# CHAPTER THREE

## MATERIALS AND METHODS

### Materials

***Sample Size.*** Ten healthy mixed breed dogs were used as the animal model study.

***Animal Selection.*** Ten systemically healthy dogs (5 males and 5 females , age range 1-5 years) were recruited for the study. Exclusion criteria consist of animals with systemic illnesses , compromised immune systems , pregnant and / or lactating dogs.

***Ethical Consideration.*** The experimental design and the use of laboratory animal were approved by the ethical committee of Faculty of Veterinary Medicine , Chiangmai University. These permission criteria are based on regulation of National Research Council. Ethical consideration of the use of laboratory animals consist of

1. To realize that laboratory animal is valuable.
2. To minimize number of laboratory animal necessary for required accuracy.
3. To use of wildlife must conform with wildlife law and conservation policy.
4. To realize that laboratory animal is as alive as human.
5. To record all activities performed on laboratory animal.

***Presurgical Therapy.*** Pyrantel pamoates (Antiminth®, Pfizer) at dose 5 mg/kg are administered orally as an antihelminthic drug. Rabies vaccines (Rabigen Mono®, Virbac Laboratories, France) and multivalent vaccines (Vanguard 5/L®, Norden Laboratories, Nebraska, U.S.A.) were subcutaneously administered as the vaccination program.

**Methods**

***Blood Collection.*** Ten ml of whole blood from each dog was obtained from cephalic vein. The blood was then transfered in a tube containing 1.4 ml of citrate phosphate dextrose (CPD) as an anticoagulant. The rest of venous blood was used to determine the animal's platelet count.

***PRP Preparation.*** To produce PRP extracts , 8.5 ml of citrated blood was centrifuged in a standard laboratory centrifuge for 10 minutes at 2,400 rpm. Subsequently , the yellow plasma (containing the platelets) was taken up into a neutral monovette with a long cannula , using an additional air-intake cannula. To combine the platelets into a single pellet , a second centrifugation step was performed with this second monovette for 15 minutes at 3,600 rpm. The plasma supernatant (containing relatively few cells) was then reduced to approximately 0.4 ml (by taking up with a second neutral monovette , a long cannula , and an air intake cannula). The pellet of platelets was resuspended in the residual 0.4 ml of plasma using a conventional shaker and transfered to an Eppendorf tube for later analysis of platelet count.(Gernot et al,2001)

***Surgical Therapy.*** The experimental areas were lower borders of mandibles at $2^{nd}$ , $3^{rd}$ and $4^{th}$ permanent premolar and $1^{st}$ permanent molar area. The radiographs were taken to certify that there were none of decidous teeth in the experimental areas. To prepare the experimental sites, the left side of mandible area was clipped and scrubbed with tincture iodine, following by wiping with 70% isopropyl alcohol. Skin incision was made extraorally at the border of the mandible at $2^{nd}$ , $3^{rd}$ and $4^{th}$ permanent premolar and $1^{st}$ permanent molar area. Periosteal flap was elevated and two artificial defects with diameter of 5 mm and 8 mm depth were performed using trephine drill, one defect

served as control and the other was added 100 μL of PRP. Both artificial defects were filled with collagen (Tissue Vlies®, Baxter AG) in order to hold blood clot and PRP in the defects. The sutures were made using an absorbable suture material (Dexon 3-0 ). Ibuprofen and doxycycline (Vibramycin®, Pfizer) were administered orally as analgesic and antibiotic for 3 and 7 days postoperation. Soft canned food was fed for 7 days postoperation. Every 2 dogs were sacrificed at 2 weeks, 4 weeks, 6 weeks, 8 weeks, and 12 weeks.

***Euthanasia Method.*** To euthanize the animals, pentobarbital sodium (Nembutal®, Sanofi, Animal Health, Thailand) at dose 25 mg/kg was injected intravenously until the animals were anaesthesized without pain or distress. Skin incision at ventral of the neck was made caudally. Common carotid artery was identified and then ligated at anterior part. Common carotid artery was cut incompletely caudally to the ligation point. The blood was then releasing from the whole body. Fixative 10% formalin was injected into the body through common carotid artery until excessive formalin drained from nostrils. Conventional suture was ligated caudal to the cutting point to prevent pouring back of excessive formalin. Left quadrants of mandibles consisted of experimental and control sites were collected by using bone saw. The rest of the euthanized animals were donated to department of veterinary pre-clinic, Faculty of Veterinary Medicine, Chiangmai University, as specimens for veterinary student course.

***Radiography.*** Radiography of left quadrants of mandibles were performed.

***Histological Section.*** The relevant part of the mandible was removed and fixed in buffered (neutral) 10 % formalin solution after the overlying soft tissue was scraped away. Several blocks were prepared by cutting the bone in cross section with a saw. Then

the specimens were dehydrated in ascending grades of alcohol and embedded in light curing resin (Technovit 7200 VLC+BPO, Kulzer & Co., Germany). Further processing was done with the Exact Cutting and Grinding Equipment (Exact Apparatebau, Norderstedt, Germany). The blocks were cut along the vertical axis of the mandibles and reduced to a thickness of 30 μm. Subsequently the undecalcified cut and ground specimens thus obtained were stained with Giemsa (Fürst et al, 2003). The histological sections were examined under microscope for both histological descriptive and histomorphometrical examination.

***Histomorphometrical Analysis.*** A couple of digital photographs of each slide was performed by taking the lower half and upper half. A public domain java image processing program inspired by national institute of health (NIH) (version 1.24) was used to perform a quantitative expression of the patterns present in sections of bones. The measurements were often in units square pixels which can be calibrated into the appropriate metric units using a stage micrometer or a standard metric scale as shown in figure 7. The square pixel unit was changed to the percent of new bone formation. The values were then calculated the mean.

Histomorphometric analysis was of greatest value when accompanied by a qualitative evaluation of the histological section.(James and Webster,1983; National Institutes of Health,2003)

***Statistical Analysis.*** The healing process at 2 weeks , 4 weeks , 6 weeks , 8 weeks and 12 weeks of both experimental and control groups will be described histologically and compared histomorphometrically using SPSS (version 10) computer software (Student indepenent T-test , $P< 0.05$ are used). Descriptive criteria of

histological sections examined under microscope are bone healing process and woven bone formation.

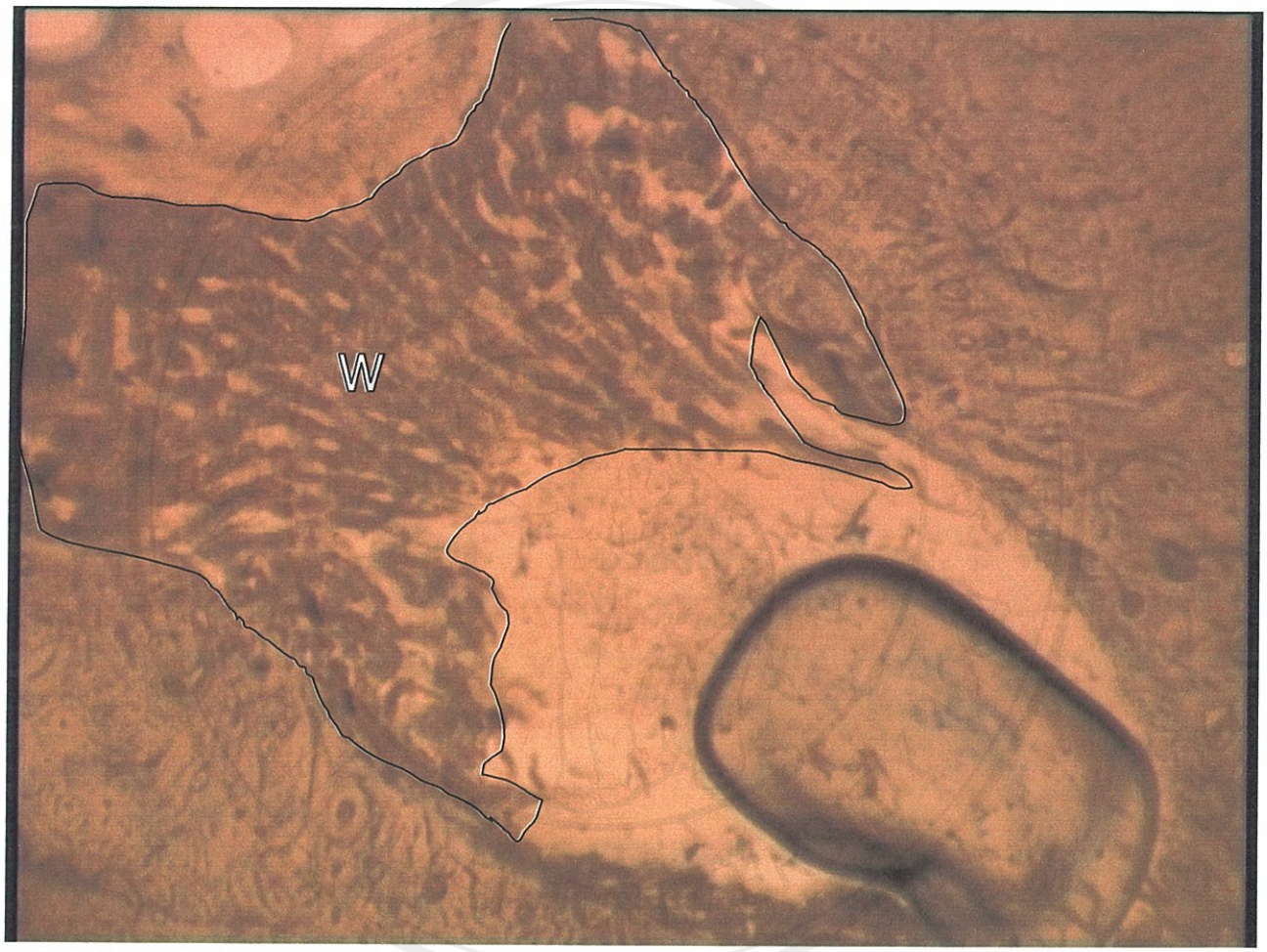


Figure 7 Woven bone (W) measured by image J (version 1.24)