ORIGINAL PAPER

RJME Romanian Journal of Morphology & Embryology http://www.rjme.ro/

Histological evaluation of the incorporation and remodeling of structural allografts in critical size metaphyseal femur defects in rats of different ages

NATALIYA ASHUKINA[1)], VALENTYNA MALTSEVA[1)], PETRO VORONTSOV[2)], ZINAIDA DANYSHCHUK[1)], OLGA NIKOLCHENKO[2)], MYKOLA KORZH[3)]

[1)]*Laboratory of Connective Tissue Morphology, Sytenko Institute of Spine and Joint Pathology, National Academy of Medical Sciences of Ukraine, Kharkiv, Ukraine*

[2)]*Department of Transplantology and Experimental Modeling with an Experimental Biological Clinic, Sytenko Institute of Spine and Joint Pathology, National Academy of Medical Sciences of Ukraine, Kharkiv, Ukraine*

[3)]*Department of Emergency Traumatology and Reconstructive Surgery, Sytenko Institute of Spine and Joint Pathology, National Academy of Medical Sciences of Ukraine, Kharkiv, Ukraine*

Abstract

Insufficient bone regeneration is a common issue for patients with extensive bone damage, therefore the use of allografts is required. With increasing life expectancy, there is a higher risk of bone repair issues after fractures or orthopedic surgical intervention. We studied incorporation and remodeling of structural allografts in critical size metaphyseal femur defects in 52 rats aged 3-month-old and 12-month-old who underwent surgeries creating a bone defect, which was either filled with a structural allograft (3-month-old – 3moAllo; 12-month-old – 12moAllo) or left empty (3-month-old – 3moE; 12-month-old – 12moE). Histological analyses were performed 14, 28 and 90 days after the surgery. The percentage of bone and fibrous tissues, and allograft relative to the defect area was evaluated. The transmission electron microscopy was carried out 14 days after allograft implantation. When the defect was empty, slower bone regeneration was observed in 12moE rats *versus* 3moE, leading to sufficient irregularities in the anatomic structure of the femur 90 days after the surgery. When a structural allograft was used, the area of the fibrous tissue was larger in the defects of 12moAllo compared with 3moAllo rats 90 days after surgery. No age-related differences were found in the allograft remodeling and structures of the osteocytes, osteoblasts, and osteoclasts over the observation period. Evident issues with bone regeneration were found in critical size defects both of 12moE and 12moAllo rats. However, the allograft use allowed the bone maintaining anatomic structure 90 days after the surgery.

Keywords: bone regeneration, bone substitute, animal model, aging, histology, transmission electron microscopy.

Introduction

Bone substitutes are used because there is a need to optimize the reparative osteogenesis in bone defects that occur because of injuries, gunshot wounds, bone tumor removals, during revision hip arthroplasty or surgical intervention on the spine. These defects are large and do not heal through bone tissue formation on their own. They are called "critical size defects" [1]. It is known that the frequency of nonunion or delayed union after bone fractures is high – from 3% to 28% [2, 3]. This causes disability in patients, which then requires additional surgical intervention, and increases the amount of money spent.

According to *United Nations* (UN) data, the number of people over 65 is increasing all over the world: from 8.7% in 2017 to potentially 15.8% in 2050; in Ukraine, 16.5% and 25.5%, respectively [4]. They are developing issues with bone turnover, which lead to decreased bone mineral density and lower bone quality [5], lowered reparative potential [6, 7]. This also makes bone repair after injuries and surgeries on the skeleton more difficult.

One way to improve bone regeneration is to use bone grafting using autologous and allogeneic bone grafts. Autografts are the "gold standard" in orthopedic surgery. However, their use is limited by the volume of material that can be obtained, additional damage in the donor site, and the impossibility of their extraction in patients with severe injuries. Therefore, bone allografts that lack living cells due to sterilization are widely used to accelerate bone healing [8]. Depending on the situation, bone allografts of different structures (cortical, cortical-cancellous, and cancellous) and shapes (structural and fragmented) can be used [9]. In 2020, the most common surgical interventions that used bone allografts were, in first place, spine fusion, in second, dental implant procedures, and, in third, joint reconstruction [10]. The rapid incorporation of the replacement materials into the bone, which is based on their osteoconductive and osteoinductive properties, is crucial for the recovery of the biomechanics of the segment of the skeleton that was operated on [11]. Nonetheless, bone quality at the moment of implantation, which decreases with age, is also a determining factor of the success of bone grafting [5].

Aim

The objective of the study was to examine age-related differences in the incorporation and remodeling of structural allografts in critical size metaphyseal femur defects in rats.

Materials and Methods

Animals

Experiments were performed in accordance with the Ukrainian Law "On the Protection of Animals from Brutal Treatment" [12], European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986, ETS 123) and Directive 2010/63/EU. The plan for the experimental study was approved by the Bioethics Committee at the Sytenko Institute of Spine and Joint Pathology, Kharkiv, Ukraine (Protocol No. 191 from 22.04.2019).

Fifty-two male white laboratory rats from the population of the Experimental Biological Clinic of the Sytenko Institute of Spine and Joint Pathology were included in the study. The rats were of two different ages: 3-month-old, with an average weight of 263.8±9.1 g (n=26) and 12-month-old, with an average weight of 446.3±10.8 g (n=26). Four or five animals lived in each cage, with a 12-hour light period, a temperature of 20–24°C, with access to water and standard diet *ad libitum*.

Study design

All animals underwent a surgery that created metaphyseal femur defects. In each age group, the rats were randomly assigned to one of the groups: empty defect (n=9, 3-month-old – 3moE; n=9, 12-month-old – 12moE) or defect with structural allograft (n=17, 3-month-old – 3moAllo; n=17, 12-month-old – 12moAllo) (Figure 1a).

Surgery

The operations were completed under aseptic and antiseptic conditions under general anesthesia (Ketamine, 50 mg/kg intramuscularly). After the fur was shaved off on the left knee and femur, the area was treated using a Betadine solution and, from the anterior–lateral access, transcortical defects of critical size were created into the distal metaphysis of the left femur using a dental burr. Cylindrical allografts of a corresponding size (3 mm diameter, 3 mm height) were placed in the defect (Figure 1, b–e). The wounds were treated with antibiotic powder, sutured in layers, and treated with a Betadine solution.

The animals were euthanized by administering a lethal dose of anesthetic (Sodium Thiopental, 90 mg/kg intramuscularly): 14 days after the surgery, three rats from each of the 3moE and 12moE groups, and seven rats from each of the 3moAllo and 12moAllo groups; 28 and 90 days after surgery – three rats from each of the 3moE and 12moE groups, and five rats from each of the 3moAllo and 12moAllo groups.

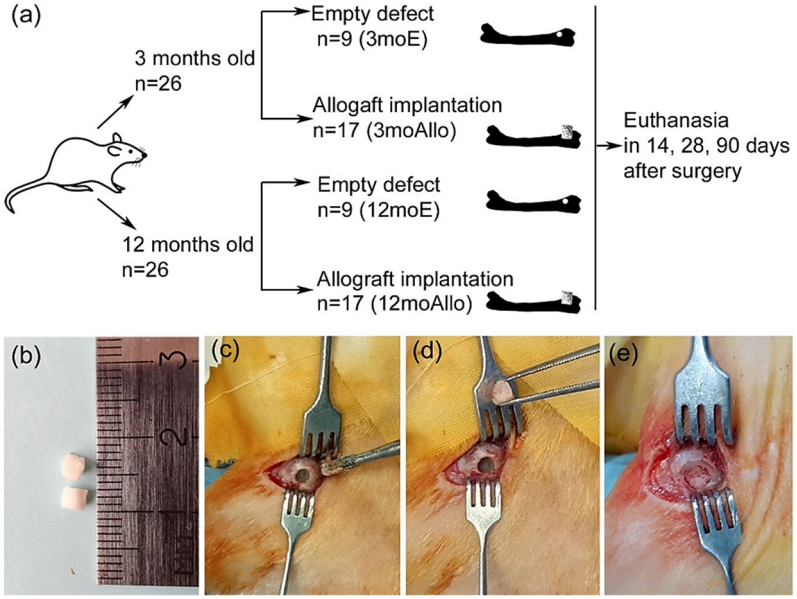

Figure 1 – ***The study design and the process of the allograft implantation: (a) Three-month-old rats (n=26) and 12-month-old rats (n=26) were divided into four groups; each rat underwent a surgery that created a metaphyseal defect in the left femur, which was then filled with an allograft or left empty; the animals were euthanized 14, 28, and 90 days after the operation, and histological analysis was performed; (b) Allografts (3 mm diameter, 3 mm height); (c) The transcortical defect was created in the distal left femur metaphysis using a dental burr and (d and e) the allograft was placed into the created defect. Allo: Allograft; E: Empty defect; mo: Months; n: No. of rats.***

Preparation of bone allografts

The allograft donors were 6-month-old rats (n=13), whose femurs were taken out after they were administered a lethal dose of anesthetic (Sodium Thiopental, 90 mg/kg intramuscularly). To remove blood remnants and to keep it sterile initially, the donor's allogeneic bone was stored in a 10% hydrogen peroxide solution for two hours. To remove fats, the bone was stored in a solution of 96% ethyl alcohol and 100% diethyl ether (1:1, *v/v*) for two hours. To decrease its antigen properties derived from the remaining non-collagen protein, the bones were stored in a solution of salts (0.45 M sodium chloride, 0.1 M disodium hydrogen phosphate) at -40°C overnight. The femurs were then taken out and dried using a convectional heater for 4–5 days. The allografts (3 mm diameter, 3 mm height) were made from distal femoral metaphysis and sterilized using radiation doses ranging from 15 to 25 kGy by used of LU-10 linear accelerator (10 MeV; 10 kW) [13]. After sterilization, four samples from each batch of implants were sterility testing using fluid thioglycolate medium for bacteria culture method. Allografts were considered sterile when there were no growing colonies in a nutrient medium.

Histology

Femurs that were operated on were taken out, cleaned of soft tissues, and fixed in 10% neutral formaldehyde for four days. After four days, the bones were taken out, washed in tap water, and decalcified in 5% trichloroacetic acid for five days [14]. Then, they were washed in 96% ethyl alcohol, and the distal metaphysis with a critical defect

was cut out of each bone. The distal metaphysis of the femur was dehydrated in isopropyl alcohols of increasing concentration (80%, 90%, 90%, 100%, 100%, 100%) and a mixture of paraffin and isopropyl alcohol (1:1, *m/v*) and embedded into the paraffin. Histological sections were stained using two methods: Hematoxylin–Eosin (HE), and Goldner's trichrome staining, which made it possible to differentiate between a mature mineralized bone matrix and an immature bone matrix [14]. The structure of the tissue and the allograft were analyzed using the Olympus BX63 light microscope (Japan). The DP73 digital camera (Olympus) and the «cellSens Dimension 1.8.1» software (Olympus, 2013) were used to take pictures.

Histomorphometry

Seven sections were obtained from each femoral distal metaphysis. For each section, the areas of newly formed tissues (bone and fibrous) and the allograft were measured using the «cellSens Dimension 1.8.1» software (Olympus, 2013). After measurement, the percentage of bone tissue (B%), fibrous tissue (F%) and allograft (A%) relative to the area of the defect was calculated.

Transmission electron microscopy (TEM)

Materials for the TEM were taken from two rats from each of the 3moAllo and 12moAllo groups, all of which were euthanized 14 days after the surgery. The allograft and the tissue connected to it were extracted from the defect area, immediately placed in the Karnovsky's fixative, and stored there for one day. Then, the materials were decalcified in the 3.7% ethylenediaminetetraacetic acid (EDTA) for two weeks at 4°C. After decalcification, the specimens were cut into fragments, subjected to post-fixation in 1% osmium tetroxide, dehydrated in first ethanol at increasing concentrations (50%, 70%, 80%, 90%, 96%, 96%) and then 100% acetone, permeated by a mix of epoxy resin and acetone (Epon and Araldite), embedded in fresh epoxy resin, and polymerized at 65°C for 18 hours. The resulting blocks were sliced into semi-thin and ultra-thin sections using an ultramicrotome. Grid post-staining was performed using lead citrate, then aqueous uranyl acetate, and lead citrate again. The sections were analyzed using the EMV-100BR transmission electron microscope (Sumy, Ukraine).

Statistical analysis

The results were presented as mean ± standard deviation (SD). The data was analyzed using the IBM Statistical Package for the Social Sciences (SPSS) Statistics 20 software. The normality of the distribution was checked using the Kolmogorov–Smirnov method. The influence of age on bone regeneration was evaluated using an unpaired *t*-test. Analysis of variance (ANOVA) with Bonferroni's *post hoc* correction was used to compare the values between the observation periods (14, 28 and 90 days). The critical level of significance was accepted to be 0.05.

Results

Bone repair in the empty defects

Fourteen days after the surgery, the formulation of granulation and fibrous tissues and woven bone was discovered in the defects of rats from both age groups (3moE and 12moE) (Figure 2a). Newly formed bone trabeculae were located on the perimeter of the defect bordering the host bone. They contained many brightly stained osteocytes, with large hypochromic nuclei. Active osteoblasts were found on the surface of the bone trabeculae. Granulation and fibrous tissues were located in the central area of the defect. Osteogenic cells and fibroblasts were found between the bundles of collagenous fibers in the fibrous tissue. Neutrophils, macrophages, undifferentiated cells, fibroblasts, and many blood capillaries were discovered in the granulation tissue (Figure 3, a and b).

The relative percentage of the tissues formed in the defect depended on the age of the animal: 12-month-old rats had a 2.4 times lower B% (p=0.0001) in comparison to the 3moE group (Figure 4b), but a 2.1 times higher F% (p=0.005) (Figure 4a). During the histomorphometry, the area of the fibrous tissue was considered to be the total area of the granulation and fibrous tissues.

Twenty-eight days after the surgery, F% significantly decreased in the defects of animals of both ages: by 3.16 times (p<0.05) in 3-month-old rats, and by 20.47 times (p<0.001) in 12-month-old rats (Figure 4). In rats of the 3moE group, the fibrous tissue was located in the central region of the defect, but for the 12moE group – mostly in the defect in the cortex (Figure 2).

The quality of the newly formed bone in the defect differed between the two groups. Lamellar bone was discovered in rats of the 3moE group. The bone matrix was stained evenly, the density of the osteocytes was uniform, the red bone marrow was located in the intertrabecular spaces (Figure 2a). In rats of the 12moE group, the newly formed bone trabeculae had an unevenly stained matrix, which reflected the imbalance of the mineralization processes. Destructive fissures and areas without cells were observed in some bone trabeculae. Yellow and red bone marrow formed in the intertrabecular spaces (Figure 2a). B% was not different in rats of different age groups (Figure 3b).

Ninety days after the surgery, B% and F% were not different in rats of different age groups (Figure 4, a–c). However, in 12-month-old rats, the area of the defect in the cortex was filled with fibrous tissue (Figure 2a). The irregularities in the anatomic shape of the distal femoral metaphysis were noted in animals of both age groups, but more evident in the 12moE group (Figure 2c).

Bone repair in the defects with allograft filling

Fourteen days after the surgery, the allografts were clearly seen in the defects of animals of both age groups. The newly formed bone and fibrous tissue were situated on the perimeter of the allograft and in its «intertrabecular spaces» (Figure 2b). The new bone formed directly on the surface of the allograft, but their matrices were of different colors after Goldner's trichrome staining and the new bone contained living cells, as opposed to the allograft (Figure 5, a and c). During TEM analysis, the cell structure in the new bone did not differ significantly in rats of different ages. Osteocytes located in the new bone had large nuclei with pores in the nuclear membrane. Their cytoplasm contained a rough endoplasmic reticulum (rER) and few mitochondria (Figure 5d). Functionally active osteoblasts with eccentric nuclei containing 1–2 nucleoli, rER, Golgi apparatus and mitochondria in the cytoplasm

were situated on the surface of new bone trabeculae (Figure 5, b and d). Osteoclasts had their characteristic structure and were found in various degrees of functional activity (Figure 5d). Using TEM, it was shown that the allograft matrix was structurally different to the matrix of the newly formed bone. In 12-month-old rats, areas of hypermineralization were discovered in the matrix of the allograft (Figure 5b). These areas were not found in 3-month-old rats (Figure 5d). In both age groups, empty osteocyte lacunae were identified in the allograft matrix.

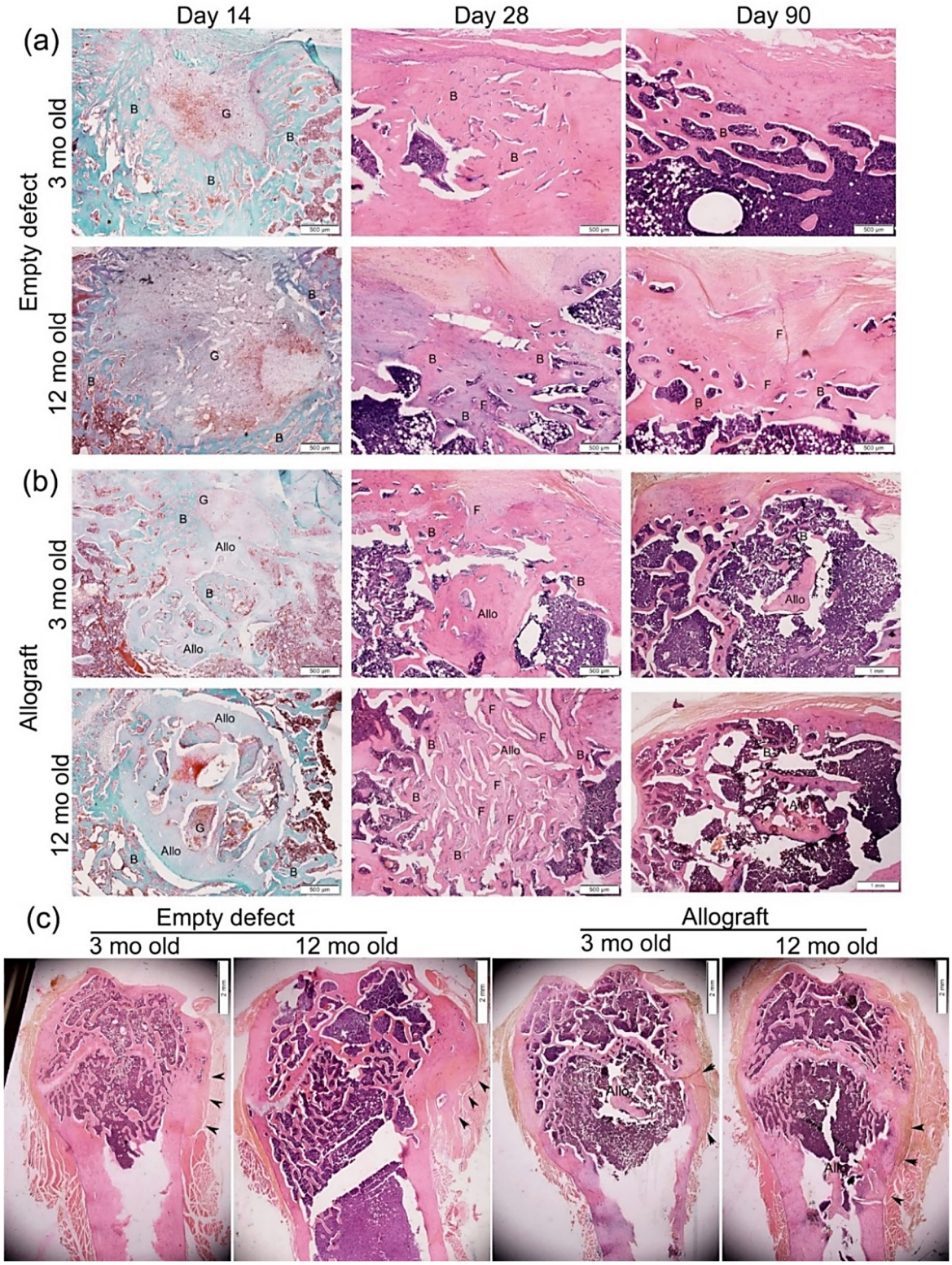

Figure 2 – ***Histological findings (day 14 – Goldner's trichrome staining; days 28 and 90 – HE staining): (a) The formulation of bone tissue is shown in unfilled defects created in the distal femoral metaphysis of rats of different ages 14, 28 and 90 days after surgery; (b) Incorporation and remodeling of structural allografts with bone formation is demonstrated; (c) The anatomic shape of the distal femoral metaphysis is shown to be disturbed 90 days after the creation of the unfilled defect; the location of the defect is indicated by arrows. Scale bar: (a) 500 µm; (b) 500 µm and 1 mm; (c) 2 mm. Allo: Allograft; B: Newly formed bone; F: Fibrous tissue; G: Granulation tissue; mo: Months.***

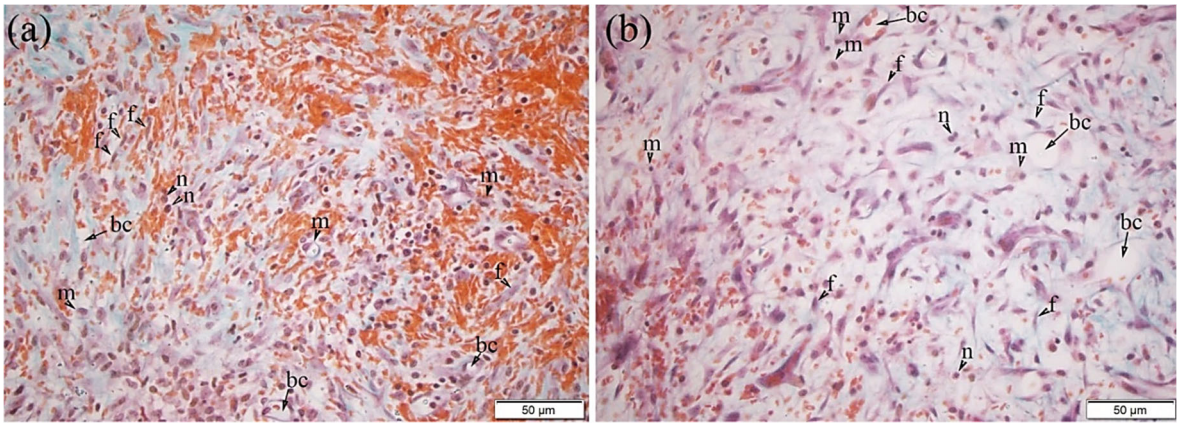

Figure 3 – ***Granulation tissue in the metaphyseal femoral unfilled defects 14 days after surgery in 3-month-old (a) and 12-month-old (b) rats. Neutrophils (n), macrophages (m), fibroblasts (f), and many blood capillaries (bc) distributed between collagen fibers (green color). Goldner's trichrome staining. Scale bar: (a and b) 50 μm.***

A% and F% were not different in rats of different age groups. B% significantly decreased by 1.4 times (p=0.035) in the 12moAllo group in comparison to the 3moAllo group. At the same time, F% in the 12moAllo rats decreased by 3.2 times (p<0.0001) compared with the 12moE group (Figure 4, a–c).

Twenty-eight days after the surgery, the allograft remained in animals of both age groups. The new bone formed directly on the surface of the allograft. Aside from the bone trabeculae, fibrous tissue of different degrees of maturity, blood capillaries, undifferentiated cells and fibroblasts were discovered in the inner region of the allograft (Figure 2b). In some places, osteoclasts could be found on the surface of the allograft. A% did not differ between groups or from the value 14 days after the surgery. B% and F% did not differ between groups of different ages (Figure 4, a–c).

Ninety days after the surgery, A% decreased by 1.5 times (p=0.007) in the 3moAllo group and by 1.7 times (p=0.038) in the 12moAllo group compared with the values 14 days after surgery. In the 3moAllo group, fibrous tissue was practically nonexistent in the defect, while in the 12moAllo group F% decreased by 2.2 times (p=0.005) compared with the value 14 days after the surgery (Figure 4). A thin cortex formed in rats of the 3moAllo group, while in the 12moAllo group, fibrous tissue was also found in the defect of the cortex (Figure 2b).

Discussions

In the conducted study, the relationship between age (three and 12 months at the beginning of the experiment) and the incorporation and remodeling of structural allografts was analyzed in rats. Critical size defects (3 mm depth, 3 mm diameter) in the distal femoral metaphysis were chosen as a model. Long bone metaphyseal defects of similar size in rats are used to evaluate bone substitute biomaterials [15–17]. A defect of critical size is defined as the smallest defect that does not heal by itself during the life of the animal or the duration of the experiment [18]. For the distal metaphysis of a rat's femur, the minimal size of a critical defect was determined to be 2.5 mm in diameter and depth [1]. When the diameter was 3.5 mm and the depth was 4 mm, stabilization using a plate was necessary [19]. We chose a diameter of 3 mm and a depth of 3 mm, so that we would not need additional fixation, but the defect would be larger than the minimum size of a critical defect [1].

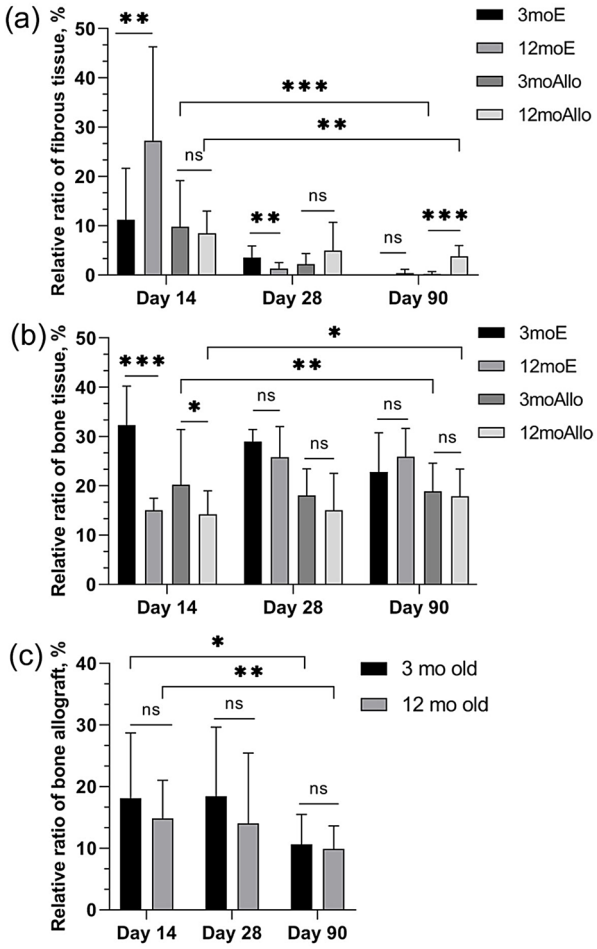

Figure 4 – ***The relative percentages of newly formed tissues and allografts in the metaphyseal femoral defects 14, 28 and 90 days after surgery in 3-month-old and 12-month-old rats: (a) Fibrous tissue; (b) Bone tissue; (c) Allografts. The data is presented as mean ± SD. Unpaired t-tests were used to compare the groups of different ages (3-month-old and 12-month-old). ANOVA with post hoc Bonferroni's correction was used to compare the values between the observation periods (14, 28 and 90 days). *p<0.05; **p<0.01; ***p<0.001; ns: Not significant. 3moE: 3-month-old, empty defect; 12moE: 12-month-old, empty defect; 3moAllo: 3-month-old, allograft implantation; 12moAllo: 12-month-old, allograft implantation. ANOVA: Analysis of variance; SD: Standard deviation.***

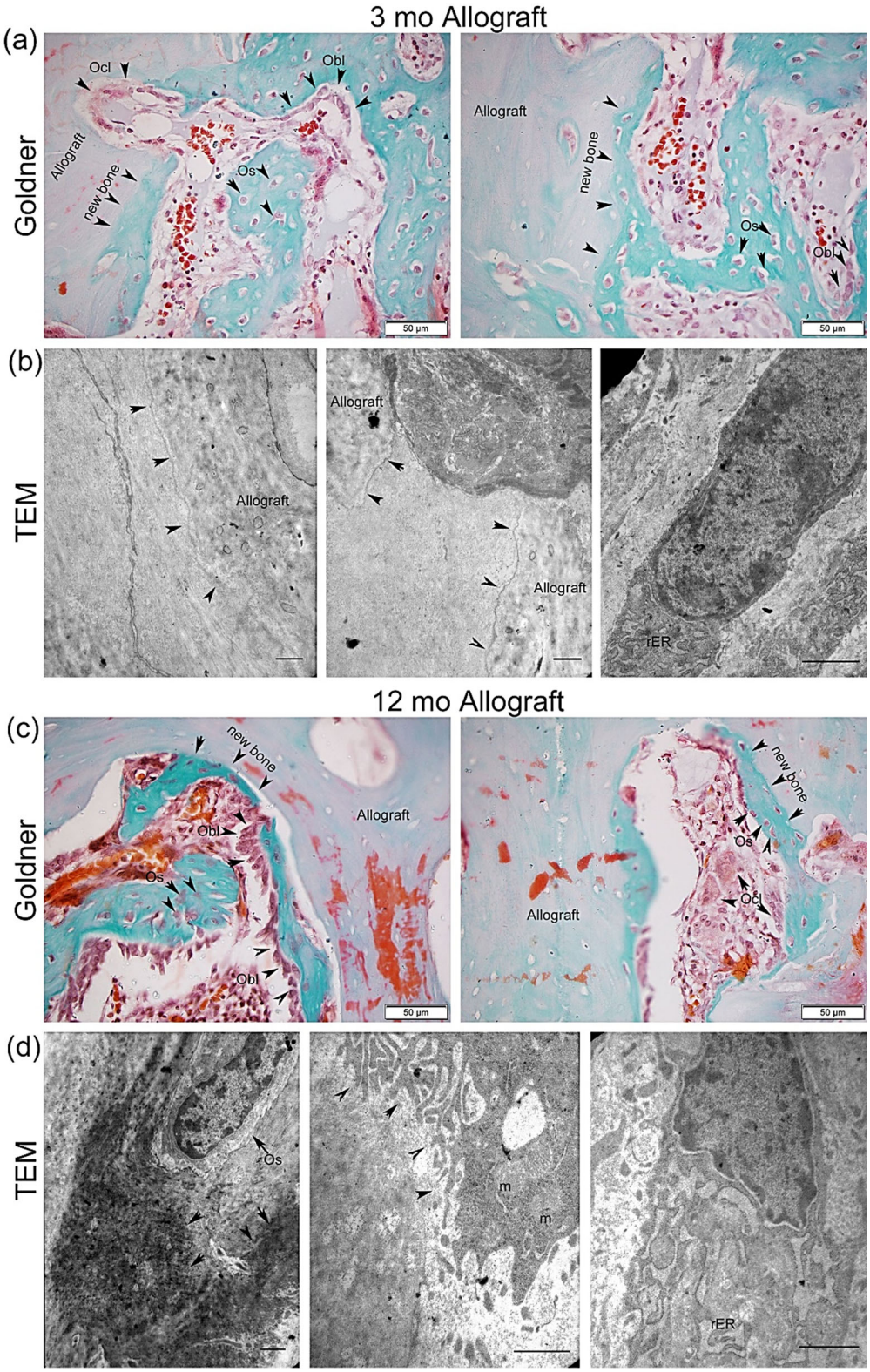

Figure 5 – ***The bone formation, incorporation and remodeling of allografts 14 days after surgery in 3-month-old (3 mo) and 12-month-old (12 mo) rats: (a) Representative Goldner's trichrome staining shows that the new bone forms directly on the surface of the allograft (arrows) in 3 mo and (c) 12 mo rats; numerous osteoblasts (Obl) on the surface of newly formed bone trabeculae, resorption of the allograft by osteoclasts (Ocl) and osteocytes (Os) in the new bone matrix can be seen; (b and d) TEM was used to illustrate the features of the allograft matrix and the absence of differences in the structure of Os, Obl, and Ocl in animals of both age groups; (b) In the two figures on the left, the arrows show the region of contact between the allograft and the new bone; on the right, the Obl with the nucleoli in its nucleus and the rough endoplasmic reticulum (rER) in its cytoplasm is shown; (d) In the first figure on the left, the arrows indicate an area with a probability of increased mineralization near the Os in the new bone; an Ocl with mitochondria (m) in the cytoplasm and a ruffled border is shown in the center; an Obl is shown to the right. Scale bar: (a and c) 50 µm; (b and d) 1 µm. TEM: Transmission electron microscopy.***

The focus of the study was on the formation of fibrous and bone tissue in the defect. It was determined that, 90 days after the creation of an unfilled defect, lamellar bone was formed in animals of both age groups, although the anatomic structure of the distal femoral metaphysis was disturbed, which was more evident in 12-month-old rats. It is probable this is connected to their slower rate of bone tissue formation 14 days after the surgery: B% was 2.4 times (p=0.0001) smaller in comparison with the 3-month-old group. F%, on the other hand, was 2.1 times larger (p=0.005). Twenty-eight days after the surgery, formation of fibrous tissues in the defect of the cortex was found in 12-month-old rats. In those conditions, putting load on the extremity can lead to instability and further deformation of the injured region [20], which was evident 90 days after the surgery. In clinical practice, this may not only limit the load bearing of the extremity, but also delay the beginning of rehabilitation for the patients.

When the defect is filled with a structural allograft, the anatomic structure of the distal femoral metaphysis was maintained 90 days after the surgery in rats of both age groups, despite the significant difference in B% between the groups [1.4 times (p=0.035) smaller in the 12-month-old rats]. This could be explained by the fact that the allograft provided mechanical integrity in the early stages (14 days) after the surgery [21].

The speed of the allograft remodeling did not depend on the age of the recipient: the size of the allograft decreased significantly in the period from 14 to 90 days by 1.7 times (p=0.038) in 3-month-old rats and by 1.5 times (p=0.007) in 12-month-old rats. About 10% of the allograft's original size was evident in the defect at the end of the experiment (90 days). The analysis of the allograft remodeling in patients was based mostly on radiographic methods, and does not paint the full picture, as fibrous tissue is not radiopaque [22]. According to histological studies of patient tissue, incorporation of structural allografts results in their incomplete remodeling and fibrous tissue formation [22–24]. Some authors believe that the formation of fibrous tissue on the surface of the allograft prevents it from remodeling fully [8]. However, others presume that the ingrowing of the fibrous tissue into the allograft allows for increased mechanical integrity. In an experimental study, an increase in the integrity of a cancellous allograft due to the ingrowth of fibrous tissue was found four weeks after filling a 2.7 mm defect in the tibial metaphysis of rats [25]. We used the same type of allograft and also observed fibrous tissue ingrowth over 14 and 28 days. The decrease in bone volume and number of trabeculae in older rats [26] could have led to the formation of a larger area of fibrous tissue in 12-month-old rats 90 days after the surgery because it was necessary to reinforce the allograft. In rats who were subjected to ovariectomy to model osteoporosis, the formation of fibrous tissue in the defect filled with an allograft was detected after 21 days [17], although the authors did not conduct the experiment further.

Another potential reason for the formation of fibrous tissue in 12-month-old rats is that the number of osteoprogenitor cells decreases with age [27]. According to a histological analysis of the biopsy from the iliac crests, the number of osteoprogenitor cells is lower in patients over 65 years of age than in patients 25–39 years of age. This is caused by the fibrosis of the osteogenic layer of the periosteum and the prevalence of adipocytes in the intertrabecular spaces [28]. Based on these findings, the increased amount of fibrous tissue in 12-month-old rats 90 days after surgery could be linked with the loss of osteoinductive properties by the allografts used in the study, because of their preliminary treatment [8, 29].

It was histologically determined that the most active incorporation and remodeling of the structural cancellous allograft in the acetabulum occurs in the first week after the allograft is implanted. After that, the unremodeled part of the allograft remains for over 10 years. This postmortem study was conducted using the bones of patients more than 65 years of age, who previously underwent a revision total hip arthroplasty [30]. Incorporation and remodeling of an implant in younger patients is less studied. In our study, remnants of the allograft (10%) were also found 90 days after surgery in rats of both age groups.

The results of the TEM regarding the remodeling of the allograft in 12-month-old rats with the formation of hypermineralized areas match with the current knowledge about the predisposition of older bone to micropetrosis [30]. Cells of the newly formed bone in rats of both age groups had a normal structure 14 days after the surgery. The smaller area of bone tissue found in 12-month-old rats in comparison with 3-month-old rats can be explained by the decrease in the functional activity of osteoblasts in cancellous bone due to age [6].

A limit of our study was that it involved rats of only two ages – three and 12 months. It is possible that the inclusion of older animals (*e.g.*, 18 months old) would demonstrate more differences in the incorporation and remodeling of the allograft and the formation of new bone.

Conclusions

It was determined that the delayed bone regeneration, which occurred at early stages in unfilled critical size defects created in the rats' distal femoral metaphysis, caused irregularities in its anatomic structure 90 days after surgery. These irregularities were more evident in 12-month-old rats. Using allografts to fill critical size metaphyseal defects facilitates the formation of bone tissue and the maintenance of the normal anatomic structure of the distal femoral metaphysis. No age-related differences were found in the time required for the allograft to remodel and the structure of the newly formed bone.

Conflict of interests

The authors declare no conflict of interests in association with the present study.

Acknowledgments

The authors are grateful to Kateryna Samoilova and Oksana Maiboroda, who provided technical support in the preparation of the allografts; Mykhaylo Ivashchenko, who proofread the manuscript.

References

[1] Tao ZS, Wu XJ, Zhou WS, Wu XJ, Liao W, Yang M, Xu HG, Yang L. Local administration of aspirin with β-tricalcium phosphate/poly-lactic-*co*-glycolic acid (β-TCP/PLGA) could enhance osteoporotic bone regeneration. J Bone Miner Metab, 2019, 37(6):1026–1035. https://doi.org/10.1007/s00774-019-01008-w PMID: 31076895

[2] Antonova E, Le TK, Burge R, Mershon J. Tibia shaft fractures: costly burden of nonunions. BMC Musculoskelet Disord, 2013, 14:42. https://doi.org/10.1186/1471-2474-14-42 PMID: 23351958 PMCID: PMC3573940

[3] Popsuishapka O, Litvishko V, Uzhegova O, Pidgaiska O. Frequency of complications at shaft fractures according to Kharkiv traumatological Medical-Social Expert Committee (MSEC) data. Orthop Traumatol Prosthet, 2020, (1):20–25. https://doi.org/10.15674/0030-59872020120-25 http://otp-journal.com.ua/article/view/200786
[4] United Nations (UN). World population prospects: the 2017 revision. UN Department of Economic and Social Affairs (DESA), 2017 [online], accessed: March 19, 2021. https://www.un.org/development/desa/publications/world-population-prospects-the-2017-revision.html
[5] Javaheri B, Pitsillides AA. Aging and mechanoadaptive responsiveness of bone. Curr Osteoporos Rep, 2019, 17(6): 560–569. https://doi.org/10.1007/s11914-019-00553-7 PMID: 31760583 PMCID: PMC6944648
[6] Becerikli M, Jaurich H, Schira J, Schulte M, Döbele C, Wallner C, Abraham S, Wagner JM, Dadras M, Kneser U, Lehnhardt M, Behr B. Age-dependent alterations in osteoblast and osteoclast activity in human cancellous bone. J Cell Mol Med, 2017, 21(11):2773–2781. https://doi.org/10.1111/jcmm.13192 PMID: 28444839 PMCID: PMC5661248
[7] Gibon E, Lu L, Goodman SB. Aging, inflammation, stem cells, and bone healing. Stem Cell Res Ther, 2016, 7(1):44. https://doi.org/10.1186/s13287-016-0300-9 PMID: 27006071 PMCID: PMC4804630
[8] Roberts TT, Rosenbaum AJ. Bone grafts, bone substitutes and orthobiologics the bridge between basic science and clinical advancements in fracture healing. Organogenesis, 2012, 8(4): 114–124. https://doi.org/10.4161/org.23306 PMID: 23247591 PMCID: PMC3562252
[9] Baldwin P, Li DJ, Auston DA, Mir HS, Yoon RS, Koval KJ. Autograft, allograft, and bone graft substitutes: clinical evidence and indications for use in the setting of orthopaedic trauma surgery. J Orthop Trauma, 2019, 33(4):203–213. https://doi.org/10.1097/BOT.0000000000001420 PMID: 30633080
[10] ***. Bone graft and substitutes market size, share & trends analysis report by material type (allograft, synthetic), by application (craniomaxillofacial, dental, foot & ankle), by region, and segment forecasts, 2021–2028. Grand View Research [online], San Francisco, CA, USA, accessed: March 19, 2021. https://www.grandviewresearch.com/industry-analysis/bone-grafts-substitutes-market
[11] Abjornson C, Brecevich A, Callanan T, Dowe C, Cammisa FP, Lorio MP. ISASS recommendations and coverage criteria for bone graft substitutes used in spinal surgery. Int J Spine Surg, 2018, 12(6):757–771. https://doi.org/10.14444/5095 PMID: 30619681 PMCID: PMC6314336
[12] ***. On the protection of animals from brutal treatment. Verkhovna Rada of Ukraine [online], revision: February 13, 2020, accessed: January 20, 2021 (in Ukrainian). https://zakon.rada.gov.ua/laws/show/en/3447-15/ed20200213#Text
[13] Korzh MO, Vorontsov PM, Slota OM, Husak VS, Vorontsova MP. Method of manufacturing implantation dehydrated bone biomaterial of allogeneic origin [online]. Patent of Ukraine No. 119699, 2019, accessed: April 6, 2021 (in Ukrainian). https://base.uipv.org/searchINV/search.php?action=viewdetails&IdClaim=260351&chapter=biblio
[14] Bumbu BA, Bumbu A, Rus V, Gal AF, Miclăuş V. Histological evidence concerning the osseointegration of titanium implants in the fractured rabbit femur. J Histotechnol, 2016, 39(2):47–52. https://doi.org/10.1080/01478885.2016.1144842 https://www.tandfonline.com/doi/full/10.1080/01478885.2016.1144842
[15] Tielinen L, Manninen M, Puolakkainen P, Kellomäki M, Törmälä P, Rich J, Seppälä J, Rokkanen P. Inability of transforming growth factor-β1, combined with a bioabsorbable polymer paste, to promote healing of bone defects in the rat distal femur. Arch Orthop Trauma Surg, 2001, 121(4):191–196. https://doi.org/10.1007/s004020000206 PMID: 11317678
[16] Roshan-Ghias A, Lambers FM, Gholam-Rezaee M, Müller R, Pioletti DP. *In vivo* loading increases mechanical properties of scaffold by affecting bone formation and bone resorption rates. Bone, 2011, 49(6):1357–1364. https://doi.org/10.1016/j.bone.2011.09.040 PMID: 21958844
[17] Dreyer CH, Rasmussen M, Pedersen RH, Overgaard S, Ding M. Comparisons of efficacy between autograft and allograft on defect repair *in vivo* in normal and osteoporotic rats. Biomed Res Int, 2020, 2020:9358989. https://doi.org/10.1155/2020/9358989 PMID: 32190690 PMCID: PMC7073494
[18] Poser L, Matthys R, Schawalder P, Pearce S, Alini M, Zeiter S. A standardized critical size defect model in normal and osteoporotic rats to evaluate bone tissue engineered constructs. Biomed Res Int, 2014, 2014:348635. https://doi.org/10.1155/2014/348635 PMID: 24738053 PMCID: PMC3967594
[19] Thormann U, Ray S, Sommer U, Elkhassawna T, Rehling T, Hundgeburth M, Henß A, Rohnke M, Janek J, Lips KS, Heiss C, Schlewitz G, Szalay G, Schumacher M, Gelinsky M, Schnettler R, Alt V. Bone formation induced by strontium modified calcium phosphate cement in critical-size metaphyseal fracture defects in ovariectomized rats. Biomaterials, 2013, 34(34):8589–8598. https://doi.org/10.1016/j.biomaterials.2013.07.036 PMID: 23906515
[20] Glatt V, Evans CH, Tetsworth K. A concert between biology and biomechanics: the influence of the mechanical environment on bone healing. Front Physiol, 2017, 7:678. https://doi.org/10.3389/fphys.2016.00678 PMID: 28174539 PMCID: PMC5258734
[21] Foster AL, Moriarty TF, Zalavras C, Morgenstern M, Jaiprakash A, Crawford R, Burch MA, Boot W, Tetsworth K, Miclau T, Ochsner P, Schuetz MA, Richards RG, Metsemakers WJ. The influence of biomechanical stability on bone healing and fracture-related infection: the legacy of Stephan Perren. Injury, 2021, 52(1):43–52. https://doi.org/10.1016/j.injury.2020.06.044 PMID: 32620328
[22] Rolvien T, Barbeck M, Wenisch S, Amling M, Krause M. Cellular mechanisms responsible for success and failure of bone substitute materials. Int J Mol Sci, 2018, 19(10):2893. https://doi.org/10.3390/ijms19102893 PMID: 30249051 PMCID: PMC6213546
[23] van der Donk S, Buma P, Slooff TJJH, Gardeniers JWM, Schreurs BW. Incorporation of morselized bone grafts: a study of 24 acetabular biopsy specimens. Clin Orthop Relat Res, 2002, (396):131–141. https://doi.org/10.1097/00003086-200203000-00022 PMID: 11859236
[24] Hooten JP Jr, Engh CA, Heekin RD, Vinh TN. Structural bulk allografts in acetabular reconstruction. Analysis of two grafts retrieved at post-mortem. J Bone Joint Surg Br, 1996, 78(2): 270–275. PMID: 8666640
[25] Tägil M, Aspenberg P. Fibrous tissue armoring increases the mechanical strength of an impacted bone graft. Acta Orthop Scand, 2001, 72(1):78–82. https://doi.org/10.1080/00016470 1753606743 PMID: 11327419
[26] Pietschmann P, Skalicky M, Kneissel M, Rauner M, Hofbauer G, Stupphann D, Viidik A. Bone structure and metabolism in a rodent model of male senile osteoporosis. Exp Gerontol, 2007, 42(11):1099–1108. https://doi.org/10.1016/j.exger.2007.08.008 PMID: 17949933
[27] Chen TL. Inhibition of growth and differentiation of osteoprogenitors in mouse bone marrow stromal cell cultures by increased donor age and glucocorticoid treatment. Bone, 2004, 35(1):83–95. https://doi.org/10.1016/j.bone.2004.03.019 PMID: 15207744
[28] Giordano V, Franco JS, Koch HA, Labronici PJ, Pires RES, do Amaral NP. Alterações na estrutura óssea relacionadas à idade [Age-related changes in bone architecture]. Rev Col Bras Cir, 2016, 43(4):276–285. https://doi.org/10.1590/0100-69912016004010 https://www.scielo.br/j/rcbc/a/dNFJDmVFntGXM8B5TqRTMmc/?lang=en
[29] Sohn HS, Oh JK. Review of bone graft and bone substitutes with an emphasis on fracture surgeries. Biomater Res, 2019, 23(1):9. https://doi.org/10.1186/s40824-019-0157-y PMID: 30915231 PMCID: PMC6417250
[30] Butscheidt S, Moritz M, Gehrke T, Püschel K, Amling M, Hahn M, Rolvien T. Incorporation and remodeling of structural allografts in acetabular reconstruction: multiscale, micro-morphological analysis of 13 pelvic explants. J Bone Joint Surg Am, 2018, 100(16):1406–1415. https://doi.org/10.2106/JBJS.17.01636 PMID: 30106822 PMCID: PMC6133218

Corresponding author
Nataliya Ashukina, PhD, Head of the Laboratory of Connective Tissue Morphology, Sytenko Institute of Spine and Joint Pathology, National Academy of Medical Sciences of Ukraine, 80 Pushkinskaya Street, 61024 Kharkiv, Ukraine; Phone +380667590789, e-mail: nataliya.ashukina@gmail.com

Received: June 29, 2021 *Accepted: October 25, 2022*