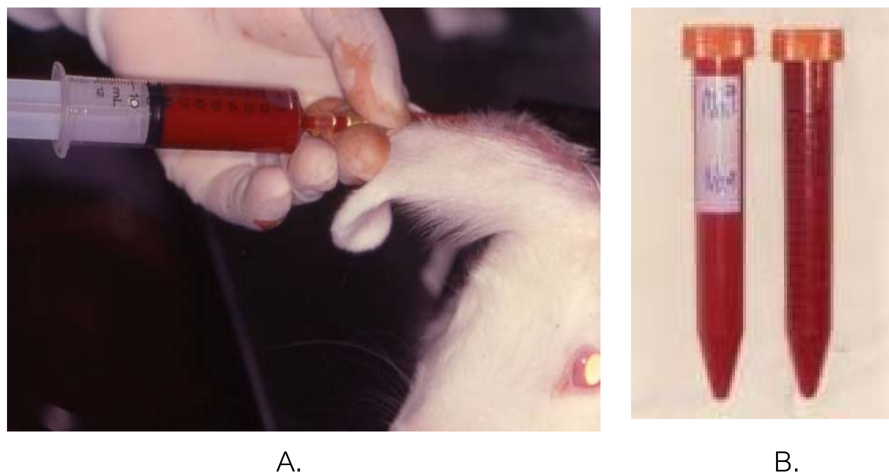
Chapter 2

Material and method

Material

<u>Platelet rich plasma (PRP) preparation</u>:

The animal, New Zealand white rabbit, was placed into a restraining box. Before blood collection, the animal was anesthetized with intramuscular injection of diazepam at a dose of 5mg/kg body weight. 10 minutes after anesthetized, 11 ml of rabbit autologous whole blood was collected from central ear artery by neddle gauge No.21 connected with 15 ml sterile syringe. After that, 11ml of rabbit whole blood was drawn into 15 ml plastic laboratory tube contained 3 ml acid citrate dextrose (ACD-A) as an anticoagulant (fig.8). One ml from 14 of rabbit whole blood/ACD-A solution was divided and drawn into the two Eppendorf tubes, 0.5 ml whole blood in each tube. One of the Eppendorf tube was counted the platelet whole blood and other would be kept at room temperature for mixed with Bio-Oss® and placed the Bio-Oss® with whole blood into control defect .



A. B.

Figure 8. A. rabbit whole blood was collected form marginal ear vein.

B.14 ml of rabbit whole blood/ACD-A solution in the laboratory tube.

The platelet rich plasma was prepared following aseptic processing procedure using 13 ml rabbit whole blood/ACD-A solution were placed into the SORVALL H-6000 A SWINGING BUCKET ROTOR® for centrifugation at Blood Bank, faculty of medicine, Prince of Songkla university (fig.9). The centrifugation speed was used following the prepared platelet concentration protocol of Blood Bank, faculty of medicine, Prince of Songkla university. The first centrifugation was made at 2,000 rpm (1170 g) for 10 minutes. After that, the rabbit whole blood was separated into two components; 1.Blood cell component (BCC) was, composed the red blood cell, WBC and platelet, formed at lower part of the tube and 2.Serum component (SEC) was, composed all of plasma and slightly platelet, was formed at upper part of the tube. The SEC and the first 3 mm upper part of BCC were collected by disposable plastic pipet. These solution was drown into the second 15 ml plastic tube and placed into the machine for second centrifugation processed. The second centrifugation was made at 4,000 rpm (4660 g) for 15 minutes. After second centrifugation, the blood component was separated into two layers: the upper layer was platelet poor plasma (PPP) and the lower layer was platelet rich plasma (PRP). All of platelet poor plasma was removed using disposable plastic pipet, only 1 ml of platelet rich plasma component was remaining.

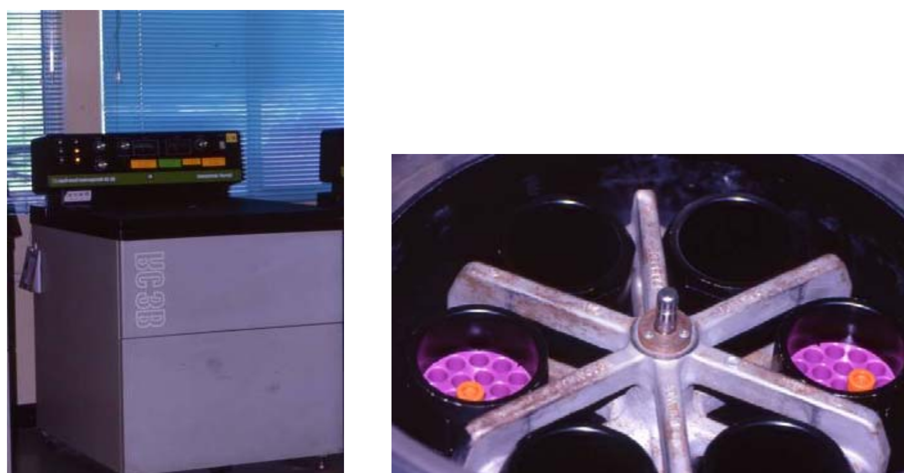


Figure 9. SORVALL H-6000 A SWING BUCKET ROTOR was used for preparation of autologous PRP.

One ml of PRP was divided and drawn into the two Eppendorf tubes, 0.5 ml of PRP in each tube. One of the Eppendorf tube was used for platelet PRP counted and other was kept in disinfected manner at room temperature (28 – 30°c) until the surgeon are ready for its used. The whole blood and PRP platelet were measured by the machine count, Sysmex K-1000 (TOA Medical electronic Co. LTD, KOBE, JAPAN), at Blood Bank of PSU (fig.10). The PRP gel preparation required initiating the coagulation process with a mixture of 10 ml of 10% calcium chloride was mixed with 5,000 units topical bovine thrombin (fig.11). The platelet rich plasma was used with in 6 hours after preparation. (Marx, et al., 1998)

Figure 10. Sysmex K1000 was used for platelet count of the rabbit whole blood and autogenous PRP.

Figure 11. The solution of 10% calcium chloride with 5000 units of bovine thrombin were used for activated PRP to form gel.

<u>Bio-Oss® preparation</u>:

Bio-Oss® spongiosa (Geistlich Phama, Wolhusen, Switzerland), 0.25 to 1 mm granule, was used in this study. 0.1 g Bio-Oss® was prepared in ASTECAIR BHA 48 (Astec Environmental System Ltd, England) sterile hood. Bio-Oss® 0.5 g was divided and placed into 5 Eppendorf tubes, each tubes contained 0.1 g Bio-Oss®. For accurately preparation the measurement machine, Sartorius® MC 210s (SARTORIUS AG GOTTINGEN, GERMANY), was used. All of 0.1 g Bio-Oss® in Eppendorf tubes were kept in disinfectant package until the surgeon are ready for its used. (fig.12)

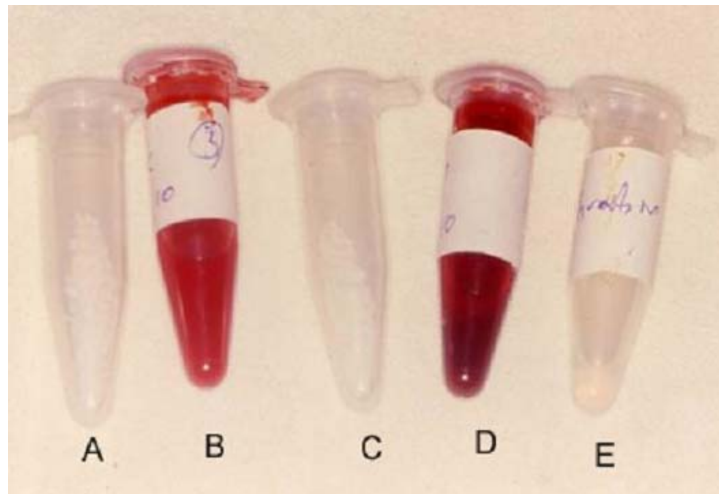


Figure 12. Bio-Oss particle (A and C), PRP (B), Whole blood (D) and thrombin (E) in Eppendorf tubes.

<u>Animal preparation</u>:

Twelve adult male and female New Zealand white rabbits, weighting 3.5 $\pm$ 0.5 kg, were used in this study. The animals were randomly divided into 3 groups, 4 animals in each group, and sacrificed after 2, 4 and 6 weeks, respectively. The animals were fed a standard rabbit diet, given water ad libitum, and kept separately in cages at The animal house, faculty of science, Prince of Songkla university. This experimental study was approved by The ethic committee for the use of experimental animals, faculty of science, Prince of Songkla university.

Method:

<u>Anesthesia and surgical method</u>:

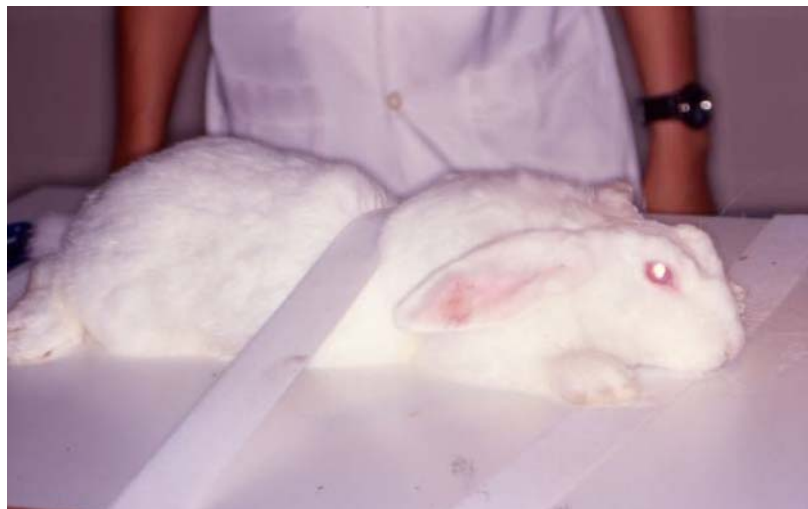


Figure 13. The rabbit was placed in supine position before anesthesia procedure.

In fig.13, the rabbit was placed on the surgical table in supine position before anesthesia procedure. Anesthesia in the experimental rabbit was administered by an intramuscular injection of ketamine HCL (25 mg/kg) and diazepam (5 mg/kg) into the gluteal region. Three minutes after that, the intravenous catheter was placed into the marginal ear vein of rabbit and intravenous anesthesia was administered with thiopental. The thiopental was started at 5mg/kg and maintain level of anesthesia with a titrate dose of thiopental (2.5 mg/kg), quid 10 minutes. The recommend for maximum dose of thiopental was 30 mg/kg. (Flecknell, 1996: 182-190) The intravenous fluid, 5% $D/N_{/2}$,in the rate 4 ml/kg/hr was given through the operation. Until the animal was placed in level of anesthesia, the calvarial region was shaved and disinfected with providone iodine, and the animal was draped to allow asptic access to the operation field. A sagittal incision from the nasal to the occipital region was performed after local infiltration of 2% lidocaine HCl with epinephrine $1{:}10^5$, 1.8 ml. The muscular dissection, plane to plane, and incision of the periosteum was performed (fig.14). Subsequently, two surgical bone defects were created in the left and right parietal bone using fissure bur in slow-speed micromotor under copious saline irrigation. The same size of left and

right parietal bone defects were made in square shape, 10X10 mm, with a depth equal to the thickness of the removed cortical bone. The square bone plugs were gently removed and avoided injury to the dura mater. Four bur hole markers were made with fissure bur, 1mm from the edge around each corner of square shape bone defect. This marking were filled with gutta-percha (Hygenic corporation), for later identification of the defect edges in the histologic sections and to improve radiographic evaluation (fig.15). A flip of a coin determined the experimental (Bio-Oss®/PRP) and the control (Bio-Oss® alone) side following: if the upper part of the coin was shown that mean the right side was selected for the experimental but the lower part of the coin was shown that mean the left side was selected for experimental , and the other side was selected for control. After that, 0.1 g Bio-Oss® was placed into 3 ml sterile syringe and mixed with 0.5 ml of PRP. The combination graft (Bio-Oss®/PRP) was activated to form gel using 0.1 ml of calcium chloride/bovine thrombin solution, this gel could be helped surgeon for easily to mold and placed the graft into bone defect. Moreover, the gel was easy to handle and did not easily migrate of Bio-Oss® particles. The combination graft of Bio-Oss® and PRP was placed into the bone defect at the experimental site. The other 0.1g Bio-Oss® was placed into 3 ml of sterile syringe and mixed with 0.5 ml autologous whole blood and placed into the control defect. Subsequently, the periosteum, muscle and skin were sutured with Vicryl® 4-0, and providone iodine was applied to the wound. After surgery, each rabbit was returned to its cage and it could be ambulatory within 2 or 3 hours after surgery. The single dose of Pethidine (10 mg/kg) was intramuscular administered for post-operative analgesic. For the post-operative antibiotic, PGS 1,000,000 units was intramucular injected for first week. All animals were fed a standard diet and water ad libitum until the date of sacrificed.

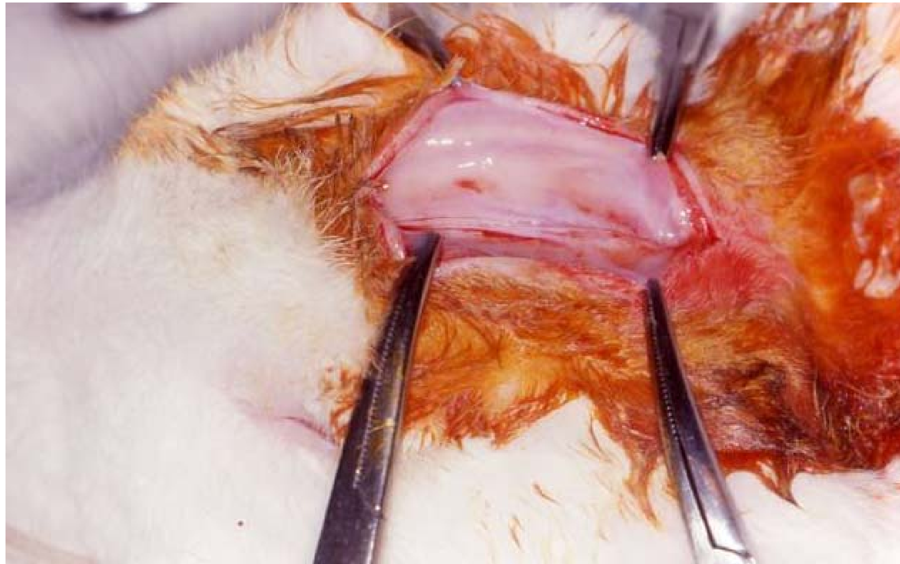
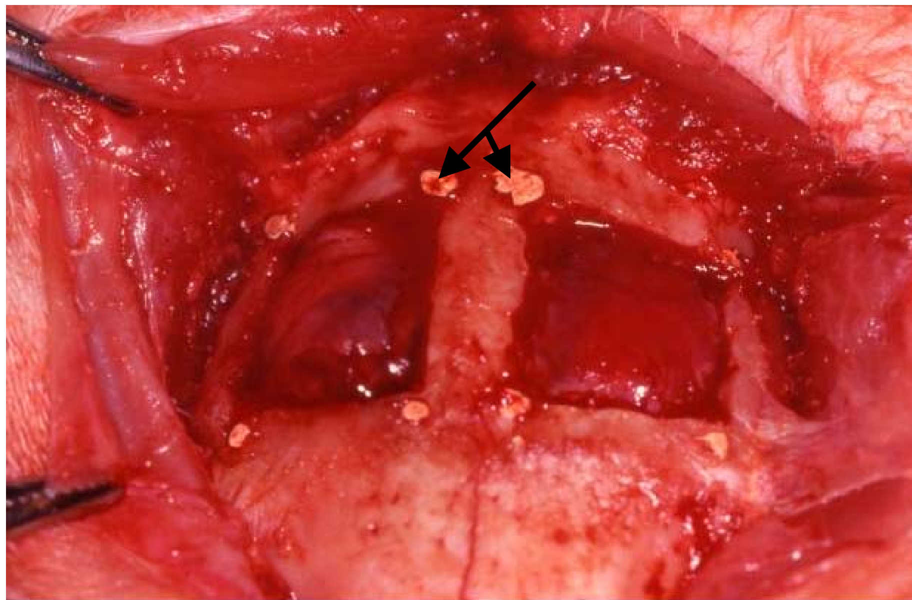
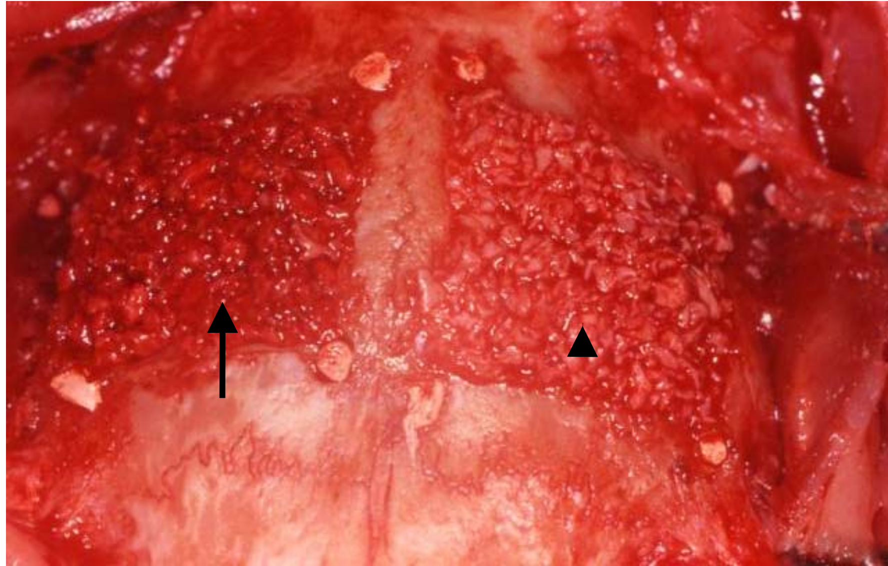


Figure 14. The sagittal incision was made from nasal to occipital part of rabbit.



A.



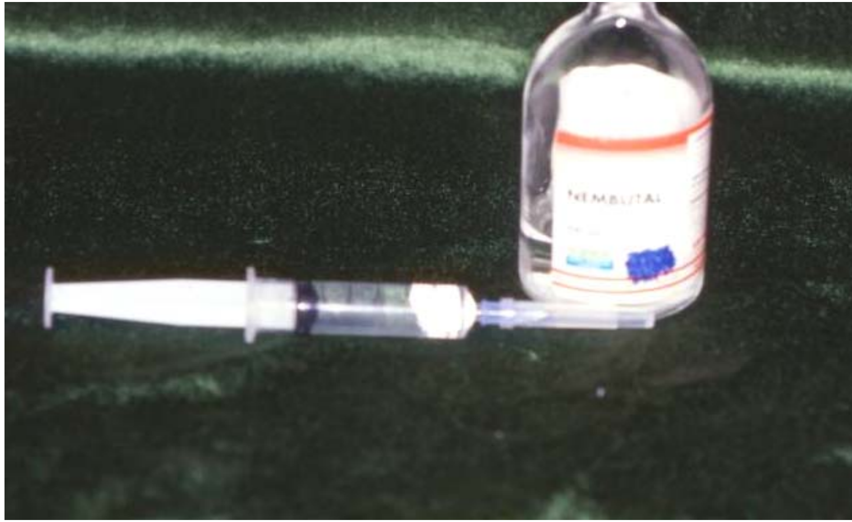
B.

Figure 15. A. The gutta-percha marker were used to locate bone defect (arrow).

B. Bio-Oss/PRP particle (arrow) and Bio-Oss alone (arrowhead) were placed into the bone defect.

Tissue processing:

The animals were sacrificed with an overdose of intravenous injection pentobarbital (Nambutal, 200mg/ml) at 2,4 and 6 weeks after surgery (fig.16). Skin incision with a periosteal flap was used to exposed the calvarial bone and the previously grafted site were excised widely. A tissue block with the experimental and control bone defects and including 2 mm of surrounding bone were removed with a fissure bur in slow-speed micromotor under copious saline irrigation. The specimens were fixed in 10% formalin solution (fig.17).



A.

B.

Fig 16. A. Nambutal solution is pentobarbital that used for sacrifice the experimental rabbit.

B. The rabbit was sacrificed by marginal ear vein injection with Pentobarbital.

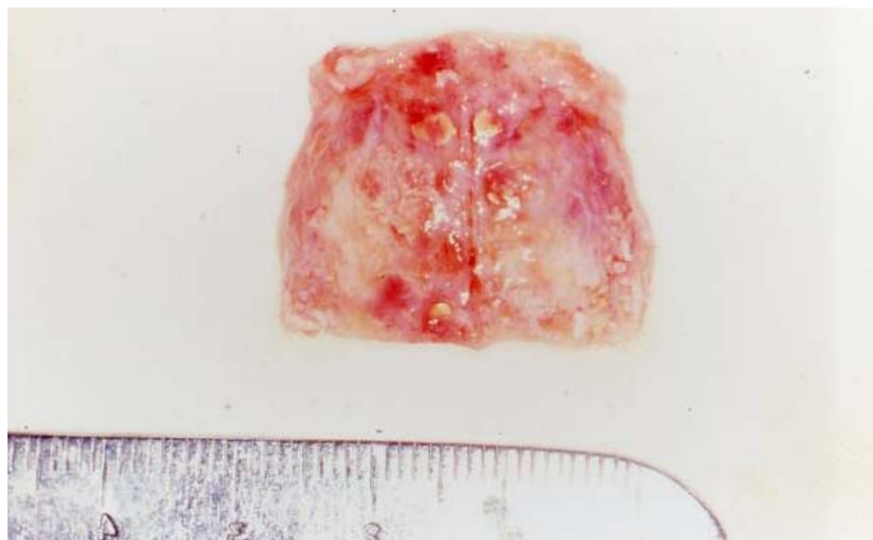


Figure 17. The specimen was harvested from rabbit cranium in group 3 (6 weeks).

<u>Radiologic method</u>:

Until all bone specimens were received, the radiographic method were performed, 5X5 mm aluminium foil, from 31 X41 mm Super Polysoft Insight Kodak X-ray film, was fixed on Kodak X-ray film. These aluminium foil was act as calibrated radiograph reference marker in radiomorphometry process. The bone specimen was contacted to 31 X41 mm Super Polysoft Insight Kodak X-ray film (Eastman Kodak Company, Rochester, N.Y.), with 5X5 aluminium foil. The bone specimen/ aluminium foil/Kodak X-ray film was placed into the parallel film holder device (fig.18), developed from the XCP device, for parallel technique radiograph (fig.19). The parallel film holder were used in all of the radiographic examination so that the same position and distance could be reproduced. The dental radiographic machine (Gendex, Gendex Co.,Illinois, USA.) with long cone tube, fixed with parallel film holder, was used (fig.20). The radiographs exposed with a fixed distance, 7inches, and setting of 55 kvp, 10 mA, 0.42 second. The X-ray beam was perpendicular to the plane of the specimen and the film. All of radiographic films were taken by the same dental radiographic machine and then processed by a automatic film processor (Dent X 9000,Dent X/Logetronics GmbH,Kornberg,Germany). Immediately after the roentgenogram, each specimen was

returned to fix in 10% formalin. All radiographs were scanned by the scanner machine (ScanJet 4C/T Scanner, Hewlett-Packard Co.,USA.), for obtain digital radiographic images of the specimens, at the radiology department, faculty of dentistry, PSU. The digital radiographic images were transferred to a computer, each roentgenogram was assessed the bone density within 10X10 mm bone defect by using Digora analysis soft wear (DIGORA Digital System, Soredex, Helsinki, Finland). The image analyzer converted the image of the selected area into digital gray values and, subsequently, these were correlated to the numbers of pixels and calculated bone density (fig.21).

Figure. 18. Bone specimen and marker were attached to the periapical film.

Figure.19. The parallel film holder that developed from XCP. This device is help the researcher to take the radiograph in parallel technique.

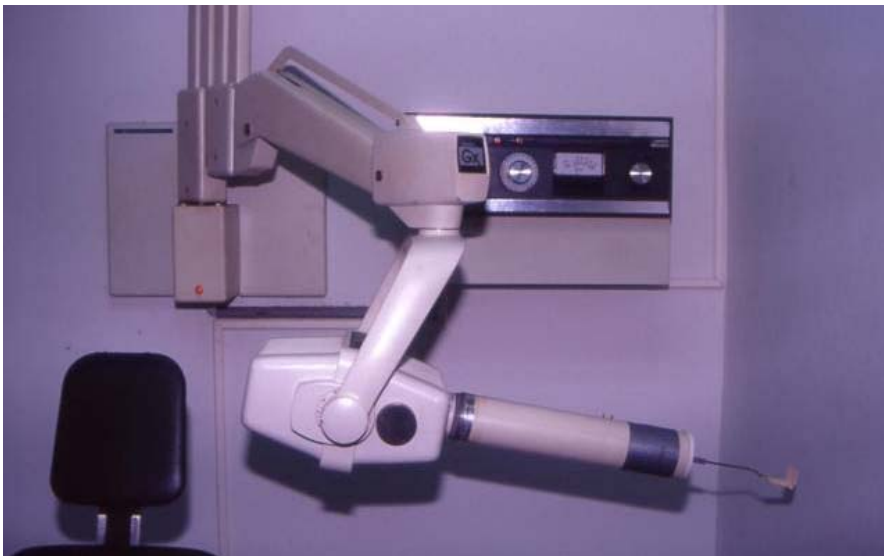


Figure. 20. Radiograph was taken with parallel technique.

Figure 21. Scanner machine (Scan Jet 4C/T) and computer with Digora analysis soft wear for radiomorphometry analysis.

Histologic method:

After complete taken the radiographic images, three bur hole marker were made, with small round bur, one millimeter from the edge around bone defect on the left side of each bone specimen. These bur hole marker were divided the bone specimen into the upper, middle and lower part. These method was provided for later identification of the left side of bone specimen on histologic analysis. The bone specimens were decalcified in 10% formic acid. After completed decalcified, each bone specimen was sutured with black silk on the previous prepared bur hole marker for identified the left side of the specimens. Each bone specimen was equally divided into the upper, middle and lower part, contained 3mm in the widely of bone defect, by blade No.10 (fig.22). The upper, middle and lower part of each bone specimen were embedded in paraffin and fixed the left site of each specimen that identified by black silk suture incorporated to the bevel side of each paraffin block. 5 μm thickness of three serial sections of each specimen were cut transversally through the experiment and control defect, thus, the experiment and control defect could be placed in the same histologic slide. (Page, et al.,1996: 309-339) Each histologic section was stained

with haematoxylin and eosin (fig.23). From this method, each bone specimen could be have 9 histologic slides (3 slides from each upper, middle and lower part of bone specimen). The three histologic slide were randomly selected for histological analysis (each of histologic slide was randomly selected from the 3 slides of each of upper, middle and lower part). For these method, it could be reduced the bias while selected the histologic slide. These three randomly selected histologic slides were analyzed under light microscope for detection of new bone formation and the degree of inflammation.

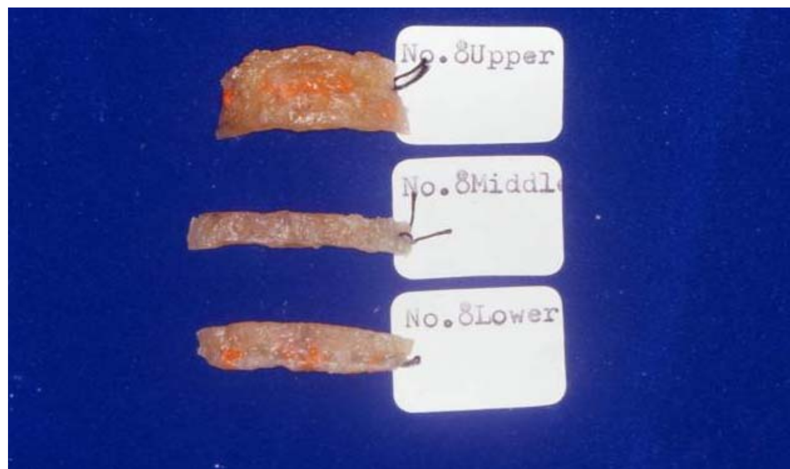


Figure 22. After decalcification process, the each of bone specimen was divided to three pieces; upper, middle and lower. The black silk sutures were located on the left side of bone specimens.

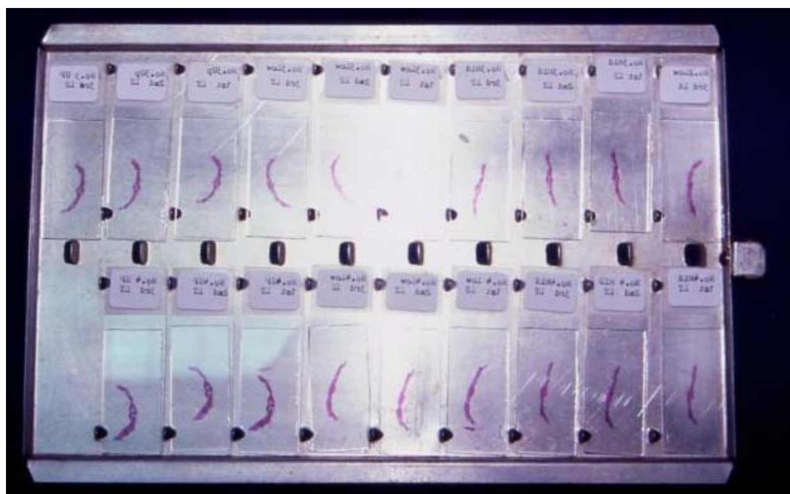


Figure 23. The 5 μm thickness of bone specimens were cut transversally through

the experimental and control defect. All specimens were stained with hematoxylin and eosin stain (H&E).

Histomorphometric analysis:

Computer-assisted histomorphometry was performed in order to measure the amount of newly formed bone in the defect. Histomorphometry measurement were performed in the three randomly selected sections form each specimen that previous described. To obtain digital histologic images of the experimental and control bone defect, a digital camera was (AxioCam MR, Carl Zeiss, light microscopy, Gottingen, Germany), attached to light microscope and using Axiovision program. Measurements were carried out directly at magnification of 4X, in the light microscope. These histologic images were transferred to a computer and measured a total bony defect area and area of new bone formation in the experimental and control site, using Zeiss KS 400 program (Carl Zeiss, Jena, Germany) (fig.24). The percentage of bone area in the experimental and control bone defect were estimated ;

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

Figure 24. The personal computer, a digital camera (AxioCam MR), light microscope and KS 400 programm were used for histomorphometric analysis.

<u>Statistic analysis</u>:

Total bony defect area, area of new bone formation and percentage bone area were measured in three times separated for reduced the bias. The data were present in square millimeter and calculate the mean values. SPSS for Window version 10 (SPSS, Korea) was used in the data analysis. The difference in amount of radiographic bone density and percentage of bone area between the experimental and control side at 2, 4 and 6 weeks were analyzed statistically with **Wilcoxon signed rank test** at level of significant of $P \leq 0.05$. The **Kruskal-Wallis test** was used to analyze the changing of bone formation between the time period (2,4 and 6 weeks) in the Bio-Oss® alone group and also in between the time period in the Bio-Oss® with PRP group at level of significant of $P \leq 0.05$. The difference in amount of platelet count between PRP and whole blood were analyzed statistically with **Wilcoxon signed rank test** at level of significant of $P \leq 0.05$. The Statistical Package for the Social Sciences (SPSS) for window, version 10 (SPSS, Seoul, Korea) was used in the data analysis.