In the literature, the term "anomaly" is often referred to myocardial bridges [10], referring them to the group of structures with negative effects.

The angiographic view of the myocardial bridges coincided with those described in the literature. Also, within this particular study a special attention was given to the non-uniformity of compression of the coronary arteries during the systole, which is suggesting the non-uniformity of the pontine compression forces determined by the orientation of the pontine myocardium, the thickness of the bridge and possibly by the pontine histoarchitectonics, and the correlation between the amount of the connective and muscular tissue.In 50% of detected cases the degree of subpontine systolic coronary reduction was below 50% and only in 16% of cases was over 75%.

However, not all patients with severe subpontine stenosis caused by myocardial bridges develop myocardial ischemia, possibly some other proischemic mechanisms, are intervening, such as subpontine coronary spasm which is so intensely attributed to the intramural trajectory by some authors [10-12]. To the genesis of subpontine coronary spasm often are attributed: stress, smoking, drug use [13, 14] and chemotherapy treatment [15]. All above-mentioned risk factors are linked with toxic injury of functional integrity of the heart. Understanding the mechanisms underlying cardiotoxicity may lead to treatment of the toxicity or to its prevention. There is a field for harm reduction strategies to be applied by reducing or eliminating effects of acute cardiotoxicants on the body.

20% of deaths attributed to coronary diseases are linked to smoking [16]. Toxic effects of smoking result from chronic exposure to numerous toxic chemicals and carcinogens, including cardiotoxicants in tobacco smoke following the combustion of tobacco in the cigarette. Conversely, products that do not involve combustion to deliver nicotine (e.g. nicotine replacement therapies (NRTs), smokeless tobacco, or electronic cigarettes) have substantially reduced levels of toxic substances [17]. In smokeless heated tobacco products (HTPs) levels of carbon monoxide, acute heart toxicant, is reduced up to 98% compared to cigarettes, as well as aldehydes (approximately by 80–95%) and VOCs (approximately by 97–99%) [18]. Non-combustible nicotine sources are considered for smoking harm reduction [19]. Giuseppe Biondi-Zoccai et al. made independent assessment of proatherosclerotic effects of cigarette smoking versus HTP use [20]. HTP had less impact than conventional cigarette on some dimensions of oxidative stress, antioxidant reserve, platelet function, and blood pressure. In addition, HTP had less acute effects on soluble Nox2-derived peptide, 8-iso-PGF2a-III (quantification of isoprostanes) and vitamin E. Also, users of HTP appeared to be more satisfied and capable of decreasing desire for continuing smoking.

Promoting smoking cessation should become an essential contributor to the treatment of patients with CVD and especially patients with coronary atherosclerosis, myocardial bridges founded after coronary angiography. In cases when patients do not quit smoking, doctors should apply harm reduction strategies, that is, to discuss with a patient the possibility of switching to an alternative products with a reduced or modified risk, while continuing efforts to quit the use of nicotine completely, since alternative products reduce, but do not entirely eliminate adverse health effects [21].

The study showed the presence of proximal to bridge atherosclerotic plaques in 32% of cases, which corresponded to data from literature, but there wasn't any correlation between the degree of pontine stenosis and the atherosclerotic stenosis degree. Thus, even if the myocardial bridges predispose to the formation of proximal to bridge coronary atherosclerosis by generating the retrograde blood flow with a traumatic mechanical effects for the intima in the proximal to bridge portion of coronary artery being a predisposing factor for coronary atherosclerotic pathology (depending on the thickness and length of the bridge) [8, 22], these structures are not able to determine the grade of coronary lesion and the grade of prepontine atherosclerotic stenosis.

At the same time, no cases of subpontine atherosclerosis were detected, all the obtained data being consistent with the information given in the literature and is suggesting the protective effect of the bridges in under the bridge coronary atherogenesis [11, 12, 23].

There was not determined any correlation between the grade of the ventricular myocardial hypertrophy and the degree of systolic compression caused by the bridges.

Although, many authors stipulate that myocardial hypertrophy predisposes to thickening of the bridge and increases the incidence of their detection on the coronary angiography, especially in the case of hypertrophic cardiomyopathy, according to obtained results only the severe hypertrophy characteristic for the hypertrophic cardiomyopathy, would increase the capabilities of the myocardial bridge to compress the artery into cardiac systole and not a hypertrophy of up to 20 mm [24].

Conclusions

The anatomical incidence of myocardial bridges is 62%, and the angiographic one – 5.3% in the analyzed cases. The discrepancy in the incidence is determined by the myocardial bridges' capacity to reduce the under-bridged arterial segment lumen.

The coronary branch with the highest predisposition to myocardial bridges is the anterior interventricular branch. This vessel was the location of the thickest bridges detected in this research.

The stenosis' degree caused by myocardial bridges varies depending on its compression force between 10-95%. The most compressive bridges were detected on the anterior interventricular branch trajectory.

No correlation was found between the degree of systolic stenosis caused by the myocardial bridges and the prepontine atherosclerosis degree. Similarly, no associations were found with the degree of the left ventricular myocardium hypertrophy.

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Authors' contributions
MT conceptualized the idea, conducted literature review, wrote the manuscript, revised and approved the final text; IC designed the research and revised the manuscript critically. Both authors revised and approved the final version of the manuscript.

Funding
This study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy, The International Hospital *Medpark* and journal publication fee was covered by *Philip Morris Sales and Marketing SRL*. 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 project was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (protocol No 55 of 03.06.2016).

Conflict of Interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.64-2.21.12
UDC: 616.62-006.6-089.87

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Comparative analysis between En-bloc resection and transurethral resection of non-muscular-invasive bladder tumors

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Manuscript received March 03, 2021; revised manuscript April 16, 2021; published online April 28, 2021

Abstract

Background: Transurethral resection of the bladder (TURB) tumor was first described by Stern and McCarthy in 1931, and is still considered the gold standard in diagnosis and treatment of non-muscle-invasive bladder cancer. The quality of TURB affects accuracy of histopathologic evaluation, and subsequently impacts the risk of recurrence and patient outcome. New methods that aim to improve the effectiveness of TURB are reviewed, and recent studies are discussed, including resection methods and image enhancement techniques.
Material and methods: Between January 2016 and April 2019, within the Urology Clinic of *Nicolae Testemitanu* State University of Medicine and Pharmacy 108 patients were surgically treated with bladder tumor pathology. Patients were divided in two groups: En-bloc resection group which includes 51 patients and transurethral resection group with 57 patients, the obtained data were comparatively analyzed.
Results: Tumor analysis showed that the majority of the patients' tumors were localized on lateral urinary bladder walls, single bladder tumors were detected in 64 (59%) cases, tumor sizes up to 3 cm were detected in 74 (69%) patients included in the study. Detrusor muscles were detected in 49 (96%) cases of En-bloc group and 45 (79%) cases of TURB group. Most recurrences occurred in patients with high-grade histological result, recurrence rate in En-bloc group occurred in 18% and in TURB group in 37%.
Conclusions: The En-bloc resection technique of non-muscle-invasive bladder tumor proved to be a safe and effective method compared to the conventional transurethral resection technique (TURB). This method provides more favorable results for obtaining better quality tumor samples (present of detrusor muscle) that allow to establish correct diagnosis and staging of the disease and reduces the number of recurrences.
Key words: En-bloc resection, non-muscle-invasive bladder cancer.

Cite this article
Vladanov I, Plesacov A, Ghicavii V. Comparative analysis between En-bloc resection and transurethral resection of non-muscular-invasive bladder tumors. *Mold Med J.* 2021;64(2):65-69. https://doi.org/10.52418/moldovan-med-j.64-2.21.12.

Introduction

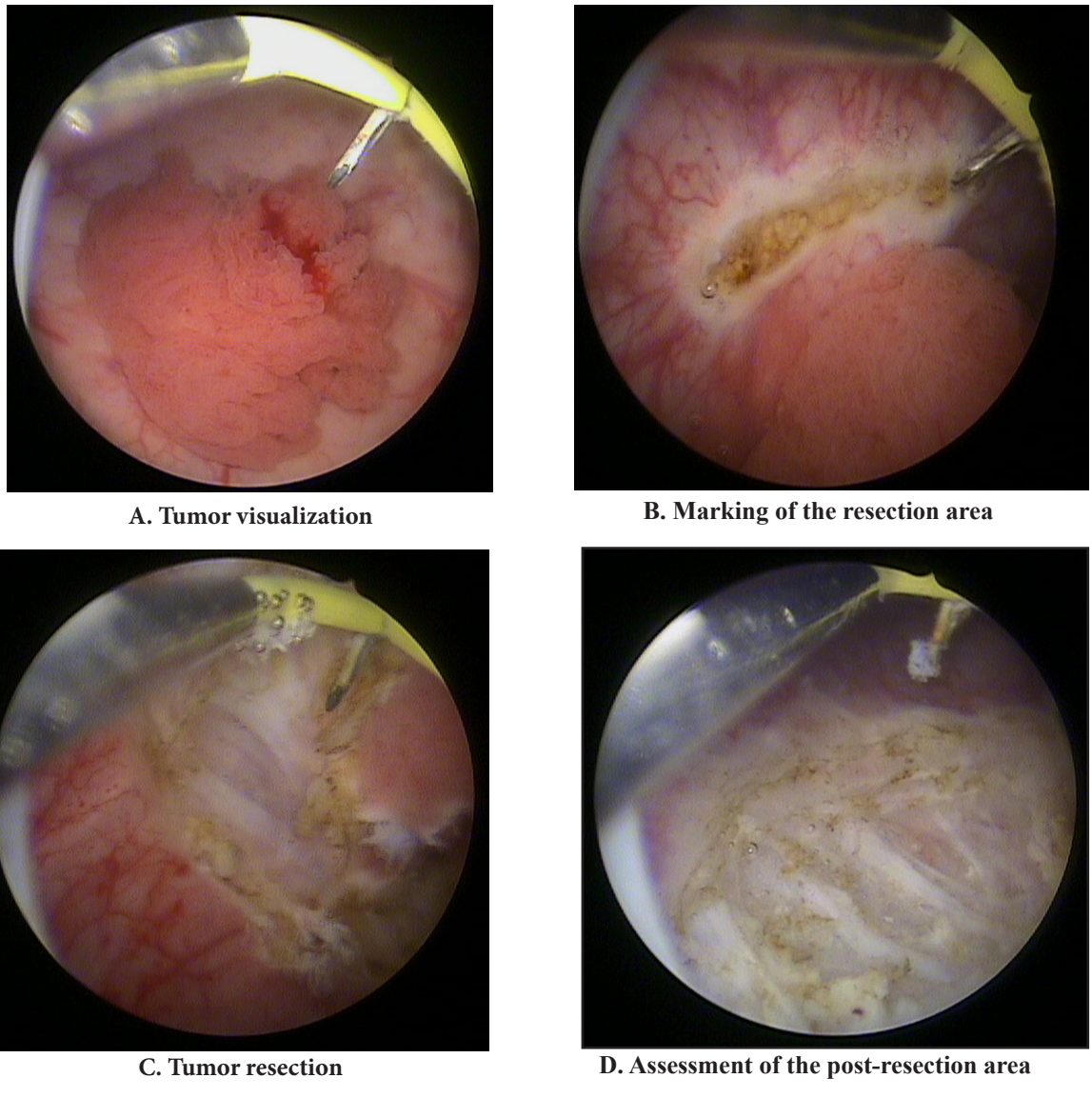
Papillary tumors confined to the mucosa and invading the lamina propria are classified as stage Ta and T1, respectively, according to the Tumor, Node, Metastasis (TNM) classification system. Flat, high-grade tumors that are confined to the mucosa are classified as carcinoma in situ (CIS) (Tis) [1]. Transurethral resection of the bladder (TURB) is the method which should be chosen for treatment of these tumors. In some cases, it should be combined with intravesical instillations. For this reason, the therapeutic tactics should be chosen regarding the non-muscle-invasive bladder cancer (NMIBC). It is important that all tumors are characterized according to their stage, grade, and other pathological characteristics, taking into account that the term "Non-muscle-invasive BC" is only a generalized definition.

In NMIBC, 60% of patients present with pTa, 30% with pT1, and 10% with (CIS) lesions [2], T1 tumors are mostly high-grade, and high-grade clinical stage T1 (high-grade T1; formerly T1G3) urothelial carcinoma of the bladder (UCB) is biologically the most aggressive phenotype among NMIBCs [3].

Transurethral resection of the bladder (TURB) tumor was first described by Stern and McCarthy in 1931, and is still considered the gold standard in diagnosis and treatment of non-muscle-invasive bladder cancer [4].

The quality of TURB affects accuracy of histopathologic evaluation, and subsequently impacts the risk of recurrence and patient's outcome. New methods that aim to improve the effectiveness of TURB are reviewed, and recent studies are discussed, including resection methods and image enhancement techniques. The goals of TURB are to obtain an adequate tissue specimen for determining tumor stage and grade (diagnosis) and to resect all visible lesions (therapeutic). Complete resection including a sample of the underlying *muscularis propria* is recommended by the guidelines of the European Association of Urology (EAU) and American Urological Association (AUA) [5, 6].

It is important to take into account that after TURB recurrences often develop. Another danger represents



A. Tumor visualization

B. Marking of the resection area

C. Tumor resection

D. Assessment of the post-resection area

Fig. 1. Operating area

the tumor understaging. To overcome these limitations, an effort has been made to optimize the surgical strategy and introduce technological improvements, including new energy sources and other methods [7]. En-bloc resection is a promising new surgical technique (Figure 1), involving circular incision of the mucosa at a safe distance from the lesion, followed by preparation and removal of the whole tumor, including the underlying detrusor muscle. Many authors believe that this 'no-touch' principle translates into better specimen quality, improved surgical radicality, and a reduced recurrence rate [7].

The aim of the study is to compare the results after En-bloc resection and transurethral resection of non-muscular-invasive urinary bladder tumors.

Material and methods

The study was performed between January 2016 and April 2019, within the Department of Urology and Surgical Nephrology of *Nicolae Testemitanu* State University of Medicine and Pharmacy and *Timofei Mosneaga* Republican Clinical Hospital where 108 patients were surgically treated with bladder tumor pathology. A transversal descriptive study was performed. The patients were selected from all amount of bladder tumors patients treated in our department by En-bloc resection and transurethral resection of the bladder tumors, according to the following criteria. The inclusion criteria were: primary non-muscular-invasive bladder cancer, patients over 18-year-old and the Eastern Cooperative Oncology Group (ECOG) score 0-2. The exclusion criteria were identified as follows: other non-urothelial tumors, severe comorbidities, ECOG score ≥ 3 and pregnancy. 108 patients were divided in two groups: En-bloc resection group which included 51 patients and transurethral resection group (control group) with 57 patients, the obtained data were comparatively analyzed. Descriptive statistics was applied. In this study the results are demonstrated as absolute and relative values.

Results

The study was done on 108 patients who were treated endourologically endoscopically by En-bloc resection (51 patients) and TURB (57 patients). No blood transfusion was required and minimal intraoperative hemorrhage during the procedure was observed. These two groups had comparable clinicopathological characteristics: gender, age, tumor grade, tumor multiplicity, tumor size, postoperative complications, histological and oncological outcomes (tab. 1, 2).

Of 108 patients included in the study, according to gender repartition, 89 (82%) were men and 19 (18%) women. The age varied between 26 to 85 years, the mean age was 65.8 years, and the majority of the patients were over 60 – 72 years (66%).

Tumor analysis showed (tab. 1) that the majority of the patients' tumors were localized on lateral urinary bladder walls, single bladder tumors were detected in 64 (59%) cases, and tumor sizes up to 3 cm were detected in 74 (69%) patients included in the study. Of 108 patients 49 (45%) were tobacco users, which is an important risk factor for the development of bladder cancer.

Only grade I and grade II complications occurred in each group (tab. 2), according to the Clavien-Dindo classification for surgical complications. Intraoperative obturator nerve reflex occurred in 10% of En-bloc group and 14% of TURB group. Bladder perforation occurred in 2 patients (4%) in En-bloc group and 3 (5%) in TURB group, which was managed by catheterization for 3-4 days. Histopathological examination showed that fragments of detrusor muscle were detected in both groups: 96% in En-bloc and 79% in TURB, thus we can see benefit of En-bloc tumors samples for an accurate diagnosis.

All interventions were performed as a one-step procedure with patient follow-up during 16-months. Most recur-

Table 1. Patient and tumor demographics

Parameters	Categories	En-bloc (n=51)	TURB (n=57)	Total (n=108)
Gender	Men, n (%)	43 (84%)	46 (81%)	89 (82%)
	Women, n (%)	8 (16%)	11 (19%)	19 (18%)
Age, years	Mean age (CI 95%)	65.4 (26-83)	66.3 (28-85)	65.8 (26-85)
Age group	18-30 years, n (%)	3 (6%)	1 (2%)	4 (4%)
	31-60 years, n (%)	16 (31%)	16 (28%)	32 (30%)
	60 years and more, n (%)	32 (63%)	40 (70%)	72 (66%)
Tobacco/Smoking	Yes, n (%)	22 (43%)	27 (48%)	49 (45%)
Tumor size	< 3 cm, n (%)	36 (71%)	38 (67%)	74 (69%)
	≥ 3 cm, n (%)	15 (29%)	19 (33%)	34 (32%)
The number of tumors	Single tumors, n (%)	31 (61%)	33 (58%)	64 (59%)
	≥ 2 tumors, n (%)	20 (39%)	24 (42%)	44 (41%)

Note: CI – Confidence Interval, En-bloc – En-bloc transurethral resection of bladder tumor, TURB – Transurethral resection of bladder tumor.

Table 2. Surgical and histological outcomes

Parameters	Categories	En-bloc (n=51)	TURB (n=57)	Total (n=108)
Histopathology grade	Low-grade, n (%)	27 (53%)	32 (56%)	59 (55%)
	High-grade, n (%)	24 (47%)	25 (44%)	49 (45%)
Detrusor muscle	**n (%)**	**49 (96%)**	**45 (79%)**	**94 (87%)**
TNM	Ta, (Tis), n (%)	32 (63%)	37 (65%)	69 (64%)
	T1, n (%)	19 (37%)	20 (35%)	39 (36%)
Recurrent rate	**n (%)**	**9 (18%)**	**21 (37%)**	**30 (28%)**
Clavien-Dindo	CD grade I	2 (4%)	3 (5%)	5 (4.5%)
	CD grade II	6 (12%)	7 (12%)	13 (13%)
Complications	ONR, n (%)	5 (10%)	8 (14%)	13 (12%)
	BP, n (%)	2 (4%)	3 (5%)	5 (4.5%)
Operation time	Minutes	34 ± 8	30 ± 11	32

Note: En-bloc – En-bloc transurethral resection of bladder tumor, TURB – Transurethral resection of bladder tumor,
Low-grade – Low-grade papillary urothelial carcinoma, High-grade – High-grade papillary urothelial carcinoma,
Tis (CIS) – Carcinoma in situ, "flat tumor", Ta – Noninvasive papillary tumor, T1 – Invades subepithelial connective tissue, CD – Clavien-Dindo,
ONR – Obturator nerve reflex, BP – Bladder perforation.

rences occurred in patients with high-grade histological result, recurrence rate in En-bloc group occurred in 18% and in TURB group in 37%.

Discussion

The initial treatment of all bladder tumors is the gold standard in treatment of non-muscle-invasive bladder cancer which is the accurate transurethral resection of the tumor. Full resection of all visible tumors with the histological examination is the standard care that must be performed [4, 8].

Kawada et al. were the first research group to present a case report on En-bloc resection using a rotational resection technique which remains the method of choice to date [9]. Until now, subsequent research approaches adopted the method described by Kawada et al. or modified it for use with different energy sources (monopolar or bipolar electrical current, Hybrid-Knife, holmium: YAG, thulium: YAG, and KTP laser), although these different energy sources yielded similar results for the evaluated parameters [10].

The specimen is of good quality and well oriented, which makes it easier for the pathologist to assess tumour stage [11]. The En-bloc resection technique seems well tolerated and feasible to use in selected cases. One major goal of En-bloc resection is to improve the quality of endoscopic bladder resection. It is assumed that high-quality resection may decrease the need for second resections and allows for better risk stratification [12]. The presence of detrusor muscle within the specimens serves as a surrogate for resection quality [13-16].

The results of the histopathological examination (tab. 2) according to the WHO/ISUP 2004 and TNM classification in booth groups have shown similar results and averaged consist: stage Ta was detected in 69 (64%) cases and stage T1 was in 39 (36%) cases of the patients, Low-grade papillary urothelial carcinoma in 59 (55%) patients and High-grade papillary urothelial carcinoma in 49 (45%) cases of patients included in the research. Detrusor muscles were detected in 49 (96%) cases of En-bloc group and 45 (79%) cases of TURB group.

A complete, high-quality TURB is associated with improved NMIBC outcomes. It was suggested that the quality of tumor resection could be surgeon related [17]. The recurrence rate was so low when TURB was performed by senior surgeons, thus showing a learning curve to perform high quality resections [18].

Recently, Hayashida et al. reported the safety and usefulness of combined endoscopic mucosal resection (EMR) and En-bloc resection in NMIBC patients, where EMR was used to remove the tumor mass that protruded from the mucosa, using a polypectomy snare similar to that used for EMR, while En-bloc resection was used to remove the residual lesion, they demonstrated that EMR combined with En-bloc resection is feasible, safe, and useful for treating patients with NMIBC [13].

According to Clavien-Dindo complication classification in both groups were registered complications grade I and II (tab. 2). The complications include: obturator nerve reflex, bladder perforation, hematuria of different intensity, urinary tract infection and urinary retention which was managed with standard approach, without additional problems. The tumor recurrence rate shows the benefit of En-bloc resection. In En-bloc group recurrence rate occurred in 18% and in TURB group – in 37%.

Age and grade were the most important prognostic factors for overall survival while the prior disease-recurrence rate and number of tumors were the most important prognostic factors for disease recurrence. Important to mention is that such factors as stage and grade were relevant for disease progression and also for disease-specific survival. T1 high-grade (T1G3) patients do poorly, with one- and 5-year disease-progression rates of 11.4% and 19.8% respectively [19].

Conclusions

The En-bloc resection technique of non-muscle-invasive bladder tumor proved to be a safe and effective method compared to the conventional transurethral resection technique (TURB). This method provides more favorable results for obtaining better quality tumor samples (present of detrusor muscle) that allow to establish correct diagnosis and staging of the disease and reduces the number of recurrences.

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IV and AP acquired, interpreted the data, drafted the first manuscript, IV performed most of the analyzed interventions, VG designed the trial and revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

Funding
The study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy. 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 was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (protocol No 4 of December 16, 2016). Written informed consent was obtained from all participants in the study.

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

REVIEW ARTICLES

https://doi.org/10.52418/moldovan-med-j.64-2.21.13
UDC: 616.124-008.46:616.74-007.17-053.2

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Cardiomyopathy secondary to Duchenne muscular dystrophy in children

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Manuscript received January 11, 2021; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: Cardiomyopathy (CM) associated with Duchenne muscular dystrophy (DMD) is a commonly recognized appearance of this neuromuscular disease, significantly increased morbidity and mortality, as well as the necessity for cardiological management. CM in DMD is defined by left ventricular (LV) systolic dysfunction and both atrial and ventricular dysrhythmias and is associated with higher mortality than other cases of pediatric dilated CMs. Notwithstanding the high rate of cardiac involvement, patients are usually asymptomatic despite significant LV dysfunction, because of likely poor mobility that masks the usual heart failure (HF) symptoms. Also, imagistic predictors are provided to be very helpful in defining early LV dysfunction, especially electrocardiogram and cardiac imaging (transthoracic echocardiography, speckle-tracking, cardiac magnetic resonance) are used to detect the onset and progression of dilated cardiomyopathy (DCM) in DMD.
Conclusions: As most DMD patients are asymptomatic for a long time of their life, so identifying predictors of HF is crucial to support these patients. Ventricular dysfunction based on the ejection fraction (EF) measurement helps to choose therapy. In the case of early DCM (LVEF≥50%) the great purpose is to prevent ventricular dysfunction incipience with first-line HF therapy with Angiotensin-converting-enzyme inhibitors (ACE-I) or angiotensin receptor blockers (ARBs). Current guidelines recommend the use of conventional HF medication in case of disease progression and DCM with Mid-Range Reduction of LV EF (40-49%). The therapeutic approach for patients with DCM and severe ventricular dysfunction (<40%) has been studied less profoundly and contemporary guidelines recommend all drugs used for HF treatment.
Key words: Duchenne muscular dystrophy, cardiomyopathy, heart failure, neuromuscular.

Cite this article
Rodoman I, Palii I, Sacara V, Gladun S. Cardiomyopathy secondary to Duchenne muscular dystrophy in children. *Mold Med J.* 2021;64(2):70-78. https://doi.org/10.52418/moldovan-med-j.64-2.21.13.

Introduction

Duchenne muscular dystrophy (DMD), defined more than 150 years ago, is the most widespread type of muscular dystrophy in children, affecting 1:3500/5000 liveborn boys [1, 2]. DMD belongs to the group of dystrophinopathies, affected by genetic mutations in the gene that codes for dystrophin and defined by variable degrees of skeletal and cardiac muscle impairment. Typically, DMD is the most severe form while Becker muscular dystrophy (BMD) is more benign form along with the X-linked DCM (XL-DCM) and the cardiomyopathy of DMD/BMD carriers [3-5].

Mutations linked to the dystrophin gene occur in the short arm of the X chromosome (Xp21.1), which describes the largest known gene, composed of 79 exons [6, 7]. Mutations causing a shift in the open reading frame occur in the lack of dystrophin, leading to the DMD phenotype. X-linked recessive inheritance model is typical for this disease; a mother-carrier has a 50% chance of transmitting it to her boys. Although most DMD mutations are inherited, spontaneous mutations can happen in up to 30% of cases [8].

The major presenting manifestation of DMD is skeletal muscle weakness. Notwithstanding a family history, this diagnostic should be suspected in boys manifesting gait disturbances occurring between two and five years of age, followed by proximal weakness, delayed psychomotor development, calf hypertrophy, and high levels of liver transaminases. The primary screening includes the dosage of the enzyme creatine phosphokinase (CPK), which is usually high (> 2000 U/L). Confirming tests range according to availability, but they embrace genetic tests that show specific gene mutations (deletions, duplications, point mutations) or muscle biopsy revealing the deficiency of dystrophin [9].

However, the heart muscle is affected by multiple muscular dystrophies. Cardiomyopathy associated with DMD is a commonly recognized appearance of this neuromuscular disease, significantly increased morbidity and mortality, as well as the necessity for cardiological management [10]. Progressive muscular damage leads to increasing muscular weakness and motor dysfunction, motor development delays, and impairment in respiratory and cardiovascular

function. Until recently, respiratory failure secondary to neuromuscular dysfunction was the most common reason for death, but at the moment dilated cardiomyopathy (DCM), arrhythmias, and congestive heart failure (HF) represent the most life-limiting heart condition in DMD [9, 11, 12]. Nevertheless, the development of respiratory care across the last few years has made cardiomyopathy (CM) an increasing cause of morbidity and mortality in DMD patients [13-15].

DMD DCM is described by left ventricular (LV) systolic dysfunction and arrhythmias, which are allied with higher mortality than other cases of pediatric dilated CMs. Quick detection and management are associated with postponed progression of LV dysfunction and advance in results. However, DMD CM remains underrecognized and undertreated broadly because the symptoms of heart failure in these patients are frequently neglected and not clinically apparent [16].

Epidemiology

The prevalence of DMD in the USA, Australia, England, and Canada is estimated to be 1 per 3500 to 50000 male births [17]. But according to the data of Sacară et al. the frequency of DMD/BMD is 9.13:100000 for the Republic of Moldova [18, 19]. The DMD onset mean age is 3–5 years and patients usually remain in the non-ambulatory stage till the age of 10–12 [15, 16]. The symptomatic HF incidence is rare early in childhood but increases at the age 10-20. Prospective studies have estimated the prevalence of DCM and noted it in the third of the patients under 14 years, half in those who were 18 years old, and almost all patients who were older than 18 years [14-17]. There have also been records of patients with X-linked DCM with low clinical evidence of skeletal muscle disease due to compensatory upregulation of the brain (B) isoform in skeletal muscle but not cardiac myocytes, although this is a unique phenotype [12, 20]. Female carriers have a chance to be influenced by this disease, although men manifest cardiac dysfunction at a significantly more youthful age than women. The awaited life prognosis with modern therapies, such as assisted ventilation is 25–30 years of age [15, 16].

Pathophysiology of DMD DCM

DMD is caused by mutations in the dystrophin gene that result in the reduction or absence of the sarcolemmal protein dystrophin, a protein that plays a fundamental role in the cytoskeleton of muscle cells by attaching intracellular structures with the extracellular matrix [21]. Some pathomechanisms have described cellular impairment initially caused by the absence of dystrophin, in both skeletal and cardiac muscles.

Dystrophin is located on the cytoplasmic side of cardiac and skeletal muscle sarcolemma and contributes to structural maintenance. It is a component of a larger glycoprotein complex and is also involved in cellular regulation and signal transduction. In cardiac muscle, the lack of dystrophin ends in instability and progressive degeneration of the muscle fibers through membrane vulnerability with continuous muscle compressions [22, 23].

Ordinarily, dystrophin presents structural maintenance for the myocyte and sarcolemmal membrane by its binding of actin at the C amino-terminus with the dystrophin-associated protein complex and sarcolemma at the carboxyl-terminus and the extracellular matrix of muscle [6, 24].

Dystrophin is additionally existing in the T-tubular membranes of cardiac myocytes and is implicated in the supporting of membrane stability and transduction of mechanical energy from the sarcomeres to the extracellular matrix. The loss of the dystrophin guides to an extreme vulnerability of the cellular membranes; cellular stress could be immediately interfered with by the absence of dystrophin, or obliquely via intracellular Ca2+ overload or oxidative stress.

Abnormal calcium handling due to the lack of dystrophin is believed to perform a significant function in the pathogenesis. Damage of cell membrane integrity and formation of breaks may result in high calcium levels in muscle fibers. Stretch-activated channels may perform abnormally leading to increased calcium entry. Elevated cytoplasmic calcium may activate proteases, such as calpain and increase the generation of reactive oxygen varieties producing injury to cellular proteins and membranes [25, 26]. Reactive oxygen kinds activate NF-KB pathways directing to increased pro-inflammatory cytokines. Inflammation may be harmful, promoting additional muscle degradation and necrosis [25, 26].

The generation of these damaging cellular pathways and Ca2+ pathways manage to dystrophic DCM [27]. As muscle syndrome advances, skeletal and cardiac myocytes necrotize, and mechanisms of repair are also not enough, with consistent gradual replacement by fibrofatty tissue [28].

DMD DCM is marked by a weaker LV wall and its progressive dilatation, reflecting the continuing myocyte damage [9, 29]. In particular, the constant mechanical stress manages to apoptosis and fibrotic replacement and scarring that progresses from the epicardium to the endocardium, starting at behind the posterior region and mitral valve apparatus. This scarring develops downward progressively via the apex and around the heart, finally attending to DCM [30-32].

Disease predictors

Contemporary 2018 DMD Care Guidelines estimated that regular cardiac follow-up is crucial for care [16]. From the time of the final diagnosis, all energy should be directed to detect the early incipience and the progression of the DCM, which is very challenging.

Notwithstanding the high rate of cardiac involvement, patients are usually asymptomatic despite significant LV dysfunction, because of likely poor mobility that masks the usual HF symptoms. Dyspnea on exertion or decreased exercise capacity goes unnoticed due to concomitant skeletal muscle weakness. Orthopnea and paroxysmal nocturnal dyspnea can be missed due to the use of nocturnal non-invasive mechanical ventilation for rest [15, 16, 33].

Early identification is so important for treatment, conditioning the life expectancy. In the non-ambulatory stage, an

asymptomatic patient's continuing check-up is necessary to determine the progress of the disease. Clinical examination remains difficult because of low BP rates, cool extremities due to reduced skeletal muscle mass. Therefore, these clinical features require a multidisciplinary evaluation to distinguish ongoing of the cardiac process.

However, enhanced survival of patients with DMD has contributed to an increase in overall the incidence and prevalence of CM [15]. Early detection is a key in preventative treatments that can delay the progressive deterioration in cardiac function and the start of overt HF symptoms.

Several biomarkers are currently used in the diagnosis and monitoring of cardiac disease. One such biomarker is cardiac troponin I, which is discharging during myocardial cell injury and classically utilized for the diagnosis and evaluation of myocardial infarctions. Also, serum biomarkers are provided to be very helpful to define HF and are currently used to evaluate the functional state in adult and pediatric patients.

Electrocardiogram and cardiac imaging are also routinely used to detect the onset of DCM and its progression [29]. These non-invasive tests provide useful information about the ventricular function, both systolic and diastolic ones.

Cardiomyopathy in DMD is described by progressive fibrosis in LV finalizing into its dysfunction and dilatation [9]. Echocardiographic precursors of cardiac dysfunction appear in 10-20 years of life and are almost completely present in all adults [33]. So, the investigation into recognizing additional, potentially modifiable predictors for CM progression is limited. Studies in *mdx* mice, a mouse model of DMD, show that dystrophin-lacking myocardium is more exposed to pressure overload of the LV than normal one [34]. Increased LV afterload may pose the already weak myocardium of DMD patients at risk for accelerated myocardial dysfunction. LV afterload is influenced by hypertension, obesity, and aortic stiffness [35].

DMD DCM and other comorbidities

Some studies have distributed cross-sectional data on the prevalence of hypertension in DMD patients [36-38]. Ricotti et al. found hypertension in 5% of the patients aged 3–15 years on steroids while in a study by Braat et al. 45% of the patients had hypertension [37, 38]. Wong et al. reported that 25.5% of patients on daily steroids aged 10–13 years had systolic hypertension [36]. In patients aged 13–16 years, they reported systolic hypertension in 10.3% of patients. One study described a correlation between low blood pressure (BP) ranges and younger age in DMD with Hispanic origin as an involving predictor [39].

Obesity is commonly found in DMD due to corticosteroids using and low mobility [40]. Prevalence of obesity has been described in up to 73% DMD patients on steroid treatment <13 years and higher body-mass index (BMI) was correlated with longer duration and greater cumulative dose in ambulant DMD patients utilizing prednisone [41, 42]. Increased BP is a well-known side outcome of corticosteroids [43]. But its utilization has led to the ambulant stage prolongation in DMD by almost 3 years [44]. It has also been correlated with a delayed onset of DMD-related cardiomyopathy [45, 46]. Corticosteroid treatment is thus an essential part of the standards of care in DMD and is prescribed from the age of 4 to 5 years onward. Extensive research analyzing BP values in DMD patients with and without steroids will therefore not be feasible, and the high percentage of patients on steroids in this study may well be the cause that steroid use was not an objective factor in the linear mixed model.

In N.M. van de Velde et al. study increased BMI, but not systolic BP was linked to early myocardial deformation defined by peak systolic global longitudinal strain (GLS) in young DMD patients < 11 years of age. The results of this study propose that factors influencing afterloads, such as increased BP and BMI, may play an important role in the degeneration of cardiac function in DMD [47].

Genotype-phenotype correlations in DCM-DMD

The dystrophin gene is the largest gene identified in humans, and its complexity and correlation to phenotype remain to be analyzed [48]. Inheritance is in an X-linked recessive manner. Genetic mutations are generally out of the frame and cover duplications, deletions, frameshift (nonsense and splice), missense, and premature stop codon [49]. Numerous attempts were tried to order DMD patients based on their genotype. Where the mutation happens within the gene has some correlation for cognition (i. e. mutations upstream of exon 30 correlates with spared cognition), but not motor function [50].

There are limited data correlations in genetic mutations and CM in DMD. It is still an open question whether variations in genotype indicate myocardial dystrophin expression, and consequently what impact they will produce on cardiac function. Therefore, Jefferies et al.'s study proved that mutations in exons 12 and 14 - 17 were associated with cardiomyopathy but mutations in exons 51 and 52 appeared to be protective against cardiac involvement [51]. However, other researches using different techniques have been uncertain [49].

Biomarkers in DMD DCM

Cardiac troponin may be a predictor of CM secondary to DMD. Troponin I is released in the circumstances of DMD heart disease, likely as a consequence of membrane integrity loss rather than a primary ischemic etiology, but there are contradictory results about their diagnostic and prognostic involvements in the DMD DCM [52-54].

High cardiac troponin levels are strongly correlated with left ventricular dysfunction and may indicate the progression of the cardiomyopathy when acute chest pain is present [55]. It also seems to correspond with the conclusion of myocardial fibrosis on cardiac MRI [56].

Recently, one study revealed that troponin I levels were significantly elevated in patients with moderate late gadolinium enhancement (LGE) related to no LGE and constant over all ages, and it is interesting, that absence of positive correlation among mild-to-severe LGE and troponin is presumably because of a reduced dystrophin amount at advanced disease stages when most of the heart muscle has already been replaced by conjunctive tissue. Therefore, tro-

ponin I could provide valuable data to control patients in the clinical practice, and extra studies are needed [56].

Based on the above, troponin can be a valuable minimally invasive outcome marker to find early myocardial disease implications and understand the origin of damage in DMD CM.

High left atrial pressure as a consequence of pulmonary hypertension and LV dysfunction induced by respiratory muscles impairment are supposed to be implicated in appearing of high levels of plasma natriuretic peptide in DMD. In patients with a non-ambulatory stage of DMD was observed mild or marked elevation of alpha-ANP levels being a sign of a lower prognosis and may be a useful predictor for disease management [57]. Villa et al. in their study reported a meaningful association among cystatin C, eGFR, and heart muscle dysfunction, contributing to the newest predictor to confirm cardiorenal syndrome in children with DMD [58].

In Carol A. Wittlieb-Weber et al. study, BNP levels measured before dying or research end were significantly higher for those patients who died compared to those living at research finish [59]. These conclusions are compatible in Cheeran et al. study, who investigated a cohort of 43 DMD patients with CM, and discovered predictors in comparing the non-living to the living cohorts including lower BMI, maximum inspiratory pressures, and cardiac serum biomarkers [60].

Imagistic predictors in DMD DCM

Electrocardiographic abnormalities

Electrocardiographic (ECG) follow-up is obligatory and valuable for the cardiological evaluation of DMD patients. Electrocardiographic irregularities commonly found in patients with DMD can correspond to fibrotic alterations seen on pathology expertise [16, 61]. But, according to some reports, the ECG frequently does not correlate with CM development [62]. In the process of LV function failing, it is possible to find more frequently sinus tachycardia, supraventricular and ventricular arrhythmias. In Fayssoil et al. study bundle branch block is a significant predictor for cardiac situations, while patients with low dystrophin level show an increased incidence of cardiac events [63].

There can be different changes on a traditional ECG: sinus tachycardia, short PR, high R waves in right precordial derivations, right ventricular hypertrophy, and Q waves in left lateral and precordial leads (D1, aVL, V5, V6), narrow and deep Q waves. The sinus tachycardia phenomenon is the most described finding in DMD. It is also essential to remark that RSr' pattern and high R waves in V1 can be normal in childhood, with no association with heart disease [64]. Also, there are some data about the correlation between the left bundle branch block and mortality in adult patients on mechanical ventilation [63]. Significant arrhythmias in the 24-h Holter ECG, especially atrial and ventricular tachycardias, were uncommon findings in patients with ejection fraction (EF) more than 35% and had low clinical applicability in patients with preserved EF [65].

Transthoracic Echocardiography/ Speckle-Tracking

Echocardiography performs the main function in LV dysfunction identification and also dynamic evaluation is required. According to the literature, local anomalies of LV function may be resolved by other imaging investigations, such as speckle tracking echocardiography (STE) or cardiac magnetic resonance (CMR) [66]. Dilated LV is assessed via standard deviations, estimated with Z-scores compared with age, BMI in DMD patients. LV dysfunction is considered lower than 55%, and fractional shortening (FS) lower than 28% [67, 68].

Correlation of 2D and 3D echo techniques for LV end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV) was significantly positive, but 3D LVEDV and LVESV were lower in comparison with 2D outcomes; while, LV EF estimation was similar in two methods [69]. According to some reports, FS is rated the best evaluator of LV systolic function, because of its independence of age and magnitude of measures [70]. In the matter of diastolic function, in DMD patients can be found the followuing echocardiographic abnormalities: high mitral A-wave velocities and lower E/A ratio, lower DTI lateral peak E-wave velocities [70].

Another predictor of LV dysfunction in DMD patients is the myocardial performance index (MPI) gained by using pulse-wave Doppler and Doppler tissue imaging. Based on the intraclass coefficient correlation, MPI obtained with Doppler tissue imaging was more reproducible.

STE is a modern technique capable to determine subclinical LV dysfunction before the overt LV EF reduction, that is recently frequently used in DMD patients. The myocardial strain is irregular in almost 50% of DMD patients, showing lower GLS values compared to the control group notwithstanding a normal LV EF [71, 72]. Furthermore, a reduction of 0.34%/year of GLS value in DMD patients according to age has been described [73]. In this study, in patients with DMD were observed differences in longitudinal (3.6%), radial (9%), and circumferential (3.8%) strains compared to healthy controls, with significantly lower values in the inferolateral and anterolateral mid-basal segments [73].

Another retrospective studies previously described circumferential and longitudinal strains in DMD patients with a greater difference for these indicators [67, 70]. However, STE analysis is often restricted in DMD because the echocardiographic picture quality is low in these patients due to chest malformations, lung hyperinflation, and limited mobility and decays by 2.5% for each 1-year in plus in age [66]. There was a suboptimal echocardiographic quality, defined as more than 30% of segments incompletely visualized, observed in 50% of 13-year-old DMD patients and 78% of 15-year-old patients [66].

Surely, LV EF, obtained by echocardiography, has been demonstrated to associate with CMR, while 2-D FS and 5/6 area length LV EF correlated strongly with CMR LV EF [26, 67].

Right ventricular (RV) function is frequently protected

in DMD patients, accompanying by LV dysfunction, presumably because of the decreased afterload of respiratory changes. In Mehmood et al. study normal RV values were reported in subjects with severe LV dysfunction, and only in few cases there have been described advanced RV dysfunction [74].

Therapeutic management for DCM-DMD

Usually, HF changes the definition to the manifestation of clinical symptoms. Most DMD patients are asymptomatic for a long time of their life, so identifying predictors of HF is essential to maintain these patients. Ventricular dysfunction based on the EF measurement helps to provide therapy. DMD DCM can be classified as normal LV EF (≥50%), mid-range (40-49%), and reduced LV EF (<40). In the following section, all cardiovascular drug therapies are described according to LV EF [75].

Early DCM (LVEF≥50%)

The great purpose is to prevent the ventricular dysfunction incipience at this stage of the disease. Because of specific therapy absence for HF in DMD, 2018 DMD Care Guidelines recommend traditional first-line HF therapy with angiotensin-converting-enzyme inhibitors (ACE-I) or angiotensin receptor blockers (ARBs) [16].

In 2005, Duboc D. reported results of five-year study for the prophylactic use of perindopril in DMD patients. This research had to estimate the perindopril effect on LV dysfunction history. In this study, 57 children aged 9.5 to 13 years with a normal cardiac test and LV EF of more than 55%, were randomized to perindopril 2–4 mg versus the placebo group. The chi-squared analysis revealed a significant advantage for patients treated to prevent the progression of DCM, described as the decrease of LV EF under 45%. After this study, ACEi has been initiated to be prescribed in prophylactic scopes [76].

DMD DCM with Mid-Range Reduction of LV EF (40-49%)

Several kinds of research have been labeled for DMD patients with moderate systolic LV dysfunction and showed some beneficial effects to preserve its appearance. Current guidelines recommend the use of conventional HF medication in case of disease progression. In 2013, Allen H. compared the outcomes of lisinopril with losartan in a randomized, double-blind, controlled trial of 22 DMD patients, that showed no difference between lisinopril and losartan in controlling ventricular function [77].

The cardioprotective impact of eplerenone in combination with ACE-I or ARB was assessed by CMR after 12 months in 42 DMD patients. In this multicenter, randomized, placebo-controlled trial, Raman S. et al. revealed that eplerenone decreased the decline of magnetic resonance assessed LV circumferential strain and LV EF at 12 months when matched to the placebo group [78]. Also, Raman S. et al. showed that early MRA therapy is effective and safe in a genetic disease with high cardiomyopathy risk [79].

Accordingly, at the ambulatory stage of the disease, before any clinical overt DCM, the preventive use of perindopril for cardioprotection is started extensively in the clinical practice and endorsed by current indication although biological consequences are still unclear. When the DCM is detectable even in case of a mild reduction of EF (>45% LVEF), fosinopril or losartan with the combination of MRA can increase ventricular function.

Also, beta blockers (BB) have been tested. In 22 patients carvedilol was administered and progressively increased over 8 weeks. This therapy modestly improved cardiac CMR-derived measured EF (41% +/− 8.3% to 43% +/− 8%; $p < 0.02$), as well as the MPI (0.55 +/− 0.18 to 0.42 +/− 0.15; $p < 0.01$) and the mean rate of pressure rise (dP/dt) during isovolumetric contraction (804 +/− 216 to 951 +/− 282 mmHg/s; $p < 0.05$) [80].

Patients with Severe Ventricular Dysfunction (<40%)

The therapeutic approach for patients with significant DCM has been studied less profoundly and contemporary guidelines recommend all drugs used for HF treatment [12, 15, 81].

Although in adult HF, the use of BBs is obligatory in declining of ventricular function, the same evidence in children is absent. Last years, some retrospective and non-randomized prospective studies have confirmed the beneficial effect of BB therapy in patients with DMD [51, 79, 82-84], while in some others this positive effect was not detected [85, 86]. Although most of the studies are retrospective and included different ages, BB in addition to ACEi demonstrated to improve 5-year and 7-year survival rates [84], and also ventricular function [82]. These contradictory conclusions have provided to the variable and frequently insufficient beginning of BB in DMD. However, BB are usually combined with ACEi/ARB when a sufficient change in heart function is not assessed with basic therapy.

Moreover, in DMD, this therapy is often designated for the appearance of autonomic dysfunction and the following predilection to arrhythmias [87]. In the contemporary literature the most frequently used drugs are: carvedilol (0.01–0.02 mg/kg) administered twice daily and slowly increased to a dose of 0.5–1 mg/kg [51,80,82,83,85], metoprolol (1 to 2 mg/kg/day) [51, 86] and bisoprolol (3–4 mg per day) [84].

In some studies, combined therapy with ACEi/BBs has demonstrated to be better to single ACEi [80, 82] in the prevention of major cardiac emergencies [83] and long-term survival [84].

End-Stage of DMD DCM

It was recently demonstrated the utility of the HR reduction approach achieved with BBs and ivabradine (2.5 mg twice daily increasing until 15 mg daily every two weeks when HR was still above 70 bpm and LVEF < 40%) in the decrease of the long-term trend of acute adverse effects in DMD patients with advanced heart implication [88]. Previously, ivabradine demonstrated the effectiveness in HR reducing and LV EF improving in a multicenter, randomized, placebo-controlled trial in children with DCM and HF symptoms [89].MRAs, spironolactone, and eplerenone are recommended in all symptomatic patients (despite treatment with an ACE-I and BB) with HF and LV EF ≤ 35%, to decrease death and hospitalization conform to Ameri-

can and European Guidelines for the management of HF in adults [75, 90].

Shortly a new mineralocorticoid receptor antagonist (MRA) called vamorolone, will be able to imitate the anti-inflammatory impact of glucocorticoids, presumably could be a real alternative to both "old MRAs" and "glucocorticoids" in the DMD therapy [91]. To date, there are no clinical studies about the use of MRAs in an advanced stage of DCM in DMD patients. Despite this, eplerenone or spironolactone are used in these patients, at the cardiologist's decision, in combination therapy with ACEi and BB, till they do not manifest hyperkalemia or renal insufficiency.

Sacubitril/valsartan, the first-in-class angiotensin receptor neprilysin inhibitor (ARNI) has recently been approved by the Food and Drugs Administration for the treatment of children with symptomatic HF and systemic LV systolic dysfunction, that was based on the reduction in the NT-proBNP levels in sacubitril/valsartan groups compared to enalapril one after 12 weeks of treatment in PANORAMA-HF trial [92].

Advanced Cardiac Therapies

Heart Transplant and Mechanical Assist Device

A potential therapy for end-stage HF in these patients is the use of a left ventricle assist device (LVAD) as destination therapy (DT) [93, 94]. LVAD treatment significantly created a reverse ventricular improvement within various mechanisms: reducing ventricular volume, LV mass, hypertrophy, and improving its function [95-101]. The use of mechanical circulatory support in DMD was described in several case reports and small series [102-107*DMD Target Therapy*

Glucocorticoid therapy has been the standard for patients with DMD. Deflazacort and prednisone are the most recommended steroids. Their introduction changed the natural history of the disease, increasing the ambulation period, preventing cardiorespiratory insufficiency, and enhancing life-expectancy [16, 44].

The curative strategies for DMD have focused on replacing dystrophin expression or moderating the processes of dystrophin deficiency.

The strategies embraced for dystrophin protein restoration are the following: nonsense readthrough, antisense oligonucleotides for exon skipping, and gene therapy. Inflammation inhibiting, reducing fibrosis, promoting muscle regeneration, and mitochondrial function facilitating are used to mitigate the dystrophic processes.

This translational investigation has managed the acceptance of the first target therapies for DMD. Ataluren (Translarna™, PTC Therapy) is the first licensed drug for DMD in Europe. Ataluren is an oral molecule that connects ribosomal RNA subunits and lets ribosomal read through of mRNA including a premature stop codon. It is recommended for the therapy of DMD with a nonsense mutation in the dystrophin gene.

Recent FDA approved AONs targeting exon 51 (eteplirsen) and 53 (golodirsen) for DMD treatment [87].

Conclusions

Information about dystrophinopathies including DMD has been rapidly increasing since the initial study by Guillaume Duchenne. It is necessary to recognize that these patients, mostly teenagers and young people, need to be sufficiently managed with the best perspectives, and not with conformism and fatality, which in general quickly condemn patients with rare diseases, particularly with poor life-expectancy prognosis. In a careful but positive way, cardiologists should be mindful of recent studies to implement the best available follow-up and management.

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IR conceptualized the idea, conducted a literature review, drafted the manuscript; IP and SG revised and approved the final text; VS interpreted the data and added the necessary information to the manuscript. All the authors approved the final version of manuscript.

Funding
The journal publication fee was covered by Institute of Mother and Child. The study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy as a PhD program. The authors are independent and take responsibility for the integrity of the data and the accuracy of the data analysis.

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

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

https://doi.org/10.52418/moldovan-med-j.64-2.21.14
UDC: 611.77.018.1+57.085

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Comparative analysis of the skin decellularization methods

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Manuscript received February 24, 2021; revised manuscript April 12, 2021; published online April 28, 2021

Abstract

Background: The extracellular matrix plays an important role in the promoting the tissue regeneration and repair. Decellularization or removal of the cells from the complex mixture of the structural and functional proteins that constitute the extracellular matrix (ECM) can be done by the physical (agitation, sonication, freeze and thaw), chemical (alkaline orchids, ionic detergents, nonionic, tri-n-butyl phosphate (TBP), hypotonic or hypertonic treatments, chelating agents), and enzymatic methods (trypsin or protease inhibitors). However, complications associated with the use of the decellularized skin have been reported, which are widespread and poorly understood. In this synthesis have been included publications, identified by the Google Search engine, National Bibliometric Tool (NBT), Pub Med databases, Web of Science, Springer, Elsevier, Wiley Online Library, Science Direct and Biosience, Biotechnology and Biochemistry. The results of the decellularization were reported in terms of the number of cells remaining in the collagen fibers depending on the duration of exposure to chemical agents.

Conclusions: The natural matrix is more widely used than synthetic material, because it has the natural structure and composition of the ECM, it naturally stimulates cell development and allows the incorporation of the growth factors and other proteins increasing cell proliferation.The assessment of the quality of decellularization techniques is done by evaluating the necrosis of the extracellulare matrix, the depletion of the collagen fibers and the remaining amount of genetic material.

Key words: decellularization, extracellular matrix, cell proliferation.

Cite this article
Macagonova O, Risnic D, Cociug A, Nacu V. Comparative analysis of the skin decellularization methods. *Mold Med J.* 2021;64(2):79-86. https://doi.org/10.52418/moldovan-med-j.64-2.21.14.

Introduction

The extracellular matrix plays an important role in the promoting of the tissue regeneration and repair [1]. With the development of the decellularization technology, the extracellular matrix in the form of a new biomaterial has attracted the attention of many researchers. The extracellular scaffold is the ideal substrate for the tissue engineering, presenting a carcass on which the body's cells survive [2, 3]. The functions of the extracellular matrix as a support material and biological substrate are the regulation of the cellular metabolism, such as the cell proliferation, morphogenesis and differentiation [4, 5]. Norton L. et al. described the decellularization technology of the use of the non-denaturing anionic detergent [2, 6]. The objectives of the decellularization process are to remove the potential immunogenic material and to obtain a biocompatible carcass for the growing cell host. Decellularization or removal of the cells from the complex mixture of the structural and functional proteins that make up the extracellular matrix can be done by the physical methods (agitation, sonication, freezing and thawing) [7, 8], chemical methods (alkaline oracids, ionic detergents, non-ionic, tri-n-butyl phosphate, treatments with hypotonic, hypertonic substances [8], (EDTA) tetrasodium salt dihydrate chelating agents), enzymatic methods (trypsin) and protease inhibitors [9].

Following the decellularization of the skin, the remaining extracellular matrix is used in the tissue engineering [10], creating a skin graft used for the wound healing, the soft tissue reconstruction in the sports medicine [11–14]. Decellularized tissue samples are frequently used with a variety of the clinical applications. In the plastic surgery, the human acellular skin matrix was used for the tear duct repair [15–18], breast reconstruction [19–21], hernia repair [22–25], in the treatment of the chronic wounds, such as trophic ulcer of the diabetic foot [26–28]. With the development of the tissue engineering, the use of decellularized products is gradually expanding, as they can function as substitutes for the traditional biomaterials (e.g., polyurethanes, PLGA (poly (lactic-co-glycolic acid)), etc.) [29, 30]. Thus, they can serve as the inductive materials for the cell invasion. Therefore, the decellularization methods fall into three broad categories: physical, chemical and enzymatic [31, 32]. Most samples are prepared using a combination of the reagents, the most popular being chemical and enzymatic techniques [33, 34]. The effectiveness of the decellularization procedure

is characterized by the following parameters: the complete character of the removal of the cells and nuclear debris, the preservation of the matrix integrity, the tissue density and the ability of the cell repopulation. The decellularized matrix must be compatible for the cells and have a repairing phenotypic building material [35]. Natural scaffolding allows the invasion, proliferation and proper secretion of the cells, which is important for their survival and regeneration of the affected tissue [36–41]. The shortcomings of some decellularization methods are: the persistence of the residual deoxyribonucleic acid (DNA), which has a significant proinflammatory effect [37], inhibitory response on the cell proliferation and the cytotoxic effect [38, 40]. Researchers have described the factors that can lead to these negative effects on the matrix, being the residual detergents, sterilizing chemicals that change the structure of the scaffold [42]. The material was synthesized based on the randomized studies, clinical and preclinical experimental cases, published between 2003 and 2020, which aimed to elucidate the results of the skin decellularization. In this synthesis, the publications accessible in English, identified by the Google Search engine, National Bibliometric Tool, Pub Med databases, Web of Science, Springer, Elsevier, Wiley Online Library, Science Direct and Biosience, Biotechnology and Biochemistry, from the databases of the life science journals and online books by the keywords are exposed in the table 1.

The literature synthesis was performed using 61 sources from PubMed, 11 – through the National Biometric Tool, 5 – Springer, 2 from ScienceDirect, 2 – Web of Science, 1 from Biosience, Biotechnology and Biochemistry, 1 – Wiley Online Library and 1 from Elsevier.

For the advanced selection of the bibliographic sources, the following filters were applied: papers published until September 2020, articles in Romanian and English. Original research journals were selected informing about studies, conducted in the clinical, preclinical and experimental conditions. After examining the titles of the articles obtained, only works containing relevant information on the skin decellularization methods were selected. The bibliography of the selected articles was also studied, in order to find all potentially significant sources of the intended purpose. The information was systematized, highlighting the main aspects of the contemporary vision on the obtaining of the extracellular dermal matrix. If necessary, the additional sources of the information were consulted to clarify some notions. Duplicate publications, articles that did not correspond to the purpose of the paper and were not accessible for viewing, were excluded from the list of the publications generated by the Search engine.

The evaluation of the decellularization of the biological scaffold for the tissue engineering was based on the cellularity of the matrix [43]. The studies were performed on the fragments of the rat, pig and human skin, taken from the back and abdomen in the first 24 hours after the euthanasia of the animals or the death of the donor. Prior to processing, in order to wash the tissue of impurities, the skin was stored in cold phosphate buffer (PBS) with antibiotic (0.1% Amikacin). Thereafter, the skin was rinsed with PBS to remove the blood residue. The maximum time between the skin sampling and tissue decellularization initiation was up to 4 hours. Deepithelated skin can be obtained by different methods: the mechanical removal of the epidermis [44], osmotic method, enzymatic method with trypsin (0.5%) and 2M sodium chloride [45, 46]. All decellularization reagents were evaluated by point of view of the mechanism of the action and the effect on the extracellular matrix [2, 44, 47]. The isolated material was characterized by the histological examination by eosin and hematoxylin staining, Massom trichrome and spectrophotometric quantification of the nucleic residue.

There are the different reagents and techniques (chemical, physical and enzymatic) of the skin decellularization and usually these methods are used in the combination to increase the effectiveness of the decellularization process. A dermal matrix is made using a three-step method. First, the epidermis is removed using a chemical process. The next decellularization process consisting of the breaking of the lipid-lipid, lipid-protein bonds, solubilization of the cell membranes, osmotic lysis of cells, dehydrating and rupture of the cell membrane finally to varying degrees, will dissociate DNA. The last one, a subsequent washing will remove any residual cellular elements or chemicals [48].

Kumar N. et al. [44] developed the successful techniques for the deepithelialization of the skin by the hypertonic solution. The composition of the substances dissolved in 100 ml of the phosphate buffer solution was as follows: 605 mg of tris base, 4 grams of sodium chloride, 202.5 mg of

Table 1. Search engines and the keywords used in the synthesis

Key words	NBT	PubMed	Web of science	Elsevier	Springer	Wiley Online Library	BBB	Science Direct
skin biomaterials	23	7468	84	2482	3518	4	20	28766
skin tissue engineering	48	479	2752	17308	40967	465	49	77409
decellularization	8	1873	1697	1900	20	2624	4	5894
skin DNA	2	57	569	1826	9	611	193	250463
ECM	32	27909	1256	107	130	51386	495	652
skin	634	791091	2200	1826	2562	1706	938	100000+
skin cellularity	2	864	77	5118	2717	6	214	332168

EDTA. The skin was washed on an orbital shaker at 37° C for 8 hours at 150 rpm. The solution was changed every 4 hours. Macroscopic and microscopic examination was performed at intervals of 4 and 8 hours [44].

Obtaining of the qualitative matrix required the combination of the reagents or the independent action of ones: 1) 0.5% sodium dodecyl sulfate (SDS) with 0.1% EDTA [3]; 2) trypsin combined with EDTA assisted of 1% triton X-100 and 0.26% tris (2-Amino-2-(hydroxymethyl)-1.3-propanediol) [46]; 3) hypertonic solution with 2-Amino-2-(hydroxymethyl)-1.3-propanediol (tris), sodium chloride and EDTA; 4) 1% triton X-100 combined with 0.25% TBP; 5) 0.5% sodium dodecyl sulfate (SDS) with 0.25% tri-n-butyl phosphate; 6) 1% or 2% sodium deoxycholate (SD) folloved by the action of the deoxiribonuclease [44]; 7) hypertonic solution of 1M sodium hydroxide (NaOH) perfects the decellularization action of the 0.25% trypsin – EDTA solution [47]; 8) freeze-thaw cycling (-80°C, six times) with ammonia water (25 mM); 9) 0.1% triton X-100 with 1.5M K Cl aqueous solution; 10) freeze-thaw cycling alone; 11) ammonia water alone; 12) triton X-100 extraction; 13) osmotic shock with 1.5M K Cl; and 14) and freeze-thaw cycling with 3M NaCl [49].

All reagents were helped by the continuous agitation at the room temperature or 37° C in a thermostat for 24 and 48 hours on an orbital shaker at 150 rpm. The solutions were changed at different intervals of 6, 12, 24 and 48 hours. Finally, the tissues were rinsed thoroughly several times with sterile buffer or the distilled water on the orbital shaker. Macroscopic and microscopic examinations were performed at 12 and 48 hours.

Results and discussion

As a result of the processing of the information identified by Google Search engine, National Bibliometric Tool, Pub Med databases, Web of Science, Springer, Elsevier, Wiley Online Library, Science Direct and Biosience, Biotechnology and Biochemistry, from the databases of the life science journals and online books according to the search criteria 879664 articles were found that address the issue of the skin decellularization. After the primary analysis of the titles, 201 articles were qualified as possibly relevant for the given synthesis. After their repeated review, 84 publications relevant to the stated purpose were finally selected.

As a result of the systematization of the literature data, it was highlighted that the normal structure of the skin served as a template after which the decellularized sample was evaluated according to twenty-one decellularization methods compared for their decellularization effects during skin scaffold preparation (table 2). The authors described the thick epidermis followed by the cellular dermal matrix. Masson trichrome staining showed a preserved cell epidermis, the dermis – with an abundance of the collagen fibers [44].

The final bibliography of the paper included 84 publications. As a result these methods were combined in fourteen separate decellularization protocols [2, 44–47, 49].

The macroscopic estimation of the deepithelialized rat skin with the hypertonic solution for an interval of 4 hours found that the epidermis was not separated from the dermis. Thus, after 6 hours the multilayered epithelium was removed more easily. However, after another 8 hours the epidermis was spontaneously separated with minimal mechanical effort and resulted in an incompletely epithelialized dermal matrix [47].

In this study, twenty-one decellularization methods were evaluated by which the incomplete acellular dermis was obtained (table 2). The treatment of the skin with the hypertonic saline after 24 hours resulted the cell-less matrix with collagen fibers with insignificant thickness. The deepithelialized skin treated with triton X-100 was characterized by more significant cell-less and with the thick collagen fibers. There were cell debris between the interstitial spaces of the collagen fibers [47]. At 48 hours, the complete acellular dermis with increased porosity was described. The treatment of the skin by SDS over 24 hours showed the cell-less membrane with the collagen fibers with the significantly preserved thickness [2]. At 48 hours, the collagen fibers were more fragile with large spaces between them. Treatment with 1% SD effectively removed the cell debris at 48 hours. Increasing of the concentration from 1% to 2% of SD, led to the expansion of the spaces between the collagen fibers. No cell nuclei were observed, and the tissue was composed of the extracellular matrix. At 48 hours,

Table 2. Overview of the techniques used in the skin decellularization

Methods	Mechanism	The effect on ECM	References
Chemical			
Alkaline and acids: ammonium hydroxide, hydrochloric acid, sulfuric acid	Solubilizes the cytoplasmic components of cells; disrupts nucleic acids	Eliminate glycosaminoglycans (GAGs), Dissociates GAGs from collagenous tissues	[50–52]
Peracetic acid	More effectively disrupts cell membranes	Preserves many of the native GAGs, preserves the structure and function of many growth factors that are resident in the ecm, including transforming growth factor-β, essential fibroblast growth factor and vascular endothelial growth factor, highly efficient in removing cellular material	[53, 54]

Non-ionic detergents			
Triton-X-100	Breaks the lipid-lipid and lipid-protein bonds, leaving the protein-protein bonds intact	Almost completely eliminates GAGs, reduced the laminin and fibronectin content	[50–52]
Ionic detergents			
Sodium duodecyl sulfate (SDS)	Solubilizes cytoplasmic and nuclear cell membranes	Removes nuclear debris and cytoplasmic proteins (vimentin); tends to disrupt the structure of the native tissue, reduced concentrations of GAG and loss of the collagen integrity	[50–52]
TBP	Tend to distort proteins	Keep resistance of of the collagen fibers, but reduce in the collagen content More effective than detergents such as Triton X-100 and sodium dodecyl sulfate (SDS), with varying effects on the preservation of ECM constituents and its native mechanical properties	[55]
Sodium deoxycholate	Solubilizes the cell and cytoplasmic membranes	Disrupts ECM components more strongly than SDS	[56]
Hypotonic and hypertonic solutions	Cell lysis by osmotic shock	Effective for cell lysis, but do not effectively remove cell debris	[50–52]
EDTA	Chelating agents that bind divalent metal ions, thereby disrupting cell adhesion to ECM	Relatively reduce the cellularity of the ECM	[57]
Solvents			
Alcohol	Dehydrating and lysing of the cells during tissue decellularization	Decreases levels of structural proteins involved in the interstitial matrix and basement membrane, with a concomitant increase in proteolytic enzymes that degrade these components	[58–60]
Glycerol	Dehydrating and lysing of the cells during tissue decellularization	Collagen fiber reassembly, increases the tissue transparency	[61]
Acetone	Removes lipids during decellularization	Damages the ECM ultrastructure	[62, 63]
Enzymatic			
Trypsin	Removes the peptide bonds of arginine and lysine	Disruption of the ECM, prolonged exposure eliminates laminin, fibronectin, elastin and GAGs, does not influence the amount of collagen in the tissue, decreases tensile strengthn of the collagen fibres	[50–52]
Endonuclease	Cleaves phosphodiester bonds within a ribonucleotide and deoxyribonucleotide chains	Difficult to remove from tissue and can invoke an immune response	[50–52]
Exonuclease	Cleaves nucleotides from the end of a nucleic acid chain	Slightly reduces ECM cellularity	[52]
Dispase	Cleaves specific peptides, especially collagen IV and fibronectin	Slightly reduces ECM cellularity	[64]
Phospholipase A2	Hydrolyzes the phospholipid component	Catalyzes the release of arachidonic acid in the cells, phospholipase A2 -arachidonic acid system is involved in the matrix-initiated signal transduction pathway in ECM, stimulates the ECM cell proliferation by homologous lectin	[58]
Physical			
Freezing	Intracellular ice crystals disrupt the cell membrane	Relative ECM disturbance	[59, 60, 51]
Freeze–thaw cycling	Cell lysis	Freeze–thaw cycling alone could not remove all the cell nuclei	[49]
Pressure	Rupture of the cell membrane	Relative disturbance of ECM	[65]
Agitation	It is used to facilitate chemical exposure and removal of cellular material	Aggressive agitation or sonication may disrupt ECM	[66]
Electroporation	The oscillation of the electric field disturbs the cell membrane	Partial cell membrane lysis	[49]

all samples treated according to the protocols showed the complete cell-less matrix with the removal of the cellular debris from the tissue [64].

The hypotonic and hypertonic solutions have been reported as the ineffective decellularizing agents [64–66]. They caused the cell lysis but did not remove the cell debris from tissues [64]. TBP treatment has led to a complete removal of the nuclear waste. TBP did not affect the resistance of the collagen fibers but led to decrease the GAGs content [67].

The cellular content of ECM has the potential to cause rejection when is grafted, therefore it must be removed before the transplantation. DNA and Gal-epitope are two main reasons why the host can respond. The epitope Gal, the α-Gal oligosaccharide (Galα1.3-Galβ1–4GlcNAc-R), is a membrane antigen present in all species, except for Old World monkeys and humans. The absence of α-gal expression in the humans and non-human primates is related to the defects in the α1.3-galactosyl-transferase gene, which catalyzes the assembly of the α-gal molecule in other animals [68]. Because humanity does not have this antigen, transplanting a xenograft leads to a host reaction, causing the graft to be rejected. Therefore, the Gal epitope must be removed from the xenografts before being transplanted [31]. Because the DNA left in the graft can cause inflammatory reactions in the host it must be removed during decellularization. Another reason why DNA needs to be removed is that it causes calcification after implantation. However, because most tissues are very dense, DNA is almost impossible to be 100% removed. Therefore, DNA remaining after the decellularization should be examined quantitatively or qualitatively, that is no image should be obtained after the staining with 4',6-diamidino-2-phenylindole (DAPI) or hematoxylin and eosin (H&E) [42, 69].

Criteria for the assessing the effectiveness of the removal of these components are suggested as follows: Decellularized ECM must have (1) less than 50 ng double-stranded DNA per mg dry weight, (2) less than 200 bp DNA length fragment and (3) no nuclear material visible by 4', 6-diamidino-2-phenylindole or hematoxylin and eosin staining [70]. In addition, the protein content remaining in the ECM must be assessed, focusing on structural proteins, such as collagen, fibronectin, laminin, GAGs and growth factors. Furthermore, the mechanical properties, including elastic recoil and tensile strength, depending on the application, must match the original tissue. In this review, decellularization techniques are evaluated for their effectiveness in these four areas, to rule out failure of complete tissue decellularization, leading to negative results after *in vivo* implantation, including a pro-inflammatory response with M1 macrophage recruitment and subsequent fibrosis [71].

The most decellularization efficacy test reports revealed positive the data on X-100 triton treatment compared to 0.1% SDS and 0.1% trypsin solutions [72–76]. Woods T. and co-authors demonstrated that the X-100 triton decellularization method in the combination with SDS or tri-n-butyl phosphate solution was the most effective, but also the most destructive in terms of the depletion of glycosaminoglycan and collagen [66]. This phenomenon was also shown in the study of Purohit S. [45] who decellularized the skin with trypsin, triton and sodium hydroxide and observed the fibrinoid necrosis, fragmentation and undulation of the fibrillar structures in the dermis which states the depletion of the dermal matrix. Although, from the protocols tested in the study [77], triton X-100 had the least harmful effect on the content of glycosaminoglycans. Crapo P. et al. [69, 78] have suggested that the denser tissues, such as the dermis, tendon and trachea require the decellularization protocols by the continuous agitation, which last from days to months. However, in the present study, the desired results were obtained after 48 hours of the treatment with the biological detergents.

Badylak S. and Gilbert T. [31] have shown that cells and cellular products cannot be completely removed from dense tissues, such as the dermis, even with the most rigorous processing methods. However, in the present study, the complete cell-less membrane was observed after 48 hours of the treatment, although SDS solubilized the cell membranes and dissociated DNA. Therefore, it is effective in the removing of the cellular material from the tissues. Sodium dodecyl sulfate has been more effective in the removing of the cell residues and cytoplasmic proteins such as vimentin from the tissue compared to other detergents, but is more aggressive for ECM [79, 80]. Dodecyl sulfate was more effective than Triton-X 100 in the removing of the nuclei from the dense tissues. SDS disrupted the native tissues and caused a decrease of GAG concentration and depleted collagen [9]. SD is very effective for the removing cellular debris. SD has been shown not to alter the structural properties of the ECM structure, as observed in Kasimir M's study [70] but tends to disrupt the structure of the tissue itself, so it should be used in a lower concentration.

Among the methods described by Hongxu Lu et al., the methods of freeze-thaw cycling with NH(4) OH and triton X-100 with 1.5M K Cl showed the best effect on the removal of cellular components from the complexes, while the other five methods could only partially remove cellular components. The ECM scaffolds prepared by these two methods had similar gross appearances and microstructures [49].

Qi Xing et al. investigated three decellularization methods: high concentration (0.5wt.%) of sodium dodecyl sulfate (SDS), low concentration (0.05wt.%) of SDS, and freeze-thaw cycling method. They found that the high SDS treatment could efficiently remove around 90% of DNA from the cell sheet, but significantly compromised their ECM content and mechanical strength. The elastic and viscous modulus of the ECM decreased around 80% and 62%, respectively, after the high SDS treatment. The freeze-thaw cycling method maintained the ECM structure as well as the mechanical strength, but also preserved a large amount of the cellular components in the ECM scaffold. Around 88% of DNA was left in the ECM after the freeze-thaw treatment. *In vitro* inflammatory tests suggested that the amount of DNA fragments in ECM scaffolds does not cause a significantly different immune response. All three

ECM scaffolds showed comparable ability to support *in vitro* cell repopulation [63, 81].

Haozhen R. et al. described the successful results if the decellularization after the treatment with SDS and Triton X-100. The total absence of the nuclear structures and removal of viable cells were confirmed by hematoxylin-eosin staining and scanning electron microscopy. Collagen was preserved after both treatments. However, the elastin content decreased to about 20% and 60%, the GAGs content decreased to about 10% and 50% and the HGF content decreased to about 20% and 60% of the native liver level after SDS and Triton X-100 treatment respectively. The Triton X-100-treated scaffolds were much superior to SDS-treated scaffolds in the supporting liver-specific function, including albumin secretion (P=0.001), urea synthesis (P= 0.002), ammonia elimination (P=0.007) and mRNA expression levels of the drug metabolism enzymes [82–84].

However, there are studies [55-61], that the enzymatic removal of epitopes from the cell surface reduces the immunogenicity of the xenograft. Thus, in the research [47–55], it has been shown that the presence of the cells in the interstitium and their necrosis after the transplantation can delay the infiltration of the host cells that affect the regeneration. It is obvious, that the specifics of the interaction between the matrix and the cells of the recipient remain to be addressed in the following researches.

Conclusions

1. The removal of the cells, proteins, DNA from the skin and the porosity of the samples, directly correlate with the exposure time in the decellularization solution.

2. The highest quality extracellular matrices were prepared using a combination of chemical and enzymatic methods.

3. It is clear that skin decellularization with sodium dodecyl sulfate was the most successful and safe method in terms of minimizing the amount of DNA and the risks of the graft rejection after the transplantation.

4. Probably the pig skin could be considered as a study substrat, being available and easily processable, taking into consideration ethical aspects and grafts safety.

5. It has been found that the tissue decellularization probably inhibits the subsequent proliferation of the fibroblasts in the matrix, this fact requires further research.

6. The natural matrix is more widely used than synthetic material, because it has the natural structure and composition of the ECM, it naturally stimulates cell development and allows the incorporation of the growth factors and other proteins increasing cell proliferation.The assessment of the quality of decellularization techniques is done by evaluating the necrosis of the extracellular matrix, the depletion of the collagen fibers and the remaining amount of genetic material.

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MO and DR proposed the concept and design of the study, selected the literature and contributed to the elaboration and writing of the manuscript text. CA and NV performed a critical analysis and helped draft the manuscript. The approval of the final version of the manuscript was read and confirmed by all the authors.

Funding
This study was supported by Nicolae Testemitanu State University of Medicine and Pharmacy, and the research project "Nanoarhitecturi în bază de GaN și matrici tridimensionale din materiale biologice pentru aplicații în microfluidică și inginerie tisulară" [GaN-based nanoarchitectures and three-dimensional matrices of biological materials for applications in microfluidics and tissue engineering] – 20.80009.5007.20 offered by the National Agency for Research and Development of the Government of the Republic of Moldova.

Conflict of interests
The authors declare the absence of a conflict of financial or non-financial interests.

Moldovan Medical Journal. June 2021;64(2):87

Professor Constantin Etco – to the 80th anniversary

22.07.1941–19.12.2017

Professor Constantin Etco was a person of high moral standing and civic responsibility, a true professional who contributed to the development of national medicine and higher medical and pharmaceutical education and proved himself a talented educator and skilled manager. He devoted himself to medicine and became over the years a scientist of international stature in the field of public health, economics, management and psychopedagogy in medicine. He developed and implemented for the first time, at *Nicolae Testemitanu* State University of Medicine and Pharmacy of the Republic of Moldova (*Nicolae Testemitanu* SUMPh), the concept of reorganizing the medical staff training system through residency and master's degree studies, contributing substantially to the health system reform.

Constantin Etco was born on July 22, 1941 in Horodiste village, Calarași district, where he completed secondary education. After school he worked for two years, then entered and graduated from Tiraspol Medical School and performed his military service. Between 1965-1971, he continued his studies at Chisinau State Institute of Medicine, Faculty of General Medicine.

Upon graduation with honors from the Institute, given his qualities of a leader and organizer, he was invited as an assistant to the Department of Social Hygiene and Healthcare Organization. Constantin Etco had the great chance to work (being also the first PhD student) under the leadership of Professor Nicolae Testemitanu during 15 years. He passed through all the stages of professional training: assistant, senior lecturer, associate professor, university professor and head of department.

Under the guidance of Professor Nicolae Testemitanu, he defended his PhD thesis – *Medical-social aspects of infant mortality from pneumonia of children in rural areas* (1979) at the Central Institute for Advanced Training of Physicians in Moscow and the thesis of doctor habilitat in medical sciences – *Conditions, ways of life of children from densely populated rural areas* (1992). The results of his research works have been widely applied in the development of prophylactic measures aimed at improving children's health.

Parallel with the scientific-didactic activity, as the dean of postgraduate studies (1993-1995), later the dean of master, doctoral and postdoctoral studies (1995-2000), professor Etco participated in the elaboration of normative acts (government resolutions, regulations) for legislating the reform of postgraduate training of University graduates through residency and master's degree training – a new form of postgraduate specialization, introduced for the first time at *Nicolae Testemitanu* SUMPh, later implementing master's degree courses, young lecturers' training in fundamental disciplines as well as doctoral programs.

Also, due to his innovative spirit, in 1997, he founded the Department of Economics, Management and Psychopedagogy at *Nicolae Testemitanu* SUMPh – a division in charge of preparing doctors for work in new economic and social conditions. As the head of the department, Constantin Etco decided to review curricula and introduced new disciplines, such as: healthcare economics, management, psychology, legal bases, medical inactivity – for students and psychopedagogy – for master/PhD students and competitors. Based on the modules developed by the mentioned department, where by 2005, there were 6 study disciplines and 8 courses, the foundation has been laid for establishing the Public Health Management School.

Constantin Etco is one of the first national promoters of economic theory development, which represents the landmark of a new research direction in the medical area and human resources management. He consolidated this branch by creating the *The Economy, Management and Psychology Association in Medicine* (in 2000) and by founding in 2003 a scientific journal *Public Health, Economics and Management in Medicine*, while the treatise *Management in the System of Health* (2006, 864 p.) remains the most important book for doctors, heads of medical institutions and PHMS master students.

Between 1986-2013, Professor Etco was the member of the Scientific Council and the Senate of *Nicolae Testemitanu* SUMPh, since 1994 – member of the Association of University Professors for Training of Healthcare Administrators of the USA, since 1998 – vice president of the League of Physicians of the Republic of Moldova.

During 2000-2017 he served as the head of the Department of Medicine and Pharmacy of the National Council of Accreditation and Attestation, promoting 1600 files of scientists to degrees and scientific titles of doctor and doctor habilitat at 36 specialties in medicine. Under the guidance of professor Etco, 36 doctoral and 4 PhD theses in medical sciences were defended, the outcomes of scientific research resulting in 760 published papers, including 35 monographs, 80 textbooks, compendiums, guides, course materials, etc. Moreover, we can even mention *Professor Constantin Etco's school*.

As the main specialist in public health and management of the Ministry of Health, he contributed to the elaboration of normative acts on the health system reform: National Health Policy of the Republic of Moldova for 2007-2021, Strategy for the development of the health system in 2008-2017 etc. Professor Etco is one of the leading authors of the laws and regulations required for the implementation of the compulsory health insurance system. The results of his research are used to develop health policies and strategies, national programs and government resolutions, representing the bedrock for the decisions of the Ministry of Health in carrying out national health system reforms. In 2017, Constantin Etco, disciple and promoter of professor Testemitanu's ideas, published and launched the homage volume – *Nicolae Testemitanu – a name that became a symbol*.

He really knew how to honor his obligations and show respect to those around him, regardless of whether they were students and master/doctoral students or scientists and colleagues. Endowed with humanity, parental patience and receptivity, he had always been able to support them through his temperate speech and exemplary behavior.

His prodigious activity was appreciated with high state distinctions and honorary titles: State Medal *Nicolae Testemitanu* (2011), Medal *Dimitrie Cantemir* (2011), Order *Glory of Labor* (2012), Laureate of *Scientist of the Year,* Award of the Academy of Sciences of Moldova (2008, 2011), Health Award *Entire Medical Career* (2016), Laureate of the *National Award* (2016), etc.

The memory of Professor Constantin Etco will be perpetuated by installing his bust on the *Alley of Brilliant Scientists and Doctors* of *Nicolae Testemitanu* SUMPh. The life and activity of professor Etco will remain an exceptional model of service, an example worth following for the younger generations.

Emil Ceban, MD, PhD, Professor
Rector of *Nicolae Testemitanu* State University
of Medicine and Pharmacy of the Republic of Moldova

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The references in the Cyrillic script should be transliterated into Latin script using the American Library Association and Library of Congress Romanization Tables as follows: А=A, Б=B, В=V, Г=G, Д=D, Е=E, Ё=E, Ж=ZH, З=Z, И=I, Й=I, К=K, Л=L, М=M, Н=N, О=O, П=P, Р=R, С=S, Т=T, У=U, Ф=F, Х=KH, Ц=TS, Ч=CH, Ш=SH, Щ=SHCH, Ъ=", Ы=Y, Ь=', Э=E, Ю=IU, Я=IA.

Immediately after the transliteration the translation of the title in English in the square brackets should follow. For example: Ivanov IV, Shchukin NF, Men'shikov VM, Ad"yunktov AM. Transplantatsiia organov i tkanei [Transplantation of organs and tissues]. Vestnik Khirurgii. 2010; 26(6):45-49. Russian.

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