CHAPTER 3

RESEARCH METHODOLOGY

Animal model

Six New Zealand White rabbits from the Laboratory Animal House, Faculty of Medicine, Chiang Mai University, were used in this study. The rabbits, weight 2 to 3 kg and approximately 12-17 months of age, were divided into two groups of three rabbits. The first group was used for limbal tissue biopsy and the second group was used for oral mucosal biopsy. The study of each rabbit was summarized in figure 3.1.

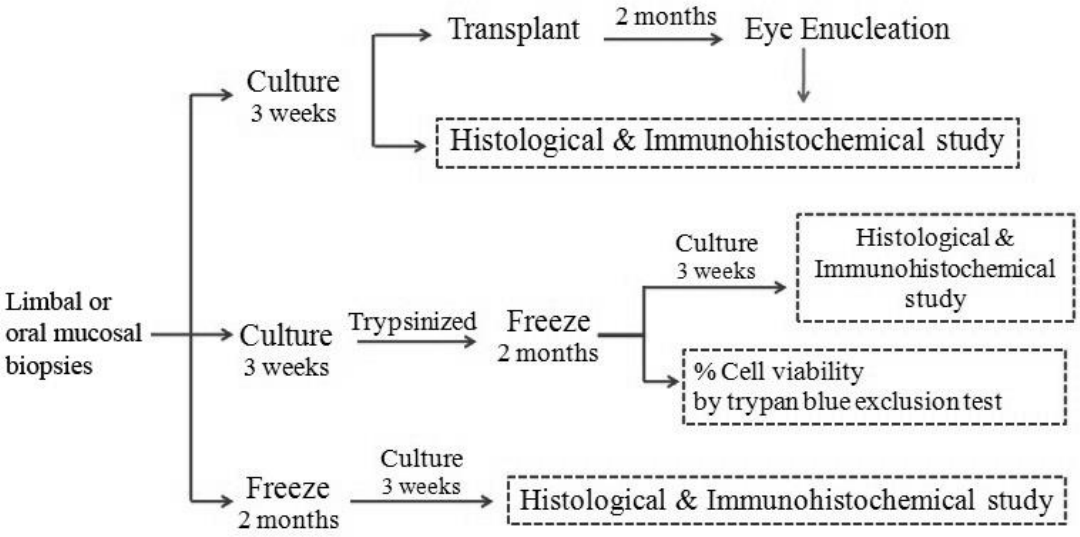

Figure 3.1 The summarization diagram of the experiment.

Preparation of 3T3 mouse fibroblast

Mouse fibroblast 3T3 cell line was purchased from Cell Lines Service (CLS) (Eppelheim, Germany). Mitomycin C (MMC) treated 3T3 mouse fibroblasts were used for co-culture with both limbal and oral mucosal epithelial tissues.

1. 3T3 mouse fibroblasts were incubated with 4 µg/ml MMC for 2 hours at 37ºC to inactivate their proliferative activity.
2. The cells were washed with Dulbecco's Modified Eagles Medium (DMEM) and incubated with 0.25% trypsin in 0.02% Ethylenediaminetetraacetic acid (EDTA) at 37ºC for 5 minutes and then plated onto 6-well culture insert.
3. The cells were allowed to adhere the culture surface for 24 hours.

Preparation of human amniotic membrane

Human amniotic membranes (HAM) were obtained from Department of Ophthalmology, Faculty of Medicine, Chiang Mai University.

1. The cryopreserved HAM was thawed under sterile condition.
2. HAM was washed with versene solution and then incubated in 0.25% trypsin with 0.02% EDTA at 37ºC for 30 minutes to remove the amniotic epithelium.
3. The amniotic epithelial cells were gently scraped using cells scraper (Corning Life Sciences, Tewksbury MA, USA).
4. The denuded HAM was placed on 24 mm culture plate inserts (Corning).
5. The culture insert was placed into 6-well culture plate containing MMC treated 3T3.

Rabbit model of limbal stem cell deficiency

The right eye of the rabbit was used for the experiment.

1. Harvesting of limbal and oral mucosal tissues

   Rabbits were sedated using xylazine hydrochloride 5 mg/ml and ketamine hydrochloride 50 mg/ml. Then, limbal or oral mucosal tissues, 2x4 mm^2 in size, were harvested by an ophthalmologist. The specimens were collected in collecting medium which is a 1:1 mixture of DMEM and Ham's F12 medium containing 10% fetal bovine serum, 50 µg/ml gentamicin, 100 µg/ml streptomycin and 3 µg/ml ampicillin and then transported to the laboratory.

2. Induction of LSCD in rabbit model

   LSCD in rabbit eye was performed by using technique previously described by Avila and colleague with some modification (Avila et al., 2001). A sterile cotton swab soaked with 1N NaOH was applied to the rabbit eye for 30 seconds. Then, the eye was washed many times with 0.9% NaCl. After alkali burns, combined steroid and antibiotic eye drop were given to the rabbit's eye twice daily for 2 weeks. The eye that show corneal opacity, vascularization and epithelial irregularly was considered to have LSCD.

These two processes were done by an ophthalmologist and a veterinarian.

Cultivation of limbal and oral mucosal epithelial cells

1. The excessive tissue was removed with scissors including sclera and conjunctiva for limbal specimens or submucosal connective tissues for oral mucosal specimens.

2. The tissue was placed with the epithelium facing down on denuded amniotic membrane that fastened on 24 mm culture insert.
3. Culture medium composes of DMEM and Ham's F12 (1:1) containing 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 20 µg/ml insulin, 100 µg/ml streptomycin and 3 µg/ml ampicillin. The culture was put in CO_2 incubator at 37ºC, 95% humidity and 5% CO_2. The medium was changed every 2 day and cell growth was observed by inverted microscope.
4. After 14 days of culture or the culture reached confluence, air-lifting method was used by lowering the medium allowing the cells expose to air for 1 week.
5. The cultured epithelial sheet was used for transplantation by an ophthalmologist. Three rabbits were transplanted with cultivated LSC epithelial sheets and other three rabbits were transplanted with cultivated oral mucosal epithelial sheets at 21 days after alkali burn. A part of the epithelial sheet was used for hematoxylin-eosin and immunohistochemical staining for the characterization of the epithelial sheets.
6. Two months after transplantation, the transplanted eyes were enucleated and the epithelial morphology and phenotype of ocular surfaces were studied using hematoxylin-eosin and immunohistochemical staining.

Preparation of paraffin block and sectioning protocol

The samples in this experiment included cultured epithelial sheets from fresh tissue biopsies, cultured epithelial sheets from cryopreserved tissues and cells, and

cornea from all transplanted eyes. Limbal and corneal regions from normal eye and oral mucosal tissue were also used as a normal control.

1. The samples were fixed in 4% paraformaldehyde at 4ºC for 2 hours.
2. Washed with 1X PBS for 5 minutes (2 times).
3. Dehydrated in increasing concentrations of ethanol; 50%, 70%, 80%, 95% and 100% respectively. Each step was done twice for 1 hour each and cleared by xylene for 1 hour (2 times).
4. Infiltrated with xylene : paraffin (2:1), xylene : paraffin (1:1) and paraffin respectively for 45 minutes each and then embedded the tissues in paraffin blocks.
5. Paraffin blocks were sectioned at 5 µm thickness on a microtome and floated on a 40°C water bath containing distilled water.
6. The sections were transferred onto cleaned glass slides and allowed the slides to dry overnight at room temperature.
7. The slides were placed in a 60°C oven for 30 minutes and then they were used in hematoxylin-eosin staining and immunohistochemical staining.

Hematoxylin-eosin staining

1. The slides were deparaffinized in xylene for 5 minutes (2 times).
2. The slides were rehydrated in ethanol; 100%, 95% and 80% respectively. Each step was done twice for 2 minutes each.
3. The slides were immersed in distilled water for 2 minutes and stained in hematoxylin for 10 minutes and then rinsed in running tap water for 5 minutes.

4. The sections were decolorized in acid alcohol 2 dips and rinsed in running tap water for 2 minutes.
5. The sections were immersed in ammonium water 5 dips and rinsed in running tap water for 10 minutes.
6. The slides were counterstained in eosin for 2 minutes and dehydrated in step up concentration of ethanol, 95% 100% respectively. Each step was done twice for 2 minutes each.
7. The sections were cleared in xylene for 2 minutes (2 times) and mounted with mounting medium.
8. The slides were observed under a light microscope.

Immunohistochemical staining

This technique was performed using a Histostain-SP kit (Invitrogen, UK). The method was described below:

1. The sections were deparaffinized in xylene for 5 minutes (2 times).
2. The sections were rehydrated in step down concentration of ethanol; 100%, 95% and 80% respectively. Each step will be done twice for 2 minutes each and immersed in 1X PBS for 10 minutes.
3. Antigen retrieval was performed using a citrate buffer solution (pH 6.0). The slides were placed in the solution and heated using a microwave oven at 600 watt for 15 minutes then cooled down to room temperature.
4. The slides were washed with 1X PBS 5 minutes (3 times).

5. Endogenous peroxidase activity was blocked in peroxidase quenching solution for 30 minutes using the mixture of 1 part of 30% H_2O_2 in 9 part of absolute methanol and washed with 1X PBS 5 minutes (3 times).
6. Nonspecific sites were blocked using serum blocking solution for 20 minutes.
7. Serum blocking solution was drained and incubated with the primary antibodies; p63 (1:100), K3 (1:100) and Cx43 (1:100) for 2 hours and then washed with 1X PBS 5 minutes (3 times).
8. The slides were incubated with biotinylated secondary antibody for 20 minutes and washed with 1X PBS 5 minutes (3 times).
9. The slides were incubated with horseradish peroxidase labeled streptavidin for 10 minutes and washed with 1X PBS 5 minutes (3 times).
10. The slides were incubated with DAB chromogen for 50 seconds and counterstained with hematoxylin for 3 minutes.
11. The slides were rinsed by distilled water and mounted with coverslip using mounting solution then analyzed under inverted microscope.

Cryopreservation by vitrification

Limbal and oral mucosal specimens for cryopreservation from sedated rabbits were collected in artificial cerebrospinal fluid (aCSF) until delivered to the laboratory.

First step of this experiment was to select the suitable CPAs formula. Limbal and buccal specimens were frozen in 3 different formulas of CPAs for 1 week. After that, the tissues were cultured and observed the growth potential. The CPAs formula that showed the highest viability was selected.

The 3 CPAs formulas include:

Formula 1: 10% DMSO, 20% FBS in DMEM

Formula 2: 25% Glycerol, 25% Propylene glycol, 20% FBS in DMEM

Formula 3: 25% DMSO, 25% Propylene glycol, 20% FBS in DMEM

Cryopreservation was done using vitrification method and divided into two parts.

1. Tissues cryopreservation:

   The tissues were frozen immediately after biopsied.

   1.1 Tissues were washed 3 times with the selected CPAs then put into cryotubes containing 0.5 ml CPAs and transferred to canisters.

   1.2 Cryotubes were stored in liquid nitrogen container by directly plunging in liquid nitrogen.

2. Cells cryopreservation:

   2.1 The cultured epithelial sheets from limbal and oral mucosal tissues were trypsinized by 0.25% trypsin and then pipetted into centrifuge tube containing 4.5 ml of DMEM.

   2.2 Cells were centrifuged at 1,200 rpm for 5 minutes.

   2.3 The supernatant was discarded and the cells were resuspended with 0.5 ml of selected CPA.

   2.4 The cell suspension was pipetted into a cryotube and transfer to a canister.

   2.5 Cryotube was stored in liquid nitrogen container by directly plunging in liquid nitrogen.

Both tissues and cells were frozen for 2 months. After that, the tissues and cells were thawed and cultured by the following protocol.

1. Cryotube was rapidly thawed at 40ºC for 1 min.
2. In case of cryopreserved limbal or oral mucosal biopsies, the tissue in a cryotube was placed into a culture dish and washed 3 times with DMEM. The excessive tissues were removed under stereo-microscope and then co-cultured with 3T3 cells on a denuded amniotic membrane in culture inserts.
3. In case of cryopreserved cells, the cells in a cryotube were poured into a centrifuge tube containing 4.5 ml of DMEM and mixed together. A small volume of the cell suspension was used for determining the cell viability by trypan blue exclusion test. The rest of cells were centrifuged at 1,200 rpm for 5 minutes. The supernatant was discarded and the pellet was dissolved in DMEM. The cell suspension was cultured by seeding onto a denuded amniotic membrane with the present of 3T3 cells.
4. After 3 weeks of culture, the epithelial sheets were processed for hematoxylin-eosin and immunohistochemical staining for the examination of the cell layers and the characteristic of the stem cell and epithelial cell markers.