

Effect of Osteoplasty with Bioactive Glass (S53P4) in Bone Healing - *In vivo* Experiment on Common European Rabbits (*Oryctolagus cuniculus*)

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Osteoplasty, is a procedure mostly applied in complicated bone fractures. Nowadays this method is widely used in primary fracture treatment while the native bone graft is progressively replaced with various synthetic bone substitutes. From the numerous bone grafts we'd like to mention a representative of ceramics, the S53P4 bioactive glass. (BonAlive®). The aim of this study was to investigate the healing process of different fracture types generated on rabbit femurs. During this experiment we used seven common European rabbits. We separated these animals into two groups; in the first group we surgically generated a total fracture in the middle 1/3 of the femur, while in the second group, we produced only a bone defect on the femur. The osteoplasty was carried out with bioactive glass and autologous bone grafts. The radiographic follow-up was immediate after the operation and after 3, 6 and 7 weeks. The animals were euthanized after 19, 20 and 21 weeks, for histomorphometric examination of the femur. It was also studied the ionic release from the used bioactive glass at physiological pH and the etching of the glass was studied by Scanning Electron Microscopy.

Key words: common European rabbit, bioactive glass, S53P4, osteoplasty

Bone is that human tissue which has the ability to heal and regenerate itself. Occasionally a bone defect is formed in various orthopedic and/or trauma pathologies; in these cases the bone fails to heal and needs bone reconstruction [1]. An ideal bone substitute should have several properties such as non toxicity, bioactivity (capability of direct bonding to living bone), biocompatibility, osteoconductivity and / or osteoinductivity, sufficient mechanical properties (loading/weight-bearing capacity), porosity allowing new bone ongrowth and ingrowth, suitable degradation rate, convenient handling properties, intraoperative mouldability and possibly, ductility permitting application by injection as a liquid or as a paste and also it is important to have the properties of in situ bone regeneration [2, 3].

From the early use of alloys and metals for implants, the coatings have attracted a special attention in order to improve the osseointegration process and to minimise the adverse effects through organism. Titanium is first and one of the most common used metals for implants, being also the most studied. Several coating methods have been developed in order to improve the implant properties. Hydroxyapatite coatings were studied for improving bone stress distribution near the dental implant-bone interface [4] or for decreasing the osteoclastic activity by functionalization the hydroxyapatite with alendronate as bioactive component [5]. The surface functionalization of metal can be performed inducing amino free groups which can react with natural bone (activation of Ti to TiOH,

followed by reaction with amino propyl triethoxysilane or ethanolamine) [6] or can consist in chemical treatment in order to generate a microporosity at the surface of metal which allow the proliferation of osteoblasts and a better integration of implant [7]. Titanium was itself use as a coating for other bone substitution materials. Recently, a new method for plasma-sprayed titanium coating to polyetherether-ketone have been reported, showing a dramatically increased for mechanical properties of polyetherether-ketone use as substitute for cortical bone [8]. Other alloys or coatings were also studied. Magnesium alloys coated with bredigite (Ca7MgSi4O16) through the combination of anodic spark deposition improved the biointegration of implant, enhancing the new bone formation and decreasing the bone inflammation [9].

Bioactive glasses are a group of synthetic silica-based bioactive materials with bone bonding properties first discovered by Larry Hench. They have several unique properties compared with other synthetic bioabsorbable bioactive ceramics, such as calcium phosphates, hydroxyapatite and tricalcium phosphate. Bioactive glasses have different rates of bioactivity and absorption rates depending on their chemical compositions. The use of bioactive glass in orthopaedics is related to its ability to release in aqueous solution at physiological pH ions which leads to the formation of hydroxycarbonate apatite layer between implant and bone, fact which improve the osseointegration process through the formation of an intimate bond between bone and implant. Important for

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clinical applications is the study of ionic release because the degradation speed of glass influences directly the formation of hydroxyapatite and biointegration of implant [10] (fig. 1). The glass coating based on phosphate bioactive glass may also act as a drug delivery system for some biological interest ions such as zinc, boron, copper. The release of these ions stimulates the formation of new tissue due to their role in the osseointegration proteins conformation and also by their direct participation to the formation of hydroxyapatite [11]. Besides their involvement in the hydroxyapatite formation, these ions present the advantage of having an antibacterial effect at the site of implantation, this effect depending by the concentration of released ions (the antibacterial effect being more pronounced with the decreasing of pH) [12]. Several types of bioactive glasses like fluoride based glass [13] or a large variety of 45S5 bioglass [14, 15] were studied in different pH media like 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) buffer in water [16], simulated body fluid or sodium phosphate buffered saline [17].

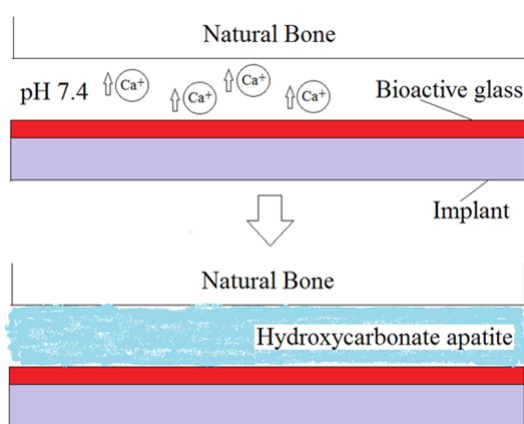


Fig. 1. Osteointegration process induced by bioactive glass coating

In vivo, the material is highly osteoconductive and it seems to promote the growth of new bone on its surface [18]. However, currently available bioactive ceramics do not satisfy every clinical application. Therefore, the development of novel design of bioactive materials is necessary [19]. The purpose of this paper is to study the effects of the bioactive glass (S53P4) - the healing process,

osteointegration properties - in different bone fractures, on rabbit models.

Experimental part

Materials and Methods

The animal testing was made into the specialized animal experimentation lab at the University of Medicine and Pharmacy Targu-Mures, Romania. A number of seven common European rabbits were used in this study. The procedure is described below and representative images during surgery are shown in figure 2.

An induction mixture of Ketamine (ketamine hydrochloride, 35 mg/kg) and Xilazin (xylazine, 5 mg/kg), was administered intramuscularly, by a qualified veterinary doctor. Then an intravenous canula was inserted in the auricular vein for further medication. The lateral right hind limb of each rabbit was shaved and prepared with antiseptic solution (10% povidone iodine) and sterile draped with the rabbit in a left lateral decubitus position. After local infiltration with lidocaine, the right femur was exposed through a lateral longitudinal incision. The femur was exposed between the muscle fibers of the *vastus lateralis* muscle.

The animals were separated into two groups, because two different operating techniques were applied. In the first group we surgically generated a total fracture in the middle 1/3 of the femur. The fracture was fixed with an intramedullary nail and two cerclage. All these implants are from stainless steel type 316L. The bone defect was filled with BonAlive® and autologous bone graft (fig. 2, b). In the second group only a bone defect was generated surgically, without a bone fracture. The bone defect was filled with bioglass type BonAlive® (fig. 2, c).

In order to evaluate the ionic dissolution of bioactive glass, glass samples were kept in water at physiological pH (7.4) for three weeks. The solution was made by dissolving in deionized water 795.0 mg/L disodium hydrogen phosphate, anhydrous (Merck), 144.0 mg/L Potassium dihydrogen phosphate (Sigma Aldrich) and 9000 mg/L sodium chloride (Sigma Aldrich) [http://himedialabs.com/TD/TS1120.pdf]. Three grams of bioactive glass platelets were kept in 10 mL of this solution. For studying the influence of ionic release in the presence of a metallic implant two samples were analysed by adding at the

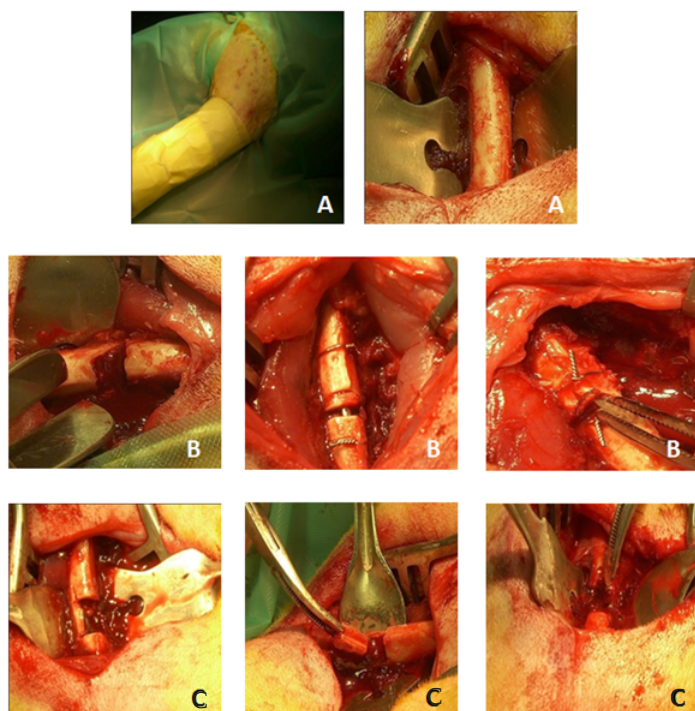


Fig. 2. Representative aspects related to the implantation of the materials during the animal testing: a) aspect during the preparation of the animals for surgery, b) aspects during the surgery for the first group of animal studies (bioactive glass S53P4 associated with metallic implants), c) aspects during the surgery for the second group of animal studies (just bioactive glass S53P4)

solution two different quantities of stainless steel, one of 1.8234 g and one of 3.0165 g.

The morphology of bioactive glass was investigated using FESEM FEI Quanta Inspect F. A Bruker S8 Tiger wavelengths dispersive X-ray fluorescence spectrometer was used for analysing the ionic release. All liquid samples were analysed using a maximum high voltage 60 kV Rhodium X-ray anode with a counter gas. All results were obtained based on QuantExpress calibration method specific for equipment, using K α lines for elements. In order to avoid boiling and evaporation of the sample an atmospheric He medium was used. Detection of Cl and S was carried out on PET crystal. LiF crystal was used for others elements. Liquid samples were analyzed into cups with Mylar (polymer) films resistant against corrosive media. Results and discussions

The radiographic follow-up was done immediately after the operation and after three, six and seven weeks (table 1). From the first group, the first rabbit's radiography (fig. 3), shows the fixation of the fracture with intramedullar nail and cerclage. The implanted bioactive glass was placed around the fracture. In a specimen from the first group, on the 7th postoperative week, the bone ends are dislocated, increased bone activity and callus formation is

Table 1
POSTOPERATIVE RADIOGRAPHY

Group/ individual	After surgery	The control radiography
I/1	immediately postop	7. week
I/2	immediately postop	-
II/1	immediately postop	6. week
II/2	immediately postop	6. week
II/3	immediately postop	6. week
II/4	immediately postop	3. week
II/5	immediately postop	3. week

seen with the activity and incorporation of the bioactive glass.

On the second group's first radiography the bone defect which is filled with bioactive glass can be observed. A control radiography was made on the 7th postoperative week: it shows increased bone activity. The bone ends are

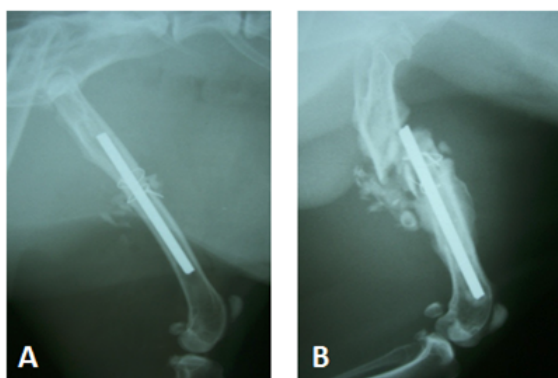


Fig. 3. Radiological results of the animal testing - first study group:
a) postoperative radiography after surgery; b) postoperative radiography at 7th week after surgery

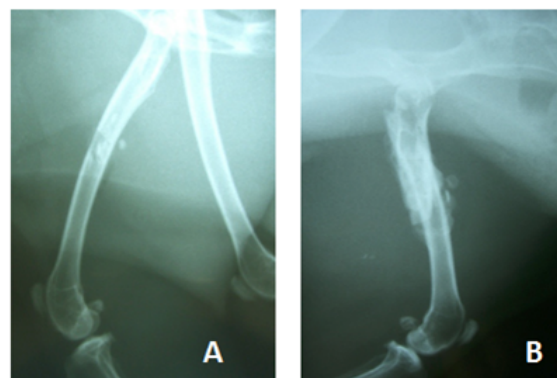


Fig. 4. Radiological results of the animal testing second study group:
a) postoperative radiography after surgery; b) postoperative radiography at 7th week after surgery

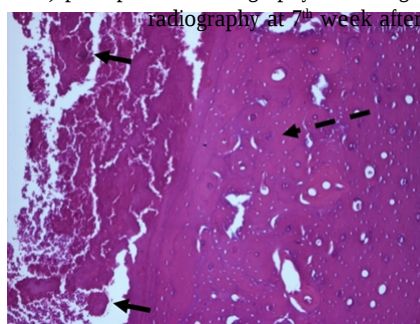


Fig. 5. New bone formation near the implanted area (ob. 10x, HE col.)

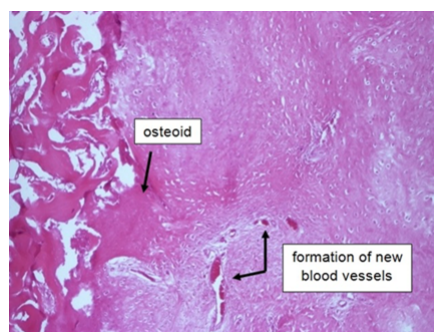


Fig. 6. Young bone tissue, with new blood vessels (ob. 10x, HE col.)

in an axial line and are well fit together. Intense callus formation can be observed (fig.4).

The rabbits were euthanized after 19, 20 and 21 weeks post operatively, and their femur was removed for histological examination. Using traditional hematoxylin and eosin stain, we were able to study the effects of bioactive glass (S53P4), the healing process and osteointegration properties. For histological exams, the femur was harvested and sections were prepared from the place of the implantation. By the microscopic examinations the partly absorbed bioactive glass granules are replaced, with new bone tissue (fig.5-dashed arrow). These granules are seen as a homogenous, circular/oval eosinophil area on the histological section (fig. 6 continuous arrow).

The mature, newly formed bone tissue is represented by a bone tissue in different stages of evolution, with a variable number of osteoblasts, osteoclasts and osteoid tissue. It shows a young, hypercellular bone tissue, with the presence of osteoblasts, osteoid substance and new blood vessel formations. These also fully replace the implanted bioactive glass (S53P4) in an experimental animal, euthanized on the 20th postoperative week.

Rabbits are one of the most commonly used animals for medical research, being used in approximately 35% of musculoskeletal research studies. In scientific literature, the fragile weight-bearing femur of rabbits, are described as a disadvantage [20, 21], which was also observed during the present study. In the course of the experiment the muscular strength of the rear leg and the fragility of the

bone generated the some difficulty. In order to determine whether a new material is suitable for the requirements of biocompatibility and mechanical stability prior to clinical use, it must undergo rigorous testing under both initial *in vitro* and then *in vivo* conditions. Testing in *in vitro* conditions is used primarily as a first stage test for acute toxicity and cytocompatibility to avoid the unnecessary use of animals in the testing of cytologically inappropriate materials. However, *in vitro* characterization is not able to demonstrate the tissue response to materials, instead being confined to the response of individual cell lines or primary cells taken from animals [22].

The present study *in vivo* experiment on common European rabbits aimed to investigate the effects of the bioactive glass S53P4: osteointegration properties and the healing process in different fracture types. In four cases bioactive glass S53P4 was used and in three cases an autograft was implanted (one piece of the removed bone was used as an autograft and then reimplanted to fill the defect).

The control radiography in studied cases, where bioactive glass S53P4 was implanted and where a bone defect was generated surgically, showed a stronger bone activity. A slower healing process was observed, where cerclage and intramedullar nail was used for the osteosynthesis. The 7th postoperative week radiography shows a worse bone healing in the case of first study group, but on the 7th postoperative week radiography for second study group a much better bone healing process can observe.

The rabbits were sacrificed after 19, 20 and 21 weeks postoperatively, and their femur was harvested and histological sections were prepared from the place of the implantation. After twenty weeks the implanted bioactive glass granules weren't absorbed totally. A study on rabbits, using bioactive glass (S53P4), at a 16th weeks follow up, bone ingrowth was noticed in the samples with large glass granules. Furthermore, new bone formation was found in the medullary cavity and also some studies shows the bone formation effect of the bioactive glass after it was used in several bone cysts [22, 23]. It can only be concluded that the composite of bioactive glass (S53P4) is biocompatible with the bone tissue within the 16th week of implantation period. Another study on rabbit model observes the new bone, directly attached to the surface of the glass granules, which had developed visible reaction zones [24]. The present studies results are in concordance with the literature. Many studies deal with the antibacterial characteristic of bioactive glass S53P4 and shows that this biomaterial can heal osteitis and can reduce the grade of bone infections [25-27]. The authors of the present study did not observe any cartilage formation or any adverse round cell tissue reactions during the histopathological examinations.

The morphology of analysed samples of bioactive glass is presented in figure 6. In the physiological pH after three weeks (Fig. 7C), a very pronounced exfoliated layer is visible at the surface of glass. In the presence of the stainless steel, this exfoliated layer is visible after two weeks which indicate an accelerated degradation in the presence of a metal. Intermediary, acicular crystals are formed at the surface (fig. 7B and F), probably due to the

Ion	Water pH 7.4, mg/ml	Water pH 7.4 and 1.1824 g stainless steel, mg/mL	Water pH 7.4 and 3.4142 g stainless steel, mg/mL
P	-	0.1	-
Ca	0.3	0.6	0.8
Si	0.4	0.2	0.3
Fe	0.3	-	-
Cu	0.081	-	-
Cl	-	0.2	0.3

Table 2
THE VALUES OF IONIC RELEASE IN STUDIED MEDIA

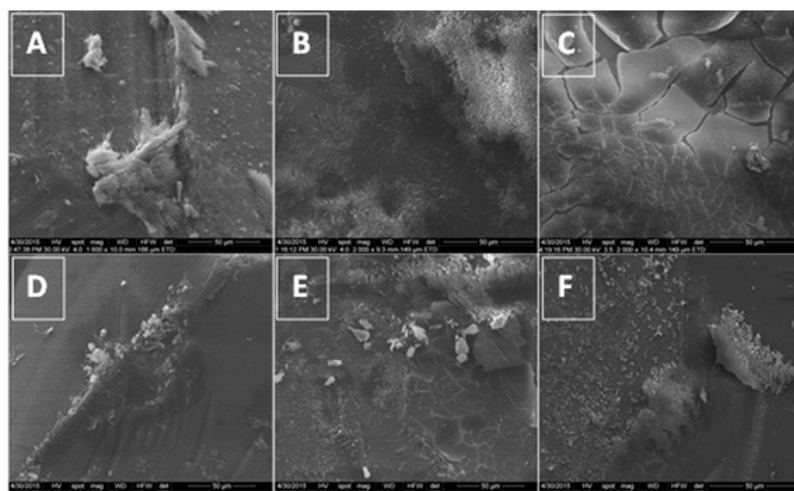


Fig. 7. Scanning Electron Microscope images of bioactive glass etched in physiological fluid for one week (A), two weeks (B), respectively three weeks (C) and in the presence of higher amount of metal after one week (D), two weeks (E) and three weeks (F)

etching of the surface in the presence of corrosive media. The presence of the same crystals after three weeks at the surface of bioactive glass kept in the presence of metal show this as an intermediary stage in the degradation process of the glass.

These observations are also supported by the analysis of the ionic release and presented in table 2. An interesting observation can be performed related with the type of ions released. Ions which are contained also by the metal alloy (Fe and Cu) are not released in the liquid media which indicate that adhere at the surface of the alloy. But Ca, Si and Cl are released in higher amounts in the present of metal. This observation indicates two advantages related to the presence of an alloy: the degradation of bioactive glass is accelerated and the release of ions that improve osseointegration process is increased, both aspects favouring the biological process.

Conclusions

From the radiographical results, a stronger bone activity was observed in the group of rabbits on which bioactive glass as utilized and where a partial was fracture induced. In cases where cerclage and intramedular nailing was performed, the healing process was inhibited by these materials, compared to the other cases, where no foreign materials where used. With the application of bioactive glass S53P4 the authors observed better results, than with an autologous bone. The bioactive glass was near totally absorbed on the histopathological sections, proving the biocompatibility of the material. Histopathological sections from the implantation area, demonstrated the osteo-conductiv property of the bioactive glass. The osteo-stimulative characteristics of the bioactive glass is confirmed by the radiological follow-up and the new young bone formation, on histopathological images. The authors did not observe any inflammatory cell reactions, that sustain the antibacterial properties of bioactive glass. The bioactive glass, as a bone graft material can be used, with good results, on a rabbit model, but the anatomical area of the intervention must be selected carefully to avoid possible complications. The studies related to ionic release from bioactive glass at physiological pH support the conclusion that these ions lead to a better osseointegration process. Also, it was demonstrated that bioactive glass S53P4 present a different biodegradation in the presence of metallic implants than in the case where is used alone into the bone.

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