Evaluation of Bone Healing of Artificial Defects in Laboratory Animals After Covering with Alloplastic Material

MANUELA CHIVU[1], CRISTIAN TANTAR[2,3], EMILIAN HUTU[1], RALUCA MONICA COMANEANU[1]*, ELENA RUSU[4]

[1]Titu Maiorescu University, Faculty of Dental Medicine, 67A Gheorghe Petrascu Str., Bucharest, Romania

[2] Lucia Blaga University of Sibiu, Faculty of Medicine, 2A Lucian Blaga Str., 550169, Sibiu, Romania

[3] Sibiu County Emergency Clinical Hospital, Sibiu, 2-4 Corneliu Coposu Blvd., 550245, Sibiu, Romania

[4] Titu Maiorescu University,Faculty of Medicine, 67A Gheorghe Petrascu Str., Bucharest, Romania

Bone defects are commonly seen in clinical practice. They are caused by different types of trauma, infections, congenital malformations and cancers. Current approaches to skeletal reconstructive surgery use biomaterials, autografts or allografts. The aim of this study was to analyze bone repair from histologic point of view. To study the repair of bone defects, we used two batches of Wistar mices (Lat Rattus Norvegicus). The 46 subjects under study were divided into two equal lots. In all subjects, a round defect with a diameter of 5 mm was surgically performed on the right and left parietal bone. In the 23 subjects in group I the defect in the left parietal bone was covered with alloplastic material (Osteoset) and the defect in the right parietal bone was not covered with osteoconductive, osteoinductive or osteogenic materials. Regarding subjects in group study II, none of the surgically created bone defects were covered with alloplastic materials. Euthanasia of the subjects included in the study was performed at 2 and 4 months respectively, at the time of surgery. Euthanasia, bone sampling and assembly for microscopic preparations were done on the same day. The histological analysis of a bone repair shows the direct correlation between the healing process and the addition of alloplastic materials (Osteoset).

Keywords: bone healing, artificial defects, alloplastic material, graft, laboratory animals

Bone defects are commonly seen in clinical practice. They are caused by different types of trauma, infections, congenital malformations, and cancers, and their restoration remains a challenge. Bone repair is the subject of intensive investigation in reconstructive surgery. Current approaches to skeletal reconstructive surgery use biomaterials, autografts or allografts, although restriction on all these techniques exists.

Bone possesses the ability to perform a wide array functions, and bone responds to a variety of metabolic, physical and endocrine stimuli. Bones (a) represent the foundation for our bodily locomotion, (b) provide load-bearing capacity to our skeleton and protection to our internal organs, (c) house the biological elements required for hematopoiesis, (d) trap dangerous metals (i.e., lead), and (e) maintain the homeostasis of key electrolytes via calcium and phosphate ion storage [1].

Bone formation occurs during different pathways, intramembranous and endochondral. In both processes, bone remodeling is required for the maintenance of all normal healthy bone, which involves a balance between osteoclastic bone resorption and osteoblastic bone formation. The most important factor that affects normal bone remodeling is the tightly controlled regulation of relation between osteoblasts and osteoclasts. The process of remodeling is regulated by a rich innervation of the skeleton, being the source of various growth factors, neurotransmitters, and hormones regulating function of the bone. Optimum treatment of fractures and/ or bone defects requires knowledge of the complex cellular interactions involved with bone healing and remodeling.

In bone defect treatments, the *gold standard* remains bone grafting [2]: bone graft could be used alone or in combination with other materials in order to promote bone healing through osteoinduction, osteoconduction, and osteogenesis. Calcium phosphate grafts have been used for most trauma and orthopedic surgery procedures. Calcium sulphates were mainly used to restore bone defects after tumor resection surgery but offer minimal structural support [3].

Osteoset (medical grade alpha-hemihydrate calcium sulfate) (Wright Medical Technology, Arlington, TN) is known as a synthetic bone graft substitute. It can be used either alone or in combination with other naturally occurring materials to fill bone voids or gaps [4]. Bone substitutes can be divided into three classes, namely (1) osteoconductive; (2) directly osteogenic and (3) osteoinductive. Osteoconductive bone substitutes, such as ceramic materials, do not actively stimulate the bone formation process, whereas directly osteogenic and osteoinductive bone substitutes do [5]. Any material considered for use as a bone substitute must meet the following requirements: a) it must be fully biocompatible; b) it must be able to serve as an anchoring surface for host cells; c) it must have a porosity that allows osteo-conduction; d) it must be progressively resorbed and replaced by new bone.

The knowledge of the bone regeneration progress under different conditions helps the clinician to choose of the optimum variant for diagnosis and treatment.

The aim of this study was to analyze bone repair from histologic point of view. The objectives considered have included the interpretation and the establishment of

* email: monica_tarcolea@yahoo.co.uk; Phone: 0723860069. *All authors have equal contributions to the study and the publications.*

relations between statistically significant-parameters used to investigate the bone regeneration.

Experimental part

To study the repair of bone defects, we used two batches of Wistar mices (Lat Rattus Norvegicus), with a balanced gender distribution, between 8 and 12 months of age and weighing between 200 and 300 g.

Biobase conditions complied with European standard vivarium and animal welfare standards, the subjects benefiting during the experiment from a controlled environment in terms of humidity, temperature and light / dark cycles; also did not have food or liquid restrictions (water).

During the course of the study, the rules, principles and ethical requirements for the conduct of biomedical research and animal experiments as well as animal protection legislation have been respected.

We conducted an exposed - non exposed study with two identical batches, the witness and study defect being present at the same subject. The 46 subjects under study were divided into two equal lots. In all subjects, a round defect with a diameter of 5 mm was surgically performed on the right and left parietal bone. In the 23 subjects in group I the defect in the left parietal bone was covered with alloplastic material (Osteoset) and the defect in the right parietal bone was not covered with osteoconductive, osteoinductive or osteogenic materials. Regarding subjects in group study II, none of the surgically created bone defects were covered with alloplastic materials. The main purpose of cranioplasty is to reconstruct or to replace the damaged or missing bone tissue for brain protection and aesthetics [6].

Eutanasia of the subjects included in the study was performed at 2 and 4 months respectively, at the time of surgery.

After radiological examination of areas where bone defects were surgically induced, dissection tissues were collected from these areas and fixed in 4% formalin for 48 h at room temperature. Subsequently, these pieces were subjected to histological processing after a slow decalcification (2 weeks in a secrete nitric acid-based

Bone formation in the surface	Central	Yes	Peripheral	Yes
		No		No
In-depth bone formation	Limited to the surface of the graft	Yes	In-depth of graft	Yes
Graft vasculature	Absent	Yes	Penetrated in depth	Yes
	Limited to the surface of the graft	Yes		
Immature bone	Central	Yes	Peripheral	Yes
Mature bone	Central	Yes	Peripheral	Yes
Host bone interface / Material used for grafting	Immature bone	Yes	Mature bone	Yes
Host bone interface / Material used for grafting	Detectable	Yes	Undetectable	Yes
Central zone	Connecting tissue	Yes	Bone	Yes
	Vase de neoformație	Yes		
Bone bridge	Thin	Yes	Thick	Yes
Osteoblasts	Absent	Yes	Present at central zone	Yes
			Peripherally present	Yes
Osteocytes	Absent	Yes	Present at central zone	Yes
			Peripherally present	Yes
Osteoclasts	Absent	Yes	Present at central zone	Yes
			Peripherally present	Yes
Bone trabeculae	Absent	Yes	Present at central zone	Yes
			Peripherally present	Yes
Haversian Channels	Absent	Yes	Present at central zone	Yes
			Peripherally present	Yes
Inflammation	Absent	Yes	Present	Yes
Granulation tissue	Absent	Yes	Present	Yes
Osteoclastic degradation of the matrix support	Absent	Yes	Present at central zone	Yes
			Peripherally present	Yes
Replace matrix support with mature bone	Absent	Yes	Present at central zone	Yes
			Peripherally present	Yes

Table 1
HISTOLOGY EVALUATION SHEET

decalcifying formula belonging to the laboratory of the Department of Pathological Anatomy of UMF Iuliu Haţieganu Cluj).

Euthanasia, bone sampling and assembly for microscopic preparations were done on the same day.

After the inclusion of the histological parts in paraffin, the separation of the paraffin blocks with the microtome and the gluing of the sections on the test glass plate was followed. The paraffin sections, which are already stretched and glued on the test glass plates, must first be treated to remove the paraffin they are soaked with, which is not miscible with either water or alcohol. For this purpose, before staining, they should be dewaxed and then hydrated. For the dewaxing, the pieces were passed through 3 baths of benzene, then 3 baths of alcohol (96, 80 and 70°). From the last alcohol, the test glass plates are passed into 2-3 glasses distilled water for hydration, then they were colored with hematoxylin-eosin (HE), with tricrom Masson and Red Sirius.

The microscopic samples were examined and photographed using a Leica microscope with an ICC50HD image acquisition system. A histological evaluation sheet [table 1] was used to interpret the microscopic samples.

We have assigned a numerical rating to each of the following criteria. Summing up the scores obtained for each parameter we obtained the healing score. The healing score was calculated for each individual subject.

The images taken from the test glass plates were assembled into a unique, panoramic image of the whole preparation using Adobe Photoshop CS6 with its *Photomerge* function. The images thus obtained were measured with the Olympus Cell^{R}A software after its calibration with the zoom ratio (40x) used for shooting.

Results and discussions

The analysis of the test glass plates identified a set of patterns of bone defect closure representing different stages of the bone regeneration process, ranging from complete absence of regeneration (Pattern 1) to complete defect ossification (Pattern 8). We discussed the patterns illustrated graphically and with concrete examples from the examined lots. Pattern 1 revealed the complete absence of any bone regeneration attempt, the defect being covered by a connective tissue / tendinous (Red Sirius). For each such case the length of the tendon area was measured (fig. 1).

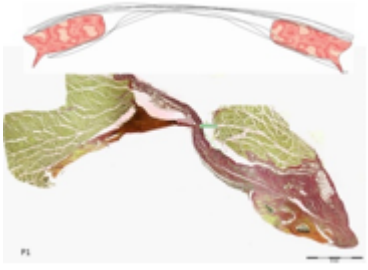

Fig. 1. Pattern 1 (P1)

In pattern 2 we have detected the presence of a central ossification nucleus flanked by tendinous conjunctive areas (HE). For each case with this pattern, the length of the ossification nucleus and the length of the lateral tendinous areas were measured, and the percent of the defect (sum of the measured lengths) was ossified (fig. 2).

In Pattern 3 we encountered 2 central ossification nuclei separated by pseudo-articulation flanked by tendinous conjunctive areas (HE). For each case with this pattern, the length of the ossification nuclei and the length of the lateral tendinous areas were measured, and the percent

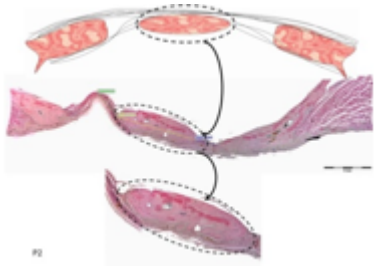

Fig. 2. Pattern 2 (P2)

of the defect (the sum of the lengths measured) was ossified (fig. 3).

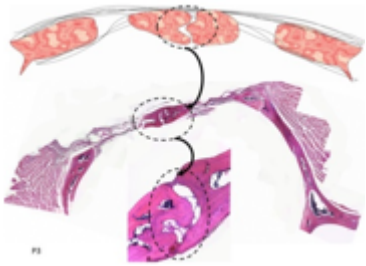

Fig. 3. Pattern 3 (P3)

Pattern 4 comprises cases with 3 or more central ossification nuclei separated from tendinous connective bridges (HE). For each case with this pattern, the length of the ossification nuclei and the length of the lateral tendinous areas were measured, and the percent of the defect (the sum of the lengths measured) was ossified. This pattern appears to represent a more advanced stage of pattern 2 (fig. 4).

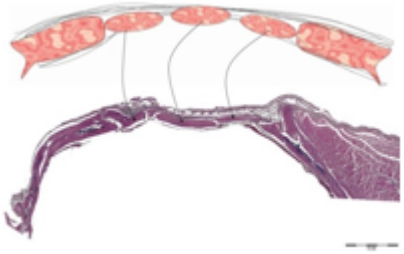

Fig. 4. Pattern 4 (P4)

In Pattern 5 we encounter a pseudo-articular central ossification nucleus (like those formed in the non-immobilized fractures) by a bone lobe that covered ½ of the defect created and flanked on one side by a conjunctive-tendinous area (HE). For each case with this pattern, the length of the ossification area and the length of the lateral tendon area were measured, as a percentage of the percentage of the defect (sum of the measured lengths) was ossified. This Pattern appears to be a more advanced stage of pattern 3 (fig.5).

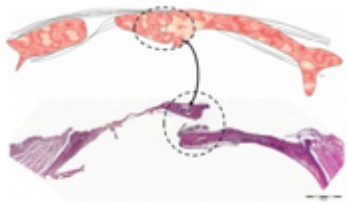

Fig. 5. Pattern 5 (P5)

The presence of a centrally separated ossification blade through pseudo-articulation (like those formed in the non-immobilized fractures) completely covers the defect created (HE). For each case with this pattern, the length of the ossification area was measured. This pattern appears to be a more advanced stage of pattern 3, following pattern 5 (fig. 6).

Pattern 7 reveals the presence of a peripherally separated osseous blade by pseudo-articulations (such as those

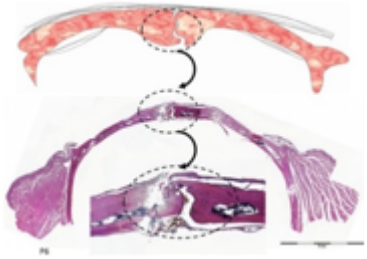

Fig. 6.Pattern 6 (P6)

formed in the non-immobilized fractures) covering entirely the defect created (HE HE). For each case with this pattern, the length of the ossification area was measured. This pattern appears to be a more advanced stage of pattern 2, following pattern 4 (fig. 7).

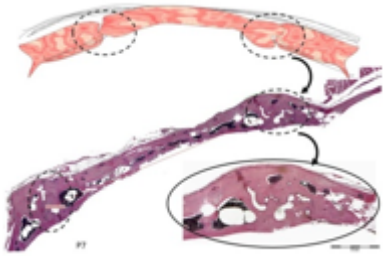

Fig. 7. Pattern 7 (P7).

Pattern 8 is the ultimate, most advanced result of all the previously described patterns. It is the histological expression of complete healing of the defect created. The former defect can still be observed due to the absence of muscle insertion (visible on the edges) on the regenerated bone area (fig. 8).

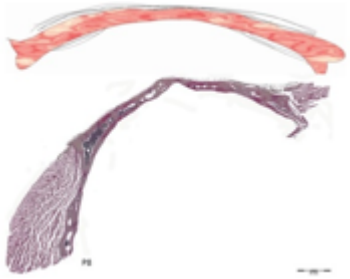

Fig. 8. Pattern 8 (P8)

Analyzing the frequency of the patterns in the two lots we can see that the P3 pattern was most frequently present, 10 times in lot 1 and 6 times in lot 2, which proves the presence of healing. Moreover, in lot 2 (evaluated at 4 months) patterns 5, 6 and 7 indicating good healing are present in 52.17% of subjects (table 2).

2 months	4 months
P1- 0	P1- 0
P2-2	P2-0
P3-10	P3-6
P 4-4	P 4-4
P5 -1	P5 -5
P6- 2	P6- 4
P7- 1	P7- 3
P 8 -0	P 8 - 1

Table 2
SYNTHESIS OF PATTERNS

The use of biomaterials for bone repaired is a common treatment option in clinical practice. Local and systemic factors are involved in favoring or inhibiting bone repair, and a fundamental role is played by the cells of macrophage lineage, which, by means of producing cytokines, mediate inflammation, bone cells differentiation, and bone cellular activity. However, the defect area is characterized by ischemia and hypoxia, the presence of inflammatory cytokines, and oxygen free radical accumulation.

Bone graft substitutes have been developed to fill and repair osseous lesions. Osteoset, a calcium sulfate (plaster of Paris) product with osteoconductive factors, has been proved effective in healing bone defects. An advantage of Osteoset is the ability to incorporate antibiotics into the calcium sulfate. Calcium sulfate as a resorbable slow drug release carrier is affordable, readily available, easy to sterilize, biocompatible, and visible under X ray.

Animal studies have demonstrated significant effect of Osteoset on bone healing. However in one randomized prospective study [7] they found moderate effect of Osteoset on bone healing in humans. They have observed that the decrease in defect volume seems to be higher at 6 weeks and the new bone formation seems to be more visible in the defects with Osteoset pellets. The reason could be the osteoconductive properties of Osteoset. [7].

Statistical analysis revealed that by using alloplastic commercial biomaterials to graft a bone defect the healing process is more advanced after four months than after two months. The evaluation of the healing process at two months revealed that engineered bone tissue using basal and complex osteogenic medium had significantly higher rates than for the groups with alloplastic commercial biomaterial. This underlines once again the osteo-conductive potential of alloplastic biomaterials and the osteogenic potential of engineered bone grafts [7, 8].

Microscopically, bone is a highly complex and specialized form of connective tissue. It is a mineralized tissue, which is composed of an organic matrix strengthened by deposits of calcium phosphate crystals; in other words bone is a natural composite material. The organic matrix is composed of collagen type I fibers (approximately 95%) and of proteoglycans and numerous non-collagenous proteins (5%). This organic matrix, calcified by calcium phosphate minerals, embeds bone cells, which participate in the maintenance and organization of bone [9]. The bone, once formed, is maintained dynamically through two different processes, modeling and remodeling, which are also employed in bone fracture recovery. The new bone is formed in bone modeling process, without prior bone resorption, while in the bone remodeling process, bone formation follows bone resorption.

Among all clinical available grafts, autologous bone is still being considered as the gold standard since all necessary properties required in bone regeneration in term of osteoconduction, osteoinduction and osteogenesis are combined [10]. Osteogenesis is the capacity to produce new bone by the osteoblasts by differentiation of osteoprogenitor cells either present in the recipient bone or coming from the graft material. This property is mainly present in autogenous grafts as compared with allografts and xenografts. Osteoinduction is the capability of the graft materials to induce formation of the bone-forming cells via differentiation of multipotent mesenchymal stem cells of the surrounding host tissues to produce osteoprogenitor cells followed by development of osteoblasts. Osteoconduction is a characteristic whereby the graft acts as a permanent and resorbable scaffold, mechanically supporting ingrowth of vessels and new bone

from the borders of the defect into and onto its surfaces. This characteristic initiates or induces new bone formation. Osseointegration is the ability to bind to the surrounding bone without an intervening layer of fibrous tissue, allowing incorporation of the graft at the host site [11, 12].

In some studies, activated osteoblasts, mature chondrocytes, and granulation tissue were evident from the first month. These histological findings indicate that Osteoset has osteoconduction properties, and no evidence of osteoinducing activity could be detected [13].

Conclusions

Bone grafting is one of the most commonly used options to treat large bone defects.

The histological analysis of a bone repair shows the direct correlation between the healing process and the addition of alloplastic materials (Osteoset).

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