# CHAPTER 2

# METHOD OF STUDY

## Materials and Methods

### 1. Chitosan-Gelatin Sponge (CGS) Preparation

#### 1.1. Preparation of Substrate Solution

Chitosan is insoluble in water but soluble at pH values under 6.5 in most acidic media. Acetic acid has been mostly used as a standard solvent for chitosan solutions. In this study, the chitosan solution were prepared by a traditional method.

Chitosan 1% in 1% acetic acid solution (w/v) 500 ml was prepared as described. 5 Grams of chitosan powder (Fluka® medium viscosity) was completely disperse in distilled water 250 ml under continuous magnetic stirring ~500 rpm. Then 2% acetic acid 250 ml was added to dilute the powder in room temperature.

5% Aqueous gelatin B solution (w/v) 200 ml was prepared as described. 10 Grams of gelatin powder from bovine skin (Sigma® medium viscosity, 225 bloom) was completely diluted in distilled water 190 ml under continuous hand stirring in T 60 $^{\circ}$C.

This 2 kinds of solution were kept in cool until crosslinking process.

#### 1.2. Crosslinking Evaluation

Gluteraldehyde solution 25 % by weight (Fluka®) was a crosslinking agent and the amount for crosslinking method were gluteraldehyde 33.5 mg/g of chitosan and 6.67 mg/g of gelatin B. (Kwunchit Oungbho, 1997b: 185)

Chitosan-gelatin B ratio was 1:10 by weight which proved to get the stable CG foam. (Kwunchit Oungbho, 1997b: 180-181) According to this ratio, chitosan 1%in 1%acetic acid solution and 5% aqueous gelatin B solution were weighed in 90 g and 180 g. Gluteraldehyde solution 25% was calculated amount for crossliking process , 30.15 mg (0.12ml) for chitosan solution and 60.03 mg (0.24 ml) for gelatin B solution.

### 1.3. Preparation of CGS

180 Grams of 5% gelatin B solution were warmed 1 hour in water bath 45 °C, added crosslinking agent 0.24 ml and stirred by hand until completely disperse. After that the crosslinking gelatin B solution was warmed again in same condition and left in room temperature for 24 hours.

90 Grams of 1% chitosan in 1% acetic acid solution was left until reaching the same temperature as room temperature, added crosslinking agent 0.12 ml and stirred by hand until completely disperse. After that the crosslinking chitosan solution was left in room temperature for 24 hours.

The crosslinking gelatin B was contained in 45°C water bath again to make liquid phase of this solution. Then mixed with the crosslinking chitosan solution and stirred until thoroughly disperse. The mixture was put into plastic culture test tubes and rapidly transferred to a freezer at -40 °C to solidify the solvent and induce solid-liquid phase separation. (**Figure 4**) The solidified mixture was maintained at that temperature at least 2 hours and was transferred into a freeze-drying vessel. (**Figure 5**) The sample was finally freeze dried for at least 30 hours, resulting in a foam as test tube shape. (**Figure 6**)

The chitosan-gelatin sponge was sliced into 8 mm diameter disks and treated by ethylene dioxide gas for sterilization. (**Figure 7**) The pore sizes of foam was determined by Scanning Electron Microscope (SEM). The CGS disks were stored in cool place until use.

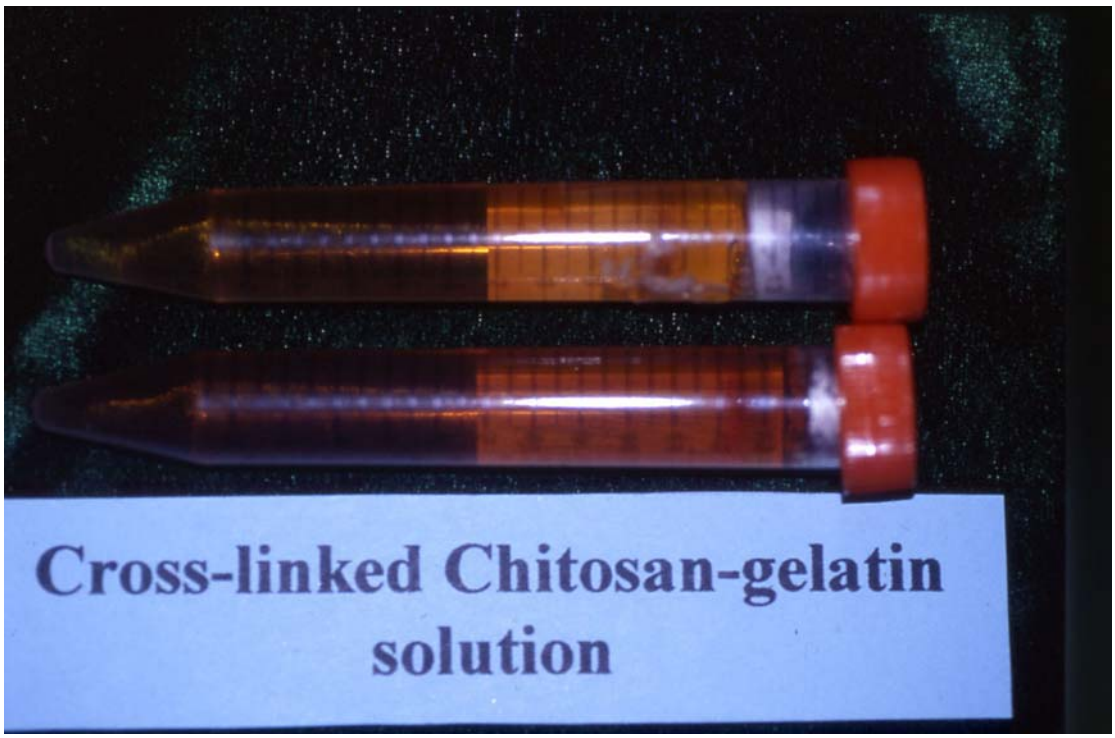


**Figure 4.** Cross-Linked Chitosan-Gelatin Solution after 24 Hours.


**Figure 5.** Freeze-Dried Chamber

**Figure 6.** Freeze-Dried Chitosan-Gelatin Foam

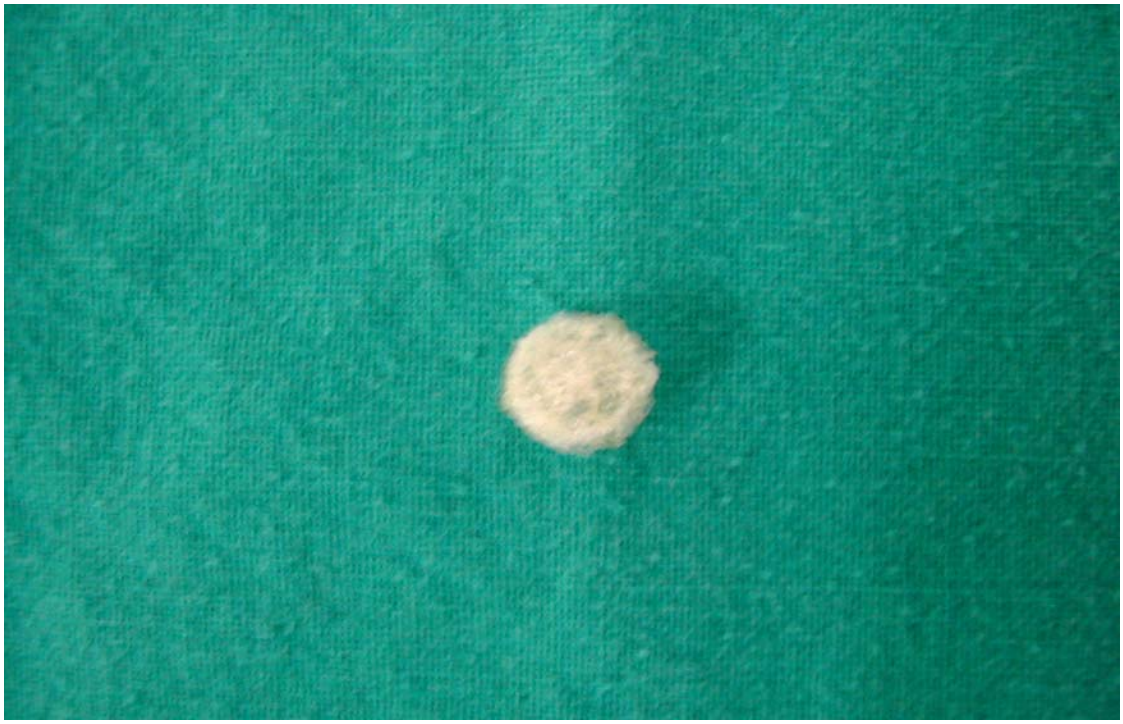

**Figure 7.** Chitosan-Gelatin Sponge Disk in 8 mm-Diameter

### 1.4. SEM Observation

The porous morphologies of CGS were studied by SEM. The sample was prepared by mounting on brass stub and applying silver paint. After that the sample was coated by gold sputtering method and examination under a JSM-5800/JSM-5800LV Graphic SEM. (Scientific Equipment Center, Graduate School, Prince of Songkla University, Hat Yai)

## 2. Animal Experiment

### 2.1. Test Animals and Animal Housing

Six adult New Zealand white rabbits aged 7 months and weighing between 2.7 and 3.5 kg obtained from local breeders were used in this study irrespective of their strain or sex. They were delivered to the laboratory not later than 1 week before the start of the experiment, and acclimatised to the housing conditions and the feed. The animals were kept in single stainless cages at 24 $C^{o}$ and 50% relative humidity in at least 12 hours light per day. A standard dry-feed diet and water were offered ad libitum. The Prince of Songkla University (Hat Yai) Animal Research Ethical Committee approved all animal protocols.

### 2.2. Anesthetic Phase

Premedication was obtained by means of an intramuscular injection of Diazepam 1 mg/kg 30 minutes before surgery. Following an induction with intramuscular injection of Ketamine 25 mg/kg and Diazepam 4 mg/kg ,then 5% $D/N_{/4}$ 4 ml/kg/h and general anesthesia was given with Thiopenthal 2.5 mg/kg/dose via marginal ear vein of the rabbits. (**Figure 8**) During the surgery, oxygen 5 Litre per minute via canular was given to the rabbits until postoperative phase. (**Figure 9**) At the beginning of surgery, PGS 50,000-100,000 U/kg was also given for prophylaxis.

**Figure 8.** The Rabbit with Intravenous Canular

**Figure 9.** The Rabbit with Oxygen Canular

**2.3. Operative Phase**

The rabbit was in prone position after that shaved and disinfected with povidone iodine over the cranium area between both ears of the rabbit which was prepared and draped in a sterile fashion. (**Figure 10**) Lidocaine 2% with adrenaline 1:100,000 approximate amount1.8 ml was injected to the skin area of the surgical field for control bleeding. Midsagittal incision 2-3 cm was made until exposing the periosteum of calvarial bone and then the periosteum was cut and reflected to expose the parietal bone area of the rabbit. (**Figure 11**) Two bony defects 8 mm in diameter were created in parietal bone approximately 5 mm between sagittal suture by means of a trephine bur. The amalgam landmark 4 points per defect was made by means of fissure bur$\phi$ 0.18 mm. (**Figure 12**) During surgery the drill holes were carefully rinsed with 0.9% NaCl solution and cleaned out in order to remove abraded particles, reduce drilling temperature and avoid bone necrosis.

The removed bone from both defects were collected and chopped in small pieces to use as autogenous graft. The CGS disk was soaked by 0.9% NaCl solution 10 minutes before use as test material. The autogenous graft site and CGS disk test site were randomly chosen as show in **Table 3.** The graft materials were placed in the defects. (**Figure 13**) Then soft tissue and skins were closed in two layers with Vicryl® 3-0.

**Table 3.** Graft Materials Randomly Selected to Test Animals

| *Number of Rabbit* | Test site | Graft material | Specimen label |
|---|---|---|---|
| 1 | 1R | Autograft | 1a |
| | 1L | CGS | 1c |
| 2 | 2R | Autograft | 2a |
| | 2L | CGS | 2c |
| 3 | 3R | CGS | 3c |
| | 3L | Autograft | 3a |
| 4 | 4R | Autograft | 4a |
| | 4L | CGS | 4c |
| 5 | 5R | Autograft | 5a |
| | 5L | CGS | 5c |
| 6 | 6R | Autograft | 6a |
| | 6L | CGS | 6c |

R = Right side L = Left side

a = Autograft c = CGS

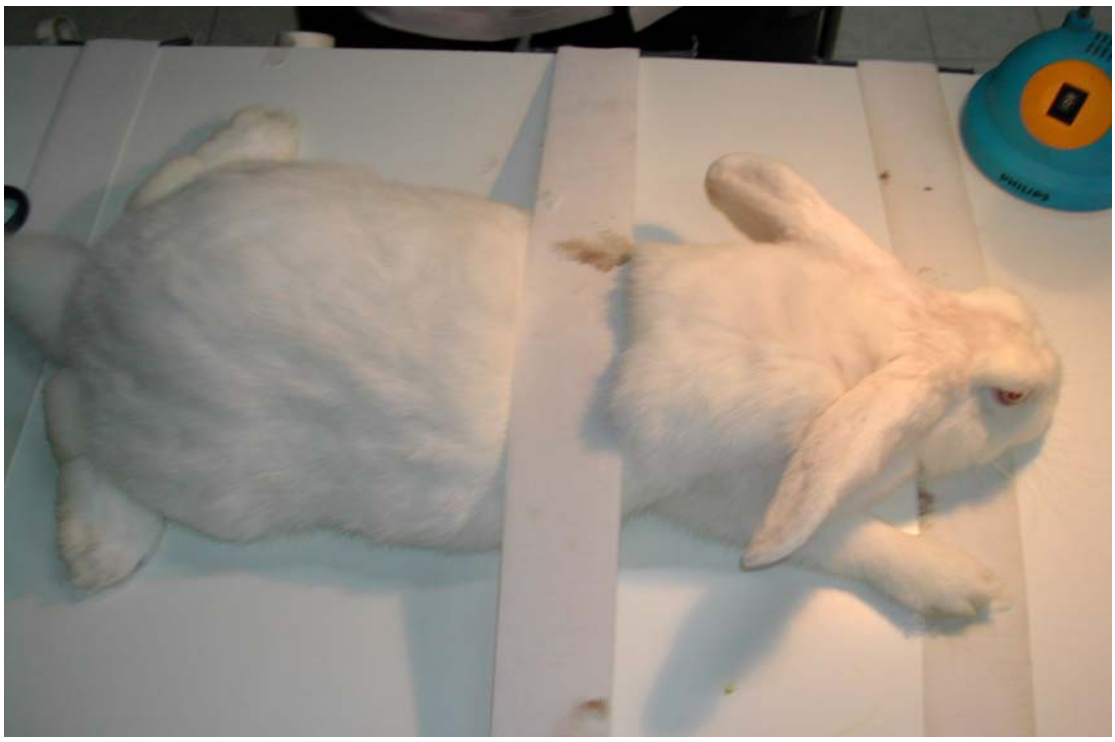

**Figure 10.** Position of Rabbit

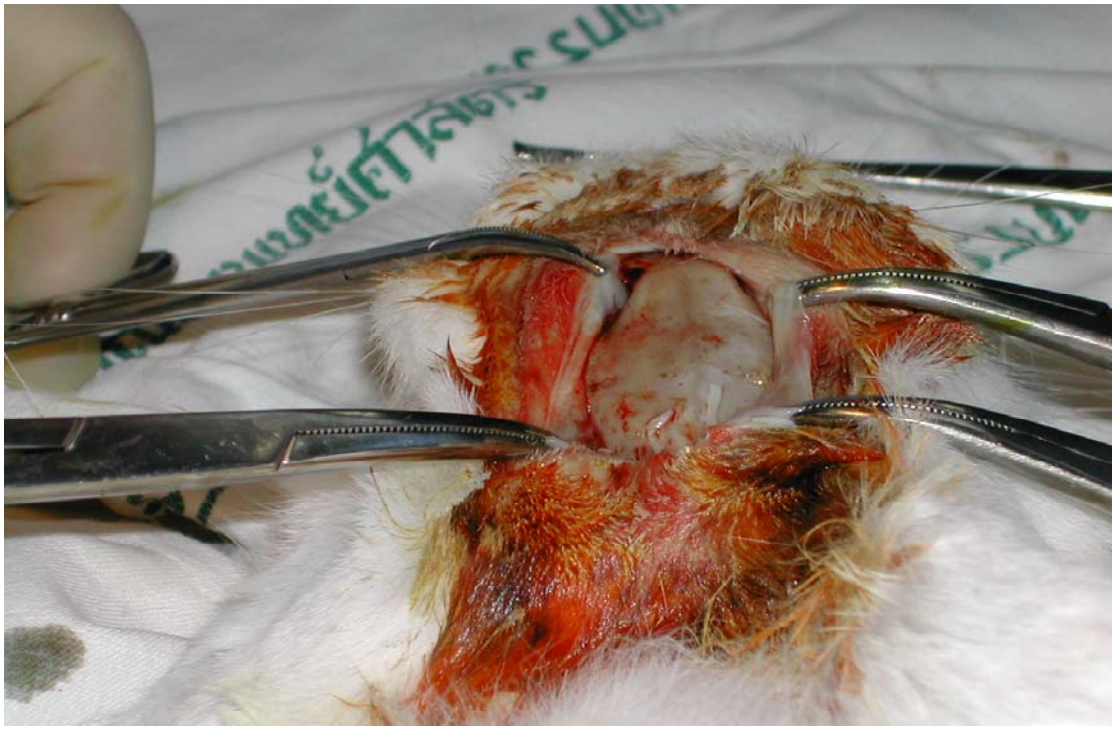

**Figure 11.** Exposure of Rabbit Parietal Bone

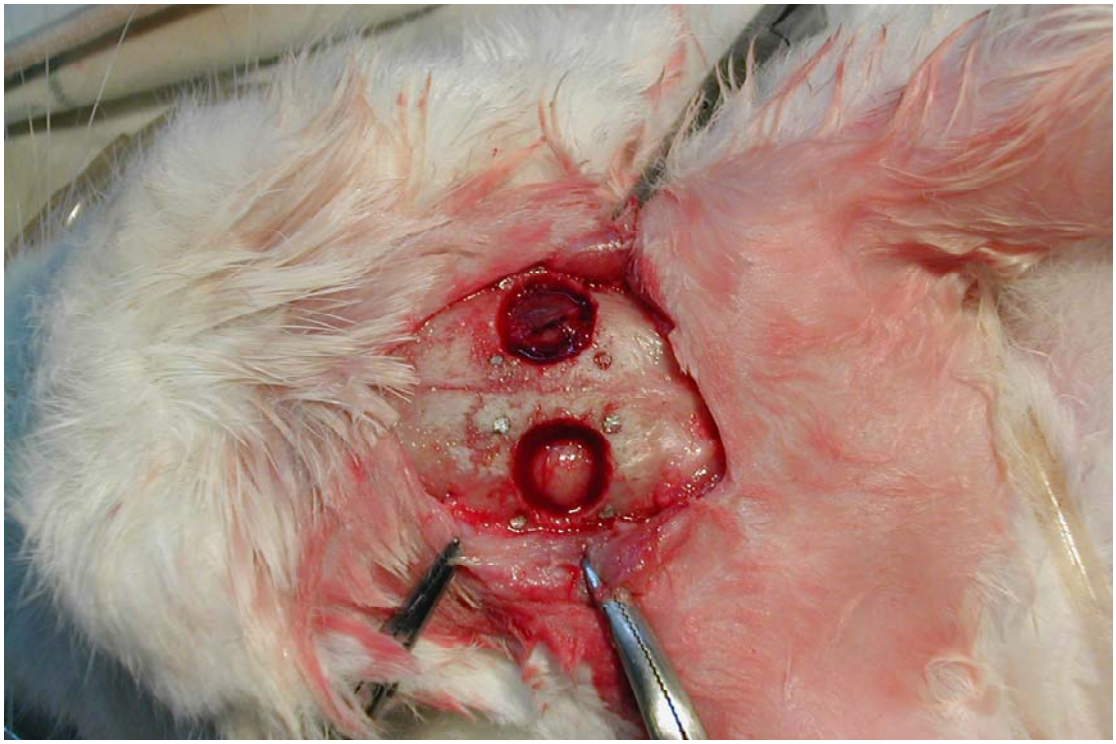

**Figure 12.** Four-Points Amalgam Landmark of Each Bony Defect

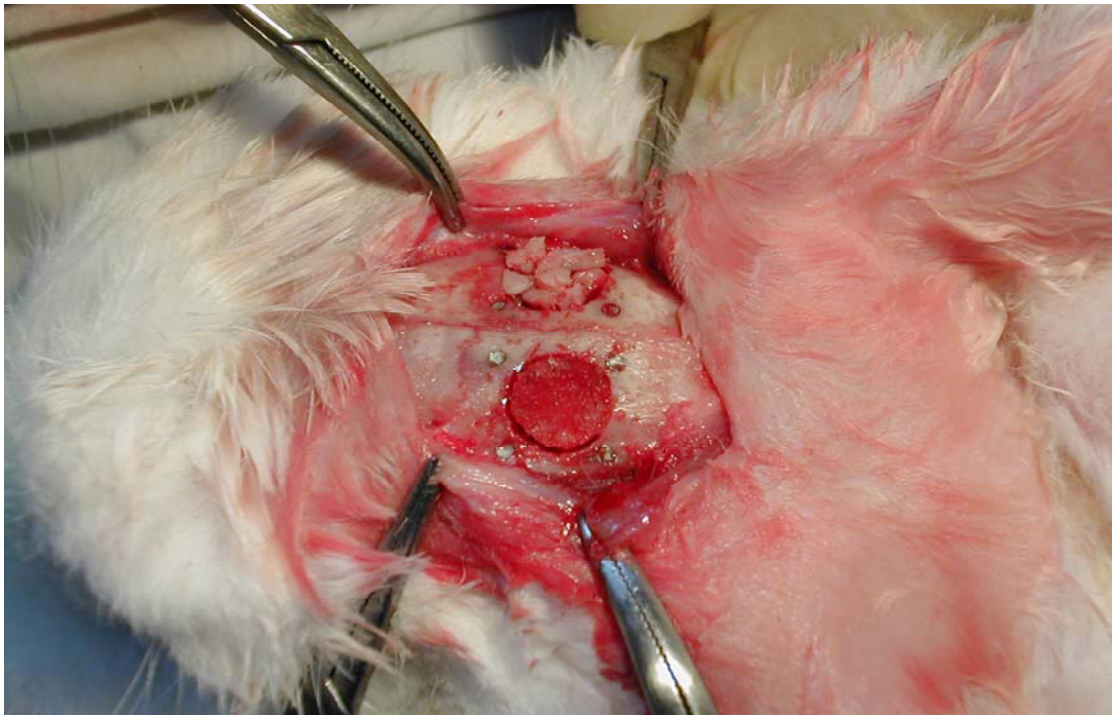

**Figure 13.** Autogenous Graft and CGS Graft in Place

**2.4. Postoperative Phase**

The rabbits were carefully observed for good recovering and then the animals were stabled in single cages, fed a standard pellet and water ad libitum, and under standard environmental conditions. As antibiotic therapy, PGS (50,000-100,000 U/kg) was administered intramuscularly and the wound was dressed 1 time per day during the three days post-operatively.

**2.5. Sacrifice Period**

Twelve weeks after surgery, the animals were pharmacologically euthanised by overdosing Pentothal intravenously. The calvarial bone plates with containing two bony defects were determined by the amalgam landmark and cut into rectangular pieces with approximate dimension of 1.5 x 3 cm by surgical bur with 0.9%NaCl rinse. (**Figure 14**) The specimens were cleaned of soft tissue and immediately immersed to 10%buffered formalin solution at least 24 hours for tissue fixation and then the

specimens were immersed into 0.145 M NaCl solution and kept at -8 $^{o}$C until use for examination.

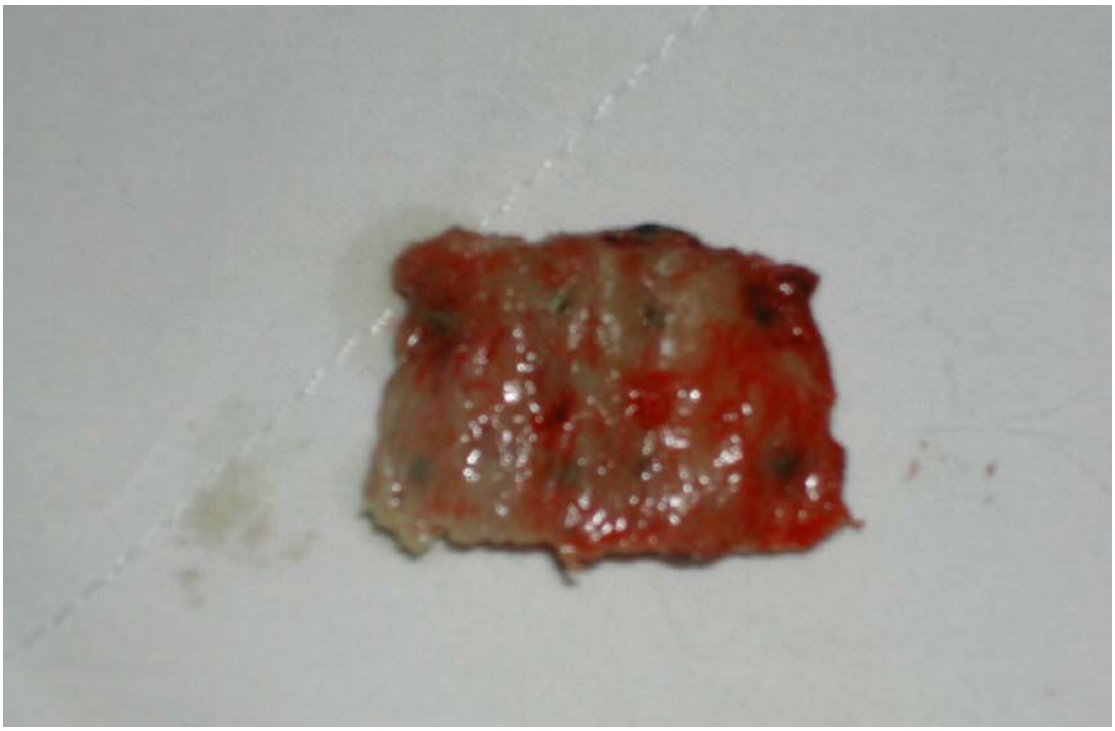

**Figure 14.** Specimen after Sacrifice Procedure

## 3. Specimens Evaluation

### 3.1. Radiographic Preparation

Radiographs of the specimens were taken before histologic sections performed on Gendex X-ray machine with 60 kvp, 10 mA , 0.22 sec and using the occlusal films (Kodak, Ultra-speed DF-49). The distance between film and x-ray tube was kept as 3.5 cm in every specimens and the aluminum 5-steps wedge was used for film calibration. The films were automatically processing by Dent-X9000 processor. The resulting radiographs were scanned using Bio-Rad® Model GS-700 Imaging Densitometer and analysed with Molecular Analyst® software.(Scientific Equipment Center, Graduate School, Prince of Songkla University, Hat Yai : **Figure 15**) The average radiographic optical density(Mean OD) or count of the pixels within the object per area of each defect was measured three times to minimize the measuring error and

calculated for comparing the amount of mineralized tissue produced in response to each type of graft material.


**Figure 15.** Imaging Densitometer Unit

### 3.2. Histological preparation

The specimens were decalcified with 10%formic acid and cut into 2 pieces, each piece containing of autogenous graft defect and CGS graft defect. Each 2 pieces of the specimen were trimmed until encroaching on the graft area, then divided into 2 pieces at the center before dehydrated in graded series of alcohol with automatic tissue processor (Leica TP 1020) and embeded in paraffin blocks. The specimen blocks were sectioned along a sagittal plane to the bone surface by using diamond saw microtome in 5 μm thickness. Three slides from one specimen block were obtained and stained with Haematoxylin-Eosin (H&E) in one mounted-glass slide as showing in **Figure 17**. In summary, 6 slides from center of each autogenous graft defect or CGS graft defect were represented to each defect and examined under light microscope. One piece of sectioned-specimen from each glass slide was randomly chosen to quantifiably

examined by using Leica Qwin Imaging Analysis (Bone laboratory, Prince Philip Dental Hospital, Faculty of Dentistry, Hong Kong University : **Figure 16**). The x5 magnification was used for histomorphometric analysis. The amount of new bone formation was determined by measuring the area containing osteoblast cells in five microscopic fields (four peripheral, one central) on a representative section from each defect. The amount of new bone formation was summed and expressed as a relative percentage area of the total field of view or ***Mean bone area%***. One representative section was measured three times to minimize the measuring error and calculated for comparing the amount of new bone formation produced in response to each type of graft material.

## 4. Statistical Analysis

A Kolmogorov-Smirnov was used to prove the normal distribution of the data and a pair *t*-test was performed on the two graft materials associated with significantly different amount of radiographic optical density and % of new bone formation area at *p* value <.05 by using SPSS program software.


Figure 16. Leica Qwin Imaging Analysis Unit

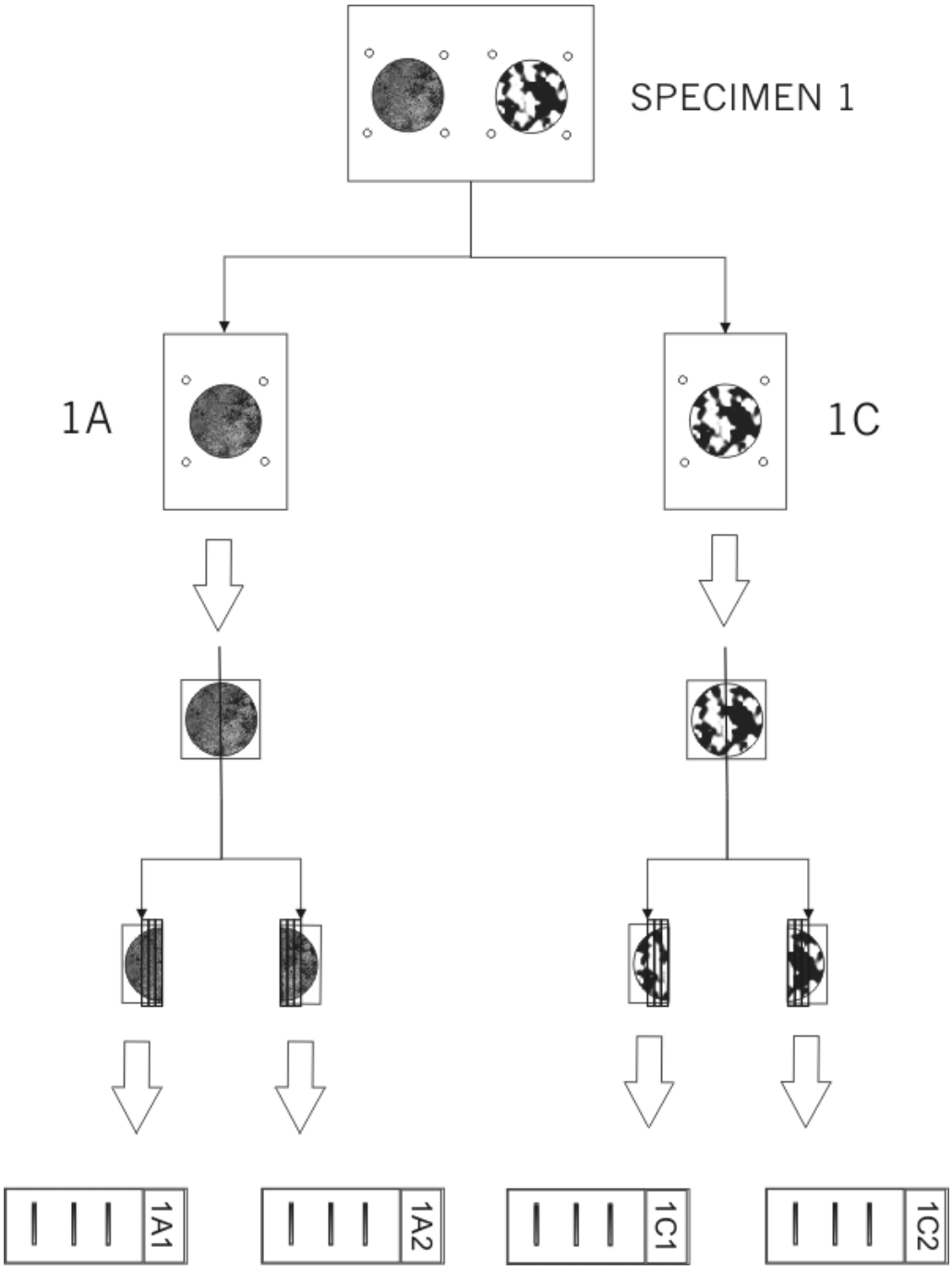


Figure 17. Slide-Labeled Diagram