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A review of animal models for bone fracture nonunion and their role in studying biological therapy efficacy

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The bone healing impairment, such as non-union fractures after injuries of long bones, lead to loss of working capacity and result in significant financial costs, which emphasizes the socio-economic significance of the problem. However, it is not known which method of modeling the non-union bone fractures is more optimal for further research into the effectiveness of biological therapy aimed at treating bone healing impairment. For a detailed study of methods of non-union fracture treatment of, it is necessary to determine the developed animal models. The objective was to analyze the existing animal models of fracture nonunion in long bones in vivo and to consider the possibility of their further use to evaluate the effectiveness of the use of modern biotechnologies for the in the management of fracture nonunion. It was found that the majority of developed animal models of atrophic long bone non-union were created using small animals, namely rats, mice, and rabbits. A more common method of modeling bone non-union is performing an osteotomy with the formation of a defect of different widths between the bone fragments and subsequent removal of the periosteum proximal and distal to the osteotomy site; damage to the endosteum or removal of bone marrow. Also, in such animal models, researchers use a silicone spacer, a polysulfone plate, or a latex-silicone foil to physically prevent fracture union. In these animal models, studies using mesenchymal stromal cells, platelet-rich plasma or bone morphogenetic protein-2 (BMP-2) have already been conducted for the management of non-union bone fractures. At the same time, the clinical results of the application of various biological therapies are ambiguous, which determines the conduct of further experimental studies, in particular, in vivo. However, there are disagreements about which in vivo modeling methods give a reproducible result and prevent bone union, which determines the need for further analysis of existing modeling tools for conducting research in this direction.

Порушення регенерації кістки, такі як незрощення переломів після травм довгих кісток, призводять до втрати працездатності й обумовлюють значні фінансові витрати, що підкреслює соціально-економічну значущість проблеми. Проте невідомо, який спосіб моделювання незрощення перелому кістки є оптимальнішим для подальшого дослідження ефективності біологічної терапії? спрямованої на лікування порушень репаративного остеогенезу. Для детального вивчення способів лікування незрощень переломів потрібно визначення розроблених експериментальних моделей на тваринах. Метою було провести аналіз існуючих експериментальних моделей незрощення переломів довгих кісток in vivo та розглянути можливість їхнього подальшого використання для оцінювання ефективності застосування сучасних біотехнологій для лікування порушень регенерації кістки. Виявлено, що переважна кількість розроблених моделей атрофічного незрощення переломів кісток створена з використанням невеликих тварин, а саме щурів, мишей та кролів. Поширенішим способом моделювання незрощення є виконання остеотомії з формуванням різної ширини дефекту між фрагментами кістки та подальшим видаленням періосту проксимальніше та дистальніше ділянки остеотомії; ушкодженням ендосту або видаленням кісткового мозку. Також у таких моделях дослідники застосовують силіконовий спейсер, полісульфонову пластину або латексно-силіконову фольгу для фізичного перешкоджання зрощення перелому. У наведених моделях уже проведені дослідження з використанням мезенхімальних стромальних клітин, збагачених тромбоцитами плазми та морфогенетичного кісткового білка-2 (BMP-2) для лікування незрощення кістки. Водночас клінічні результати застосування різної біологічної терапії є неоднозначними, що обумовлює проведення подальших експериментальних досліджень, зокрема, моделювання in vivo. Проте існують розбіжності щодо того, які способи моделювання дають відтворюваний результат і перешкоджають зрощенню кістки, що і визначає необхідність подальшого аналізу існуючих засобів моделювання для проведення досліджень у цьому напрямі.

Ключові слова. Щур, кроль, миша, остеотомія, стегнова кістка, великогомілкова кістка, періост, регенерація кістки.

Keywords. Rat, rabbit, mice, osteotomy, femur, tibia, periosteum, bone regeneration

Introduction

Complications that occur after traumatic fractures of long bones (non-union, delayed union, and false joint) due to impaired reparative osteogenesis are an urgent medical and social problem. Despite the ability of the bone to heal with the restoration of the original structure, in certain cases, in particular, and after massive injuries due to gunshot wounds, fusion does not develop resulting in permanent functional disorders, which require long-term treatment and significant financial costs. According to Kharkiv traumatological MSEC, the incidence of non-unions after treatment of diaphyseal fractures of the limbs ranges from 3 to 28.6 %, depending on the location, the osteosynthesis used, and the complexity of the injury [1]. In Great Britain, according to approximate estimates, the annual cost of treating non-union reaches 320 million pounds for a total population of 67 million [2]. Researchers from the USA report 4.9 % of non-unions per year after fractures of long bones of various locations (most often after fractures of both bones of the tibia or femur). The complexity of combat trauma increases the risk of non-unions up to 31 % of cases [3]. Risk factors include the number of simultaneous fractures, the use of nonsteroidal anti-inflammatory drugs together with opioids, and surgical treatment [4]. In the national database of Scotland, the frequency of non-unions after bone fractures is about 1.9–9 %, with the highest number at the level of the tibia and more often in people aged 35–44 years than in patients of older age groups [5], which emphasizes the urgency of the problem.

An important task for doctors in case of failed fracture healing is to correct the mechanical conditions and biological factors triggering this. The first is solved with the help of osteosynthesis. To improve the classical surgical methods of treatment of non-union fractures, it is proposed to use bioengineering approaches, among which only autologous transplants have become widespread in clinical practice in the case of atrophic non-unions [2, 6]. However, their known disadvantages (non-sufficient amount of material, rapid resorption, the need for additional surgical intervention, and pain during removal from the patient) led the scientific community search for other biological materials that could potentially promote osteogenesis. In particular, it has been proposed to use autologous fibrin [7], allo-implants [8], mesenchymal stromal cells (MSCs) [9], platelet-rich plasma, growth factors [10], etc.

However, in order to improve the methods of treatment of non-unions using bioengineering technologies, it is first of all necessary to choose an experimental model that will allow to reproduce the mechanisms of reparative osteogenesis disorders that occur after long bone fractures. For this, experimental in vivo modeling is carried out using small laboratory animals.

Purpose: to conduct an analysis of existing experimental models of non-union of long bone fractures in vivo and to consider the possibility of their further use to evaluate the effectiveness of biotechnologies in the treatment of bone regeneration disorders.

Material and methods

The literature analysis was performed in the PubMed database using Mesh keywords for the following search query: “Fractures, Ununited” AND “Fracture Healing” AND (“Ankle Fractures” OR “Femoral Fractures” OR “Tibial Fractures” OR “Humeral Fractures” OR “Elbow Fractures”) AND “Disease Models, Animal”. Inclusion criteria were original experimental studies with available full text in English. The exclusion criteria were studies of infected fractures and oncological diseases. The search depth was 10 years.

Results and their discussion

After performing the search, 28 articles were selected, which mainly considered the use of mice, rats or rabbits to study non-union of long bone fractures.

Prerequisites for the development of experimental models of fracture non-union in vivo

One of the key stages in the healing process of a bone fracture is the restoration of the vascular network — neovascularization. However, recently, as reported by the authors of a recently published literature review [11], there is increasing evidence that bone calluses at the site of non-union can be well vascularized and express such a proangiogenic mediator as vascular endothelial growth factor factor (VEGF). And dysregenerative disorders are due to reduced expression of pro-osteogenic cytokines — bone morphogenetic proteins (BMP). The authors [11] express an opinion about the need to stimulate osteogenesis at the stage of bone callus remodeling, rather than vascularization, with the help of VEGF in the early stages of fracture healing, because even atrophic non-unions can be vascular [12].

At the same time, atrophic non-union models are more promising for preclinical studies because this type of non-union is more likely to require transplantation or additional biological therapy, while hypertrophic non-unions can only be treated with surgery [12].

There is an opinion that during the treatment of bone non-unions, it is necessary to stimulate not only osteogenesis, but also chondrogenesis, since the healing of most fractures proceeds by intramembranous and endochondral bone formation [13]. However, there are difficulties in determining the stage of regeneration at which stimulation will be effective, which requires complex studies, including the use of morphological analysis methods. This determines the need for experimental research in this direction.

The use of an experimental model of small animals, in which the fracture is performed by open osteotomy and fixed intramedullary with a Kirchner needle, will probably allow to determine the stages of reparative osteogenesis disorder, at which the use of biological therapy (growth factors, MSCs, bioengineered structures, etc.) will be effective for stimulating chondro- and osteogenesis, in contrast to the model with a closed fracture, which involves the reproduction of a critical defect. One of the modern concepts of treatment of aseptic non-unions that occur after intramedullary osteosynthesis of a diaphyseal fracture is to achieve union in situ without removing the rod [14, 15]. This is due to avoidance of repeated traumatization and development of complications. The described experimental model provides a possibility of checking fracture healing in such cases without repeated surgical intervention.

Models of non-union of bone fractures in animals

Rats. It is proposed to model non-union of the tibia in rats by open osteotomy of the middle third of its diaphysis and subsequent cauterization of the periosteum 2 mm proximal and distal to the fracture site with intramedullary fixation with a 0.8 mm Kirchner needle [16]. This is due to the proven importance of a well-vascularized periosteum as a source of osteogenic cells for successful fracture healing [11]. The given model on female Sprague-Dawley rats aged 8 weeks allowed to achieve non-union in all animals at the 8th week of observation, which was confirmed by radiography and histology [16]. Other researchers in male Wistar rats after osteotomy of the femoral diaphysis removed the periosteum near the fracture site and performed fixation with a Kirschner pin to obtain non-union at the 6th week after the intervention [17].

On male Wistar rats aged 4–5 months the tibial non-union model was recreated by its osteotomy, removal of the periosteum and endosteum, and fixation with an external fixator. Characteristic signs of atrophic non-union were detected using radiography, histology and micro-computed tomography (micro-CT) after 8 weeks [18]. Another model of femur non-union in 12-week-old Fischer 344 rats was proposed, in which, in addition to removing the periosteum around the fracture site, bone marrow ablation was performed, and the fracture was fixed with a 0.8 mm Kirchner needle. The absence of fracture union was confirmed on the 12th week after the intervention radiologically, histologically with the help of micro-CT [19]. In a recent study comparing the reproducibility of rat femoral non-union models in the case of simple osteotomy combined with periosteum removal and bone marrow removal, the authors concluded that the third option, when both periosteum and bone marrow had been removed, was a more reliable model [20].

Another approach to inhibiting the influence of soft tissue on fracture healing was to wrap the femoral diaphysis with latex-silicone foil after the osteotomy, creating a 0.38-mm gap and fixing with a 5-hole plate. In male Sprague-Dawley rats aged 6 months this resulted in non-union of the femur at week 10 after the intervention [21]. On male Wistar rats, the researchers created a non-union model using a spacer that was inserted into a defect of a critical size of the femur, which made it possible to achieve non-union 4 weeks after the intervention, and 4 weeks after removing the spacer, this condition was preserved [22]. Another study on male Sprague-Dawley rats aged 4 months also used a silicone spacer, which was placed in a 3 mm wide gap after the femoral osteotomy. The fracture was stabilized with a 4-screw plate and non-union was confirmed at 4 weeks post-intervention using micro-CT scanning and histological analysis [23]. In 13-week-old female SASCO Sprague-Dawley rats, researchers obtained non-union 8 weeks after making a critical size defect (8 mm) in the mid-diaphysis of the femur and fixation with a radiolucent polysulfone plate [24].

Mice. Models of bone non-union for mice were proposed similar to those described for rats [16, 17]. In particular, we tested the reproduction of a violation of femoral bone consolidation in 9-week-old CD1 mice using a transverse partial osteotomy (50 % of the diameter) of the middle third of the diaphysis, using a 25-gauge needle as an intramedullary fixator and cauterizing the periosteum 2 mm proximal and distal to the fracture site. The achievement of non-union was confirmed histologically on the 9th week after the intervention [25]. Another femoral fracture simulation in 10-week-old male DT mice involved the use of a 4-screw plate to fix a critical diaphyseal defect, 1.6 mm in length, and achieving non-union by week 5 post-intervention [26]. To study the effect of ischemia on the occurrence of non-union, the researchers performed resection of the femoral artery

in male 129J/B6 mice aged 10–14 weeks before tibial fracture, which made it possible to obtain non-union at the 4th week of observation in the case of unstable fractures, but not in cases of their fixation [27].

Rabbits. In order to achieve non-union of the rabbit tibia, the researchers suggest combining its osteotomy with the removal of a 2 mm fragment of the diaphysis, periosteum and endosteum around the fracture area and fixation with a plate. This made it possible to achieve impaired consolidation of bone fragments at the 8th week after the intervention in female New Zealand rabbits [28–30].

In addition, in New Zealand rabbits, to achieve non-union of the tibia, it was proposed to perform large defects (10 mm long) in the metadiaphyseal zone with subsequent removal of the periosteum 5 mm proximal and distal to the injury site and stabilization with 2 Kirchner needles. The authors verified the model at the 6th and 12th weeks using radiographic and histological analyzes [31]. Creating a defect of the same size in rabbits and cauterizing only the periosteum 2 mm either side of the defect also resulted in non-union of the radial fracture 4 weeks after the intervention. The authors classified the atrophic type of non-union using radiography and histology [32].

Therefore, atrophic non-union of a fracture at the level of the diaphysis of long bones in animals is modeled, mainly, by performing an open osteotomy, with the formation of defects of a critical size between the fragments; placement of spacers in the gap after osteotomy; cauterization/removal of periosteum, endosteum, bone marrow, which are sources of cells for angiogenesis and osteogenesis. In each of the models, either intramedullary Kirchner rods, or plates with screws, or external devices are used to stabilize the fracture. The most common way to model non-union is to perform an osteotomy with the formation of a different width of the defect between the ends of the bone and subsequent removal of the periosteum proximal and distal to the ends of the defect. This is since periosteum cells are more involved in fracture regeneration (callus formation) than endosteum cells [33]. In the published works, the use of animals of both sexes is given without any preference. However, there is no agreement among researchers as to which of the models gives a more reproducible result and reliably creates the conditions for the occurrence of bone non-union. Because of this, there is a need to continue the development of experimental models that will allow studying the mechanisms of bone regeneration disorders with further development of dysregeneration treatment strategies.

Variants of using experimental models in vivo to study the therapeutic effect of biological therapy in case of non-union of fractures

The gold standard in the treatment of aseptic non-union of fractures is the use of bone autografts with stable fixation of fragments [2, 6]. To date, a diamond concept of non-union treatment has been developed, which outlines four treatment conditions — three biological (MSC, growth factors, and osteoinductive scaffolds) and one biomechanical (fracture stabilization) [34].

The use of cultured MSCs is considered one of the promising directions of biological treatment of aseptic fracture non-unions. There is experience of successfully using MSCs from adipose marrow in preclinical studies to optimize bone regeneration in mice [35, 36] and rats [37]. Another variant of cell therapy is the use of already differentiated cells in the osteogenic direction. Thus, injection of osteoblasts with intercellular matrix 6 hours after a fracture, promoted fracture union at the 12th week in the rat femur non-union model, in which bone marrow ablation and periosteum removal near the fracture site were performed [19, 20]. The need for further experimental research in this direction is confirmed by the results of a recently published systematic review, which revealed only three cases of their use in clinical practice for the treatment of non-unions, in which young patients participated after tumor resection [6]. There is little information on the use of MSCs from adipose marrow in older people for the treatment of non-unions [6].

Regarding the use of platelet rich plasma (PRP) in patients with non-union of bones, there are already more results of successful clinical use. For example, stabilization of a fracture (aseptic non-union with a tibial bone defect, type B according to ASAMI) using the Ilizarov device with the simultaneous injection of PRP made it possible to obtain faster healing of the fracture and remove the device [10]. In a systematic review that included 13 clinical trials of patients with delayed union or non-union long bone fractures, intraoperative PRP administration helped 146 of 155 achieve union after 4.64 months, and in only injections — in 144 out of 183 after 5.15 months. [38]. However, it remains unclear how many PRP injections are needed and for how long to achieve the best therapeutic effect in the case of impaired bone fracture healing. In experimental studies in rabbits (tibia non-union model with a 10-mm long defect), filling with a gelatin base with hydroxyapatite and VEGF resulted in significantly better fracture union results by histological and radiographic

parameters compared to rabbits with an unfilled defect 6 weeks after the intervention [31]. In a similar model of non-union of rabbits, the use of allografts with the addition of VEGF accelerated the osseointegration of the latter by the 12th week of observation [39].

Another direction of biological therapy to optimize bone tissue regeneration in the case of fractures is the use of bone morphogenetic proteins in combination with various matrices, which are the only approved biological drugs to accelerate fracture healing in the United States [13]. This, in turn, is due to the mixed results of clinical trials using various biological therapies. To stimulate osteogenesis, the use of BMP-2 in the treatment of non-union fractures is currently being experimentally investigated. In the rat femur dysregeneration model, it was proven that the removal of newly formed tissue from the non-union area followed by the use of a fibrin matrix with the simultaneous administration of rhBMP-2 contributed to the formation of a significantly larger volume of bone tissue compared to animals that did not receive rhBMP-2 [23]. But delayed treatment of a non-union fracture may be less effective. In particular, in the rat femur non-union model, 8 weeks after the fracture, the researchers removed tissue from the critical defect area (8 mm long), wrapped the ends of the bone with a polycaprolactone nanofibrous mesh and filled it with alginate with BMP-2. In the comparison group, animals were subjected to the same procedures during the execution of the model. Bone formation in the critical defect area was worse in rats with delayed treatment [24]. In rabbits with a 2 mm long tibial defect, endosteum and periosteum removed near the defect, and fixed with a 5-hole plate, the use of rhBMP-2 improved fracture healing, compared to rabbits without such treatment, 7 weeks after the intervention [30]. However, the clinical use of BMP-2 for the treatment of non-union long bone fractures is not mandated by protocols due to conflicting results regarding efficacy. It is currently recommended only for the treatment of open fractures of the diaphysis.

Thus, the study on animals of the mechanisms of the development of complications that occur after traumatic fractures of long bones (non-union, delayed union, false joints) to determine the effectiveness of the latest treatment strategies is an important part of preclinical trials. Despite the ability of the bone to heal with the restoration of the original structure, in certain cases, in particular, after massive injuries due to gunshot wounds, fusion does not occur and permanent functional disorders develop, requiring long-term treatment and significant financial costs. According to the results of preclinical tests, promising areas of biological therapy can be the use of cultured MSCs induced to differentiate into osteoblasts or chondroblasts; PRP, VEGF and BMP-2 on different carriers (e. g., allo-implants, autologous fibrin, gelatin, hydroxylapatite). However, the clinical use of these biotechnologies for the treatment of non-union fractures of long bones is not provided by the protocols due to conflicting results regarding efficacy and safety, which necessitates conducting large-scale experimental studies, in particular, *in vivo* modeling.

For this purpose, experimental models on small laboratory animals (rabbits, rats, mice) are described, tested and used today, which are recognized as useful due to their ease of use, ease of reproduction, economic and ethical justification. The results of published studies demonstrate the suitability of such models for elucidating the biological factors that lead to the development of dysregenerative processes in bone and the emergence of fracture healing complications, for preclinical testing of the effectiveness of the treatment strategies being created. However, despite the large number of models of non-union in small animals, in each of them, researchers and developers allow a flaw in the form of fracture union, which necessitates the further search for a better model of bone non-union in animals.

Conclusions

Various experimental models have been developed on rats, mice, and rabbits, which have proven the possibility of fracture non-union.

Most of the proposed models are based on intramedullary fixation of the fracture with expansion of the diastasis between the fragments and disruption of the endosteum and periosteum.

The given experimental models can be used for the development of innovative technologies for the treatment of regeneration disorders in view of the achievements of regenerative medicine.

Due to the expansion of biotechnological factors that require experimental research, the development of experimental animal models should be continued.

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

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A REVIEW OF ANIMAL MODELS FOR BONE FRACTURE NONUNION AND THEIR ROLE IN STUDYING BIOLOGICAL THERAPY EFFICACY

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