CHAPTER III

RESEARCH METHODOLOGY

3.1 Conceptual framework of research

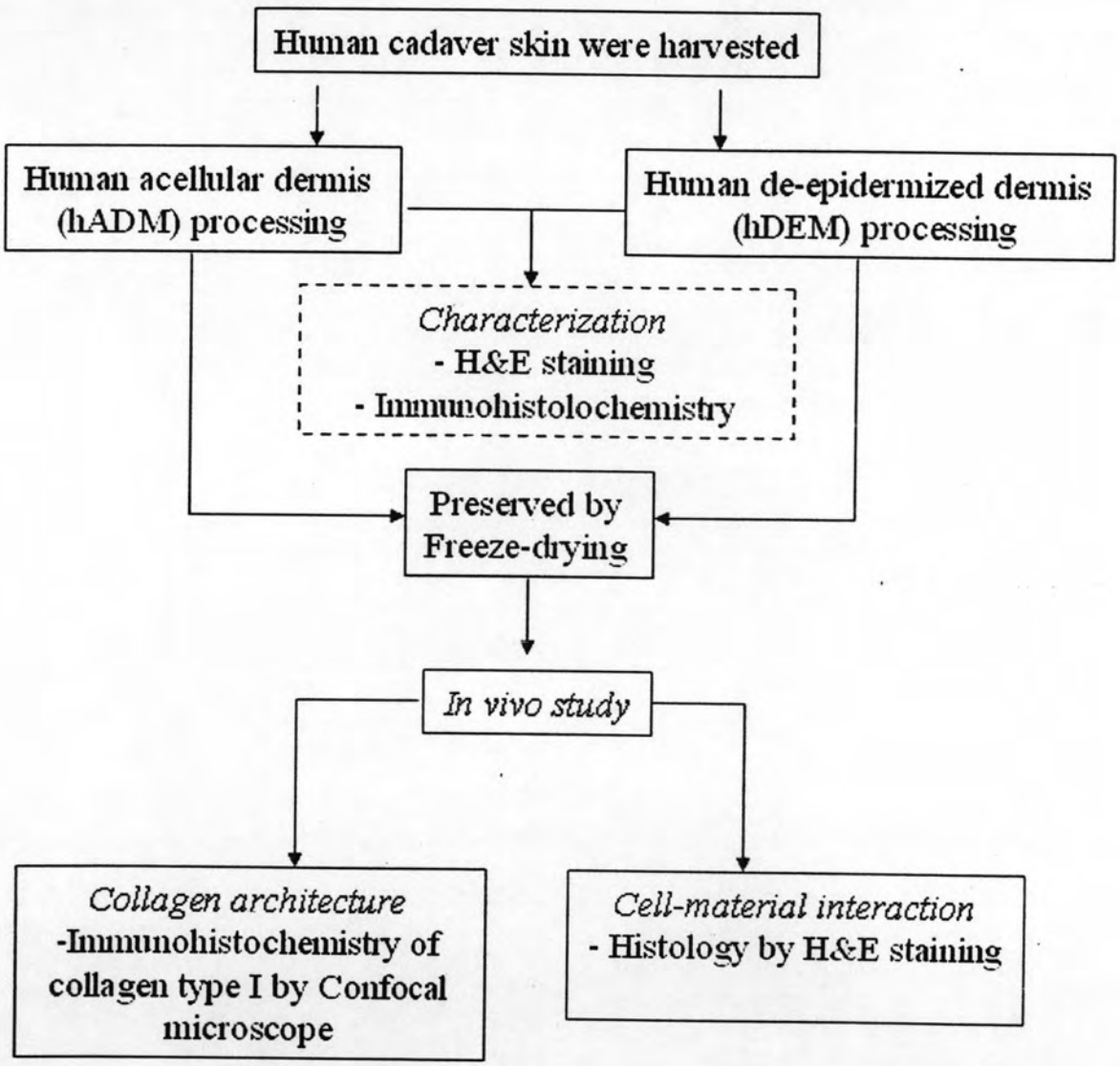

Figure 3.1: Conceptual framework of research

3.2 Materials

- Dulbecco's modified eagle medium, DMEM (10%medium + L-glutamine + AB, Hyclone, Utah, USA)
- Human cadaver skin from donated cadavers at Department of Anatomy, Chulalongkorn Memorial hospital (Bangkok, Thailand)
- Hoechast 33258 (2-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2, 5-bi-1H-benzimidazole) from (Fluka, Germany)
- Mouse skin fibroblasts (L929 or murine fibroblasts)
- Sodium Azide (NaN_3) (Labchem, Australia)
- Sodium Chloride (NaCl), Potassium Chloride (KCl) (BDH, UK)
- di-Sodium Hydrogen orthophosphate-12hydrate (Na_2HPO_4) (BDH, UK)
- di-Sodium Hydrogen orthophosphate (K_2HPO_4) (BDH, UK)
- Glycerol ($C_3H_8O_3$) (BDH, UK)
- Choloform ($CHCl_3$) (BDH, UK)
- Methanol (CH_3OH) (BDH, UK)
- Absolute Ethanol from (BDH, UK)
- Sodium Dodecyl Sulfate (SDS) from (Fisher Scientific, USA)
- Trypsin from hog pancreas (101 Unit/mg, lot number 1166819 31605364, Fluka, Switzerland)
- Trypsin-EDTA (0.25% trypsin with EDTA·Na, Gibco BRL, Canada)
- Thiopental Sodium for Injection BP 1g (Batch No. 1C 314/47, Jagsonpal Pharmaceuticals Ltd., Haryane, India)
- 1% aqueous solution Eosin Y (lot. 360610) (Bio optica, Milan, Italy)
- Mayer's Hematoxylin (lot. 350610) (Bio optica, Milan, Italy)
- Xylene ($C_6H_4(CH_3)_2$) (BDH, UK)
- Dulbecco's modified eagle medium, DMEM (10%medium + L-glutamine + AB, Hyclone, Utah, USA)
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (USB corporation, Cleveland, OH, USA)

- Polyclonal Rabbit Anti-Mouse Immunoglobulins/FITC Rabbit F(ab')2 (lot.00028846) (DakoCytomation, Dako, Denmark)
- Anti-Human Collagen Type I (lot.0603025487) (Chemicon International, USA)

3.3 Equipment

- 4-digit balance (Sartorious, Germany)
- Fourier Transform Raman (FT-Raman) Spectrophotometer (Perkin Elmer, Spectrum GX, UK)
- Freeze-dryer (Martin Christ, alpha 2-4 LSC, Germany)
- Magnetic stirrer (Wisestir MSH-10, Daihan scientific)
- Light microscope (Olympus, USA)
- pH meter (Professional Meter PP-50, Germany)
- Scanning electron microscope (Joel Ltd., JSM-5400, Tokyo, Japan)
- Spectrofluorometer (Perkin Elmer, Victor 3, UK)
- Micropipette 100µl, 1000 µl, 5000 µl (Eppendorf, USA)
- Tissue Processor (Leica TP-1020, Leica, Germany)
- Tissue paraffin embedded machine (Leica EG-1150H, Leica, Germany)
- Rotary microtome (Leica RM-2265, Leica, Germany)
- Automatic tissue stainer (Leica Auto Stainer XL, Leica, Germany)
- Frozen rotary microtome (Leica CM-1880, Leica, Germany)

3.4 Method

3.4.1 Human Acellular Dermis protocol

a. Skin Harvesting
   - Human cadaveric skin was harvested from donated cadavers at department of anatomy , Chulalongkorn Memorial hospital, Bangkok, Thailand
   - Soak 70% alcohol with Batadine® solution on the cadaver's skin
   - Use blade No.23 to dissect the skin into 10x10 cm^2 pieces
   - Wash the skin in sterile 1M NaCl with Batadine® solution

b. Sterilization (Glycerol Treatment) [10]
   - Remove hair and adipose tissue in dermis layer
   - Wash again with 1M NaCl with betadine solution 2-3 times
   - Put in 95% Glycerol in PBS buffer with amphotericin B, 100 iu/ml penicillin,100 µg/ml streptomycin 3-4 weeks at 4°C

c. De-epidermization (NaCl solution) [9]
   - Use forceps to transfer the skin into sterile 1M NaCl at 4°C for 48-96 hours (until epidermis separate from dermis)
   - Use forceps remove epidermis from dermis
   - Wash de-epidermized dermis (hDED) with PBS buffer 2-3 times

d. Remove fat
   - Put hDED into Chloroform : Methanol (2:1 v/v) solution at 20 times of hDED weight to extract fat and stir for 2 hours
   - Wash with distilled water 3-4 times

e. De-celluralization [8]
   - Soak hDED with 0.25% Trypsin with 0.02% sodium azide and stir in magnetic stirrer 400-450 rpm at 4°C for 2-3 hours
   - Enzyme was changed and hDED was stored at 4°C for 1 week

3. The specimens were taken out and immerged into 10% formalin for H&E staining (2 rats/4 specimens), for SEM investigation (2 rats/4 specimens)
4. The rest of the specimens (2 rats/4 specimens) were soaked with Frozen Section Medium (Richard-Allan Scientific), covered with aluminum foil and kept them under -80°C for preparing frozen sections and immunohistochemistry staining

3.5.3 Preparation *in vivo* specimens for H&E staining

1. After immerging the specimens in 10% formalin for 24-48 hours.
2. Then the specimens were preceded in tissue processor (Leica TP-1020) over night.
3. After tissue processing, the specimens were embedded in paraffin by tissue paraffin embedded machine (Leica EG-1150H).
4. Then the specimens were cut 5 μm in thickness with rotary microtome (Leica RM-2265).
5. The sections were H&E stained in automatic tissue stainer (Leica Auto Stainer XL).
6. The slides were examined by a light microscope (Olympus) at 100X, 400X. Fibroblast cell (stained by hematoxylin to bluish purple color) and Collagen fibers (stained by eosin to pink color).
7. The picture were observed and recorded at 4X, 10X, 20X, and 40X under light microscope (Olympus, USA).

3.5.4 Preparation *in vivo* specimens for immunohistrochemistry

1. The specimens were divided into 3 section planes (shown in figure 3.2)
2. Then performed frozen section (thickness 5 μm) in frozen rotary microtome (Leica CM-1880) at -50°C
3. The sections were collect in negative ions slides
4. The sections were fixed with cold acetone (CH_3COCH_3) at -2°C for 10 minutes, then left them to dry at room temperature

3.5 Animal study (*In vivo* study)

We supposed to investigate the cell-materials *in vivo* interaction by using subcutaneous implantation on the back of 4-week-old female Wistar Rat (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 procedure, Act 1986) under aseptic condition. We divided specimens into 2 groups; hADM and hDED and collected the results in 1-week, 2-week and 4-week by using 6 rats each groups (n=6). After removing the specimens, we suppose to investigate cell-material interaction and collagen architecture.

3.5.1 Subcutaneous implantation

1. All the specimens (10x10x1 mm) were lyophilized and sterilized by ethylene oxide treatment (15% ethylene oxide, 85% CO_2, 5.5 atm, 55°C).
2. Dehydrated them with distilled water 24 hours at 4°C before use.
3. 4-week-old female Wistar rats were divided into 3 groups.
4. Rats were injected with Thiopental Sodium (60 mg/kg, 6% solution, Jagsonpal Pharmaceuticals Ltd., Haryane, India) before implantation.
5. After removing hair on the rat's back, make a pocket about 2 cm wide on the dorsum, then subcutaneous implanted 2 specimens each rat (left and right) and the wound was stitched using nylon sutures.
6. Feeding all the rats with normal procedure and specimens were collected after 1-week, 2-week and 4-week.

3.5.2 Collecting specimens

1. After 1-week, 2-week and 4-week implantation, the specimens will be taken out for investigation by injected the rats with overdose Thiopental Sodium (200 mg/kg) until they died.
2. Cut out the skin on the back which consists of the specimens and take a picture of fresh specimens

- Wash hADM with PBS buffer and put into 0.5% SDS in PBS buffer stir in magnetic stirrer for 2-3 hours and then wash again with PBS buffer for 2-3 times

f. Preservation
   - Put hADM in the plastic tray with distilled water, and then put it into Freeze dryer (Alpha 2-4 LSC, Martin Christ, Germany)
   - Frozen at -40°C overnight and then lyophilized for 24 hours

3.4.2 Human De-epidermized dermis protocol

- Following process a.-f. in 3.4.1 except process c.

3.4.3 Histological examination (Hematoxylin & Eosin staining)

The specimens were stained by using routine H&E staining to investigate the orientation and appearance of the eosin-stained collagen fiber bundles within hADM and hDED. By dividing each group with 2 specimens, the specimens were cut into 3 sections (2 edges and 1 center, Figure 3.2) and processed by:

1. Dehydrated sample with increasing series of alcohol concentration by using tissue processor (Leica TP-1020), and then embedded in paraffin cassette in tissue paraffin embedded machine (Leica EG-1150H)
2. Paraffin-embedded specimens were cut at 5 μm thickness with rotary microtome (Leica RM-2265)
3. After removing the paraffin, and stained by automatic tissue stainer (Leica Auto Stainer XL)
4. The slides were examined by a light microscope (Olympus) at 100X, 200X 400X. Fibroblast cell (stained by hematoxylin to bluish purple color) and Collagen fibers (stained by eosin to pink color)
5. The picture were observed and recorded at 4X, 10X, 20X, and 40X under light microscope (Olympus, USA)

5. 3% normal horse-serum was dropped on the specimens and left at room temperature for 20 minutes
6. After that anti-human collagen type I (Chemicon International, USA) was dropped and left at room temperature for 60 minutes
7. Then, the specimens were washed by PBS buffer twice 3 minutes each
8. The secondary polyclonal rabbit anti-mouse immunoglobulins / FITC (DakoCytomation, Dako, Denmark) was dropped and left at room temperature and incubated for 30 minutes
9. The sections were mounted and investigated on Confocal microscope (Figure 3.2)

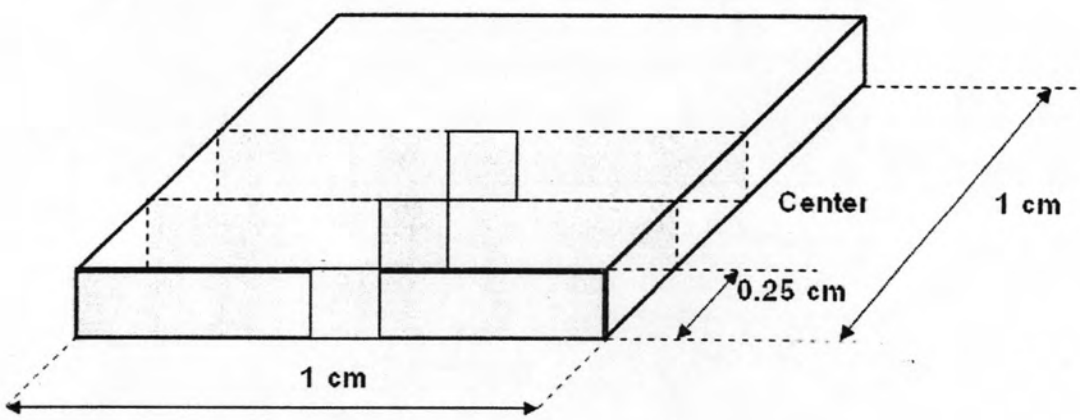

Figure 3.2: The investigation planes of the specimens