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Medical Sciences

Peculiarities of the Healing of the Defect of Diaphyses of a Long Bone of the Skeleton after the Implantation of -Tricalcium Phosphate of Different Microscopic Structure and Geometry

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ABSTRACT. **The aim of this work is to compare the healing process of the defect of compact bone tissue after the implantation of osteoplastic materials based on -tricalcium phosphate, which differ by manufacturer, geometrical shape and microscopic structure. The experiment was held on 48 white Wistar rats. In the middle third of the diaphysis of the femur we reproduced the perforated defect in diameter of 2.5 mm to the medullary canal, which in the animals of the first group was filled with the osteoplastic material «ChronOS™» (block), and in the animals of the second group - with «Calc-i-oss®» (granules). Fragments of the injured bones were studied on the 15th and 30th day by the methods of light microscopy with morphometry and by scanning electron microscopy. It was found that regardless of the geometric shape and the microscopic structure, -tricalcium phosphate shows high biocompatibility, osteoconductive properties, good integration with bone tissue of the regenerate and almost equal rate of resorption and replacement by the bone tissue. However, the microscopic structure of -tricalcium phosphate («ChronOS™») significantly affects the microscopic structure of bone tissue of the regenerate, which manifests itself in the specificity of its geometric shape.** *© 2017 Bull. Georg. Natl. Acad. Sci.*

Key words: rats, bone, β-tricalcium phosphate, reparative osteogenesis

Because of the similarity of the chemical structure with the bone tissue and biological inertness, osteoplastic materials based on β-tricalcium phosphate are widely used for optimization of reparative osteogenesis. β-tricalcium phosphate, in the form of granules, blocks or cylinders, demonstrated excellent characteristics for the replacement of bone defects in traumatology, spinal surgery and dentistry [1, 2]. Today, many promotional publications about osteoplastic materials based on β-tricalcium phosphate prove their safety and biocompatibility. However, the information about other determining properties of β-tricalcium phosphate, such as dynamics of the rate of its biodegradation, replacement by bone tissue of the regenerate for certain commercial drugs, is completely absent, but for others there is a significant divergence in data [3 -5]. However, it is clear that such divergence is probably influenced by many factors. For example, the size of the defect, bone tissue regeneration potential, the characteristics of the osteoplastic material (manufacturer, geometric shape, total porosity, pore size, design, size, tissues (blood, bone marrow) or liquids (saline solution) for impregnation before implantation and so on) [6, 7]. All this indicates that for a more predictable dynamics of rate of formation of bone tissue of regenerate and resorption of osteoplastic materials, which differ by the manufacturer, geometric shape and microscopic structure we need experimental models of bone defects, on which the osteoplastic materials that optimize reparative osteogenesis could be studied and compared with each other under standard conditions and by various methods of investigation. It should also be noted that the majority of works devoted to the morphological research of β-tricalcium phosphate were conducted on the bones of the skull and cancellous bones [8, 9]. However, the information on studies of the comparative impact of different by the manufacturer, geometrical shape and microscopic structure β-tricalcium phosphate on the dynamics of healing of the compact bone tissue defect was not found in the scientific literature. In addition, in the morphological studies of bone regeneration process after implantation into its defect of the overwhelming amount of osteoplastic materials based on β-tricalcium phosphate, the electron microscopic characteristics of the bone tissue of the regenerate are absent [10, 11]. Therefore, the aim of our study was to compare the healing process of experimental defect of the compact bone tissue after the implantation of osteoplastic materials based on β-tricalcium phosphate, which vary by manufacturer, geometrical shape and microscopic structure using histological, morphometric and electron microscopic techniques.

Materials and Methods

The experiment was performed on 48 white Wistar rats eight months of age with the weight of 250±10 g. All procedures were agreed with the Commission on Biomedical Ethics of Sumy State University (Minutes № 4/14 of 06.11.2015). The study protocol was done according to the provisions «European Community Directive of 24 November 1986 on the maintenance and use of laboratory animals for research purposes». Before surgery, animals were initially injected with 2.5 mg/kg of acepromazine intramuscular and in 5 minutes 75 mg/kg of ketamine intramuscular (Calypsol, Gedeon Richter, Budapest-Hungary). After the induction of the animals in anesthesia, a defect of the medullary canal with diameter of 2.5 mm was reproduced under aseptic conditions in the middle third of the femoral diaphysis using a portable drill with a spherical cutter at low speed with cooling. Further, the experimental animals were divided into 2 groups:

Group 1 (24 rats), where the defect without rigid fixation was filled with the osteoplastic material «ChronOS™» (Synthes, Switzerland), which is a pure β-tricalcium phosphate in the form of block with a total porosity of 70%, with the macropore size from 100 to 500 microns and micropores to 10 microns (Fig. 1);

Group 2 (24 rats), where the defect without rigid fixation was filled with the osteoplastic material «Calc-i-oss®» («Degradable Solutions Dental», Switzerland), which is a synthetic granular material (1-1.6 mm), which is made of pure β-tricalcium phosphate (β-phase purity of > 99%, Ca / P - 1.5) with a total porosity of 50% and the size of micro pores is from 1 to 6 μm (Fig. 2).

Before the implantation the blocks of «ChronOS™» and the granules of «Calc-i-oss®» were moistened with the rat's own blood (which was taken from the tail vein) to fill pores, remove residual air from the material and ensure the necessary consistency which would permit easy cutting of the materials by scalpel and thus modeling the shape of the defect.

After entering the bone defect of osteoplastic material the wound was tightly stitched with silk thread through all the layers of soft cover, the seam was treated with 3% alcohol solution of iodine. Then, during the next 3 days after operation for prevention

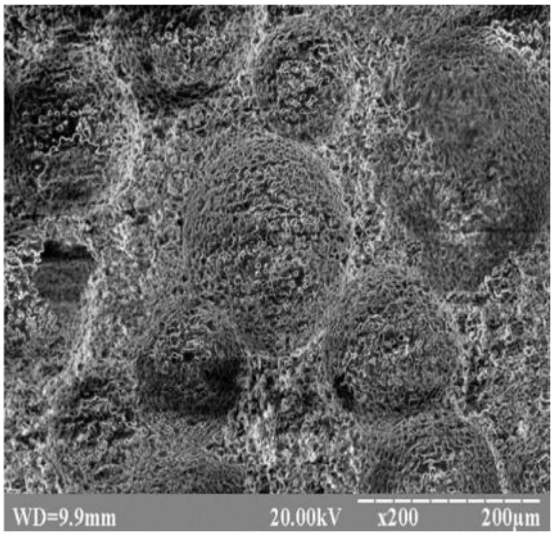

Fig. 1. Microstructure of the osteoplastic material «ChronOS™». Visible numerous pores. Electronic scanning image. X 100.

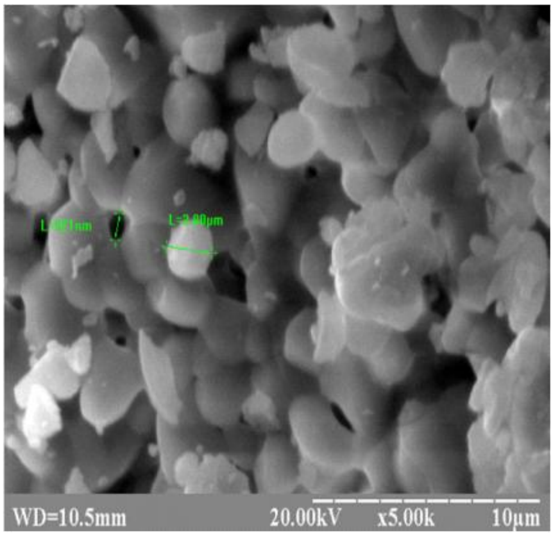

Fig. 2. Microstructure of the osteoplastic material «Calc-i-oss®». Visible crystals of the mineral in size of 2 microns and micro pores between them in size of 921 nm and above. Electronic scanning image. X 1000.

of septic complications the after-operation seam was treated with an alcohol solution of iodine and for analgesia ketorolac was injected intramuscularly at a dose of 0.6 mg 2 times a day.

Next on the 15th and 30th day after surgery animals were taken out of the experiment by decapitation under deep ether anesthesia, followed by a study of injured bones using light microscopy with morphometry and scanning electron microscopy.

For light microscopy, we extracted the fragments of femoral bones from the site of implantation of osteoplastic material and fixed them in 10 % solution of neutral formalin. After washing with water, the bone samples were subjected to decalcification in 5 % aqueous solution of Trilon B (Edetic acid), dehydrated in alcohols of increasing concentration and poured into paraffin. Histological sections were made at Sannomiya microtome «Reichert», stained with hematoxylin-eosin, analyzed at the light microscope «OLIMPUS» and photographed by digital camera.

Morphometric analysis consisted in identifying in the site of the defect of the area of bone and connective tissues, and remnants of osteoplastic material which was performed using the program for image processing «Video-Test» and «Video-Size».

For scanning electron microscopy we extracted the fragments of the femur from implanted osteoplastic material and placed samples in glutaraldehyde holder. In one day, the samples were washed in phosphate buffer, fixed in 1% OsO_4 solution and dehydrated in ethanol of increasing concentrations. Further the bone fragments were glued on metal tables with electricity conductive adhesive, sprayed with carbon dust in standard vacuum installation of VUP-5 type and examined with an electron microscope «SEM 106-I».

Using light and scanning electron microscopy we established morphological characteristics of newly formed tissue-specific structures of the regenerate, the nature of their interaction with osteoplastic material «ChronOS™» and «Calc-i-oss®». In addition, by using these methods, we investigated the state of the structure of adjacent to the site of implantation maternal bone in order to establish or refute postoperative complications due to the presence or absence of signs of necrobiosis and necrosis of osteocytes. The resulting digital values were treated statistically by calculating the arithmetic mean (M) and its standard error (m). The significance of differences between the indicators of the animals of the first and second

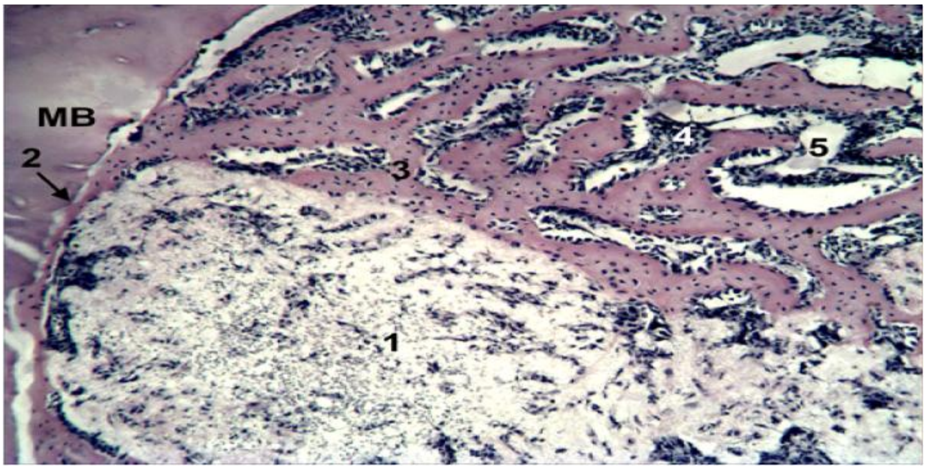

Fig. 3. The area of the defect of femur of a rat on the 15th day after the implantation of «Calc-i-oss®». A large fragment of osteoplastic material (1) with the foci of fibrogenesis, which is separated from the maternal bone (MB) by a thin layer of the bone tissue (2). In the inter-gully space of the bone tissue of the regenerate (3) there is the connective tissue (4) and small remnants of «Calc-i-oss®» (5). Haematoxylin and Eosin staining. X 100.

groups, of the 15th and the 30th days was evaluated using Student t-test with the use of statistical computer program MS Excel XP. The differences were considered significant at $p<0.05$.

Results

On the 15th day of the experiment in the defect area of the animals of the first and second groups between the osteoplastic materials and the mother bone there was a thin layer of connective and rough-fiber bone tissues, which was in close contact with the implants. Here, the osteogenic cells formed the connective and bone tissue of the regenerate not only on the outer surface of the osteoplastic materials but also inside their peripheral and central sections. In the animals of the second group in the defect area there were places where between large and small fragments of granules of «Calc-i-oss®» and bone tissue of regenerate there was a thin layer of connective tissue, as well as places of direct contact of the bone tissue with the osteoplastic material. The bone tissue of the regenerate in the animals of the first group occupied 41.11±1.87%, and in the animals of the second – 47.32±2.29% of the total area of the defect ($p<0.05$). In the animals of the second group the bone tissue of the regenerate was presented by the bone trabeculae, containing in its composition a large number of osteoblasts, osteocytes and integrated fragments of the osteoplastic material «Calc-i-oss®». Bone trabeculae formed small- and large-looped mash structures, in the inter-trabecular spaces of which there were located the remains of the osteoplastic material and the connective tissue of the regenerate (Fig. 3).

The bone tissue in the animals of the first group, which was formed on the outer surface of «ChronOS™», was also presented by mesh works of the bone trabeculae. However, the bone tissue, which was formed inside of the osteoplastic material «ChronOS™» by shape and size repeated the pores of the implant, had specific rounded shape and contained numerous osteoblasts and osteocytes. At the same time, in some places the pores of the ceramic material of rounded form were completely filled with the bone tissue, in the others it occupied only the peripheral sections, and in the central part of the pore there was located osteoid or connective tissue. In the composition of the osteoid there were found osteogenic cells, preosteoblasts/osteoblasts, as well as collagen fibers, which germinated to the micropores of the implant. Osteogenic cells had round, oval or elongated shape and size from 4 to 8 microns, were located haphazardly, not having regular interrelation (Fig. 4).

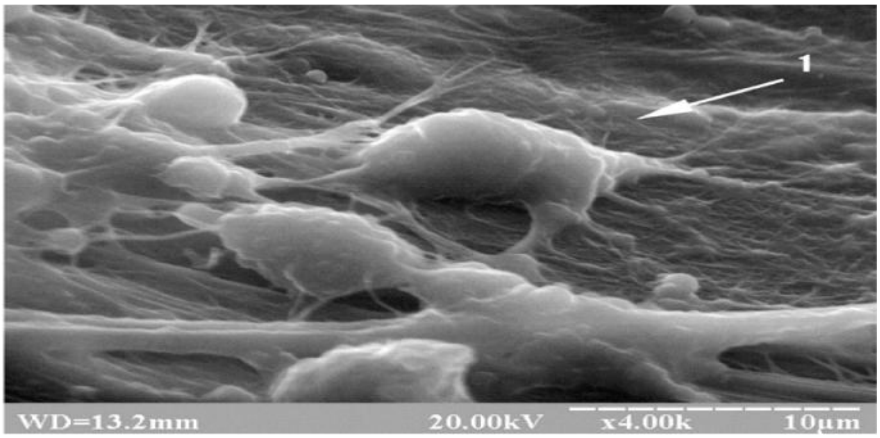

Fig. 4. The area of the defect of femur of a rat on the 15th day after the implantation of «ChronOS™». Osteogeni cells on the surface of osteoid tissue, which is located in the central part of the pore of osteoplastic material. Electronic scanning image. X 4000.

Connective tissue of the regenerate was built of the fibroblasts, collagen fibers and blood vessels, contained in the inter-trabecular spaces of the bone tissue of the regenerate directly on the outer surface and inside of the osteoplastic materials and occupied 19.14±1,2% of the total area of the defect in the animals of the first and 19.11±0.95% –in the animals of the second group ($p>0.05$). Osteoplastic materials «ChronOS™» and «Calc-i-oss®» on the histo-preparations, stained with hematoxylin and eosin, had light (gray) color, occupied 39.75±1.55% and 33.57±1.53% of the total area of the defect ($p<0.05$). The signs of aseptic inflammation in the area of the defect were not detected, and the adjacent to the site of implantation of osteoplastic materials maternal bone contained typical osteocytes, the bodies of which were located in the bone lacunae and the spikes in bone tubules.

On the 30th day of the experiment in the animals of the first and second groups the most part of the osteoplastic materials «ChronOS™» and «Calc-i-oss®» was located in the central area of the defect. Inside their remnants, as on the 15th day of the experiment, there were osteogenic cells with the foci of fibro- and osteogenesis, sometimes there occur also osteoclasts. In the area of the defect of the animals of the second group the bone tissue of lamellar and rough fibrous structure in the peripheral parts of the defect contained integrated into its structures small fragments of «Calc-i-oss®», and in the inter-gully spaces – the remains of osteoplastic material together with the connective tissue. Large fragments of «Calc-i-oss®» were located in the central parts of the defect, on the surface of which there was formed not only the bone tissue but also the connective tissue of the regenerate. Unlike them, in the animals of the first group in the peripheral parts of the defect the bone tissue was represented by solid margins without remains of the implant and had mostly lamellar structure.In the central parts of the defect the bone tissue of the regenerate according to the pores of ceramic material had a look of specific rounded formations, between which there were the remains of osteoplastic material and a small amount of connective tissue (Fig. 5).

In the composition of the bone tissue of the regenerate of the animals of the first and the second groups there occurred a significant amount of osteoblasts and osteocytes. The latter, in size of about 10-15 microns, were located in the bone lacunae and had short and long spikes (Fig. 6).

The area of the bone tissue compared with the 15th day of the experiment in the animals of the first and second group increased by 32.67% ($p<0.05$) and 21.76% ($p<0.05$) and was 54.53±2.6 % in the first and

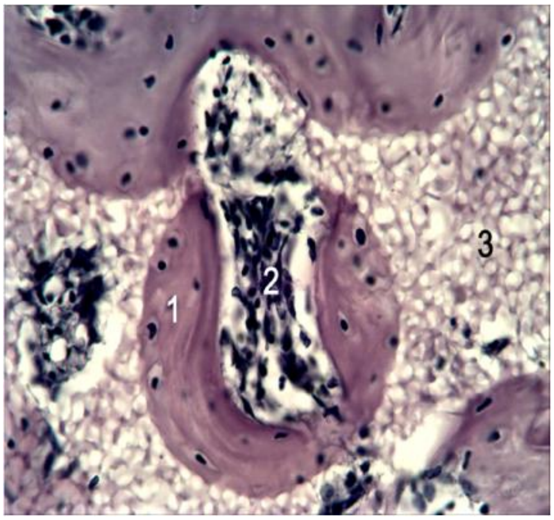

Fig. 5. The area of the defect of femur of a rat on the 30th day after the implantation of «ChronOS™». To the bone tissue of theregenerate of specific rounded shape (1) from the inner part of the pore of the implant the connective tissue (2) is adjacent, and from the outside the remnants of the osteoplastic material (3). Haematoxylin and Eosin staining. X 400.

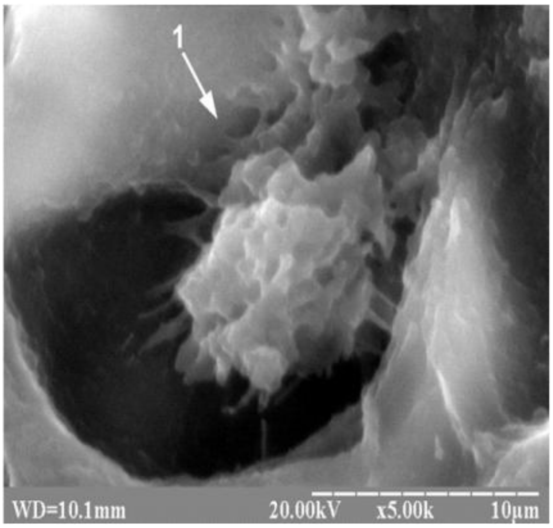

Fig. 6. The area of the defect of femur of a rat on the 30th day after the implantation of «Calc-i-oss®». Osteocyte (1) in the composition of rough-fiber bone tissue, which was formed on the surface of osteoplastic material. Electronic scanning image. X 5000.

57.62±1.64% – in the second case (p>0.05).The connective tissue in the animals of the first and the second group was built of the fibroblasts, collagen fibers, blood vessels and was directly in contact with the formed bone tissue and osteoplastic materials. The area of the connective tissue compared to the 15th day of the experiment in the animals of the first and the second group decreased by 1.77% (p>0.05) and 25.48% (p<0.05), and of the osteoplastic material by 32.92% (p<0.05) and 16,2% (p<0.05) and amounted to 18.8±0.8 and 14.24±1.08% (p<0.05) in the first and 26.67±1.15 % and 28,13±1.27% (p>0.05) – in the second case. It should also be noted that on the 30th day of the experiment, as in the previous term of supervision in the area of implantation of osteoplastic materials «ChronOS™» and «Calc-i-oss®» the signs of inflammatory response were absent, and in the adjacent to the site of implantation maternal bone there were lacunae with typical osteocytes.

Discussion

The conducted study with the help of light and scanning electron microscopy showed that the dynamics of the healing of the defect of the femoral shaft diaphysis had both similarities and differences depending on implanted into its cavity calcium phosphate osteoplastic material.

To common features we can refer those that osteoplastic materials «ChronOS™» and «Calc-i-oss®» during the whole period of the experiment demonstrated their high biocompatibility, as evidenced by the absence the inflammatory process in the site of the defect, of necrosis and necrobiosis of osteocytes in the composition of the maternal bone.These results are consistent with the data of most researchers [3, 4]. Furthermore, in all the periods of observation there was found high tropism of osteogenic cells to osteoplastic materials, as evidenced by their location and formation of foci of fibro- osteogenesis, both on the outer surface and inside «ChronOS™» and «Calc-i-oss®». It should be noted that the established fact is also the evidence of good integration of tissue-specific structures of the regenerate with osteoplastic materials and manifestation of their osteoconductive properties. This is connected with the fact that one of the definitions of osteoconduction is the ability of osteogenic cells to use osteoplastic material as a platform for attaching and generating on the surface and in its cavities of tissue-specific structures of the regenerate [12].

With the help of light and scanning electron microscopy there was found another common feature of the implanted calcium phosphate osteoplastic materials «ChronOS™» and «Calc-i-oss®». Thus, in the area of their implantation at all stages of observation there were detected the signs of only desmalosteogenesis, which was evidenced by neoplasm in the cavity of the defect the bone and connective tissues and absence of cartilaginous.

One of the most important properties of calcium phosphate osteoplastic materials is their ability to resorption and replacement by the tissue-specific structures of the regenerate. In the literature there are works on the impact of β-tricalcium phosphate on healing of the bone defects. But such studies were conducted on the bones of the skull and cancellous bone, and the results of these studies either lack the data of morphometric parameters or have significant. For example, Eftekhari H. et al without noting the morphological indicators in its study, observed the resorption of β-tricalcium phosphate and formation of connective and mostly bone tissue of the regenerate on the surface and inside the granules of the osteoplastic material on the 30th day after its implantation into the defect of the epiphysis of the femurs of rats. [5]. Though, Berchenko H. N. et al found the formation of bone trabeculae on the surface of β-tricalcium phosphate granules only in certain areas, but mostly there occurred active tumors on the surface and inside of the implant of friable connective tissue on the 30th day after its implantation into perforated defect of the tibia of rats [13].

During conducting the research of compact bone tissue defect we also observed gradual resorption of implanted in its cavity osteoplastic materials «ChronOS™» and «Calc-i-oss®» and their replacement by bone tissue of the regenerate. At the same time, morphometric study made it possible to find out the rate of resorption of osteoplastic materials and their replacement by tissue-specific structures of the regenerate depending on the period of observation. Thus, on the 15th day of the experiment, the predominance in rate of resorption of osteoplastic material (by 18.4% ($p<0.05$)) and formation of the bone tissue of the regenerate (by 13.14% ($p<0.05$)) was in the area of implantation of «Calc-i-oss®», and the amount of connective tissue (19.14±1,2% and 19,11±0.95%) in the animals of both groups was similar. However,on the 30th day of the experiment most of morphometric parameters got equal. It was evidenced by the area of osteoplastic materials «ChronOS™» (26.67±1.15 %) and «Calc-i-oss®» (28.13±1.27 %) and the bone tissue of the regenerate (54.53±2.6 % and 57.62±1.64 %), which in the animals of both groups became almost equal. And only the amount of connective tissue in the area of implantation of «Calc-i-oss®» (14.24±1.08%) was significantly lower (by 27.66% ($p<0.05$)) than in the area of implantation of «ChronOS™» (18.18±0.8%).

A vivid distinction between the studied osteoplastic materials became the geometric shape of the bone tissue of the regenerate, which was formed in the area of implantation of «ChronOS™» and «Calc-i-oss®».Thus, in the area of implantation of «Calc-i-oss®» the bone tissue had no significant features, besides of presence of integrated into its structures remnants of the osteoplastic material. Unlike «Calc-i-oss®», in the area of implantation of «ChronOS™» the geometric shape of the bone tissue had a fundamental difference. In all periods of surveillance the bone tissue of the regenerate was mainly represented by specific individual and interrelated rounded formations, which in shape and size were direct reflections of the pockets of osteoplastic material «ChronOS™». This fact indicates that the pores of the implant served as the conductor for the vessels, cellular elements and bone tissue, which in turn is the proof of the osteoconductive impact of the osteoplastic material «ChronOS™» on the reparative osteogenesis. Here, it should be noted that the described and vividly demonstrated structure of the cells of the bone tissue did not depend on which of the calcium-phosphate osteoplastic material was implanted into the defect of the femoral shaft diaphysis.

Conclusion

Therefore, regardless of the geometric shape and microscopic structure of β-tricalcium phosphate exhibits its high biocompatibility, osteoconductive properties, good integration with bone tissue of the regenerate and almost the same speed of resorption and replacement by the bone tissue.However, the microscopic structure of β-tricalcium phosphate («ChronOS™») significantly affects the microscopic structure of bone tissue of the regenerate, which manifests itself in the specificity of its geometric shape.

სამედიცინო მეცნიერებანი

ჩონჩხის გრძელი ძვლის დიაფიზების დეფექტის მკურნალობის თავისებურებები ხვადასხვა მიკროსკოპული სტრუქტურისა და გეომეტრიის - ტრიკალციუმის ფოსფატის იმპლანტაციის შემდეგ

ა. კორენკოვი

სუმის სახელმწიფო უნივერსიტეტი, ადამიანის ანატომიის დეპარტამენტი, სუმი, უკრაინა

(წარმოდგენილია აკადემიის წევრის ნ. მითაგვარიას მიერ)

წინამდებარე ნაშრომის მიზანია კომპაქტური ძვლოვანი ქსოვილის დეფექტის მკურნალობის პროცესის შედარება ოსტეოპლასტიკური მასალის იმპლანტაციის შემდეგ, -ტრიკალციუმის ფოსფატის საფუძველზე, რაც განსხვავდება მწარმოებლის, გეომეტრიული ფორმისა და მიკროსკოპული სტრუქტურის მიხედვით. ცდა ჩატარდა ვისტარის 48 თეთრ ვირთაგვაზე. ბარძაყის დიაფიზის შუა მესამედში წარმოიქმნა 2,5 მმ დიამეტრის პერფორირებული დეფექტი ხერხემლის არხისკენ, რომელიც პირველი ჯგუფის ცხოველებში შეივსო ოსტეოპლასტიკური მასალით "(ChronOS™)" (ბლოკი) და მეორე ჯგუფის ცხოველების შემთხვევაში კი, შეივსო "Calc-i-oss®" (გრანულები) მასალით. დაზიანებული ძვლების ფრაგმენტები შესწავლილ იქნა მე-15 და 30-ე დღეს სინათლის მიკროსკოპის მორფომეტრიით და სკანირებადი ელექტრონული მიკროსკოპიით. აღმოჩნდა, რომ გეომეტრიული ფორმისა და მიკროსკოპული სტრუქტურის მიუხედავად, -ტრიკალციუმის ფოსფატმა აჩვენა მაღალი ბიოთავსებადობა, ოსტეოკონდუქტიურობის თვისებები,

რეგენირებად ძვლის ქსოვილთან კარგი ინტეგრაცია და თითქმის თანაბარი რეზორბცია და ძვლოვანი ქსოვილით ჩანაცვლება. თუმცა, -ტრიკალციუმის ფოსფატის (ChronOS™) მიკროსკოპული სტრუქტურა მნიშვნელოვან გავლენას ახდენს რეგენირებადი ძვლის ქსოვილის მიკროსკოპულ სტრუქტურაზე, რაც ვლინდება მისი გეომეტრიული ფორმის სპეციფიკურობაში.

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