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The effect of mesenchymal stromal cells, their microvesicles and platelet-rich plasma on pathological changes in pancreatic tissue in acute necrotizing pancreatitis in rats

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Abstract

Background. *Improving treatment outcomes for acute necrotizing pancreatitis (ANP) remains the most difficult and unresolved problem for surgeons and intensive care specialists.*

Objective. *To evaluate the effect of mesenchymal stromal cells (MSCs), MSC-derived microvesicles (MSC MVs) and platelet-rich plasma (PRP) on morphological and immunohistochemical characteristics of the pancreas in ANP in rats with regard to the route and timing of PRP administration, as well as separate and combined application.*

Material and methods*. The effects of MSCs, MSC MVs and PRP on the morphological and immunohistochemical characteristics of the pancreas in ANP in rats* were studied, taking into the account the *route (intraperitoneal/intravenous) and time of administration (6 and 24 hours*

from the start of disease modeling), as well as their separate or combined use (PRP and MSCs, PRP and MSC MVs). The study was conducted on 72 adults Wistar rats. Acute pancreatitis was induced by the administration of a 0.3 ml of 5% solution of non-ionic polyethylene glycol octylphenol ether detergent into the caudal part of the rat pancreas. Organ and tissue sampling was performed on the 3rd day from the beginning of the disease modeling. Histological changes in pancreatic tissues were studied by hematoxylin and eosin staining the preparations. Immunohistochemical staining of pancreatic tissue with anti-TGF-β1 and anti-SCARD1 primary monoclonal antibodies was performed followed by studying the nature of their expression.

***Results**. A comparative assessment of the effects of MSCs, MSC MVs and PRP on the histomorphological and immunohistochemical changes in pancreatic tissue in ANP in rats, found that more pronounced regeneration and neoangiogenesis processes were observed with the intravenous delivery of MSCs and MSC MVs, regardless of the timing of administration (6 or 24 hours from the onset of the disease). The obtained data suggested the immunomodulatory and pro-regenerative effects of MSC and MSC MVs produced by potentiating the polarization from inflammatory M1 macrophages to anti-inflammatory M2, as evidenced by a pronounced immunohistochemical reaction to TGF-β1, which is secreted predominantly by macrophages of the M2 phenotype.*

***Conclusion**. The experimental use of MSCs and MSC MVs to treat ANP at early stages of the disease provides more pronounced processes of repair and neoangiogenesis in pathologically altered pancreatic tissue.*

Keywords: microvesicles, mesenchymal stem cells, acute pancreatitis, immunohistochemistry, pancreas, intravenous, intraperitoneal

Conflict of interest The authors declare no conflict of interest

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ANP, acute necrotizing pancreatitis
AP, acute pancreatitis
CD, cluster of differentiation
DAB, diaminobenzidine
FBS, fetal bovine serum
IHC, immunohistochemistry
MP, macrophage
MSC, mesenchymal stromal cell
MV, microvesicle
PBS, phosphate buffered saline
PRP, platelet-rich plasma (plasma enriched with soluble platelet factors)
SCARD1, Scavenger Receptor Class D Member 1
TGF-β 1, transforming growth factor beta-1

Introduction

Currently, cell therapy is considered as one of the means of treating acute necrotizing pancreatitis (ANP) [1]. The therapeutic effect of bioproducts of cellular origin occurs in three directions: the differentiation and replacement of damaged tissue cells, production of biologically active molecules, and interaction with immune cells [1–3].

At an early stage of ONP, macrophages (MPs) penetrate the pancreas, are activated and differentiated into the proinflammatory phenotype M1, secreting inflammatory cytokines and mediators, causing local inflammation and necrosis in the pancreas [4]. Depending on the functional phenotype, macromolecule markers appear on MPs, which are called the cluster of differentiation (CD).

CD68 (cluster of differentiation 68, macrosialin, SCARD1) is a lysozyme-associated membrane protein expressed on the surface of monocytes and macrophages and is a common marker for detecting macrophages in rat and mouse tissues [5, 6]. CD68 plays a role in the phagocytic activity of tissue macrophages both in intracellular lysosomal metabolism and in extracellular cell-cell and cell-pathogen interactions. It is believed that the presence of enzymatic activity in this marker prevents membrane damage by lysosomal enzymes due to their degradation [5].

Transforming growth factor-beta1 (TGF-β1) belongs to the family of dimeric polypeptides and is widely represented in tissues. The sources of TGF-β1 are mainly monocytes and MPs, it content being constant [7]. This polypeptide is secreted mainly by activated MPs of the M2 phenotype, it controls regenerative and neoplastic processes, the development of fibrosis, and also performs the function of stimulating and regulating the angiogenesis during the healing process [4, 8]. TGF-β1 has been shown to be involved in the restoration and regeneration of rat liver [9], pancreas in acute pancreatitis (AP) [10-12], blood vessels [13], heart [14]; it also plays a role in the cartilage regeneration [15] and promotes the healing process of nerve tissues [16].

The ability of mesenchymal stromal cells (MSCs) and their microvesicles (MSC MVs) to interact with immune cells allows us to consider options for their use as a promising option for the treatment of experimental ANP. The analysis of data from scientific literature indicates an active investigating search for optimal timing, dosages, and routes of delivery for bioproducts of cellular origin in various pathologies. In general, the results of this issue studies and the opinions of different authors are rather ambiguous and sometimes contradictory, therefore, a comprehensive study of the therapeutic potential of cellular origin bioproducts in ANP remains relevant.

The study objective was to evaluate the effects of mesenchymal stromal cells, their microvesicles, and plasma enriched with soluble platelet factors (PRP) on the morphological and immunohistochemical characteristics of the pancreas in rats with acute necrotizing pancreatitis, depending on the route and time of administration, as well as separate and combined use.

Material and methods

Experimental studies were conducted at the vivarium of the Belarusian State Medical University in accordance with 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), as well as in accordance with the "Regulations on the Procedure for the Use of Experimental Animals in Scientific Research and the Educational Process at the Belarusian State Medical University". The animals were kept in a designated box for one week to adapt to new conditions; before the study, all animals were weighed and examined for signs of disease.

Experimental design

The experimental study was conducted on 72 mature male Wistar rats weighing 275–380 g. The nature of the experimental studies is presented in Table 1.

Table 1. Experimental studies on animals

Characteristics of animal groups (rats)	Number of animals
Intact animals	6
Control group of animals	
K. Acute experimental pancreatitis without treatment	6
Experimental groups of animals	

$O_{1.}$ Acute experimental pancreatitis, treatment with intravenous saline solution	6
$O_{2.}$ Acute experimental pancreatitis, regional treatment PRP 24 hours after modeling the disease	6
$O_{3.}$ Acute experimental pancreatitis, regional treatment with MSCs at 24 hours after modeling the disease	6
$O_{4.}$ Acute experimental pancreatitis, regional treatment with MSC MVs at 24 hours after modeling the disease	6
O_{5} Acute experimental pancreatitis, treatment with intravenous MSCs 24 hours after modeling the disease	6
$O_{6.}$ Acute experimental pancreatitis, treatment with intravenous MSC MVs at 24 hours after modeling the disease	6
$O_{7.}$ Acute experimental pancreatitis, treatment with intravenous MSCs 6 hours after modeling the disease	6
$O_{8.}$ Acute experimental pancreatitis, treatment with intravenous MSC MVs at 6 hours after modeling the disease	6
$O_{9.}$ Acute experimental pancreatitis, treatment with regional PRP during modeling and intravenous MSCs 6 hours after modeling the disease	6
$O_{10.}$ Acute experimental pancreatitis, treatment with regional PRP during modeling and intravenous MV MSC 6 hours after modeling the disease	6

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 simulate the necrotizing form of AP, all animals underwent standard laparotomy and 0.3 ml of 5% non-ionic detergent of polyethyleneglycol octylphenol ether (Triton X-100, manufactured by Carl Roth GmbH & Co. KG, Germany) was injected into the caudal (gastric-splenic) part of the pancreas using an insulin syringe.

Methodology for obtaining bioproducts of cellular origin

Bone marrow MSCs were obtained from rat femurs by perfusion with DMEM/F12 medium (Elabscience, China) containing 5% fetal bovine serum (FBS). The collected cell suspension was washed by centrifugation at 1500 rpm for 20 minutes. MSCs were seeded in

DMEM/F12 medium containing 10% FBS in T25 culture flasks (Sarstedt, Germany). After large colonies of MSCs were formed at the bottom of the plastic flasks, the cells were removed with 0.25% trypsin-EDTA (Elabscience, China) under a Leica DM2500 inverted microscope (LEICA Microsystems, Germany) at a magnification of x25, washed by centrifugation and recultured in new culture flasks at a concentration of 3000/cm^2.· MSCs of 2-3 passages were used in the study. The cell mass of MSCs was increased to 80% confluent growth in DMEM/F12 nutrient medium with the addition of 10% FBS and the antibiotic solution (streptomycin/penicillin, Sigma, USA). MSCs were brought to a concentration of 200 thousand/mL and used in experiments in vitro.

Extracellular MSC MVs were obtained by differential centrifugation with the selection of different centrifugation speed modes, time, and filtration with a solution [17]. At the first stage, MSCs were seeded in a T75 culture flask (Sarstedt, Germany) at a concentration of 3000/cm^2 in a complete nutrient medium DMEM/F12 with 10% FBS. The cells were cultured in a CO_2 incubator (Esco, Singapore) at +37°C and 5% CO_2 until the confluence on the growth surface of more than 90%. At the second stage, to enrich the MV culture medium, the cell culture was washed with phosphate-buffered saline (PBS, Elabscience, China) and the culture was transferred to a serum-free DMEM/F12 medium. The cultivation was continued for 48 hours to create conditions of mild cellular stress that do not cause necrotic cell death and promote the release of high-molecular substances. The third stage involved directly obtaining the MV fraction from the MSC culture fluid. The resulting conditioned medium was centrifuged for 20 minutes at 3000 rpm at +4°C to remove large particles, cells, and cellular debris. The supernatant containing MV was collected and precipitated by centrifugation in 1.5 ml Eppendorf tubes at 14,500 g for 30 minutes at +4°C (MicroCL 21R microcentrifuge, Thermo Scientific,

USA). Two thirds of the supernatant volume were removed, the lower third of the volume was combined from two tubes. The MV suspension was brought to a volume of 1.5 ml with the PBS, previously filtered through a 0.2 nm syringe membrane filter, and centrifuged at 14,500 g for 30 minutes at a temperature of +4°C. The supernatant was removed, leaving 250 μL of sediment in a test tube, combined into one test tube, brought to 1.5 ml with PBS and centrifuged in the same mode. The resulting MV sediment was resuspended in 1 ml of PBS (from the initial cell culture volume of 10 ml) and used in the experiments.

Allogeneic PRP was obtained using a modified method of R. Yamaguchi et al. [18].

Method of regional (intraperitoneal) administration of cellular origin bioproducts in the treatment of acute necrotizing pancreatitis in rats

In animals of groups O_2, O_3, and O_4, after the completion of the AP modeling stage, a catheter made of transparent thermoplastic implant-free non-toxic polyvinyl chloride was inserted into the abdominal cavity to the pancreas through a counter-opening on the anterior abdominal wall in the left lower quadrant according to the technique we developed (Patent for invention BY 24210 dated 28.12.2023). Surgical procedures were performed under sterile conditions; antibiotic prophylaxis was not performed. In the postoperative period, all animals were given pain relief by intramuscular administration of ketorolac at a rate of 4.8 mg/kg/day of animal weight.

Treatment of acute necrotizing pancreatitis in experiment

For animals of group O_1, the treatment was initiated 24 hours after the start of the ANP modeling; in addition to pain relief, 0.9% NaCl

solution was administered intravenously in a volume of 2 ml once a day. For animals of group O_2, the above treatment was supplemented with regional (intraperitoneal) administration of PRP in a volume of 150 μL twice every 6 hours through a previously installed catheter (Patent for invention BY 24225 dated 07.02.2024) on the 1st day after modeling. For animals of group O_3, the treatment during the same period was performed by regional administration of MSCs on the 1st day after modeling at a dose of $1x10^6$ cells/kg in 1.0 ml of the prepared solution through a previously installed catheter (Patent for invention BY 24535 dated 03. 02.2025). In group O_4, the standard infusion therapy with saline solution was combined with the regional administration of 1.0 ml of prepared solution of MSC MVs through a previously installed catheter on the first day from the onset of the disease (Patent for invention BY 24542 dated 05.03.2025). The MV dose was calculated as equivalent to (obtained from) 1 million MSCs. To determine the most effective route of administration (intravenous or intraperitoneal/regional), the timing of AP treatment (6 or 24 hours from the onset of disease modeling), isolated or combined use of MSCs, MSC MVs and PRP, the animals of groups O_5 and O_7 were administered MSCs intravenously at a dose of $1x10^6$ cells/kg in 1.0 ml of the prepared solution 6 hours (group O_7) and 24 hours (group O_5) hours after disease modeling; animals of groups O_6 and O_8 were administered intravenously MSC MVs in a volume of 1.0 ml of the prepared solution 6 hours (group O_8) and 24 (group O_6) hours after disease modeling. In animals of group O_9, after modeling the disease, before suturing the laparotomy wound, PRP was regionally administered to the pathologically altered part of the pancreas in a volume of 300 μL; after 6 hours, the above treatment was combined with intravenous administration of MSCs at a dose of $1x10^6$ cells/kg in 1.0 ml of the prepared solution. In animals of group O_{10}, the local administration of

PRP in a volume of 300 μL during modeling of the ANP was supplemented after 6 hours with intravenous administration of MSC MVs in a volume of 1.0 ml of the prepared solution.

On the 3rd day from the start of the ANP modeling, the animals were taken out of the experiment by euthanasia (with intraperitoneal sodium thiopental at a dose of 200 mg/kg). Organs were collected from all animals at the control periods for pathomorphological studies.

Macroscopic and histological evaluation of rat pancreas

Macroscopic changes in the pancreas and abdominal cavity were visually examined and photographed on the 3rd day after the creation of the ANP model. Pancreatic tissue was collected for pathomorphological examination. The material was fixed in a 10% solution of neutral buffered formalin (pH=7.2) for 24 hours. The obtained samples were dehydrated in alcohols of increasing concentration (70-96%). Then the samples were placed in liquid paraffin. Handling histological specimens was carried out using the Leica TP 1020 station, with subsequent production of paraffin blocks on the Leica EG 1160 automatic embedding station. From all paraffin blocks, 3 μm thick sections were made and stained with hematoxylin and eosin. Using the Motic EASYSCAN PRO (Motic, China) histological scanner, full-slide digital scans of histological preparations were performed, which analysis and video capture of visual fields for illustrative material were made using the Aperio ImageScope (Leica, USA) software.

Immunohistochemical study of rat pancreas

Immunohistochemical (IHC) staining of autopsy material from rat pancreas for subsequent analysis of the expression pattern of biomolecular markers was performed using primary monoclonal

antibodies against TGF-β1, Scard1 (Wuhan Fine Biotech Co., China). Secondary antibody complexes (peroxidase-antiperoxidase) according to the manufacturers' specifications were used as visualization systems, and diaminobenzidine (DAB) was used as a chromogen. Negative controls included tissue slides without primary antibody staining.

For each IHC marker, a staining protocol was developed with the selection of the optimal antigen unmasking mode, dilution of primary antibodies, choice of visualization system, and chromogen exposure time (Table 2). The sections were washed with running water, stained with Mayer's hematoxylin, and embedded in Canadian balsam.

Table 2. Monoclonal antibodies used for immunohistochemical staining of rat pancreas

Primary antibody, clone	Origin	Unmasking buffer, pH	Visualizing system	Dilution	Exposure time to chromogen	Positive control
TGF-β1	Rabbit	pH=9.0	Universal	1:600	5 minutes	Internal
Scard1 (CD-68)	Rabbit	pH=9.0	Universal	1:600	5 minutes	Internal

The Aperio ImageScope software was used to analyze the expression of the assessed markers. Tissues expressing the corresponding proteins were used to control expression; the primary antibody was excluded for negative control.

The results of the IHC study of molecular biomarkers were interpreted based on the localization and intensity of tissue staining with the DAB chromogen. The criteria for assessing the staining intensity were the following: negative staining (-), weak intensity (+), moderate intensity (++), and pronounced intensity (+++).

Results

Previously, we had conducted experimental studies that revealed significant systemic changes in this model of ANP, and also revealed the ability of MSCs, MSC MVs, and PRP to have a therapeutic effect on systemic parameters in this disease in experimental animals [19].

In animals of all groups, clinical and behavioral manifestations of the disease were of the same type. During the first day, the rats showed hypodynamia, somewhat inhibited behavior, decreased exploratory reaction, and they stopped eating. No effusion was seen at autopsy of the animals withdrawn from the experiment on the 3rd day from the beginning of abdominal AP modeling. The pancreas was edematous, loosely fused with the stomach, spleen, and loop of the small intestine, its lobulation was smoothed out, the tissue was gray.

When studying morphological changes in the pancreas of animals of groups K and O_1, the severity of necrotic changes was variable, but as early as in this period in animals of the control group, we noted the initial signs of organization among the remaining ductal and acinar structures of destroyed lobules in the pancreas with polymorphic cell infiltration with a significant number of fibroblasts, lymphocytes, and eosinophils. Histologically, on the 3rd day in the non-treated pancreatic tissue of animals with ANP (group K), the severity of necrotic changes varied from subtotal necrosis to focal necrosis in the marginal sections of the pancreas lobules, but initial processes of necrotic detritus resorption were already noted at the site of necrotic tissue. Fibroblast proliferation, collagen fiber synthesis, proliferation of glandular structures with swollen epithelium, edema, and inflammatory infiltration were also noted (Fig. 1A). Widespread steatonecrosis was observed in the adjacent tissues of the omentum (Fig. 1B).

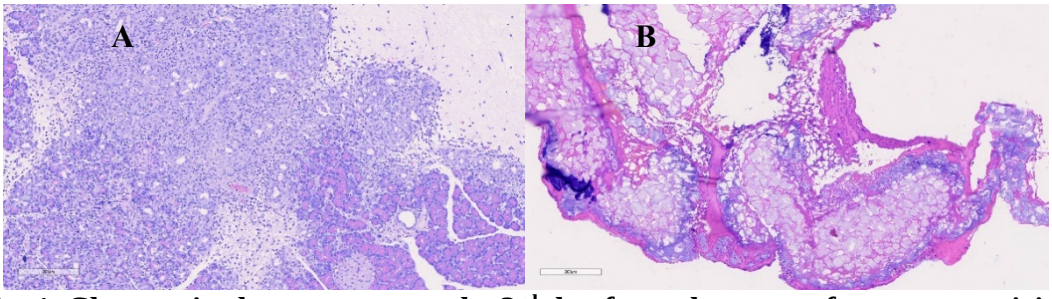

Fig. 1. Changes in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in animals of group К. Hematoxylin and eosin stain. A, digital zoom x4, G 500nm; B, digital zoom x2, G 1 mm

In group O_1 animals, which underwent the basic treatment, structural damage to the pancreatic tissue was noted during the same period; foci of necrosis of entire lobules, polymorphic cell inflammatory infiltration, pronounced edema, fibroplasia and loss of zymogen were seen (Fig. 2A). Pronounced acute discomplexation and dystrophic changes in pancreatic acinocytes were detected (Fig. 2B). Structural damage to the pancreatic lobules was accompanied by vascular thrombosis and multiple microhemorrhages (Fig. 2C, D).

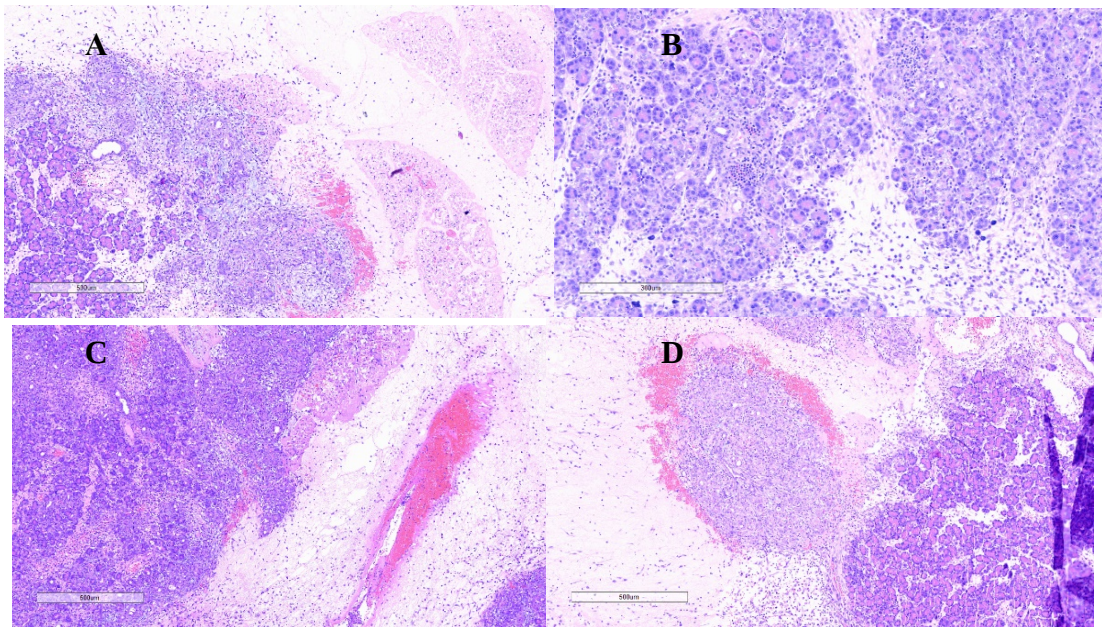

Fig. 2. Changes in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in animals of group O_1. Hematoxylin and eosin stain. A, digital zoom x4, G 500 nm; B, digital zoom x8, G 300nm; C, digital zoom x4, G 500 nm; D, digital zoom x4, G 500 nm

When studying the regional impact of the bioproducts of cellular origin on morphological changes in the pancreas of animals of groups O_2, O_3 and O_4, the foci of steatonecrosis and foci of necrosis in the pancreatic tissue were found on the 3rd day from the onset of the disease modeling. The reparation processes were more pronounced, the detritus resorption at the site of pancreatic necrosis foci was completed, signs of regeneration were determined in the form of pronounced proliferation of fibroblasts with an insignificant amount of collagen fibers. Pronounced proliferation of fibroblasts was noted both on the side of the alteration foci, and also in the interlobular stroma.

In the O_2 group of animals where PRP was used, the foci of steatonecrosis and small foci of necrosis in the pancreatic tissue, interstitial edema with polymorphic-cellular inflammatory infiltration were observed on the 3rd day, and fibrin thrombi were found in the lumen of the veins (Fig. 3A). But as early as in this period, a kind of organization in the damaged structures of the pancreas, pronounced fibroplasia, lympho-leukocyte infiltration in the necrosis zone were noted (Fig. 3B). Edema of the pancreatic tissue, a significant number of fibroblasts, delicate fibrous stroma, lymphoid infiltration with the formation of lymphoid follicles were seen (Fig. 3C).

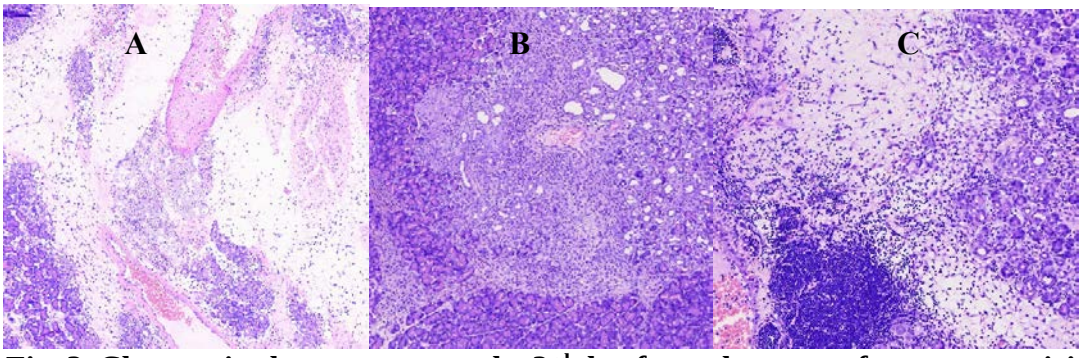

Fig. 3. Changes in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in animals of group O_2. Hematoxylin and eosin stain. A, digital zoom x8, G 300 nm; B, digital zoom x8, G 300 nm; C, digital zoom x4, G 500 nm

In the animal groups O_3 and O_4, in which MSCs and MSC MVs were respectively used regionally for the treatment,, the reparation processes were more pronounced, the detritus resorption was completed, the signs of organization among the remaining ductal and acinar structures of the destroyed lobules were noted at the site of pancreatic necrosis foci, with polymorphic cell infiltration and a significant number of fibroblasts, lymphocytes, and eosinophils (Fig. 4).

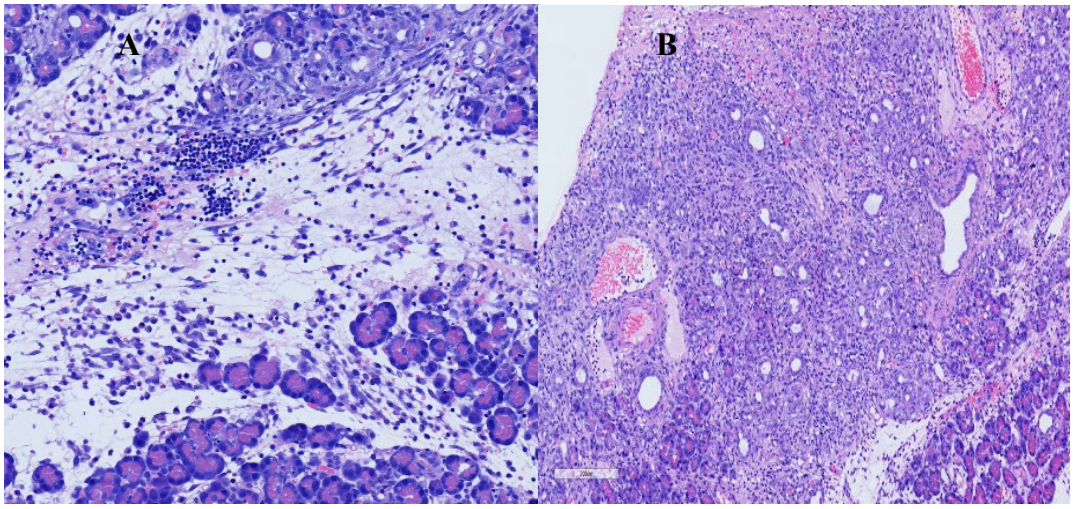

Fig. 4. Changes in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in animals of groups O_3 and O_4. Hematoxylin and eosin stain. A, group O_3, digital zoom x15, G 300 nm; B, group O_4, digital zoom x10, G 200 nm

The comparative analysis of the pancreas pathomorphology in rats with ANP, with regard to the route of administering the bioproducts of cellular origin, the more pronounced processes of regeneration and neoangiogenesis were observed with the intravenous route of delivering MSCs and MSC MVs, regardless of the time of administration (6 or 24 hours).

Histologically, on the 3rd day in animals of group O_5, where MSCs were administered intravenously 24 hours after the onset of disease modeling, the interstitium edema with polymorphic cellular inflammatory infiltration was noted in the pancreatic tissue, small foci of steatonecrosis and foci of necrosis were detected, fibrin thrombi were found in the

venous lumen (Fig. 5A). The pancreatic stroma was delicately fibrous with a significant number of fibroblasts and lymphoid infiltration (Fig. 5B). In the pancreatic necrosis zone, the pronounced lympholeukocytic infiltration with fibroplasia and organization was determined (Fig. 5C).

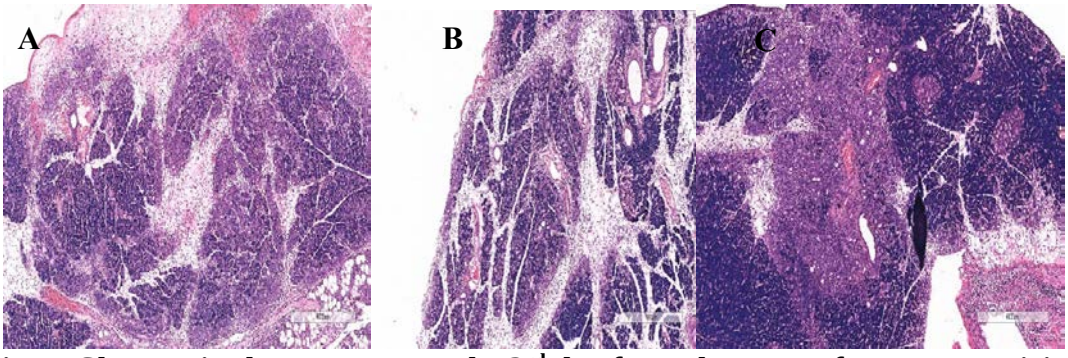

Fig. 5. Changes in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in animals of group O_5. Hematoxylin and eosin stain, digital zoom x6, G 600 nm

During the same period, necrosis, steatonecrosis, stromal edema, inflammatory infiltration, vascular congestion, marginal leukocytes, and leukodiapedesis were histologically observed in the pancreatic tissue of group O_6 animals (Fig. 6A). Alongside with these changes, pronounced reparative processes were observed in the pancreas: detritus resorption was completed, and regeneration signs in the form of pronounced proliferation of fibroblasts with a moderate amount of collagen fibers were observed (Fig. 6B).

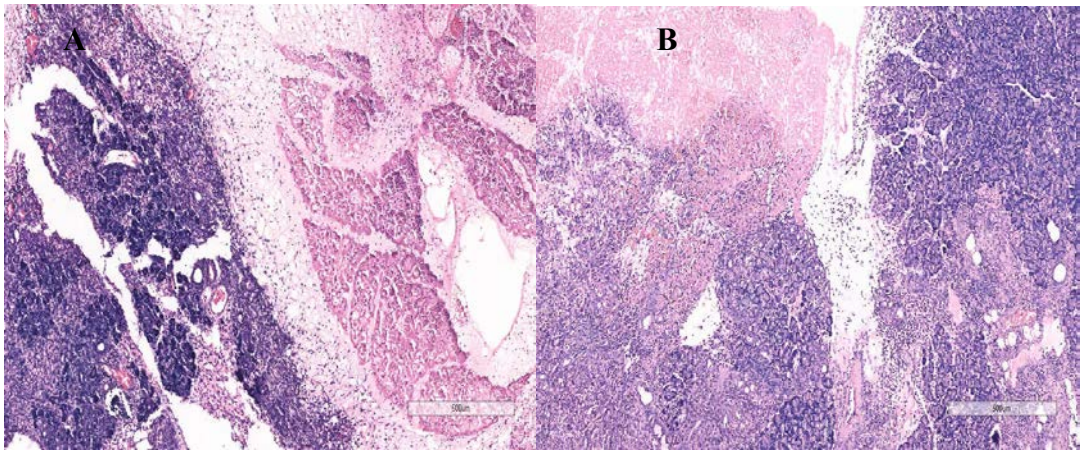

Fig. 6. Changes in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in animals of group O_6. Hematoxylin and eosin stain, digital zoom x6, G 500 nm

With intravenous administration of MSCs 6 hours after the onset of disease modeling (group O_7), the complete resorption of the necrosis zone with signs of neoangiogenesis organization and polymorphic cell infiltration were observed in the pancreas as well as a significant number of fibroblasts, lymphocytes, eosinophils among the remaining ductal and acinar structures of the destroyed lobules (Fig. 7A). The perifocal reaction zone was more pronounced, small foci of steatonecrosis remained in the peritumor zone, and mucoidization of the newly formed stroma with foci of fibrinoid necrosis wass noted in the necrosis organization zone (Fig. 7B).

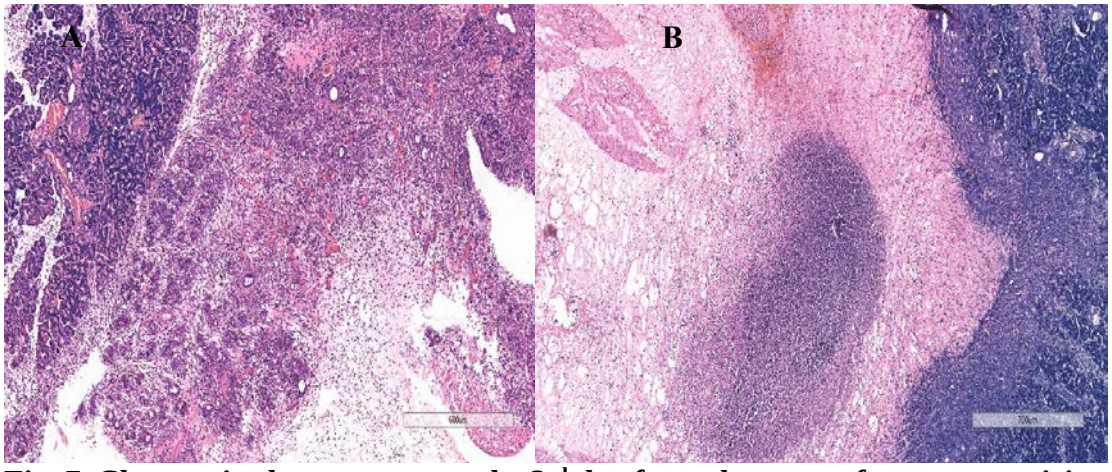

Fig. 7. Changes in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in animals of group O_7. Hematoxylin and eosin stain. A, digital zoom x6, G 600 nm; B, digital zoom x6, G 700 nm

In O_8 group, a massive deposition of fibrin with signs of organization was observed on the surface of the serous membrane of the pancreas, a reparative reaction was seen in the underlying sections, occasional eosinophils in the infiltrate content were noted in the area of damage. A reparative reaction was determined in the subcapsular sections of the damage area, occasional eosinophils in the infiltrate content, and initial signs of neoangiogenesis were noted (Fig. 8A, B). Massive necrosis, stromal edema, inflammatory infiltration, vascular congestion were observed in the pancreatic tissue, and newly formed vessels appeared in the marginal zone of damaged pancreatic lobules (Fig. 8C). Alongside with necrosis zones, young connective tissue with polymorphic cell inflammatory infiltration in the interlobular space of the pancreas was visualized (Fig. 8D).

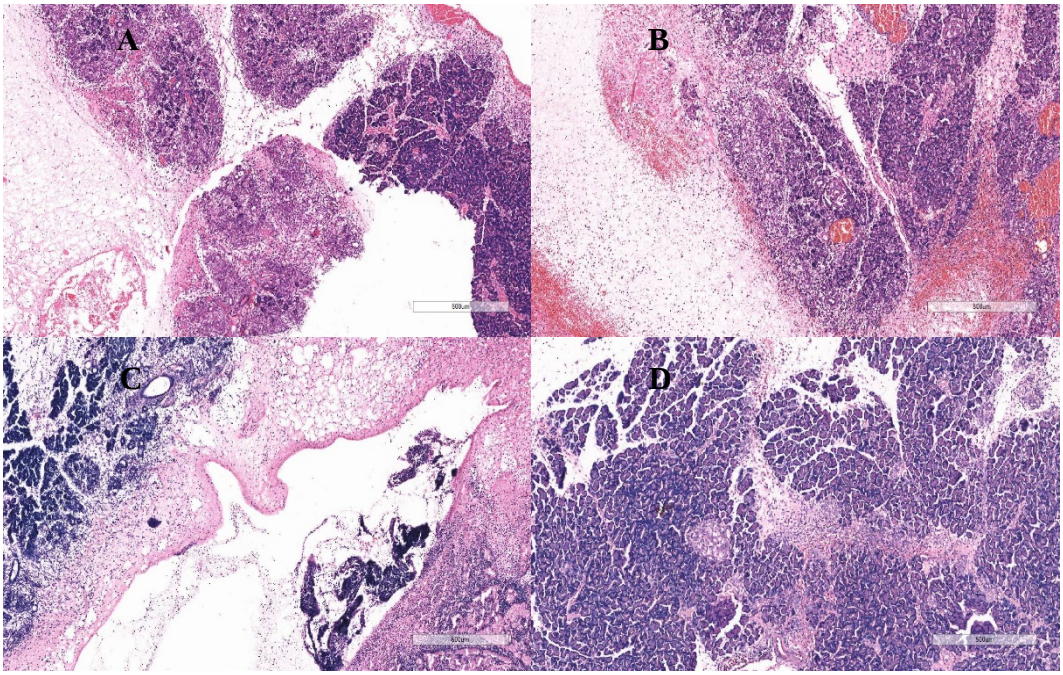

Fig. 8. Changes in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in animals of group O_8. Hematoxylin and eosin stain. A, B, C, digital zoom x6, G 600 nm; D, digital zoom x6, G 500 nm

With the combined use of PRP and MSCs (group O_9), PRP and MSC MVs (group O_{10}), the general nature of pathological changes in the pancreas was similar: foci of necrosis with detritus resorption, signs of regeneration in the form of pronounced proliferation of fibroblasts with a moderate amount of collagen fibers, neoangiogenesis in the marginal zone of damage were noted (Fig. 9).

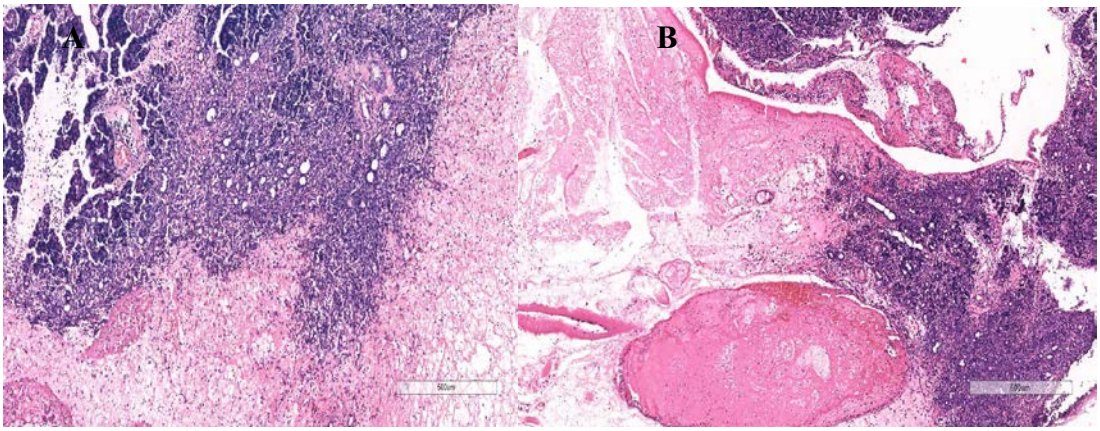

Fig. 9. Changes in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in animals of groups O_9 and O_{10}. Hematoxylin and eosin stain. A, group O_9, digital zoom x6, G 500 nm; B, group O_{10}, digital zoom x6, G 600 nm

In an IHC study of TGF-β1, it was found that in animals in the untreated group (K) and in groups where bioproducts of cellular origin were applied regionally (O_2, O_3, and O_4), the moderate (++) expression of TGF-β1 was observed in the zones of damage and the beginning of organization (Fig. 10) when compared to the animal groups that were treated intravenously.

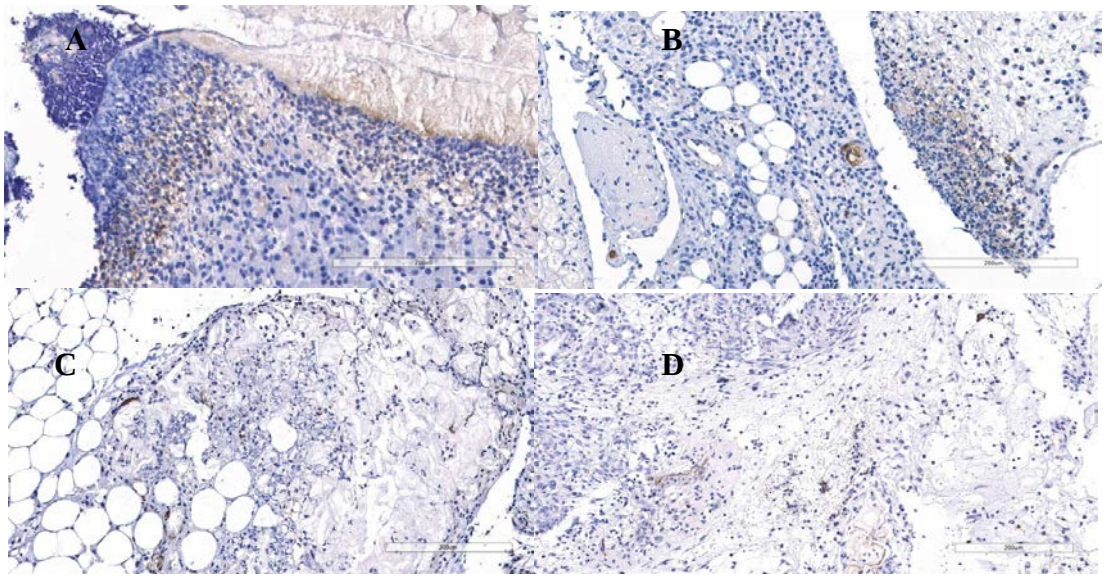

Fig. 10. Moderate expression of TGF-β1 in the area of pancreatic steatonecrosis on the 3rd day from the onset of acute necrotizing pancreatitis in experimental animals. A, group К, digital zoom x15; B, group O_2, digital zoom x10, C, group O_3, digital zoom x10; D, group O_4, digital zoom x10

In all experimental groups, where the treatment was performed by means of intravenous administration of MSCs and MSC MVs (O_{5-10}), regardless of the time of administration from the onset of disease modeling, the pronounced (+++) expression of TGF-β1 was observed in the edematous stroma of the pancreas, in the zones of organization and neoplasm of vessels, as well as at the border of steatonecrosis and preserved gland tissue (Fig. 11).

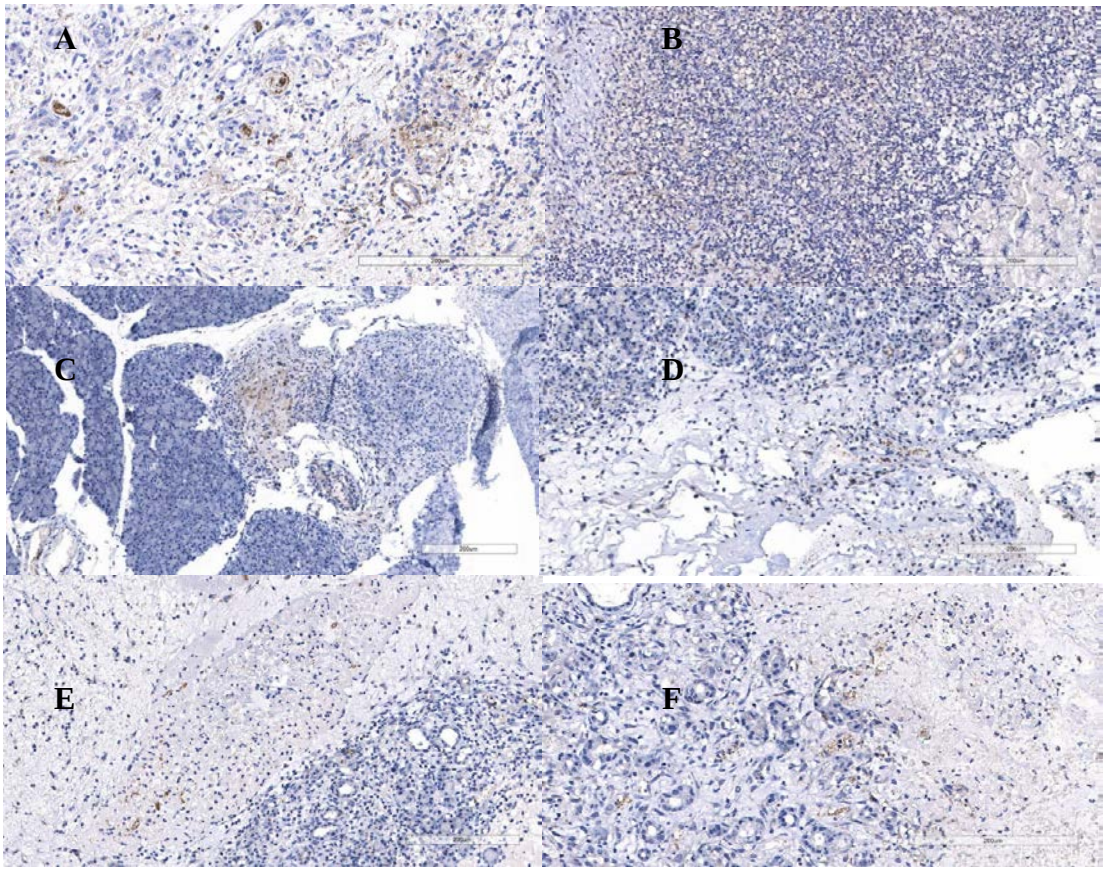

Fig. 11. Pronounced expression of TGF-β1 in the pancreas on the 3rd day from the onset of acute necrotizing pancreatitis in experimental animals. A, group O_5, in the zone of organization and neoangiogenesis, digital zoom x15; B, group O_6, in the area of steatonecrosis, digital zoom x10; C, group O_7, in the area of steatonecrosis, digital zoom x10; D, group O_8, in the area of steatonecrosis, digital zoom x10; E, group O_9, in the zone of neoangiogenesis, digital zoom x10; F, group O_{10}, in the zone of neoangiogenesis, digital zoom x10

In a comparative IHC assessment, the lowest rates of CD68 expression intensity (single cells expressed the marker, negative staining) were noted in groups K and O_2 (Fig. 12).

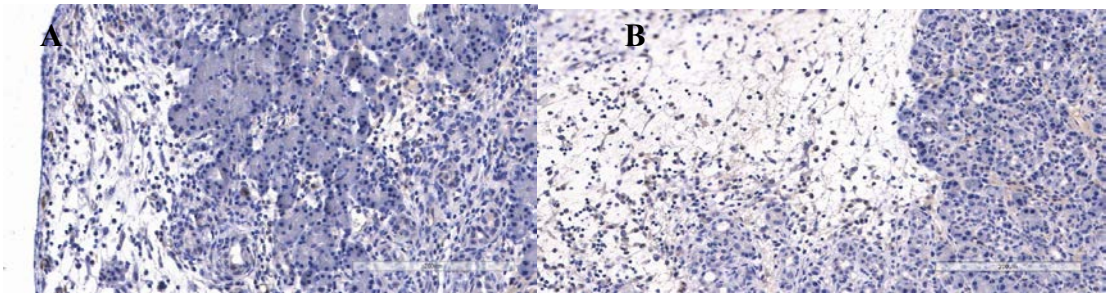

Fig. 12. Single expression of CD68 (SCARD1) in pancreatic macrophages on the 3rd day from the onset of acute necrotizing pancreatitis in experimental animals. A, group K, in the marginal zone of damage, digital zoom x10; B, group O_2, in the interlobular space, digital zoom x10

Moderate expression of SCARD 1 in the zones of the beginning organization in the pancreas was observed in groups O_3 and O_4 (Fig. 13).

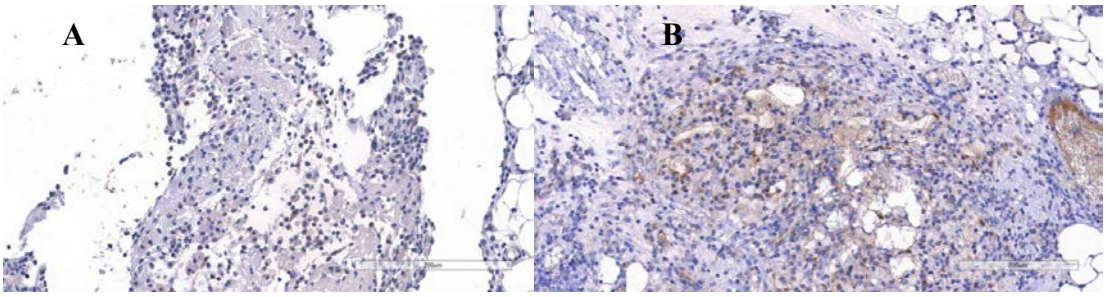

Fig. 13. Moderate expression of CD68 (SCARD1) in pancreatic macrophages on the 3rd day from the onset of acute necrotizing pancreatitis in experimental animals. A, group O_3, in the area of steatonecrosis, digital zoom x10; B, group O_4, in the zone of neoangiogenesis, digital zoom x10

On the contrary, in groups $O_{5\text{-}10}$, at the border of steatonecrosis and the preserved pancreatic tissue, a pronounced IHC reaction of SCARD 1 was observed in the zones of the beginning organization and neoformation of vessels (Fig. 14).

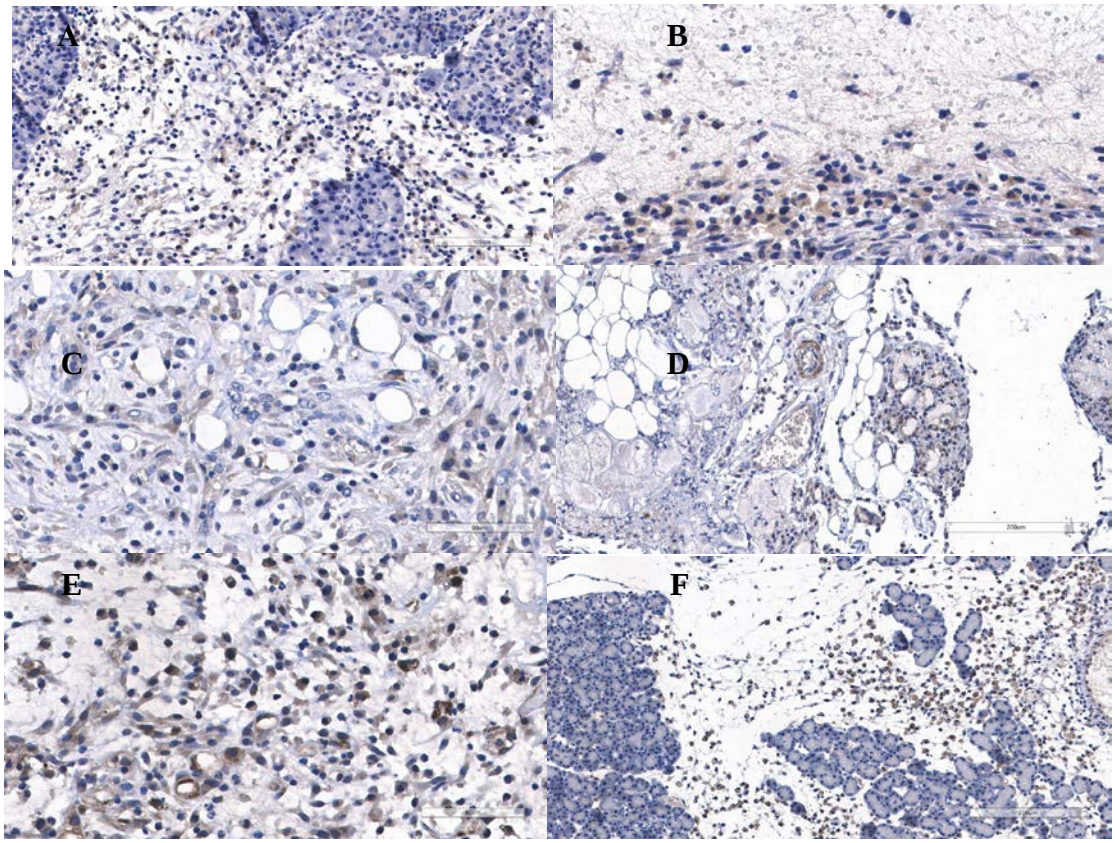

Fig. 14. Pronounced expression of CD68 (SCARD1) in pancreatic macrophages on the 3rd day from the onset of acute necrotizing pancreatitis in experimental animals. A, group O_5, in the interlobular space, digital zoom x10; B, group O_6, in the marginal zone, digital zoom x 10; C, group O_7, in the zone of neoangiogenesis, digital zoom x15; D, group O_8, in the marginal zone, digital zoom x10; E, group O_9, in the zone of neoangiogenesis, digital zoom x15; F, group O_{10}, in the interlobular space, digital zoom x10

Discussion

The pathophysiology of ANP includes the activation and release of pancreatic enzymes into the interstitial space. It has been shown that in the initial phase of the disease, intra-acinar activation of trypsinogen occurs with its conversion to trypsin, followed by the activation of other enzymes, which lead to endothelial dysfunction and ischemia of the pancreatic tissue [20]. In addition to the activation of pancreatic enzymes in the pancreas and their release into the bloodstream, in ANP, the

infiltration of the pancreas with immune cells begins within minutes, including MPs, neutrophils, dendritic, mast cells, natural killer cells, as well as T and B lymphocytes [8]. MPs play a decisive role in the ANP pathogenesis. In particular, having high plasticity, they are quickly polarized, differentiated into various phenotypes and perform various functions in AP. There are two functional phenotypes of MPs: classically activated (M1) and alternatively activated (M2) cells [4]. In ANP, MPs, migrate to the pancreas under the impact of various stimuli, and then are polarized to the M1 phenotype, which secretes inflammatory mediators IL-1β, IL-6, IL-12 and tumor necrosis factor alpha (TNF-α), initiate and enhance inflammation, which ultimately causes severe AP with the development of organ failure. In contrast, M2 MPs play an anti-inflammatory, immunoregulatory and pro-fibrotic role by secreting IL-4, IL-10, IL-13 and transforming growth factor-β [4, 21, 22]. An imbalance in MP polarization, especially a variant in which the M1 to M2 ratio significantly increases, is one of the mechanisms underlying the development of ANP [22]. In view of the above, a targeted MP therapy, using cell-derived bioproducts aimed at changing the polarization of MPs from the pro-inflammatory phenotype M1 to the anti-inflammatory M2, is attractive for the treatment of AP.

In our study, the comparative histomorphological and immunohistochemical assessments of the pancreas condition with regional administering the bioproducts of cellular origin in ANP in rats, it was established that the use of MSCs, MSC MVs, and PRP had a high therapeutic efficacy in the early stages of the disease compared to control animals where no treatment was administered. When studying morphological changes in the pancreas of animals of groups K, O_1, O_2, O_3, and O_4, the foci of necrosis in the pancreas tissue were observed on the 3rd day from the beginning of the experiment, but as early as in this

period, the initial signs of organization were noted. In animals where MSCs and MSC MVs were used regionally (groups O_3 and O_4, respectively) for the treatment, these processes were more pronounced, the detritus resorption at the site of pancreas necrosis foci was already completed, the signs of regeneration were noted in the form of pronounced proliferation of fibroblasts with an insignificant amount of collagen fibers. These data were confirmed by a moderate (++) immunohistochemical reaction of SCARD1 (CD68), a common marker of MPs and TGF-β1, which is secreted predominantly by anti-inflammatory MPs M2 [4]. The lowest rates of SCARD1 expression intensity were observed in groups K, where treatment was not used, and $O_{2,}$ where PRP was used for treatment, single cells expressed the marker. When comparing the morphological and immunohistochemical characteristics of the pancreas with regard to the routes of administering the bioproducts of cellular origin, the more pronounced ("+++" expression of TGF-β1 and SCARD1) reparation and neoangiogenesis processes were observed with the intravenous method of MSC and MSC MV delivery, regardless of the time of administration (6 or 24 hours). Our data are consistent with the results of other studies. In their experiment, AM Roch et al. (2020) found that intravenous administration of MSCs in mice with AP increased the proportion of anti-inflammatory M2 phenotype MPs in the pancreas [23]. It can be assumed that intravenously administered MSCs and their MVs act as moderators of peripheral blood monocytes/MPs migrating to the site of inflammation in the pancreas. There is accumulated evidence that MSCs promote tissue regeneration through the secretion of growth factors and modulation of the immune response rather than by replacing damaged cells [24]. Studies by K.H. Jung (2011) showed that in the presence of MSCs, the secretion of IL-10 and the expression of the transcription factor Foxp3 increase, which

promotes the differentiation of activated T cells into regulatory T lymphocytes (Tregs), which stimulate the differentiation of MPs into the anti-inflammatory M2 phenotype [25, 26].

Thus, our data indicate an immunomodulatory and pro-regenerative effect of MSCs and MSC MVs by potentiating polarization from inflammatory M1 MPs to the anti-inflammatory state of M2 MPs.

Conclusion

The experimental use of mesenchymal stromal cells and their microvesicles for the treatment of acute necrotizing pancreatitis in the early stages of the disease provides more pronounced processes of reparation and neoangiogenesis in pathologically altered pancreatic tissue.

In conclusion we can summarize the obtained results as follows:

1.The model of acute necrotizing pancreatitis created by introducing 0.3 ml of a 5% solution of the non-ionic detergent of polyethyleneglycol octylphenol ether into the gastrosplenic part of the rat pancreas allows the necrotizing form of the disease to be reproduced in animals. The changes revealed are pathogenetically characteristics of a severe form of acute pancreatitis, which justifies the expediency of using this experimental model to study the effect of new treatment methods for this disease.

2.In a comparative assessment of the effect of mesenchymal stromal cells, their microvesicles and plasma enriched with soluble platelet factors on the histomorphological and immunohistochemical characteristics of the pancreas in acute necrotizing pancreatitis in rats, with regard to the route (regional/intravenous) and time of administration

(6 and 24 hours from the onset of the disease), it was established that more pronounced processes of reparation and neoangiogenesis were observed with the intravenous method of delivery of mesenchymal stromal cells, their microvesicles, regardless of the time of administration (6 or 24 hours).

3.No difference was found in the intensity of reparative processes in the pancreatic tissue of experimental animals in the treatment of acute necrotizing pancreatitis between the separate and combined (plasma enriched with soluble platelet factors and mesenchymal stromal cells; plasma enriched with soluble platelet factors and microvesicles of mesenchymal stromal cells) intravenous use of the bioproducts of cellular origin.

4.The data we have obtained indicate an immunomodulatory and pro-regenerative effect of mesenchymal stromal cells, and their microvesicles by potentiating the polarization from inflammatory macrophages M1 to anti-inflammatory macrophages M2, as evidenced by a pronounced immunohistochemical reaction to TGF-β1, which is secreted predominantly by macrophages of the M2 phenotype.

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