Evaluation of the Bone Repair Capacity and the Cytotoxic Properties of a Particular Xenograft: An Experimental Study in Rats

Partiküler Xenograftın Kemik Yenileme Kapasitesi ve Sitotoksik Özelliğinin Değerlendirilmesi: Sıçanlar Üzerinde Deneysel Çalışma

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ABSTRACT Objective: Bone grafts have been widely used in osseous defects in medicine and dentistry (e.g. periodontology, implantology). This study aims to assess bone and tissue response to a xenograft (Unilab Surgibone) after the first and third month of implantation and its cytotoxicity in vitro. **Material and Methods:** Fifteen rats, each of which having a 5 mm critical-size, symmetrical, circular defects in the corpus of the mandible were recruited. The experimental defects were filled with the biomaterial whereas defects on the opposite side were used as controls (sham-operated group). After histological follow-up, serial sections were prepared for evaluation under light microscope. The cytotoxicity of xenograft was examined in vitro. **Result:** Xenografts did not show any cytotoxicity for the cells. There was no callus formation in the implantation site at the first and third months, and Unilab Surgibone material was surrounded with fibrous tissue. Dense collagenous tissue was observed in the control site at the first and third months. **Conclusion:** Although there was no evidence of bone building, the biomaterial seems biocompatible and can be considered as a space filler for such applications in dentistry.

Key Words: Bone and bones; cytotoxicity tests, immunologic; dental prosthesis repair; osteogenesis

ÖZET Amaç: Kemik greftler kemik defektlerinde tıp ve diş hekimliğinde yaygın olarak kullanılmaktadır (örneğin periodontoloji, implantoloji). Bu çalışma bir xenografta (Unilab Surgibone) implantasyondan sonraki birinci ve üçüncü ayda kemik ve doku yanıtını değerlendirmeyi amaçlamaktadır. **Gereç ve Yöntemler:** Mandibula korpusunda 5 mm kritik boyutlu simetrik dairesel defektlere sahip 15 sıçan çalışmaya alınmıştır. Çalışmaya esas defektler biyomateryal ile doldurulurken, karşı taraftaki defektler kontrol olarak kullanıldı. Histolojik takip sonrasında, ışık mikroskobu altında değerlendirme için seri kesitler hazırlandı. Xenograft sitotoksisitesi in vitro incelendi. **Bulgular:** Xenograftlar hücreler için herhangi bir sitotoksisite göstermedi. İmplantasyon alanında birinci ve üçüncü ayda kallus oluşmadı, ve Unilab Surgibone malzemesi fibröz doku ile çevrildi. Kontrol alanında birinci ve üçüncü ayda yoğun kollajenöz doku gözlendi. **Sonuç:** Her ne kadar kemik yapımına dair hiçbir kanıt olmasa da, biyomateryal biyouyumlu görünmektedir ve diş hekimliğinde bu tür uygulamalar için bir boşluk doldurucu olarak düşünülebilir.

Anahtar Kelimeler: Kemik ve kemikler; sitotoksisite testleri, immünolojik; diş protezi tamiri; osteogenez

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Bone repair is a major challenge for reconstructive surgery. The reconstruction of segmental mandibular defects following trauma or ablative surgery remains controversial because no single method results in the replacement of tissue that precisely matches the quantitiy or structural qualities of autogenous mandibular bone. Moreover, current

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methods of reconstruction have their attendant risks.[1,2]

All applications in tissue engineering techniques over the past decade have enabled the *in vivo* regeneration of living bone in many animal models[1]. Ideally, bone grafts or substitutes should be sterile, non-toxic, and should also show no immunological reactions. Indeed, they should be fully integrated into the recipient bone and no evidence of encapsulation by connective tissue should be seen.[3] Various types of xenografts have been used in medicine, dentistry, and also in periodontology. One of the xenografts is Unilab Surgibone, which is currently being used succesfully in medicine and implantology. Moreover, its osteoconductive properties are also known.[4] Unilab Surgibone is obtained from freshly sacrificed calves which is partially deproteinized and processed by the manufacturers. It is available in varius shapes like tapered pins, blocks, cubes, granules, circular discs and pegs.[5] Bovine bones have been the most preferred xenograft materials, basically because they are easily obtainable and there are no great ethical considerations. Additionally, they have the great advantage of practically unlimited availability of source/raw material. Partially deproteinized and defatted preparations (e.g. Unilab Surgibone) reduce antigenity and cause mild immune response.[6]

The goal of the present study was to assess whether Unilab Surgibone was effective on the mandibular bone repair in rats. We also evaluated it from the cytotoxicity point of view.

MATERIAL AND METHODS

The study received approval of the Local Ethical Committee of Cumhuriyet University. Unilab Surgibone (Missisauga, Ontario, Canada L4W 4N8) is an extracellular composite matrix of hydroxyapatite and collagen of bovine bone. The chemical structure is similar to hydroxyapatite (Ca10 (PO4) 6 (OH)2) but contains 29% protein (collagen). The particular form (mean granular diameter between 600 and 800μ) used in the present study is commercially available. Surgical procedure was performed under sterile conditions. At the first step, general anaesthesia was applied with Ketamin hydrochloride (3-10 mg/kg body weight) and xylazin chloride (90 mg / kg body weight). Then, a linear incision was made through the skin, subcutaneous tissues, and masseter muscle parallel to the inferior border of the mandible. The buccal and lingual surfaces of the mandible were exposed with an elevator, and a 5 mm circular, full thickness critical-sized bone defect was created in the body of mandible using a specific drill under saline irrigation. Then, the defects on the right side were filled with the particular Unilab Surgibone material whereas the opposite defects acted as controls (sham-operated group). A total number 15 Wistar albino rats were included in the present study. While seven of the animals were sacrificed at the end of first month, the remaining eight rats were sacrificed three months after the experimental procedure. All of the animals were sacrificed under ether anesthesia. Thereafter, implantation tissues were repositioned and sutured with a suitable suture. No clinical complications were seen, and extreme caution was taken for rats during the experimental procedure. To quantify the early phase of bone healing, rats were sacrificed after the first and third months following to experiment. The related sites of the mandible were removed containing the surrounding normal bone tissue. Specimens were fixed in 10% buffered neutral formalin for 24 hours and decalcified in formic acid- hydrochloride acid combination for 24 hours.[7] After rinsing with tap water, specimens were dehydrated in the increasing concentrations of ethanol, embedded in paraffin and 5-7 mm thick sections were taken at the transverse plane and stained with hematoxylin (Bio-Optica, catalog no: 05-M06007)-eosin (Bio-Optica, catalog no:05-M10002) (H-E) and Van Gieson (V-G) (Bio-Optica, catalog no:04-030802). Hematoxylin-eosin and Van Gieson staining methods were used according to staining procedures suggested by Allen and Mallory.[8,9] After these procedures, specimens were histologically evaluated under the light microscope for bone and cellular tissue response. Bone formation was classified according to a previously developed classification method.[10] No or minimal bone healing with fibrous tissue interposition was graded as 0, partial bone healing with occasional fibrous tissue in-

growth was graded as 1, and complete bone healing bridging the defect was graded as 2. Data were analysed using Fishers exact test and and $p< 0.05$ was chosen as the level of statistical significance.

CYTOTOXICITY OF XENOGRAFT

Cells

A mouse connective tissue fibroblast cell line, L929 (ATCC cell line, NCTC clone 929) was cultured in Dulbecco's minimum Eagle medium (DMEM) (Gibco, Eg-genstein, Germany) supplemented with 10% fetal calf serum (Gibco, Germany) and 2mM glutamine. No antibiotics were added to the cell culture medium. The cultures were cultivated in an incubator at 37^0 C with 5% CO_2, until cell monolayers attained confluence, which occured after seven days. Assays were always performed in the exponential growth phase of the cells.

Agar Diffusion Method

Agar diffusion tests were performed according to international standards (International Standard ISO 10993-5 1999). Briefly, cultures were harvested using 0.25% trypsin solution (Gibco, Germany). Cells from stock cultures were seeded in 35mm diameter of cell culture petri dishes (Nunc, Wiesbadan, Germany) at a density of $1x10^6$ cells per petri dish, and subcultured once a week. After the formation of confluent cell layer, the medium was removed and replaced with complete medium containing 1.5 % agarose (FMC BioProducts, Rockland, ME, USA). After solidifying the agarose, the cells were stained with a vital dye (neutral red; Sigma). During experimental procedures, cells were protected from light to prevent cell damage elicited by photo-activation of the stain. Xenograft was applied on diameter of 6mm. Four replicate dishes and four additional dishes containing positive and negative control materials were prepared for the experimental material. Physiologic saline solution was used as negative control, while absolute phenol was used as the positive control. After an exposition period of 24 h at 37^0 C, cell responses were evaluated by inverted microscope observation. In this study, cell lysis was scored as follows: 0 = no cell lysis detectable;1= less than 20% cell lysis; 2= 20% to 40% cell lysis; 3= >40% to <60% cell lysis; 4= 60% to 80% cell lysis; 5= more than 80% cell lysis. For each sample, one score was given and the median score value for all parallels from each sample was calculated for the lysis zone. Cytotoxicity was then classified as follows: 0-0.5= non-cytotoxic; 0.6-1.9=mildly cytotoxic; 2.0-3.9=moderately cytotoxic; 4.0-5.0=markedly cytotoxic. The median (instead of the mean) was then calculated to describe the central tendency of the scores, because the results were expressed as an index in a ranking scale. Statistical analysis was accomplished using analysis of variance (ANOVA).

RESULTS

Cytotoxicity of Unilab Surgibone on Cells

The xenograft evaluated in our study showed no cytotoxicity for the cells (Figure 1). There was no decolorization zone around the samples. Although the cells were directly in contact with the xenograft in the culture media, they did not show any signs of injury, and preserved their morphological characteristics and wholeness like those seen in the controls. Overall, lysis index score was 5 (markedly cytotoxic) for the positive control group (Figure 2), while it was 0 (non-cytotoxic) for negative control group (Figure 3).

Histological Analysis

In all specimens, minimal inflammation was detectable either macroscopically or microscopically. On the other hand, there was no necrosis since there was no cytoplasmic and or nuclear abnormalities and tumor formation in the defective area (Figure 4-7). There was no necrosis. Bone formation was mainly confined to the edges of the defect (Figure 4b, 5b, 7). The defect area in the control (sham-operated) group was filled with fibrous tissue at the first and third months. There was no callus formation in the implantation site at first and third months, and Unilab Surgibone material was surrounded with fibrous tissue. By month three, multinuclear giant cells were detected around the implant. There was no statistically significant difference regarding bone regeneration between two defects at each observation period ($p> 0.05$;Tables 1 and 2).

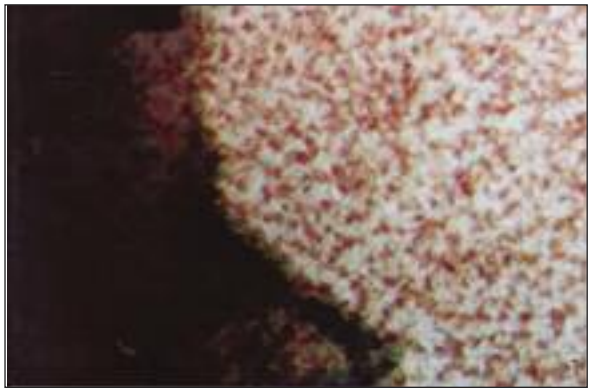

FIGURE 1: There is no cytotoxicity for the cells.

FIGURE 2: Extensive cytotoxicity in the positive control group.

DISCUSSION

The present investigation revealed the histological outcome of the mandibular bone repair by application of a xenograft within different observation periods. As well known, the critical size defect has been defined as the smallest intraosseous wound that is not healed by bone building during the lifetime of the animal.[11,12] These defects were used for testing the biomaterials and therefore have a great importance.[7,13,14] Moreover, this model can be of significance and can contribute additional information in some clinical situations such as periodontology and implantology.

Bone repair in rat mandible using different biomaterials is one of the most interesting study areas. This is because of the attempts to find out the best materials with improved effects on bone regeneration. In addition, evaluations on this site of the body can improve our knowledge and be helpful in understanding and evaluating bone repair in similar bony structures such as periodontal and peri-implantal bone defects.

FIGURE 3: No cytotoxicity in negative control group.

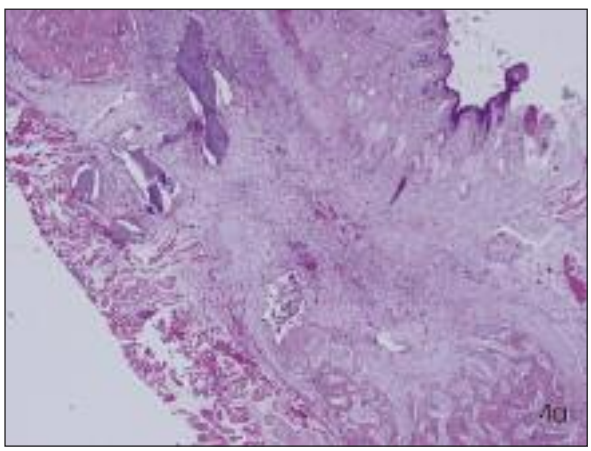

FIGURE 4a: Remnants of the xenograft surrounded by fibrous tissue at 30 days. H-E 40X.

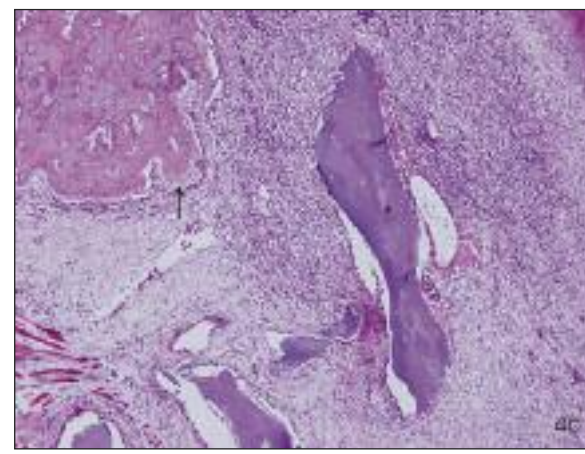

FIGURE 4b: Limited new bone formation (←) in the edges of the defect. Remnant of the xenograft surrounded by fibrous tissue (*). H-E, 100X.

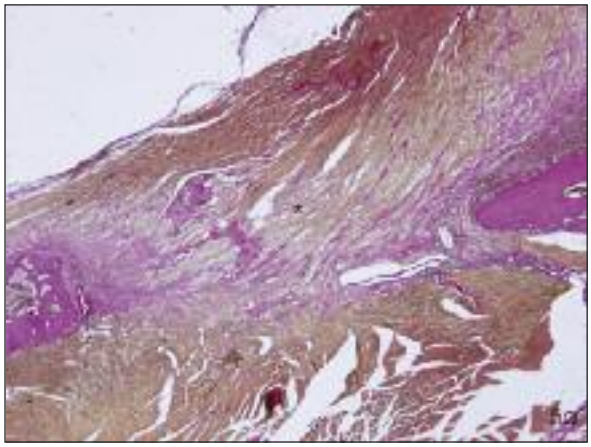

FIGURE 5a: The sham-operated group. The defect area with dense fibrous connective tissue (*) after 30 days. V-G, 40X.

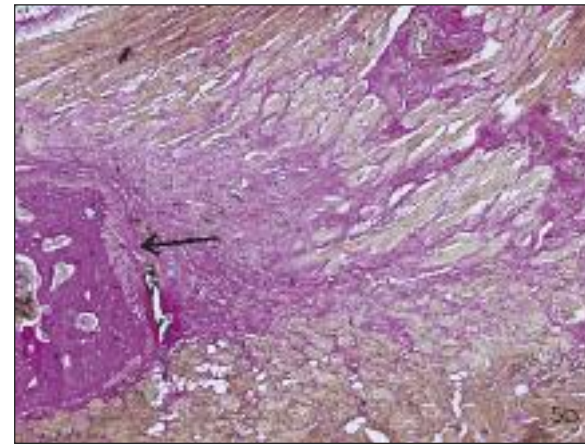

FIGURE 5b: The sham-operated group. The confined bone formation (←) in the edges of the defect after 30 days. V-G, 100X.

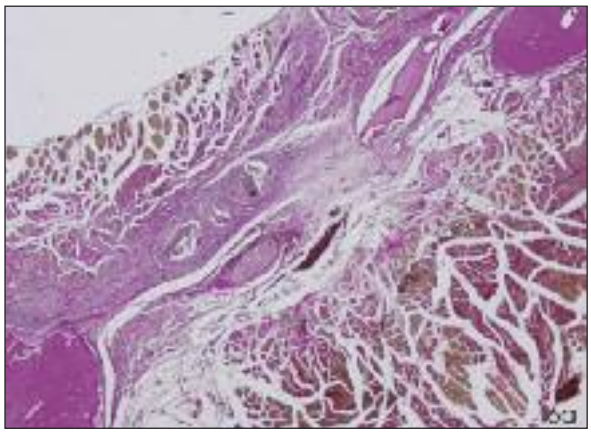

FIGURE 6a: A dense, fibrovascular tissue (*) in the site of xenograft implantation on 3rd month. V-G, 40X.

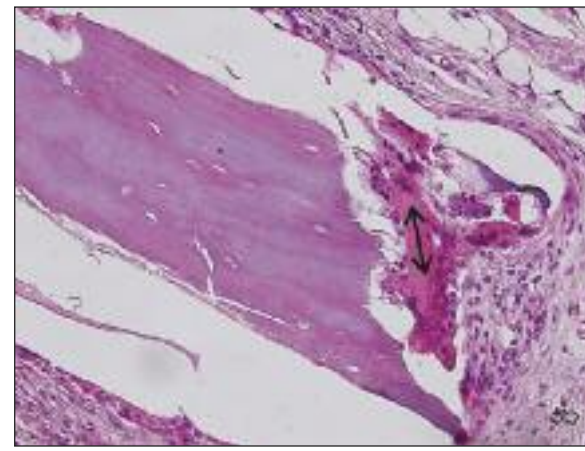

FIGURE 6b: Multinuclear giant cell (↔)in the implantation site. H-E, 400X.

Several studies have been conducted to assess mandibular bone repair using osteoinductive or osteoconductive biomaterials with or without guided bone regeneration (GBR).[15-17] The common reason is to establish a better grafting material which can be used for regeneration and filling of the bone defects. To our knowledge, the xenografts have osteoconductive properties ensuring bone ingrowth into grafted materials.[8,18,19] There are various types of xenografts used in medicine and dentistry. An investigated form is the Bio-Oss which has been tested both experimentally.[20,21] and clinically.[22] There are some studies that investigated the outcomes of Bio-Oss and Emdogain combination.[23] On the other hand, Biocorall is a natural xenograft

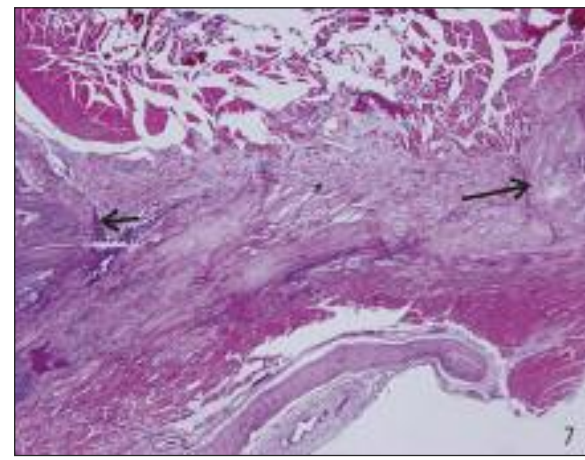

FIGURE 7: The sham-operated group. High amounts of fibrous connective tissue (*) in the defect area or 3rd month. The confined bone formation (←) in the edges of the defect H-E, 40X.

TABLE 1: Bone healing scores at the 1st month.

Groups	0	1	2
Control	7*	0	0
Experiment	7	0	0

*p > 0.05.

TABLE 2: Bone healing scores at the 3rd month.

Groups	0	1	2
Control	8*	0	0
Experiment	8	0	0

*p > 0.05

that has been investigated both experimentally and clinically.[24,25]. The outcomes pointed out the fact that the material showed osteoconductive properties, with resorption being evidenced together with the bone ingrowth. Our experiment also revealed that the material exhibits osteoconductive properties.

Our study is the fist one in the literature. It can contribute new information concerning the critical sized defect healing in the mandibular area, and furthermore, in the periodontal area.

The implantation of a biomaterial into the recipient bone generally results in tissue response. The response to injury depends on various factors, including the extent of injury, the loss of basement membrane, blood- material interactions, provisional matrix formation, the extent or degree of cellular necrosis, and the extent of the inflammatory reaction. These results, in turn, may affect the extent or degree of granulation tissue building, foreign body reaction, and fibrosis or fibrous capsule development.[26] By the first and third months, multinuclear giant cells were detected within the implantation site. Giant cells are short-living cells which are formed by fusion of macrophages[27] and a constant source of newly emigrated monocytes is essential for the production of new giant cells.[28] The observed multinuclear giant cells showed similar morphological characteristics seen in the foreign body giant cells (FBGC). These cells are osteoclast-like cells. Both cell types develop from a common precursor.[29] Since foreign body giant cells (FBGC) are the fusion products of monocytic precursors, which are also the precursors to macrophages,[30,31] the presence of such leukocytes in the wound healing compartment may be of central importance in driving the tissue reaction to the material. These results are similar to a previous investigation. It was reported that Unilab Surgibone was tested in critical sized defects in rats for one month and the biomaterial was seen biocompatible and useful.[32] Moreover, in the case of the cytotoxic activities, the xenograft evaluated here showed no cytotoxicity for the cells. Therefore, it seems as a useful material.

In conclusion, the material used in this study does not enhance bone building; and it seems only to be a beneficial bone filling material in the treatment of bone defects. Unilab Surgibone could be used as a bone-filler material in the treatment of traumatic and post-traumatic skeletal complications (e.g delayed unions, non-unions), defects due to bone removal (e.g. bone tumors, congenital diseases) or low bone quality (e.g. osteoporosis, osteopenia). Further studies are needed to expand the knowledge in this area.

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