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Regional use of extracellular microvesicles of mesenchymal stromal cells in acute necrotizing pancreatitis in an experiment

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Abstract

Background. *The significance of the problem of treatment of acute pancreatitis is due to an increase in the incidence with an increase in the number of necrotizing forms, accompanied by a high incidence of severe complications and high mortality.*

Objective. *To identify the impact of regionally used extracellular microvesicles of mesenchymal stromal cells on the endogenous intoxication markers in acute necrotizing pancreatitis in the experiment.*

Material and methods*. Acute pancreatitis was induced by the introduction of a 0.3 ml of 5% solution of non-ionic polyethylene glycol octylphenol ether detergent into the caudal part of the rat pancreas. The study was conducted on 42 adult Wistar rats, which were randomly divided into 4 groups. Group I (n=6) included intact animals, Group II (control group) (n=12) included rats with pancreatitis without treatment,*

Group III (n=12) consisted of rats with pancreatitis treated with analgesia + infusions of 0.9% sodium chloride solution (saline), Group IV (n=12) included rats with pancreatitis treated with analgesia+ saline infusions + regional application of extracellular microvesicles of mesenchymal stromal cells. Cells were obtained from the bone marrow of healthy animals. Microvesicles were obtained by differential centrifugation under sterile conditions. Microvesicles were administered one day after the pancreatitis induction through the catheter installed into the pathologically altered part of the pancreas. The dose of microvesicles was calculated as equivalent to (derived from) 1 million mesenchymal stromal cells. The hematological parameters, markers of the systemic manifestation of the pathological process (alpha-amylase, aspartate aminotransferase, alanine aminotransferase), the endogenous intoxication markers (lipid peroxidation activity, nitric oxide level), the systemic inflammatory response markers (tumor necrosis factor-alpha, interleukin-6) were studied on the 3rd and 7th day from the start of disease modeling.

***Results**. Regional use of extracellular microvesicles of mesenchymal stromal cells in the treatment of acute experimental necrotizing pancreatitis at an early stage helped to normalize the level of blood platelets, reduce enzymeemia, elements of endogenous intoxication (interleukin-6, tumor necrosis factor-alpha), and the nitric oxide level.*

***Conclusion**. The early application of extracellular microvesicles of mesenchymal stromal cells in the treatment of acute necrotizing pancreatitis in an experiment has a positive effect on parameters, which are key links of pathogenesis and leading markers of this disease severity.*

Keywords: extracellular microvesicles, mesenchymal stem cells, acute pancreatitis, systemic inflammatory response syndrome, pancreas

Conflict of interest The authors declare no conflict of interest

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AEP, acute experimental pancreatitis
ANP, acute necrotizing pancreatitis
AP, acute pancreatitis
AST, aspartate aminotransferase
CS, cytokine storm
EV, extracellular microvesicle
IL, interleukin
LPO, lipid peroxidation
MDA, malonic dialdehyde
MOF, multiple organ failure
MSC, mesenchymal stromal cell
NO, nitric oxide
PBS, phosphate buffered saline
ROS, reactive oxygen species
SIRS, systemic inflammatory response syndrome
TNF-α, tumor necrosis factor alpha

Introduction

The incidence of acute pancreatitis (AP) over the past decade has remained at a high level and has no tendency to decrease [1]. In 15–30% of cases, the disease has a severe course with high mortality from 15 to 40% [2]. Such a high incidence of adverse outcomes, especially in the early stages of acute severe pancreatitis, is largely due to severe endogenous intoxication, which ineffective and untimely correction underlies the development of multiple organ failure [1, 2].

The cell therapy using mesenchymal stromal/stem cells (MSCs) for various diseases is considered a new promising trend in experimental and clinical medicine. The biological and therapeutic activity of MSCs is

associated with the availability of their sources (bone marrow, adipose tissue, umbilical cord, etc.), as well as proven anti-inflammatory, immunomodulatory and regenerative effects in a number of pathological conditions [3]. The use of MSC-based biotherapeutics in medicine is currently expanding; MSCs have now become the subject for hundreds of clinical trials. Cell therapy for acute pancreatitis was first described experimentally by K.H. Jung et al. in 2011 [4]. The use of MSCs in experimental AP is aimed at providing an anti-inflammatory effect by increasing cell resistance to pyroptosis (cell death associated with the inflammatory process) and reducing the severity of inflammation. Under experimental conditions, it was found that the use of MSCs in rats with AP leads to a decrease in pancreatic edema and hemorrhages, reduces the level of cell necrosis, and suppresses signs of systemic inflammation [5].

Meanwhile, the use of MSCs has its limitations, including because they are poorly stored and require administration within 2–4 hours after obtaining in vivo. When administered to patients, most cells die within 2–5 days; as a result of cell destruction, undesirable respiratory and inflammatory reactions may occur in patients; with systemic intravenous administration, most stem cells settle in the lungs [6]. The mechanism of MSC action is still the subject of debate. It is assumed that MSCs have a contact effect, secrete extracellular microvesicles (MVs) or soluble factors, through which they exhibit their therapeutic effectiveness. Currently, some drugs based on extracellular microvesicles obtained from MSCs are already undergoing clinical trials as a new trend for the use of bioproducts of cellular origin in various pathological conditions [7]. Studies have shown that the resulting microvesicles affect key aspects of biological processes, including membrane transport and horizontal transfer of genetic material (various proteins, ribonucleic acid). According to the minimum criteria, the term “extracellular microvesicles”

is a general term for particles naturally released from the cells bounded by a lipid bilayer and unable to replicate because they do not contain a functional nucleus [8]. Extracellular microvesicles are obtained from the culture medium while maintaining MSCs in vitro under conditions of mild cellular stress that does not cause cell death. In this case, MVs are detached from the cells and carry sections of the outer membrane of MSCs; they are stable and can be stored frozen for 1 year without loss of physiological activity. MVs have the biological activity inherent in MSCs: they stimulate angiogenesis, modulate immune responses, suppress apoptosis, and enhance cell proliferation [7–9]. The use of MSC MVs has proven beneficial in the treatment of joint damage (osteoarthritis) in mice [10], traumatic injury and model Alzheimer's disease in rats, myocardial infarction in rats [11-12], modeled allergic asthma in mice, and pulmonary arterial hypertension, induced liver fibrosis in mice, acute pyelonephritis in mice [13-16], skin burns in rats [17]. The effectiveness of MSC MVs in acute pancreatitis has not been studied.

Thus, along with MSCs for anti-inflammatory and regenerative therapy in acute experimental pancreatitis, it is advisable from a scientific point of view to study the results of using MVs obtained from MSCs. The widespread prevalence of AP and the unsatisfactory results of medical care for patients with this pathology indicate the relevance of the proposed developments for the purpose of their subsequent implementation into clinical practice as new, more effective means and method of treatment.

The objective was to identify the impact of regional use of extracellular microvesicles of mesenchymal stromal cells on the endogenous intoxication parameters in acute necrotizing pancreatitis in the experiment.

Material and methods

Experimental studies were conducted at the base of the Belarusian State Medical University (BSMU) vivarium in accordance with the international rules and principles of the “European Convention for the Protection of Vertebrate Animals Used for Experiments and for Other Scientific Purposes” (Strasbourg, March 18, 1986), as well as in accordance with the “Regulations on the procedure for the use of experimental animals in research work and the educational process at the Belarusian State Medical University.” The animals were kept in a dedicated box for one week to adapt to new conditions; before the start of the study, they were weighed and examined for signs of disease.

Experimental design. An experimental study was conducted on 42 mature male Wistar rats weighing 275–380 g; for the study conduct the animals were randomly distributed into four groups: group I (n=6) included intact animals, group II (control) (n=12) included rats with acute necrotizing pancreatitis (ANP) without treatment, group III (n=12) included rats with ANP treated with: anesthesia + infusion of 0.9% sodium chloride solution, group IV (n=12) consisted of rats with ANP who received the following treatment: anesthesia + infusion of 0.9% sodium chloride solution + regional administration of MSC MVs. Hematological parameters, markers of the systemic manifestations of the pathological process (alpha-amylase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), markers of systemic inflammatory response (TNF-α, IL-6), markers of endogenous intoxication (lipid peroxidation (LPO) activity, nitric oxide (NO) level).

Before the start of the experiment, 6 rats were randomly selected for a preliminary assessment of laboratory parameters. The obtained data

did not differ from normal physiological parameters in the representatives of the laboratory animals used and therefore were accepted as the norm.

Modeling of acute necrotizing pancreatitis

To perform the manipulations, the animals were anesthetized with sodium thiopental (manufactured by JSC Sintez, Kurgan, Russian Federation) at a dose of 45 mg/kg of animal weight. In order to induce the necrotizing form of AP, a standard laparotomy was performed on all animals and 0.3 ml of 5% nonionic detergent of polyethylene glycol-octylphenol ether (Triton X-100, manufacturer Carl Roth GmbH &Co. KG, Germany) was injected into the tail (gastrosplenic) part of the pancreas (PG) with an insulin syringe.

Method for regional administration of extracellular microvesicles of mesenchymal stromal cells in the treatment of acute necrotizing pancreatitis

After creating a model of AP, a catheter made of transparent thermoplastic implantable non-toxic polyvinyl chloride was inserted into the abdominal cavity through a contra-aperture on the anterior abdominal wall in the left lower quadrant in animals of group IV, according to the method we had developed (Fig. 1). The proximal end of the catheter was placed between the gastrosplenic part of the pancreas (the site of injection of nonionic detergent) and the spleen so that the side hole was directed towards the pancreas. The proximal end of the catheter was fixed to the stomach with a submersible U-shaped suture, so as to plug the proximal outlet and ensure that the injected drug flows only through the side hole directly to the site of the pathological process in the pancreas. After completion of the manipulation, the pancreas was carefully immersed in the abdominal cavity, the anterior abdominal wall was sutured tightly

with single-row interrupted sutures (Invention No. 24210 of December 28, 2023, RB).

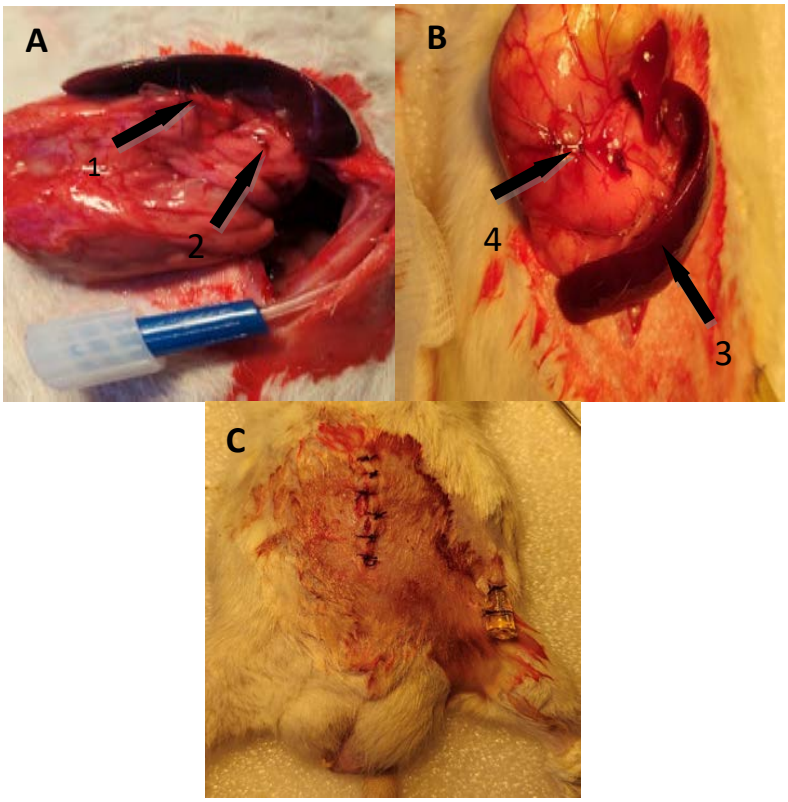

Fig. 1. Method of placing the catheter for regional administration of drugs in the treatment of acute experimental pancreatitis in a rat model. A. The proximal part of the catheter is placed between the gastrosplenic part of the pancreas and the spleen (1, Proximal part of the catheter; 2, Pancreas); B. The proximal end of the catheter is fixed to the stomach with a submersible U-shaped suture (3, Spleen; 4, U-shaped suture on the stomach); C, View of a postoperative wound and a catheter pulled out through a counter-aperture on the skin

Surgical procedures were performed under sterile conditions and no antibiotic prophylaxis was administered. In the postoperative period, all animals were given pain relief with intramuscular administration of ketorolac at the rate of 4.8 mg/kg of animal weight per day.

Experimental treatment of acute necrotizing pancreatitis

In order to study the effect of MSC MVs on early pathological systemic changes in AP, the treatment began 24 hours from the start of

the experiment. In addition to pain relief, animals of group III were intravenously administered a 0.9% NaCl solution in a volume of 2 ml once a day, and in animals of group IV, the above treatment was supplemented with the regional administration of suspension of MSC MVs in a volume of 1.0 ml of the prepared solution through an installed catheter. The dose of microvesicles was calculated as equivalent to that obtained from 1 million mesenchymal stromal cells.

On the 3rd and 7th days from the start of AP modeling, the animals were removed from the experiment by euthanasia. Blood samples were taken from all animals at the control time for laboratory tests. Initially, anesthesia was performed with an intraperitoneal injection of sodium thiopental at a dose of 45 mg/kg of animal weight. The chest was opened and the blood samples in a volume of 4.5 ml were taken from the heart by puncture for biochemical, hematological, and serological studies. For euthanasia, after blood sampling, the animal was additionally given an intraperitoneal injection of sodium thiopental at a dose of 200 mg/kg.

Method for obtaining extracellular microvesicles of rat mesenchymal stromal cells and their characteristics

MSC samples were obtained from the bone marrow of Wistar rats. MSCs were isolated by cell adhesion to plastic on T25 culture flasks (Sarstedt, Germany). Visual control of cells was ensured by using an inverted DM 2500 Leica microscope (Leica, Germany) at x25 magnification. The isolated MSCs were identified by assessing the degree of expression on the membrane surface of CD90 (BIO-RAD, cat. 158952) and CD45 (BD Biosiences, USA), identifying MSC molecules. Data recording and analysis were performed using a FACSCantoII flow cytometer and FACSDiva 8.0 software (Becton Dickinson, USA).

To isolate and characterize MVs from the culture medium, according to the minimum criteria for the study of extracellular vesicles, a basic characterizations of the cultured cells, the cultivation conditions, and the culture fluid collection were performed [18].

At the first stage, MSCs were seeded into a T75 culture flask at a concentration of 550 thousand cells/flask in complete nutrient medium DMEM/F12 with the addition of 10% fetal bovine serum (FBS) and an antibiotic solution (streptomycin/penicillin). The cells were cultured in a carbon dioxide incubator while maintaining a temperature of 37°C and 5% carbon dioxide content for 2 days until confluency on the growth surface was 95–100%.

At the second stage, to enrich the culture medium with microvesicles, the cell culture was washed with a solution of phosphate-buffered saline (PBS) and the culture was transferred to the serum-free medium DMEM/F12 (10 ml). Cultivation was continued for 48 hours to create conditions of mild cellular stress that did not cause necrotic cell death and promoted the release of extracellular MVs.

The third stage involved directly obtaining the MV fraction from the MSC culture fluid. MVs were obtained using the differential centrifugation method under sterile conditions.

Thus, the standard for the amount of MVs per animal was their preparation from 10 ml of conditioned medium from one culture flask (T75) and diluted in 1 ml of PBS solution.

Microvesicles were identified using a FACSCantoII flow cytometer relative to latex calibration particles - Negative Control Compensation Particles Set and controls. The MV population was assessed after identifying a logical “gate” of microvesicles (particles from 300 to 1000 nm) in Dot/Plot analysis, focusing on their location relative to the calibration particles by direct and side light scattering and cutting off the

“noise” region located below the threshold sensitivity level of the device. The number of MVs was estimated as the number of recorded "events" in the selected area. Between 100,000 and 150,000 particles were analyzed per sample. To identify the microparticle population from other instrument signals (“noise”), controls were used: phosphate-buffered saline filtered through a 0.2-μm syringe filter and an unstained microvesicle population. For immunophenotypic characterization of rat MSC MVs, antibodies against CD90 and CD45 were used and the degree of their expression on the membrane surface was assessed.

During the experiment, the following hematological parameters were studied: red blood cell count, hemoglobin, hematocrit, platelet count, and white blood cell count. Hematological studies were performed on a veterinary automatic hematological analyzer IVet-5, Norma Instruments Zrt. (Hungary). Manufacturer's control materials were used to calibrate the analyzer.

Using the biochemical analyzer A-25, BioSystems (Spain) and reagent kits for it (Diasens, Republic of Belarus and Fenox Medical Solutions, Republic of Belarus), the biochemical parameters of animal blood serum: ALT, AST, alpha-amylase were studied.

Nitric oxide content was determined using a reagent kit of Bioassay Technology Laboratory (China) in accordance with the manufacturer's specification. The IL-6 concentration in serum samples was determined using the Fine Test RAT IL-6 Elisa reagent kit (China). Serum TNF-α levels were determined using a Cloud-Concord reagent kit (China).

LPO activity was assessed based on the accumulation level of lipid peroxidation secondary products: malonic dialdehyde (MDA). MDA content in animal blood hemolysates was determined according to the method by T. Asakava and S. Matsushita (1980) [19].

Statistics

The results obtained were subjected to statistical processing using the IBM SPSS Statistics 23 software with a preliminary check of the compliance of the studied variables with a normal distribution using the Kolmogorov–Smirnov test. Quantitative data are presented as medians (Me) and interquartile range (Q25;Q75). To assess the statistical significance of differences between groups, the nonparametric Mann–Whitney U test was used. Differences were considered statistically significant if the probability of error was $p<0.05$.

Results

According to the results of our previous studies, the ANP model, created by introducing 0.3 ml of a 5% solution of Triton X-100 into the pancreas tissue, allowed us to reproduce in animals a form of the disease with a histological pattern of the pancreas tissue necrosis and pathomorphological changes in the heart, lungs, liver and kidneys, as well as with the development of endogenous intoxication and significant changes in systemic parameters [20, 21].

In the present study, data were obtained from which it follows that there was a statistically significant decrease in the number of platelets in the animals of groups II and III. As shown in Fig. 2, on the 3rd day in the blood of animals of groups II and III, where MSC MVs were not used, thrombocytopenia was most pronounced, and the number of platelets was statistically significantly lower than in the blood of intact animals ($p<0.05$). In animals of group IV, where MSC MVs were used for treatment, the platelet level on the 3rd day was the highest and did not differ from this parameter in intact animals: (734.5 (574.50; 843.75) and 553.0 (428.25;751.0) $\times 10^9$ /L, respectively; $p>0.05$).

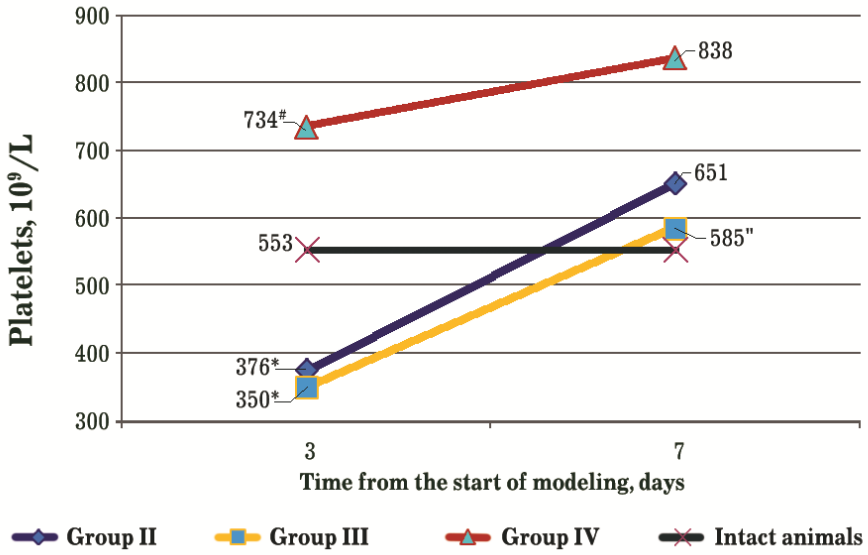

Fig. 2. Dynamics of platelet levels in the blood of experimental animals (n=6 in each subgroup; * – significant difference when compared with intact animals; # – significant difference when compared with the results of group III animals on day 3; ″ – statistically significant difference between the results in the group on days 3 and 7)

Further throughout the experiment, an increase in platelet levels was observed in all animals, and on the 7th day this parameter was not statistically significantly different from that in intact animals ($p>0.05$). It was found that after the use of MSC MVs in animals of group IV, on the 7th day, the platelet level was the highest (838.5 (531.50;1055.25) $\times 10^9$ /l).

In experimental animals, a premature activation of pancreatic enzymes and their diversion into the systemic circulation was observed, which is an important early component of the AP pathogenesis. On the 3rd day from the start of modeling the severe AP in group II animals (without treatment), the highest α-amylase activity was observed, which was statistically significantly different from this parameter in intact animals (904.0 (845.0;1339.50) and 819.0 (499.75;980.0) units/mL, respectively; $p<0.05$). Meantime, in group III, α-amylase activity was 744.0 (721.50;855.0) units/mL; and in group IV animals, where MSC MVs were used for treatment, α-amylase activity was 873.50

(748.50;1051.75) units/mL without statistically significant differences from normal values ($p>0.05$). Subsequently, after 7 days of observation, in animals of groups II and III, α-amylase activity did not change, and in group IV, changes in this parameter did not have statistically significant differences between groups.

To determine the effect of MSC MVs on hepatocyte cytolysis induced by endotoxins in severe AP, a study was carried out on the dynamics of the main intracellular enzymes (AST and ALT), which serve as indicators of liver tissue damage. On the 3rd day the blood serum AST level was higher in animals of groups II and III compared to that in intact animals. In animals of group IV at that time the AST values were below normal (99.15 (84.55;125.53) units/L and 124.50 (96.25;156.0) units/L, respectively; $p>0.05$) (Fig. 3). Subsequently, an increase in this parameter was observed in animals of all experimental groups, however, in animal group III, where saline solution was used for treatment, the AST level increased most intensively ($p=0.006$) and on the 7th day it was 2.2 times higher than this parameter value in intact animals: 275.0 (228.25;311.50) and 124.50 (96.25;156.0) units/L, respectively; $p<0.05$.

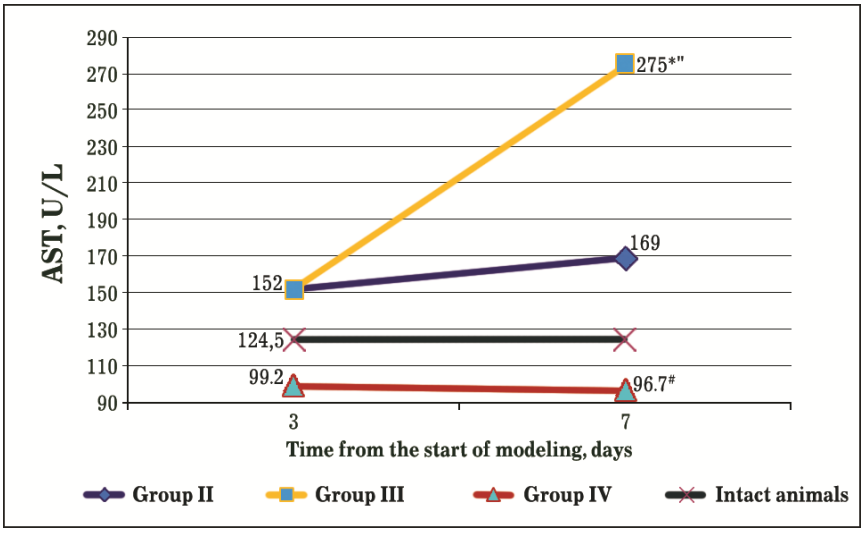

Fig. 3. Dynamics of aspartate aminotransferase in the blood of experimental animals (n=6 in each subgroup; * – significant difference when compared with intact animals; # – significant difference when compared with animals of groups II and II on day 7; ″ – significant differences between the results in the group on days 3 and 7)

In animal group IV where MSC MVs were used, the AST level on the 7th day was the lowest 96.7 (92.55;115.13) units/L) and was statistically significantly lower than the values of this parameter in group II and group III animals ($p<0.05$). When studying the ALT blood serum level in the animals of all groups, we found that the activity of this enzyme changed in different directions and was not statistically significantly different from the values of intact animals ($p>0.05$).

To evaluate the effect of MSC MVs on the development of systemic inflammatory response syndrome (SIRS) in severe AEP, the changes in the levels of cytokines TNF-α and IL-6 were studied over time. On the 3rd day, as shown in Fig. 4, in group IV animals, where the basic therapy was combined with the administration of MSC MVs, the IL-6 concentration was statistically significantly lower compared to this parameter in animals of groups II and III, and 2.6 times lower than the values of intact animals (98.2 (71.30;102.75) and 258.30 (209.30;333.25) pg/ml, respectively; $p<0.05$).

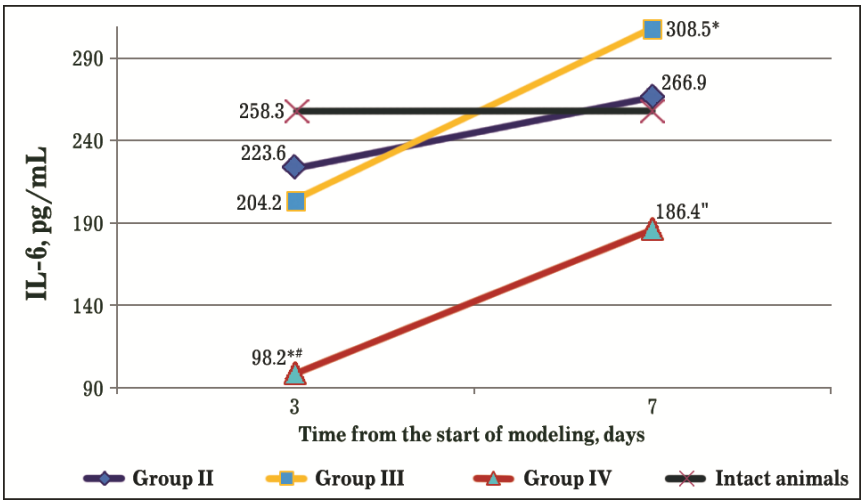

Fig. 4. Dynamics of interleukin-6 concentration in the blood serum of experimental animals (n=6 in each subgroup; * – significant difference when compared with intact animals; # – significant difference when compared with the results in animals of groups II and III on day 3; ″ – significant difference between the results in the group on days 3 and 7)

Subsequently, without statistically significant differences between the groups, an increase in this parameter was noted; and only in animals of group III (standard therapy), the IL-6 concentration by the 7th day of the experiment was the highest and differed from the values of intact animals: (308.50 (232.33;406.43) and 258.30 (209.30;333.25) pg/mL, respectively; $p<0.05$).

In animals of group II (without treatment), the most significant increase in the concentration of TNF-α was observed; on the 3rd day this parameter was statistically significantly higher than in the group of intact animals: (1.73 (1.47;1.87) and 1.45 (1.32;1.77) pg/mL, respectively; $p<0.05$) (Fig. 5).

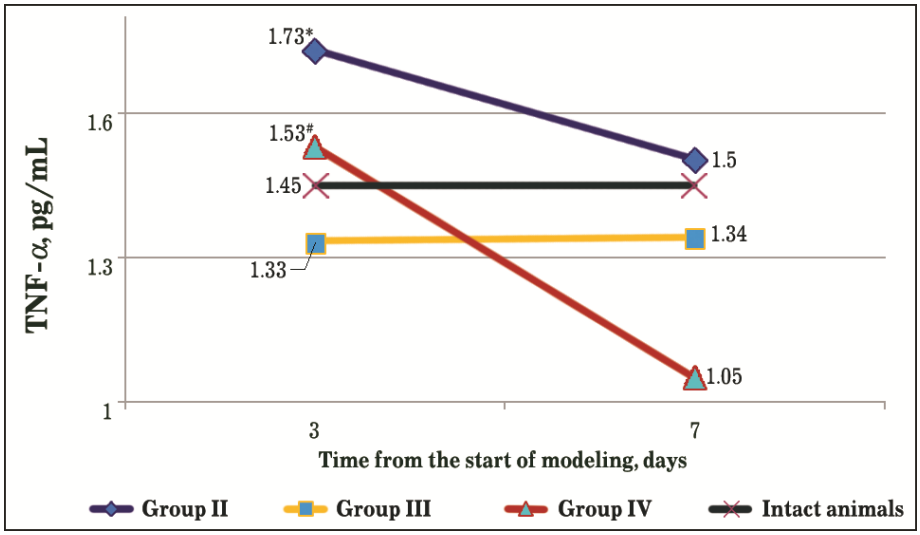

Fig. 5. Dynamics of tumor necrosis factor alpha concentration in the blood serum of experimental animals (n=6 in each subgroup; * – significant difference when compared with intact animals; # – significant difference when compared with the results of group II animals on day 3)

No significant increase in TNF-α concentration in group III animals was observed throughout the experiment. On the 3rd day of the experiment, in group IV, where MSC MVs were used, the concentration of TNF-α did not differ from the intact animal values: (1.53 (1.08;1.95) and 1.45 (1.32;1.77) pg/mL, respectively; $p>0.05$). On the 7th day, the concentration of TNF-α in group II decreased from 1.73 (1.47;1.87) pg/mL to 1.50 (1.16;1.76) pg/mL, but remained higher than in animals of groups III and IV: 1.34 (1.21;2.47) and 1.05 (0.81;2.94) pg/mL, respectively; $p>0.05$.

According to the data obtained in the investigations, the blood MDA level in animals of group II on the 3rd day after the creation of the AP model was higher than in intact animals: 21.60 (19.40;22.45) and 18.65 (16.50;19.53) μmol /mL, respectively; $p>0.05$, which indicated an increase in the intensity of free radical oxidation. In animals of group III, despite the treatment, on the 3rd day there was an increase in the blood MDA level to 19.95 (18.10;21.80) μmol/mL, then throughout the experiment the blood MDA level continued to increase and its content

reached its maximum values by the 7th day: 21.25 (19.68;22.38) μmol/mL. In group IV animals, the use of MSC MVs did not prevent the MDA accumulation; on the 3rd day of the experiment, its values were higher than in animals of groups II and III, and 2 times higher than the values of intact animals: 36.2 (34.98;39.0) and 18.65 (16.50;19.53) μmol/mL, respectively; $p<0.05$. Subsequently, there was an increase in this parameter to 45.64(36.03;56.36) μmol/ml; and on the 7th day it was higher than in other groups of animals ($p<0.05$).

The cytotoxic effect in severe forms of AP is due to the activation of oxygen-dependent mechanisms and the synthesis of nitric oxide. These studies showed that on the 3rd day after the creation of the necrotizing form of AP, the blood serum concentration of nitric oxide in group I animals was statistically significantly (81%) higher than the values in intact animals: 29.22 (19.42;35.48) and 16.04 (14.31;23.54) μmol/mL, respectively; $p<0.05$ (Fig. 6).

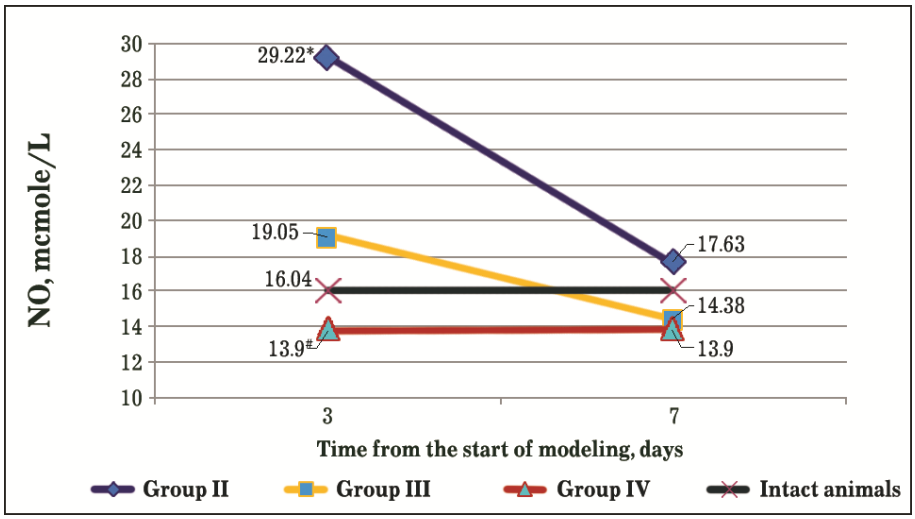

Fig. 6. Dynamics of the nitric oxide level in the blood of experimental animals (n=6 in each subgroup; * – significant difference when compared with intact animals; # – significant difference when compared with the results of groups II and III animals on day 3)

The use of basic therapy for the treatment did not prevent the increase in the blood serum concentration of nitric oxide in animals of group III on the 3rd day of the experiment to 19.05 (14.78;23.30) μmol/mL. Further, throughout the experiment, a decrease in the level of this parameter was observed in animals of groups II and III, and on the 7th day the value of this parameter in the both groups did not differ statistically significantly from the values in intact animals ($p>0.05$). The use of MSC MVs contributed to a decrease in the blood serum content of nitric oxide in animals; and after 72 hours from the start of the experiment it was lower compared to that in intact animals: 13.90 (11.8;15.65) and 16.04 (14.31;23.54) μmol/mL respectively; $p>0.05$, there was no further increase in this parameter.

Discussion

According to modern concepts, the key pathogenetic component of AP is the cytokine storm (CS) syndrome with the development of systemic inflammatory response syndrome [22]. The study of the action of inflammatory mediators and methods of their inhibition is the main direction for improving the complex treatment of acute pancreatitis. Systemic inflammatory response syndrome is a complex cascade of activation of proinflammatory mediators (prostaglandins, leukotrienes, cytokines (TNF-α, IL-1β, IL-6, IL-8, platelet-activating factor)), phospholipase A2, neutrophils, platelets, monocytes and adhesion molecules [23].

It is believed that the migration of leukocytes into the interstitium is one of the key moments in the SIRS development. The content of leukocytes increases in the pancreas within an hour, and in the lungs after 3 hours [24]. According to some authors, TNF-α is one of the main factors in triggering the cascade reactions that contribute to the damage to

the pancreatic parenchyma and the development of multiple organ failure [25]. There is also an opinion that the primary source of proinflammatory mediators are the acini cells themselves. In response to hyperstimulation or direct damage to the acinar cell, the synthesis of IL-1β, IL-6, IL-8 occurs, as well as the activation of an important pro-inflammatory mediator - platelet activating factor [26]. The latter attracts neutrophils, which migrate to the lesion through the vessel wall, and also causes platelet adhesion and aggregation. After the activated neutrophil penetrates the tissue, a “respiratory burst” occurs with the release of large quantities of proteolytic enzymes, oxygen free radicals and cytokines that can destroy cells and attract new leukocytes, including monocytes. Monocytes transform into active macrophages, which also produce large amounts of proinflammatory mediators and soluble receptor proteins [27]. The release of a significant amount of cytokines (IL-1β, TNF-α) can activate leukocytes in the systemic circulation and microvasculature of the liver, kidneys, lungs, and other organs, which again induces the synthesis of inflammatory mediators (CS) [28]. This results in a sepsis-like state characterized by fever, leukocytosis, and release of acute phase proteins such as C-reactive protein, complement components, and ferritin.

Cytokine TNF-α is one of leading mediators of inflammation in AP. Its concentration in the blood serum of patients correlates with the severity of the AP course and systemic complications. [29]. Our study found that in the III (control) group of animals, where no treatment was used, the most significant increase in the concentration of TNF-α was observed; on the 3rd day this figure was 1.2 times higher than in the group of intact animals, which correlates with data from other authors. In animals where MSC MVs were used for treatment, the concentration of TNF-α on the 3rd day of the experiment did not differ from intact animal values. Subsequently, a decrease in this parameter was noted; and on the

7th day, the concentration of TNF-α in these animals was 43% lower than the values in the control group.

The level of IL-6 reflects the activity of all pro-inflammatory cytokines, therefore, the level of IL-6 is used to study systemic changes occurring under the effect of cytokines. Long-term persistence of elevated blood serum IL-6 concentrations in patients with destructive pancreatitis correlates with a high incidence of complications and mortality [30]. In their study, Z. Dambrauskas et al. (2010) found that severe AP was associated with overexpression of proinflammatory cytokines, and IL-6 is one of the markers for differential diagnosis between mild and severe AP [31]. The use of MSC MVs in experimental animals reduced the systemic inflammatory response, which was observed in the dynamics of IL-6 values. In this group of animals, on day 3, the concentration of IL-6 was significantly lower compared to the values of this parameter in animals of the control group and 2.6 times lower than the values of intact animals.

The half-elimination of cytokines from the bloodstream is 3.2–7.5 minutes, and in case of CS, there required their constant entry into the peripheral bloodstream from the site of inflammation and from the cells of the inflamed endothelium of blood vessels, which become the main producers of cytokines [23, 32]. Activation and death of endothelial cells leads to clinical manifestations of endovasculitis, increased permeability of vascular walls, production of reactive oxygen species (ROS) and nitric oxide, heparanase, endothelin, the release of damage-associated molecular patterns (DAMPs), swelling and dysfunction of tissues and organs [23, 33]. The interaction of nitric oxide with ROS results in the formation of peroxynitrite, which is an extremely cytotoxic product that can damage the endothelium [34]. It causes vasodilation, disrupts oxygen transport and leads to a deterioration in the body's oxygen status. Nitric oxide also stimulates platelet sequestration, increases the permeability of

cell membranes, causes metabolic and structural damage to endothelial cells, and has a cytotoxic effect [35]. As previously found, the interaction between ROS and nitric oxide plays a key role in the development of severe forms of pancreatitis [36]. According to our data, on the 3rd day after the creation of a severe form of AP, the blood serum nitric oxide concentration in group I animals (without treatment) was 81% higher than the values in intact animals. The use of MSC MVs contributed to the fact that the blood serum content of nitric oxide in animals after 72 hours from the start of the experiment did not differ from the values of intact animals and was 2.1 times lower than in the control group (without treatment).

The processes of lipid peroxidation are closely related to SIRS. The products of LPO themselves have pronounced toxicity and destructive action. [36]. There are opinions that it is the LPO processes that are one of the main causes for the development of destructive changes in organs and tissues, and the volume of these changes is directly proportional to the LPO intensity [37]. However, according to our data, the use of MSC MVs did not prevent the MDA accumulation, since on the 3rd day of the experiment its values were higher than in animals of groups II and III, and 2 times higher than the values in intact animals.

Conclusion

Experimental use of extracellular microvesicles of mesenchymal stromal cells at the early stage of severe acute necrotizing pancreatitis helps to normalize the blood platelet level, reduce enzymeemia, the level of elements of the systemic inflammatory response and endogenous intoxication (TNF-α, IL-6 and nitric oxide levels), which form the basis of the pathogenesis of severe acute pancreatitis, which indicates a positive

effect of extracellular microvesicles of mesenchymal stromal cells on the above mentioned parameters of the pathological process in this disease.

Based on the results of our study we can make the following conclusions:

1. In a group of animals where extracellular microvesicles of mesenchymal stromal cells were used for treatment, the level of platelets on the 3rd day was 2 times higher than in the control group (376.0 (301.5;784.0) $\times 10^9$/l) and did not differ from this parameters values in intact animals (734.5 (574.50; 843.75) and 553.0 (428.25; 751.0) $\times 10^9$ /L, respectively; $p>0.05$, Mann–Whitney U Test).

2. Combination of standard therapy with administering the extracellular microvesicles of mesenchymal stromal cells prevented the increase in the blood serum level of aspartate aminotransferase in the animals, which may suggest a cytoprotective effect of extracellular microvesicles of mesenchymal stromal cells on the cellular structures of the liver. On the 3rd day in animals of this group, aspartate aminotransferase values were below of intact animals: (99.15 (84.55; 125.53) and 124.50 (96.25; 156.0) units/L, respectively, $p>0.05$, Mann–Whitney U Test); and no further increase in the level of this enzyme was observed.

3. Use of extracellular microvesicles of mesenchymal stromal cells decreased the systemic inflammatory response, as demonstrated by the changes in interleukin-6 and tumor necrosis factor-alpha values over time. In this group of animals, on the 3rd day, the concentration of interleukin-6 was statistically significantly lower compared to this parameter value in animals of the control group and 2.6 times statistically significantly lower than its values in intact animals (98.2 (71.30;102.75) and 258.30 (209.30;333.25) pg/mL, respectively ($p<0.05$, Mann–Whitney U Test). After 72 hours from the start of the experiment, the

concentration of tumor necrosis factor alpha in animals where extracellular microvesicles of mesenchymal stromal cells were used for treatment did not differ from the intact animal values: 1.53 (1.08;1.95) and 1.45 (1.32;1.77) pg/mL, respectively; $p>0.05$, Mann–Whitney U Test. Subsequently, a decrease in this parameter was noted; and on the 7th day the concentration of tumor necrosis factor alpha was 43% lower than the values of the control group (1.50 (1.16;1.76) and 1.05 (0.81;2.94) pg/ml, respectively; $p>0.05$, Mann–Whitney U Test).

4. Regional use of extracellular microvesicles of mesenchymal stromal cells prevented the increase of the blood serum concentration of nitric oxide in experimental animals. After 72 hours from the start of the experiment, the value of this parameter did not differ from that in intact animals (13.90 (11.8;15.65) and 16.04 (14.31;23.54) μmol/mL, respectively; $p>0.05$, Mann–Whitney U Test) and was 2.1 times statistically significantly lower than in the control group (29.22 (19.42;35.48) μmol/mL; $p <0.05$, Mann–Whitney U Test).

5. The treatment of acute necrotizing pancreatitis using extracellular microvesicles of mesenchymal stromal cells did not prevent the malondialdehyde accumulation, since on the 3rd day of the experiment its values were statistically significantly higher than in animals of groups II and III, and 2 times higher than its values in intact animals: 36.2 (34.98;39.0) and 18. 65 (16.50;19.53) μmol/mL, respectively; $p<0.05$, Mann–Whitney U Test. Subsequently, in the group where extracellular microvesicles of mesenchymal stromal cells were used, there was an increase in this parameter to 45.64 (36.03;56.36) μmol/mL, and on the 7th day it was statistically significantly higher than in other groups of animals ($p<0.05$, Mann–Whitney U Test).

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