

Regeneration of Bone Defects Using Bioactive Glass Combined with Adipose-derived Mesenchymal Stem Cells

An experimental *in vivo* study

CRISTIAN TRAMBITAS¹, ANCA MARIA POP^{2*}, ALINA DIA TRAMBITAS MIRON³, DORIN CONSTANTIN DOROBANTU⁴, FLAVIU TABARAN⁵, BOGDAN CORDOS⁶, BOGDAN ANDREI SUCIU¹, KLARA BRINZANIUC¹

¹ University of Medicine, Pharmacy, Sciences and Technology of Tirgu Mures, Department of Anatomy and Embryology, 38 Gheorghe Marinescu Str., 540139, Tirgu Mures, Romania

² University of Medicine, Pharmacy, Sciences and Technology of Tirgu Mures, Faculty of Medicine, 38 Gheorghe Marinescu Str., 540139, Tirgu Mures, Romania

³ University of Medicine, Pharmacy, Sciences and Technology of Tirgu Mures, Department of Medical Informatics and Biostatistics, 38 Gheorghe Marinescu Str., 540139, Tirgu Mures, Romania

⁴ University of Medicine, Pharmacy, Sciences and Technology of Tirgu Mures, Department of Plastic Surgery, 38 Gheorghe Marinescu Str., 540139, Tirgu Mures, Romania

⁵ University of Agricultural Sciences and Veterinary Medicine, 3-5 Calea Manastur, 400372, Cluj Napoca, Romania

⁶ University of Medicine, Pharmacy, Sciences and Technology of Tirgu Mures, Department of Experimental Studies, 38 Gheorghe Marinescu Str., 540139, Tirgu Mures, Romania

Large bone defects are a medical concern as these are often unable to heal spontaneously, based on the host bone repair mechanisms. In their treatment, bone tissue engineering techniques represent a promising approach by providing a guide for osseous regeneration. As bioactive glasses proved to have osteoconductive and osteoinductive properties, the aim of our study was to evaluate by histologic examination, the differences in the healing of critical-sized calvarial bone defects filled with bioactive glass combined with adipose-derived mesenchymal stem cells, compared to negative controls. We used 16 male Wistar rats subjected to a specific protocol based on which 2 calvarial bone defects were created in each animal, one was filled with Bon Alive S53P4 bioactive glass and adipose-derived stem cells and the other one was considered control. At intervals of one week during the following month, the animals were euthanized and the specimens from bone defects were histologically examined and compared. The results showed that this biomaterial was biocompatible and the first signs of osseous healing appeared in the third week. Bone Alive S53P4 bioactive glass could be an excellent bone substitute, reducing the need of bone grafts.

Keywords: bioactive glass, regeneration of bone defects, adipose-derived stem cells, osteogenic differentiation

Bone tissue is normally capable of regeneration, which means that most injuries or bony defects will heal spontaneously, without major interventions. Despite the high innate regenerative capacity of bone, large osseous defects fail to heal and remain a clinical challenge, requiring surgical interventions. Healing such defects requires the formation of large amounts of bone in an environment often hostile for osteogenesis, due to damage to surrounding tissue and loss of vascularity [1-3]. The majority of bony injuries are represented by fractures that heal without the formation of scar tissue, bone being regenerated and its properties restored, with newly formed tissue being usually hard to distinguish from adjacent injured bone [4]. There are cases in which bone repair is uncertain, for example fractures of tibia, where up to 13% have a delayed union or fracture non-union. In other clinical conditions, bone regeneration is required in large quantities which exceed the potential of self-healing, such as large bone defects created by trauma, skeletal abnormalities, infection or tumors [5, 32-35].

Bone tissue engineering is an alternative solution for the repair of large bone defects, by providing a template to guide hard tissue regeneration. The standard tissue engineering approach includes the use of scaffolds, mesenchymal stem cells and growth factors, called the triangular concept, which will offer a bridging material guiding the regeneration of the new tissue [6, 7]. Scaffolds used in bone regeneration are broadly classified into

biological, mineral such as glass and ceramic materials and polymer scaffolds. The ideal scaffold for bone regeneration must be biocompatible, should allow cells infiltration and induce an osteogenic response [8, 9]. Bioactive glasses are synthetic, biocompatible materials, available commercially in different formulations which received a lot of attention during the last decade due to their osteoconductive and osteoinductive properties (fig. 1).

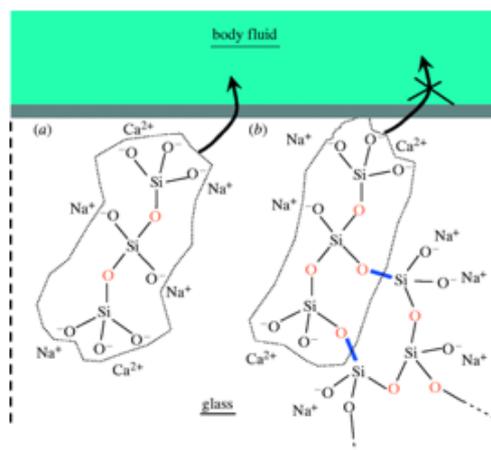


Fig. 1. Chemical structure of bioactive glasses; a) a three-membered silicate chain without covalent links will be relatively fast dissolved; b) a part of a five-membered ring with additional covalent cross-links through the additional Si-O bonds [10]

*email: ancapop98@yahoo.com, Phone: +40 747 363336

Data from scientific literature showed that the regenerative potential of bioactive glasses are influenced by several factors, including the fabrication method, composition, microstructure and pore characteristics, in addition to loading with growth factors or stem cells. Isolation and use of stem cells enriched regenerative medicine with new promising treatment possibilities to address large skeletal defects, may facilitate clinical trials and enlarge the therapeutic options for reconstructive surgery. [11,12] Despite certain limitations, animal bone defect models provided essential information for the future design of bioactive glasses and the use of in vivo models for testing certain formulations is considered to be an important step before clinical trials; it enables the understanding of mechanisms and biological performances of these materials in the physiological environment [13-16].

The main objective of this study was to examine, by using an experimental animal model, the differences in the healing of critical-sized calvarial bone defects using bioactive glass combined with adipose-derived mesenchymal stem cells with natural healing of similar bone lesions. We intended to compare through histologic methods the cell dynamics and regenerative processes.

Experimental part

Material and methods

This study was approved by the Ethics Committee of our university, based on decision Nr. 137/10.11.2016. We used 16 male Wistar rats with a weight of 550-600 g, maintained in the animal facility for one week prior to the experiment, with unrestricted access to water and food. The study design considered that each animal will receive a study defect located on the right side and a control defect located on the left side of the calvaria, in order to reduce the number of animals included in the experiment.

Experimental phases used to obtain the bone defects

The animals were anesthetized with a combination of ketamine 80mg/kg and xylazine 10 mg/kg and maintained in this condition during the entire procedure. The surgical sites were shaved, scrubbed with betadine and draped. Using an aseptic technique and sterile instruments, a cranial incision was made along the midline, in an anterior-posterior direction. We removed the subcutaneous tissue and periosteum in order to expose the calvaria. Using a round sterile trephine bur with a diameter of 4.5 mm bilateral full-thickness defects were created in the parietal bones. The bur was frequently cooled with sterile saline solution in order to avoid overheating and extreme care was taken not to damage the dura mater, an essential condition for the re-ossification of the defect. In each case the right side defect was filled with bioactive glass granules and 10 microliters of nutritive solution containing around 1.750.000 stem cells. We used Bon Alive S53P4 bioactive glass, which is composed of 53% SiO₂, 23% Na₂O, 20% CaO and 4% P₂O₅. This is a bioresorbable material which is completely transformed into bone after a few years, enough time for the normal bone remodeling and growth mechanisms to replace the resorbed biomaterial. In contact with body fluids, Bon Alive S53P4 releases sodium, calcium, silica and phosphorus ions which create an alkaline environment that has an important antimicrobial effect. Each left side defect was considered as negative control. The periosteum and skin were repositioned and closed with sutures. The animals were monitored daily for 1 week regarding wound healing, clinical signs of infection and food intake. After 1, 2, 3 and 4 weeks the animals were

euthanized by intra-peritoneal administration of ½ doses of ketamine and xylazine previously used for anesthesia, followed by intra-cardiac injection with 0.1 ml of T61 (embutramide 200mg, chlorhidric tetracaine 5 mg, mebezonium iodide 50 mg). The defects with surrounding tissue were harvested and sent for histologic examination.

Protocol for adipose-derived stem cells harvesting

The animals were anesthetized and prepared following the same protocol as described above and fragments of 1 cm³ of well vascularized subcutaneous tissue from the dorsal aspect of the interscapular region were obtained. These were introduced into sterile culture medium, using 100 mL solution containing 50 mL DMEM (Dulbecco Modified Eagle Medium) + 10% FBS (Fetal Bovine Serum) + 2% antibiotic/antifungal substance and maintained in this environment for 30 min. The donor sites were closed with sutures and the animals received an intramuscular analgesic substance for pain control. The tissue specimens were sliced into 1 mm³ fragments and introduced in collagenase type I solution, filtered to eliminate the detritus and centrifuged for 5 min at 1000 rpm. Afterwards, the fragments were placed in ammonium chloride in order to remove the red blood cells, centrifuged again for 5 min at 1000 rpm and placed into flasks at 37°C in an atmosphere with 5% CO₂.

Histologic protocol

The histologic protocol consisted of the following steps: fixation in 10% formaldehyde solution for 3 days, decalcification in ethylenediaminetetraacetic acid (EDTA) 14% for 2 weeks, dehydration in increasing concentrations of ethanol and embedded in paraffin. After sectioning into 5 microns thick slices, the specimens were stained with hematoxylin and eosin and examined under a microscope (Olympus BX50, Olympus Japan) connected to a CCD camera. Each specimen was evaluated by an experienced specialist.

Results and discussions

The histological analysis at 1 week are presented in figure 2 and figure 3. In the control group the defect was partially limited by adult bone tissue and contained granulation tissue in different evolutive stages. On the periphery adipose tissue and a small quantity of loose connective tissue were seen, with moderate inflammation (fig. 2). In the study group, the bone lesions were filled with adipose and fibrous tissue in different evolution stages; areas of translucent foreign material which remained unstained were observed. On the periphery there was a moderate inflammatory reaction, with a direct contact between bioactive glass and fibrous connective tissue in most of the cases (fig. 3).

After 2 weeks in the control group there were only few differences compared to the first week. The presence of oriented connective fibers at the periphery of the defect could be noticed (fig.4). In the study group, the main observation was the presence of type I collagen fibers.

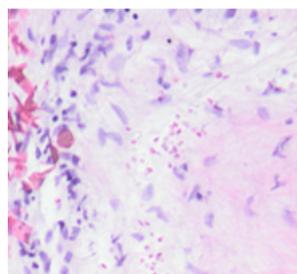


Fig. 2. Histological aspect after 1 week obtained from control site. The presence of granulation tissue and loose connective tissue with moderate inflammatory reaction was noticed. (H&E stain)

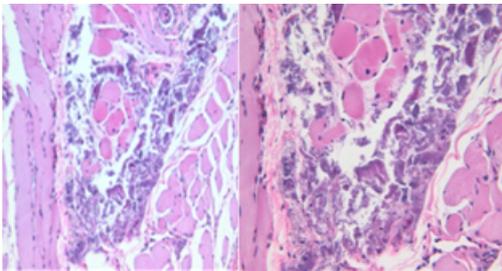


Fig. 3. Histological aspect after 1 week obtained from bone defects filled with bioactive glass and stem cells. The direct contact between the biomaterial and bone is evident, there is a moderate inflammation and small quantities of fibrous tissue. (H&E stain)

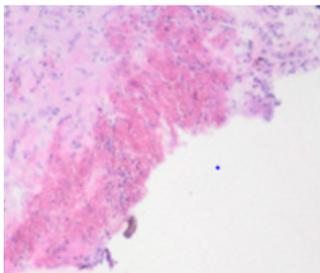


Fig. 4. Histological aspect after 2 weeks obtained from control site, characterized by the presence of connective fibers at the periphery. (H&E stain)

The granules of bioactive glass were still visible, due to the lack of integration in the surrounding tissues. There was a mild inflammatory reaction without signs of infection or material rejection. In the implant zone there was a

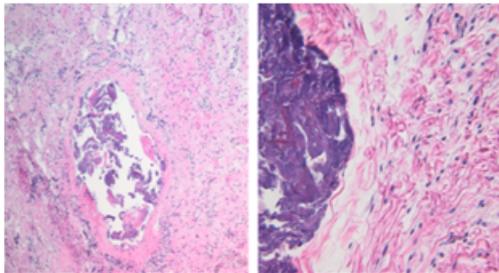


Fig. 5. Histological aspect after 2 weeks obtained from bone defects filled with bioactive glass and stem cells. Type I collagen fibers are visible, together with granules of bioactive glass, which were not integrated in the surrounding tissue. (H&E stain)

significant population of stem-like cells compared to the control site (fig. 5).

In control group, the first signs of healing could be seen after 3 weeks due to the presence of collagen fibers and fibroblasts, with minimal inflammation (fig. 6). In the study group, the implant zone was well bordered and blood vessels were present (fig. 7).

The fourth week showed the following changes: in the control group there were signs of healing represented by the presence of collagen fibers and small blood vessels, with a persistent minimal inflammatory reaction (fig. 8).

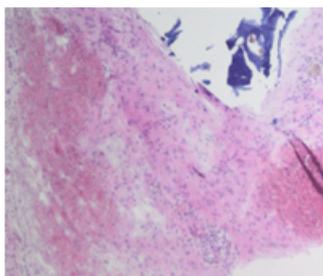


Fig. 6. Histological aspect after 3 weeks obtained from control site, showing the presence of collagen fibers and reduced inflammation. (H&E stain)

In the study group at 4 weeks mature fibrous tissue, without cells with stem morphology was noted. There were thick collagen fibers and an intense vascular proliferation (fig. 9) The presence of blastic cells in the second and third

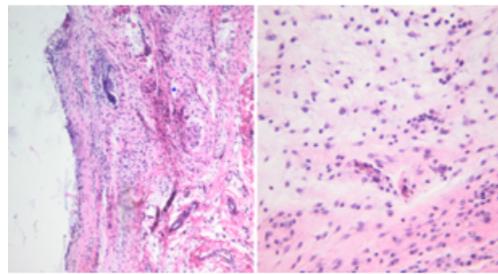


Fig. 7. Histological aspect after 3 weeks obtained from bone defects filled with bioactive glass and stem cells showed the first signs of angiogenesis. (H&E stain)

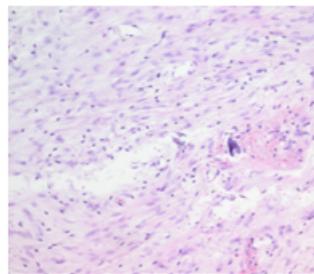


Fig. 8. Histological aspect after 4 weeks obtained from control site, which confirms the progression of the healing process. (H&E stain)

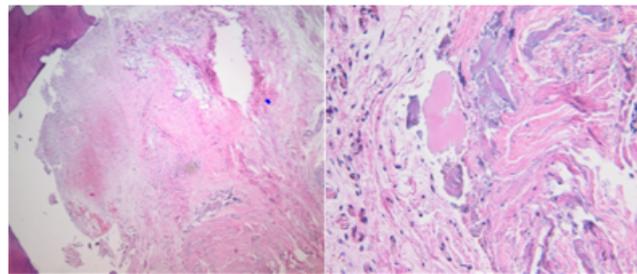


Fig. 9. Histological aspect after 4 weeks obtained from control site and bone defects filled with bioactive glass and stem cells (H&E stain)

week and their regression in the fourth week showed their differentiation.

The final goal of tissue engineering is to repair, replace and restore the biological properties of the absent, dysfunctional or damaged tissue; in this respect, during the last decade, bone regeneration has gained much interest and important resources from tissue engineering researchers. A promising approach developed by numerous studies was the combination of scaffold biomaterials with stem cells and growth factors, intending to develop a material that could successfully replace and repair the bone defects [9, 17, 18]. Biological scaffolds for bone regeneration use materials as chitosan and collagen, but there is an increasing tendency to combine them with mineral agents as hydroxyapatite, in order to have a more precise copy of the natural bone. [19] Unfortunately, the use of these biomaterials limits the extent to which the physical properties of the scaffold can be tailored. Therefore, mineral scaffolds, such as ceramic materials or glass were considered more promising [20, 21].

The bone-bonding properties of the silicate bioactive glass 45S5 were revealed almost 50 years ago and since then the 3-dimensional glass forming SiO₂ network was the most researched glass used for medical applications. It was shown that this glass forms a carbonate-substituted hydroxyapatite layer on its surface, when in contact with body fluids, which resembles the mineral constitution of bone and determines a borderless contact with the bone

tissue. [22] This bioactive glass has osteoconductive and osteoinductive properties, as it supports the new bone growth at the interface with the host tissue; it develops from periphery to center as well as from the implant center to the bone-implant interface. This osteogenic induction was attributed to the sodium and calcium ions and soluble silica, which concentration was considered to be critical. The favorable effects of bioactive glasses implants are not limited to osseous regeneration, as it was shown that they induce an increased vascularity in bone lesions of earlier irradiated calvaria, where the circulation was compromised [21].

A lot of research focused on tissue regeneration after using mesenchymal stem cells isolated from adipose tissue, which proved to have similar characteristics with the stem cells derived from the bone marrow [23]. The cells isolated from the stromal fraction of fat tissue became promising candidates for tissue engineering procedures as they are abundant and their source is available being easily harvested, with minimal morbidity [24]. When developing a bone defect model, the size of the lesion proved to be of utmost importance, especially the critical size. In the rat calvaria, lesions with a diameter of 5 mm are most commonly used, based on the research conducted by Bosch et al [25] who concluded that this dimension allows the induction of two defects per animal with concomitant avoidance of the sagittal suture to span the defect. Hollinger et al [26] considered that an 8 mm critical-size defect in rats is more suitable, as they demonstrated at least 10% new bone formation after 15 months in the 5 mm defects [27]. A critical-size defect is defined as a lesion in bone which cannot heal totally without additional intervention, such as bone graft, within the lifetime of the experimental animal or the time period of a scientific investigation [28, 29]. The size depends on the species and on anatomical site; in rats it is 8 mm and these critical-size defect models are widely used in bone healing experiments. Recently, a critical-size defect was defined as a segmental bone deficiency of a length exceeding 2-2.5 times the diameter of the affected bone [30]. The calvarial bone defect model became popular among researchers due to the fact that a standardized defect can be generated, which can be further analyzed by histologic and radiographic techniques [18]. Furthermore, these sites allow the placement of biomaterials without the need of external fixation, due to the support offered by the dura mater and skin. The drawback of this type of model is represented by the inability to evaluate the performance of the tested biomaterial under physiological mechanical loading, which is an important aspect in clinical situations. Sacak et al [31] confirmed that bioactive glass was suitable for the induction of in vivo osteogenic differentiation of adipose-derived stem cells in a critical size calvarial defect model. Their results showed that the quality of newly formed tissue was similar to that obtained by bone grafting alone and it was not significantly different from that obtained after using bioactive glass alone.

Conclusions

Tissue engineering techniques suitable for bone regeneration and repair proved to be applicable in animal bone defect models designed to study new osteoinductive scaffolds or biomaterials. Our study confirmed that in rat animal models, the 5 mm calvarial bone defects do not heal spontaneously. The biomaterials used as implants were highly biocompatible, as no signs of rejection were

recorded and the first signs of healing were observed after three weeks. Therefore, we consider that Bone Alive S53P4 bioactive glass is an excellent bone substitute, which could be used as a replacement of the bone graft. In vivo animal models represent an inevitable phase before any clinical use of a new treatment method, as they allow a better understanding of the material's performance in a physiological environment.

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