Chapter 4

Discussion

Discussion

Researchers in oral and maxillofacical surgery are effort to improve bone grafting material. An ideal bone substitute should be biocompatible and gradually replaced by new bone. It should have osteoconductive/osteoinductive properties. (Jensen, et al.,1996) Osteoconduction is the act or process of stimulating osteogenesis. Osteoinduction is defined as transformation of non-osseous connective tissue cells into osteogenic and chondrogenic cells. Osteoconduction is characterized as bone growth by apposition from the surrounding bone. This process provides a physical matrix or scaffolding suitable for deposition of new bone. (Khan, Tomin and Lane, 2000; Kim and Lim, 2001) The most common osteoconductive bone graft materials are xenogenic grafts, such as Bio-Oss®. (Jensen, et al.,1996; Klinge, et al.,1992; Skoglund, Hising and Young, 1997) However, Bio-Oss® was no the ideal bone graft material, because there has only osteoconductive property. (Artzi and Nemcovsky,1998; Boyne, 1997: 3-21; Khan, Tomin and Lane, 2000; Paolantonio, et al.,2001; Proussaefs, et al.,2003; Ruhaimi,2001) Growth factors are an alternative way to improve and acceleration both soft tissue and bone healing. (Herndon, Nguyen and Glipin, 1993; Kawase, et al.,2003; Khan, Bostrom and Lane, 2000; Lee, 1997; Lind, 1996; Mundy, 1996; Rudkin and Miller, 1997; Schliephake, 2002) In our current knowledge suggested that platelets contain angiogenic, mitogenic and vascular growth factors, especially TGF-β and PDGF, in their alpha-granules. (Marx, 1999: 71-82; Marx, 1998) TGF-β_1 and TGF-β_2 have been shown to improve bone formaiton, inhibit bone resorption, osteoclast formation, and accelerate rapid maturation of collagen in early wound healing. PDGF increase the population of wound healing cells and recruits other angiogenic growth factors to the wound site. (Herndon, Nguyen and Glipin,1993; Khan, Bostrom and Lane, 2000; Lind,

1996; Mundy, 1996) It is therefore a reasonable hypothesis that increasing the concentration of platelets in a bone defect may lead to improved wound healing. To prepare PRP, in this study, freshly collected rabbit whole blood was centrifuged at 2000 rpms for 10 minutes as the first step and then, after discarding the RBC fraction, at 4000 rpms for 15 minutes as the second step. The platelet density in the resulting PRP preparation was increased by 1297.44% when compared to the whole blood. This percentage increase in platelet density was higher than that obtained by **Marx RE** (Marx, et al.,1998), **Landesberg R** (Landesberg, Roy and Glickman, 2000) and **Aghaloo TL** (Aghaloo, et al.,2002) This discrepancy could be due to a variation of the procedures for centrifugation of the original plasma samples (e.g. force, time, etc.). Additionally, the operator may alter the final concentration by increasing or decreasing the volume into which the platelet bottom is suspended. Moreover, **Froum SJ** (Froum, et al.,2002) suggested the PRP platelet concentration will depend upon three factors: (1) the total number of platelets in the original sample; (2) the recovery rate of the system used and (3) the final volume of plasma into which the platelets are suspended. The content of growth factor rise in linear relationship with the number of platelet. **Marx RE** (Marx, et al.,1998) presented the platelets sequestered by the centrifugation process showed an intense uptake of both PDGF and TGF-β monoclonal antibodies, thus confriming the presence and retention of these growth factors in the PRP preparation. **Okuda K** (Okuda, et al., 2003) showed the significant correlation between platelet counts and growth factors, PDGF and TGF-β, in PRP. We believed that those growth factors are significantly concentrated along with platelets count in the PRP preparation and could be improve bone regeneration in this study. (Garg, 2001; Marx, et al., 1998; Sonnleitner, Huemer and Sullivan, 2000; Weibrich, Kleis and Hafner, 2002) However, the level of PRP growth factors in this study have not been specifically determined and have not been study in the biological effects on the cells culture. The technique for PRP preparation that used in this study could not be induce significant PRP growth factor release. **Ekback G** (Ekback, et al.,2002) reported that increasing platelet concentration by centrifugation does not significantly induce P-selectin expression, which is a marker

of platelet degranulation. **Lacoste E** (Lacoste, Martineau and Gagnon,2003) reported that the mean growth factor concentrations detected in supernatants from non-activated PRP were not difference significantly from growth factor concentrations reported in plasma from whole blood. They were suggesting that the technique for PRP preparation does not induce significant growth factor release.

The results obtained from radiomorphometric analysis showed that the radiodensity in the experimental site was slightly greater than control site in 2 and 4 weeks, these result due to the property of PRP gel. These gel prevented the migration of Bio-Oss® particles and easily to handle while placed Bio-Oss® particle into bone defect. The property of PRP gel in handling the particulate graft material were dramatically improved by the addition of the thrombin-activated PRP. The resultant fibrin formation consolidated the graft, allowing it to be cut into conveniently sized blocks that could be easily carried to and inserted into the defect. Our finding was consistent with many study that suggested PRP gel improved the handling properties of the graft material with which it was combined, thereby facilitating graft placement and stability. (Anitua, 1999; Froum, et al.,2002; Kassolis, Rosen and Reynold, 2000; Marx, et al., 1998; Shanaman, Filstein and Danesh-Meyer, 2001; Whitman and Berry, 1998) From above mention, PRP gel might be made the radiodensity in experimental site greater than control site in 2 and 4 weeks. In contrast, the radiodensity of control site was greater than experimental site at 6 weeks. These results may be explained by the fact that new bone formation in the control site was greater than experimental site in the 6 weeks. Thus, these radiodensity were consistent with the histomorphometric data in 6 weeks. The histologic study, in both site, showed the bone formation was direct deposition on the surface of the Bio-Oss® particles. The histology result were shown the slightly inflammatory cells infiltration in bone defect in 2 weeks group, but not inflammatory cell was found in 4 and 6 weeks group, these result confirmed the biocompatible of Bio-Oss® material. In experimental sites, the regenerated bone was characterized by the direct apposition of new bone formation to the Bio-Oss® particles. The newly formed bone tissue showed the centripetal of bone in growth. This

characteristic was found in the osteoconductive material, such as Bio-Oss®. (Boyne,1997: 87-100; Hollinger, et al.,1990; Klinge, et al.,1992; Ruhaimi, 2001) Moreover, in the central portion of the experimental defect, an area of connective tissue could be observed, which harbord large numbers of graft particles. The histologic observations suggest that PRP in combination with Bio-Oss® did not appear to enhance the new bone formation. In present study, the multinucleated giant cells was not seen in the histologic specimen. We have not seen the resorption of Bio-Oss® particle in histologic specimen. In addition, the observation time period (2,4 and 6 weeks) in this study may not be sufficient for Bio-Oss® to completely disappear, but the question still arise whether this material indeed is resorbable, as other studies have indicated. It is controversial whether it is completely resorbable or only bio-degradable. **Klinge B** (Klinge, et al.,1992) reported almost total resorption of Bio-Oss® granules implanted in 5 mm rabbit skull defects after 14 weeks, whereas **Skoglund A** (Skoglund, Hising and Young,1997) described Bio-Oss® particles being present in the graft area in all of their six patients regardless of observation time (9 to 44 months). They concluded that this material seemed to degrade slowly rather than being resorbed. **Young C** (Young, Sandstedt and Skoglund,1999) reported newly formed bone was seen adjacent to Bio-Oss® without prior resorption of the Bio-Oss® in rabbits after 12 weeks.

The results from histomorphometric analysis showed the new bone formation in control site was greater than experimental site in 2, 4 and 6 weeks. In addition, the bone formation in both site increased along with the time period. However, this study did not show a significant increase in bone formation with the addition of PRP to Bio-Oss® particles in the histomorphometrically and the radiomorphometrically in noncritical sized defects in the rabbit cranium. From histomorphometric result, the bone formation in experimental site were less than control site in all time periods. This could be due to the absence of osteoblastic cells in the anorganic bovine bone and lack of an osteoinductive effect of PRP. (Roldan JC, et al.,2004; Wironen, Jaw and Fox, 2000) The potential of PRP to enhance bone formation was still controversial. Many studies reported more favorable outcomes following the use of PRP, suggesting that PRP may

improve new bone quality and quantity as a result of more rapid consolidation and graft mineralization. (Anitua, 1999; Fennis, Stoelinga and Jansen, 2002; Froum, et al.,2002; Garg, 2001; Marx, et al.,1998) However, other reports indicate that PRP may not be effective when used with bone substitutes. **Wironen JF** (Wironen, Jaw and Fox, 2000) have shown that PRP is not osteoinductive when added to demineralized bone matrix placed in pouches created in the recti abdomini muscles of athymic nude rats. More specifically related to this study, **Terheyden H** (Terheyden, 2000) compared rhOP-1 and PRP in bilateral sinus grafts that used 100% anorganic bovine bone as a grafting material. The PRP was not effective in producing bone regeneration, whereas in the contralateral sinus the rhOP-1 was effective. **Shanaman R** (Shanaman, Filstein and Danesh-Meyer, 2001) presented three cases reports study for evaluated the effect of PRP in combination with allogenic bone graft to enhance bone regeneration in alveolar ridge defects exhibiting both vertical and horizontal bone loss prior to the placement of dental implants. They suggested, the addition of PRP did not appear to enhance the quality or quantity of new bone formation over that reported in comparable guided bone regeneration (GBR) studies without PRP. **Froum SJ** (Froum, et al.,2002) presented the study to test the efficacy of PRP in three bilateral sinus graft cases with grafts of anorganic bovine bone that contained mineral or no autogenous bone. Their histomorphometric result indicated that the addition of PRP to the grafts did not make a significant difference either in vital bone production or in interfacial bone contact on the test implants. Also, the present study, we found that PRP did not potential to enhance bone formation when combined with Bio-Oss® particle in rabbit calvarial model. Mpreover, several authors have explained the mode of action of platelet growth factors. In review, platelet degranulation and release of growth factors occurs within 3 to 5 days, and the growth factor activity may end in as soon as 7 to 10 days. (Garg, 1999: 83-101; Marx, et al.,1998; Marx, 1999: 71-82;) Therefore, it seem to be that the PRP growth factors may be effective to enhance bone regeneration, when they used with autogenous bone. However, the present study, we use a nonvital bone substitute as a graft material for two reasons. First, reports using autogenous bone in the bone

regeneration model have shown PRP to have a positive effect on bone formation. Second, we felt it important to determine if PRP would have a similar positive effect on a noninductive, nonvital graft material. Also, from the result of these study, PRP may not produce the desired stimulatory response because of the vital bone cells in autogenous bone are needed for this stimulation were not occur. The comparison study in a mixture of bovine HA (Bio-Oss®, Geistlich Phamaceutical, Wolhusen, Switzerland) with PRP and Bio-Oss® alone in experimental model for evaluated new bone formation. **Zechner W** (Zechner, et al., 2003) said that " Furst et al, failed to detect a stimulatory effect on the number of bone to implant contact. They presumed the cause to be the poor osteoregenerative potential of the local bone stock of the sinus floor". **Yildirium M** (Yilidirium, et al.,2000) studied in 38 human biopsy samples taken after sinus floor elevation with autogenous venous blood plus Bio-Oss® and implant placement and reported in same data. **Zechner W** (Zechner, et al.,2003) were concluded the osteoregenerative potential of local bone, ie, its capacity to regrow, is a major factor in determining the effectiveness of growth factors, both autogenous and recombinant. To develop their stimulatory action, they apparently need a local bone stock with an adequate cellular reactivity in terms of preosteoblast count and the angiogenetic potential.

In addition, the explanation for the different healing responses observed clinically may relate to differences in concentration of PDGF or TGF-β with in the PRP between studies. Variations in concentration of PDGF are known to influence bone healing, and, as with other growth factors, such as BMPs, local variations in concentration may be a function of the type of carrier of delivery system. (Jiang, et al., 1999; Lee, 1997; Shanaman, Filstein and Danesh-Meyer, 2001) **Jiang D** (Jiang, et al., 1999) were indicated that by 15 minutes, maximal PDGF-BB adsorption to the material had occurred. This presented a reasonable time period for a clinician to add the solubilized PDGF-BB to the bone graft material and attain optimal results. However, their haven't been the study that demonstrated the appropriated time for solbilized the growth factors of PRP into Bio-Oss®. Moreover, a study using the rat calvarial model

showed that in certain concentrations, PDGF may actually interfere with certain BMPs, resulting in impaired bone regeneration. The complex biochemical interactions that may potentially occur at a molecular level between different growth factors contained within PRP and the host during wound healing are still not clear and require further study to better understand the effect of PRP on osseous regeneration. Although, autologous PRP, polypeptide growth factors can be obtained from an animals source or can also be genetically engineered. (Aghaloo, Moy and Freymiller, 2002; Fennis, Stoelinga and Jansen, 2002; Zechner, et al., 2003) However, their haven't been the study that demonstrated the effect of the animal platelet growth factors in stimulate bone formation like human platelet growth factors. In addition, **Schwartz Z** (Schwartz, et al.,2000) suggested that the small amounts of protein were present in deproteinized cancellous bovine bone in close association with the mineral phase. The results of these study indicate that the deproteinized cancellous bovine bone particles examined contain proteins and that at least some of these proteins are bioactive factors like TGF-β and BMP-2. However, these bioactive factors might be interaction to the growth factors in rabbit PRP. Moreover, the platelet growth factors that released during degranulation are chemotaxis and mitogenesis of mesenchymal cells, angiogenesis for capillary ingrowth, enhance bone cell replication, but not differentiated function of osteoblast. (Hock and Canalis,1994; Marden, et al.,1993) In addition, the platelet growth factors are powerful chemoattractive and proliferative agent for smooth muscle cells, endothelial cells and fibroblast than osteoblast. (Khan, et al.,2000; Lind,1996; Sanchez, Sheridan and Kupp,2003) Also, the excessive connective tissue formation were seen in the experimental site than control site in this study. The excessive fibroblasts which had migrated into the centre of bone defects in the experimantal site may inhibit osteogenesis by producing factors that hamper differentiation of osteogenitor cell populations. (Dahlin, et al.,1994; Nyman, et al.,1982; Ogiso, et al.,1989)

The use of bovine thrombin to activate PRP to formed gel has been associated the risk of life threatening. **Landesberg R** (Landesberg, Moses and Karpatkin, 1998) suggested that oral and maxillofacial surgeons should reconsider the used of bovine

thrombin because of bovine thrombin might be associated with the development of antibodies to factor V, XI and thrombin, with the potential development of life threatening coagulopathies. This phenomenon does not appear to be dose dependent. The risks associated with the use of bovine thrombin have led many surgeons to avoid its use. Practitioners who have placed PRP might also consider recalling these patients to evaluate the patient's PTT, PT, TT for 6 months or longer.

In this study, we used calvarial defects that had previously been proven to be good models for investigation the effect of bone graft material. (Kleinschmidt and Hollinger, 1992: 133-146; Schmitz and Hollinger, 1986) The calvarial bone and the facial bone are pure membranous bone. Morphologically and embryologically, the calvarial develops from a membranous precursor and thus resembles the membranous bone of the face. The biologic inertness of the skull as compared to other bone can be attributed to a poor blood supply and a relative deficiency of bone marrow. The middle meningeal artery provides the main cranial blood supply. A calvarial wound model has many similarities to the maxillofacial region. Anatomically, the calvarial consists of two cortical plates with regions of intervening cancellous bone similar to the mandible, also physiologically of the cortical bone in the calvarial resembles as atrophic mandible. Because the most severe test of a bone implant follows implantation in a skull defect, the calvarial has been a frequent site for the testing of bone repair materials. In addition, we selected the rabbit as the animal model in this study because of the rabbit is widely used as an experimental animal because of cheaper, easily to keep, the physiologic bone healing is like as a human, but 3 times higher than human (Roberts, et al., 1987) easily for manipulation and ethically better accepted for experiment than other animal such as goat, sheep, dog, monkey. Moreover, the histologic bone specimen is small also it would have been helpful for analysis the bone formation in the same histologic slide. The rabbit calvarial defect, compared with other experimental bone defects, is a suitable model for studying bone regenerative materials because of its effective accessibility and the lack of fixation requirements. (Ahn, et al., 2003; Kleinschmidt and Hollinger, 1992: 133-146; Kleinschmidt, et al.,1993; Schmitz and Hollinger, 1986) In addition, the defects are reproducible and native, and induced

healing processed have been well characterized. The “critical sized defect” (CSD) which implies that the defect does not heal by itself during the lifetime of the animal. Investigations of the ability of bone graft materials to obtain complete bone healing of a defect necessitates use of a CSD to exclude spontaneous bony regeneration of the defect. However, a critical sized cranial defect in the rabbit model is 15 mm. (Kleinschmidt and Hollinger, 1992: 133-146; Schmitz and Hollinger, 1986) Therefore, the two critical sized defects, that used in this study, could not be created in each of rabbit cranium due to the small size of the cranium. However, the most effective way to evaluate the effects of PRP on the bone formation in a calvarial bone graft is to study the effect in bilateral calvarial bone grafts, with the addition of PRP being the only controlled variable. Also, we decided to use two non critical sized defect in the same animal because of in this way it was possible to have the best control experimental model because both treament could be carried out in the same animal, with the same surgical procedure and also, the same condition of the healing process. Moreover, this study was only investigated the early events of bone regeneration in rabbit calvarial because of the majority of the bone regeneration took place within the first month of healing. Also, the significant differences might have seen in early wound healing periods (2, 4 and 6 weeks) between Bio-Oss® alone and Bio-Oss® with PRP. Thus, it possible to use a noncritical sized defect in this study.

The precise identification of the former defect borders is necessary for adequate stereologic and histologic evaluation. A gutta-percha marking was therefore made around all defects. However, no difficulties were observed in identifying the former edges in the present study because of the short observation period. Therefore, the developed gutta-percha marking seems more useful for studies involving a longer observation period. (Jensen, et al., 1996; Pallesen, et al.,2002)

The sample size was small, consisting of only 4 rabbits in each time period of 2, 4 and 6 weeks. This small sample size may have contributed to the lack of statistical significance for evaluated the effect of PRP to improve bone formation when combined with Bio-Oss®. In addition, the calvarial defect in the rabbit, which we used, was

relatively shallow in terms of its depth, and it is not exactly oral bone. These may be limiting factors in terms of applying the results derived from this study to a case involving a clinically larger bone defect. Therefore, further investigations are required using the clinically large bone defect model. Although the in vitro effects of growth factors, including those deived from platelets, are well documented in the literature,some problems are still unresolved. In the clinical setting their efficacy depends on such factors as growth factor dosage, mixing ratios and carrier material, the effect of each platelet growth factors such as PDGF, TGF-β, IGF-I on cell biology which still need the further studies.