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Stimulation of periosteal bone formation with platelet-rich plasma in a rat model of femoral atrophic non-union

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Objective. To investigate the effect of local injection of platelet-rich plasma (PRP) on periosteal bone formation in a rat model of femoral atrophic non-union. Methods. The study was conducted on 11 rats. Atrophic non-union was modelled by performing a mid-shaft femoral osteotomy with intramedullary Kirschner wire fixation, followed by periosteal stripping (2 mm) at both ends of the osteotomized bone and their separation with a silicone spacer. On day 7 post-surgery, 5 animals received a local PRP injection into the injury zone. Radiography was performed at weeks 2 and 4. After 8 weeks, euthanasia was performed, and the operated femurs were harvested for histological analysis. Results. In the atrophic non-union model, a loose connective tissue capsule of varying thickness without signs of inflammation was found around the spacer in all rats. Acellular areas were identified within the cortex. The structure of the periosteum and endosteum near the osteotomy site was disrupted. In 5 out of 6 animals, no signs of bone formation were observed near the spacer from either the periosteal or endosteal zones. Following PRP injection, a higher density of capillary-type vessels was observed within the capsule. Areas of cartilage with hypertrophic chondrocytes were identified, indicating endochondral ossification. In 4 rats, formed bone tissue was recorded on the fragments, predominantly on one side, in both periosteal and endosteal zones. The bone tissue was cancellous in the periosteal zone and woven bone in the endosteal zone. Conclusion. Local PRP injection into the injury zone on day 7 in a rat model of femoral atrophic non-union with previously disrupted periosteum positively affects periosteal bone formation at the fragment ends.

Мета. Вивчити вплив локального введення збагаченої тромбоцитами плазми на періостальне кісткоутворення в моделі атрофічного незрощення перелому стегнової кістки щурів. Методи. Дослідження проведено з використанням 11 щурів, яким змоделювали стан атрофічного незрощення перелому шляхом виконання остеотомії в середній третині стегнової кістки з інтрамедулярною фіксацією спицею Кіршнера, руйнування періосту (2 мм) обох країв остеотомованої кістки та відокремлення їх силіконовим спейсером. На 7 добу 5 тваринам після втручання локально вводили PRP у зону ушкодження. Рентгенографію виконували на 2 та 4 тиждень. Через 8 тижнів після операції здійснили евтаназію та вилучили оперовану стегнову кістку для гістологічного аналізу. Результати. У моделі атрофічного незрощення у всіх щурів навколо спейсера виявлено сполучнотканинну капсулу різної товщини без ознак запалення. У кортексі визначали безклітинні ділянки. Структура періосту й ендосту поблизу зони остеотомії — зруйнована. У 5 з 6 тварин не виявили жодних ознак кісткоутворення поблизу місця розміщення спейсера як з боку періосту, так і ендосту. У разі введення PRP у сполучнотканинній капсулі густіше розміщувалися судини капілярного типу. Визначено ділянки хрящової тканини з гіпертрофованими хондроцитами, що свідчить про процес енхондральної осифікації. У 4 щурів зафіксовано сформовану кісткову тканину на уламках, переважно на одному як у періостальній, так і в ендостальній зонах. Кісткова тканина — губчаста в періостальній зоні та грубоволокниста — в ендостальній. Висновок. Введення PRP в зону ушкодження на 7-му добу в моделі атрофічного незрощення перелому з попередньо зруйнованим періостом стегнової кістки щурів позитивно впливає на періостальне кісткоутворення на краях уламків. Ключові слова. Щур, атрофічне незрощення, перелом стегнової кістки, збагачена тромбоцитами плазма (PRP), періостальне кісткоутворення, гістологія, енхондральна осифікація.

Keywords. Rat, atrophic non-union, femoral fracture, platelet-rich plasma (PRP), periosteal bone formation, histology, endochondral ossification

Introduction

Non-union or delayed union of long bone fractures is one of the serious complications that leads to the loss of limb functionality and/or worsens the quality of life for patients. The causes of this complication are considered to include bone defects, osteomyelitis, high-energy trauma, disruption of bone regeneration mechanisms, systemic diseases such as diabetes, etc. [1], as well as fracture treatment with metallic fixators [2]. Non-unions most commonly occur in fractures of the humerus (10–15%), femur, or tibia (up to 12.5%) [2]. As a result of military activity in Ukraine, there has been a notable increase in high-energy injuries that are often accompanied by long bone fractures and bone defects [3, 4]. This trend indicates that the number of cases of non-union among those affected may rise in the future.

The main task in the treatment of atrophic non-unions is achieving stable fixation, removal of scar tissue that hinders union, and replacing the formed defect with autografts or allografts [5]. Additionally, the use of various angiogenesis promoters and cell differentiation agents in the osteogenic direction during surgical interventions is being developed. These orthobiological methods include the use of platelet-rich plasma (PRP), autologous mesenchymal stem cells (MSCs) from bone marrow, bone morphogenetic proteins (BMPs) [1, 5], or their combinations [6]. The goal of using these methods is to accelerate fracture healing, although their effectiveness is still being researched both clinically and experimentally. This is particularly due to the lack of clinical studies with Level I evidence [5].

Platelet-rich plasma contains platelets from which several growth factors are released (platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), and insulin-like growth factor (IGF-1)), which positively influence vascularization at the site of non-union and then stimulate the differentiation of MSCs in the direction needed for bone healing [7]. The periosteum plays a key role in reparative osteogenesis due to the presence of osteogenic precursor cells and a large number of blood vessels in its structure. Disruption of its structure and biological activity is considered one of the factors contributing to the formation of atrophic non-unions [8]. The growth factors present in PRP are potentially capable of stimulating periosteal cell activity, angiogenesis, and restoring its osteogenic potential, as evidenced by research combining periosteal stem cells and PRP to stimulate bone regeneration [10]. However, the exact mechanisms of PRP action in the case of non-union fractures are still unknown, and the timing of its application remains unclear [9].

A recent systematic review encompassing 24 clinical studies investigating molecular and biological disruptions in the regenerate of individuals with non-unions reported a suppression of BMP-7 expression in these cases [11]. This protein belongs to the TGF-β superfamily and induces the differentiation of MSCs into osteoblasts [12]. Additionally, signaling pathways of BMP and matrix metalloproteinases are likely involved in the development of non-unions [11]. At the tissue level, aseptic atrophic non-union leads to disturbances, the mechanisms of which are also almost unstudied. One of the main differences between atrophic non-union and hypertrophic non-union is the cell death in the cortical fragments at the site of non-union [11]. Therefore, targeting MSCs, the precursors of osteoblasts, for further bone formation, and the appearance of monocytes, precursors of osteoclasts, for resorption of the parent cortical bone in the periosteal zone, may be a promising treatment approach for non-unions. This may potentially support the use of orthobiological methods, specifically PRP.

Purpose: To study the effect of locally administered platelet-rich plasma on periosteal bone formation in a model of atrophic non-union of femoral fractures in rats.

Materials and Methods

Experimental research was conducted in accordance with the requirements for humane treatment of laboratory animals, as regulated by the Law of Ukraine "On the Protection of Animals from Cruelty" (No. 3447-IV dated 21.02.2006) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986) [13, 14]. The experimental research plan was approved by the Bioethics Committee at the State Institution Professor M. I. Sytenko Institute of Spine and Joint Pathology of the National Academy of Medical Sciences of Ukraine (protocol No. 224 dated 13.06.2022).

The study involved 11 male white rats, aged 6 months (body weight ranging from 325 to 550 g), from the experimental biological clinic of the State Institution Professor M. I. Sytenko Institute of Spine and Joint Pathology of the National Academy of Medical Sciences of Ukraine. A model of atrophic non-union of fractures was created in all animals by performing osteotomy in the middle third of the femur with intramedullary fixation using a Kirschner wire, destruction of the periosteum (2 mm) on both ends of the osteotomized bone using an electrocautery device, and separation of the bone fragments using a silicone spacer. The rats were divided into two equal groups: 1 — model of atrophic non-union of the fracture (n = 6); 2 — model with local PRP injection in the injury zone on the 7th day after the intervention (n = 5).

Surgical interventions were performed under aseptic and antiseptic conditions, with general anesthesia (ketamine, 50 mg/kg body weight, intramuscularly). After shaving the right thigh and disinfecting the area with the antiseptic Betadine, osteotomy was performed in the middle third of the femur through a lateral approach using a circular burr (Fig. 1, a). Subsequently, the surgical area was flushed with the antiseptic agent Decasan. To destroy the periosteum on both ends of the osteotomized bone, its coagulation was performed 2 mm distal and proximal to the osteotomy site using a high-frequency electro-surgical unipolar generator HF-1760 Model DTC-03 (Fig. 1, b).

Next, the femoral bone fragments were separated, one end of the Kirschner wire was inserted into the bone marrow canal of the distal fragment, and a silicone spacer (diameter 7 mm) was placed over the wire through a pre-made hole in the center. Then, after stretching the limb muscles, the other end of the wire was inserted into the bone marrow canal of the proximal fragment, and both osteotomized bone parts were brought together with the silicone spacer in between (Fig. 1, c, d). The Kirschner wires ranged in length from 17 to 22 mm and in diameter from 1.5 to 2.0 mm; they were selected during the procedure based on the individual size and shape of the bone canal in each animal (Fig. 1, a). The wounds were treated with the antibiotic Bicillin, and the muscles and skin were sutured layer by layer with single knot sutures (MEFIL No. 2 suture material). The skin around the surgical area was treated with Betadine.

Seven days after the surgery, 6 rats were injected with 0.3 ml of PRP into the injury zone. This time point was chosen based on the stages of the reparative osteogenesis process, where the first stage is traumatic inflammation (lasting approximately 7 days) [15]. Since PRP injections can potentially intensify inflammation, the injection was done after the inflammation stage had ended. Moreover, in a previous study, we demonstrated that injecting PRP on the 7th day after implantation of a bone allograft in a critical-size femoral defect in older rats, where bone formation was suppressed, promoted the remodeling of the graft and the replacement of bone tissue [16].

To obtain PRP, 8 ml of venous blood was collected from two donor rats into an 8.5 ml vacuum tube containing anticoagulant. The blood samples were centrifuged for 10 minutes at 1500 rpm in a laboratory clinical centrifuge OPn-3.02 DASTAN, after which the plasma was collected using an automatic pipette with a sterile tip.

Four weeks after the surgical procedure, all rats were euthanized by decapitation under ether anesthesia for histological and biochemical analysis. The method of euthanasia was chosen due to the need to obtain blood for biochemical studies.

Radiological assessments were conducted on all rats at both two and four weeks following surgical intervention. The imaging was done under general anesthesia (ketamine 50 mg/kg body weight, intramuscularly). Digital radiographs were obtained using the OPERA T90cex radiological diagnostic system.

For *histological examination*, after decapitation, the operated femora were removed from the animals, cleaned of soft tissues, and fixed for 4 days in 10 % neutral formalin. After washing in running water, the femora were decalcified in a 5 % formic acid solution for 5 days and then transferred to 70 % ethyl

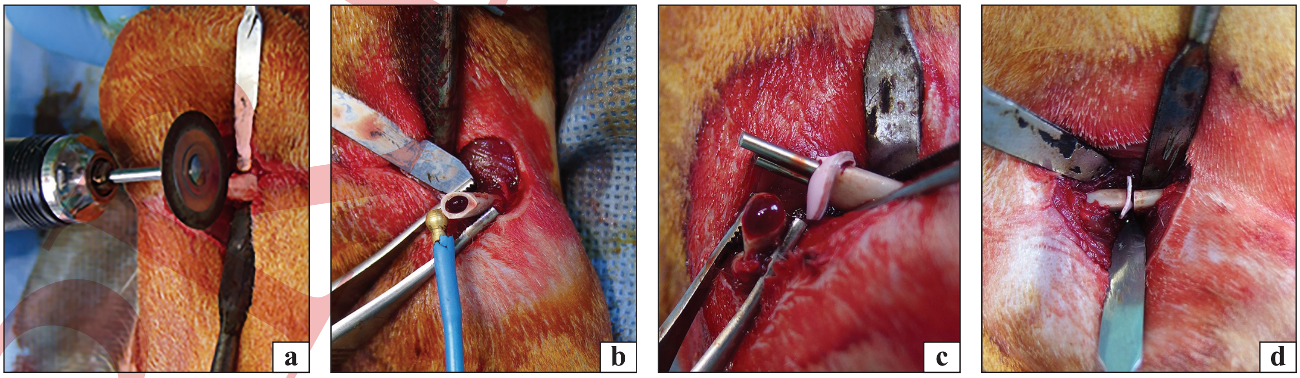

Fig. 1. Stages of surgical intervention to model atrophic non-union of the femoral fracture in rats: a) performing osteotomy of the femoral bone using a circular milling cutter; b) destruction of the periosteum of one bone fragment using a coagulator; c) insertion of the other end of the Kirschner wire with the silicone spacer into the proximal bone fragment; d) apposition of the bone fragments with the spacer between them

alcohol. The Kirschner wires were removed from the bones, and a fragment of the diaphysis with the osteotomy site was cut out. The obtained samples were dehydrated in increasing concentrations of isopropyl alcohol, then infiltrated with a mixture of isopropyl alcohol and paraffin, followed by series of paraffin baths and embedding in paraffin. Longitudinal sections were made using a Reichert sliding microtome and stained with hematoxylin and eosin. The structure of the cells and the intercellular substance in the osteotomy area and around it were analyzed using a light microscope Olympus BX63. Digital photography was performed using a DP73 Olympus digital camera, with the software Cell Sens Dimension 1.8.1.

Statistical Analysis

To assess the effect of PRP on periosteal bone formation, data from each rat were classified as either “bone formation present” or “bone formation absent”, and the results were compared between the groups using the χ^2 test for categorical data. The difference between groups was considered significant if $p < 0.05$. SPSS Statistics was used for analysis.

Results

Radiographic Analysis

After modeling atrophic non-union, on the radiographs of all rats with/without PRP injection on the 7th day, the position of the intramedullary fixator (Kirschner wire) was correct at 2 weeks, providing alignment of the right limb axis. The bone fragments were adapted, and a gap was visible between them, with a radiologically transparent silicone spacer placed in the gap (Fig. 2, a, b). In the rats of the non-union model, no significant changes were observed after 4 weeks compared to the previous observation period (Fig. 2, b). In the PRP-treated group, periosteal regeneration was observed predominantly in one of the bone fragments (Fig. 3, d).

Histological Analysis. Model of Atrophic Non-Union of the Femur. Four weeks after osteotomy in the middle third of the femur diaphysis, intramedullary fixation with a Kirschner wire, and the placement of a silicone spacer between the bone fragments, microscopic examination revealed the area consisting of the spacer and fragments of the original cortex of the femur, both proximally and distally (Fig. 3, a). The spacer was not present in the histological samples due to the dissolution of silicone during the paraffin removal process in xylene before staining. Around the spacer, a connective tissue capsule of varying thickness was found in all rats, without signs of inflammation (Fig. 3, c). The capsule was widest near the bone fragments, and its thickness decreased as it moved away from the external surface of the bone. The connective tissue capsule was connected to the external surface of the bone fragments slightly away from their edges. It consisted of parallel bundles of collagen fibers with fibroblasts with basophilic

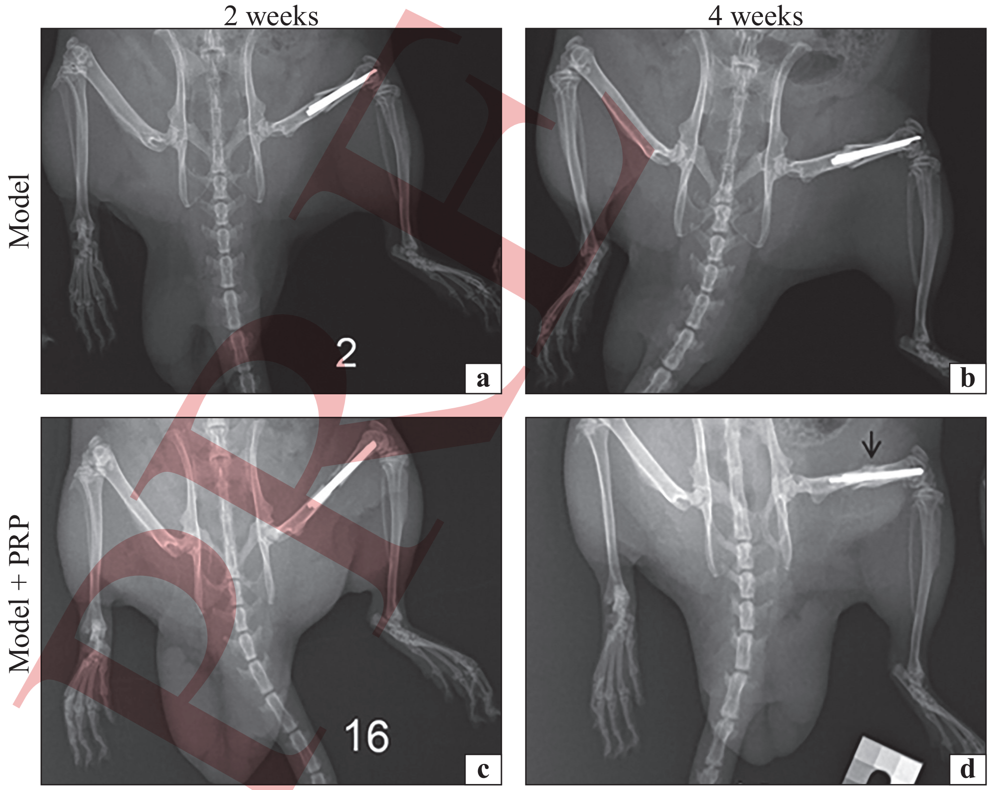

Fig. 2. X-rays of rats with atrophic non-union of the femoral bone with/without PRP injection on day 7, taken at 2 and 4 weeks post-surgery. Kirschner wires are positioned in the bone marrow canal at 2 weeks (a, c). Model: No signs of regenerate formation in the osteotomy zone at 4 weeks (b). Model+PRP: Periosteal regenerate in the distal bone fragment at 4 weeks (d)

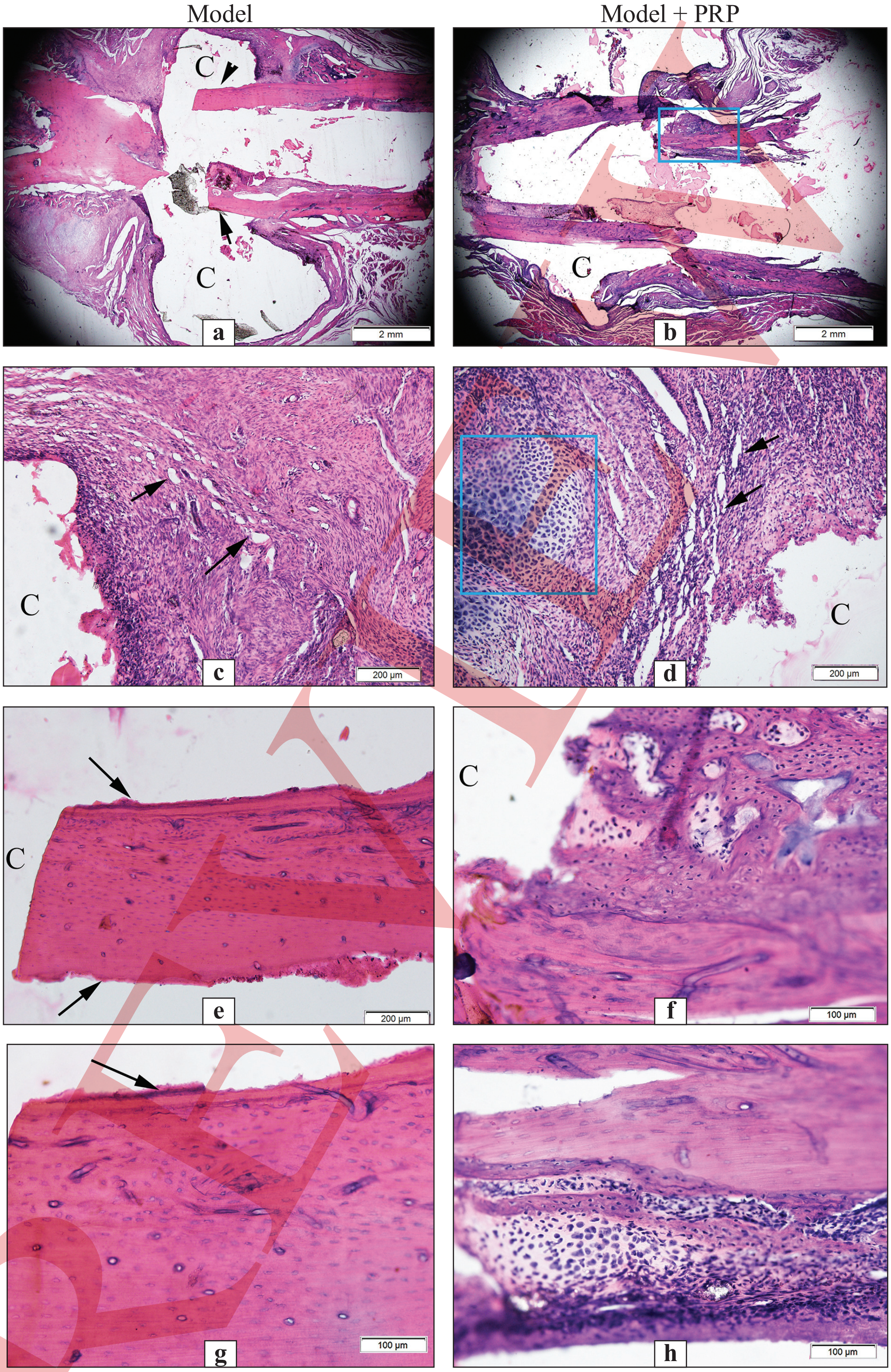

Fig. 3. Fracture sites 4 weeks after femoral osteotomy in rats with intramedullary fixation using Kirschner wire, separation of osteotomized bone fragments by a silicone spacer (S), and periosteal coagulation with/without PRP injection on the 7th day. The cavity from the spacer is surrounded by a connective tissue capsule of varying thickness (a, b). Non-union model (a, c, f, i): Fragments of the connective tissue capsule surrounding the silicone spacer with capillary-type vessels (arrows) in the thickness of the capsule (c). The ends of the osteotomized femoral bone fragments in rats with destroyed periosteum (f). Absence of newly formed tissues in the periosteal and endosteal zones (arrow) (f). Empty lacunae without osteocytes in the cortical matrix (i). Model + PRP (b, g, e, h): Capsule with signs of enchondral ossification (d). Periosteal and endosteal regenerates on one of the bone fragments (b). Newly formed trabeculae of spongy bone tissue in the periosteal zone (g). Fibrous bone tissue in the endosteal zone (k). Absence of osteocytes in lacunae in the bone matrix (g, k). Hematoxylin and eosin staining

nuclei tightly arranged between them (Fig. 3, c). In the periosteum, where the capsule was wider, areas of vascularization with capillary-type vessels of varying diameters were sometimes observed (Fig. 3, c).

In the cortical matrix near the osteotomy site, acellular areas without osteocytes were found, with a predominance of empty lacunae or debris inside them. Some areas exhibited uneven eosinophilic staining of the matrix (Fig. 3, e, i). No periosteal or endosteal structures were found near the osteotomy site where coagulation was performed during the surgical procedure (Fig. 3, e).

In one rat, bone formation was observed at the ends of the bone fragments on one side. In the remaining 5 out of 6 rats (83%), no bone formation was observed near the spacer site, neither in the periosteum nor in the endosteum.

Outside the injury zone (>2 mm from the osteotomy site), newly formed trabecular bone was found primarily in the periosteum, and in some rats, also in the endosteum. This bone consisted of fine trabeculae and osteocytes within the matrix. Osteoblasts with cuboidal shapes and basophilic nuclei were found on the surface of the bone trabeculae. In the periosteum and endosteum, where live osteocytes were absent in the cortical matrix, osteoclasts were observed.

In rats with the model of atrophic non-union of fractures, which received a PRP injection, a zone of osteotomy with two fragments and a cavity from the spacer surrounded by a connective tissue capsule was detected after 4 weeks, similar to animals without PRP injection (Fig. 3, b). In 80 % (4 out of 5) of the animals, bone tissue formation was observed on the fragments, primarily on one of the fragments (Fig. 3, b), both in the periosteum and in the endosteum. The significance of these changes compared to the model was confirmed (χ^2 (2, n = 11) = 4.41, p = 0.036). The bone tissue was spongy in the periosteal zone and coarse fibrous in the endosteal zone (Fig. 3, g, k). In two animals, the formation of bone regenerate was more intense than in others, with complete remodeling of one fragment. Just as in the model, the cortical matrix at the ends of the fragments contained empty lacunae without osteocytes, and areas without new tissue were found at the ends of the fragments in each rat. The connective tissue capsule had a similar structure as in the model (Fig. 3, d), but in animals with more pronounced tissue formation, capillary-type blood vessels were denser in the capsule, and areas of cartilage tissue with hypertrophic chondrocytes were found, indicating endochondral ossification.

Discussion

As a result of the transverse osteotomy at the mid-diaphyseal level of the femur, fixation with a Kirschner wire, periosteal coagulation, and placement of a silicone spacer, atrophic non-union of the fracture was achieved 4 weeks after surgery. At the same time, this is only a localized disturbance, meaning that normal periosteal function is preserved at a distance of 2 mm from the damage zone, confirmed by the presence of a formed periosteal regenerate of spongy bone tissue. The histological characteristics of the damage area correspond to those found in cases of atrophic non-union of fractures [11]: fibrous capsule around the spacer, absence of periosteal and endosteal regenerate, death of osteocytes in the cortical matrix of the fragments, and the absence of osteoclasts due to impaired vascularization. Radiographically, no signs of regenerative processes were observed in the damage zone.

In similar models of atrophic fracture non-union in small animals [17-23], periosteal destruction was performed, but in combination with the destruction of the endosteum [24], bone marrow [25, 26] to improve reproducibility. Another model similar to ours used latex-silicone foil [27] to isolate the fragments, but our approach uses a simpler method of fragment isolation with a silicone spacer placed on a Kirschner wire. Results have also been published using a silicone insert [28, 29] or a polysulfone plate [30], though without periosteal coagulation.

Our methodological approach in the created model resulted in atrophic non-union in 5 out of 6 rats. The worse results in one animal were likely due to insufficient periosteal coagulation, leading to new bone tissue formation at the ends of the fragments. This suggests that the proposed model can be successful in studying methods of treating atrophic non-unions.

The clinical effectiveness of PRP has not been fully proven, as indicated by an analysis of two systematic reviews [9, 31]. This is due to the heterogeneity of existing clinical data, as well as an incomplete understanding of the mechanisms of PRP action at the non-union site. In general, PRP is used in combination with bone grafts or MSCs, as well as in the form of transcutaneous local injections into the non-union fracture site. In most studies, PRP application had a positive effect on fracture healing, with only two studies finding no effect, and one showing worse results compared to recombinant BMP-7 [9, 31]. The use of PRP during surgery promoted faster bone healing and reduced pain on the visual analog scale in the PRP group (n = 16) compared to the non-PRP group in patients with tibial diaphyseal non-unions [15]. In the case of a single transcutaneous PRP injection (n = 14) into the site of oligotrophic non-union in patients with diaphyseal fractures of long bones, healing was accelerated by approximately 3 weeks (19.07 weeks vs. 16.7 weeks), which is 13 % faster compared to using intramedullary fixation with a bone graft (n = 15) [17]. In our study, we anticipate that this effect of PRP may be due to its positive impact on the restoration of periosteal bone formation, which we experimentally verified.

The injection of PRP on day 7 in the model of atrophic non-union of femoral fractures in rats positively influenced the course of the regenerative process, which manifested in the formation of bone regenerate in the periosteal and endosteal zones at the edges of the bone fragments, where the periosteum had previously been destroyed. However, this process had an uneven character, likely related to the precision of PRP injection into the damage zone.

In a similar study on the effects of PRP in a non-union model in rabbits, where a spacer was inserted between the fragments of the tibia for 3 weeks, and after its removal, the cavity was filled with a synthetic bone implant (Coragraft) with or without PRP, it was found that such a combination ensured faster bone healing (radiologically and histologically) at 3, 7, and 11 weeks compared to using only Coragraft [32]. However, the use of PRP alone without Coragraft did not show similar results. The results of using PRP in such a non-union model under conditions of periosteal coagulation and during the acute phase of the fracture, as achieved in our study, are unknown.

The results obtained in our study are consistent with other experimental studies using PRP. For example, when PRP was injected during the formation of a tibial fracture in rabbits, the cortical regenerate was more mature at 6 and 12 weeks [33, 34]. In diabetic type I rats, an increase in cell differentiation in the periosteum was observed during femoral fracture healing with the injection of PRP starting from day 12 of the experiment, which may indicate an effect of PRP on the periosteum function or on the cells of adjacent tissues under systemic disturbances in the body [35].

Conclusions

Transverse osteotomy of the femur in rats with periosteal coagulation at 2 mm from the edges of the fragments, placement of a silicone spacer between the fragments, and intramedullary fixation with Kirschner wires, results in atrophic non-union of the fracture in 83% of cases after 4 weeks.

The injection of PRP into the damage zone on day 7 in this model has a positive effect on periosteal bone formation at the edges of the fragments with previously destroyed periosteum, but predominantly in only one of the fragments. This indicates the need for the development of a more accurate method for PRP injection into the damage zone.

Conflict of Interest. The authors declare no conflict of interest.

Prospects for Further Research. Further experimental studies on the combined use of bone morphogenetic proteins with platelet-rich plasma for stimulating periosteal bone formation in non-union fracture models.

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Contributions of the Authors. Vorontsov P. M. — concept and design of the study, editing of the final version of the article; Maltseva V. Ye. — histological analysis, writing of the draft, and editing of the final version of the article; Danyshchuk Z. M. — histological analysis; Nikolenko O. A. — study design, experimental modeling in rats; Kovtun V. V. — experimental modeling in rats; Laponin S. I. — experimental modeling in rats. All authors reviewed and approved the final version of the article.

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STIMULATION OF PERIOSTEAL BONE FORMATION WITH PLATELET-RICH PLASMA IN A RAT MODEL OF FEMORAL ATROPHIC NON-UNION

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