REPAIR OF ARTIFICIAL BONE DEFECTS USING GUIDED BONE REGENERATION WITH BIO OSS AND ENAMEL MATRIX DERIVATIVE. I. LIGHT MICROSCOPIC STUDY ON LONG BONES IN RABBITS

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Summary


Guided bone regeneration (GBR) is most effective when the material used has osteogenic, osteoinductive and osteoconductive properties. Xenografts have limited biological properties, so a solution for diversification the properties of Bio Oss® was sought by combining with Emdogain®. The purpose of the present work was to study the process of GBR involving a combination of Bio Oss and Emdogain® compared with their independent use. An in vivo experiment was conducted with 10 New Zealand white rabbits. Artificial bone defects in the tibias and femurs of each rabbit were created. Depending of the used grafting materials, six experimental groups were formed: groups 1 and 2 (Bio Oss®); groups 3 and 4 (Emdogain®) and groups 5 and 6 (Bio Oss® + Emdogain®). In control groups (A and B), osseous coagulum was used as grafting material. Prior to apposition and soft tissue suturing, all bone defects were covered with Bio Gide® membrane. Half of the rabbits (groups 1, 3, 5 and control group A) were euthanised at the end of the third, and groups 2, 4, 6 and control B – of the fourth month. There were no significant quality differences between experimental and control groups by the end of month 3 or 4. Osteoblasts, osteoclasts, osteoid and woven bone were found in all the samples studied. Maturing woven and lamellar bone were found in samples treated with Bio Oss® + Emdogain®. Newly formed bone was in close contact with Bio Oss® particles. Connective tissue around the Bio Oss® particles was found only in the groups in which it was the sole material used. There was neither evidence for degradation of Bio Oss® particles, nor for inflammation. The combination of osteoconductive properties of Bio Oss® with osteoinductive potential of Emdogain® benefited the formation of new woven bone, its transformation into lamellar and the osteointegration of Bio Oss® particles.

Key words: bone, Bio Oss®, Emdogain®, histology, osteoconduction, osteoinduction, xenografts
INTRODUCTION

The medico-biological grounds of bone grafting are three primary biological properties, namely osteogenesis, osteoinduction and osteoconduction. Only autogenous bone (autograft) possesses these three properties at the same time (Pappalardo et al., 2007). Therefore, having in mind this biological complexity, autografts are the materials of choice. From a medical point of view however, this is not always a rational solution due to the need for second operation for its collection, the risk from complications, patients’ discomfort, problems related to insufficient autograft amount (Nasr et al., 1999). For correction of small defects – alveolar bone volume preservation after tooth extraction or interdental septum resorption, the use of an autograft is not justified (Pappalardo et al., 2007). In such instances, bone substitutes are utilised.

Biological disadvantages are overcome with xenografts (Baldini et al. 2011). Deproteinised mineralised bovine bone manufactured under the trade name Bio Oss® is a xenograft, which is currently among the most commonly used bone substitutes in dental practice (Baldini et al., 2011). This is mainly due to its osteoconductive properties (Hammerle et al., 1998; Hallman et al., 2002). There is a certain discrepancy in data about the effect of Bio Oss on new bone formation. According to some researchers, Bio Oss particles are incorporated in the newly formed bone, and are in close contact with the new woven and/or lamellar bone (Pettinicchio et al., 2012). Others claim that particles are surrounded by both newly formed bone and connective tissue (Carmagnola et al., 2003; Gisakis et al., 2012) and thirds – no new bone is formed around Bio Oss particles (Carmagnola et al., 2000). This provided reason to seek ways for diversification of biological properties of Bio Oss. Several studies report for combinations between Bio Oss and fibrinogen-thrombin complex (Zitzmann et al., 2001), autogenous bone (Urban et al., 2013), platelet-rich plasma (Froum et al., 2002), growth factors (Roldan et al., 2004a,b). The experiments for combining Bio Oss with growth factors as bone morphogenetic protein were evaluated as rather expensive and not very successful, as these factors are released in a cascade-like manner under natural conditions – a phenomenon, which could hardly be reproduced after mixing with Bio Oss – and due to irrelevant clinical results (Halman & Thor, 2008).

Endogain® is a preparation consisting of enamel matrix derivative (EMD) from developing pig teeth, water and propylene glycol alginate (PGA) carrier. It is a typical xenograft. Its biological properties are evaluated as osteogenic (Bosshardt, 2008) or osteoinductive (Shimizu-Ishiura, 2002). According to Esposito et al. (2009) these properties are attributed to its BMPs. In vivo experiments have shown that the use of Endogain® and guided bone regeneration (GBR) improve bone regeneration and bone volume (Donos et al., 2003).

Regardless of the multiple reports on bone substitutes and GBR, the attempts for finding a suitable xenograft or xenografts combinations for replacement of autografts are still continuing. They aim at achieving the best possible results without posing risk for immune or other diseases in the host together with the possibility for harvesting the needed amounts without adverse effects on the health and psychics of subjects.

The purpose of the present investigations was to perform a light microscopic histological evaluation of guided bone
reparation using Bio Oss® resorbable membrane, and bone substitutes Bio Oss®, Emdogain® (either solely or in combination) in artificial tibial and femoral bone defects in rabbits. It is hypothesised that the osteoconductive properties of Bio Oss® would add to the osteoinductive potential of Emdogain® for achieving a better bone formation quality compared to the use of Bio Oss® only.

MATERIALS AND METHODS

All experimental operative interventions were compliant to Directive 2010/63/EU on the protection of animals used for scientific purposes.

Experimental animals

The experiments were conduced in 10 New Zealand White male castrated rabbits, weighing 4–5 kg, 6 months of age. Animals were previously vaccinated and treated against ecto- and endoparasites. They were housed in individual cages under conditions compliant to animal welfare requirements.

Before the interventions, hair of hindlimbs was clipped and operation field was aseptically prepared. To reduce the risk of infection, the animals were preoperatively treated i.m. with 7 mg/kg gentamicin.

The induction in general anaesthesia was done with i.m. tiletamine hydrochloride/zolazepam hydrochloride (Zoletil®, Virbac Sante Animale, France) – 15 mg/kg. Fifteen minutes later, 5 mg/kg xylazine hydrochloride (Xylazin®, Alfasan International B.V., Dopharma, Netherlands) was i.m. applied and anaesthesia was maintained with 2–3 vol % isoflurane (AErran®, Baxter d.o.o., Ljubljana, Slovenia) through a mask. Fluid therapy was administered during the time of the operation with physiological saline at 10 mL/kg/h (Natrium Chlorid Braun 0.9% (B. Braun Melsungen AG, Melsungen, Germany).

The animals were fixed in lateral recumbency. The surgical approach to the proximal tibial metaphysis was medial, and to the proximal femoral metaphysis – lateral. After reaching the cortical bone, four bone defects (cavities) per animal were created, total of 40 defects.

Experimental design

Bone defects were created with bone cutters, outer diameter 4 mm, at 5 mm depth under continuous saline flush cooling.

The distribution of experimental and control specimens according to used grafting materials and the experimental period duration is presented in Table 1. Bone defects of experimental groups 1 and 2 were filled with Bio Oss®, particle size 0.25–1 mm (Geistlich Pharma AG), whereas the defects of groups 3 and 4 were filled with Emdogain® (Straumann Emdogain®, Institut Staumann AG, Switzerland). Mixture of both materials, prepared ex tempore (0.3 mL Emdogain® is added to the flask containing 0.5 g Bio Oss®), was placed in the bone defects of experimental groups 5 and 6. Control and experimental defects were covered with resorbable membrane Bio Gide® (Geistlich Pharma AG).

Soft tissues were sutured – the peristeum and muscles with a thinner continuous absorbable suture and skin wound – with thicker simple interrupted non-absorbable sutures. Animals were treated post operatively for 5 consecutive days with 10 mg/kg enrofloxacin (Baytril® 5%, Bayer Animal Health GmbH, Leverkusen, Germany). They were housed in individual cages, fed pelleted food at a daily amount according to manufacturer’s recommenda-
Water was offered *ad libitum* in automated watering troughs mounted inside the cages.

The animals were euthanised by intravenous injection of Euthanasin N until effect – half of them by the end of the 3rd month and the other half – by the end of the 4th month (Table 1).

**Preparation of specimens for light microscopy**

Specimens 10×10×5 mm in size were obtained for light microscopy. After fixation with 10% formalin for 30 days, they were washed in running tap water for 24 h, demineralised in 8% nitric acid as per classic methods. To protect fibrinous structures after bone demineralisation, the specimens were placed in 5% sodium sulphate solution for 24 h. The embedding was done in celloidin after dehydration in an ascending ethanol series (12 h in each concentration, the last one in absolute ethanol) followed by 12-hour stay in a 1:1 mixture of absolute ethanol and ether. Material impregnation was done gradually in 2%, 4% and 8% celloidin dissolved in ether+ethanol, 5 days in each concentration. It was waited until the working solution concentration became between 8% and 16%, and then samples were placed in fresh 16% celloidin solution. Cross sections of 10 µm were stored in 70 °C ethanol and were stuck onto glass slides immediately prior to staining with Mayer's acid haemalum.

**RESULTS**

The post operative period was smooth, without complications. Two weeks after removal of sutures, the operative zones were not oedematous or reddened. Skin cicatrices were visible, without infection or inflammation.

In general, Bio Oss® particles were relatively well distinguished from the adjacent tissues. Their homogeneous, yet porous structure could be observed at numerous sites. Most structure and cell elements, accepted as histomorphological criteria (presence of connective tissue, osteoblasts, osteoclasts, woven and lamellar bone) were established in both control and experimental groups. In all studied specimens, there were no inflammatory cells. Data about osteoclastic resorption of Bio Oss® particles were not available.

The membranes were resorbed completely, without remnants.

Specific histological data are presented together for experimental and controls groups by the end of the 3rd and 4th month

<table>
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<th>Xenografts</th>
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<td>4 months</td>
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A specific finding was the presence of connective tissue, represented mainly by irregularly arranged collagen fibres around Bio Oss® particles (Fig. 1). It restricted the immediate contact between the xenograft and woven bone on months 3 and 4 (Fig. 2). In some areas, connective tissue spicules could be seen between particles and woven bone (Fig. 3) and in others, woven bone was immediately contacting Bio Oss® particles. The limited woven and lamellar bone areas were mainly the periphery of the bone defect, adjacent to healthy bone. No connective tissue cells could be found. Resorption of Bio Oss® particles was lacking.

In experimental groups 3 and 4, where Emdogain® was used as xenograft, osteoblasts and osteoclasts were also present. There is evidence for woven bone in specimens obtained by months 3 and 4. The immature woven bone was built from a large amount of disordered collagen fibres and osteogenic cells. Disordered collagen fibres formed a dense network. Until the end of the third post operative
In experimental groups 5 and 6, grafted with mixture of both tested xenografts, Bio Oss® particles were surrounded by woven or lamellar bone. The findings with adjoining lamellar bone were more frequent in histological preparations by the end of the 4th month. The contact bet-

Fig. 3. Light microscopy findings in Group 1 (Bio Oss+Bio Gide, 3rd month). Fibrous spicules (D) of woven bone (B) overgrown towards a Bio Oss particle (A). Bar = 70 μm.

Fig. 4. Light microscopy findings in Group 3 (Emdogain+Bio Gide, 3rd month). Clusters of osteoblasts, presence of collagen fibres and amorphous substance.

Fig. 5. Light microscopy findings in Group 5 (Bio Oss+Emdogain+Bio Gide, 3rd month). Close contact between a Bio Oss particle (A) and newly formed woven bone (C). B – osteoblasts. Bar = 40 μm.

month, there were neither osteons nor lamellar structures. The amorphous intercellular substance was abundant (Fig. 4). At some locations, limited areas with lamellar bone could be seen on specimens collected by month 4.
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*Fig. 6.* Light microscopy findings in Group 5 (Bio Oss+Emdogain+Bio Gide, 3rd month). Newly formed lamellar bone (B) in contact with a Bio Oss particle (A). C – osteoblasts; D – osteocytes. Bar = 60 μm.

...ween woven or lamellar bone and Bio Oss® was close, immediate. Fig. 5 presents a specimen from the 3rd month, where a xenograft Bio Oss® particle was in intimate contact with woven bone. The porosity of the particle was visible. Woven bone was represented by irregular collagen fibres. Bone cells could be observed in the adjacent tissue. Fig. 6 and 7 illustrate an intimate immediate contact between newly formed lamellar bone and Bio Oss® particles. By the end of the 4th month, osteons undergoing maturation were established. The number of osteoblasts and lamellar bone amount increased. Haversian canals (Fig. 8) could be observed. Around the osteoclasts, resorption lacunae not involved in Bio Oss® resorption were present. New bone formation was particularly marked in cortical bone.

In samples from control groups A and B, newly formed bone with small areas of woven bone could be observed, but lamellar bone with formed Haversian systems were predominant. In the centre of each system, a Haversian canal containing a small blood vessel could be seen, with concentric bone tissue lamellae, among which osteocytes, located within lacunae were compressed. At some locations, parts of projections lying within the ductules of the common microcanal system, passing through the intercellular matrix were seen.

**DISCUSSION**

The present study was aimed to establish, by light microscopy, the bone reaction to two xenografts Bio Oss® and Emdogain® in an experimental study with rabbits. The body weight of experimental animals was appropriate with regard to long bone volume. This is a prerequisite for an experimental design using large bone defects. The rabbits were neutered because the trial was aimed to investigate bone repair processes. Thus, the effect of testosterone, which is involved in bone metabolism, was excluded and bone healing was protected from additional hormonal influences and was mainly dependent on the relationship between the graft and host.
Bone grafts were applied through GBR and a barrier membrane. This approach was based on the acknowledged fact that the independent use of bone substitutes was unsatisfactory and that the healing of defects covered with membrane yielded better results (Jensen et al. 1995), including the case with Bio Oss® (Hammerle et al., 1998).

The analysis of data from the present xenograft implantation in experiment tibial and femoral bone defects in rabbits had two objectives: to evaluate the alterations in the xenograft itself, as well as the host reaction (formation of new structures and their relationship with transplanted xenograft, especially Bio Oss®). With regard to the xenograft, none of examined experimental specimens involving Bio
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Oss®, i.e. experimental groups 1, 2, 5 and 6 (Table 1) has shown signs of resorption events.

The results corresponded to previously published reports, according to which the host response to GBR with the used xenografts was normal from medico-biological point of view (Froum et al., 1998; Valentini et al. 2000; Karabuda et al., 2001; Wallace & Froum, 2003). The membranes have fulfilled their role and were completely resorbed. In repaired bone defects, there were no tissue elements from the adjacent soft tissues.

The specifics of this extraoral, subcutaneous bone experiment with barrier membranes possibly caused the used barrier membranes to remain unrevealed and well protected throughout the observation period. This way, bone repair events were protected from infection and inflammation, similar to the observations of other researchers when the membrane was removed or its integrity – impaired. Similar complications were determined as an important cause for the poor results from intraoral experimental and clinical observations (Dahlin et al., 1995).

The lack of data for inflammation in bone defects repaired with xenografts could be explained with some of their biological properties. In specimens treated with Emdogain®, this could be attributed to its proven antimicrobial effect (Spahr et al., 2002). The fact that there were no signs of inflammations in the other experimental groups further supports the biological compatibility of Bio Oss with host tissues, as also reported by other researchers (Liu et al., 2011).

Histomorphological data about host response to used xenografts indicated that GBR occurred without complications. There were no cell inflammatory infiltrates, in agreement with earlier reports (Piattelli et al. 1999; Orsini et al. 2007; Pettinicchio et al. 2012).

Bone defects repair in the different experimental and control groups occurred in a different manner and had a different histomorphological outcome.

After the independent use of Bio Oss®, some particles were surrounded by connective tissue observed as irregularly arranged collagen fibres. For others, they were at the particle-woven bone interface, or in intimate contact with woven bone. This could raise two possible interpretations: in some areas the connective tissue development has either occurred earlier than that of woven bone at particles’ surface or there, the mineralisation of collagen fibres and their transformation into woven bone was delayed. An additional argument supporting the latter hypothesis was the lack of fibroblasts, specific for connective tissue and the presence of osteoblasts. In intraosseous dental implants, a connective tissue capsule isolating the implant from the adjacent bone was reported and interpreted as impaired osteointegration (Baldini et al. 2011). This could hardly happened in the present study due to the lack of micromotility of implants, which according to the authors had caused the formation of fibrous capsule. Therefore, the artefact observed around Bio Oss® could be evaluated as delayed osteointegration but not as impaired osteointegration of xenograft particles. The independent use of Emdogain® resulted mainly in woven bone formation. At some sites, limited areas of lamellar bone were observed by the end of the 4th month. It is acknowledged that the enamel matrix derivatives (Emdogain®) possessed osteoinductive properties (Bosshardt, 2008). In the context of this study, it is not important whether they were due to BMP-2 (Esposito et al., 2009) or the main con-
stitute of Emdogain® – amelogenin (Venezia, 2004) or other proteins from developing pig teeth (Boyan et al. 2006). The osteoinduction potential is sufficient for accumulation of cells, but the process of new bone formation is not enough active without the presence of a grid through which they penetrate, proliferate, undergo transformation into bone formation cells. This hypothesis is also confirmed by data that EMD initially resulted in increased amount of bone. The amount of newly formed bone decreased by the end of the first month (Shimizu-Ishura et al., 2002). Furthermore, there is evidence that the independent EMD application did not improve the status of intraosseous defects (Zucchelli et al., 2003).

The combination of Bio Oss® and Emdogain® results in faster and more intensive bone formation and remodelling. The result is woven bone formation and its transformation into lamellar bone, presence of osteons and Haversian canals. This could be attributed to the biological properties of Bio Oss® – a biologically compatible product (Baldini et al., 2011), whose appropriate pore size and length (Piattelli et al. 1999; Indjova, 2010) serves as a grid for penetration and transformation of mesenchymal cells, and their accumulation and differentiation was potentiated by osteoinductive biopotential of Emdogain®. Other researchers also reported a more intense osteogenesis in cortical bone (Araujo et al., 2008). This is imperative for minimisation of volume changes after tooth extraction, an important condition for alveolar bone volume preservation for subsequent implantation.

Apart the combination of osteoconductive biological properties of Bio Oss® with osteoinductive ones of Emdogain®, a third phenomenon could also be involved in resulting bone formation and remodelling. Pettinicchio et al. (2012) affirmed that apart being available in unlimited amount, non-autogenous grafts could serve as carriers of drugs, hormones, growth and progenitor cells. In their study, Jung et al. (2003) added rhBMP-2 to deproteinised mineralised bone (Bio Oss®). The mixture served for treatment of bone defects around intraosseous implants. The combination improved bone regeneration and it was accepted that Bio Oss® was an appropriate carrier of rhBMP-2. The hypothesis that Bio Oss® is a suitable vehicle for Emdogain® gel due to its solid consistency and porous structure is therefore consistent.

Our results agreed with those from a clinical experiment, providing clinical and radiological evidence that the addition of Emdogain® to Bio Oss® improved the repair processes in intraosseous defects (Zucchelli et al., 2003).

The results from the present study did not however correspond to those of Donos et al. (2004, 2005), suggesting that Emdogain® did not contribute with osteoinduction potential to Bio Oss® when mixed prior to application in periodontal bone defects, including in cases with GBR. The different results could be attributed to the fact, that Donos et al. (2004, 2005) applied the combination for treatment of resorptive alterations of the periodontium whose complex morphological structure probably impeded further the reconstruction events (Baldini et al., 2011).

CONCLUSIONS

On the basis of the results from the present study, it could be concluded that:

1. The osteointegration of Bio Oss® particles was better when the combination of Bio Oss® and Emdogain® was applied for repair of artificial bone de-
fects as compared to using Bio Oss® or Emdogain® only.

2. Bio Oss® could be assessed as a suitable carrier of Emdogain®, as the osteoconductive properties of the former xenograft were added to the osteoinduction potential of the latter biomaterial.

3. No evidence that any form of resorption of Bio Oss® has occurred, could be presented.

4. The healing after application of Bio Oss® or Emdogain®, used either independently or together for guided bone regeneration was not accompanied by inflammatory reactions.

5. The lack of inflammatory reaction and the immediate contact between the particles and newly formed bone structures proved the biological and osteoconductive properties of Bio Oss®.

ACKNOWLEDGMENTS

The experiment have been financially supported by Grant No. 8/2012, Medical University – Sofia, Council of Medical Science.

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Paper received 03.07.2013; accepted for publication 11.09.2013

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