Effects of the Enamel Matrix Derivative on Bioactive Glass in Rat Calvarium Defects

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ชื่อวิทยานิพนธ์	ศึกษาผลของสารอีนาเมลเมทริกซ์เดริเวทีฟที่มีต่อไบโอเอคทีฟกลาส ในรอยวิการกะโหลกศีรษะหนูทดลอง
ผู้เขียน	นายพิษณุ โพธิจรรยากุล
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ปีการศึกษา	2551

บทคัดย่อ

ปัจจุบันการนำความรู้ทางด้านวิศวกรรมเทคโนโลยีด้านการปลูกถ่ายถูกนำมาใช้ในการให้การรักษาในผู้ป่วยมากขึ้น โดยการศึกษานี้มีจุดประสงค์เพื่อดูผลของสารอีนาเมลเมทริกซ์เดริเวทีฟที่มีต่อผลึกไบโอเอคทีฟกลาส การทดลองนี้ทำการศึกษาในหนูทดลองทั้งสิ้นจำนวน 20 ตัว แบ่งออกเป็น 4 กลุ่ม กลุ่มละ 5 ตัว โดยกลุ่มที่ 1, 2 และ 3 ทำการสร้างรอยวิการที่กะโหลกศีรษะขนาดเส้นผ่าศูนย์กลาง 5 มิลลิเมตรจำนวน 2 รู โดยรูหนึ่งทำการปลูกกระดูกสังเคราะห์ไบโอเอคทีฟกลาสผสมกับสารอีนาเมลเมทริกซ์เดริเวทีฟและอีกรูหนึ่งปลูกถ่ายเฉพาะกระดูกสังเคราะห์ไบโอเอคทีฟ-กลาส กลุ่มที่ 4 รูหนึ่งทำการใส่เฉพาะสารอีนาเมลเมทริกซ์เดริเวทีฟและอีกรูหนึ่งไม่ได้รับการปลูกถ่าย กลุ่มที่ 1 ทำการเลี้ยงหนูเป็นเวลา 2 สัปดาห์ กลุ่มที่ 2 ทำการเลี้ยงหนูเป็นเวลา 4 สัปดาห์ กลุ่มที่ 3 และ 4 ทำการเลี้ยงหนูเป็นเวลา 8 สัปดาห์ หลังจากนั้นทำการเก็บตัวอย่างชิ้นเนื้อกะโหลกศีรษะมาทำการตรวจทางภาพถ่ายรังสีร่วมกับการตรวจทางพยาธิวิทยา จากผลการทดลองจะพบว่าภาพถ่ายรังสีมีค่าเฉลี่ยการทึบแสง (mean optical density) ของด้านที่ทำการปลูกถ่ายด้วยกระดูกสังเคราะห์ไบโอเอคทีฟกลาสผสมกับสารอีนาเมลเมทริกซ์เดริเวทีฟสูงกว่าด้านที่ทำการปลูกถ่ายเฉพาะกระดูกสังเคราะห์ไบโอเอคทีฟกลาสที่เวลา 2 และ 4 สัปดาห์ และที่ระยะเวลา 8 สัปดาห์ พบว่าด้านที่ทำการปลูกถ่ายด้วยกระดูกสังเคราะห์ไบโอเอคทีฟกลาสผสมกับสารอีนาเมลเมทริกซ์-เดริเวทีฟกับด้านที่ทำการปลูกถ่ายเฉพาะกระดูกสังเคราะห์ไบโอเอคทีฟกลาสมีค่าเฉลี่ยการทึบแสงใกล้เคียงกันและจากผลการตรวจทางพยาธิวิทยาชิ้นเนื้อด้านที่ได้รับการปลูกถ่ายด้วยกระดูกสังเคราะห์ไบโอเอคทีฟกลาสผสมกับสารอีนาเมลเมทริกซ์เดริเวทีฟมีค่าการสร้างกระดูกใหม่ที่มากกว่าด้านที่ทำการปลูกถ่ายเฉพาะกระดูกสังเคราะห์ไบโอเอคทีฟกลาสในช่วงเวลา 2, 4 และ 8 สัปดาห์ และที่ระยะเวลา 8 สัปดาห์พบว่าด้านที่ได้รับการปลูกถ่ายด้วยกระดูกสังเคราะห์ไบโอเอค-ทีฟกลาสผสมกับสารอีนาเมลเมทริกซ์เดริเวทีฟมีค่าการสร้างกระดูกใหม่มากเป็นอันดับที่1 รองลงมาคือด้านที่ทำการปลูกถ่ายด้วยสารอีนาเมลเมทริกซ์เดริเวทีฟเพียงอย่างเดียว ส่วนด้านที่เป็นรอยวิการที่ไม่ได้รับการปลูกถ่ายและด้านที่ทำการปลูกถ่ายกระดูกสังเคราะห์ไบโอเอคทีฟกลาสเพียงอย่างเดียวมีค่าการสร้างกระดูกใหม่มากเป็นอันดับที่ 3 และ 4 ตามลำดับ

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ABSTRACT

Replacement of local bone loss is a significant clinical challenge. Tissue engineering-based bone grafting has emerged as a viable alternative to biological and synthetic grafts. The purpose of this study was to evaluated an effect of enamel matrix derivative (Emdogain ®) on bioactive glass in enhancing bone formation in rat calvarium defects. **Material and methods**; Twenty rats were used in this study. In all animals, two standardized critical- size calvarial defects (CSD) was created surgically. The animals were randomly allocated into 4 groups of 5 animals each. Group AI: One calvarial defect was filled with bioactive glass plus enamel matrix derivative (EMD), while the contralateral defect was bioactive glass alone. The healing period was 2 weeks. Group AII and AIII: The animals were treated in the same manner as in Group AI but with a healing period of 4 weeks and 8 weeks, respectively. And Group B: One calvarial defect was filled with enamel matrix derivative (EMD), while the contralateral defect was empty as control. The healing period was 8 weeks. Formation of new bone was evaluated radiomorphometry and histomorphometrically. **Results**; No defect completely regenerated with bone. Bioactive glass particles were observed of both sides in groups AI, AII and AIII. The radiomorphometry, in group AI and AII bioactive glass with EMD had more mean optical density than bioactive glass alone but in group AIII, mean optical density was nearly the same. The histological analysis revealed that bioactive glass with EMD in group AI, AII and AIII presence more percentage of new bone formation than bioactive glass alone. At 8 weeks, bioactive glass with EMD had highest percentage of new bone formation, EMD alone, CSD and bioactive glass alone, respectively.

Conclusion; Bioactive glass with enamel matrix derivative had more new bone formation in the rat calvarial bone defects when compare with Bioactive glass alone at 2, 4 and 8 weeks. Enamel metrix derivative alone used in the rat calvarial bone defects also had more new bone formation compared with unused defects. Due to the small number of rats in this study, to confirm the result, further study needs to be preformed.

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Pisanu Potijanyakul

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CHAPTER 1

INTRODUCTION

Replacement of local bone loss is a significant clinical challenge. There are a variety of techniques available to the surgeon to manage this problem, each with its own advantages and disadvantages. Several treatment procedures including bone grafts, guided tissue regeneration, combined approaches, and growth factors have been suggested for regenerative bone defect.

The treatment of bony defects with various grafting materials has provided a baseline for what can be achieved in reference to regenerative efforts to create bone fill. While the use of autograft material is the preferred technique, Autogenous cancellous bone grafts produce the most successful and predictable results.[1] Free bone grafts act mostly as scaffolds and are thus more osteoconductive than osteoinductive even though osteogenic activity may have remained in the spongious part of the graft.[2] The major disadvantage of autogenous grafts is the need for a second surgical site and the morbidity resulting from harvesting. Allogeneic bone is non-vital osseous tissue taken from one individual and transferred to another individual of the same species. There are three forms of allogeneic bone: fresh frozen, freeze-dried and demineralized bone matrix (DBM). Allograft has disadvancetage of eliciting an immunological response due to genetic differences and the risk of inducing transmissible diseases.[3] Xenogeneic bone grafts consist of skeletal tissue that is harvested from one species and transferred to the recipient site of another species.[4, 5] These grafts can be derived from mammalian bones and coral exoskeletons. Bovine derived bone has been commonly used,[6, 7] even though other sources are such as porcine or murine bone are available. Xenogeneic bone was popular in the 1960's but fell into disfavour due to reports of patients developing autoimmune diseases following bovine bone transplants.[2, 8] The re-introduction of these products in the 1990's comes after the development of methods to deproteinate bone particles.[9] This processing reduces the antigenicity making these implants more tolerable to host tissues.[10] Their disadvantage is the concern with the possibility of future bovine spongiform encephalopathy due to potential slow virus transmission in bovine-derived products.[11, 12] Since other alternative biomaterials exist, bovine derived products should probably be avoided until the concerns regarding potential slow virus transmission are clearly addressed.

Bone grafting procedures are undergoing a major shift from autologous, allogeneic and xenogeneic bone grafts to synthetic bone graft substitutes. Such synthetic materials could also play a very important role in tissue engineering,[13] serving as bioactive scaffolds. Bioactive glasses are groups of synthetic silica-based bioactive materials with bone bonding properties first discovered by Larry Hench in early 1970s. Bioactive glass has the property to promote adsorption and concentration of proteins utilized by osteoblasts to form a mineralized extracellular matrix and thus, promote osteogenesis by allowing rapid formation of bone. The material's ability is to act like a hemostatic agent and to maintain the blood clot in the defect. There are references in the literature stating the positive properties and effects of bioactive glass as allowing the formation of bone–tissue bond.[14] Bioactive glasses are hard, solid (non-porous) and can be regarded as a three-dimensional silica (SiO_2) network. Silicon dioxide (also known as silicate) forms the main component. By varying the proportions of sodium and calcium salts, phosphates, and silicon dioxide, forms can be produced that are soluble *in vivo* (solubility being proportional to the sodium oxide content) right through to those that are essentially nonresorbable.[15] Bioactive glasses have different bioactivity and resorption rates depending on their chemical compositions. The critical feature for the rate of bioactivity is a SiO_2 content less than 60% in weight. Bioactive glasses possess both osteointegrative, osteoconductive properties and osteostimulatory capacity.[16, 17]

The basis of the bonding property of bioactive glasses in their chemical reactivity in body (tissue) fluids, a series of chemical reactions occurs, which results in the formation of a hydroxyapatite layer to which bone can bond. Three general processes are leaching, dissolution and precipitation. The leaching reaction occurs via ion exchange mechanism. Sodium is leached from the glass and is replaced with protons from the solution. An important aspect of this reaction is that the local pH is driven from an acidic value to a neutral or slightly basic pH, which can be more supportive of healing. Concurrent with the leaching reaction, dissolution of the glass silica network occurs as hydroxyl groups attack the silicon-oxygen bonds (Si-O-Si), releasing silicic acid and other elements (Ca, Na, P). As silicic acid is released into solution, silanol groups from a hydrated layer at the glass surface. The silanol (Si-OH) groups undergo a rearrangement via a polycondensation reaction to produce a silica gel. The silica-rich gel has a large surface area and a negative surface charge. This leaves behind a silica-rich gel approximately 200 μm thick. This gel serves as a nucleation site for the formation of a calcium phosphate (CaP) layer. The CaP

phase that accumulates at the surface of the silica gel is initially amorphous, but subsequently crystallizes to a hydroxycarbonate apatite structure by incorporating carbonate anions from the solution within the amorphous CaP phase. Organic species in the local environment such as collagen, chondroitin sulfate, and glycosaminoglycans, are incorporated into this bioactively forming layer. Osteoblasts are attracted to the hydroxycarbonate apatite and release organic constituents, followed by mineralization.(Figure 1.) The end result is a bonded interface consisting of a series of layers: glass - silica gel - hydroxycarbonate apatite - bone. A mechanically strong bond between bioactive glass and bone forms as a result of a silica-rich gel layer that forms on the surface of the bioactive glass when exposed to physiologic aqueous solutions. Within this gel Ca^{2+} and PO_4^{2-} ions combine to form crystals of hydroxyapatite (HA) similar to that of bone.[3, 18] It has been reported that leaching reaction of bioactive glass particles *in vivo* results in the formation of niches in which bone can form.[19] This bone is apparently unconnected to the surrounding bone (Figure 2.), and these islands of bone may form nuclei for the formation of additional bone, which may eventually become confluent with the surrounding native bone. When used as a preformed implant they have significantly greater mechanical strength when compared to calcium phosphate preparations such as ceramic HA.[15] They have several unique properties compared with other synthetic bioresorbable bioactive ceramics, such as calcium phosphates, hydroxyapatite and tricalcium phosphate (TCP).

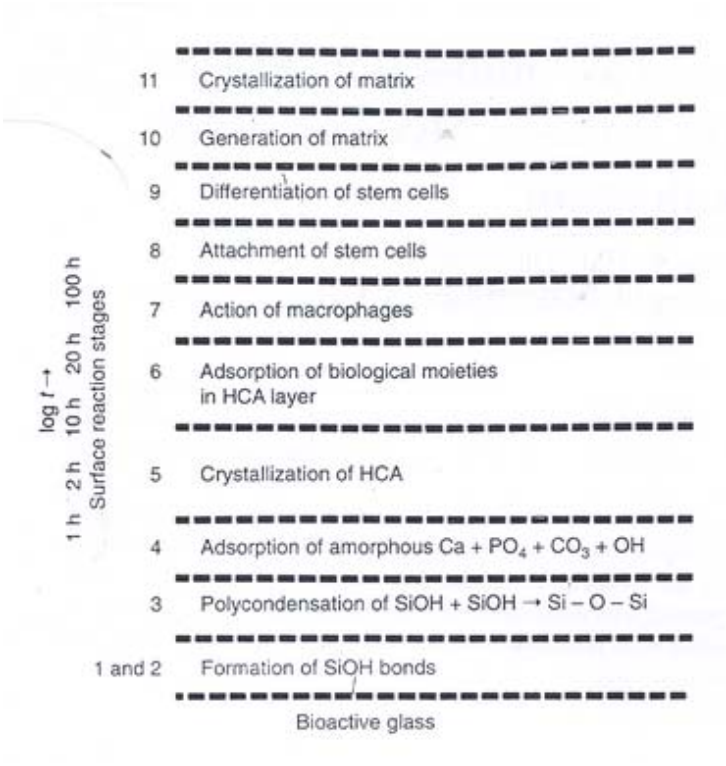


Figure 1. Sequence of interfacial reactions involved in forming a bond.[20]

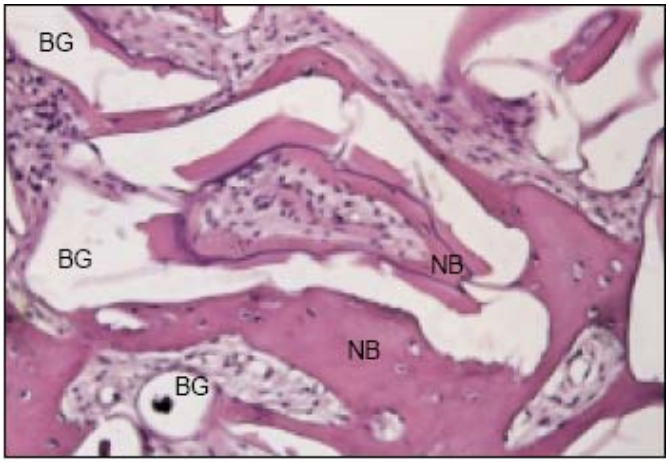


Figure 2. Micrograph of bioactive glass particles (BG). The core of the granule has been excavated, and new bone tissue deposition on the outer and inner surface can be observed (NB) (hematoxylin-eosin stain, original magnification x 200).[21]

Factors affecting dissolution of bioactive glass to be used as tissue engineering scaffolds

The solution parameters of initial pH, ionic concentration and temperature have a large effect on the rate of scaffold dissolution and even type of calcium phosphate precipitated. Ionic concentration and therefore pH, will obviously change with time as dissolution progresses and this will in turn affect the dissolution rate. If pH rises above a critical value, cytotoxicity will occur.

Three types of media have been used during bioactive glass dissolution experiments:

i) Tris-buffer, a simple organic buffer solution.

ii) Simulated body fluid (SBF), a tris buffer containing similar ion concentrations to that of human blood plasma.

iii) Alpha-MEM and D-MEM, culture media that contain both the inorganic and biological organic components of blood plasma.

Bioactive glass underwent a faster surface reaction and exhibited a larger hydroxycarbonate apatite (HCA) crystal size in Tris solution than it did in SBF or culture medium. However, Pereira *et al*. found that HCA nucleated faster in SBF than in Tris, which are put down to the far higher initial phosphorus concentration (40 ppm) in SBF, thus aiding HCA nucleation.[22] A similar result was found by Tsura *et al*.,but they suggested that the dissolution of Ca^{2+} ions from materials would increase the degree of supersaturation in the solution(SBF), which would already be supersaturated regarding HCA precipitation and hence make it much easier for HCA to be precipitated.[23]

Increasing the pH or Ca^{2+} concentration of SBF containing porous silica gel-glass reduced the induction time for hetetogeneous HCA formation. Reaction kinetics of surface layer formation have been found to alter in culture media containing serum. This dues to the adsorption of serum proteins onto the surface of the bioactive glass forming a barrier to nucleation of the HCA layer.[24]

Several *in vitro* studies have shown the nontoxicity of bioactive glass, its positive influence on osteoblast culture in term of studies suggest a direct and very positive effect of soluble silicon on mineralization, inhibitory capacity on fibroblast proliferation and ability to form calcification foci in periodontal ligament fibroblasts. The cellular activity of bone growing cells is enhanced on a bioactive glass surface[25], and the attachment and spreading time of certain fibroblasts are slowed down on the bioactive glass surface compared with an inert surface.[26]

Histologic studies in animals have shown that bioactive glass implanted in non-periodontal sites is biocompatible and incorporates into the bone tissue thus producing an alkaline pH at the implantation site.[16] Animal studies using the primate model demonstrate bone apposition in direct contact to particles of bioactive glass. The effect of bioactive glass on cellular activity observed in tissue culture studies may contribute to the inhibition of epithelial downgrowth noted in the repair of bony defects in a primate model.[26, 27] The mechanism of this phenomenon may be related to rapid collagen attachment to the silica-gel surface of the reactive bioactive glass particle. The rapidly formed connective-tissue seal may serve to block epithelial downgrowth. *In vivo*, the material is highly osteoconductive and it seems to promote the growth of new bone on its surface. The bioactive glass was mostly resorbed and replaced by bone and the remaining granules were in close contact with bone.[28] There is a dynamic balance between intramedullary bone formation and bioactive glass resorption. The effect of bioactive glass particle in medullary bone around titanium implant, show increase in reactive medullary bone formation.[29]

In an animal model, the speed of bone growth around bioactive glass particles was much faster than bone formed around hydroxyapatite particles.[30] In addition, the bone formed around bioactive glass was much denser and more mature compared with bone formed around hydroxyapatite particles. When compared with autologus bone in the augmentation of surgically created defects in the canine rib model, bioactive glass led to more bone than autologous bone alone, and 50/50 mixtures of bioactive glass and autologous bone led to twice as much bone formation in the 6-week experiment than autologous bone alone.

Recent studies of molecular biology have shown that bioactive glass induces a high local turnover of bone formation and resorption. Many osteoporotic fracture patients are candidates for concurrent treatment with bisphosphonates and bioceramic bone graft substitutes. Since osteopromotive silica-based bioactive glasses induce accelerated local bone turnover, adjunct antiresorptive agents may affect the process. However, a recent study showed that an adjunct antiresorptive therapy (zoledronic acid) is even beneficial for bone incorporation of bioactive glass. And have study that shown the activity of the bioactive glass material was found even to overshadow the effect of BMP-2 gene therapy. Based on these observations, bioactive glasses are a promising group of unique biomaterials to act as bone graft substitutes.[31]

Biograns (Orthovita, Implant Innovations, Palm Beach Gardens, FL, USA) is a resorbable amorphous bioactive glass supplied in granules that are approximately 300–355 μm in diameter. They consist of 45% SiO_2, 24.5% CaO, 24.5% Na_2O and 6% P_2O_5. It is conceivable that this material appears to resorb by dissolution rather than by osteoclastic activity.

Bone regeneration techniques increasingly rely on the use of exogenous molecules able to enhance tissue formation in pathologic and traumatic defect. Bioactive glass may be an attractive vehicle for delivering osteogenic agents to regenerative sites. Enamel matrix derivatives (EMD) are harvested from around developing teeth in carefully selected young pigs and, following special processing procedures. They are the major component of commercially available Emdogain® (Biora AB, Malmö, Sweden). (Figure 3.)

Figure 3. Emdogain® (Biora AB, Malmö, Sweden).

Most of the initial work with this material has been aimed at regenerating periodontal attachment apparatus lost due to periodontitis, but other applications are being explored. The main biological effects of EMD have been attributed to their predominant protein, amelogenin, with the remaining fraction comprising less-characterized factors. Amelogenin is not a classic growth factor, but rather a cell-adhesion matrix-bound protein. Specific amelogenin gene products are thought to have activity as epithelial–mesenchymal signaling molecules. In addition, there is evidence that the alternate splice variant of the amelogenin gene, leucine-rich amelogenin peptide may also have direct signaling activities on cementoblasts, and, thus at least by implication, osteoblasts.[32]

The enamel matrix derivative was generally believed to regulate the initiation, propagation, termination and maturation of the enamel hydroxyapatite crystallites. Other findings indicate that the enamel matrix also has a function outside the developing enamel. Enamel matrix proteins are temporarily deposited onto the dentinal root surface and provide an initial and essential step in the formation of acellular cementum. Autoradiographic and scanning electron microscopy studies provide additional evidence that, following apoptosis of hertwig epithelial root sheath (HERS) cells and deposition of the enamel matrix proteins onto the dentin surface, the cementogenesis process is initiated and kept modulated by these proteins. Subsequently, when cementum has been laid down onto the enamel-matrix-covered dentin surface, an attachment apparatus will develop. Immunological and immunohistochemical methods both show that enamel matrix proteins are present in acellular cementum, accentuating the importance of these proteins in the cementogenesis process.[33]

Enamel matrix derivative has been used clinically for periodontal regeneration, and its therapeutic effectiveness has been variously attributed to amelogenin, nonamelogenin enamel matrix proteins and growth factors. While EMD may induce periodontal regeneration, the precise mechanism of this is not known.[34] Enamel matrix proteins have been suggested to exert its influence locally by stimulating cellular activation in cell culture that resembles process critical for healing. Enamel matrix derivative effects on cells depend on their phenotype and differentiation stage. *In vitro* studies show that EMD can increase matrix production, proliferation and bone nodule formation of periodontal ligament cell cultures and the proliferation and differentiation of human and murine osteoblast cell lines, with the stimulation of

phenotypic bone markers in some osteoblast cell lines.[35-38] Narukawa M *et al*. studied that EMD stimulates osteoblastic differentiation via the induction of mRNA of osteogenesis-related transcription factors. And the study *in vitro* of Galli C *et al*., found significantly higher quantity of OPG and lower amount of RANKL and alkaline phosphatase activity and osteocalcin production were enhanced in cultures. Mineralized nodules appeared bigger and more numerous in cultures.[38] EMD stimulates not only the transcription level but also the maturation stage.[39] In contrast, some studies show no effect of EMD on osteoblastic differentiation, although other growth factors were stimulated.[32, 40]

The search for downstream target genes has also revealed EMD response elements in the rat bone sialoprotein gene promoter that may mediate the effects of EMD on bone sialoprotein gene transcription. Bone sialoprotein is a unique marker of early osteogenic differentiation that can regulate the formation of mineral crystals. Amelogenin increases expression of bone sialoprotein gene through a tyrosine kinase pathway.[41] A cDNA microarray study examining EMD-mediated changes in gene expression in periodontal ligament cells *in vitro* has reported the downregulation of genes involved in the early inflammatory phases of wound healing, while simultaneously upregulating genes encoding growth and repair-promoting molecules. The *in vitro* treatment of cementoblasts with EMD was found to decrease osteocalcin expression and to increase osteopontin expression. In 2006 Reseland JE *et al*. found that EMD had a positive effect on factors involved in mineralization *in vitro*, causing an increased alkaline phosphatase (ALP) activity in the medium as well as increased expression of osteocalcin and collagen type I. Several hundred genes are regulated by EMD in primary human osteoblasts. There appear to be similarities between the effects of EMD and parathyroid hormone (PTH) on human osteoblasts. Some study about expression pattern of several mRNAs and proteins upon EMD stimulation also indicates a secondary osteoclast stimulatory effect, suggesting that the osteogenic effect of EMD.[35] And it has been reported that EMD can induce osteoclast formation through RANK-RANKL interactions in mouse marrow cultures.[42, 43] It indicate that EMD plays an important role in the production of RANKL in osteoblasts during the process of osteoclastogenesis, suggesting that it may participate in bone regeneration through bone formation and bone resorption.[43] Narukawa M *et al*. in 2007 found EMD induced osteocalcin and type II collagen mRNA in undifferentiated mesenchymal cells.[39] Hattar S *et al*.(2005) finding bioactive granules coated with Emdogain revealed significantly higher protein production than the bioactive granules alone.

That have the ability to support the growth of osteoblast-like cells *in vitro* and to promote osteoblast differentiation by stimulating the expression of major phenotypic markers.[44]

Overall, the data support the positive effect of EMD on osteoblast differentiation, although further studies are needed to clarify which molecules in EMD stimulate osteogenesis and to define their precise modes of action.[32]

COMPOSITION OF THE ENAMEL MATRIX PROTEINS

The major fraction of the enamel matrix proteins is composed of three matrix proteins, corresponding to amelogenin, enamelin, and sheathlin (also called ameloblastin or amelin). The amelogenin, a family of hydrophobic proteins and insoluble in water at neutral pH, is account for more than 90% of the organic constituent of the enamel matrix. The amelogenin has remained remarkably well-conserved through evolution, suggesting that it may have great functional importance. The second largest component of the enamel matrix proteins is the enamelin, it also contains several proteinases. Since the enamelin was found to contain serum proteins, the more general term "nonamelogenin" is now commonly used to describe this high-molecular-weight fraction. It includes proline-rich enamelin, tuftelin, and tuft proteins. Two enzymes, corresponding to human matrix metalloproteinase 20 (MMP-20) and Enamel matrix serine proteinase 1 (EMSP1), have been purified and the cDNA cloned from developing porcine teeth. These proteins are all present in EMD. Although early immunoassay studies could not identify the presence of growth factors in EMD, nominal levels of transforming growth factor β1 (TGF-β1) have been detected immunologically. In addition, by using the bone morphogenetic protein (BMP) binding protein noggin, investigators have identified BMP-2 and BMP-4 like in an osteoinductive fraction of enamel extracts.[33, 45] The presence of BMP-6-like molecules in EMD were determined later.[39] The osteogenic activity of EMD may be mediated by the induction of BMP-like molecules, as EMD induce Cbfa1, Runx2, Sox9 expression and the phosphorylation of mothers against decapentaplegic homolog 1 (Smad1), both of which can be blocked by the BMP inhibitor, noggin.[32, 39]

EMDOGAIN® FORMULATION[33]

A commercial enamel matrix derivative (Emdogain®, Biora AB, Malmö, Sweden) received FDA approval and is now available for the treatment of periodontal defects. It is a purified acidic extract of developing embryonal enamel derived from six month-old piglets. Its purpose is to act as a tissue-healing modulator that would mimic the events that occur during root development and to help stimulate periodontal regeneration. (Figure 4.)

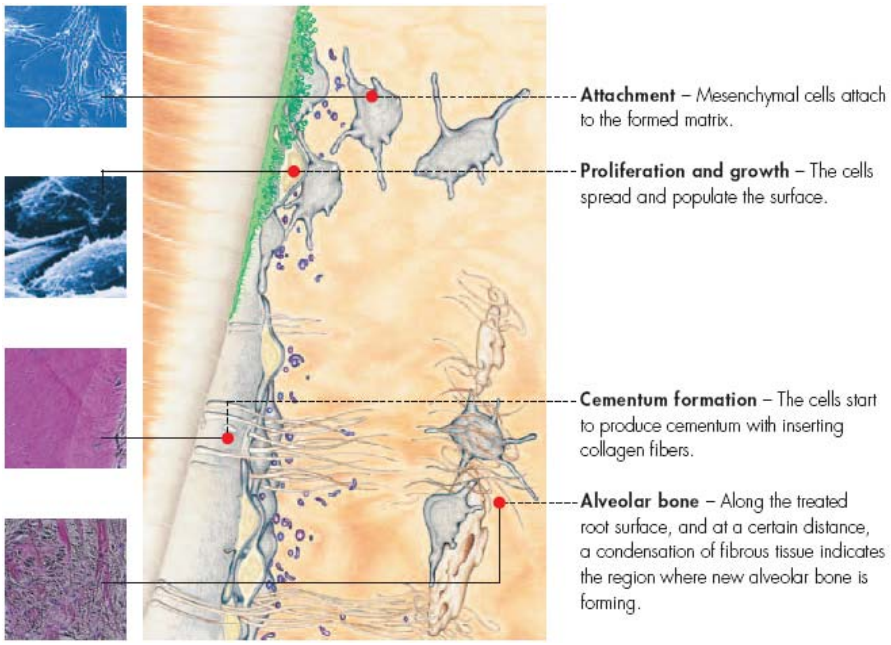


Figure 4. Cells that have effected from enamel matrix derivative in periodontal lesion

In Vivo studies

Factors influencing healing of the defect in animal models.

1 . *age* Immature animal can more actively repair an osseous defect than an older one; therefore, a true test for a bone repair material should involve an adult animal.[46]

2. *wound size* An experimental bony wound used to assess repair should, therefore, be large enough to preclude spontaneous healing. An experimental bony wound of this nature may be termed a critical size defect (CSD). The CSD may be defined as the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal.[46]

Histological evaluation of the effects of enamel matrix protein derivative on bone formation, it has been demonstrated that EMD possesses an osteo-promotive effect on bone and medullary regeneration during wound healing of injured long bones.[47] EMD is also an effective biological matrix for enhancing new trabecular bone formation in rat femer[48]and skull.[49] However EMD when used alone in the defect site, dose not significantly promote bone healing in rabbit modal.[50-52] The effects of enamel matrix protein derivative next to implant contact in the dog model have positively on bone healing after guided bone regeneration[52]same as the study Boyan BD (2000) that studied in calf muscle of nude miced, enamel matrix derivative mixed with active demineralized freeze dried bone allograft induce new bone formation. But when combination EMD with inactive demineralized freeze dried bone allograft or used alone did not induce new bone that indicate EMD is not osteoinductive but it is osteopromotive , due in part to its osteoconductive properties.[37, 53] Donos N.(2005) he found enamel matrix derivative mixed with deproteinized bovine bone mineral (xenografts) and graft at lateral aspect of mandibular ramus did not positively affect the amount of new bone formation.[54] And in 2006 he used deproteinized bovine bone mineral mixed with enamel matrix derivative implant in pectoralis muscle of rat not have bone-inductive properties.[53] Murai in 2005 studied the effects of the enamel matrix derivative and β-tricalcium phosphate on bone augmentation in calvarium of rabbit, found the mixture of enamel matrix derivative and β-tricalcium phosphate not have significant to increase in the amount of new tissue and mineralized bone but tend to increase the new bone[55] at least partly, involves stimulation of bone remodeling.[35]

In the Clinical studies

Emdogain® has been tested as a new chemical entity with a safety program encompassing single- and multiple dose studies (intravenous and subcutaneous), local irritation tests (subcutaneous and topical), and *in vitro* tests for cell toxicity and mutagenicity. No adverse results were found. Enamel matrix proteins are expected to be recognized as " self " by the human immune system, because everyone is exposed to them during tooth development in early childhood. The protein in Emdogain® is of procine origin with very high homogeneity to the human amelogenins. Emdogain® is purified through a number of processing steps, including solvent and heat treatment, ultrafiltration, sterile filtration and freeze drying.

In the physiologic environment of the periodontal lesion with neutral pH and body temperature, the viscous vehicle solution become watery and rapidly leaves the application site, while the protein change to its natural.

Ten occupationally exposed employees from the laboratories where Emdogain was developed and procedued were tested for type IV sensitization by using skin tests result to negative(buffer) and positive (histamine) controls. No reaction was seen either immediately or within the 48 hours following testing. One year later the same employees volunteered for a challenge injection of Emdogain® protein and blood sample were taken before and a month after this injection. The result was again unchanged; no immunoresponse was elicited.[56]

All clinical studies with Emdogain® have included active questioning of patients for subjective adverse reactions and have required dentists to note any objective reactions. Comparisons in clinical studies between the test surgeries with Emdogain® and identical control surgeries without Emdogain® show no differences in type or frequency of common procedure-related experiences such as root sensitivity, mucosal irritation and so on. When certain antibiotics were used, well-known side effects (eg. Skin reactions and gastrointestinal problems) were detected.[56, 57]

Karl J. Zeren in 2006 had reported a surgical technique that preserves anterior esthetics by immediate implant placement. This approach capitalizes on the potential for freeze dried bone allograft (FDBA) and EMD to assist in bridging the osteogenic " jumping distance" to achieve ridge preservation and bone augmentation. The composite graft material is considered for predictably resolving osseous voids and promoting implant integration.[58]

Objective :

The aim of this study is to evaluate an effect of enamel matrix derivative (Emdogain®) on bioactive glass in enhancing bone formation in rat calvarium defects.

CHAPTER 2

MATERIALS & METHODS

This study has been approved by the animal experiment ethical committee of Prince of Songkla University.

Animals

Twenty, male, 5-month old, albino rats of the Wistar strain were used in the study.The animals were fed rat chow and water, and kept separately in cages at the animal house 24ºC and 55% relative humidity in at least 12 hours light per day.

Anaesthesia and surgery

The animals were given atropine 0.05 mg/kg before anaesthetized with intramuscular injection of Tiletamine and Zolazepam 20 mg/Kg about 5 minutes prior to surgery. Hair over the calvarium was shaved and disinfected with betadine solution. The animal was then draped to allow aseptic access in the operation field.

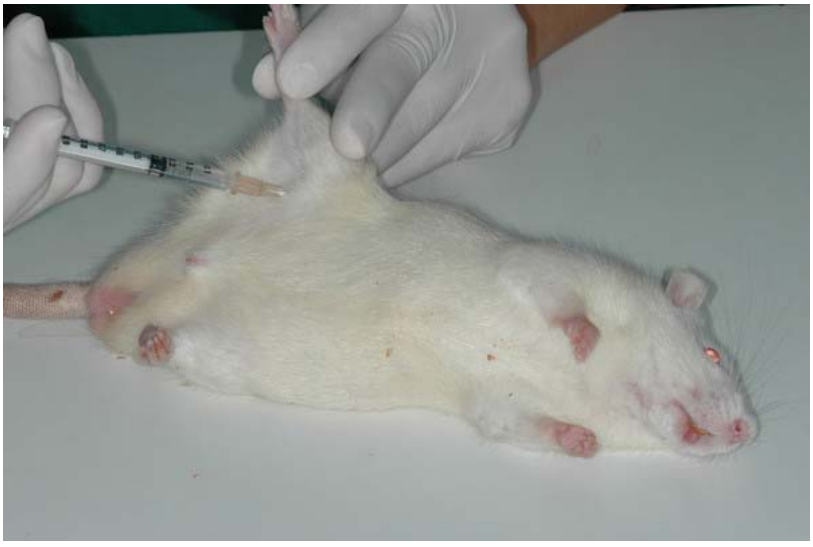


Figure 5. The rat was injected with Tiletamine and Zolazepam before the operation.

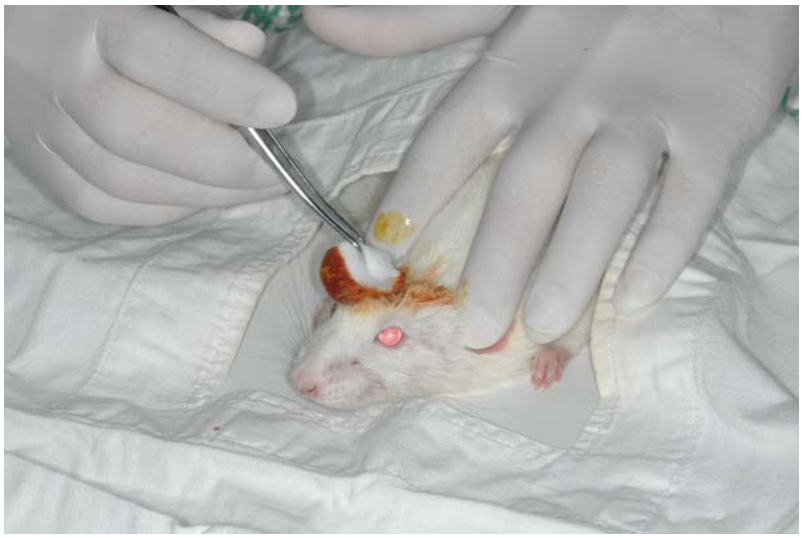


Figure 6. The rat was disinfected with betadine solution and draped.

A midline incision was cut through the skin of the calvaria, and then the cranial skin flap was elevated. The subcutaneous fascia was divided, periosteal flaps were reflected bilaterally and the cranial vertex was exposed.

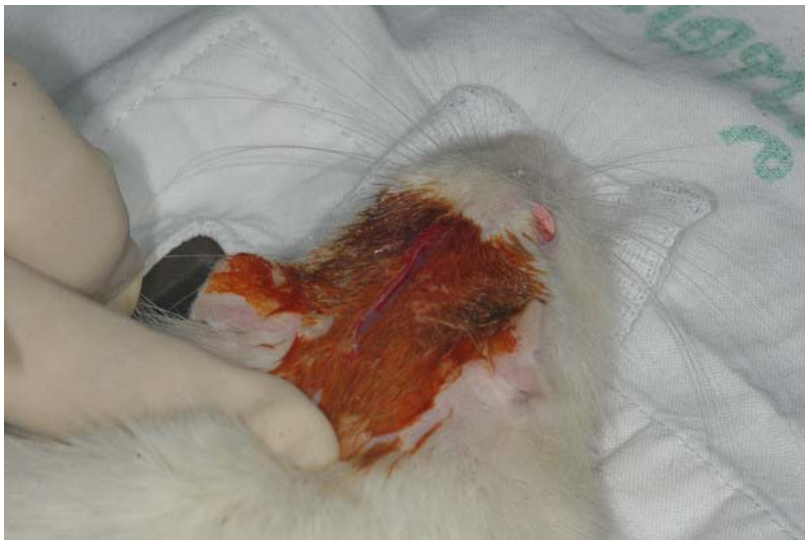


Figure 7. A midline incision was through the skin of the calvaria.

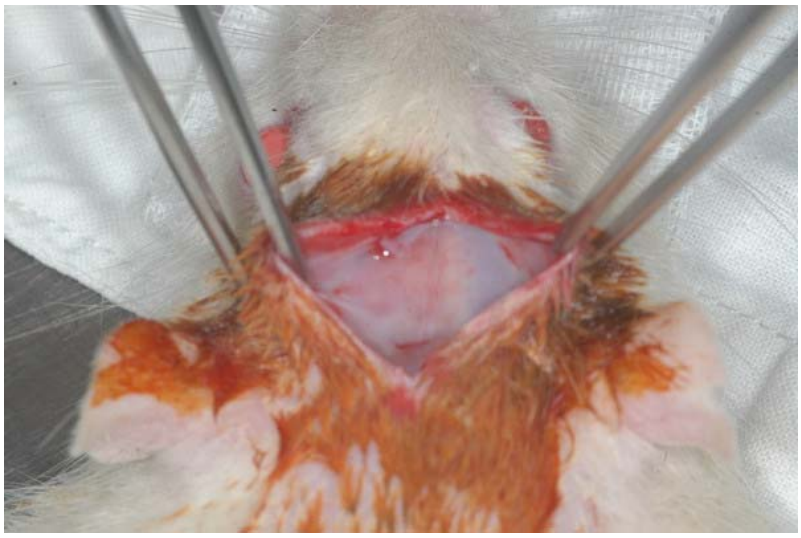


Figure 8. A cranial skin flap was elevated.

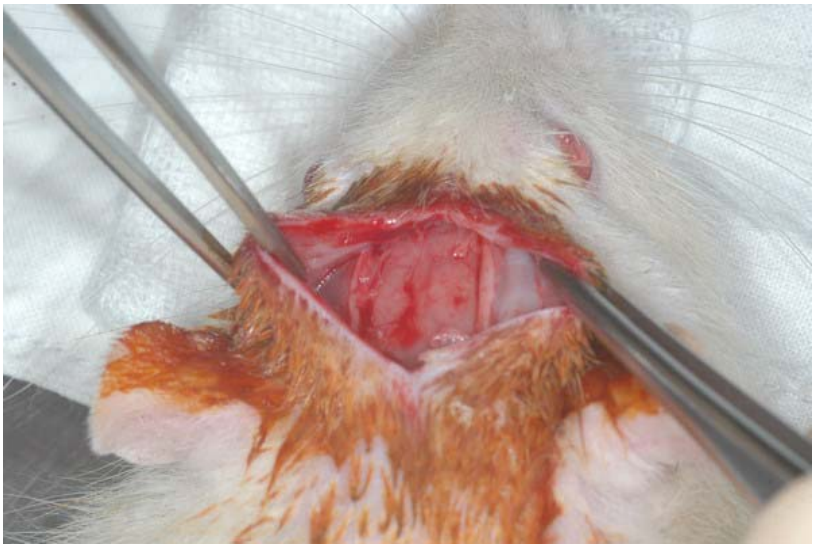


Figure 9. The subcutaneous fascia was divided and periosteal flaps were reflected bilaterally.

Two bone defects were created in the left and right parietal bone using trephine bur (5.0 mm. diameter) in slow-speed micromotor under copious saline irrigation with depth equal to the full thickness of the calvarial bone. The 2 pieces of calvarial bone were removed carefully to avoid injury to the dura mater.

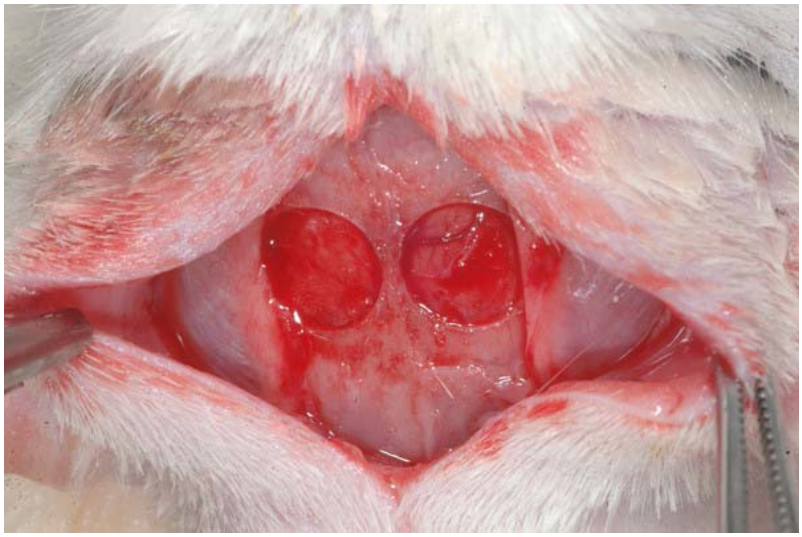


Figure 10. Two bone defects were created in the left and right parietal bone.

Reference markers were made with round bur 2 mm. anterior and 2 mm. posterior to the margin of each defect, both of which were located on a saggital axis bisecting the surgical defect. Gutta-percha was filled in the marker holes as radiographic landmarks.

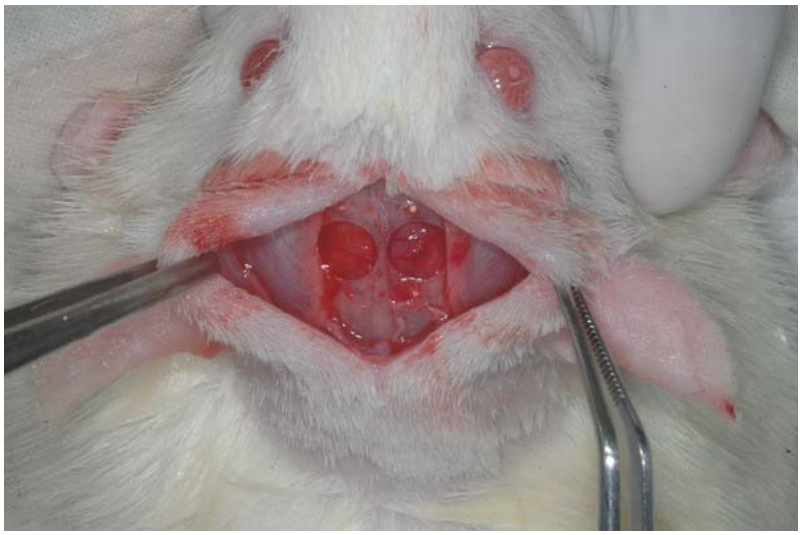


Figure 11. Reference markers were made 2 mm. anterior and 2 mm. posterior to the margin of defects.

The rats were randomly allocated into group A and group B, 15 rats and 5 rats respectively. In group A, bioactive glass 0.04 g mixed with EMD 0.01 cc.(Emdogain® Gel, Biora AB, MalmÖ, Sweden) by spatula was filled randomly in one side of the defect and the contralateral side was filled with bioactive glass 0.04 g alone. Healing period in group A was devided into 3 subgroups (2 weeks, 4 weeks and 8 weeks), 5 rats per subgroup. In group B EMD 0.01 cc. was randomly filled in one side of the defect, the contralateral side was empty. The healing period of group B is 8 weeks.

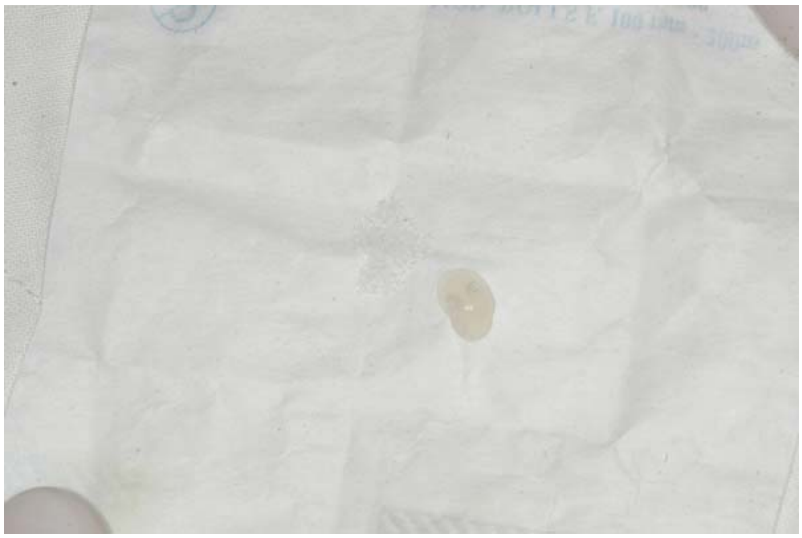


Figure 12. Biograns® (Bioactive glass) And Emdogain® (Enamel matrix derivative).

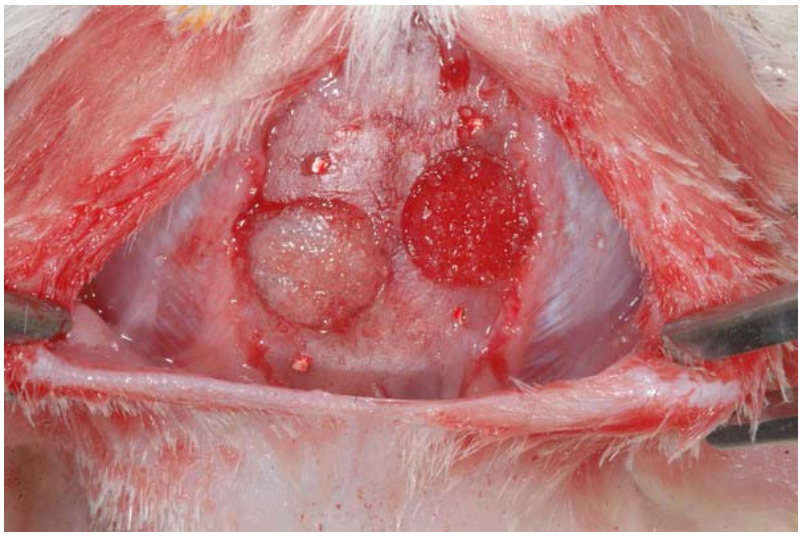


Figure 13. Bioactive glass mixed with the enamel matrix derivative on the right side and bioactive glass alone on the left side.

Soft tissue and skin were closed with 4-0 Vicryl® resorbable sutures (Ethicon). At the end of the surgical procedures, all animals were received a single intramuscular injection of antibiotic (Ampicillin, 100 mg/Kg). All animals were fed with standard diet and water filled with Ibuprofen, 20-40 mg per Kg. three day post-operation and pure water until the date of sacrifice.

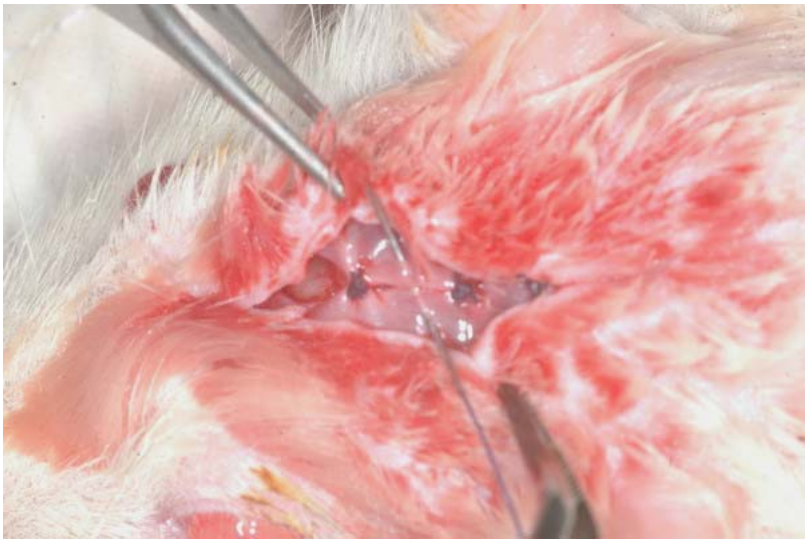


Figure 14. The flap was closed layer by layer.

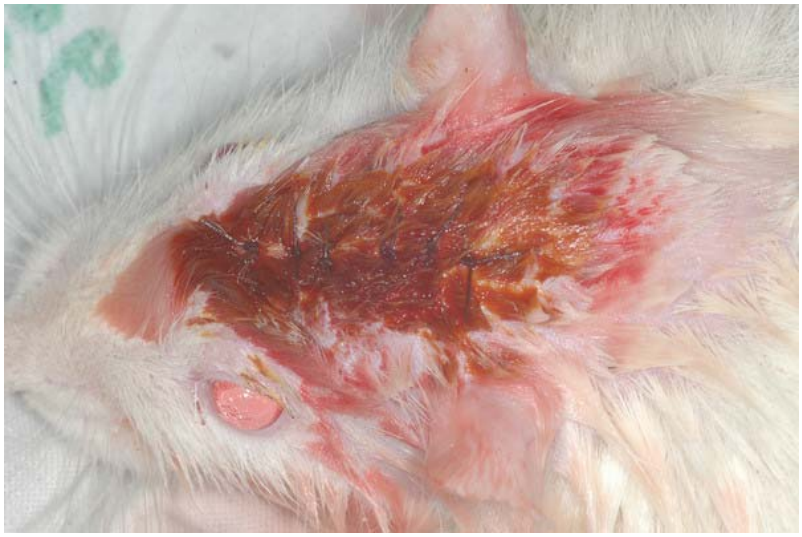


Figure 15. After operation the wound was applied with betadine solution.

Qualitative Evaluation

The rats were sacrificed by overdose intraperitoneal injection of Pentobabitone (Nembutal®) and calvarial bones were resected, including a 3 mm. margin of normal bone around the surgically treated area. Sample of the soft tissue and periosteum were also collected. The specimens were visually examined for the signs of inflammation. Osteogenesis was assessed both radiologically and histomorphologically.

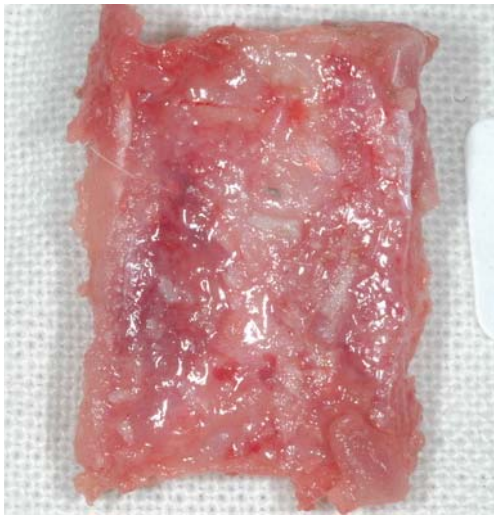


Figure 16. The specimen from rat cranium (Periosteum site).

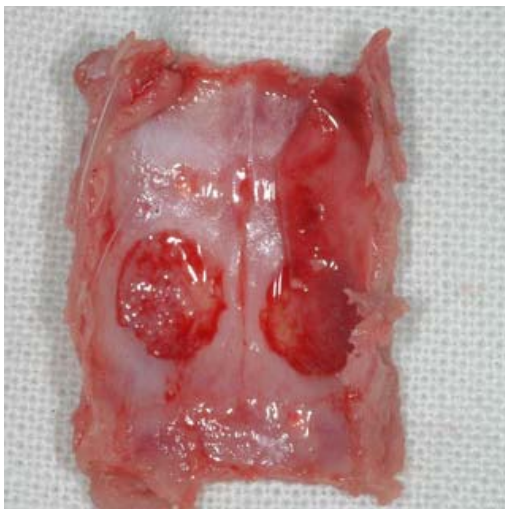


Figure 17. The specimen from rat cranium (dura site).

Quantitative Radiodensitometry

Radiographs of all specimens were taken using Gendex X-ray machine with 75 kvp, 10 mA, 0.26 sec and the Super Polysoft Insight Kodak X-ray film (Kodak Ultra-speed , Eastman Kodak company, Rochester, NY). The distance between the film and focal spot were kept on 50 cm in every subject. An aluminum 5 steps wedge was used for film calibration. The films were automatically processed using by Dent-X9000 processor. The radiographs were scanned using Bio-Rad® Model GS-700 Imaging Densitometer (BIO-RAD Laboratories Ltd, Hemel Hempstead, UK) to obtain digital radiographic images of the specimens and analysed with Molecular Analyst® software. The average radiographic optical density (Mean OD) was measured and calculated for comparing the amount of mineralized tissue produced in response to each type of graft material.

Figure 18. Radiograph was taken with parallel technique.

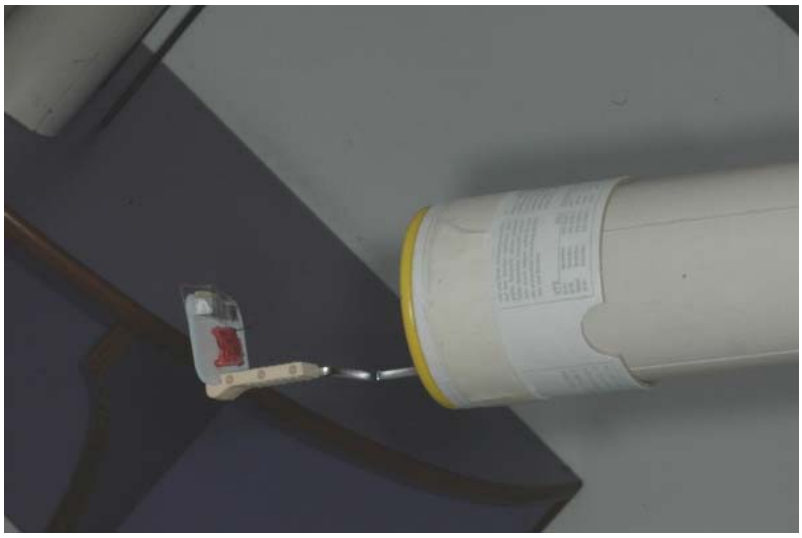


Figure 19. Bone specimen and aluminum steps wedge were attached to the periapical film.

Figure 20. Imaging Densitometer Unit.

Histologic preparation

All specimens were fixed with 10% buffered formalin solution and decalcified with 10% formic acid. After completion of decalcification, each bone specimen was divided into 4 pieces as shown in figure 21.

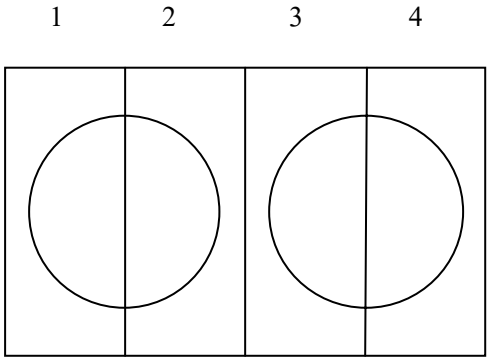


Figure 21. The specimen was divided to make histologic.

All bone specimens were embedded in paraffin and serially cut in 5 μm thick. Each histologic section was stained with haematoxylin and eosin (H&E) stain. These slides were studied blind under light microscope for detection of new bone formation, soft tissue reaction and the degree of inflammation.

Histomorphometric evaluation

Computer-assisted histomorphometry was performed in order to measure the amount of newly formed bone in the defect. To obtain digital histologic images, a digital camera (AxionCam MR , Carl Zeiss , light microscopy , Gottingen , Germany) was attached to light microscope and using Axiovision program. Measurements was carried out directly at the magnification of 5×, by the light microscope. These histologic images were transferred to a computer and measured region of bony defect area and new bone formation in the experimental and control site, using Image-Pro Plus 5.0 program The percentage of new bone area in the experimental and control bone defect were estimated ;

$$\% \text{ new bone} = \frac{\text{new bone area}}{\text{Total bone defect area}} \times 100$$

Data analysis : The analyses were performed by using commercial SPSS software (version 14.0, Standard Software Package Inc., USA). Calculations of mean numbers for each specimen, follow by computation of group mean number and standard deviation, were carried out in all measurements.

CHAPTER 3

RESULTS

Clinical observation

After the animals were grafted in the calvarial bone defects. Three rats were excluded from the study due to postoperative complication and wound infection. Two excluded rats were from group AI (Bioactive glass + EMD vs. Bioactive glass alone, healing period 2 weeks) and one from group AII (Bioactive glass + EMD vs. Bioactive glass alone, healing period 4 weeks). All other animals remained healthy during the observation period and all implantation sides healed uneventfully. After full recovery, they were able to eat the pellet food and drink water.

Gross Examination

After sacrifice, the specimens were examined and found that 3 samples from the group AIII (Bioactive glass + EMD vs. Bioactive glass alone, healing period 8 weeks) and 2 samples from the group B (Enamel matrix derivative alone vs. Empty) had inflammation. Other samples revealed the graft appeared well incorporated in experimental and control side in group A (Bioactive glass + EMD vs. Bioactive glass alone). But in group AIII were better than group AII and group AI, respectively. In group AI, the graft had been showed the same incorporated in experimental and control side, but less than group AII. The consolidation of Biograns® particle was showed as same as between the experimental and control side in each specimen, but in group AIII the Biograns® particle was shown more consolidation than group AII and the latter was shown more consolidation than group AI.

In group AI (Bioactive glass + EMD vs. Bioactive glass alone, healing period 2 weeks), Bioactive glass particles were observed in both side of bone defect in all specimens and showed well incorporated with sounding bone. The defects area had flat surface and bone defect margins of both side could be observed. (Figure 22)

In group AII (Bioactive glass + EMD vs. Bioactive glass alone, healing period 4 weeks), Bioactive glass presented homogenously in the whole area of bone defects with hard consistency. The defect area had flat surface and bone defect margins of both side still be observed. (Figure 23.)

In group AIII (Bioactive glass + EMD vs. Bioactive glass alone, healing period 8 weeks), Bioactive glass was filled with dense bone-like tissue in all specimens. The defect area had flat surface. The dense bone-like tissue also extended over the edge of bone defects. (Figure 24.)

In group B (Enamel matrix derivative alone vs. Empty, healing period 8 weeks),

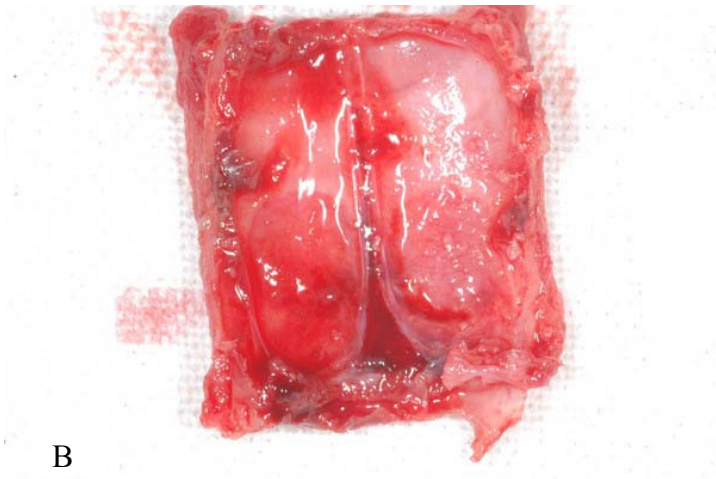
Specimens were occupied by soft fibrous tissue in all of 3 specimens. The surface of the defect area was flat and soft in consistency. The dense bone-like tissue also extended over the edge of bone defects. (Figure 25.)

The gutta-percha markers were showed a biocompatible material because there were no sign of infection at the markers. These markers were helpful for location the bone defect margin especially from the radiographs. Although, the bone defect margin of both side could be observed in all specimens, the bone defect margin of the specimens in group B were more obviously to detect than in group AI, AII and group AIII respectively.

Rostral

A Caudal

Rostral

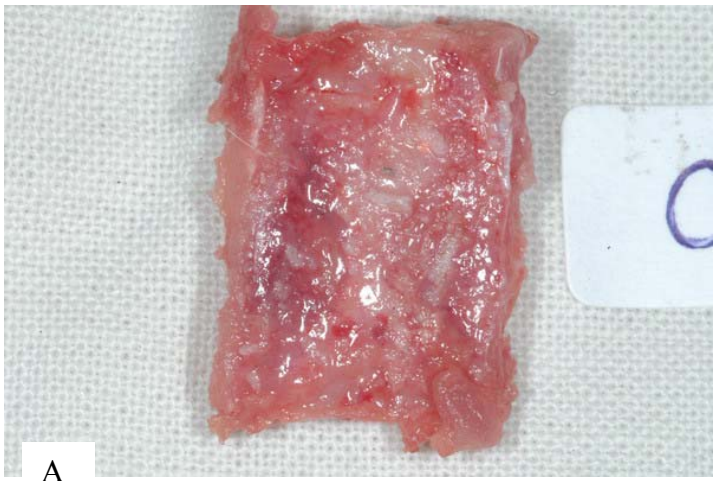


B

Caudal

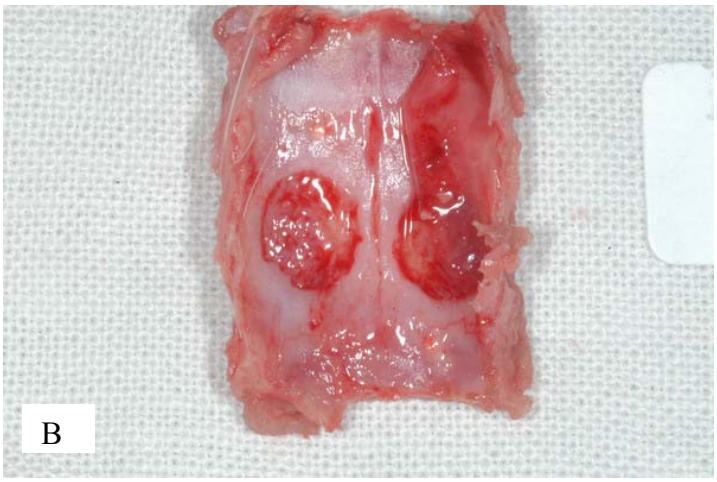
Figure 22. Specimen of rat calvarial 2 weeks after grafting with bioactive glass mixed with enamel matrix derivative and bioactive glass alone (Group AI). On the periosteal side of the rat calvarial (A). On the endocranium side of the rat calvarial (B).

Rostral



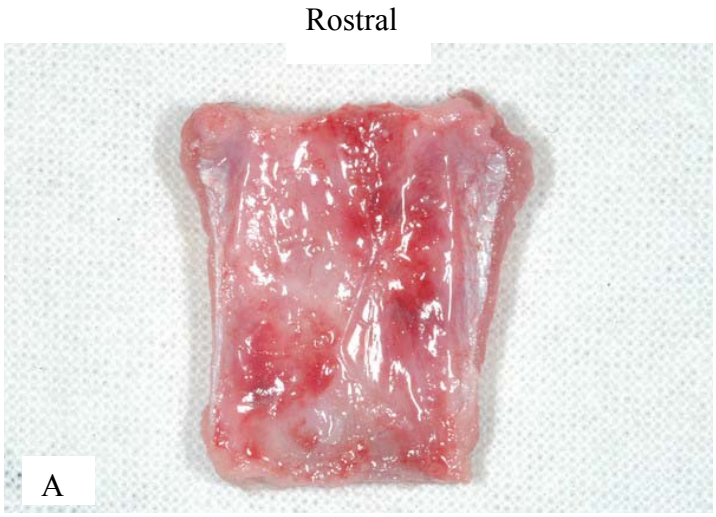
Caudal

Rostral



Caudal

Figure 23. Specimen of rat calvarial 4 weeks after grafting with bioactive glass mixed with enamel matrix derivative and bioactive glass alone (Group AII). On the periosteal side of the rat calvarial (A). On the endocranium side of the rat calvarial (B).



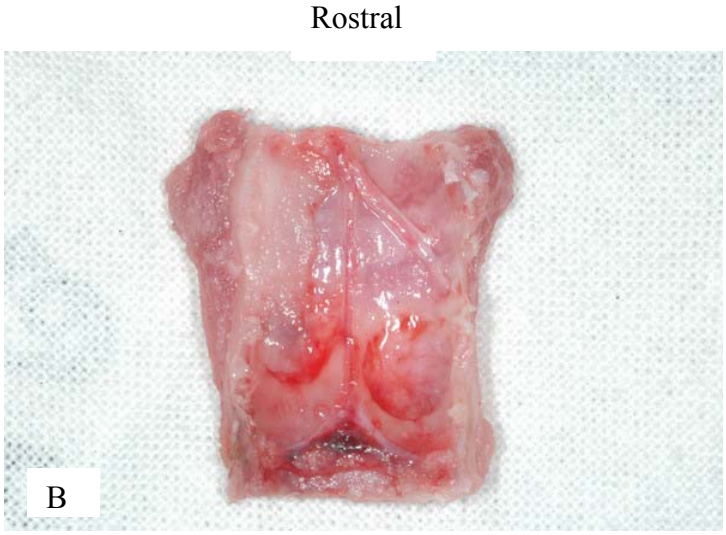
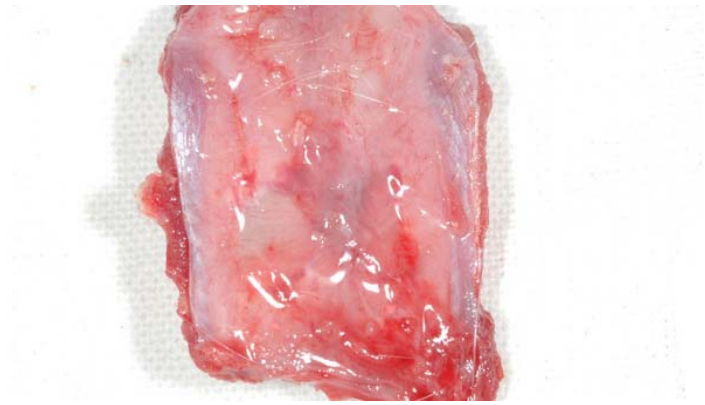


Figure 24. Specimen of rat calvarial 8 weeks after grafting with bioactive glass mixed with enamel matrix derivative and bioactive glass alone (Group AIII). On the periosteal side of the rat calvarial (A). On the endocranium side of the rat calvarial (B).

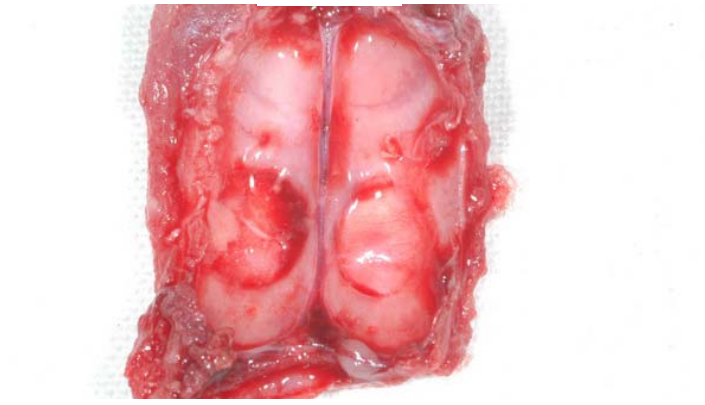
Rostral



A

Caudal

Rostral



B

Caudal

Figure 25. Specimen of rat calvarial 8 weeks after grafting with enamel matrix derivative alone and empty defect (Group B). On the periosteal side of the rat calvarial (A). On the endocranium side of the rat calvarial (B).

Radiographic Evaluation

In the period of 2, 4 and 8 weeks, from radiographic evaluation, well-delineated round bone defects were observed together with the radiopaque areas of bone graft materials in situ. (Figure 26, 27) The gutta-percha markers could also be observed in all specimens. The radiodensity of all the defects, experimental and control sides, seemed to appear the same as shown in Table 1.

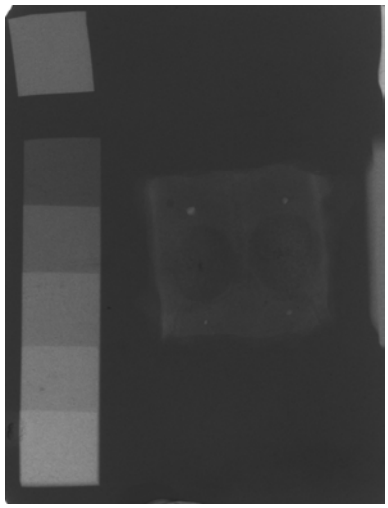


Figure 26. Radiograph of group A grafted with bioactive glass plus enamel matrix derivative and bioactive glass alone. The defect margins were observed, experimental side (right) was shown a radiopacity as well as the control side (left).

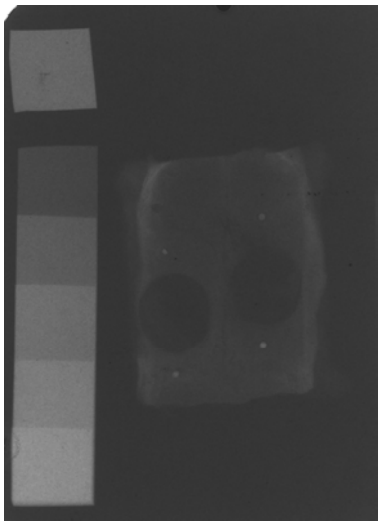


Figure 27. Radiograph of group B grafted with enamel matrix derivative alone and control side(empty cavity) healing period 8 weeks. The defect margin were observed, experimental side (left) was shown a radiopacity as well as the control side (right).

Radiomorphometric analysis

The values, established to characterize the amount of mineralized tissue produced in response to all type of graft material, were represented as the average radiographic optical density (Mean OD). Each defect side was measured for Mean OD value and calculated for the average and standard deviation as listed in Table 1.

Table 1. The data of radiomorphomeric optical density in group A and B

n = 5

Group	Week	Mean optical density (Mean± SD)	
		Experiment	Control
AI	2	0.607 ± 0.031	0.597 ± 0.015
AII	4	0.627 ± 0.035	0.601 ± 0.050
AIII	8	0.677 ± 0.087	0.678 ± 0.063
B	8	0.626 ± 0.015	0.641 ± 0.007

Group A Experimental side = Bioactive glass + Enamel matrix derivative
Control side = Bioactive glass only

Group B Experimental side = Enamel matrix derivative only
Control side = Empty

The data was shown that the radiodensity in the experimental side was slightly more radiolucent than the control side in group A 2 and 4 weeks. But in group AIII and B the radiodensity in the control side was slightly more radiolucent than the experimental side.

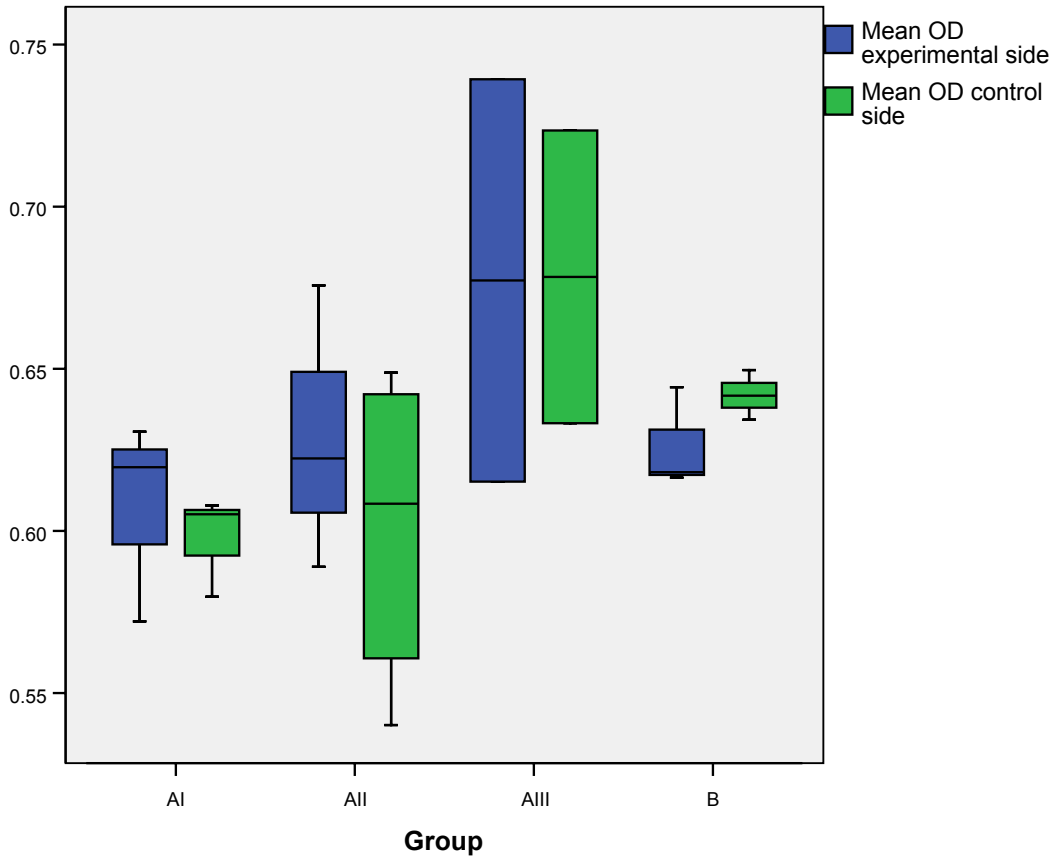


Figure 28. Distribution of mean optical density in rat's calvarial specimens.

Histological Evaluation

Microscopically, there was only minimal bone growth in the defect area with no bridging of the defect in all studied. **Group AI** Specimen of rat calvarial 2 weeks after grafting with bioactive glass mixed enamel matrix derivative (Figure 29, 30) and bioactive glass alone (Figure 31, 32). The bone edge of the defect presented an irregular morphological appearance, with small areas of reparative bone neoformation in both the groups except only one specimen in the control side. A large number of residual of bioactive glass particles were observed with osteoprogenitor cells permeating the intergranular spaces. Fibrous connective tissue was found in the both defects.

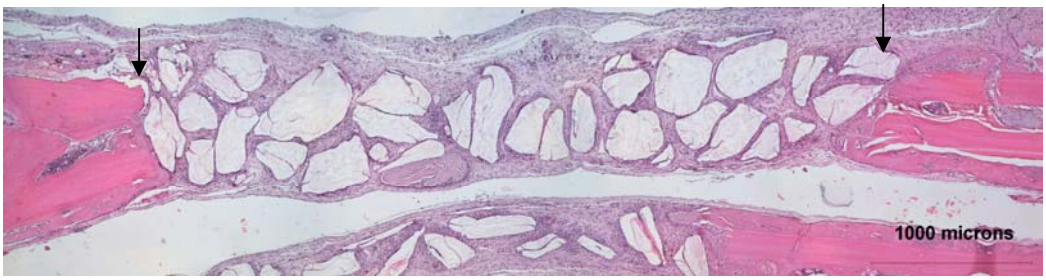


Figure 29. Saggital histological section through the calvaria showing defect from the critical size bone graft with bioactive glass plus enamel matrix derivative after 2 weeks. Note the bony ingrowth from the margins defect and have new bone supradural region (Specimens stained with Hematoxylin and Eosin). Arrows indicate the margins of the defect prepared initially.

Figure 30. Healing of defect group AI (2 weeks) after grafted with bioactive glass plus enamel matrix derivative (experimental side), demonstrating dense fibrous tissue around the particles and new bone deposited at the margin of the defect. (Specimens stained with Hematoxylin and Eosin, original magnification ×5)

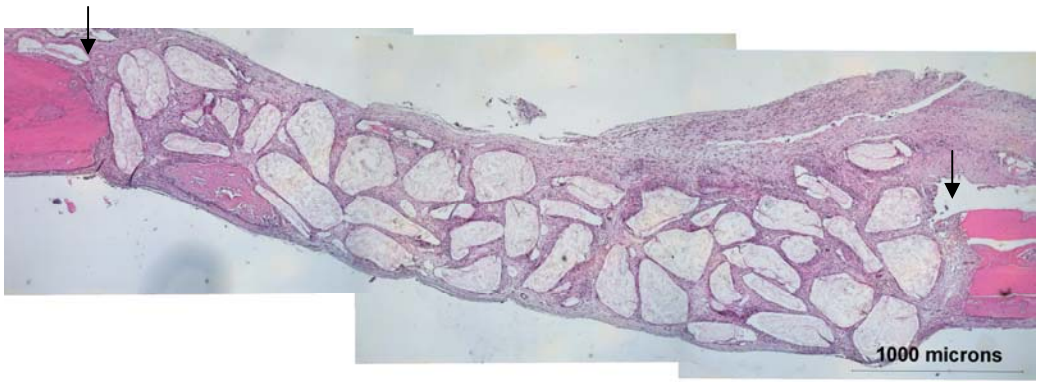


Figure 31. Saggital histological section through the calvaria showing defect from the critical size bone graft with bioactive glass alone after 2 weeks. (Specimens stained with Hematoxylin and Eosin). Arrows indicate the margins of the defect prepared initially.

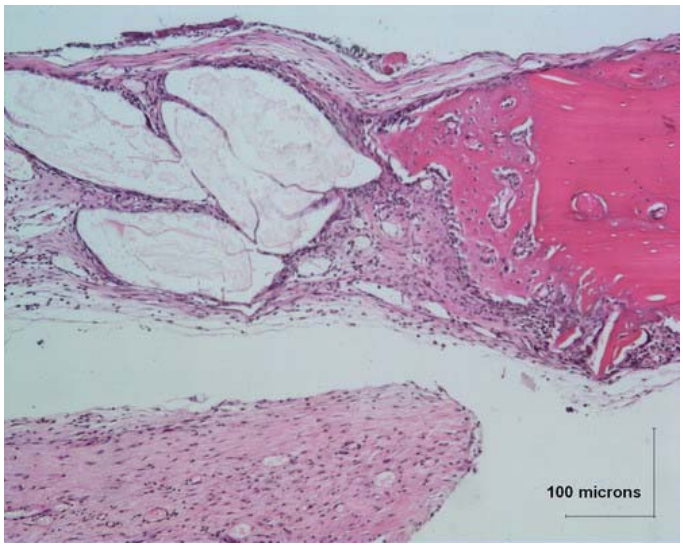


Figure 32. Healing of defect group AI (2 weeks) after grafted with bioactive glass alone (control side), demonstrating dense fibrous tissue around the particles and newly formed bone deposited at the margin of the defect with an orientation that differs from that of the pre-existing bone. The bioactive glass particles were seen in the defect (Specimens stained with Hematoxylin and Eosin, original magnification ×10).

Group AII Specimens of rat calvarial bone 4 weeks after grafting with bioactive glass mixed enamel matrix derivative (Figure 33, 34) and bioactive glass alone (Figure 35, 36). The histological observations were similar to the 2 weeks observation. At the bone edges of the defects, bone neoformation with limited expansion, the new bone extended from the defect margin and grown into the central portion of the bone defect. In the fibrous and organized stroma there were vascular proliferations. The particles of the material frequently presented complete and incomplete penetration and there was deposition of noncollagenous amorphous material on them. Progressive osteoprogenitor cell invasion into these intragranular spaces were observed (Figure 34, 36). In experimental group, there was one specimen presented an area of mineralization in supradural region.

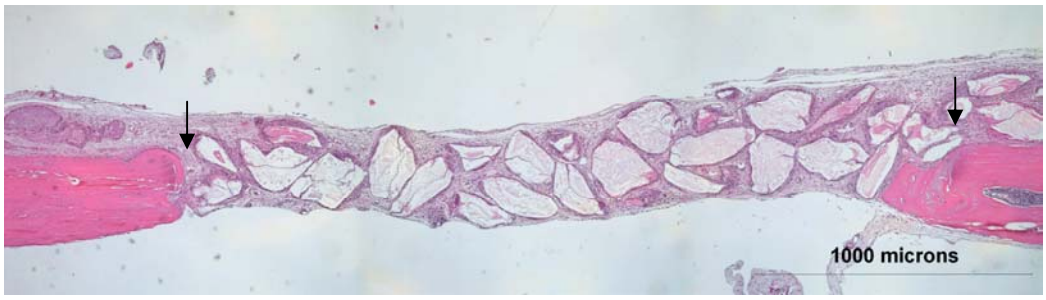


Figure 33. Saggital histological section through the calvaria showing defect from the critical size bone grafted with bioactive glass plus enamel matrix derivative after 4 weeks. (Specimens stained with Hematoxylin and Eosin). Arrows indicate the margins of the defect prepared initially.

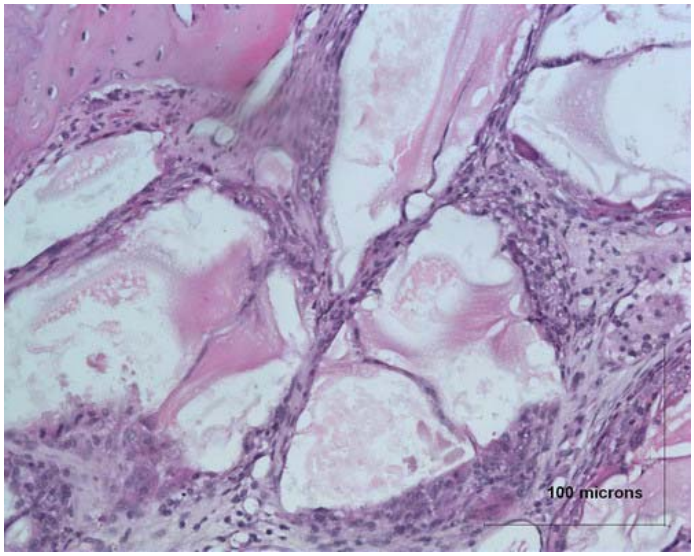


Figure 34. A specimen from group AII (4 weeks) after grafted with bioactive glass plus enamel matrix derivative (experimental side), demonstrating numerous fibroblasts.The bioactive glass particles were presented fragmented appearance, presenting osteoprogenitor cell infiltration into these fissures. Deposition of dense connective tissue was also presented in these areas(Specimens stained with Hematoxylin and Eosin, original magnification ×20)

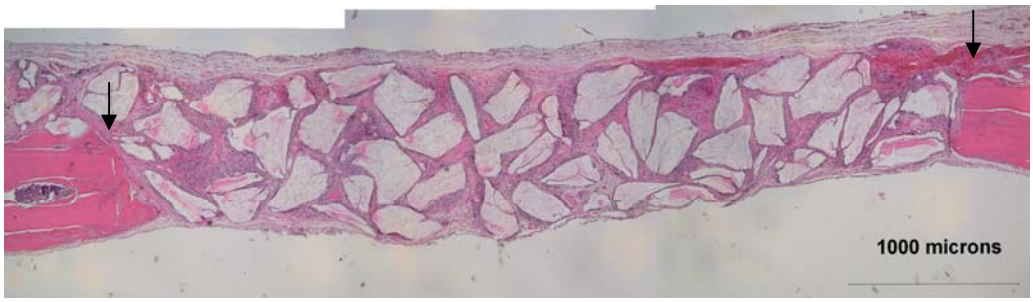


Figure 35. Saggital histological section through the calvaria showing defect from the critical size bone grafted with bioactive glass alone after 4 weeks. (Specimens stained with Hematoxylin and Eosin). Arrows indicate the margins of the defect prepared initially.

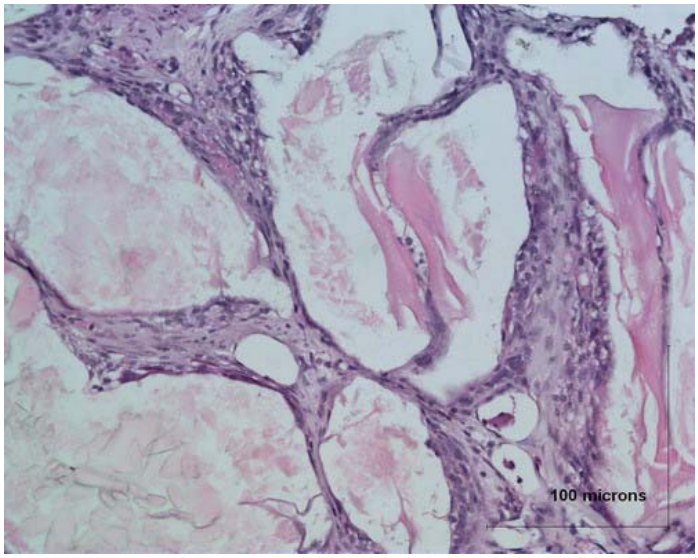


Figure 36. A specimen from group AII (4 weeks) after grafted with bioactive glass alone (control side), Bioactive glass particle showing cracks and excavated centers with invasion of osteoprogenitor cell. Amorphous material can also be observed in some excavated centers.(Specimens stained with Hematoxylin and Eosin, original magnification, ×20)

Group AIII Specimen of rat calvarial 8 weeks after grafting with bioactive glass mixed enamel matrix derivative (Figure 37, 38) and bioactive glass alone (Figure 39, 40). At the bone edges of the defect, bone neoformation with expansion in direct contact with the particles of the bioactive glass was noted. A lower number of residual bioactive glass particles were observed within the bone defects and a greater amount of dense, organized connective tissue. At this time, the particles appeared to be more fragmented. The histologic appearance showed an increase in bone formation over the time, noticeable that the new bone formation found in 8 weeks was greater than in 4 and 2 weeks, respectively. In a few cases, there were areas of mineralization in supradural linear plates without any explanation. However, in experimental groups there were more new bone at the edge of the defects. Moreover, the histologic examination revealed the amount of new bone ingrowth on experimental sides was greater than the control sides.

Figure 37. Saggital histological section through the calvaria showing defect from the critical size bone grafted with bioactive glass plus enamel matrix derivative after 8 weeks. (Specimens stained with Hematoxylin and Eosin). Arrows indicate the margins of the defect prepared initially.

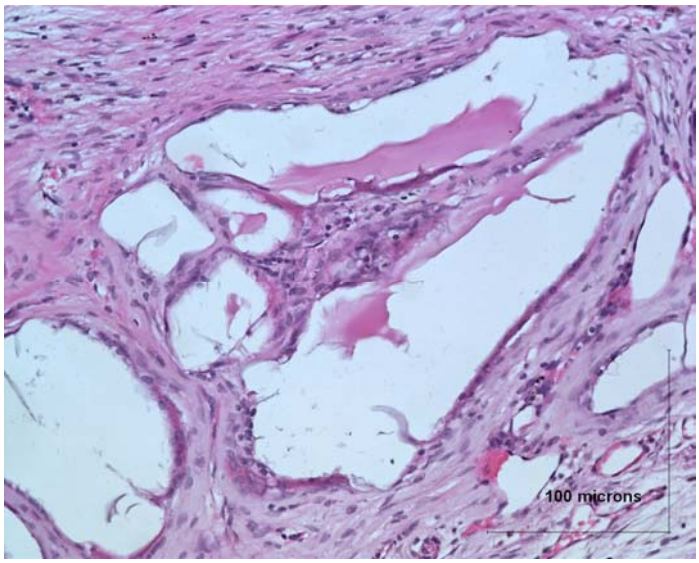


Figure 38. A specimen from group AIII (8 weeks) after grafted with bioactive glass plus enamel matrix derivative (experimental side), bioactive glass particles presented with cracked appearance and osteoprogenitor cell infiltration into these fissures. (Specimens stained with Hematoxylin and Eosin, original magnification, ×20)

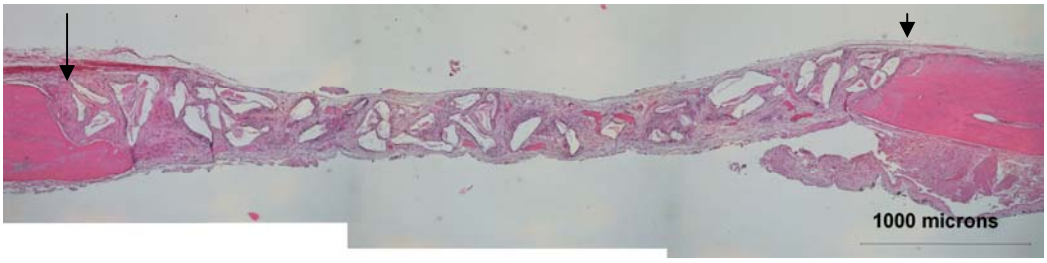


Figure 39. Saggital histological section through the calvaria showing defect from the critical size bone grafted with bioactive glass alone after 8 weeks. (Specimens stained with Hematoxylin and Eosin). Arrows indicate the margins of the defect prepared initially.

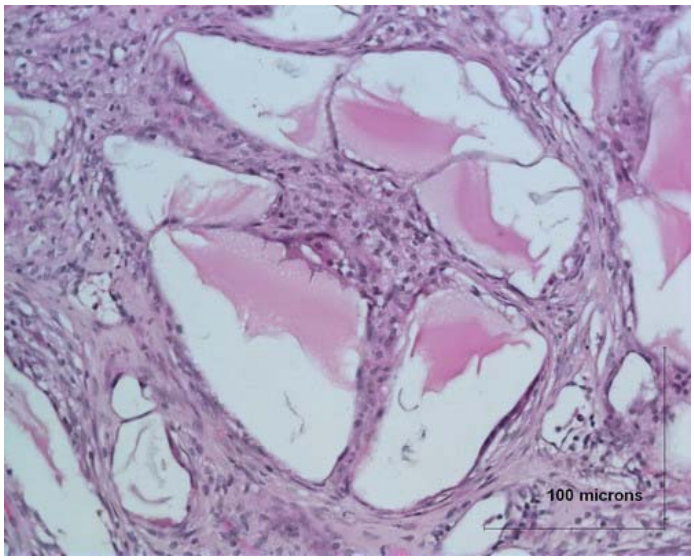


Figure 40. A specimen from group AIII (8 weeks) after grafted with bioactive glass alone (control side), dense connective tissue was covering the particles. Fibrous septation and deposition of noncollagenous amorphous matter were presented in the particles. (Specimens stained with Hematoxylin and Eosin, original magnification, ×20)

Group B Specimens of rat calvarial 8 weeks after grafting with enamel matrix derivative alone (Figure 41, 42, 43) and without grafting (Figure 44, 45, 46), Histologic evaluation of these specimens revealed a thin fibrous across the defect with small amounts of new bone being occasionally seen at the bony margin of the defect (Figure 43, 46). In the central areas of the defect, the connective tissue appeared to be thinner with a smaller number of collagen fibers. In two specimens, there were small new bone formations in supradural regions. All specimens were well vascularized and rich in fibroblasts with oriented collagen fibers.

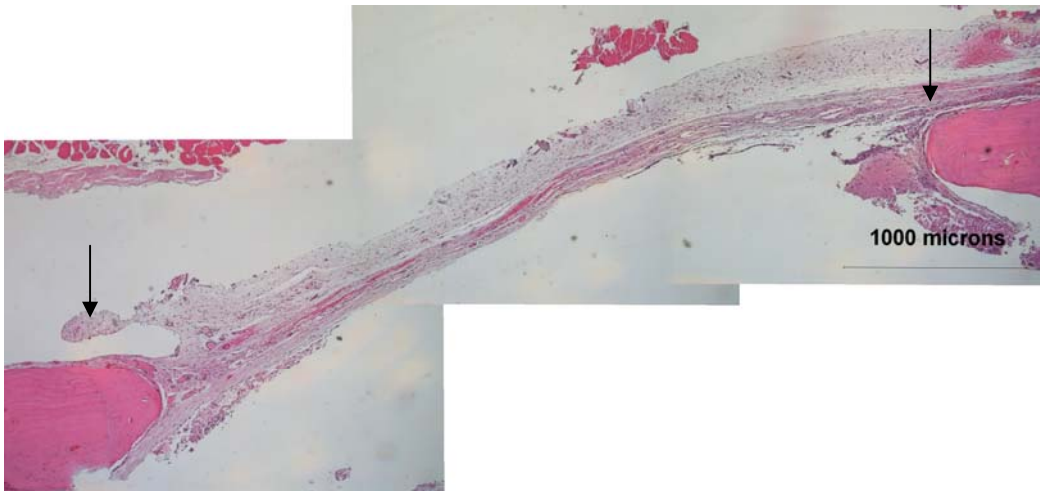


Figure 41. Saggital histological section through the calvaria showing critical size defect filled with enamel matrix derivative alone after 8 weeks. Surgical defect with fibrous connective tissue was thinner than the original calvaria. Interstitial spaces were completely filled with dense connective tissue (Specimens stained with Hematoxylin and Eosin). Arrows indicate the margins of the defect prepare initially.

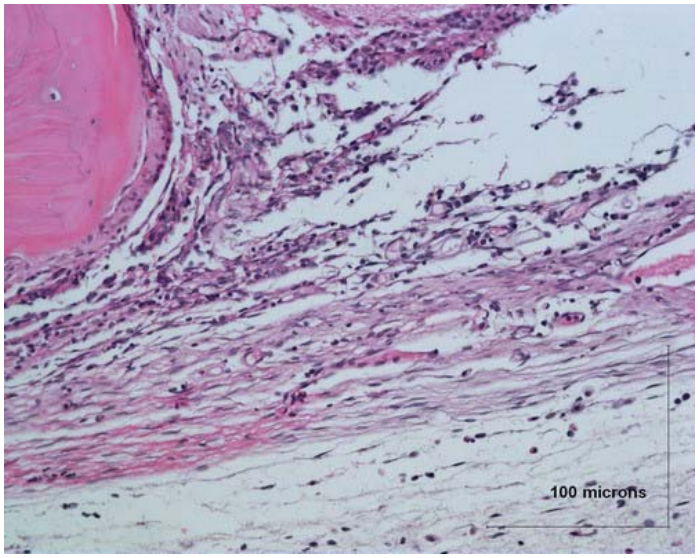


Figure 42. A specimen from group B at 8 weeks filled with enamel matrix derivative (experimental side). New bone was observed at the defect edges. (Specimens stained with Hematoxylin and Eosin, original magnification, ×20)

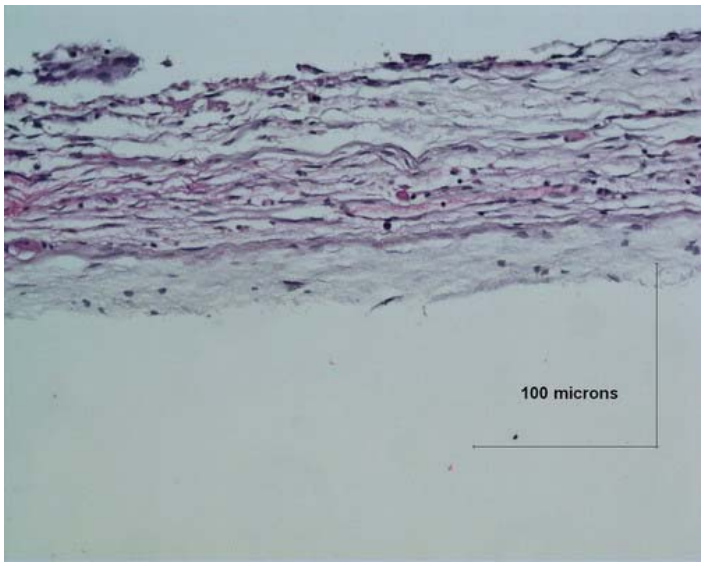


Figure 43. A specimen from group B at 8 weeks filled with enamel matrix derivative (experimental side), demonstrating areas of hyalinization in plates (Specimens stained with Hematoxylin and Eosin, original magnification, ×20)

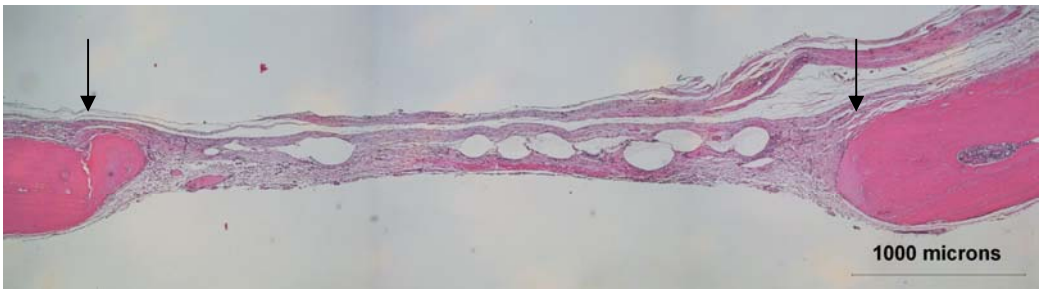


Figure 44. Saggital histological section through the calvaria showing critical size defect without grafting after 8 weeks. Surgical defect with fibrous connective tissue was thinner than the original calvaria. Interstitial spaces were completely filled with dense connective tissue (Specimens stained with Hematoxylin and Eosin). Arrows indicate the margins of the defect prepared initially.

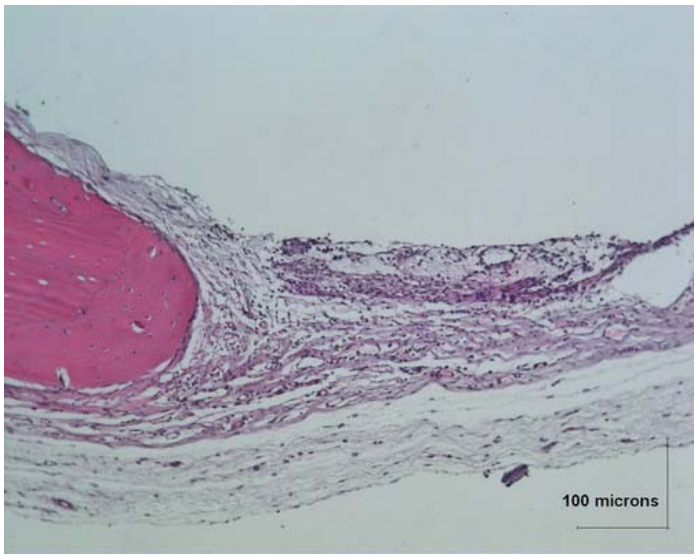


Figure 45. A specimen from group B at 8 weeks (control side), surgical defect with fibrous connective tissue was thinner than the original calvaria. Interstitial spaces were completely filled with dense connective tissue.(Specimens stained with Hematoxylin and Eosin, original magnification, ×10)

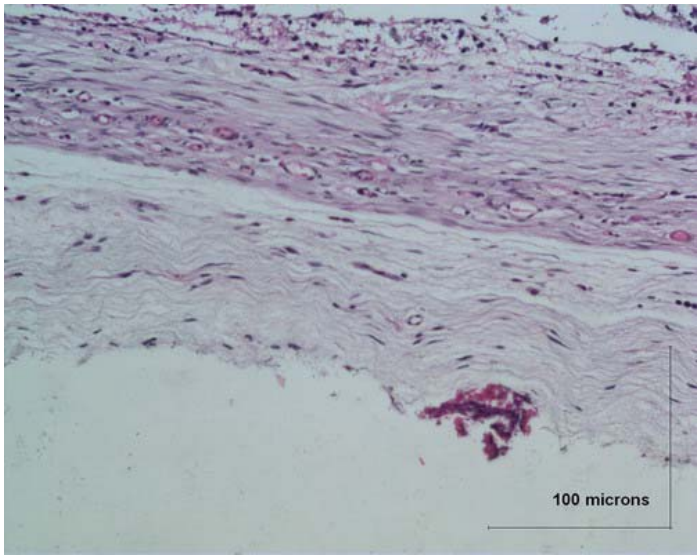


Figure 46. A specimen from group B at 8 weeks (control side), demonstrating areas of hyalinization in plates (Specimens stained with Hematoxylin and Eosin, original magnification, ×20)

Histomorphometric analysis

The histomorphometric analysis demonstrated mean new bone formation that revealed in percentage of new bone area (±SD) in Table 2. **In group AI,** at 2 weeks postsurgery, mean new bone formation that revealed in % new bone area (± SD) for bioactive glass with EMD (experimental) and bioactive glass alone (control) were 5.34 ± 3.79, 3.64 ± 1.84 respectively.

In group AII, at 4 weeks postsurgery, mean % new bone area (± SD) for bioactive glass with EMD (experimental) and bioactive glass alone (control) were 7.29 ± 3.21, 7.14 ± 7.01 respectively.

In group AIII, at 8 weeks postsurgery, mean % new bone area (± SD) for bioactive glass with EMD (experimental) and bioactive glass alone (control) were 17.98 ± 7.78, 7.05 ± 2.42 respectively. And in group B, for only enamel matrix derivative (experimental) and empty (control) were 12.12 ± 5.50, 7.78 ± 3.83 respectively. The data was shown that the percentage of new bone area in the experimental side was more than the control side in all of group A and group B.

Table 2. The data of histomorphometric analysis (percentage of new bone area) in group A and B.

n = 5

Group	**Week**	**Percentage of new bone area (Mean± SD)**	
		Experiment	**Control**
AI	2	5.34 ± 3.79	3.64 ± 1.84
AII	4	7.29 ± 3.21	7.14 ± 7.01
AIII	8	17.98 ± 7.78	7.05 ± 2.42
B	8	12.12 ± 5.50	7.78 ± 3.83

Group A Experimental side = Bioactive glass + Enamel matrix derivative
Control side = Bioactive glass only

Group B Experimental side = Enamel matrix derivative only
Control side = Empty

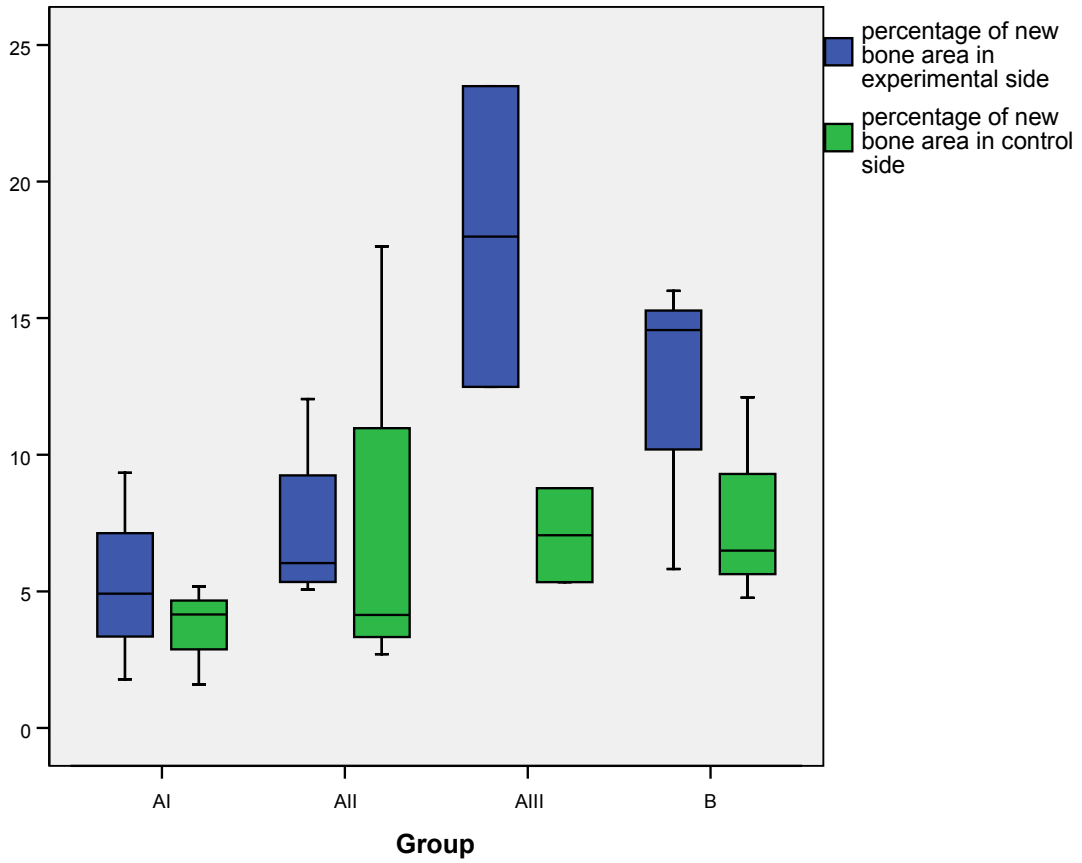


Figure 47. Distribution of percentage of new bone formation from histomorphometry analysis.

In summery, The data showed that the mean percentage of new bone in the experimental side was slightly better than the control side in 2, 4 and 8 weeks in group A and group B. In group AIII the difference of new bone between the experimental side and the control side was more than the other groups.

CHAPTER 4

DISCUSSION

In the past decade, tissue engineering-based bone grafting has emerged as a viable alternative to biological and synthetic grafts. The biomaterial component is a critical determinant of the ultimate success of the tissue-engineered graft. Because no single existing material possesses all the necessary properties required in an ideal bone graft. Enamel matrix derivative (EMD) is a mixture of proteins with amelogenin as a major component that has been demonstrated to promote periodontal regeneration. Recently, it has been shown that EMD can induce the formation of acellular cementum, collagenous fibers and alveolar bone. However, its influence may not be limited to the cementum. Using an *in vitro* wound model, it has been reported that the EMD can enhance wound-filling for all cell exposed to it in culture medium when compared to untreated condition.[59] EMD have influence bone metabolism through the activation of growth factor.[60] Takayama *et al*. reported that EMD promoted the osteogenic differentiation of pluripotent mesenchymal cells. This effect was mediated by BMP-like molecules present in the EMD.[61] Schwartz *et al*. showed that EMD can affect early stages of osteoblastic maturation by stimulating proliferation, but as cells mature in the lineage, EMD also enhances differentiation.[37] Jiang *et al*. reported that EMD enhanced gene expression of collagen I and IL-6 in primary mouse osteoblasts.[62] Moreover, EMD has been reported to sitmulate the biosynthesis and regeneration of trabecular bone and the volume fraction of mineralized tissue appeared to be higher in EMD- applied bone than in controls.[47] EMD has also been found to be an effective matrix for enhancing new trabecular bone induction and resulting attachment of orthopedic prostheses to the recipient bone.[48] In an animal study on dogs, Casati *et al*. evaluated the effect of EMD on bone healing of dehiscence-like osseous defects around dental implants with histomorphometry. The authors compared the use of EMD alone, EMD plus bioabsorbable barrier membrane, and bioabsorbable barrier membrane alone to a control group in the treatment of surgically created dehiscence-like defects around dental implants. The results showed that the group treated with EMD plus bioabsorbable barrier membrane had a statistically significant higher bone-implant contact percentage than the control group. The authors concluded that EMD

might positively influence bone healing around dental implants; however, EMD alone did not show any statistically significant effect.[52] Same as the result of Cornelini *et al*, in a study in rabbit tibia, there was no difference in bone regeneration between EMD treated and control sites.[50] The present study was designed to histometrically evaluate the effect of EMD and bioactive glass on bone healing of critical-size defects in the calvaria of rats. Bone defect's size is in agreement with previous studies of critical-size defects in various animal models.[46] It has to be realized that the critical-size defect model represents a challenging situation for bone formation as opposed to models using self-contained and/or dehiscence-type defects. The results of the present experiment had demonstrated that bioactive glass plus EMD had more new bone area than as was seen in bioactive glass alone after healing period 2 , 4 and 8 weeks and the mean optical density found the experimental side (bioactive glass plus EMD) had more optical density than control side (bioactive glass alone) at 2 and 4 weeks. But at 8 weeks, the mean optical density of experimental side and control side is nearly the same. After a healing period of 8 weeks, the percentage of new bone in the group of bioactive glass plus EMD is highest, EMD alone, control group and bioactive glass alone respectively. The amount of new bone formation comparing between bioactive glass alone group and the control group was nearly the same in 8 weeks but when there is more resorption of bioactive glass in the later period, the amount of new bone formation might be much greater. The slow resorption of the bioactive glass particles probably accounted for the greater amount of newly formed bone observed in control group when compared with bioactive glass alone group. According to MacNeill *et al*, graft materials that require extended time periods for complete resorption will reduce the total amount of newly formed bone due to their continued presence.[63] When EMD was apply alone, the magnitude of new bone formation was higher than control group. This observation is in the same result as the recent observation in which positive influence on bone formation was seen following the application of EMD on calvarial defects of rats.[49] Bioactive glass has documented osteoconductive and osteostimulatory properties. Hence, its use has been recommended in order to provide scaffold for the regeneration of various bone defects. In the present model, the narrow granule size range of 300-355 μm leads to an interfacial ion exchange throughout the particles, followed by a specific cellular response.[19, 21] Like other bioactive glass particle of similar composition but different size range, not only do they form a Ca-P rich layer on their outer surface, which is responsible for the extensive osteoconductive properties, but the particles themselves are eroded internally by phagocytosing cells entering via

small cracks.[64] Most of the bioactive glass underwent resorption over the time, which reduced their diameter and widened the entrance to the internal lumen. When bioactive glass combined with EMD 17.98 % of new bone area was observed. This finding show the highest of new bone formation at 8 weeks when compare with another group. The observation from the present study showed that the group of bioactive glass plus enamel matrix derivative presented more new bone formation correspondence with more time period. From this result we can apply to use in tissue engineering, the use of ceramic composites as matrices and scaffolds for drug delivery, carrier material for enamel matrix derivative. In the study of Cornelini R. *et al* found the enamel matrix derivative alone after implanted in the tibia of rabbit 4 weeks is fully resorbed.[50] But in this study, there was more bone formation in 8 weeks, this might be the effect of enamel matrix derivative. Though bioactive glass was not directly compared with other carrier systems for enamel matrix derivative driven bone formation, it would be a promising carrier material with the potential to adsorb enamel matrix derivative.

In this study, bioactive glass could have provided a protected coagulum, preventing the decrease in the regeneration space and also helped to keep the EMD in place. The combined bioactive glass plus EMD provided more percentage of new bone formation when compared to bioactive glass alone, the magnitude of the values indicated some advantage to the combination. This result indicate that EMD is an osteopromotive agent.

From this study, it showed that the association of bioactive glass plus EMD had positively influence bone healing since a greater percentage of new bone area was observed when compared to the other. The data also indicate that EMD involved with bone healing even in the absence of PDL cells. More studies are necessary to evaluate the benefits of EMD associated with other regenerative procedures such as bone grafts and guided bone regeneration and also to clarify the specific components and mechanisms of action behind the observed effects.

The finding of the present study should be considered with caution due to the small sample size. In addition, direct extrapolation of data obtained from animal studies to humans should be interpreted cautiously.

CHAPTER 5

CONCLUSION

From the result of this study, we may be concluded that Biograns® with enamel matrix derivative made more new bone formation in the rat calvarial bone defects when compare with Biograns® alone at 2, 4 and 8 weeks. Enamel metrix derivative alone used in the rat calvarial bone defects also made more new bone formation compared with unused defects. Due to the small number of rats in this study, to confirm the result, further study needs to be preformed.

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APPENDIX

APPENDIX

Histomorphometric analysis

The Image-Pro Plus 5.0 program was used for the histomorphometric analysis. The most central histological section of each surgical defect was selected for the statistical evaluation. Each section was initially inspected using a light microscope (×5 objective) and saved as a digital image. A composite digital image was then created by combining 3–4 smaller images because it was not possible to capture the entire defect in one image at the level of magnification that was used. (Fig. 1)

Figure 1. Four capture images of the histological section merged to create a single composite image comprising the entire length of the surgical defect.

The following criteria were used to standardize the histomorphometric analysis of the composite digital image: 1) The captured images of each histological section were merged on the computer screen to create a single composite image comprising the entire length of the surgical defect. The total area (TA) to be analyzed was then delineated on the composite image. It corresponded to the calvarial bone defect where the surgical defect had been previously created.

2) The newly formed bone area (NFBA) was also delineated (Fig. 2). The total area (TA) was considered 100% of the area to be analyzed (Fig. 3). The percentage of NFBA was calculated according to the following formula:

$$\% \text{ new bone} = \frac{\text{new bone area}}{\text{Total bone defect area}} \times 100$$

The values of percentage of NFBA of each animal were used to calculate the means and standard deviations of each control and experimental group.

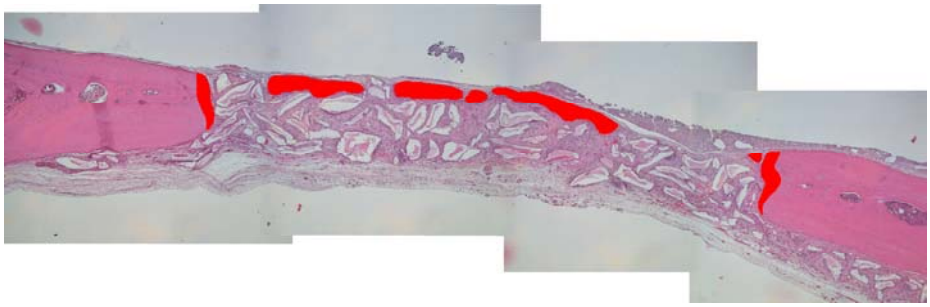

Figure 2. Calculation of the newly formed bone area (NFBA).

Figure 3. Calculation of the total area (TA).

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