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# Histomorphometric Studies of the Healing Process in Artificial Bone Defects in Rabbit Long Bones

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Histomorphometry was used for evaluation and quantitative measurement of the types of new bone formation in artificial defects created in long bones of rabbits. The aim of the study is monitoring the amounts of new woven and lamellar bone formed in artificial bone defects treated with various combinations of Bio Oss and Emdogain under guided bone regeneration. Forty bone defects were created in the hindlimbs of 10 rabbits. The amount of woven and lamellar bone increased between post treatment months 3 and 4 both after independent and combined application of both xenografts. The amount of lamellar bone was the greatest in natural healing with coagulum and after treatment with combination of Bio Oss+Emdogain. Bio Oss+Emdogain combination could be used to preserve the volume of alveolar bone and at the same time to create an environment for placing intraosseous dental implants when the available healing time was over 4 months.

*Key words*: experimental model, histomorphometry, Bio Oss, Emdogain

## Introduction

The survival of dental implants is closely related to the achieved primary stability [9]. It is a function of the amount and quality of bone at the site of implantation and more specifically, the compact bone thickness and presence of lamellar bone [7]. For oral dental implants, the main structural elements relevant for the primary and later, for the secondary stability, are the newly formed woven and lamellar bone [10].

For evaluation of new bone formation, various techniques are used: quantitative computed tomography, histological and histomorphometric examinations [6].

Quantitative computed tomography has the disadvantage of providing no information about the ratio between bone components, e.g. for the fine bone structure. By means of histology, the type of the newly formed bone elements could be established although no data about quantitative ratios are available [6]. Histomorphometry allows

the determination of the amount and relative proportion of the new bone formation (woven and lamellar bone) and other structures (connective tissue, transplant materials) on histological specimens.

The reports with quantitative histomorphometric data are only few [9]. What is more, to the best of our knowledge, there are no data from histomophometrical assessment of defect healing with different combinations of Bio Oss and Emdogain of more than 4 months' duration.

The aim of the present study was to monitor the time course of quantitative parameters of bone defect healing in a rabbit experimental model, notably the relative proportions of formed woven and lamellar bone in groups treated with guided bone regeneration and different combinations of Bio Oss and Emdogain xenografts over 4 months.

## Materials and Methods

**Experimental animals.** Ten New Zealand White rabbits were used as experimental subjects. The long bones of hindlimbs (femur and tibia) were used. Five rabbits were euthanised by the end of the 3rd month and the other five – by the end of the 4th month. The experiments were approved by the Research Ethics Committee at the Medical University – Sofia (permit No. 77/02. 05. 2012).

**Experimental design.** Bone defects were created with a bone cutters with outer diameter 4 mm, labelled at 5 mm for achieving a uniform depth of penetration.

**Anaesthesia protocol.** The induction in general anaesthesia was done with 15 mg/kg i.m. Zoletil. Fifteen minutes later, 5 mg/kg Xylazine was applied i.m. Anaesthesia was maintained with Isoflurane through a mask until the end of the surgery. All operative interventions were done under strict aseptic conditions.

**Tibial and femoral grafts.** Skin incisions' size was up to 3 cm. Experimental rabbits were fixed in lateral recumbency. After successive dissection of tissues, the cortical surface of the underlying bone was exposed. One bone defect was created in each of the two femurs and tibias per animal – a total of 4 bone defects in a rabbit, 40 for all 10 animals. The total number of experimental defects was 36, distributed into 6 groups and filled with Bio Oss, Emdogain or Bio Oss+Emdogain. The total number of control defects was 4 – distributed in 2 groups, healed with coagulum. The tissues over the operative defects were sutured in layers. During the first 3-5 post operative days, the rabbits were treated with Baytril at a dose of 1 ml/10 kg body weight.

**Histology and histomorphometry.** The animals were euthanised by intravenous injection of Euthanasin "N" until cessation of respiratory and cardiac activity. The respective regions of the limbs were dissected and surrounding soft tissues – removed. The specimens were stored in 10% formalin. Preparations for light microscopy were made.

Histomorphometric evaluation of bone defect healing treated with various xenograft(s) was conducted on permanent preparations for light microscopy. The ImageJ software, developed by the National Institute of Health, USA was used for this purpose. ImageJ is an open-source software that could be used on a computer with Java pre-installed for processing and morphometric analysis of images. All useless elements in the analysed field that are not relevant and could influence adversely the final result, are removed. A monochromatic mask of the analysed area is then created. This allows the measurement of the number of pixels in the studied field. The mask analysis function is used for determination of the area of studied objects in pixels. The results are entered into a table for calculation of the relative share of studied objects from the entire visible field.

**Statistical analysis.** Values are presented as mean ± standard deviation. The normal pattern of distribution within a group was tested with the Kolmogorov-Smirnov test. The differences between means were assessed using GraphPad InStat 3.0 statistical software.

## Results

The central parts of the histological preparations corresponding to central parts of the bone defect were chosen for evaluation of new bone formation quality in order to decrease the influence of the natural bone around the defect [10]. All experimental and control groups exhibited changes in the amount of formed woven and lamellar bone between the 3rd and the 4th month ($p < 0.001$), **(Table 1)**.

**Woven and lamellar bone volume by post treatment month 3**

The time course of the two types of newly formed bone – woven and lamellar was the same during the first observation period. The smallest proportion of newly formed bone structures was established in defects healed with Emdogain only, followed by those healed with Bio Oss only, the combination Bio Oss+Emdogain and finally, in specimens healed naturally with coagulum.

At the end of the 3rd month, the differences between the relative shares of woven bone were statistically significantly different between defects treated with Bio Oss+Emdogain vs. Emdogain only ($p < 0.001$) and between the proportion of the control group versus the treatment groups with self-administration of Bio Oss and Emdogain ($p < 0.001$). There were no considerable difference between the woven bone volumes in Bio Oss+Emdogain and control groups ($p>0.05$), as well as between those with either

**Table 1.** Relative proportion of woven and lamellar bone in bone defects with respect to the applied treatment and post treatment period, and statistical significance of differences between the 3rd and the 4th month

| **Xenografts and coagulum→** | **Bio Oss Bio Gide** | | **Emdogain Bio Gide** | | **Bio Oss Emdogain Bio Gide** | | **Coagulum Bio Gide** | |
|---|---|---|---|---|---|---|---|---|
| **Periods ► Structures ▼↓** | **month 3** | **month 4** | **month 3** | **month 4** | **month 3** | **month 4** | **month 3** | **month 4** |
| **Woven bone** | 16.58 ±1.00 | 23.75 ±1.14 | 13.66 ±1.07 | 18.50 ±1.45 | 28.58 ±1.17 | 32.75 ±1.22 | 36.50 ±1.24 | 42.17 ± 1.03 |
| **Month 3 vs. month 4** | $p < 0.001$ | | $p < 0.001$ | | $p < 0.001$ | | $p < 0.001$ | |
| **Lamellar bone** | 10.58 ±1.17 | 28.08 ±1.17 | 8.83 ±0.83 | 16.58 ±1.17 | 19.67 ±1.23 | 33.25 ±1.49 | 28.67 ±1.67 | 52.83 ±1.19 |
| **Month 3 vs. month 4** | $p < 0.001$ | | $p < 0.001$ | | $p < 0.0011$ | | $p < 0.0011$ | |

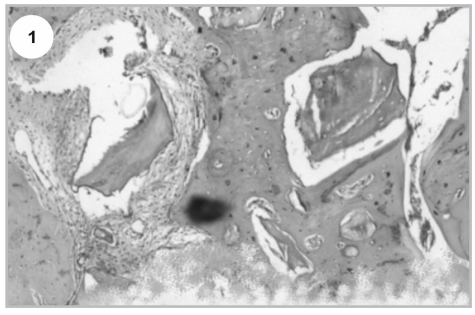


**Fig. 1.** Histology of a section of bone defect treated with GBR+Bio Oss+Emdogain 3 months after the start of the experiment

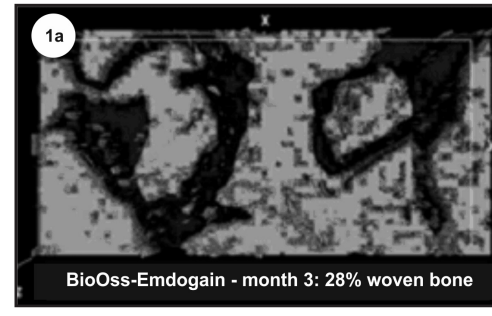


**Fig. 1a.** Histomorphometry of woven bone formed in the defect treated with GBR+Bio Oss+Emdogain 3 months after the start of the experiment

Bio Oss or Emdogain only ($p > 0.05$) and Bio Oss+Emdogain vs Bio Oss - ($p>0.05$), (**Figs. 1** and **1A**)

By the end of the 3rd month, the differences of relative amounts of lamellar bone were comparable to those observed for the woven bone (**Figs. 2 and 2A**).

**Woven and lamellar bone volume by post treatment month 4**

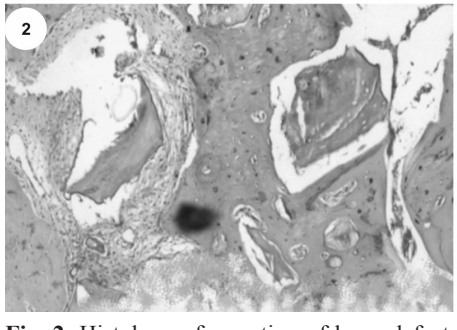


**Fig. 2.** Histology of a section of bone defect treated with GBR+Bio Oss+Emdogain 3 months after the start of the experiment

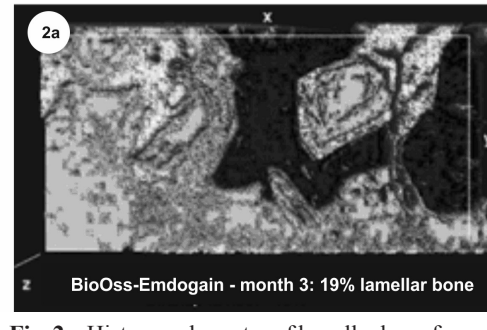


**Fig. 2a.** Histomorphometry of lamellar bone formed in the defect treated with GBR+Bio Oss+Emdogain 3 months after the start of the experiment

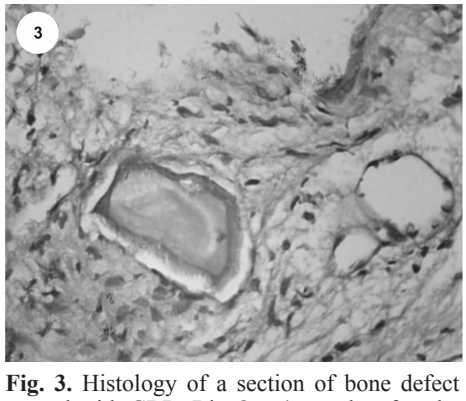


**Fig. 3.** Histology of a section of bone defect treated with GBR+Bio Oss 4 months after the start of the experiment

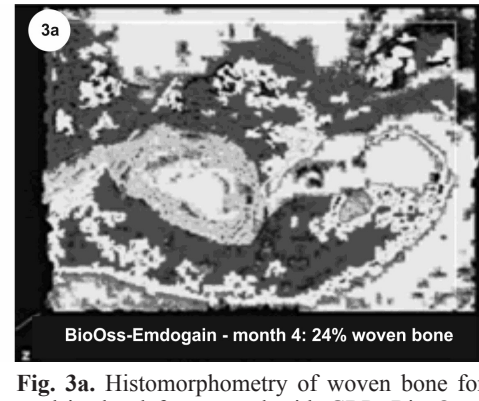


**Fig. 3a.** Histomorphometry of woven bone formed in the defect treated with GBR+Bio Oss 4 months after the start of the experiment

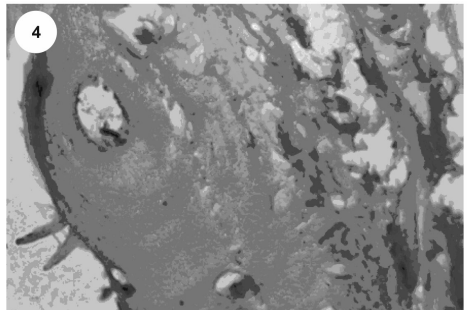


**Fig. 4.** Histology of a section of bone defect treated with GBR+Bio Oss 4 months after the start of the experiment

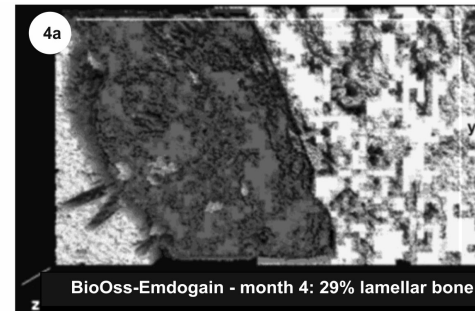


**Fig. 4a.** Histomorphometry of lamellar bone formed in the defect treated with GBR+Bio Oss 4 months after the start of the experiment

By the end of the 4th month, the relative proportions of woven and lamellar bone formation increased from the group treated with Emdogain, through the groups with Bio Oss and Bio Oss+Emdogain until the control group with naturally healed defects.

Four months from the beginning of the experiment, the differences between the relative amounts of woven bone differed very significantly among all experimental groups as well as between each of experimental groups vs controls ($p < 0.001$) (**Figs. 3** and **3A**).

At this time interval, the comparison of relative shares of lamellar bone in the different groups showed statistically significant differences between the groups of defects healed with Bio Oss+Emdogain and Emdogain only ($p < 0.001$), as well as between control defects and the groups with either Bio Oss or Emdogain only ($p < 0.001$). No significant differences were found out between the groups treated independently with Bio Oss and Emdogain ($p > 0.05$), and between Bio Oss+Emdogain vs Bio Oss only ($p > 0.05$) (**Figs. 4** and **4A**).

## Discussion

In all studied groups (experimental and control), the differences between newly formed woven and lamellar bone by the end of the 3rd month were very different from those observed one month later at a very high level of statistically significance ($p < 0.001$ – **Table 1**) and reflect the effect of experiment's duration. Similar results are reported by numerous investigators [2].

In all groups, osteogenesis was expressed at a various extent. Initially, by the end of the 3rd month, all groups exhibited enhanced formation of woven bone as compared to lamellar bone (**Table 1**). Woven bone is formed relatively rapidly – at a daily rate of 30-50-60 μm. That is why it is irregularly organised. It also contains cells. These features speak about a immature bone structure. Its mechanical strength is low, partly compensated by its relatively good mineralisation. Although with a small mechanical strength, the biological significance of woven bone is substantial. It is gradually replaced by lamellar bone [4] (**Table 1**). By the 4th month, the opposite event was observed: the amount of lamellar bone increased as compared to the woven bone. Lamellar bone is the main component of mature cortical and trabecular, cancellous bone. Lamellae are formed slowly (at a daily rate of < 1.0 μm). Its collagen network is well arranged and mineralised. Lamellar bone is composed by numerous unidirectionally oriented layers, termed lamellae [4].

On the basis of our results (**Table 1**) the greatest proportion of newly formed woven and lamellar bone was observed in defects healed naturally with coagulum, which affirms the leading importance of the guided bone regeneration [11]. One of advantages of placing a barrier membrane over the coagulum was its stabilisation. Thus, a natural spatial scaffold, facilitating the osteogenesis, is created [3]. Despite the advancement of biotechnologies and bone tissue engineering, the developed and widely implemented grafts Bio Oss and Emdogain could not compete with natural healing within the framework of this experiment as the quality of newly formed bone was concerned.

The results obtained with the combination Bio Oss+Emdogain were attributed both to the synergic effect of the advantages of each of the two grafts [14, 15] – osteoconductive properties of Bio Oss and osteoinductive properties of Emdogain [3], as well as to the biological properties of the latter superimposed on Bio Oss. Additionally, at the time of the preliminary mixing of both materials, the staining of Bio Oss granules with blood from the wound that could impair the adhesion, proliferation and differentiation of osteoblasts on the graft's surface, is avoided [8]. The presence of molecules resembling bone morphogenetic proteins (BMP) in Emdogain contribute to attraction, proliferation and differentiation of osteogenic cells' precursors into osteogenic cells. The studies provide evidence for the presence of limited amount of organic proteins in Bio Oss, regardless of the deproteinisation of the material. According to their biological behaviour, they are determined as BMP-2 [12]. The osteoinductive effect of Bio Oss is not exhibited as it is small, but the addition of Emdogain with its osteoinductive properties rendered the array of biological properties of the combination more complete and diverse. The more obvious osteogenesis, established previously with the combination of both grafts, allowed assuming that Bio Oss was an appropriate carrier of Emdogain.

The greater although statistically insignificant amount of the formed woven and lamellar bone with Bio Oss compared to Emdogain could be attributed to the higher porosity of the graft resembling the structure of the natural cancellous bone, its osteoconductive properties and biological compatibility. The Bio Oss granules placed inside the defect separate the space into smaller compartments. Thus, vascularisation and osteogenesis are facilitated [11]. At the same time, the excessive compression of Bio Oss granules impairs these events [1], which is a probable explanation for the reduced bone formation rate when defects are filled with Bio Oss only compared to healing with the combination.

The smaller amount of newly formed woven and lamellar bone in the group treated with Emdogain could be the decreased intensity of the initial active formation of new bone after the 14$^{th}$ day observed by Shimizu-Ishiura et al. [13] as well as the probability for predominance of osteoconductive properties of grafts reported by Hockers et al. [5] which were not present in Emdogain.

## Conclusion

The histomorphometric studies demonstrated repair of bone in all control bone defects and in defects healed with the combination Bio Oss+Emdogain. The relative proportion of mature bone structures, which are essential for the stability of implants, was significantly greater in control groups followed by Bio Oss+Emdogain combination compared to the independent use of either of grafts. The duration of healing was also of primary significance. The proportion of lamellar bone was statistically significantly higher by the 4$^{th}$ compared to the 3$^{rd}$ month. Regardless of the success of bone tissue engineering, the healing quality with xenografts Bio Oss and Emdogain was inferior to repair under GBR and coagulum.

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