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Application of mesenchymal stem cells in severe acute experimental pancreatitis

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Abstract

Background. *The significance of the problem of acute pancreatitis is due to an increase in the incidence with an increase in the number of common forms of pancreatic necrosis, accompanied by a high incidence of severe complications.*

Aim. *To determine the effect of regional application of mesenchymal stromal cells on the systemic manifestations of severe acute experimental pancreatitis.*

Material and methods*. This experimental study was carried out on 42 adults Wistar rats. Acute pancreatitis was induced by administering 0.3 ml of 5% solution of non-ionic polyethylene glycol octylphenol ether detergent into the caudal part of the pancreas. The animals were randomly divided into 4 groups: Group I (n=6) consisting of intact animals, Group II (control group) (n=12) of rats with untreated*

pancreatitis, Group III (n=12) of rats with pancreatitis treated: anesthesia + infusions of 0.9% sodium chloride solution (saline), and Group IV (n=12) of rats with pancreatitis treated: anesthesia + infusions of saline + regional application of mesenchymal stromal cells. Animals were taken out of the experiment by euthanasia on the 3rd and 7th day. The hematological parameters, markers of systemic manifestation of the pathological process (pancreatic amylase, aspartate aminotransferase, alanine aminotransferase, urea, creatinine), markers of endogenous intoxication (lipid peroxidation activity, nitric oxide level), markers of systemic inflammatory response (C-reactive protein, tumour necrosis factor-alpha, interleukin-6) have been evaluated.

Results. *The application of mesenchymal stromal cells in the early stages of acute pancreatitis made a favourable effect on the platelet count, the level of glycemia, helped to reduce the content of endogenous intoxication elements (malonic dialdehyde, nitric oxide) and of those of the systemic inflammatory response (interleukin-6, tumor necrosis factor-α, C-reactive protein), which are key links in the pathogenesis of severe acute pancreatitis.*

Conclusion. *Comparison of different treatment regimens for acute experimental pancreatitis has shown that the early use of mesenchymal stromal cells has a systemic positive effect and confirms the therapeutic efficacy of the method in the treatment of this disease.*

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

Conflict of interest The authors declare no conflict of interest

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AEP, acute experimental pancreatitis
ALT, alanine aminotransferase
AP, acute pancreatitis
AST, aspartate aminotransferase
BT LAB, Bioassay Technology laboratory
CRP, C-reactive protein
Hb, hemoglobin
HCT, hematocrit
IL, interleukin
LPO, lipid peroxidation
MDA, malonic dialdehyde
MOF, multiple organ failure
MSCs, mesenchymal stromal cells
NO, nitric oxide
SIRS, systemic inflammatory response syndrome
TNF-α, tumor necrosis factor alpha

Introduction

Treatment of severe forms of acute pancreatitis (AP) remains the most difficult and unsolved problem for surgeons and intensive care specialists. In 15–20% of cases, the AP development is of severe course, and changes in pancreatic tissues are necrotizing by nature [1]. With this form of AP, a marked endogenous intoxication of the body quickly develops, and intravital imaging techniques reveal disorganization of the pancreatic tissue structure and its necrosis. An avalanche-like release of damage-associated molecular patterns (DAMPs) and pro-inflammatory cytokines is manifested by a systemic inflammatory response syndrome (SIRS) followed by the dysfunction of various organs and multiple organ failure (MOF) in the next 24 hours from the onset of the disease [2]. Thus, the generalization of the inflammatory process in necrotizing AP is

associated with the release into the systemic circulation of various pathological biologically active substances that initiate and maintain endogenous intoxication, which allows us to consider them as markers for monitoring when studying the efficacy of new approaches and methods of treatment.

Using mesenchymal stromal cells (MSCs) in inflammatory processes is a new trend in regenerative medicine. MSCs were first isolated and characterized by A.J. Friedenstein et al. in 1966 [3]. In 2006, the International Society of Cell Therapy formed the minimum criteria for identifying MSCs, including adhesiveness to plastic, expression of CD73, CD90 and CD105, the lack of expression of hematopoietic and endothelial markers CD34, CD45, as well as CD11b, CD14, CD19, CD79a, HLA-DR. In addition, MSCs are characterized by the ability to an induced differentiation into the cells of adipose, bone and cartilage tissue in vitro. The clinical efficacy of cell therapy with MSCs is currently the subject of active discussion [4]. An experimental cell therapy for AP was first described by K.H. Jung et al. in 2011 [5].

The use of MSCs in experimental AP is aimed at providing an anti-inflammatory effect by increasing the cell resistance to pyroptosis (the inflammatory process-associated cell death) and reducing inflammation [6]. 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 the signs of systemic inflammation. Moreover, the anti-inflammatory effects of the administered cells do not depend on the model of AP induction, the source of MSCs used (bone marrow, adipose tissue, MSCs of human origin), or the route of administration (intravenous or intraperitoneal) [7]. An important objective indicator of a decrease in the inflammatory process in AP under the effect of MSCs may be a decrease in the peripheral blood levels of inflammatory

cytokines (interleukin-1β (IL-1β), IL-6, tumor necrosis factor alpha (TNF-α)), amylase, lipase, myeloperoxidase activity, increasing levels of anti-inflammatory cytokine IL-4 and IL-10 [7, 8]. In addition, paracrine growth factors secreted by MSCs have an anti-apoptotic effect on various types of pancreatic cells. The ability to have immunomodulatory, anti-inflammatory, anti-apoptotic effects, and to differentiate into cells of various tissues makes MSCs a potentially effective tool for the treatment of AP [9].

The objective was to determine the effect of regional application of mesenchymal stromal cells on systemic changes in acute experimental pancreatitis.

Material and methods

Experimental studies were carried out on the basis of the vivarium of the Educational Institution Belarusian State Medical University (EI BSMU). The study protocol was approved at the Ethics Committee Meeting. All investigations were conducted in conformity with the international rules and principles of “The European Convention for the Protection of Vertebrate Animals Used for Experimental and for Other Scientific Purposes” (Strasbourg, 03/18/1986), as well as in accordance with the “Regulations on the procedure for the use of experimental animals in scientific research and educational process at the Belarusian State Medical University." Before the start of the experiment, the animals were kept in a dedicated box for one week to adapt to new conditions. During the experiment, the animals were kept individually in cages. Before the study, all animals were weighed and carefully examined for possible pathology and signs of disease. Animals with detected pathology were discarded.

The experimental study design

The experimental study was performed on 42 sexually mature male Wistar rats weighing 275-380 g. The animals were kept in accordance with the standards for individual housing.

The animals were randomly divided into four groups: Group I (n=6) contained intact animals, Group II (control) (n=12) included rats with acute experimental pancreatitis (AEP) without treatment, Group III (n=12) included rats with AEP who received the treatment with anesthesia + infusion of 0.9% sodium chloride solution (saline), Group IV (n=12) included rats with AEP who received the treatment with anesthesia + saline infusion + regional (local) administration of MSCs. On days 3 and 7 from the beginning of the AEP modeling the following parameters were assessed: hematological parameters, the pathological process systemic manifestation markers (pancreatic alpha-amylase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine), markers of endogenous intoxication (LPO activity, nitric oxide (NO) level), markers of systemic inflammatory response (C-reactive protein (CRP), TNF-α, IL-6).

Before the start of the main experiment, 6 rats were randomly removed for the preliminary assessment of laboratory parameters and anatomy of visceral organs. The obtained data did not differ from normal physiological parameters in representatives of the laboratory animal species used; therefore the data from this group of animals were accepted as the norm.

Modeling of acute experimental pancreatitis

All manipulations were performed in animals anesthetized with sodium thiopental (manufactured by Sintez OAO, Kurgan, Russian Federation) at a dose of 45 mg/kg of animal weight. To reproduce AEP, a

laparotomy was performed and 0.3 ml of 5% nonionic detergent polyethylene glycol octylphenol ether (Triton X-100, manufactured by Carl Roth GmbH & Co. KG, Germany) was injected into the tail (gastrosplenic) part of the pancreas with an insulin syringe as a standard for all the animals.

Method of regional (local) administration of mesenchymal stromal cells in the treatment of acute experimental pancreatitis

In group IV animals, after completing the AP modeling stage, a catheter made of transparent thermoplastic implant-non-toxic polyvinyl chloride was inserted into the abdominal cavity through a counter-aperture on the anterior abdominal wall in the left lower quadrant according to the method we had developed [10]. Surgical procedures were performed under sterile conditions. In the postoperative period, all animals were anesthetized with intramuscular administration of ketorolac at the rate of 4.8 mg/kg of animal weight per day; antibiotic prophylaxis was not performed.

Treatment of acute experimental pancreatitis

To assess the effect of MSCs on early pathological systemic changes in AP, the treatment began at 24 hours from the start of the experiment. Animals of group III, in addition to pain relief, were intravenously injected with a 0.9% NaCl solution in a volume of 2 ml once a day, and for animals of group IV, the above treatment was supplemented with a regional administration of MSCs on the 1st day after modeling at a dose of $1*10^6$ cells per 1.0 ml of the prepared solution through a previously placed catheter.

Animals were removed from the experiment by euthanasia on the 3rd and 7th days from the start of AP modeling. Blood and organs were

collected from all animals at control periods for laboratory and pathomorphological studies, respectively. 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 blood was taken from the heart by puncture for biochemical, hematological, and serological studies in a volume of 4.5 ml. After receiving blood, the animal was additionally given an intraperitoneal injection of sodium thiopental at a dose of 200 mg/kg for euthanasia.

Obtaining a culture of rat mesenchymal stromal cells and their characteristics

Bone marrow (BM) MSC samples were obtained from Wistar rats kept in the vivarium of the Belarusian State Medical University. Femur BM samples were obtained under aseptic conditions after euthanasia of rats by a single intra-abdominal injection of a lethal dose of 3% sodium thiopental solution. The cells were washed in MEM nutrient medium with 1% fetal bovine serum (FBS). BM MSCs were isolated by perfusion from the femur and transferred to a nutrient medium.

MSCs were isolated by cell adhesion to plastic on T25 culture flasks (Sarstedt, Germany). For this purpose, BM cells were cultured in nutrient medium DMEM/F12 (Elabscience, China), with the addition of fetal bovine serum (FBS, 10%) (Gibco, USA). The resulting BM mononuclear cells were centrifuged at 1500 rpm for 20 minutes, the supernatant was removed, 1–2 ml of complete nutrient medium (CNM) was added and resuspended. Mononuclear cells were seeded into T25 culture flasks at a concentration of 500 thousand cells/cm^2 in CNM containing DMEM, 10% FBS, antibiotic mixtures (streptomycin/penicillin). The attached cells were washed with a phosphate-buffered saline solution, pH 7.4 (FBS, Gibco, USA) after 2

days, and CNM was added. The medium was changed every 3–4 days until the confluency on the growth surface reached 80–90%. After the formation of large colonies of the primary culture at the bottom of plastic flasks, the cells were subcultured for the first passage. To do this, the attached MSCs were washed with FBS, then a solution of 0.25% trypsin was added to the washed cells for 3–5 minutes at +37°C, the enzyme was inactivated by adding CNM with FBS, the cell suspension was collected and pelleted by centrifugation at 1500 rpm for 20 minutes. The supernatant was removed, and the cell pellet was resuspended in CNM. MSCs were counted and their viability assessed with 0.4% trypan blue.

For the first passage, the cells were seeded into T25 flasks in 5 ml of CNM at a concentration of 3000 cells/cm^2, a total of 75,000 cells/flask, and cultured until 80% confluency was achieved. Next, the cells of the first passage were transferred to the second and subsequent passages or frozen with 20% DMSO at -196°C in liquid nitrogen until required. The visual control of cells was carried out using an inverted Leica microscope DM 2500 (Leica, Germany) at x25 magnification.

To identify the isolated MSCs, the degree of expression was assessed 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 FACSCantoII flow cytometer and FACSDiva 8.0 software (Becton Dickinson, USA).

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

Biochemical studies were performed using a biochemical analyzer A-25, BioSystems (Spain), and reagent kits for it (Diasens, Republic of Belarus and Fenox Medical Solutions, Republic of Belarus). The studied biochemical parameters of animal blood serum included: creatinine, urea, glucose, CRP, ALT, AST, alpha-amylase.

Linked immunosorbent assay. The concentration of IL-6 in serum samples was determined using the Fine Test RAT IL-6 reagent Elisa Kit (PRC). Serum TNF-α levels were determined using a Cloud-CloneCorp reagent kit (PRC). Nitric oxide content was determined using a Bioassay Technology Laboratory reagent kit (BT LAB, PRC) in accordance with the manufacturer's instructions.

Lipid peroxidation assessment. The intensity of lipid peroxidation was assessed by the level of accumulation of LPO secondary products, malondialdehyde (MDA). The MDA content in blood hemolysates was determined by means of T. Asakava and S. Matsushita method [11].

Statistics

Statistical processing of the obtained results was carried out using the IBM SPSS Statistics 23 software with a preliminary check of the studied variables for compliance with a normal distribution using the Kolmogorov–Smirnov test. Quantitative characteristics are presented as medians and interquartile range (Me (Q25;Q75)). To assess the statistical significance of differences between groups, the nonparametric Mann–Whitney U test was used. A nonparametric alternative to the t-test for dependent samples was the Wilcoxon signed-rank test. Differences were considered statistically significant if the probability of error was $p<0.05$.

Results

Our previous studies have shown that the AEP model created by introducing 0.3 ml of a 5% Triton X-100 solution into the pancreas tissue allows us to reproduce in animals a form of the disease with a histological pattern of the glandular tissue necrosis and pathomorphological changes in the heart, lungs, liver and kidneys, as well as with significant changes in systemic parameters (increased levels of pro-inflammatory cytokines, CRP, accumulation of lipid peroxidation products, NO) and the development of endogenous intoxication [12, 13].

From the data obtained, it follows that in animals of all groups during the entire observation period there were no significant changes in hematological parameters, with the exception of a decrease in the number of platelets. As shown in Fig. 1, on day 3, in the blood of animals of groups II and III where MSCs were not used, thrombocytopenia was pronounced: the platelet count was statistically significantly lower than in the blood of intact animals (Mann–Whitney U Test, $p<0.05$).

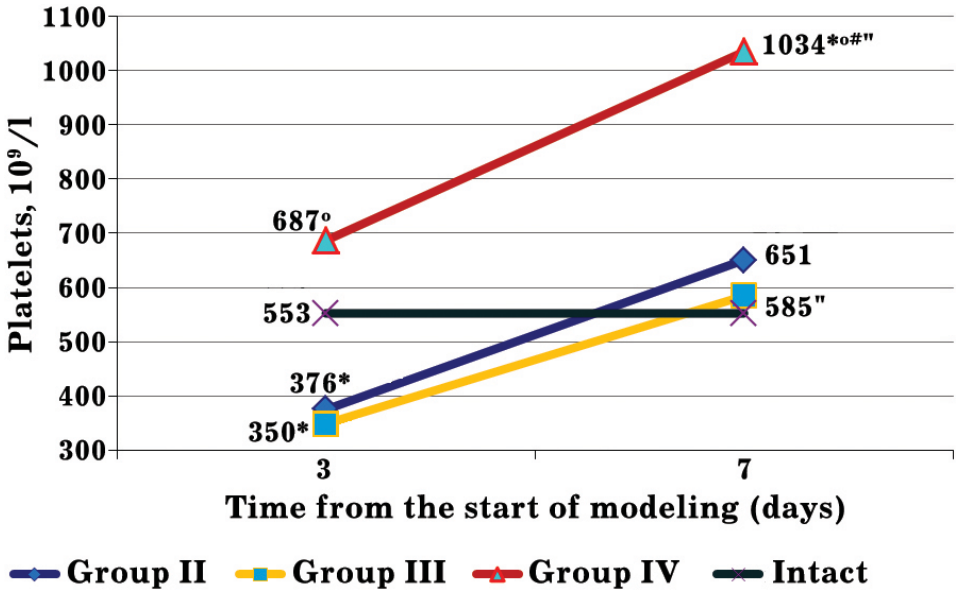

Fig. 1. Dynamics of platelet levels in the blood of experimental animals (n=12 in each group; * significant in comparison with intact animals; ° significant in comparison with day 3 in groups; # significant in comparison with day 7 in groups; ″ significance of differences between days 3 and 7 in the group)

In animals of group IV, where MSCs were used for the treatment, the level of platelet count on the 3rd day was the highest and did not differ from this value in intact animals (687.50 (510.50; 779.0) and 553.0 (428.25;751.0) x10^9/L. respectively; Mann - Whitney U - Test , p=0.631). Subsequently, throughout the experiment, an increase in platelet levels was observed in the blood of all animals, and on day 7 this parameter did not differ significantly from that in intact animals (Mann–Whitney U-Test, p>0.05). Meanwhile, after the use of MSCs in animals of group IV, on the 7th day the platelet level turned out to be the highest (1034.5(972.50;1227.25) x10^9/L).

A premature activation of pancreatic enzymes and their diversion into the systemic circulation is an important early link in the pathogenesis of AP, which was observed in experimental animals (Fig. 2).

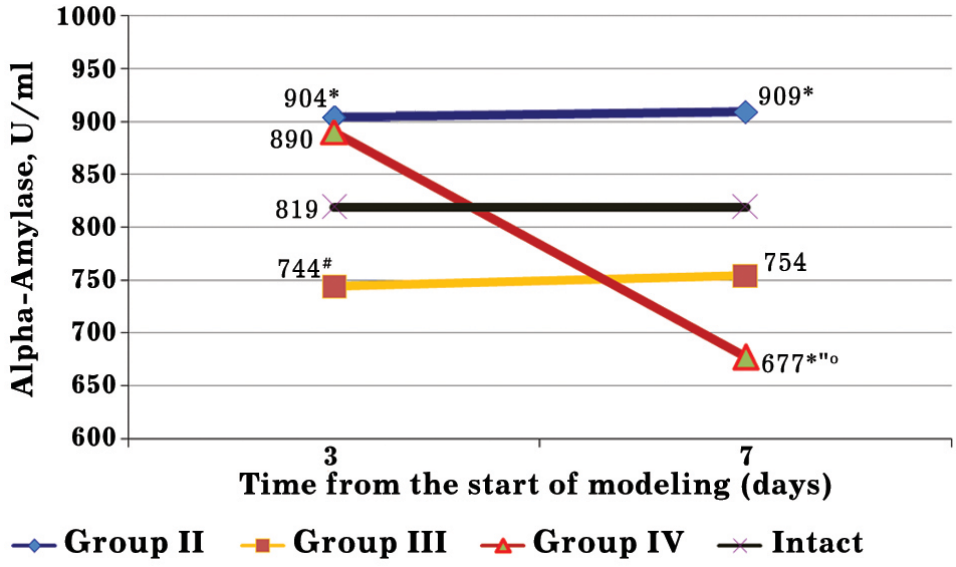

Fig. 2. Dynamics of the level of alpha-amylase in the blood of experimental animals (n=12 in each group; * significant in comparison with intact animals; # significant in comparison with day 3 of animals of group II; ° significant differences between days 3 and 7 in the group; ″ significant in comparison with day 7 of animals of group II)

From Fig. 2 it follows that on the 3rd day from the start of AP modeling 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; Mann-Whitney U-Test, p=0.047). Meanwhile, in group III, α-amylase activity was 744.0 (721.50;855.0) units/mL, and in group IV animals, where MSCs were used for treatment, α-amylase activity was 890.0 (800.0;1429.25) units/mL without statistically significant differences from normal values (819.0 (499.75; 980.0) units/mL; Mann-Whitney U-Test, p=0.286). Subsequently, after 7 days of observation, α-amylase activity did not change in animal groups II and III. In group IV, on the contrary, there was a statistically significant decrease in this parameter (Wilcoxon test, p=0.028); and by the end of the experiment, α-amylase activity was significantly different from that in intact animals (677.0 (603.75;816.25) and 819.0 (499.75;980, 0) units/mL, respectively; Mann-Whitney U Test, p=0.039).

In order to study the effect of MSCs on the cytolysis of hepatocytes during AED, the changes in the main intracellular enzymes (AST and ALT), which serve as indicators of damage to liver tissue, were comparatively studied. When studying the levels of ALT and AST in the blood serum of animals in all groups, we found that the activities of these enzymes during the experiment changed in diverse directions and was not statistically significantly different from those in intact animals (Mann–Whitney U Test, p>0.05).

When studying urea and creatinine, no significant changes in these parameters of the kidney nitrogen excretory function were detected.

According to international recommendations, one of the criteria for determining the AP severity is the glycemia level; in this regard, in order

to assess the efficacy of the compared treatment methods, we studied the changes of this parameter over time (Fig. 3).

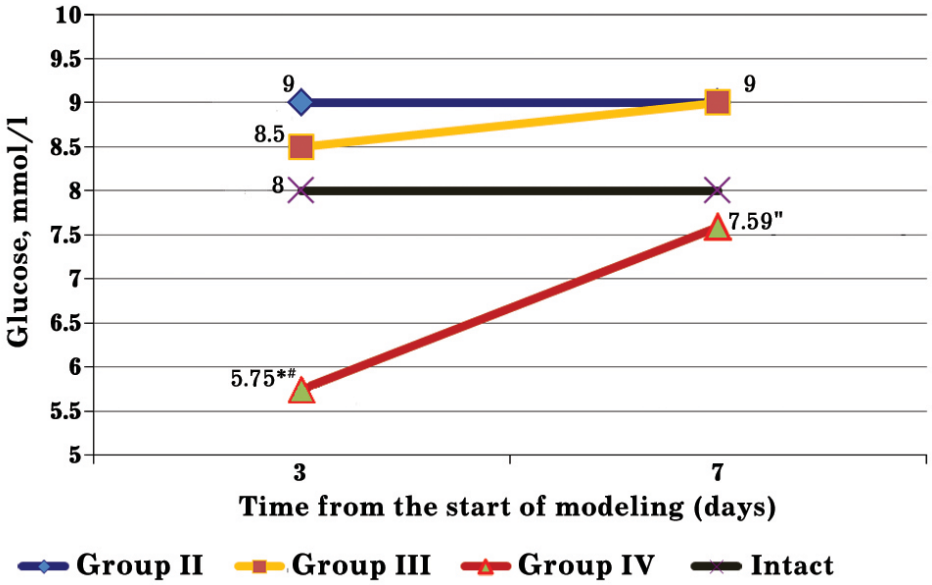

Fig. 3. Dynamics of glucose levels in the blood of experimental animals (n=12 in each group; * significant in comparison with intact animals; # significant in comparison with day 3 of animals of groups II and III; ″ significant differences between days 3 and 7 in the group)

On the 3rd day after AP modeling, in groups II and III, there was an increase in blood glucose levels compared to intact animals; the values of this parameter remained high throughout the experiment. On the 3rd day of the experiment, in animal group IV, where MSCs were used for treatment, the glucose level was statistically significantly lower than the values of this parameter in intact animals (5.75 (4.89;6.47) and 8.0 (6.75;8.25) mmol/L, respectively; Mann-Whitney U-Test, p=0.016). By the 7th day of the experiment, an increase in blood glucose levels had been observed in this group (from 5.70 (5.70;5.90) to 7.59 (7.11;8.63) mmol/L; Wilcoxon test, p=0.046); by that time this parameter practically did not differ from that of the intact anima group (Mann–Whitney U Test, p=0.936). The absence of hyperglycemia at AEP early stage in animal group IV may indirectly indicate the cytoprotective effect of MSCs on the cellular structures of the pancreas.

In order to assess the effect of MSCs on the initiation of SIRS in AEP, the levels of the cytokines TNF-α, IL-6 and CRP were determined over time. In animal group IV where basic therapy was combined with the administration of MSCs, the concentration of IL-6 on day 3 was statistically significantly lower compared to groups II and III and 3 times lower than the values of intact animals (88.0 (71.65;155.95) and 258.30 (209.30;333.25) pg/mL, respectively; Mann–Whitney U- Test, p=0.033). Subsequently, there was an increase in this parameter without statistically significant differences between the groups. However, only in animals of group III (standard therapy), the concentration of IL-6 by the 7th day of the experiment had been 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; Mann–Whitney U Test, p=0.048).

We found that in animal group II (control), where no treatment was used, the most significant increase in the TNF-α level 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; Mann–Whitney U-Test, p=0.048) (Fig. 4). On the 7^{th} day, the concentration of TNF-α in this group decreased, but remained higher than in group III and statistically significantly higher than in animals of group IV (1.50 (1.16;1.76) and 1.04 (0.90;1.10) pg/mL respectively; Mann–Whitney U Test, p=0.019).

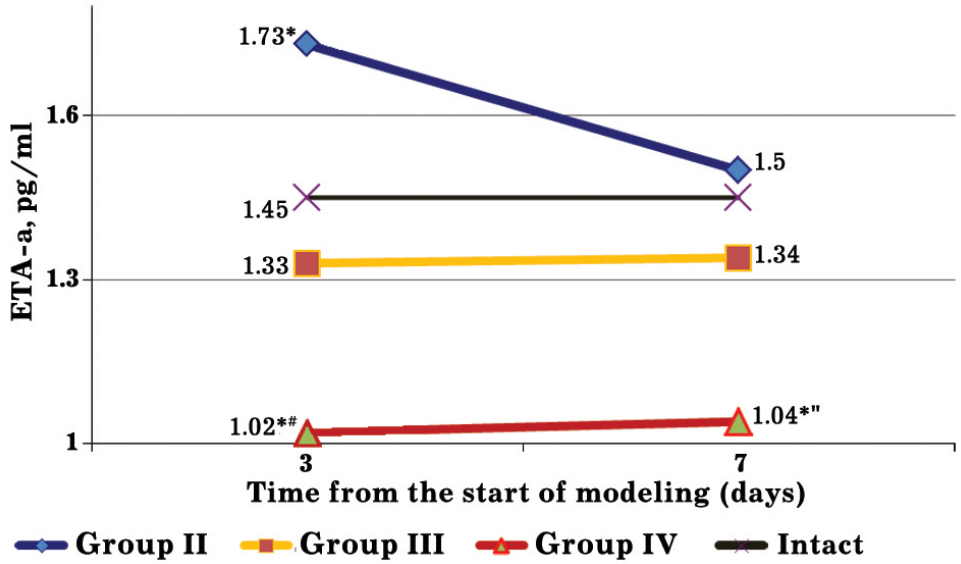

Fig. 4. Dynamics of tumor necrosis factor-α concentration in the blood serum of experimental animals (n=12 in each group; * significant in comparison with intact animals; # significant in comparison with day 3 of animals of group II; ″ significant in comparison with day 7 in animals of groups II and III)

In animals of group III, no significant increase in the concentration of TNF-α was observed throughout the experiment, and its values did not differ from that of intact animals (Mann–Whitney U Test, $p>0.05$). In group IV, where MSCs were used, on the 3rd day of the experiment the concentration of TNF-α was lower by 42% than intact animal values (1.02 (0.85;1.24 and 1.45 (1.32;1.77)) pg/mL respectively; Mann–Whitney U Test, $p=0.011$) and no further increase in this parameter was observed in these animals.

At 72 hours after AP modeling, in animals of group II, there was a significant increase (2-fold) in the CRP level compared to the norm (0.40 (0.30; 0.55) and 0.20 (0.20;0.30) pg/mL respectively; Mann–Whitney U Test, $p=0.004$). Subsequently, a decrease in this parameter (from 0.40 (0.30;0.55) to 0.30 (0.0;0.30) pg/mL, Wilcoxon test, $p=0.491$) was observed, but on the 7th day it remained higher than in the group of intact animals (Mann–Whitney U-Test, $p=0.005$) (Fig. 5).

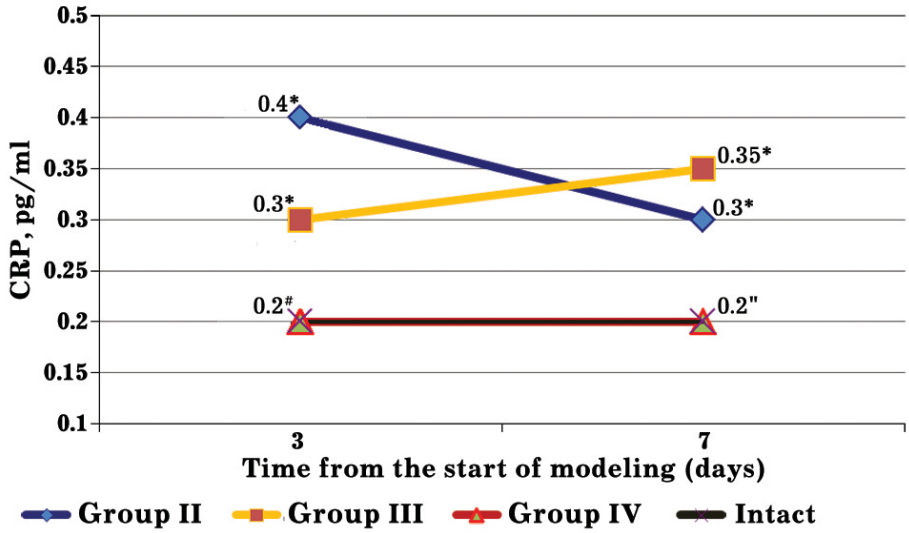

Fig. 5. Dynamics of the level of C-reactive protein in the blood serum of experimental animals (n=12 in each group; * significant in comparison with intact animals; # significant in comparison with day 3 in animals of groups II and III; ″ significant in comparison with day 7 in animals of groups II and III)

When studying the CRP level of in the blood serum of group III animals on the 3rd day from the start of AP modeling, a statistically significant increase in this parameter was also noted compared to that in intact animals (0.30 (0.0;0.30) and 0.20 (0.20;0.30) pg/mL, respectively; Mann–Whitney U Test, p=0.005). Subsequently, despite the baseline therapy in this group, there was an increase in the CRP level to 0.35 (0.30;0.40) pg/mL (Wilcoxon test, p=0.491); and by the 7th day its value had 75% exceeded the figure in intact animals (Mann–Whitney U Test, p=0.009). On the contrary, in group IV animals for which the baseline therapy was supplemented with the administration of MSCs, the CRP level did not differ from the values of intact animals throughout the experiment (0.20 (0.20;0.30) and 0.20 (0.20;0.30) pg/mL, respectively; Mann–Whitney U Test, p=0.523) and was statistically significantly lower than the values in groups II and III (Mann–Whitney U Test, $p<0.05$).

As is known, free radical mechanisms and lipid peroxidation processes are closely associated with the SIRS development; and the pancreas has the lowest level of antioxidants in the body [14]. The

intensity of LPO is reflected by the concentrations of intermediate (diene conjugates) and final (MDA) oxidation products. As our studies have shown, the MDA content in the blood of animals of group II on the 3rd day after creating the AEP model was higher than in intact animals (21.60 (19.40; 22.45) and 18.65 (16.50; 19.53) μmol/mL, respectively; Mann–Whitney U Test, p=0.109), which indicates an increase in the intensity of free radical oxidation (Fig. 6). In animal group III, despite the treatment, there was an increase in the blood content of MDA to 19.95 (18.10;21.80) μmol/mL on the 3rd day; then throughout the experiment, the blood MDA level continued to increase and by the 7^{th} day its content had reached its peak values (21.25 (19.68; 22.38) μmol/mL). On the 3rd day of the experiment, the MDA values in animal group IV, where MSCs had been used for treatment, were lower than in animals of groups II and III. Subsequently, there was a slight increase in this parameter; but on the 7th day, it was lower than in group III (20.95 (15.40; 25.18) and 21.25 (19.68; 22.38) μmol/mL, respectively; Mann–Whitney U Test, p=0.873). The latter indicates a favourable effect of MSCs on the lipid peroxidation during AEP.

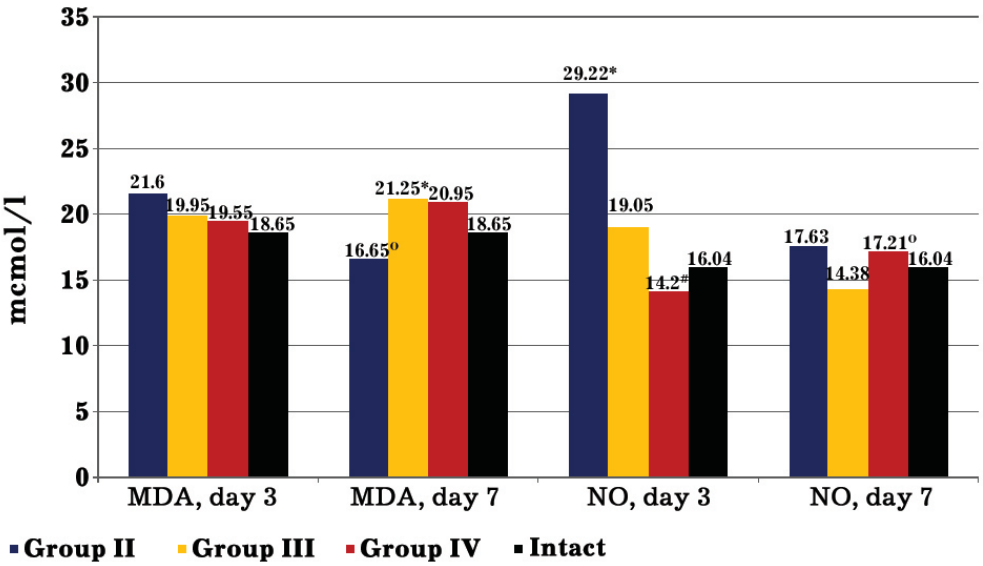

Fig. 6. Dynamics of the level of malonic dialdehyde and nitric oxide in the blood of experimental animals (n=12 in each group; * significant in comparison with intact animals; # significant in comparison with day 3 in animals of group II; ° significant in comparison with day 3 in the group)

In the development of AP, the main cytotoxic effect is due to the activation of oxygen-dependent mechanisms and the NO synthesis. According to the data obtained, on the 3rd day after the AP model, the blood serum NO level in the animals of group II statistically significantly exceeded (by 81%) the values in intact animals (29.22 (19.42;35.48) and 16.04 (14.31;23.54) μmol/mL, respectively; Mann-Whitney U Test, p=0.004) (Fig. 6). The use of baseline therapy for the treatment did not prevent the increase of blood serum NO level in the animals of group III to 19.05 (14.78; 23.30) μmol/mL on the 3rd day. 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 this parameter in the both groups was not statistically significantly different from the values in intact animals (Mann–Whitney U Test, p>0.05). The use of MSCs contributed to the fact that the blood serum NO content in the animals at 72 hours from the start of the experiment was the lowest (compared to that in intact animals (14.20 (11.35;16.05) and 16.04 (14.31;23.54)

μmol/mL, respectively; Mann-Whitney U Test, p=0.201). The content of NO in the blood serum after 7 days did not differ between different groups of animals. Thus, a decrease in the intensity of lipid peroxidation processes and a decrease in the NO level at early stages after regional administration of MSCs confirms their therapeutic efficacy in AEP.

Discussion

The AP pathogenesis has not been completely studied to date. However, the progress in uncovering some molecular pathogenetic mechanisms is obvious, which creates the prerequisites for the targeted development of new methods and means of specific treatment for AP. According to current concepts, the pathophysiology of AP includes the activation and release of pancreatic enzymes (amylase, lipase) with the subsequent launch of a cascade of events leading to SIRS. It is this syndrome that is responsible for progressive damage to the pancreatic parenchyma, its necrosis, and the MODS development [2]. Our study revealed that when modeling AP, the level of amylase in the peripheral blood increased significantly in experimental animals compared to intact ones, and the administration of MSCs decreased the level of this enzyme, which is consistent with the data obtained by other authors [7, 15–18].

In an experiment, it was previously shown that the severity of AP manifestations is interconnected with impaired microcirculation in the pancreas [19]. Aggressive substances released from dead secretory cells of the pancreas damage the vascular endothelium of both the pancreas itself and vital organs [20]. Functional failure of the endothelium leads to the activation of its procoagulant properties [21]. The interaction of platelets with the endothelium plays one of the leading roles in the AP pathogenesis [22]. The studies of some authors has also shown that changes in the number of platelets may indicate the severity and

unfavorable outcome of AP. The platelet count was significantly reduced in patients with severe and extremely severe AP, while patients with mild and moderate severity of the disease did not differ in this characteristic between themselves and the control group [23]. On the 3rd day, in the blood of animals where MSCs were not used, there was a marked thrombocytopenia: the platelet count was statistically significantly lower than in the blood of intact animals. In animals of group IV, where MSCs were used for treatment, the platelet level on the 3rd day was the highest and did not differ from that in intact animals.

Damage to the pancreatic parenchyma in severe AP can lead to necrosis of the acini, the death of β-cells and contribute to the development of hyperglycemia [24]. Assessing glycemic levels on the 3rd day of the experiment, we found that the use of MSCs in AP led to a decrease in blood glucose levels compared to the control group, and it was statistically significantly lower than the values of this parameter in intact animals. The information obtained gives reason to believe that the absence of hyperglycemia in AEP may indicate the cytoprotective effect of MSCs on the cellular structures of the pancreas.

Studying the action of inflammatory mediators and methods of their inhibition is the main direction for improving the complex treatment for AP. SIRS 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, adhesion molecules. 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 MODS development of 25]. There is also an opinion that the primary source of proinflammatory mediators is the acinar cells themselves. In response to hyperstimulation or direct damage to the acinar cells, the synthesis of IL-1β, IL-6, IL-8

takes place, as well as the activation of an important pro-inflammatory mediator, a platelet activating factor [26]. The latter attracts neutrophils, which migrate to the affected site through the vessel wall, and also causes the adhesion and aggregation of platelets. After the activated neutrophil has penetrated 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 white blood cells, 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 in the microvasculature of the liver, kidneys, lungs, and other organs, which again induces the synthesis of inflammatory mediators (cytokine cascade). Numerous studies have shown that in groups of animals with induced AP, the levels of pro-inflammatory cytokines TNF-a, IL-1, IL-6, IL-15, IL-17 significantly increased [7–9]. After administration of MSCs to animals, the level of expression of these inflammatory markers decreased, and the level of cytokines with anti-inflammatory activity (IL-4, IL-10) increased after the administration of MSCs [7–9, 28]. Another mechanism of MSC-mediated immunomodulation is their ability to potentiate the transition from the inflammatory M1 macrophages to the anti-inflammatory state of M2 macrophages [29]. At the same time, MSCs suppressed the functions of activated M1 macrophages and increased the activity of M2 macrophages, which had an anti-inflammatory effect. D. Qian et al. (2017) in their study showed a significant decrease in the level of amphoterin (HMGB1) during the treatment of AEP with MSCs compared to the control [30]. TSG-6 released by MSCs has an anti-inflammatory effect, suppressing the production of proinflammatory cytokines by macrophages [16]. In our

study, in the group of animals where MSCs were used, on the 3rd day of the experiment the concentration of TNF-α was 42% lower than the control values and there was no further increase in this parameters in these animals. During the same period, the IL-6 concentration was statistically significantly lower compared to the control group and 3 times lower than the values of intact animals. When studying CRP in a group of animals for which the standard baseline therapy was supplemented with the MSC administration, the level of this marker throughout the experiment did not differ from the values in intact animals and was statistically significantly lower than the values in the control group.

A damaged acinar cell releases free oxygen radicals and lipid peroxidation products, which can activate neutrophils with subsequent potentiation of SIRS and microcirculatory disorders. In addition, LPO products themselves have pronounced toxicity and destructive effects. There are opinions that it is the processes of LPO that are one of the main causes for the development of destructive changes, and the volume of these changes is directly proportional to the intensity of LPO [14]. Other authors experimentally demonstrated that the MSC administration to animals with AP led to a decrease in the MDA level and an increase in the activity of glutathione peroxidase and superoxide dismutase, which characterize the state of the antioxidant system [7–9, 28]. One of the MSC antioxidant mechanisms, according to Z. He et al. (2016), is the stimulation of the hyaluronate-binding protein TSG-6 (TNF-α-induced protein-6) secretion [16]. During AEP in animals that were injected with MSCs with the knockdown of the TSG-6 gene, the MDA level was higher, and the parameters the antioxidant system state were reduced. This suggested a relationship between the production of TSG-6 by MSCs with the oxidative stress suppression and the damage to pancreatic tissue. The interaction of NO with superoxide anions results in the formation of

peroxynitrite which is an extremely cytotoxic product. It causes vasodilation, disrupts an oxygen transport, and leads to a deterioration in the body's oxygen status [31]. In our study, the regional administration of MSCs to laboratory animals reduced the intensity of lipid peroxidation processes and reduced the blood serum level of NO at an early stage of AP, which confirms the therapeutic effect of this method in treating the disease.

Conclusion

A comparison of various treatment regimens for acute experimental pancreatitis showed that the early use of mesenchymal stromal cells have a beneficial effect on the platelet count, the glycemia level, helps to reduce the contents of endogenous intoxication elements (malonic dialdehyde, nitric oxide) and those of the systemic inflammatory response (interleukin-6, tumor necrosis factor alpha, C-reactive protein), which are key links in the pathogenesis and leading markers of the acute pancreatitis severity.

Based on the results, we can make the following conclusions:

- In animals of group IV, where mesenchymal stromal cells were used for the AEP treatment, the platelet count in blood on the 3rd day of the experiment was 83% higher than in the group of animals with acute experimental pancreatitis without treatment and did not differ from that parameter in intact animals (687.50 (510.50;779, 0) and 553.0 (428.25;751.0) $x10^9$/L, respectively; Mann–Whitney U Test, p=0.631). Using the mesenchymal stromal cells at the same time of the experiment led to the hyperglycemia correction. The glucose level was statistically significantly lower than the values of this parameter in the animals of the control group (5.75 (4.89;6.47) and 9.0 (6.50;11.50) mmol/L, respectively; Mann-Whitney U Test, p=0.016). Animals in this group also showed a statistically significant decrease in amylasemia; by the end of the experiment, α-amylase activity in the blood serum had decreased from

890.0 (800.0;1429.25) units/mL to 677.0 (603.75;816.25) (Wilcoxon test, p=0.028).

- On the 3rd day of the experiment, the concentration of tumor necrosis factor alpha was 42% lower in the blood serum of animals, where baseline therapy was combined with the administration of mesenchymal stromal cells, compared to that in intact animals (1.02 (0.85;1.24 and 1.45 (1.32;1.77) pg/mL, respectively; Mann-Whitney U Test, p=0.011); and there was no further increase in this parameter. And the concentration of interleukin-6 was statistically significantly lower compared to groups II and III and 3 times lower than the values of intact animals (88.0 (71.65;155.95) and 258.30 (209.30;333.25) pg/mL, respectively; Mann–Whitney U Test, p=0.033). In the blood serum of group IV animals, the level of C-reactive protein throughout the experiment did not differ from the values of intact animals (0.20 (0.20;0.30) and 0.20 (0.20;0.30) pg/mL respectively; Mann–Whitney U Test, p=0.523) and was statistically significantly lower than the values in groups II and III (Mann–Whitney U Test, $p<0.05$).
- On the 3rd day of the experiment, the blood serum malonic dialdehyde level was lower in the animal group where mesenchymal stromal cells were used for treatment, compared to that in the animals of other groups and did not differ from the values of the intact animals (19.55 (18.40;20.7) and 18.65 (16.50;19.53) μmol/mL, respectively; Mann–Whitney U Test, p=0.109), and at 72 hours from the start of the experiment, the blood serum NO content of the animals was twice lower than the values in the control group (14.20 (11.35;16.05) and 29.22 (19.05) 42;35.48) μmol/mL, respectively; Mann–Whitney U Test, p=0.019).

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