CHAPTER IV

EXPERIMENTAL WORK

The experimental work can be divided into three main parts: (1) Materials and reagents, (2) Equipments, and (3) Experimental procedures. The experimental procedures are subdivided into seven parts: (i) Acellular dermis (ADM) preparation, (ii) Determination of fat content, (iii) Determination of cell removal (DNA assay), (iv) Histological examination, (v) Scanning electron microscopic (SEM) analysis, (vi) Fourier transform Raman (FT-Raman) spectroscopic analysis, and (vii) Animal study. All experimental procedures are summarized in Figure 4.1.

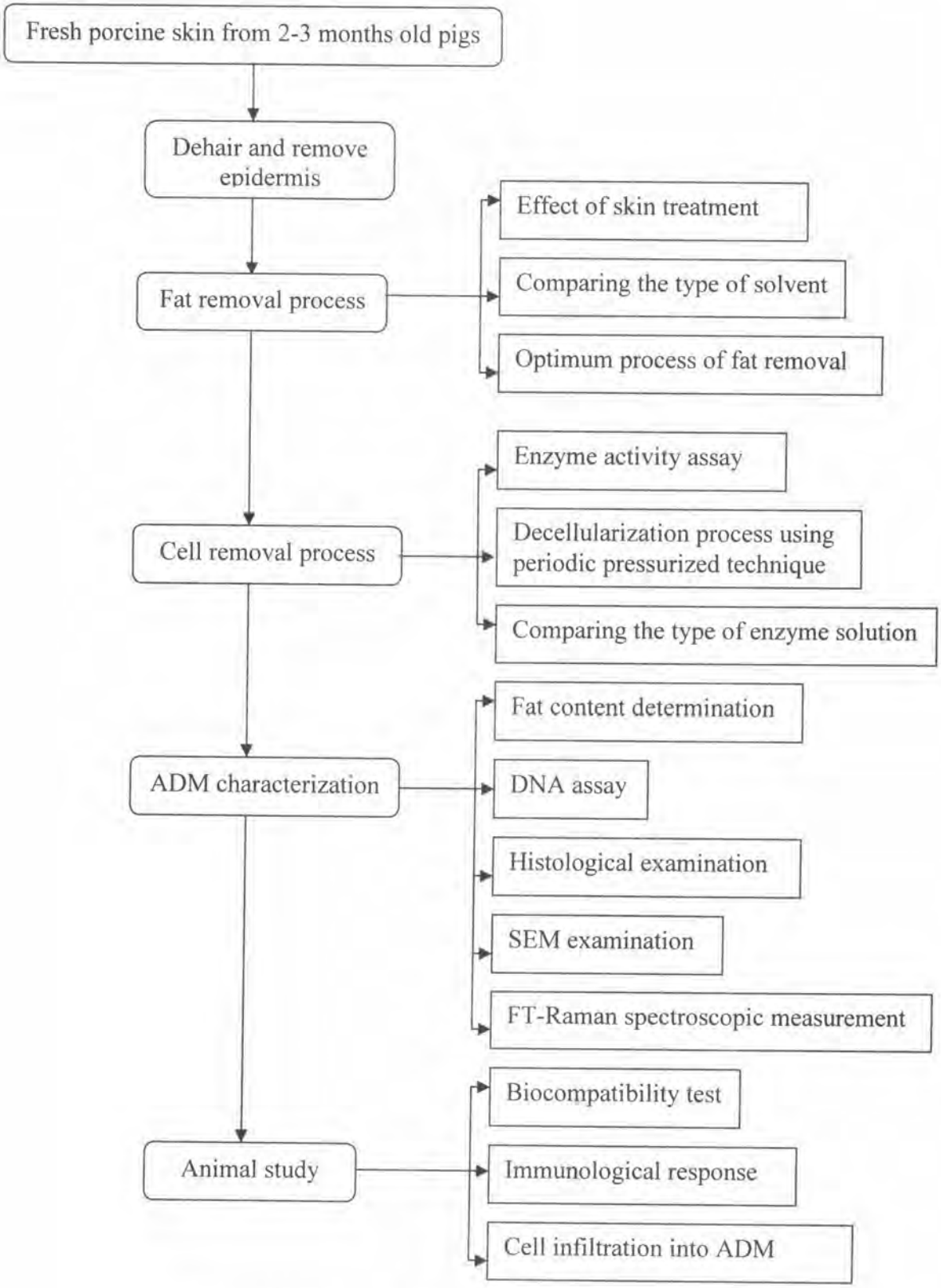

Figure 4.1 Schematic diagram of experimental procedures.

4.1 Materials and Reagents

1. Fresh porcine skin from 2-3 months old pigs was obtained from local slaughterhouse. Porcine skin was kept at -20°C until use.
2. Trypsin from hog pancreas (95 Unit/mg, lot number 1166818), Glycerol, Hexamethyldisilazane, Hoechst 33258 (2-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2, 5-bi-1H-benzimidazole), and Disodium hydrogen phosphate heptahydrate were obtained from Fluka (Germany).
3. Dispase II from Bacillus polymyxa (0.5 Unit/mg, lot number 12131100) was purchased from Roche Applied Science (Germany).
4. Sodium citrate and methanol were supplied by Fisher Chemicals (UK).
5. Sodium dodecyl sulphate (SDS) and sodium chloride were supplied by Ajax Finechem (Australia).
6. Sodium azide was obtained from Asia Pacific Specialty Chemicals (Australia).
7. Glycerol was obtained from Sigma (USA).
8. Chloroform and formaldehyde were supplied by BDH (UK).
9. Dihydrogen phosphate monohydrate and Ethyl alcohol were supplied by Merck (Germany).
10. Sodium sulphide was purchased from Panreac Quimica SA (Spain).

4.2 Equipments

1. Periodic pressurized apparatus made of stainless steel, as shown in Figure 4.2.
2. Fourier Transform Raman (FT-Raman) Spectrophotometer (Perkin Elmer, Spectrum GX, UK)
3. Lyophilizer and -40°C freezer (Thermo Electron Corporation, Heto PowerDry LL3000, USA)
4. 4-digit balance (Mettler Toledo, AG204, Switzerland)
5. pH meter (Sartorius PP-50, Germany)
6. Scanning electron microscope (Joel, JSM 5400, Japan)
7. Magnetic stirrer (Eyela RNC-3, Germany)
8. Micropipette (Pipetman P20; P200; and P1000, USA)
9. Spectrofluorometer (Perkin Elmer, Victor 3, UK)
10. Optical microscope (Olympus, USA)
11. Microcentrifuge (2,236g) (Hitachi Koki, Chibitan II, Japan)
12. 96-black well plate (BMG Labtechnologies, Germany)

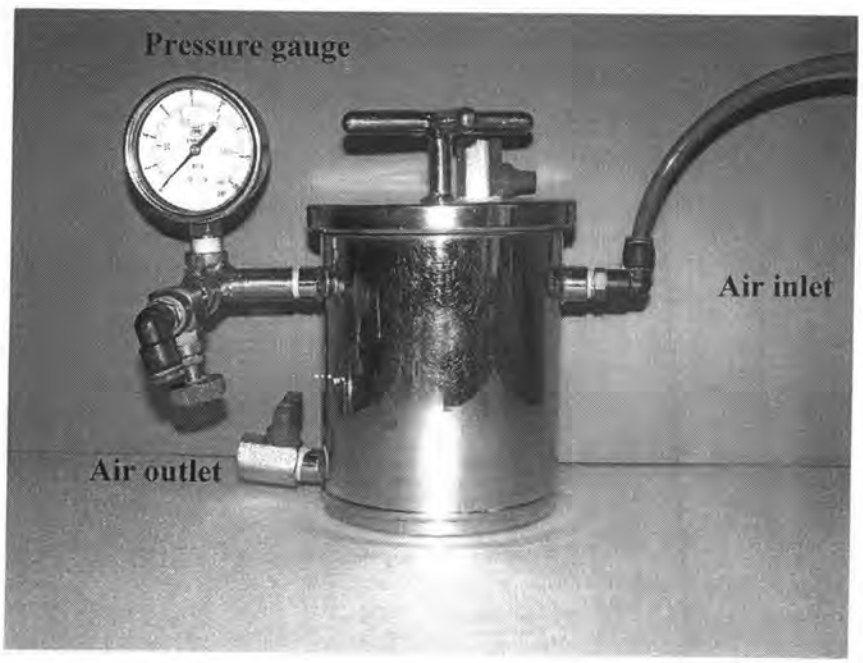

Figure 4.2 Periodic pressurized apparatus

4.3 Experimental procedures

4.3.1 Preparation of acellular dermis from porcine skin

4.3.1.1 Dehair and de-epidermis process

Fresh porcine skin was cut in pieces (3x3x0.1 cm^3) and washed thoroughly with water. After complete cleaning, subcutaneous fat was excised off. In order to remove hair, the skin was treated in 20% (w/v) sodium sulphide at room temperature for 5 min and scraped by forceps. To remove epidermis, dehaired skin was soaked in 1 M of NaCl solution at room temperature for 24 hr, followed by 85% (w/v) glycerol solution for 14 days, then distilled water for 24 hr.

4.3.1.2 Fat removal process

De-epidermis skins were further divided into nine groups (FR1-FR9) in order to investigate the optimal process of fat removal. In general, each group was treated into 2 steps; pretreatment with freeze thaw, and solvent extraction. Samples were freeze thaw and then soaked in a solvent with a continuous stirring. The number of freeze thaw cycles, type of solvent, and soaking time in the solvent were varied for each group as summarized in Table 4.1. For one cycle of freeze thaw, samples were rapidly frozen in liquid nitrogen for 90 sec, then followed by thawing at room temperature for 4 min. In this experiment the ratio of sample weight to volume of solvent was 1/20 (1 g of samples in 20 ml of solvent). After the treatment, samples from each group were extensively washed with PBS buffer for 6 hr.

Table 4.1 Fat removal treatment of de-epidermis porcine skin

Groups	Freeze thaw (number of cycles)	Solvent	Soaking time
FR1	-	Hexane	12 hr
FR2	5	Hexane	12 hr
FR3	10	Hexane	12 hr
FR4	15	Hexane	12 hr
FR5	10	Chloroform/ Methanol	12 hr
FR6	10	Chloroform/ Methanol	3 hr
FR7	10	Chloroform/ Methanol	2 hr
FR8	10	Chloroform/ Methanol	1 hr
FR9	10	Chloroform/ Methanol	20 min

4.3.1.3 Cell removal process

4.3.1.3.1 Enzyme activity assay

The activity assay of enzyme was preformed at various time (0-24 hr) under constant temperature and pH. The 0.9 ml of 0.1 M phosphate buffer (at pH 7.4) and 0.1 ml of 1% trypsin solution were mixed in a test tube and shaken by a vortex mixer. The 1 ml of 0.2% (w/v) azo-casein solution was added to the mixing solution and further shaken. The solution was incubated in water bath at the room temperature for 20 minutes. After that, the 2 ml of 10% (w/v) trichloroacetic acid solution (TCA) was added to the solution for precipitation of non-hydrolyzed substrate, and then the solution was put in a ice bath at about 4°C to stop the hydrolysis reaction. Finally, the solution was centrifuged at 3,000 rpm (1,660g) for 20 minutes to separate the solid and liquid phases. The absorbance of the sample at the wavelength of 440 nm was measured using a UV-VIS spectrophotometer. Blank test was prepared by adding the TCA solution to the substrate before azo-casein solution was added.

4.3.1.3.2 Decellularization of skin using periodic pressurized technique

This experiment was designed to study the influence of pressurized periods, enzyme refreshment, and type of enzyme solution (trypsin and dispase II) on the efficiency of cell removal. De-fat skin was divided into six groups (CR1-CR6). The treatment for each group was summarized in Table 4.2. CR1-CR5 groups were treated in 1% (w/v) trypsin solution (56 unit/g as seen in Appendix A) while 0.24% (w/v) dispase II solution (241 unit/g as seen in Appendix A) was used in CR6 treatment. CR1 and CR2 treatments were performed under continuous stirring. CR3-CR6 groups were treated in a periodic pressurized apparatus. The pressurized period conditions, i.e. number of pressurized periods, and period time, were varied as shown in Figure 4.3. This therefore results in differences in total treatment time of decellularized process. For CR2-CR6 groups, enzyme solution was also refreshed as indicated in Table 4.2. The ratio of sample weight to volume of enzyme solution was 1/25 (1 g of samples in 25 ml of enzyme solution). In addition, 0.02% (w/v) sodium azide was added in every treatment group to prevent bacterial growth. After the treatment, ADM was then extensively washed and stored at -20°C.

Table 4.2 Cell removal treatment of de-fat skin

Group	Enzyme	Enzyme refreshment	Pressurized periods	Treatment time
CR1	Trypsin	-	-	24 hr
CR2	Trypsin	Every 6 hr	-	24 hr
CR3	Trypsin	Every 6 hr	24	12 hr
CR4	Trypsin	Every 1.5 hr	36	3 hr
CR5	Trypsin	Every 45 min	36	3 hr
CR6	Dispase II	Every 1.5 hr	36	3 hr

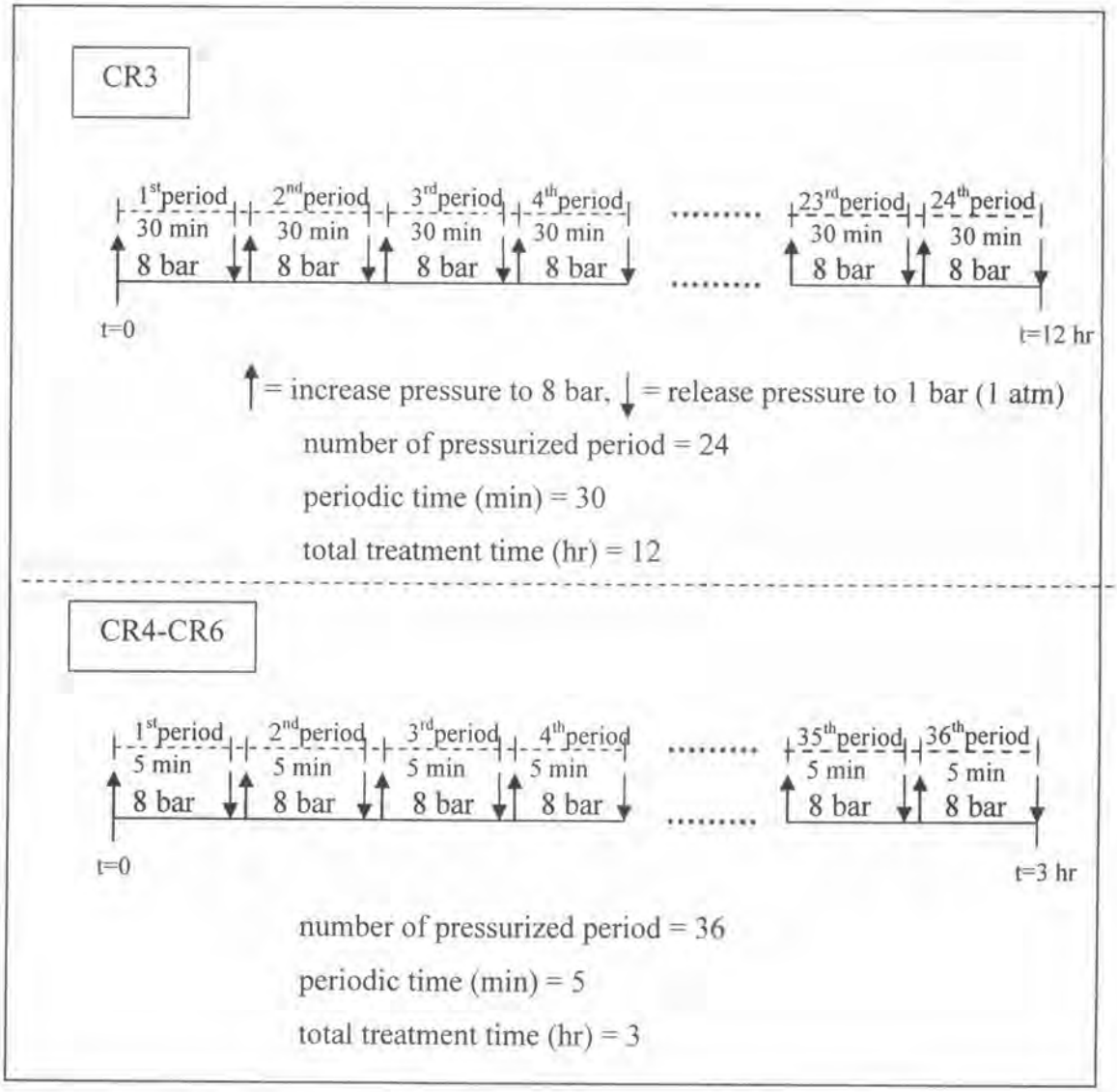

Figure 4.3 Treatment pattern of periodic pressurization

4.3.2 ADM characterizations

4.3.2.1 Fat determination

The fat content of skin was determined by method of Folch [17]. The skin was dried and ground, following by a treatment with chloroform/methanol (2/1 v/v) solvent. The ratio of solvent volume and sample weight was 20:1. The mixture was stirred for 15-20 min at room temperature and then filtrated to separate the liquid phase. 4 ml of water (0.2 times volumes of chloroform/methanol solvent) was added

to the solution to separate methanol from chloroform. After vortexing for a few seconds, the solution was centrifuged at 450g (2,000 rpm) for 15 min. The upper phase (water/methanol) was removed and the lower phase (chloroform/fat) was left in an oven for 4 hr to evaporate chloroform. The dried fat was cooled in a desiccator at room temperature. The weight of dried fat was measured. The percentage of fat removal was determined using following equation.

$$\text{Fat removal (\%)} = \left(\frac{W_R - W_S}{W_R}\right) \text{x } 100 \qquad (1)$$

where W_R represents the weight of dried fat of fresh skin and W_S represents the weight of dried fat of de-fated skin.

4.3.2.2 DNA assay

The percentage of cell removal was determined by measuring the amount of DNA using Hoechst 33258 fluorescence dye [16]. Each group of samples ground with a mortar and pestled in liquid nitrogen. One hundred mg of ground sample were transferred to a micro centrifuge tube and 1 ml of 0.02% (w/v) SDS in saline–sodium citrate (SSC) was added to lyses cells. The mixture was homogenized and incubated at 55°C for 6 hr with occasional mixing in order to digest attached cells. After incubation, centrifugation was performed at 2,236g (10,000 rpm) for 10 min. 50 µl of the supernatant were transferred to another micro centrifuge tube and 950 µl 0.02% (w/v) SDS in SSC solution was added. 100 µl of the digest was mixed with 100 µl Hoechst 33258 fluorescence dye (1 µg/ml). The mixed solution was transferred to 96 black well plates and fluorescence intensity was determined with a fluorescence spectrophotometer (VICTOR^3 Perkin-elmer USA) at the excitation and emission wavelengths of 355 and 460 nm, respectively. Three measurements were repeated for each sample. The untreated skin was used as a reference. The calibration curves were performed using L929 cells (see Appendix B). The percentage of cell removal was calculated as follows.

$$\text{Cell removal (\%)} = \left(\frac{C_R - C_S}{C_R} \right) \text{x } 100 \qquad (2)$$

where C_R represents the number of cells in the reference and C_S represents the number of cells in ADM samples.

4.3.2.3 Histological examination

ADM samples were first dehydrated with an increasing series of alcohol concentration and then embedded in paraffin. Paraffin-embedded ADMs were sectioned at a thickness of 5 μm. After removing the paraffin, samples were stained with hematoxylin and eosin, then samples were examined by a light microscope at a magnification of 400x to observe fibroblast cells (stained by hematoxylin to bluish purple color) and collagen fibers (stained by eosin to a pink color).

4.3.2.4 Scanning electron microscopy (SEM)

ADM samples and fresh skin (without treatment) were freeze-dried prior to morphology observation using a scanning electron microscope (SEM, Joel JSM 5400). Samples were loaded onto copper studs and coated with gold for 4 min at 10 mA under a pressure of 0.1 Torr. ADM morphology was examined under an accelerating voltage of 12–15 kV.

4.3.2.5 FT-Raman spectroscopy

ADM samples after treatment with various conditions were washed with PBS buffer of pH 7.4 and freeze-dried prior to FT-Raman analysis. FT-Raman spectra were measured using a Perkin-Elmer spectrum GX with the excitation wavelength of 1,064 nm using Nd:YAG laser. The backscattered light was collected at 180°. The laser power was adjusted to 500 mW and 128 scans at 4 cm^{-1} resolution were accumulated.

4.3.3 Animal study

4.3.3.1 Subcutaneous implantation

ADM samples produced via group CR4 (dispase model) and CR5 (trypsin model) were freeze-dried and steriled by ethylene oxide treatment. This process employed a mixture of 15% ethylene oxide and 85% carbon dioxide at a pressure of 5.5 atm and a temperature of 55 ^{o}C in a short sterilizing cycle of 30 minutes. Sterized samples were rehydrated by soaking in normal saline solution for 24 hr. The sterilized ADM (10x10x1 mm^3 each) were subcutaneously implanted onto the back of 4-week-old female Wistar (National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand). All animal experiments were performed in accordance with *Home office guidelines on the scientific use of animals* (Scientific procedures, Act 1986) under aseptic conditions. A midline incision was made on the dorsum of rats and the pocket was generated on the skin. The ADM was covered with the skin flap and the wound was stitched using nylon sutures. The implanted samples were removed at 1-week, 2-week, and 4-week postoperatively (n = 3). The retrieved sample was fixed in 10% (v/v) formalin for at least 3 days prior to histological and scanning electron microscopy (SEM) examination.

4.3.3.2 Observation of cell infiltration

To investigate the cells infiltrated into scaffolds, implanted ADM were examined using SEM and histological examinations. For histological, ADM samples were prepared as described in 4.3.2.3. In the case of SEM, implanted samples were serially dehydrated by series of ethanol composed of 30%, 50%, 70%, 80%, 90%, and 95% for 5 min at each concentration and 15 min for 100% ethanol. Dehydrated scaffolds were dried by adding 100 µl of hexamethyldisilazane (HMDS) which was left to evaporate at room temperature. Dried scaffolds were cut to observe cell infiltration in cross-sectional plane (see Figure 4.4). C1 plane represented the sample edge, C2 plane was at the depth of 0.25 cm from the edge, and C3 plane was at the center of implanted samples.

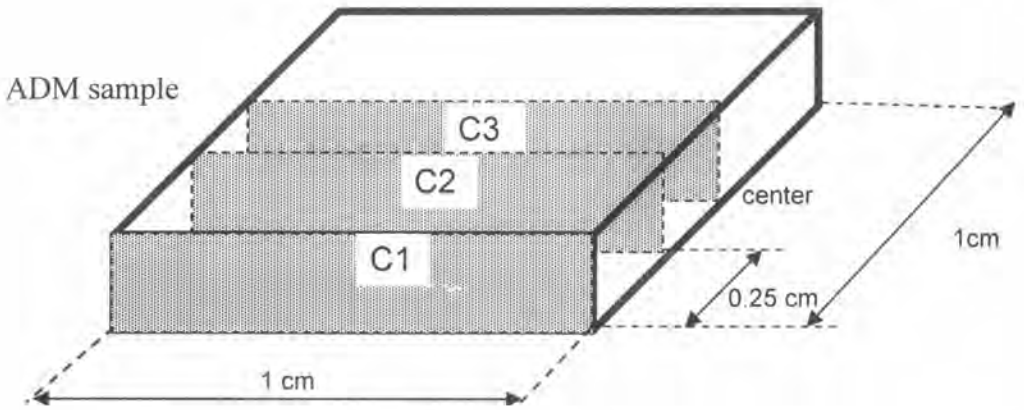

Figure 4.4 Schematic diagram of sectional direction on implanted ADM samples prior to cell infiltrated observation

4.3.4 Statistical analysis

Significant levels were determined by paired t-test. All statistical calculations were performed on the SPSS system for Windows (version 13.0, Statistical Package for Social Sciences (SPSS), Chicago, IL, USA). P-values of <0.05 and <0.01 were considered.