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Morphological effectiveness of platelet-rich plasma application in the experimental treatment of post immobilization extra-articular contractures of the mandible

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ABSTRACT

Aim: The purpose of the study was to prove the effectiveness of platelet-rich plasma (PRP) application in the treatment of post-immobilization extra-articular contractures of the mandible by modeling the specified pathology and conducting morphological analysis of experimental material.

Materials and Methods: The study involved an experiment conducted on 60 male WAG rats aged 9-11 months. Four groups were formed. Group 1 included 6 intact rats that were not subjected to any interventions and were withdrawn from the experiment one month after its initiation. Group 2 included 18 rats with a mandibular fracture in the angle region which was treated over the course of one month using an immobilizing muzzle. After removal of the muzzle, extra-articular mandibular contracture was diagnosed. All rats were withdrawn from the experiment one month after its initiation. Group 3 included 18 rats with mandibular fractures that were treated over the course of one month using an immobilizing muzzle. After removal of the muzzle, post-immobilization extra-articular mandibular contracture was diagnosed. Following this diagnosis, 6 rats were withdrawn from the experiment. The other rats received PRP injections into the contracture area every three days for 15 days. After completion of the treatment, these rats were also withdrawn from the experiment. Group 4 included 18 rats with a mandibular fracture. After modeling a mandibular fracture, PRP was injected into the soft tissues surrounding the fracture through the available holes in the immobilizing muzzle every five days for one month. After a month, the immobilizing muzzle was removed from the rats, among which 6 rats were randomly selected and withdrawn from the experiment. The other rats continued to be injected with PRP every three days for 15 days, after which the animals were withdrawn from the experiment. The material for morphological examination consisted of masseter muscle samples. Histological, histochemical, immunohistochemical, morphometric and statistical methods were used.

Results: The comprehensive morphological study showed that PRP in the masseter muscle had antifibrotic and anti-inflammatory effects, reduced the severity of alternative changes in muscle fibers and increased their regenerative potential, reduced the severity of hemodynamic disorders, and increased the number of vessels. The therapeutic effect of PRP was more pronounced in cases where it was applied both during the treatment of mandibular fracture using an immobilizing muzzle for one month and for an additional 15 days after its removal, compared to animals in which PRP was applied only during the 15 days following muzzle removal.

Conclusions: The comprehensive morphological study of experimental material conducted by the authors confirmed the effectiveness of platelet-rich plasma in the treatment of post-immobilization extra-articular contractures of the mandible, thereby expanding the available arsenal of treatment methods for this pathology.

KEY WORDS: experiment, morphological effectiveness, platelet-rich plasma, post-immobilization extra-articular contractures, mandible

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INTRODUCTION

The mandibular fracture is one of the most common fractures of the face (70-80%) compared to any other bone of the cranium [1]. One of the complications after mandibular fracture treatment is post-immobilization extra-articular contractures [2]. Post-immobilization contractures can be a significant problem for patients, as they can result in pain, limited mandible range of motion and reduced

function. Also, they can have a substantial impact on quality of patient's life [3].

The pathogenesis of mandibular post-immobilization extra-articular contractures is difficult and multifactorial [4]. Numerous researchers associate their development with damage to the masticatory muscles, which include the masseter, temporal muscle, and the lateral and medial pterygoid muscles [5]. Our previous studies revealed changes

in the morphofunctional state of the parenchymal and stromal components of the masseter muscle in patients with post-immobilization extra-articular contractures of the mandible [6].

The available range of conservative and surgical treatment methods for extra-articular post-immobilization contractures of the mandible does not allow for full restoration of mandibular function or significant improvement in patients' quality of life [7]. This fact highlights the relevance of conducting comprehensive experimental studies on laboratory animals, which will allow for the investigation of the disease pathogenesis, the development of new treatment methods, and the evaluation of their effectiveness.

AIM

The purpose of the study was to prove the effectiveness of PRP application in the treatment of post-immobilization extra-articular contractures of the mandible by modeling the specified pathology and conducting morphological analysis of experimental material.

MATERIALS AND METHODS

The study involved an experiment conducted on 60 male WAG rats aged 9-11 months, carried out at the Experimental Biological Clinic of Kharkiv National Medical University.

Group 1 included 6 intact rats that were not subjected to any interventions and were withdrawn from the experiment one month after its initiation.

Group 2 included 18 rats in which a mandibular fracture in the angle region was modeled using an original technique previously described by the authors [8]. The diagnosis of the modeled fracture was performed by visual inspection of the mandible in the affected area and using the diaphonization method [9]. The latter was applied to 6 randomly selected rats that were withdrawn from the experiment on the first day after fracture modeling. In 12 rats, the mandibular fracture was treated over the course of one month using an immobilizing muzzle designed by the authors (Fig. 1). After removal of the muzzle, extra-articular mandibular contracture was diagnosed on the side where the fracture had been previously modeled. All rats were withdrawn from the experiment one month after its initiation.

Group 3 included 18 rats with mandibular fractures. The fracture modeling and diagnostic procedures were similar to those in Group 2. In 12 rats, the mandibular fracture was treated over the course of one month using an immobilizing muzzle similar to that used in Group 2. After removal of the muzzle, post-immobilization extra-articular mandibular contracture was diagnosed. Following this diagnosis, 6 rats were withdrawn from the experiment. The other 6 rats received PRP injections into the contracture area every three days for 15 days. After completion of the treatment, these rats were also withdrawn from the experiment.

Group 4 included 18 rats with a mandibular fracture, the modeling procedure and diagnosis of which were



Fig. 1. Group 2 rat under anesthesia with a modeled mandibular fracture and an immobilizing muzzle.

similar to groups 2-3. After modeling a mandibular fracture in rats, PRP was injected into the soft tissues surrounding the fracture through the available holes in the immobilizing muzzle every five days for one month. After a month, the immobilizing muzzle was removed from the rats, among which 6 rats were randomly selected and withdrawn from the experiment. The other 6 rats continued to be injected with PRP every three days for 15 days, after which the animals were withdrawn from the experiment.

In rats of groups 3 and 4, 2 ml of blood was collected from the vessels of the tail in special tubes. Centrifugation of the blood resulted in 0.5 ml of PRP.

The material for morphological examination consisted of masseter muscle samples taken from intact rats in group 1 and from rats in groups 2-4, specifically from the area where the mandibular fracture had been modeled. Tissue fragments of the masseter muscle were fixed in a 10% formalin solution. Tissue consolidation was achieved by passing the samples through alcohols of increasing concentration, Nikiforov's solution (96% ethanol and diethyl ether in a 1:1 ratio), and chloroform, followed by paraffin embedding. Serial sections with a thickness of $4-5 \times 10^{-6}$ m were prepared from the paraffin blocks for subsequent staining with hematoxylin and eosin, picrofuchsin according to Van Gieson, and using the Rego method.

Immunohistochemical study was performed on Super Frost Plus adhesive slides ("Menzel", Germany). The Master Polymer Plus Detection system (Peroxidase, DAB chromogen) (Master Diagnostica, Spain) was used, citrate buffer (pH 6.0) and EDTA buffer (pH 8.0) were used for high-temperature processing of antigen epitopes. Immunohistochemical study was performed using a rabbit monoclonal antibody to vimentin (clone SP20, Master Diagnostica, Spain).

Microslides were examined using a ZEISS Primostar 3 microscope (Carl Zeiss, Germany) equipped with an integrated color digital camera and a BRESSER Science TFM-301 Trino microscope with a BRESSER Full HD camera (Bresser GmbH, Germany).

Morphometric analysis was performed using the Labscope software. In the masseter muscle, in the field of view of the microscope $\times 100$, the following parameters were assessed: specific volumes (%) of parenchymal and stromal components; absolute number of blood vessels; specific volume of altered muscle fibers. The immunohistochemical reaction was evaluated by counting the absolute number of Vimentin⁺-cells in stroma of the masseter muscle in the field of view of the microscope $\times 1000$.

The data in the groups were statistically processed using the PAST software (version 4.15, Natural History Museum, University of Oslo, Norway). The mean values in the groups

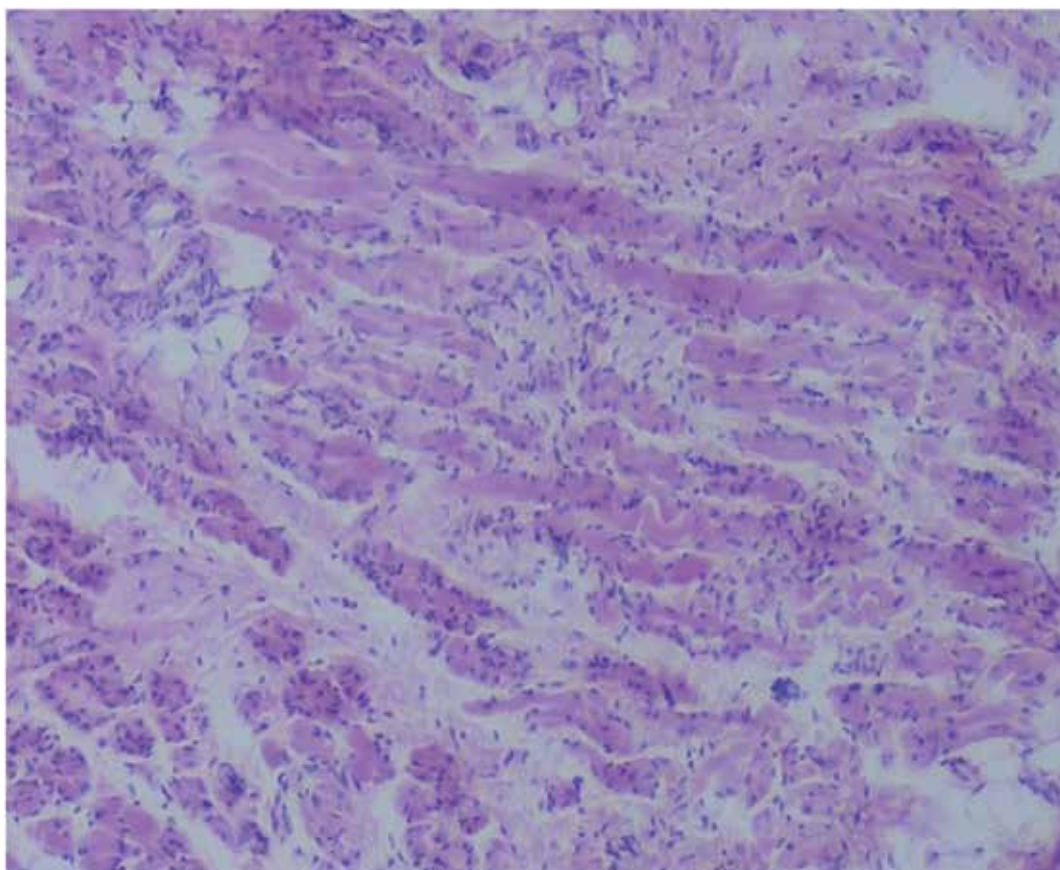


Fig. 2. Atrophic and hypertrophic muscle fibers in the masseter muscle of a Group 4 rat. Hematoxylin and eosin staining, $\times 100$.

were compared using the Mann-Whitney U test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

In rats that were withdrawn from the experiment after one month, overview microscopy of the masseter muscle showed that in group 1, the muscle fibers had a uniform thickness. In groups 2 and 3, the muscle fibers appeared thinned, indicating the development of atrophic changes. In group 4, the muscle fibers exhibited uneven thickness due to the presence of both atrophic and hypertrophic fibers, with the latter predominating (Fig. 2).

In groups 2-4, compared to group 1, the muscle fibers exhibited ischemic, dystrophic, and necrotic changes. These alterations were manifested by wavy deformation of the muscle fibers, uneven hematoxylin and eosin staining of the cytoplasm and nuclei, and the absence of nuclei. Rego staining revealed that altered muscle fibers appeared black (Fig. 3). Morphometric analysis showed that the specific volume of altered muscle fibers in groups 2 and 3 did not differ significantly ($p > 0.05$). In group 4, this parameter was significantly lower compared to groups 2 and 3 ($p < 0.05$) (Table 1).

The stromal component of the masseter muscle in groups 1-4 was characterized by the presence of connective tissue, blood vessels, and nerve fibers. The absolute number of blood vessels in groups 2-4 was significantly lower compared

to group 1 ($p < 0.05$), which negatively affected tissue trophism. There was no significant difference ($p > 0.05$) in the absolute number of blood vessels between groups 2 and 3, whereas group 4 showed a significantly ($p < 0.05$) higher value compared to groups 2 and 3 (Table 1).

In the stroma of the masseter muscle in group 1 rats, few diffusely distributed cellular elements were observed, represented by lymphocytes, monocytes, histiocytes, and fibroblastic lineage cells. In groups 2-4, compared to group 1, the above-mentioned cellular infiltration was focal-diffuse and its severity increased (Fig. 4). The indicated infiltration was moderately pronounced in group 4 and maximally pronounced in groups 2 and 3.

Immunohistochemical examination revealed Vimentin⁺-cells, which are mesenchymal cells responsible for the production of connective tissue components, among the above-mentioned cellular infiltration (Fig. 5). Compared to group 1, the absolute number of such cells was significantly higher in groups 2-4 ($p < 0.05$). There was no significant difference ($p > 0.05$) in the number of Vimentin⁺-cells between groups 2 and 3 ($p > 0.05$), while group 4 showed a significantly lower value compared to groups 2 and 3 ($p < 0.05$).

In groups 2-4, compared to group 1, hemodynamic disturbances were observed in stroma of the masseter muscle. These were manifested by edema, vascular hyperemia, the presence of acute thrombi in the lumen of some vessels, and the formation of small- and large-focal hemorrhages.

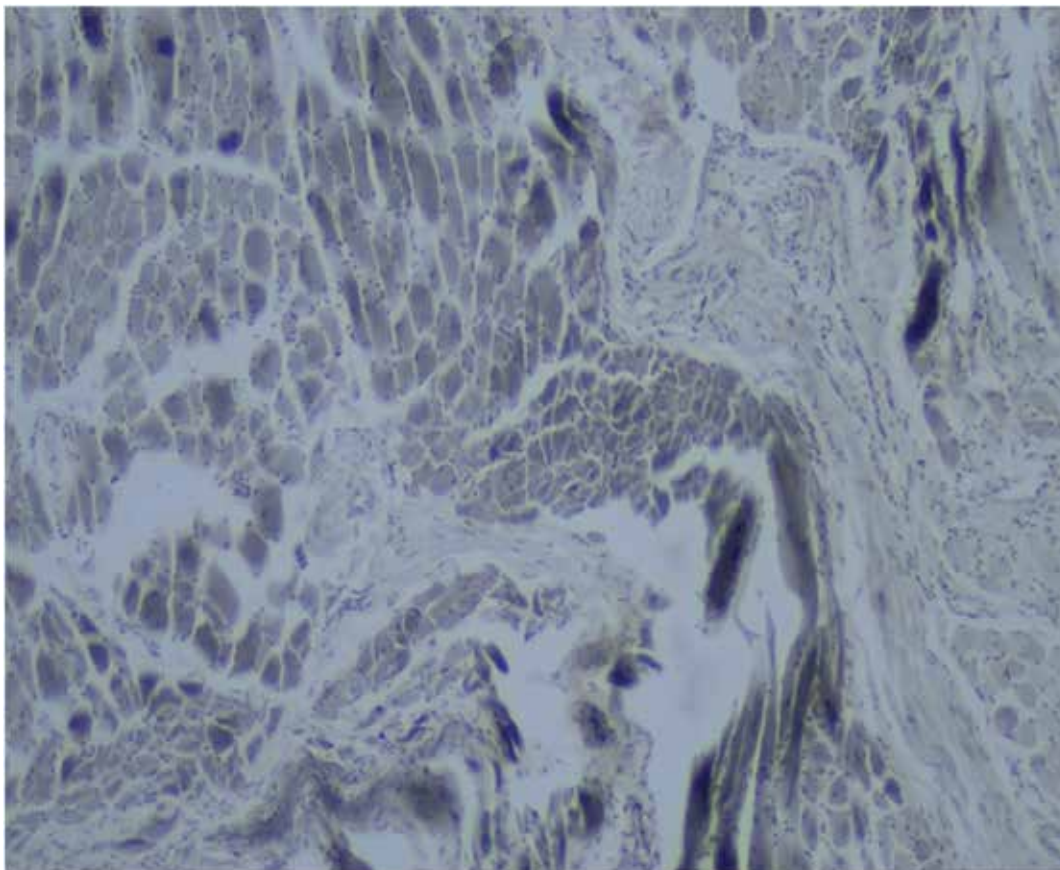


Fig. 3. Altered muscle fibers stained black in the masseter muscle of a Group 3 rat. Rego staining, $\times 100$.

Table 1. Mean values of morphometric parameters in rats of Groups 1-4 withdrawn from the experiment after 1 month

Group	Name of the morphometric parameter				
	Specific volume of parenchyma	Specific volume of stroma	Absolute number of blood vessels	Specific volume of altered muscle fibers	Absolute number of Vimentin ⁺ -cells
1	89.3±1.8%	10.7±1.8%	12.8±1.1	-	15.8±1.3
2	43.6±2.4% ¹	56.4±2.4% ¹	4.8±0.3 ¹	26.7±2.0%	34.6±2.3 ¹
3	44.1±2.6% ¹	55.9±2.6% ¹	4.2±0.4 ¹	25.8±2.1%	38.5±2.7 ¹
4	66.9±4.1% ^{1,2,3}	33,1±4.1% ^{1,2,3}	7.5±0.8 ^{1,2,3}	4.9±0.5% ^{2,3}	25.3±2.6 ^{1,2,3}

Note: ¹ – statistically significant difference compared to group 1; ² – statistically significant difference compared to group 2; ³ – statistically significant difference compared to group 3.

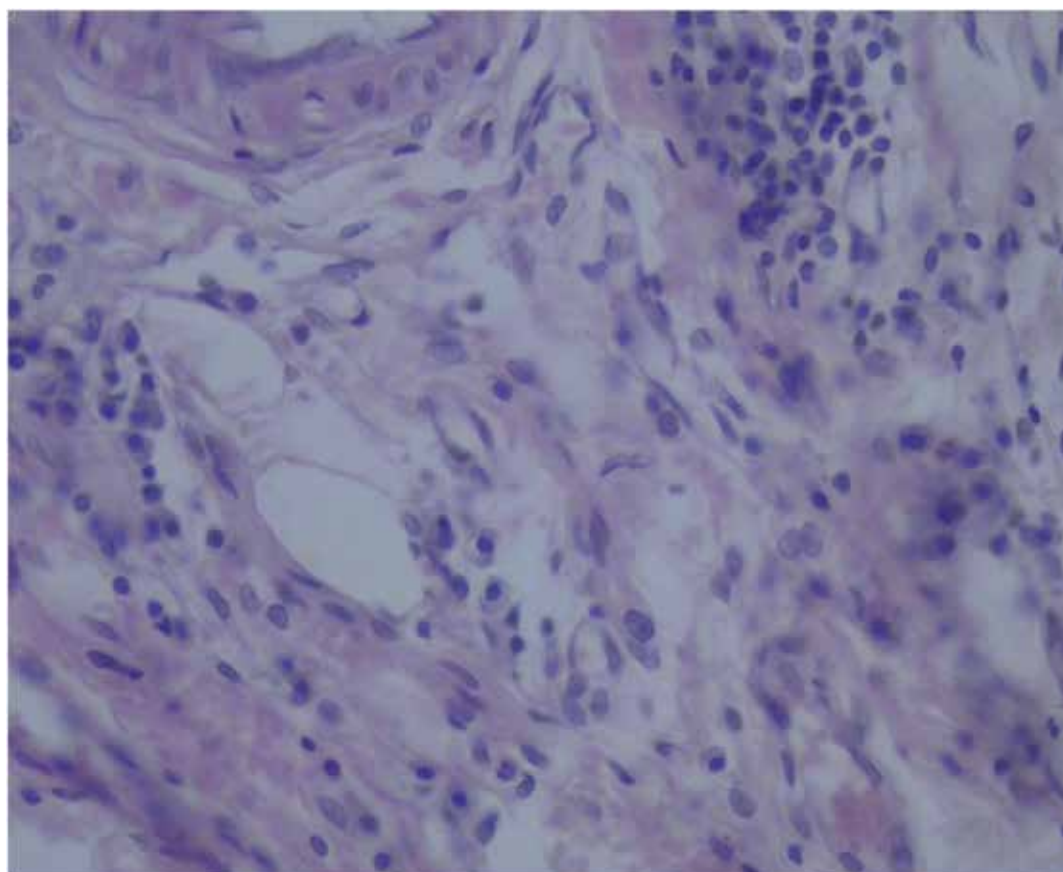


Fig. 4. Diffuse polymorphic cellular infiltration in stroma of the masseter muscle of a group 2 rat. Hematoxylin and eosin staining, ×400.

These changes were most pronounced in groups 2 and 3, while in group 4 they were moderately expressed.

In morphometric studies, the masseter muscle in groups 2-4 compared to group 1 was characterized by a decrease ($p < 0.05$) in the specific volume of parenchyma and an increase ($p < 0.05$) in the specific volume of stroma. There were no significant differences ($p > 0.05$) in the specific volumes of parenchyma and stroma between groups 2 and 3. In group 4, compared to groups 2 and 3, the specific volume of parenchyma was higher ($p < 0.05$), while the specific volume of stroma was lower ($p < 0.05$). The observed increase in the specific volume of stroma in groups 2-4

was due to the excessive content of connective tissue in the stroma, along with the presence of adipocyte clusters between the connective tissue fibers (Fig. 6).

In rats that were withdrawn from the experiment after 1 month and 15 days, compared to the previous period, in group 2 the specific volume of altered muscle fibers in the muscle parenchyma increased ($p < 0.05$) (Table 2). In group 3 the specific volume of altered muscle fibers decreased ($p < 0.05$). In group 4, no alternative changes were recorded in the muscle parenchyma at all.

Compared to the previous period in rats that were withdrawn from the experiment after 1 month and 15 days, the number

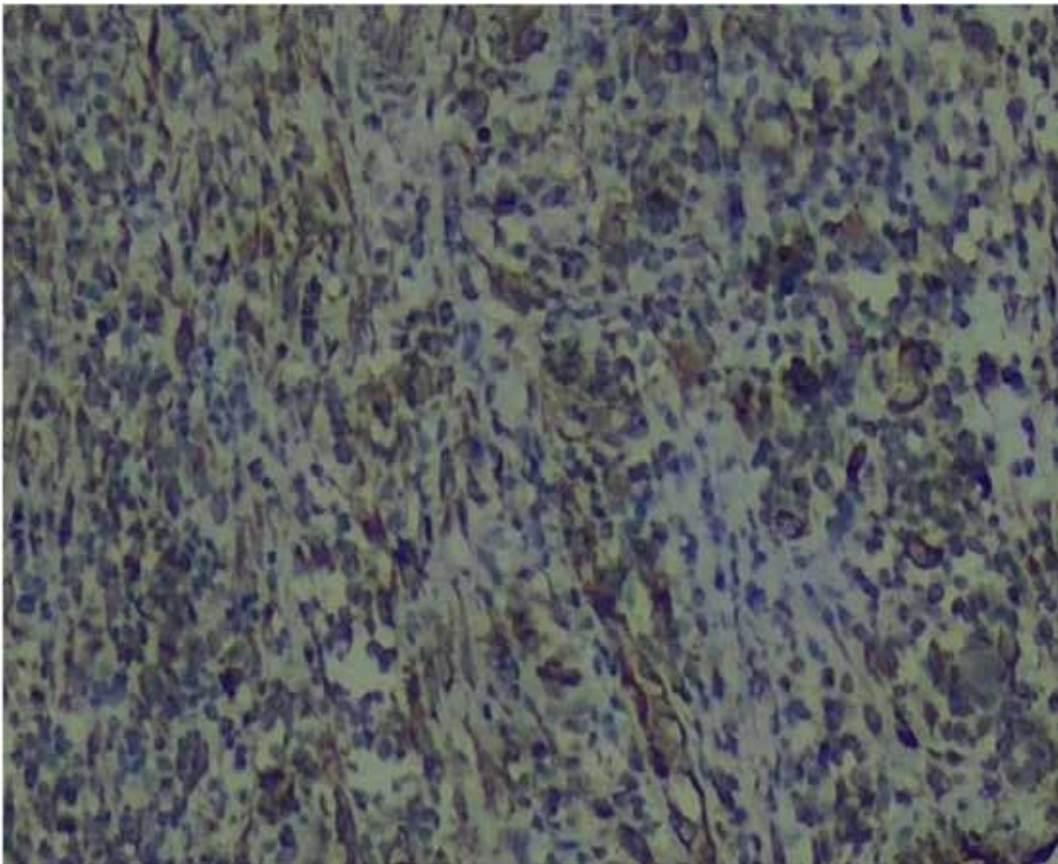


Fig. 5. Vimentin⁺-cells in stroma of the masseter muscle of a group 2 rat. Immunohistochemical staining with monoclonal antibody to vimentin, $\times 400$.

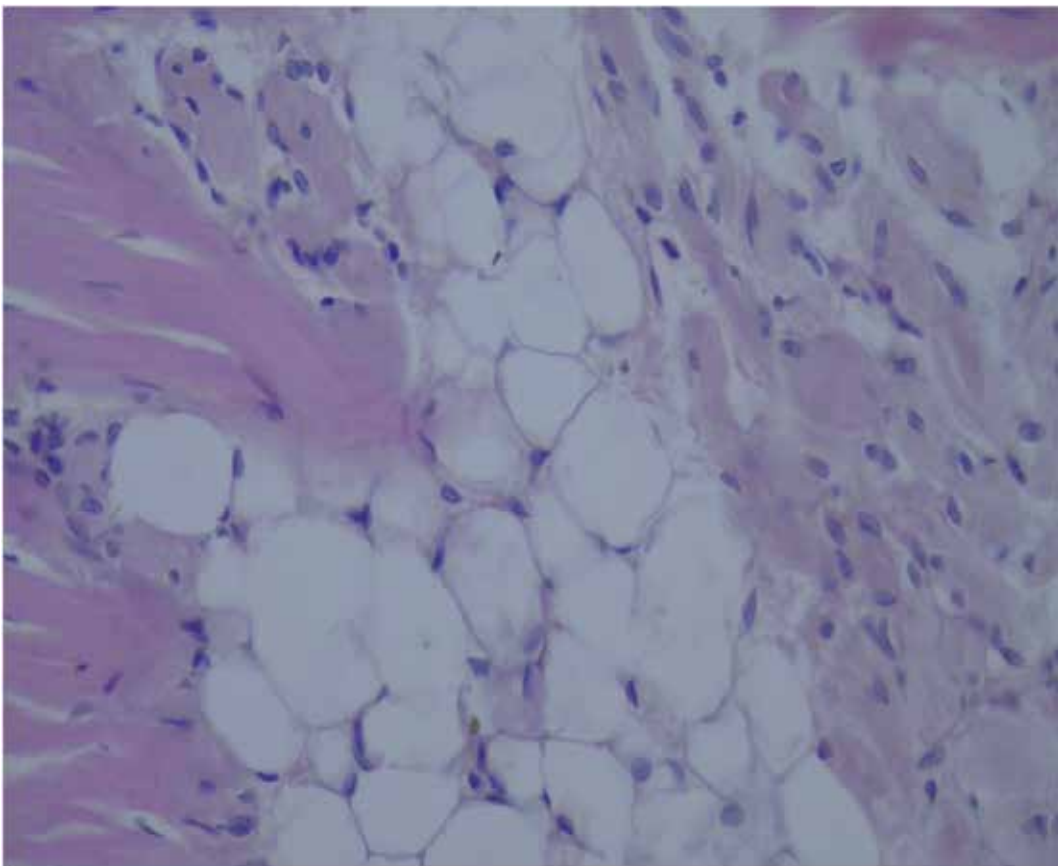


Fig. 6. Excessive connective tissue content with clusters of adipocytes in stroma of the masseter muscle of a group 2 rat. Hematoxylin and eosin staining, $\times 400$.

Table 2. Mean values of morphometric parameters in rats of groups 2-4 withdrawn from the experiment after 1 month and 15 days

Group	Name of the morphometric parameter				
	Specific volume of parenchyma	Specific volume of stroma	Absolute number of vessels	Specific volume of altered muscle fibers	Absolute number of Vimentin ⁺ -cells
2	35.4±2.4% ^{1,4}	64.6±2.4% ^{1,4}	3.2±0.3 ^{1,4}	39.4±2.3% ⁴	47.2±2.4 ^{1,4}
3	65.8±4.0% ^{1,2,4}	34.2±4.0% ^{1,2,4}	10.1±1.0 ^{1,2,4}	5.3±0.4% ^{2,4}	29.4±1.4 ^{1,2,5}
4	86.2±4.0% ^{1,2,3,4}	13.8±4.0% ^{1,2,3,4}	17.4±1.1 ^{1,2,3,4}	-	14.4±1.1 ^{1,2,3,4}

Note: ¹ – statistically significant difference compared to group 1; ² – statistically significant difference compared to group 2; ³ – statistically significant difference compared to group 3; ⁴ – statistically significant difference compared to the previous period.

of vessels in stroma in group 2 decreased ($p < 0.05$). In group 3, this indicator increased ($p < 0.05$), but had a lower ($p < 0.05$) value compared to the indicator in group 1. The indicator in group 4 increased ($p < 0.05$) compared to the previous period and the indicator in group 1.

In stroma of the masseter muscle, compared to the previous period, hemodynamic disturbances increased in group 2, decreased in group 3, and were not observed at all in group 4.

Polymorphic cellular infiltration in stroma of the masseter muscle in rats withdrawn from the experiment after 1 month and 15 days, compared to the previous period, increased in group 2 and decreased in group 3. In group 4, the degree of infiltration corresponded to that observed in group 1.

Among the polymorphic cellular infiltration, immunohistochemical analysis revealed Vimentin⁺-cells, the number of which increased ($p < 0.05$) in group 2 compared to the previous period. In group 3, the absolute number of Vimentin⁺-cells decreased ($p < 0.05$), although it remained significantly higher ($p < 0.05$) compared to the value in group 1. In group 4, this parameter also decreased ($p < 0.05$) and was significantly lower ($p < 0.05$) compared to the value in group 1.

In rats withdrawn from the experiment after 1 month and 15 days, the specific volume of parenchyma in group 2 decreased ($p < 0.05$), while the specific volume of stroma increased ($p < 0.05$), compared to the previous period. In groups 3 and 4, the specific volume of parenchyma increased ($p < 0.05$), and the specific volume of stroma decreased ($p < 0.05$). However, compared to group 1, the specific volume of parenchyma remained lower ($p < 0.05$), and the specific volume of stroma remained higher ($p < 0.05$). In group 4, compared to group 3, the specific volume of parenchyma was higher ($p < 0.05$), and the specific volume of stroma was lower ($p < 0.05$).

DISCUSSION

PRP is a biological hemoderivative product obtained from blood in which platelets are present in a higher concentration than basal levels [10]. It has shown promising results in promoting tissue regeneration, repairing bone defects, treating skin wounds, plastic surgery, sports injuries etc. [11].

The comprehensive morphological study of experimental material conducted by the authors confirmed the effectiveness of PRP in the treatment of post-immobilization extra-

articular contractures of the mandible. The therapeutic effect of PRP was more pronounced in cases where it was applied both during the treatment of mandibular fracture using an immobilizing muzzle for one month and for an additional 15 days after its removal, compared to animals in which PRP was applied only during the 15 days following muzzle removal.

PRP exhibits an antifibrotic effect, which was morphologically manifested by a decrease in the specific volume of stroma in the masseter muscle, one of the main components of which is connective tissue. In the conducted study, the use of PRP led to a reduction in the number of Vimentin⁺-cells in stroma of the masseter muscle, which include, among others, fibroblastic lineage cells. These cells are actively involved in the production of connective tissue components [12].

Studies conducted by other researchers have shown that PRP reduces vimentin expression by interstitial cells, thereby suppressing sclerotic processes in organs [13]. The antifibrotic properties of PRP are also attributed to the suppression of fibroblast-to-myofibroblast transition via VEGF-A/VEGFR-1-mediated inhibition of TGF- β 1/Smad3 signaling [14]. The platelets of PRP may release proteins (such as fibroblast growth factor or hepatocyte growth factor) that are known to inhibit myofibroblasts. It has also been proven that matrix metalloproteinases contained in platelets can degrade fibrillar collagen, thereby reducing the severity of sclerotic changes in organs [15].

In the study conducted by the authors, it was found that PRP normalizes the intensity of immune cell infiltration in stroma of the masseter muscle in animals. It is well known that interstitial inflammation leads to the development of sclerotic changes in organs. Therefore, the normalization of connective tissue content in stroma of the masseter muscle may be attributed to the anti-inflammatory effect of PRP.

The anti-inflammatory effect of PRP is attributed to its influence on the production of pro-inflammatory cytokines and the morphofunctional state of immune cells [16]. PRP decreased the lymphocyte number. In PRP the platelets release a considerable amount of RANTES (a major monocyte chemoattractant) from its alpha-granules. RANTES also inhibits many cytokines, decreases in the concentration of lipoxin A4. PRP alters macrophage phenotype, reduces cyclooxygenase-2 expression, and normalizes prostaglandin E2 levels [17].

The use of PRP in the study led to a reduction in the severity of alternative changes in muscle fibers of the masseter muscle and also stimulated muscle fibers regeneration, as evidenced by an increase in the specific volume of parenchyma. According to the literature, PRP enhances the morphofunctional state of muscle cells and their regenerative potential [18, 19].

PRP is a source of angiogenic growth factors (transforming growth factor- β , platelet-derived growth factor, insulin-like growth factor-1, vascular endothelial growth factor, epidermal growth factor, and others), which stimulate the formation of new blood vessels and activate the morphofunctional state of vascular endothelial cells, thereby improving

tissue trophism and oxygenation [20, 21]. In our study, the application of PRP led to a reduction in the severity of hemodynamic disturbances in the masseter muscle and an increase in the number of blood vessels in its stromal component.

CONCLUSIONS

The comprehensive morphological study of experimental material conducted by the authors confirmed the effectiveness of platelet-rich plasma in the treatment of post-immobilization extra-articular contractures of the mandible, thereby expanding the available arsenal of treatment methods for this pathology.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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