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Changes in femoral bone mineral density with allograft during regeneration depending on biological therapy and rat age

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In orthopedic and trauma surgery, bone defects are an increasing clinical problem in daily practice. To fill them, many advantages have bone allografts (BA), namely a large stock of transplantation material. Purpose of the study: to evaluate changes in bone mineral density (BMD) of the femur of rats after filling a distal metaphyseal defect with an bone allograft in combination with the simultaneous administration of mesenchymal stromal cells (MSCs) or platelet-rich plasma (PRP) depending on age. Methods. The model of the hole defect in the metaphysis of the femur in rats (aged 3 and 12 months) investigated BMD in terms of filling BA, including, with the simultaneous administration of MSCs or PRP during surgery. Results. In rats with an unfilled defect — the immutability of BMD during the experiment. Compared with 3-month-old rats in 12-months-old rats with BA BMD was 1.11 times higher on the 14th day ($p < 0.05$), and on the 90th day it was 1.11 times lower ($p < 0.05$), on the 28th day it was not differed. In the 3-month-old rats with A and MSC showed a 1.20-fold lower BMD ($p < 0.05$) on the 28th day, and on the 90th day BMD did not differ compared to the group with BA. BMD in 12-month-old rats with BA and MSC did not differ from the group with BA. In in 12-month-old rats with BA and PRP BMD was 1.18 times ($p < 0.05$) higher on the 28th day and on the 90th day — 1.14 times ($p < 0.05$) compared to the BA group, and in 3-month-old rats did not differ for all terms. Conclusions. In the case of filling a bone defect with a bone alograft in 12-month-old rats, the increase of BMD of the femur is slower than in 3-month-old rats. The use of BA with MSCs in younger rats causes a delay in bone regeneration on the 28th day, but does not disrupt this process, according to BMD on 90th day compared to rats with BA, and in older rats does not significantly change BMD during the study. Filling defect BA with PRP promotes the growth of femoral BMD in older rats from the 28th day, but in younger rats it does not cause significant changes compared to the group with BA.

В ортопедичній та травматологічній хірургії дефекти кісток становлять усе більшу клінічну проблему в щоденній практиці. Для їх заповнення кращими вважаються кісткові алоімплантати (А) через більшу доступність порівняно з автоімплантатами. Мета. Оцінити зміни мінеральної щільності кісткової тканини (МЩКТ) стегнової кістки щурів після заповнення дефекту дистального метафізу алогенним кістковим імплантатом у поєднанні з одночасним введенням мезенхімальних стромальних клітин (МСК) або збагаченої тромбоцитами плазми крові (PRP) залежно від віку. Методи. На моделі дірчастого дефекту критичного розміру в дистальному метафізі стегнової кістки у щурів (віком 3 та 12 міс.) досліджено МЩКТ стегнової кістки в умовах заповнення дефекту А, в тому числі, з одночасним введенням МСК або PRP під час втручання на 14, 28 та 90 добу. Результати. У тварин із незаповненим дефектом МЩКТ стегнової кістки не змінилася протягом експерименту. Порівняно з 3-міс. у 12-міс. щурів із А МЩКТ вища в 1,11 разу на 14-ту добу ($p < 0,05$), а на 90-ту — нижча в 1,11 разу ($p < 0,05$), на 28-му добу не відрізнялася. У 3-міс. тварин з А + МСК виявлено нижчий показник МЩКТ у 1,20 разу ($p < 0,05$) на 28-му, а на 90-ту добу МЩКТ не відрізнявся порівняно з групою з А. У 12-міс. щурів із А + МСК показники МЩКТ не відрізнялися від групи з А. У 12-міс. щурів із А + PRP показник МЩКТ вищий у 1,18 разу ($p < 0,05$) на 28-му добу та в 1,14 разу — на 90-ту добу ($p < 0,05$) порівняно із групою з А, а у 3-міс. не відрізнявся на всі терміни. Висновки. У разі заповнення кісткового дефекту алоімплантатом у 12-міс. щурів зростання МЩКТ стегнової кістки відбувається повільніше, ніж у 3-міс. Використання А з МСК у молодших особин викликає затримку регенерації кістки на 28-му добу, проте не порушує цей процес, згідно з показниками МЩКТ на 90-ту добу порівняно з тваринами з А, а у старших щурів істотно не змінює МЩКТ протягом дослідження. Заповнення дефекту А з PRP сприяє зростанню МЩКТ стегнової кістки у старших щурів з 28-ї доби, а у молодших не викликає значущих змін порівняно з групою з А. Ключові слова. Кістковий алоімплантат, дефект, моделі тварин, регенерація кістки, мінеральна щільність кісткової тканини, мезенхімальні стромальні клітини, збагачена тромбоцитами плазма крові.

Key words. Bone allograft, defect, animal model, bone regeneration, bone mineral density, mesenchymal stromal cells, platelet-rich plasma

Introduction

The need to fill bone defects in orthopedic and trauma surgery is an increasing clinical and organizational problem [1]. One of the causes of these defects is the surgical treatment of bone infections and tumors, which mostly requires further bone reconstruction [2]. In addition, impaired healing of fractures after high-energy trauma or in conditions of osteoporosis often requires additional treatment for bone tissue formation [3].

Reconstruction of bone defects can be achieved using autografts, allografts and biomaterials based on ceramics, synthetic or natural polymers [4].

Among the above-mentioned osteoplastic raw materials, allografts have many advantages, which, in particular, are characterized by a large reserve of primary material of the desired configuration. Also, at the same time, there is no damage to the patient's bone tissue, less pain, and a shorter duration of the operation than in the case of using autografts [5]. According to P. Feltri et al. [6], the use of bone autograft leads to fewer complications, reoperations, and failures than other methods in the treatment of large diaphyseal defects, and it is the "gold standard" for bone regeneration, but there is a need to develop other methods of replacing bone defects.

To study the quality of newly formed bone tissue after filling a critical defect with a bone allograft and under the conditions of simultaneous use of various biological therapy techniques, a non-invasive method such as two-photon X-ray absorptiometry can be successfully used to determine bone mineral density (BMD). This indicator does not allow to assess the structure of newly formed bone tissue but can indirectly reflect the mineralization process during bone regeneration [7].

Purpose: to evaluate changes in bone mineral density of the femur of rats after filling the distal metaphyseal defect with an allogeneic bone implant in combination with simultaneous administration of mesenchymal stromal cells or platelet-rich plasma depending on age.

Material and methods

Experimental studies were conducted in compliance with the requirements of humane treatment of experimental animals [8, 9] after approval by the local bioethics committee (protocol No. 191 dated 22.04.2019).

140 white rats were used in the experiment. Of these, 70 were 3 months old (young, sexually mature individuals undergoing bone formation) and 70 were 12 months old (older animals with complete bone formation undergoing bone tissue remodeling). All rats were given a critical-sized defect in the distal metaphysis of the femur and randomly divided into eight groups of 15 each:

– I and II — 30 animals (3 and 12 months old), defect without filling;

– III and IV — 30 individuals (3 and 12 months old) — with filling with bone allograft;

– V and VI — 30 rats (3 and 12 months) — with filling with bone allograft with simultaneous injection of mesenchymal stromal cells (MSC);

– VII and VIII — 30 animals (3 and 12 months) — with filling with bone allograft with simultaneous injection of platelet-rich plasma (PRP).

Donors of adipose tissue for obtaining MSCs, blood for obtaining PRP and allografts were 20 rats, 10 individuals of each age (3 and 12 months);

Defect formation and implant placement were performed under aseptic and antiseptic conditions under general anesthesia (ketamine, 50 mg/kg, intramuscularly). We selected a defect diameter of 3 mm, depth of 3 mm, which does not require additional fixation and exceeds the minimum size of a critical defect [10]. Cylindrical allografts with a diameter of 3 mm and a length of 3 mm were placed in the defect area of rats of groups III–VIII. In groups V–VIII, during surgical intervention, MSCs or PRP were also injected into the defect cavity before the installation of the allografts. After local antibiotic treatment, the muscles and skin wound were sutured in layers, and the surgical area was treated with an antiseptic.

Bone allografts with a diameter of 3 mm and a length of 3 mm were made using hollow drills from the metaphyses of femurs of 3-month-old white rats, which ensured their standard dimensions, then packed in polyethylene, sterilized by radiation "Accelerator LU-10" (Scientific research complex "Accelerator" National Scientific Center Kharkiv Institute of Physics and Technology).

MSCs were obtained from adipose tissue from the omentum of rats, according to the principles outlined by B. M. Buehrer and B. Cheatham [11]. After 7–9 days of cultivation, the cells were removed and transferred to sterile microtubes in an amount of 1.0 × 106 cells per 0.5 ml of culture medium for injection into defects of rats of groups V and VI.

To obtain PRP, blood was collected from 20 individuals of 3 and 12 months of age, 10 animals in each group. At the same time, the rats were removed from the experiment, 8 ml of venous blood was collected into an 8.5 ml vacuum tube with anticoagulant. The tube was centrifuged in a laboratory clinical centrifuge OPn–3.02 1500 rpm for 10 min (first stage),

then plasma (supernatant fraction) was collected from it with a sterile pipette, transferred to a graduated sterile tube, which was centrifuged again at 3000 rpm for 10 min (second stage). Next, platelet-poor plasma (supernatant fraction) was collected with a pipette, and the PRP left at the bottom of the tube was used for injection into the defect of the femur of rats of groups VII and VIII in a volume of 0.2 ml.

To study the BMD of the femurs, rats were removed from the experiment 14, 28 and 90 days after surgery, 5 animals from each group were decapitated under ether anesthesia. After that, the operated femurs were isolated and cleaned of soft tissues, fixed in 10 % neutral formalin solution for 4 days, then washed with tap water and dried with paper towels. The BMD of the operated femurs of rats was determined using two-photon X-ray absorptiometry (DXA) on an Explorer QDR bone densitometer (Hologic, USA). Scanning was performed in the "detail Lumbar Spine" mode simultaneously for 7 bones, after which each of them was analyzed separately: the area was determined and the Region of Interest (R1-R7) was set, Figure.

Statistical data processing was performed using the IBM SPSS Statistics 20 software. The results are given as median and quartiles (Me; Q1, Q3). To compare the obtained data, the Mann–Whitney analysis method was used. The difference was considered statistically significant if $p < 0.05$.

Results

Unfilled defect. The study of BMD in experimental animals of both age groups with an unfilled defect of the distal metaphysis of the femur showed the absence of a significantly significant time course of this indicator during the study. In 12-month-old rats, the value of BMD of the femur was greater than in 3-month-old rats on the 14th day of the experiment by 1.22 times ($p < 0.05$), on the 28th day — by 1.07 times ($p < 0.05$), and on the 90th day there was no difference (Table).

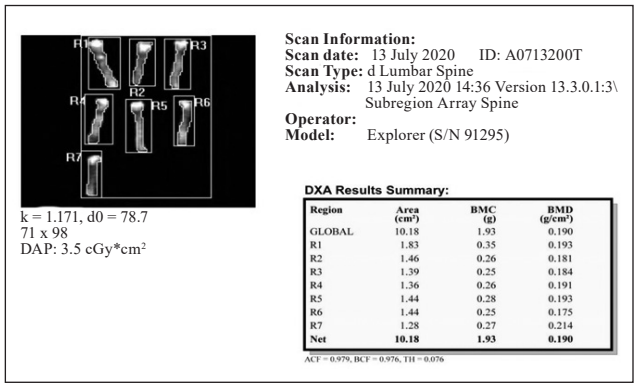

Figure. Dual-energy X-ray absorptiometry densitogram of femurs of white rats with bone allografts 14 days after surgery

Allograft. In 3-month-old animals with an allograft in the defect, BMD values were 1.2 times higher ($p < 0.05$) on the 28th day, 1.30 times higher on the 90th day ($p < 0.05$) compared to the 14th day. Compared to 3-month-old rats, in 12-month-old rats with an allograft, BMD was 1.11 times higher on the 14th day ($p < 0.05$), and 1.11 times lower on the 90th day ($p < 0.05$), but did not differ on the 28th day. During the experiment, a change in BMD values in 12-month-old animals was detected only on the 90th day, when it significantly increased by 1.13 times ($p < 0.05$) compared to the 28th day of the study (Table).

Allograft + MSC. In the case of filling the defects of the distal femoral metaphysis of rats with an allograft and simultaneous administration of MSCs, in 3-month-old individuals a lower BMD index was found in 1.20 times ($p < 0.05$) on the 28th, and on the 90th day BMD did not differ compared to the group with an allograft of the same age. During the experiment in 3-month-old animals on the 90th day an increase in BMD was observed in 1.28 times ($p < 0.05$) compared to that on the 14th ($p < 0.01$) and in 1.19 times compared to that on the 28th day of the experiment ($p < 0.01$). In 12-month-old rats with an allograft and MSCs no statistically significant difference was recorded compared to the group with an allograft and with 3-month-old animals with similar filling of the defect at all studied periods (Table).

Allograft + PRP. In 3-month-old animals with allograft and simultaneous administration of PRP, BMD data did not differ compared to the group with allograft for all observation periods. A gradual increase in the analyzed indicator was observed during the experiment. Thus, on the 28th day of the experiment, BMD in rats of this group was higher than on the 14th by 1.18 times ($p < 0.05$), and on the 90th day 1.32 times higher than on the 14th ($p < 0.05$) and 1.12 times higher than on the 28th day of the experiment ($p < 0.05$). In 12-month-old animals with allograft and simultaneous administration of PRP, BMD data did not differ compared to the group with allograft for all observation periods. A gradual increase in the analyzed indicator was observed during the experiment. Thus, on the 28th day of the experiment, BMD in rats of this group was higher than on the 14th by 1.18 times ($p < 0.05$), and on the 90th day 1.32 times higher than on the 14th ($p < 0.05$) and

1.12 times higher than on the 28th day of the experiment ($p < 0.05$). In 12-month-old animals, the BMD index was 1.18 times higher ($p < 0.05$) on the 28th and 1.14 times higher ($p < 0.05$) on the 90th day compared to the group with an allograft. During the experiment, in 12-month-old rats, a higher BMD index was found on the 28th and 90th day compared to the 14th day by 1.18 times ($p < 0.05$) and 1.29 times ($p < 0.05$), respectively (Table).

Discussion

The use of MSCs and PRP to optimize the healing of bone defects and fractures is one of the current methods of biological therapy in modern orthopedics. C. G. Park et al. [12] showed that the combination

Table

Bone mineral density indicators of the femur of rats of different ages with types a hole defect filling (g/cm2, (Me; Q_1, Q_3))

Type of defect filling	Age of rats (months)	Time after surgery (days)		
		14th	28th	90th
unfilled defect (n = 30)	3	0.149 (0.138; 0.157)	0.168 (0.165; 0.170) $p_3 > 0.05$	0.158 (0.157; 0.170) $p_3 > 0.05$ $p_4 > 0.05$
	12	0.182 (0.178; 0.201) $p_1 < 0.05$	0.180 (0.175; 0.197) $p_1 < 0.05$ $p_3 > 0.05$	0.171 (0.169; 0.187) $p_1 > 0.05$ $p_3 > 0.05$ $p_4 > 0.05$
allograft (n = 30)	3	0.172 (0.167; 0.182)	0.208 (0.187; 0.211) $p_3 < 0.05$	0.218 (0.215; 0.227) $p_3 < 0.05$ $p_4 > 0.05$
	12	0.191 (0.183; 0.193) $p_1 < 0.05$	0.175 (0.162; 0.185) $p_1 > 0.05$ $p_3 > 0.05$	0.197 (0.188; 0.202) $p_1 < 0.05$ $p_3 > 0.05$ $p_4 < 0.05$
allograft + mesenchymal stromal cells (n = 30)	3	0.161 (0.158; 0.169) $p_2 > 0.05$	0.173 (0.162; 0.176) $p_2 < 0.05$ $p_3 > 0.05$	0.206 (0.193; 0.228) $p_2 > 0.05$ $p_3 < 0.01$ $p_4 < 0.01$
	12	0.171 (0.167; 0.195) $p_1 > 0.05$ $p_2 > 0.05$	0.183 (0.171; 0.206) $p_1 > 0.05$ $p_2 > 0.05$ $p_3 > 0.05$	0.198 (0.186; 0.199) $p_1 > 0.05$ $p_2 > 0.05$ $p_3 > 0.05$ $p_4 > 0.05$
allograft + platelet-rich plasma (n = 30)	3	0.161 (0.158; 0.169) $p_2 > 0.05$	0.190 (0.178; 0.204) $p_2 > 0.05$ $p_3 < 0.05$	0.213 (0.205; 0.217) $p_2 > 0.05$ $p_3 < 0.01$ $p_4 < 0.01$
	12	0.174 (0.168; 0.197) $p_1 > 0.05$	0.206 (0.199; 0.219) $p_1 > 0.05$ $p_2 < 0.05$ $p_3 < 0.05$	0.224 (0.204; 0.233) $p_1 > 0.05$ $p_2 < 0.05$ $p_3 < 0.05$ $p_4 > 0.05$

Notes: p_1 — probability of difference when comparing indicators in rats of different ages under the same treatment conditions for the same period after the intervention; p_2 — probability of difference when comparing indicators in animals of the same age for the same period of the experiment with the group with a defect filled only with allograft; p_3 — probability of difference when comparing indicators in individuals of the same age and filling the defect with those on the 14th day after the intervention; p_4 — probability of difference when comparing indicators in animals of the same age and filling the defect with those on the 28_{th} day after the intervention.

of these two methods accelerates the engraftment of structural bone allografts in rabbits compared to the use of PRP alone. B. E. Liebig et al. [13] claim that the use of PRP or MSCs together with synthetic grafts and bioengineered constructs has a positive effect on the healing rate of bone defects and fractures. In our study, we first analyzed the change in femoral BMD in rats without filling the defect with an allograft and using biological therapy methods (MSCs or PRP). The results of the analysis of BMD indicators indicate slow and incomplete healing of the defect at the final observation period, regardless of the age of the rats. In older individuals, the BMD indicator is higher than in younger ones only at the early stages of the experiment, which is consistent with our previous results of histological analysis [14] and biochemical assay of blood serum indicators, which reflected the processes of bone mineralization in these rats [15, 16].

In the case of filling a critical-sized defect in the femur with an allograft without PRP or MSCs, the increase in BMD during the experiment was detected only in younger animals. This statement is consistent with the findings of D. Chaverri et al. [17], who indicate an increase in markers of bone formation in the blood serum in the case of filling the defect with an allograft. The results obtained coincide with the data of A. L. Foster et al. [18], in which the stability of the location of the affected bone sections is indicated as a necessary condition for proper bone tissue recovery, including in animal experiments, which in our case is provided by a bone allograft.

Filling the distal femoral metaphysis defect with an allograft and simultaneous injection of MSCs resulted in lower BMD values on the 28th day of the experiment only in younger rats compared to the allograft group, which may indicate a delay in bone formation. In older animals, BMD values did not differ from the allograft group at all observation times. However, at the end of the study, BMD values in younger animals also did not differ from the allograft group, which is consistent with the results of histological analysis [19] and assessment of biochemical indicators that reflect bone remodeling in rats under experimental conditions [20, 21]. A possible reason for the lower BMD values in younger individuals in the case of filling the defect with an allograft with simultaneous injection of MSCs is the delay in bone tissue regeneration caused by immune inflammation mediated by T- and B-cells due to the reaction to the injection of foreign genetic material [22]. This is also consistent with the results of histological studies by S. Prat et al. [23], which showed that the remodeling of bone allografts in the defect in combination with MSCs under certain conditions may be incomplete and accompanied by the formation of connective tissue. In rats with filling the defect with an allograft and simultaneous injection of PRP, changes in BMD values depending on age were also found.

In younger animals, they did not differ from the allograft group, while in older animals, they were higher at 28 and 90 days. However, BMD values between younger and older rats with PRP did not differ at 90 days, which is consistent with the results of our previous histological analysis [24]. This also confirms the beneficial effect on bone regeneration of the combination of PRP with allograft for older animals, because when using only allograft, BMD in older animals is lower at 90 days than in younger animals due to the slowing of bone tissue regeneration with age. However, the combination of allograft with simultaneous administration of PRP in younger animals does not significantly affect the healing of the bone defect.

Conclusions

The BMD of the femur of rats with an unfilled hole defect of critical size of its distal metaphysis of both age groups did not change significantly during the 90 days of the experiment according to the results of measurement by the method of two-photon X-ray absorptiometry.

In the case of filling a similar defect with an allograft in older individuals, the growth of BMD of the femur occurs more slowly than in younger ones: in 3-month-olds from the 28th day, and in 12-month-olds on the 90th compared to the 14th day. In older rats, the BMD index is lower compared to younger ones on the 90th day of the study.

The use of allograft in combination with MSCs in a critical-sized defect in younger animals causes a delay in bone regeneration on day 28, but does not disrupt this process, according to BMD indicators on day 90 compared to rats with allograft, and in older animals does not significantly change femoral BMD during the study. Filling the defect with allograft with simultaneous administration of PRP promotes the growth of femoral BMD in older rats from day 28, and in younger ones does not cause significant changes compared to the allograft group.

Conflict of interest. The authors declare the absence of a conflict of interest.

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CHANGES IN FEMORAL BONE MINERAL DENSITY WITH ALLOGRAFT DURING REGENERATION DEPENDING ON BIOLOGICAL THERAPY AND RAT AGE

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