CHAPTER 3

MATERIALS AND METHODS

3.1 Overview

This study was divided into three phases. The first phase was the production and preparation of PHA films. This was followed by the surgery to insert the PHA films into the selected animal model and eventually the preparation of the microscopic thin sections for histomorphometric analysis.

3.2 Production of PHA

3.2.1 Bacteria strain

Pseudomonas putida in this study was provided by Professor Irene K.P. Tan from the Institute of Biological Sciences, University of Malaya.

3.2.2 Preservation of the bacteria

400 ml of nutrient agar was prepared in a Schott bottle and was autoclaved for 15 minutes at 121 ºC. After that, 0.8 ml of filter-sterilised 0.02 mg/ml Cycloheximide was added into the sterilised but still molten nutrient agar

solution. The presence of Cycloheximide is to prevent subsequent growth of fungi in the agar medium. The solution was poured out into Petri dishes at approximately 1 cm thickness and left to set.

In order to preserve the bacteria, single colonies grown on the nutrient agar plate were taken to grow in nutrient broth for 24 hours. After that, 0.5 ml of this culture and 0.5 ml of 80 % glycerol were transferred into sterile Eppendorf tubes and stored in - 20 ºC.

The whole process was implemented in a sterile condition where apparatus and materials used were autoclaved or filtered in advance. Due to the high temperature of the autoclave (121 ºC), certain materials e.g. Cycloheximide were filtered through 0.2 μm membrane filters instead of autoclaved. All microbiological procedures were carried out in the laminar-flow to prevent contamination.

3.2.3 Inoculum

A single colony from nutrient agar plates were streaked onto nutrient agar slants in bijou bottles, placed in a 30 ºC incubator for one day to allow cell growth, and then stored in 4 ºC until use (within one week). The entire slant of the strain was used as an inoculum. The slants were to be added to the modified rich medium (Section 3.2.4)

3.2.4 Media and growth conditions

PHA biosynthesis was carried out using a two-stage cultivation of *Pseudomonas putida* by using one litre Erlenmeyer flask, shaken at 240 rpm at 30˚C. Initially, the inocula were added to 400 ml modified rich medium. The modified rich medium was one in which the meat extract and peptone were substituted with nutrient broth (Doi *et al*., 1989) (Table 3.1). After 24 hours, the cells were harvested by centrifugation at 3000 rpm for 10 minutes at 15 ˚C using the Beckman J2-M1 centrifuge. The harvested cells were then washed with sterile normal saline once before transferring into a one litre Erlenmeyer flask containing 400 ml nitrogen-limiting E2 medium (Lageveen *et al.*, 1988) (Table 3.2 and Table 3.3). 0.5 % saponified palm kernel oil (SPKO) was added into the nitrogen-limiting E2 medium, as the sole carbon source to promote PHA production. After 48 hours, the cells were harvested by centrifugation at 3000 rpm for 10 minutes at 15 ˚C and washed with sterile distilled water once. The washed cells were resuspended in methanol in a glass petri dish and dried in oven at 60 ˚C to constant weight.

Table 3.1 Modified rich medium (modified from Doi *et al.*, 1989)

Yeast extract	10 g
Nutrient broth	15 g
Ammonium sulphate	5 g

*per 1000 ml of distilled water

Table 3.2 E2 medium (Lageveen *et al.*, 1988)

$NaNH_4HPO_4.H_2O$	1.73 g
$K_2HPO_4.3H_2O$	3.75 g
KH_2PO_4	1.85 g
MT solution (see table below)	1.00 ml
$MgSO_4.7H_2O$	0.25 g
SPKO (0.5% w/v)	5.00 g

*per 1000 ml distilled water

Table 3.3 Microelement (MT) stock solution

$FeSO_4.7H_2O$	10.00 g
$CaCl_2$	2.00 g
$ZnSO_4.7H_2O$	2.20 g
$MnSO_4.4H_2O$	0.50 g
$CuSO_4.5H_2O$	1.00 g
$NH_4Mo_7O_{24}.4H_2O$	0.10 g
$Na_2B_4O_7.10H_2O$	0.02 g

*per 1000 ml of 1N HCl

3.2.5 Palm kernel oil

The palm kernel oil (PKO) that was used in this study was kindly given by Southern Acids (M) Bhd., Klang, Selangor. This is the oil extracted from the nut of the oil palm (*Elaeis guineensis Jacq*) fruits (Figure 3.1).

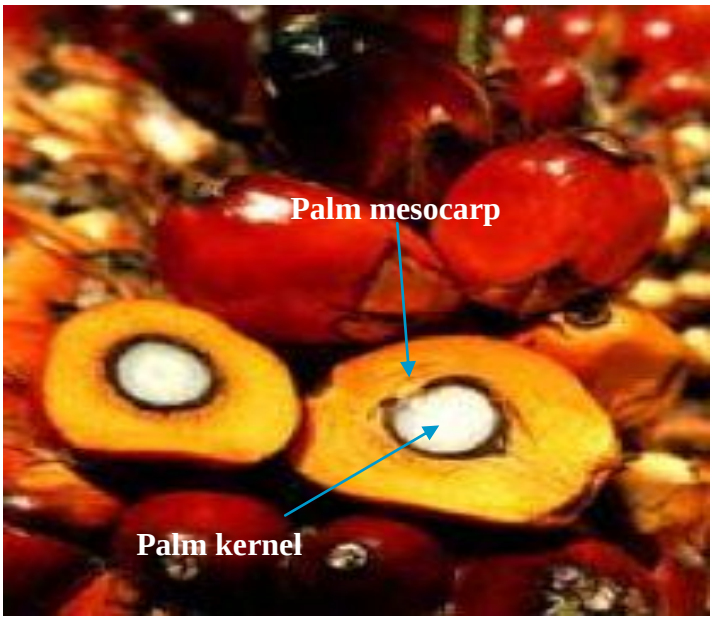

Figure 3.1 Oil palm fruit *(website: www.biofuelmerchants.com/id60.html)*

3.2.6 Saponification of palm kernel oil (SPKO)

Ethanolic sodium hydroxide solution was prepared by dissolving 2.8 g of sodium hydroxide in 100 ml of ethanol. Then, 8 g of PKO was added to the ethanolic sodium hydroxide solution. The mixture was then gently refluxed for 1 hour at 65 ˚C. The excess ethanol was then evaporated by rotary evaporation, using the Eyela Rotary Evaporator. Upon evaporation, semi solid sodium salts of fatty acids were formed. The salts were then further dried to a constant weight in an oven at 60 ˚C and stored at room temperature for future use.

3.2.7 Extraction, purification and casting of PHA

1 g of dried cells were suspended in 100 ml chloroform and refluxed at 70 ˚C for 4 hours. The extract was then cooled to room temperature. After that, it was filtered through, using Whatman filter paper no. 1. The excess chloroform was allowed to evaporate to about 10 ml under reduced pressure, using the Eyela Rotary Evaporator. The polymer in the concentrate was then precipitated by adding the solution drop-wise into 100 ml of stirred methanol for 5 minutes. The solution was left overnight without stirring to allow the polymer to settle under gravity. The next day, the methanol-chloroform mixture was decanted and the precipitated polymer was dissolved again in 10 ml of chloroform. It was poured into a glass petri dish and the remaining solvent was allowed to evaporate in a fume cupboard. Upon evaporation, a polymer film was obtained.

The PHA films were stored at room temperature for 12 weeks to allow for stabilisation.

3.3 Sterilization of the PHA films

The PHA films produced were cut approximately into the size of 19 mm × 27 mm and individually packed into the sterilization pouch. The PHA films were then sterilized in the ethylene oxide gas chamber for 6 hours.

PHA had been sterilized using ethylene oxide without causing any significant physical changes (Williams and Martin, 2002).

3.4 Study on biocompatibility of PHA and bone reaction

Biocompatibility and bone reaction will be assessed in an animal model, using histomorphometric analysis.

3.4.1 Animal model

12 mature New Zealand white female rabbits, around 10 - 12 months old and weighing between 2.5 kg to 3.5 kg were used. The site of investigation was the body of the mandible.

This study was approved by the Animal Care and Use Committee, Faculty of Medicine, University Malaya (Ethics code: PM/20/06/2006/NIH(R)).

3.4.2 Surgical procedures

The surgical tools and consumables were prepared before the surgery (Figure 3.2). Prior to surgery, each rabbit was premedicated according to their weight, with an intra-mascular injection of ketamine (30-40 mg/kg body weight) and xylazine (3-5mg/kg body weight).

The perimandibular region on both sides were shaved, extending down to the neck. The intended submandibular incision area was marked. The estimated position of the defect is shown in Figure 3.3. Local anaesthesia (lignocaine hydrochloride 2 % w/v) was given along the incision marking to control bleeding

and to enable early post operative analgesia. The incision was made to expose the body of the mandible.

A full thickness bone defect approximately 15 mm × 10 mm in size was created. The continuity of the lower border of the mandibular was not disturbed. A lead foil was used as a template to ensure consistency in the creation of the bone defect (Figure 3.4). The osteotomy was implemented using a fissure bur on an electric-motor driven dental handpiece under extensive cooling with normal saline. The same surgical procedure was carried out on both sides of the rabbit's mandible.

Subsequently, a PHA film of size 19 mm × 27 mm was wrapped over the defect on one side (Figure 3.5); while on the contra-lateral side, the defect was left empty as a control. All wounds were closed with absorbable sutures.

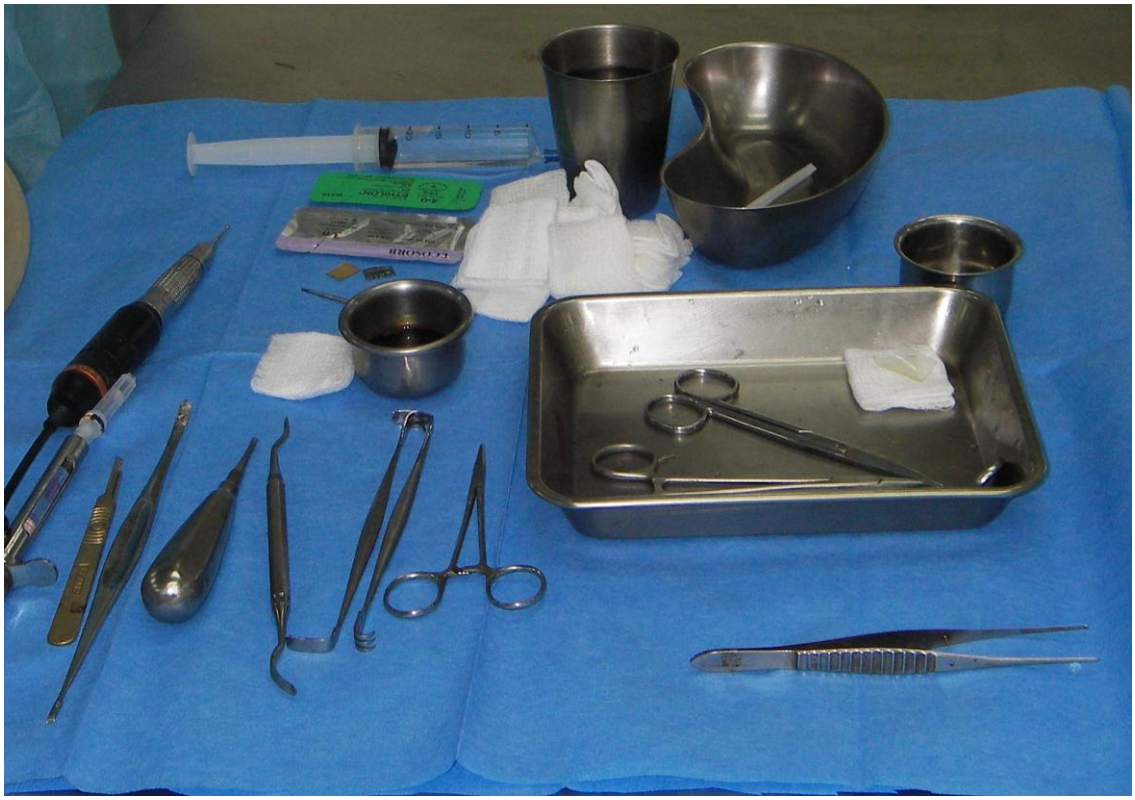

Figure 3.2 Surgical tools and consumables used for the insertion of PHA.

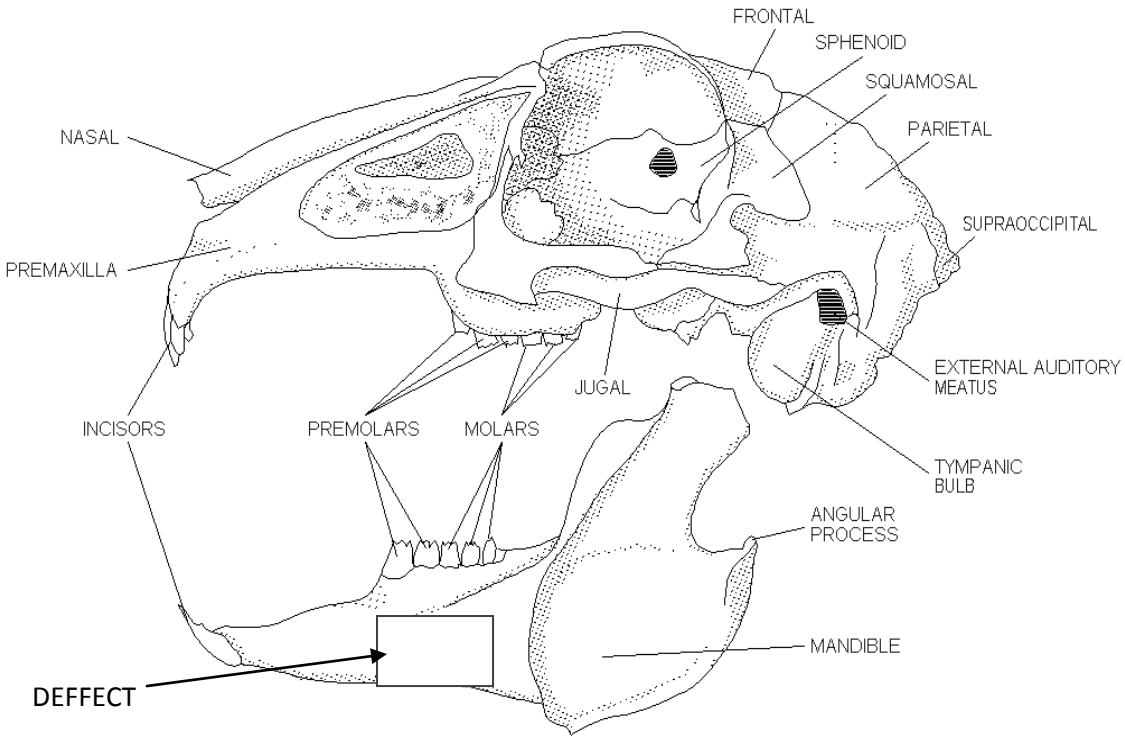

Figure 3.3 Rabbit skull – disclosing the position of the defect at the body of the mandible (*adapted and modified from* website http://cas.bellarmine.edu – Bellarmine University).

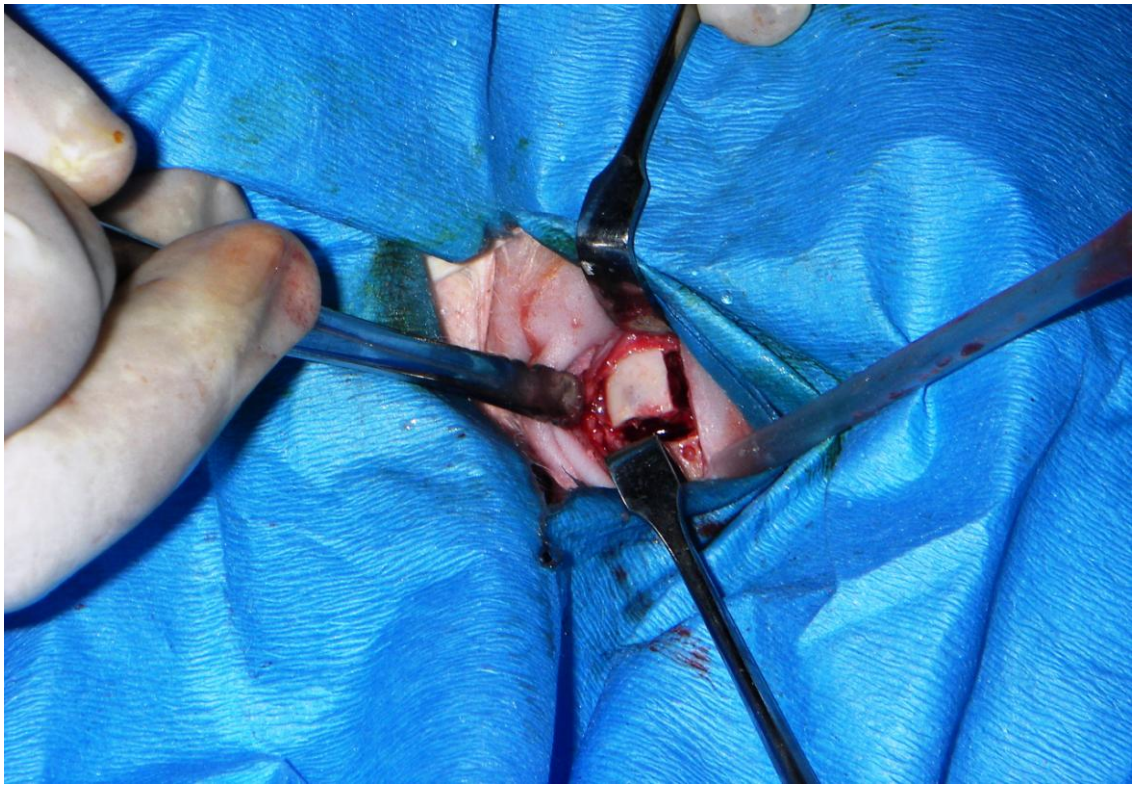

Figure 3.4 The excision of bone from the mandible to create a defect. A lead foil was used to gauge the ideal size of the defect.

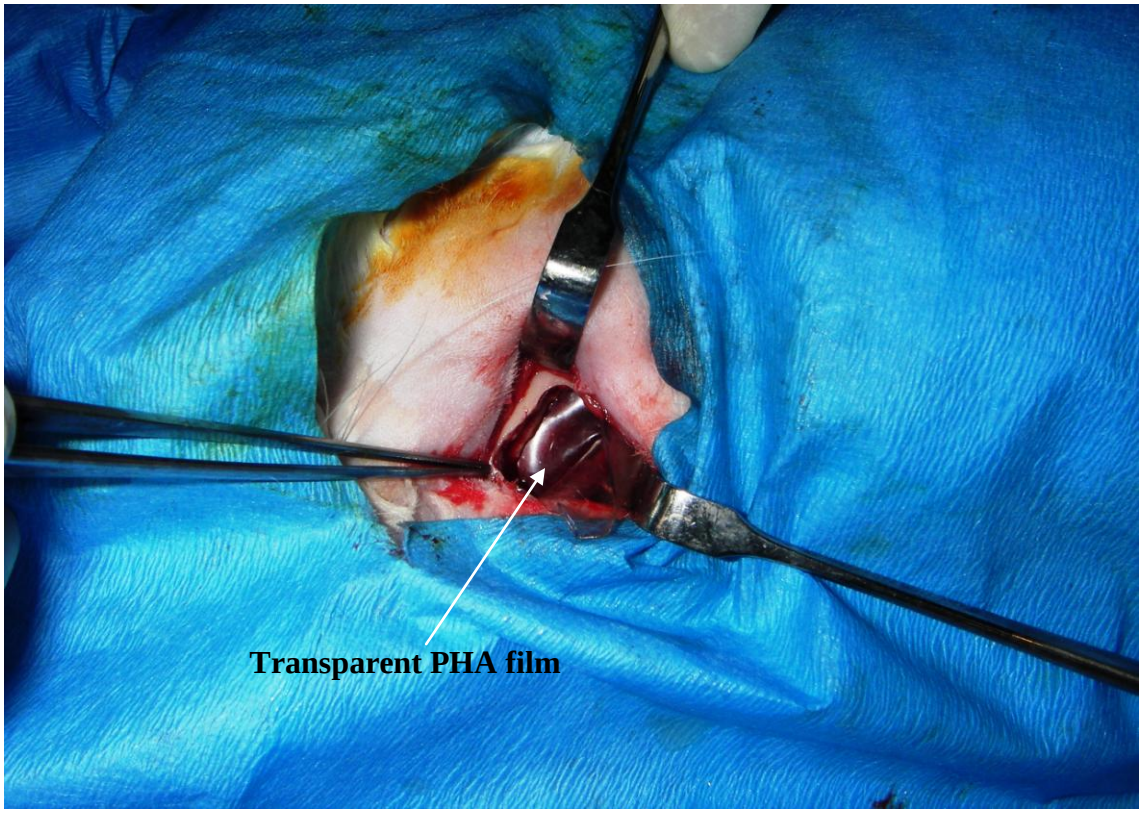

Figure 3.5 The PHA film was placed into the defect and wrapped over it.

3.5 Postoperative care

The rabbits received analgesics (meloxicam - Metacam®) and antibiotics (sulphamethoxazole 200 mg, trimethoprin 40 mg - Kombitrim®) for 4 days post operatively. Each rabbit was individually caged and received food pellet and water.

3.6 Euthanasia

The rabbits were sacrificed at three weekly intervals starting from three weeks after surgery and ending at 12 weeks (Table 3.4). Three rabbits were

sacrificed each time (Table 3.5). The euthanasia was done at the Animal Experiment Unit, Medical Centre of University of Malaya. The rabbits were sacrificed with an intravenous overdose of pentobarbital (Nembutal) (25-30 mg/kg body weight). The defects and the surrounding tissues were harvested and prepared for histological examination.

Table 3.4 Summary of the animals and defect profile in this study

Rabbit	Weight (kg)	Right Mandible	Left Mandible
1	3.20	PHA	Negative
2	2.80	Negative	PHA
3	2.95	PHA	Negative
4	3.10	PHA	Negative
5	3.10	Negative	PHA
6	3.00	PHA	Negative
7	2.80	Negative	PHA
8	2.80	Negative	PHA
9	2.90	Negative	PHA
10	2.70	Negative	PHA
11	2.90	PHA	Negative
12	2.60	PHA	Negative

Table 3.5 Test period before euthanasia

Animal	Test period
Rabbit 1	12 weeks
Rabbit 2	
Rabbit 3	
Rabbit 4	9 weeks
Rabbit 5	
Rabbit 6	
Rabbit 7	6 weeks
Rabbit 8	
Rabbit 9	
Rabbit 10	3 weeks
Rabbit 11	
Rabbit 12	

3.7 Fixation

The harvested defects and surrounding tissues were fixed in 4% buffered formalsaline (Table A-2) for about 4 weeks before histological processing.

3.8 Radiography of excised bone specimens

Radiographs of all the excised specimens containing the bone defects were taken using a Siemens Dentotime (exposure settings: 70 kV and 7 mA, time: 0.32

s, length: 150 mm). These radiographs were necessary to assist in locating the site of the bone defects within the excised specimens.

3.9 Histological processing

The Exakt Cutting Grinding technique (Exakt Vertriebs GmbH, Germany) was employed to prepare thin undecalcified sections of the specimens for histological evaluation. The steps involved are detailed below:

3.9.1 Dehydration and infiltration process

The specimens were processed using the Dehydration & Infiltration System (EXAKT-510). These specimens were dehydrated in an ascending series of alcohol rinses, cleared in xylene, and infiltrated by immersing them in different percentage of ethanol and Technovit 7200 VLC embedding media (Table 3.6). Technovit 7200 VLC is a light sensitive resin, so an enclosed environment was required for the infiltration steps. The purpose of this process is to infiltrate the tissue from organic and inorganic residues and also to dehydrate the tissue.

Table 3.6 Dehydration and infiltration process

Steps	Reagent	Duration (Days)	
1.	60% Alchohol/H_2O	9	
2.	80% Alchohol/H_2O	9	
3.	90% Alchohol/H_2O	9	
4.	Absolute Alchohol I	9	
5.	Absolute Alchohol II	9	
6.	70% / 30% Alchohol/Technovit 7200	9	
7.	50% / 50% Alchohol/Technovit 7200	9	
8.	30% / 70% Alchohol/Technovit 7200	9	
9.	100% Technovit 7200 I	9	Placed in vacuum
10.	100% Technovit 7200 II	9	
11.	100% Technovit 7200 III	45 or more	

3.9.2 Embedding/ Blocking (Light Polymerization Unit)

After the infiltration process, the specimens were embedded in fresh Technovit 7200 VLC within a plastic mould (Embedding Mould/ large/ 16 mm), using the Light Polymerisation Unit (EXAKT 520). Two to three specimens were fitted into one plastic mould (Figure 3.6). The mould together with the specimens was then place in the light polymerization unit where the photo curing resins were cured under the blue or yellow light 4 hours alternately (Figure 3.7). During polymerization, moulds containing the embedding material must be kept cool by being placed in running cold water until the resin sets.

Figure 3.6 Specimens in plastic moulds.

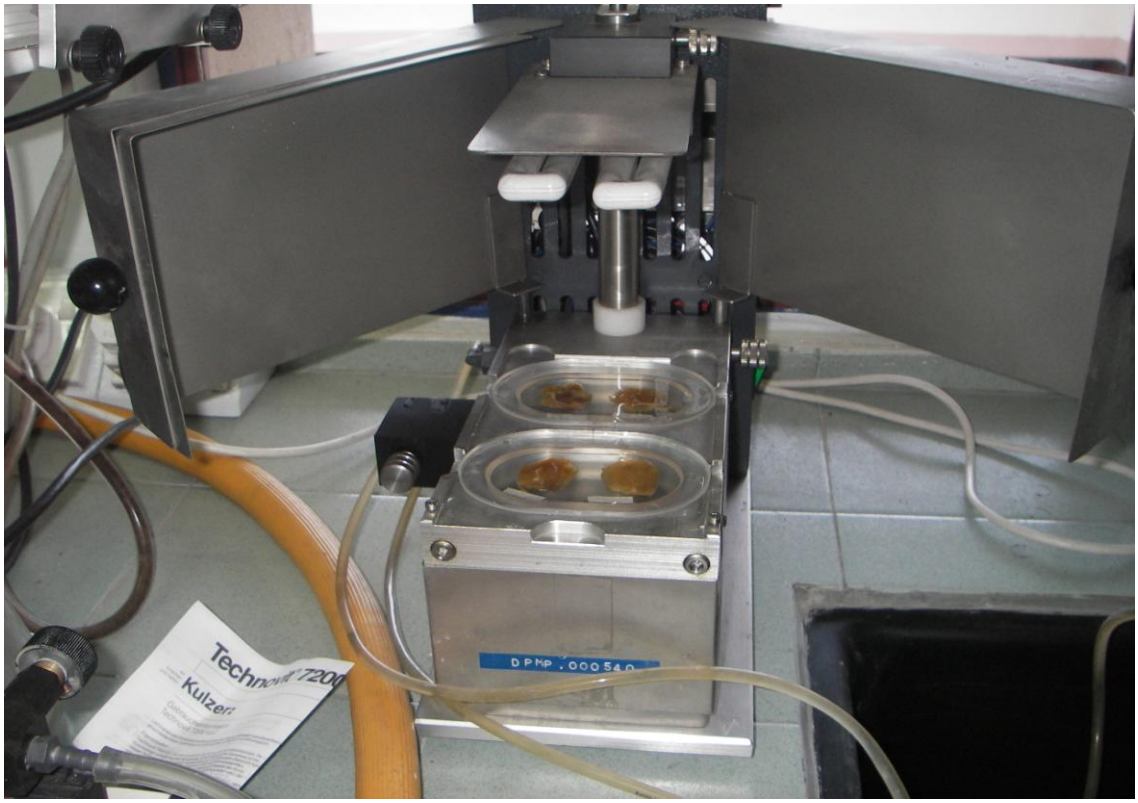

Figure 3.7 Plastic moulds with specimen placed in the Light Polymerization Unit (EXAKT 520).

3.9.3 Attachment of first slide to specimen block

The surface of a slide (50 mm × 100 mm) was roughened with sand paper (Wet Grinding Paper K1200) to create a rough surface in order to attach the specimen block. The adhesive was prepared by mixing Syrup 1 and Syrup 2 with the ratio of 2:1 and Technovit 4000 powder [Technovit 4000 Resin/ Cold-curing (contains 1500 g powder, 1000 ml syrup I, 500 ml syrup II, measure-spoon)] was added into the mixture (1 portion of S1:S2 to about 5 - 6 portions of T4000 powder). The adhesive was mixed quickly and it was applied to the back of the specimen block and was attached to the rough surface of the slide. It was allowed to harden for at least 15 minutes (Figure 3.8).

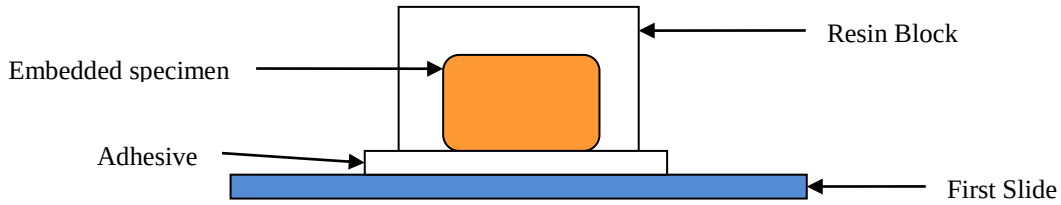

Figure 3.8 Attachment of first slide to the specimen block with adhesive (mixture of S1 + S2 + Technovit 4000).

3.9.4 Gross grinding of slide block

The grinding machine (EXAKT 400 CS) was set up with sand paper (Wet Grinding Paper K800) (Figure 3.9). The slide block (Figure 3.8) was attached

onto the block holder using vacuum suction. Weights were added to the block holder on the grinding machine to pull down the slide block onto the grinding plate. A few oscillations were allowed before lifting off the block holder by removing the weight. The purpose of this coarse grinding was to expose the specimen and also to obtain an evenly flat surface. Then, the thickness of the resin block attached to the first slide was measured with a micrometer.

Figure 3.9 Grinding Machine (EXAKT 400 CS) - set up with sand paper.

3.9.5 Preparation of sandwich slide block (with Adhesive Press)

The thickness of a fresh and clean slide (50 mm × 100 mm) was measured before attaching it to the top of the Adhesive Press (EXAKT 402). Then, some adhesive (Technovit 7210 VLC) was spread evenly on the surface of the slide

block. The slide block with adhesive was then placed on the base holder of the Adhesive Press. The base together with the slide block was raised up to come into contact with the new slide, avoiding bubbles formation at the same time. The switch was turned on to allow curing of the adhesive for 15 minutes. Finally, a sandwich block was formed and the thickness of the whole block was measured (Figure 3.10).

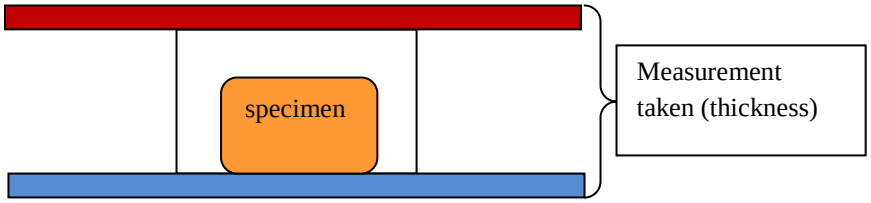

Figure 3.10 The thickness of the sandwich formed was measured.

The thickness of the adhesive (Technovit 7210 VLC) (Figure 3.11) was obtained by the following equation:

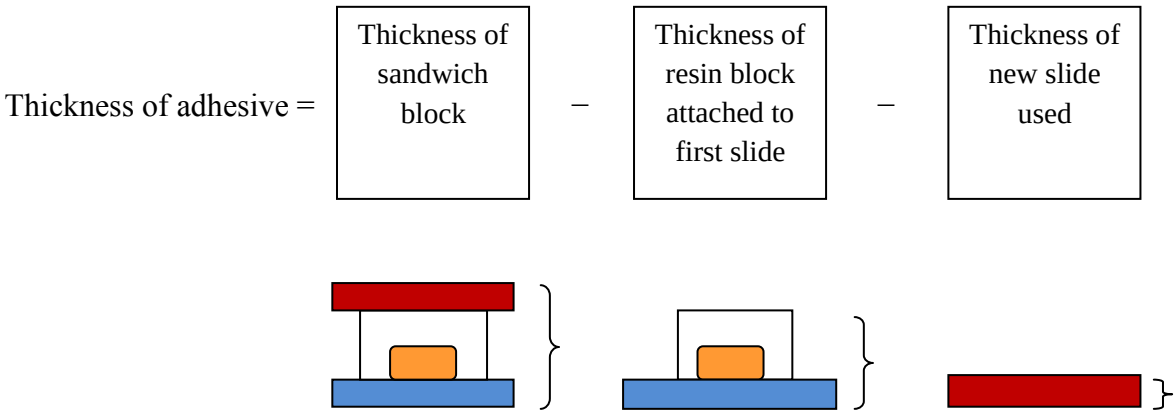

Figure 3.11 Thickness of adhesive (Technovit 7210 VLC) between the resin block and the new slide used.

3.9.6 Sectioning

All specimen blocks were oriented for cutting in a mesio-distal direction. With customary wire or band saws there is an active cutting line in the contact region between cutting band and the specimen. This process is known as CL or Contact Line. When the EXAKT Contact Point CP Technology is used for cutting, the specimen oscillates during cutting. Thus, there is localized contact between the cutting band and the specimen at all times during the active cutting process (EXAKT).

A suitable slide holder was attached to the cutting machine (EXAKT 300) (Figure 3.12) with the aid of an Ellen key. Then, the sandwich block was attached to the holder using vacuum. The block holder together with the sandwich block was advanced towards cutting edge of the blade using the micron dial.

In order to set to zero position:

1. The sandwich block was advanced till the inner surface of the slide touches the cutting band.
2. The cutting machine was turned on to test the frictional sound produced between the initial contacts of the cutting band with the first slide of the sandwich block. The micron dial was turned till the frictional sound stopped- this is the zero point.

The thickness of section was set by turning the micron dial. Before cutting, block holder clamp was removed; the top knob and the screw within were tightened.

The switch was turned on to allow cutting and oscillation process. The slicing of the sandwich block was commenced by bringing the sandwich block towards the running blade and then it was released.

The thin section obtained was then measured with the micrometer. The thickness of the tissue embedded could be obtained by subtracting the thickness of the adhesive (Technovit 7210 VLC) that lay beneath the tissue.

Figure 3.12 The Cutting Machine (EXAKT 300).

3.9.7 Fine grinding (with EXAKT Grinding System)

This grinding system (EXAKT 400 CS and EXAKT AW110) was designed to achieve highest plane parallelism and excellent surface quality especially when grinding materials consisting of hard and soft components.

The grinding machine was set up with sand paper (Wet Grinding Paper K800). The thin section was attached to the block holder of the grinder. The grinder was calibrated to grind the section till desired thickness was obtained.

3.9.8 Polishing

For polishing, the sand paper was replaced with the polishing paper (K4000) to ensure a smooth and satisfactory planarity of the section. The histological sections prepared using the Exakt Cutting grinding achieved a thickness of approximately 40 to 90 microns.

3.10 Histological analysis

A total of 72 thin sections (Figure 3.13) were obtained for histological evaluation:

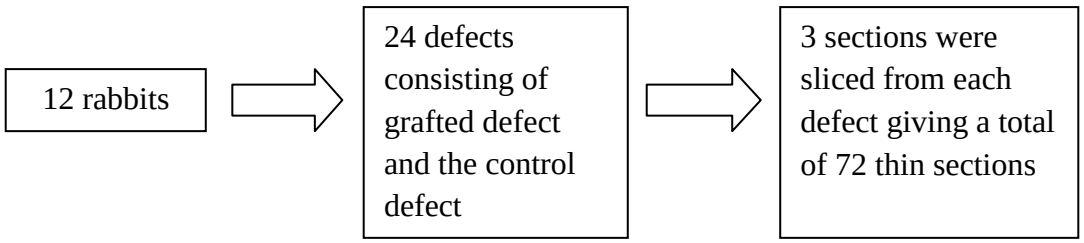

Figure 3.13 A summary of the total amount of thin sections in this study.

Twenty-four thin sections were stained using the PAS technique while the remaining forty eight sections were stained with haematoxylin and eosin.

3.10.1 Staining technique

3.10.1.1 Haematoxylin and Eosin staining

The Mayer Haematoxylin solution (Batch no.: F1227D8) and 1 % Aqueous Eosin solution (Batch no.: F01588) were supplied by Labchem Sdn. Bhd.

Forty-eight thin sections were immersed in Mayer Haematoxylin for 8 minutes. The excess stain was then rinsed off and stained with Blue in saturated Aqueous Lithium Carbonate for 15 seconds. The solution was washed in running water for 5 minutes and then followed by immersion in 1% Aqueous Eosin for 8

minutes. The thin sections were rinsed in water to remove excess stain and were allowed to air dry for 24 hours before viewing under the microscope.

3.10.1.2 Periodic Acid-Schiff (PAS) technique

The PAS stain is a histochemical reaction in that the periodic acid oxidizes the carbon to carbon bond forming aldehydes which react to the fuchsin-sulfurous acid which form the magenta colour.

The reagents were prepared as followed (Table 3.7 and Table 3.8):

Table 3.7 1.0 % Periodic acid

Periodic acid	1 ml
Distilled water	100 ml

*****mix well. Solution remained stable for 1 year.

Table 3.8 Schiff's reagent

Basic/diamond Fuchsin	1 g
Potassium or sodium metabisulphite	1 g
Concentrated HCl	20 ml
Activated charcoal	2 g
Distilled water	200 ml

The distilled water was boiled and removed from flame to avoid excessive effervescence before adding the basic fuchsin. The solution was mixed and cooled down to 50 ˚C. Hydrochloride acid was added and the solution was cooled down to room temperature. Then, sodium metabisulphite was added and mixed together with the solution and was kept in the dark for 18 to 24 hours. After that, activated charcoals were added, shake for 1 minute and then filtered. The solution was stored in a dark bottle at 4˚C and the solution remains stable for 1 month.

In this study, PAS was used to stain 24 thin sections. The thin sections were hydrated with distilled water. This was followed by oxidation in 1% periodic acid for 5 minutes and was rinsed off with distilled water. Then, the sections were flooded with Schiff's reagent for 30 minutes and were washed in running tap water for 5 minutes. After that, the sections were stained with Harris Haematoxylin for 30 seconds and washed in running tap water for another 3 minutes. Finally, the thin sections were allowed to air dry before viewing under the microscope.

3.11 Histological study

All sections were examined for host tissue response and healing within the area of the critical bone defects in the PHA and control sites. Any signs of chronic inflammation, foreign body giant cell reaction, osteoclastic activity, osteoblastic reaction, new bone formation or resorption were taken into account.

3.12 Histomorphometric study

A light microscope (Nikon Eclipse E400) was used. The sections were viewed and photomicrographs were taken with a digital camera (Evolution MP Cooled, 5 Megapixel) attached to the microscopic system. Each section was systematically photographed at magnification × 100 or × 200, capturing the images oriented along the entire 15 mm × 10 mm bone defect.

Bone histomorphometry was performed using the point-counting technique. A rectangular grid with points 5 cm × 5 cm apart was superimposed on the digitized images. The entire grafted area for the test and control sites were systematically sampled and quantified. In the 3-, 6- 9- and 12-week specimens, the volume fractions (%) of newly formed bone, of residual grafting PHA material, and of soft tissue/marrow space occupying the defects were determined at × 100 or × 200 magnifications. The parameters scored were tabulated as follow:

1. newly-formed bone
2. host bone
3. connective tissues/marrow spaces
4. implanted PHA
5. others

A score chart was obtained from this analysis. The average mean new bone volume for PHA group at different time intervals of sacrifice were calculated. These values were compared to the respective average mean new bone volume calculated for the control groups (Figure 4.9).

The mean new bone volume was calculated using the formula shown below:

$$\text{Mean new bone volume} = \frac{\text{Score of New Bone formed}}{\text{Total scores}} \times 100\ \%$$

The average of the mean new bone volume were then calculated from the same time interval.

$$\text{Average mean new bone volume} = \frac{\text{Total mean new bone volume of the same time interval}}{3}$$

3.13 Statistical analysis

Data were presented as average mean value ± standard deviation (SD) of each group. Statistical comparisons were performed using Student's t-test with a confidence level of 95 % ($p < 0.05$) considered statistically significant (Johnson and Kuby, 2000).