CHAPTER III

MATERIALS AND METHODS

Specimens

In vitro studies

Twenty ml of heparinized human umbilical cord blood per sample were collected from 15 full – term healthy pregnant women with informed consent. Twenty ml of heparinized sheep whole blood collected from PB were obtained from the Faculty of Veterinary, Chulalongkorn University and the National Institute of Animal.

In vivo studies

Twenty ml of heparinized whole blood from PB of the recipient lamb who received $CD34^+$ human donor HSC after in utero transplantation was drawn from the external jugular vein of the lamb.

Materials

1. Autoclave tape (3M, USA)
2. Aluminum foil (Diamond ®, USA)
3. Coplin staining jar (Vertical staining jar, Wheaton Product No.900620)
4. Diamond – tipped scribe
5. Disposable gloves
6. Forceps
7. Glass coverslips:22 ×22 mm, 22 ×30mm (MENZTEL GLASER ®,Germany)
8. Glass pipettes : 1ml, 5 ml, 10 ml (Witeg , Germany)
9. Graduated cylinder : 250, 500, 1,000 ml
10. Humidified chamber
11. Laboratory sealing film (Whatman ®,USA)
12. Microscope slide box
13. Needle (Vacutainer System Precision Glide ™ ,UK)
14. Needle for animal (18 G for adult animals, 20G for lambs)
15. Pasture pipettes 20, 200, 1,000 µl

16. Polystyrene conical centrifuge tube : 15, 50 ml (FALCON ®,USA)
17. Reagent bottles :250, 500 ml (Duran ®, Germany)
18. Slide (Super Frost , Germany)
19. Slide film (Eritchome 400 ,Kodak)
20. Syringe 60 ml
21. Test tube rack
22. Timer

Equipment

1. Autoclave (All American)
2. Biohazard laminar flow hood (FASTER BHA 48,Italy)
3. Computer assisted Flow Cytometer analysis (Macintosh computer -system)
4. Dark room
5. 3Dual – bandpass (DAPI/TRITC) and single bandpass filters (Vysis Inc., Framingham,MA,USA)
6. Fluorescence microscope equipped with an appropriate filter set and camera (Olympus , BX 50, Japan), Andrology unit, Department Obstetrics and Gynecology, Faculty of medicine, Chulalongkorn University
7. Flow Cytometer : FACSCalibur ™, Part number 34012420
   : FACSort ™, Part number 34011590 (Becton Dickinson , USA)
8. HyBrite ™ instrument (Vysis Inc., Framingham,MA,USA)
9. Light microscope (Olympus, Japan)
10. Liquid nitrogen tank
11. Low – speed centrifuge (Kokusan, Japan)
12. Microlitre pipette : 1 – 20 , 50 – 1,000 μl and sterile tips (Eppendrof)
13. pH – meter (Orion 420, 2 point calibration)
14. Printer (Hewlett Packard DeskJet 1600 CM)
15. Refrigerator : 4 °C , -20 °C (Sharp)
16. Software Requirement for Flow Cytometry analysis
    - Macintosh system software, version 8.6
    - Cell Quest software, version1.0 or later for acquision and analysis

- FACSComp software, version 1.0 or later for instrument set up
- FACSConvert software, version 1.0 or later for generate data will be analyzed

17. Ultrasound machine (Aloka SSD 2000)

Reagents

1. General reagents
   - 1.1 Absolute ethanol
   - 1.2 Acetic acid (Merk, Germany)
   - 1.3 Bovine serum albumin
   - 1.4 Clorox (5%Sodium hypochlorite)
   - 1.5 Distill water
   - 1.6 Dulbecco's medium
   - 1.7 DMSO
   - 1.8 Fetal calf serum (Gibco BRL, Germany)
   - 1.9 Histopaque®-1077 (Sigma ,USA)
   - 1.10 Heparin (LEO)
   - 1.11 Hydrochloric acid (Merk, Germany)
   - 1.12 Percoll solution (Sigma,Germany)
   - 1.13 Potassium chloride (Merk, Germany)
   - 1.14 Potassium dihydrogen phosphate (Merk, Germany)
   - 1.15 Sodium chloride (Merk, Germany)
   - 1.16 Sodium dihydrogen phosphate anhydrous (Merk, Germany)
   - 1.17 Sodium hydro xide(Merk, Germany)
   - 1.18 Tryphan blue dye

2. Reagents of Flow Cytometry
   - 2.1 Bovine serum albumin (BSA)
   - 2.2 CD 45 PerCP conjugated monoclonal antibodies
   - 2.3 Clorox (5%Sodium hypochlorite)
   - 2.4 1% Paraformaldehyde

2.5 PBS (Phosphate buffer saline)

2.6 Sheath fluid(Haema -Line® 2(Serono beaker diagnotics) sigma , USA)

3. Reagents of CD43 + Progenitor donor cell isolation

3.1 CD34 + Multi Kit (microbeads)

3.2 Reagent Cryopreservation medium

3.3 FcR Blocking

3.4 Hank Buffered salt solution (HBSS) (Gibco BRL)

3.5 Mini/Midi MACS starting kit (Magnetic cell separation units (MACS)

4. Reagent of FISH analysis

4.1 CEP® 16 satellite spectrum orange TM DNA probe kit

4.2 4,6-diamidino-2-phenylindole (DAPI ; Sigma,USA)

4.3 1,4-diazobicyclo [2,2,2] octane (DABCO: Sigma , USA)

4.4 Ethanol series solution (70%, 90% and 100%)

4.5 Formamide (Unilab, Australia)

4.6 Glycerol (Mallinckrodt, USA)

4.7 Immersion oil

4.8 0.02% pepsin in o.01 NHCL

4.9 Rnase, Dnase free (Boehringer Mannheim)

4.10 Nail varnish : colorless acetone-base solvent

4.12 20 × SSC (Sodium chloride , trisodium citrate;MerkGermany)

Methods

In vitro Study of mixing experiment

1. Collection of blood sample
   1.1 Human UCB donor samples used in this study were 20 ml of blood samples in heparinized tubes from full term pregnant women with informed consent at King Chulalongkorn Memorial Hospital during the years of 2000-2002. The required screening tests included VDRL, HBsAg and anti-HIV.
   1.2 Sheep blood samples (recipient) were collected for 20 ml in heparinized tubes from the external jugular vein of normal adult sheep at the Faculty of Veterinary, Chulalongkorn University and the National Institute of Animal.

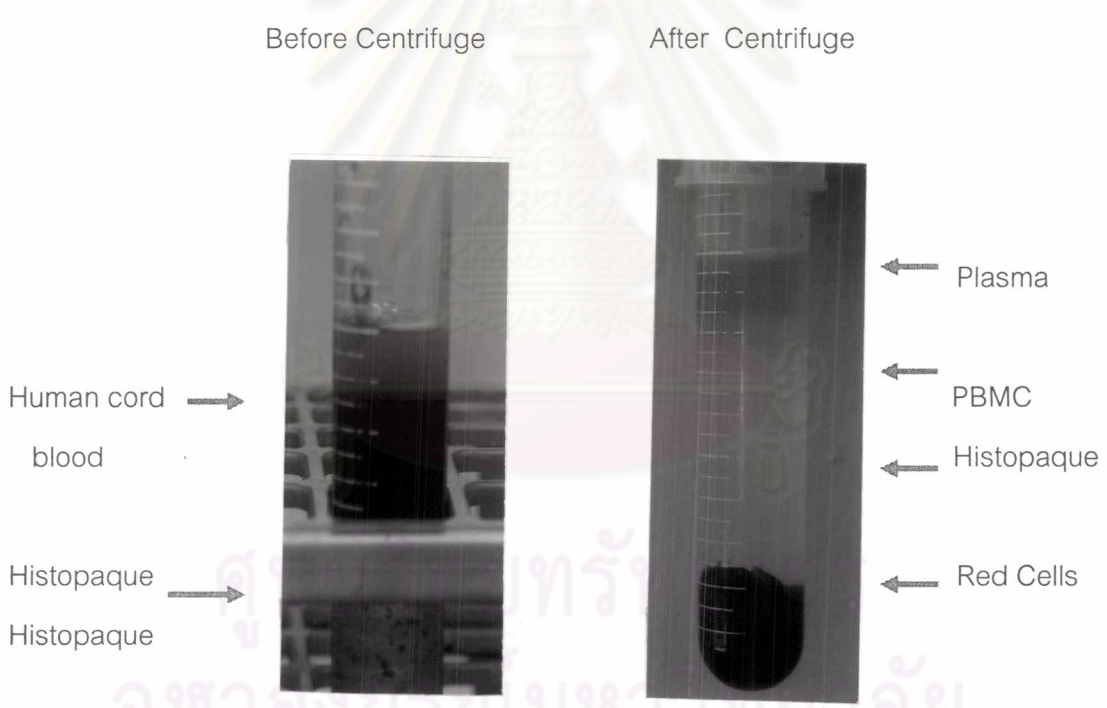
2. Human lymphocyte preparation
   2.1 Each 15 ml polypropylene centrifuge tube contained 5 ml histopaque(Ficoll/hypaque)1077 at room temperature.
   2.2 Human umbilical cord blood 9 ml was carefully transferred over on histopaque in each tube taking care not to mix these two parts together (human UCB and histopaque). The cap of the tube was tightly placed.
   2.3 Centrifuged tube at 1,500 rpm for 30 minutes at room temperature.
   2.4 Removed the top plasma layer (yellow band of the platelet) with a pipette. Discarded this layer into a container of Clorox.
   2.5 Used a swirling with the pipette, gently collected the next layer buffy coat (white band of peripheral mononuclear cell: PBMC) from each tube. The next layer was the residual of histopaque so that tried not to collect of this layer included the red cell layer which sits at the bottom of the tube (See attach diagram)
   2.6 Immediately transferred each PBMC band to a new polypropylene 5 ml centrifuge tube. Added PBS 3 ml to each tube for washing and

centrifuged 1,500 rpm for 10 minutes at room temperature.

2.7 Removed supernatant and kept pellets after centrifuged. Added an Additional 1 ml PBS to this last tube and mixed cells by vortex. Repeated steps 2.6-2.7 until the pellet is white.

2.8 Put aliquot cell suspension on the counting chamber of hemocyto -meter and counted the cells. Calculated the total cell counts for mixing experiment

2.9 Remained human lymphocyte was cryopreserved in freezing medium and kept in liquid nitrogen.



3. Sheep lymphocyte preparation

3.1 Collected sheep blood 20 ml into a syringe containing heparin which was anticoagulant and mixed well by inverting the syringe several time.

3.2 Collected blood was transferred into a centrifuge tube and centifuged at 1750 g at room temperature for 15 minutes to separate the buffy coat at the interphase between serum and erythocytes.

3.3 Collected the buffy coat from 20 ml. sheep blood using a pipette to minimize sticking of the cells. The buffy coat should be diluted to a final volume of 18 ml in PBS.

3.4 Mixed well to create a single cell suspension or granulocytes which remained at the interphase of the following gradient.

3.5 Placed 3 ml of 60%Percoll solution (sigma,USA)in 15 ml conical tube. Carefully overlaid with 9 ml of the buffy coat suspension , taking care not to disrupt the discontinuous gradient.

3.6 Centrifuged the gradients at 1750 rpm at room temperature for 30 min and avoided using a brake to stop the rotor.

3.7 There should be four distinct layers visible after centrifugation erythocytes and granulocytes (bottom), the layer of the percoll and then lymphocytes, monocytes and platelets forming a discrete white band at the interphase between the Percoll and PBS.

3.8 Used a pipette to collecte cells at the interphase , resuspended the cells in PBS and washed twice at 1500 g for 10 min until supernatant became clear.

3.9 Put an aliquot cells suspension on the counting chamber of hemocytometer and counted the cells . Calculated the total cell counts for mixing experiment.

3.10 Remained sheep lymphocytes was cryopreserved in freezing medium and kept in liquid nitrogen.

4. Mixing experiment between human – sheep lymphocytes

After calculated the total cell count of human and sheep lymphocytes, established a dilution series between human lymphocytes(donor) and sheep lymphocytes (recipient) in 5 ratio 1:100,1:500, 1:1,000, 1:5,000 and 1:10,000 for detecting of 0.01% to 1% of donor cells by Flow Cytometry and FISH analysis.

5. Flow Cytometry analysis

FACSCalibur ™ (Becton Dickison, Thailand) were used in Flow Cytometric analysis. The absolute number of lymphocytes were analyzed range between 5,000 to 10,000 cells for each sample of mixing experiment between human and sheep lymphocytes to determine the sensitivity of human cell concentrations in chimerism detection. Lymphocytes were gated on light scatter characteristics in combination with CD 45 expression.

5.1 Direct staining cells for Flow Cytometry analysis

5.1.1 Pipetted 10 μl of human CD 45 PerCP monoclonal antibody into each 5 ml tube for the total of 5 tubes.

5.1.2 Added the mixing of human – sheep lymphocyte 5 ratio into each tube (step 5.1.1). Then these tubes were incubated for 30 minutes in dark room at room temperature for single cell.

5.1.3 Centrifuged 10 minutes at 1,500 rpm, then removed supernatant and resuspended pellet with PBS 2 ml.

5.1.4 Repeated step 3 again but not resuspended pellet with PBS.

5.1.5 Fixed the stained cells by addition of 500 μl 1% paraformaldehyde in each tube and incubated for 30 min. in dark room at room temperature. Then, human lymphocytes (CD45) were measured by Flow Cytometric analysis.

5.2 Fluorescence activated cell sorter (FACS)

Cells were analyzed by using the cell sorter FACStar Plus ™ (Becton Dickinson, Thailand)

5.2.1 New 50 ml propylene tubes were precoated with 4% bovine serum albumin or fetal calf serum to collect sort cells.

5.2.2 Placed the propylene tubes on ice / refrigerator for at least 1 hour before using.

5.2.3 Installed the collection tubes with 4% bovine serum albumin – coated into the instrument before sorting cells.

5.2.4 Setting a sort gate CD 45 (human lymphocytes).

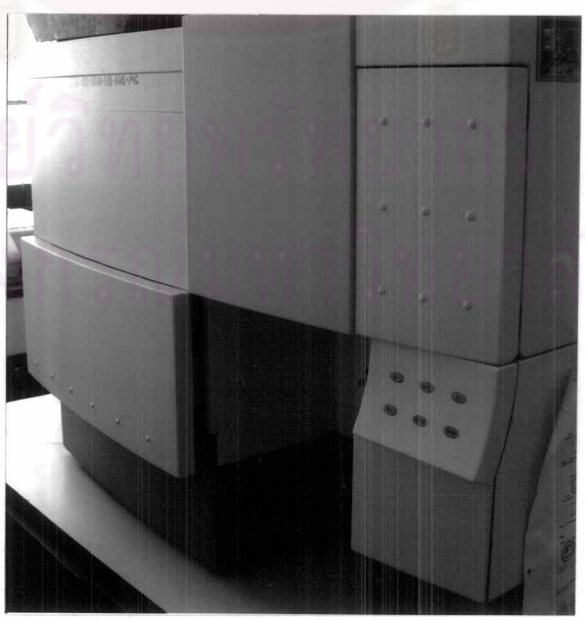
5.2.5 Set the sort count that determined a minimal sort 30,000 cells.

5.2.6 Selected the sort mode

5.2.7 One part of the sorted cells were reanalyzed by Flow Cytometry to verify quality.

5.2.8 After sorting cells, removed carefully the collection tubes from the instrument.

5.2.9 Softly spun the collection tubes at 300 rpm for 10 min because the sorted sample was diluted. Therefore, it should make to concentrate before required for further rapid FISH analysis.



Flow Cytometry :FACSCalibur

6. Chromosome specific DNA probes for FISH analysis

The DNA probe used in this study recognized the satellite DNA of the centromeric region of the human chromosome16(16q11.2, locusD16Z3).This probe could detect chromosome 16 which labeled with fluorescent CEP 16 spectrum orange.

7. FISH analysis

7.1 Slide preparation

Each mixture of human- sheep lymphocytes different concentrations was smeared on a clean glass slide at room temperature and allowed to air – dry. Then fixation of slides were used to preserve morphology by methanol / acetic fixative agent. One set of slides for each FISH procedure were consisted of 5 slides of mixed human – sheep lymphocytes and one slide of human lymphocytes control.

7.2 Pretreatment of material on the slides

7.2.1 All slides were baked at 65 ^{o}C (Program # 5 of Hybrite) for 3 hours.

7.2.2 Treated each slide with 0.02% pepsin in 0.01NHCL at 37^{o}C for 5 min.

7.2.3 Washed each slide in deionized water 2 times, for 5 min.

7.2.4 Treated each slide with RNase in moist chamber at 37^{o}C for 30 min. This treatment served to remove endogenuous RNA and improved the signal to noise ratio in hybridizations with DNA target.

7.2.5 Washed each slide in deionized water 2 times, for 5 min.

7.2.6 Dehydrated each slide through an ethanol series (70%,90%,100%) for one minute and air dry.

7.3 Probe preparation

This procedure was prepared in the dark room, while the slides were dehydrated through the ethanol series.

Preparation of CEP 16 probe mixed the following in an eppendrof tube

Component	Volume / 5 slides
Deionoized water	29 μl
CEP 16 (Hybridized buffer)	21.4 μl
CEP16 Probe	0.5 μl
Total	50.9 μl (10 μl/ 1 reaction)

The mixture probe was vortexed 3 seconds. Then put on microcentrifuge at room temperature for short time (1-3 seconds).

7.4 Setting the Hybrite Parameter.

Moistened two strips for a towel water and placed through along the heating surface of the HyBrite unit. And then turned on the HyBrite for choose program #1 the melt temperature to 75 °C for 5 minutes. Set the hybridization temperature to 37 °C for 16-18 hours (overnight).

7.5 Simultaneous Hybridization

7.5.1 Applied 10 μl mixture probe to target site on the slide.

7.5.2 Added glass coverslip (22×22 mm) , sealed the edge with rubber cement and mark area by diamond – tipped scribe.

7.5.3 Placed the specimen slides on the HyBrite surface

7.5.4 Closed the lid of the HyBrite and start the Hyb / melt program. The program would well begin the melt phase of the program and carry on the process through the 16-18 hours hybridization.

7.6 Post – Hybridization washing

7.6.1 Removed the rubber cement and cover slip from the slides

7.6.2 Immersed slides in three changes of 0.1 × SSC at 60 °C water bath. Each jar was contained 50 ml 0.1 × SSC.

7.6.3 Dehydrated through an ethanol series (70%,90%, 100%) for each one minute and followed air dry in dark room at room temperature.

7.7 Mounting Slides

After air dry , mounted the slides with applying 10 μl of DAPII counterstain (Vysis Inc., Framingham,MA,USA) or antifade medium to the target area of the slides. Placing a glass coverslip over on the slides should avoid the air bubble. Then sealed the slides with nail varnish and stored in the dark prior to signal examination.

7.8 Visualization and photography

FISH signals were analyzed with the epifluorescence microscope (Olympus BX 50, Japan), equipped with an appropriate filter sets: single band pass filter for DAPI II SpectrumAqua ™ ,SpectrumGreen ™,SpectrumOrange ™ (DAPI, Aqua,FITC and Texas red respectively) and a triple band pass filter (DAPI/FITC/TRICT). Selected a minimum of 15 good quality , complete human and sheep lymphocytes spreads with well defined, non overlapping for cell enumeration and analysis.

The CEP 16 signal would be visible as a distinct fluorescent signal located near the centromere region of the chromosome 16 visualized of spectrum orange by dual band pass filters (Vysis Inc.,Framingham,MA,USA). Human chromosome 16 was recognized by an orange fluorescent spot in nucleus. The slide was stained and counted for the presence of human lymphocyte. The photography of fluorescence microscopy was recorded by the Kodak Ekatachrome 400 slide film for FISH analysis.

In vivo study of human-sheep chimeric in utero transplantation

1. Creation of human – sheep chimeras

To perform in utero transplantation study of CD 34 $^{+}$ HSC from HCB into fetal sheep, preimmune fetal sheep at 48 to 54 days gestation (term 145 days) was transplnated with hematopoietic cells derived from CD 34 enrichment of human UCB by an intraperitoneal injection under ultrasound guidance. This study was approved by our institutional within committee. A series of at two ultrasound guided, intraperitoneal transplant was performed spared 1 week apart.

2. Preparation of cord blood cells

2.1 Human UCB collection procedure was described as shown in methods of collection of blood sampling.

2.2 After the collection, the bag was weighed and a small sample of blood was set aside for further assessment of complete blood counts, cell viability and microbiological test (aerobic and anaerobic cultures).

2.3 Diluted cord blood containing ACD anticoagulant 1:4 with PBS containing 2 mM. EDTA and carefully layered 35 ml of diluted cell suspension over 15 ml of Ficoll-hypaque.

2.4 Centrifuged for 35 minutes at 400 g, 20^{o}C in a swinging bucket rotor (without brake).

2.5 Aspirate the upper layer, leaving the mononuclear cell layer undisturbed at the interphase.

2.6 Carefully collected interphase cells and washed twice in Iscove's Modified Dulbecco's medium supplemented with fetal calf serum (IMDM) /2%FCS).Then centrifuged for 10 min at 200g, 20^{o}C.

2.7 Resuspended cell pellets in a final volume of 300 μl buffer medium per 10^{8} total cells. For less than 10^{8} total cells, used 300 μl

3. CD 34 progenitor cell isolation strategy

The isolation of HSC was performed by positive selection of CD 34 expressing cells were assayed by immunomagnetic beads. NormallyHSC presented at a frequency of about 0.1% -0.5% in cord blood. For immunomagnetic beads separation,this procedure was done at the National Blood centre,Thai Red Cross Society,described briefly as the following. CD 34 HSC were magnetically labeled with a conjugated primary monoclonal antibody coupled to beads. The magnetically labeled cells were enriched on positive selection columns in the magnetic field.

3.1 Magnetic CD 34 monoclonal antibody was done by placed 100 μl FcR Blocking Reagent / 10^8 total cells in column.

3.2 Added CD 34 100 μl microbeads / 10^8 total cells in column. Mixed well to create suspension for each 5 minute in refrigerator.

3.3 Washed and resuspended cells with buffer. The column should be adjusted to a final volume of 1 ml.

3.4 Before magnetic separation, the column was prewashed with buffer medium.

3.5 Placed cells into pre- separation filter to remove clump. Before use, filter should be washed with buffer.

3.6 Placed cells into column and washed 3 times with buffer, adjusted a final volumn of sample 400 μl, aliquot was taken for phenotypic assessment assay to monitor loss of progenitor cells and bacteria fungal cultures.

3.7 Placed remained cells in freezing medium and kept in liquid nitrogen.

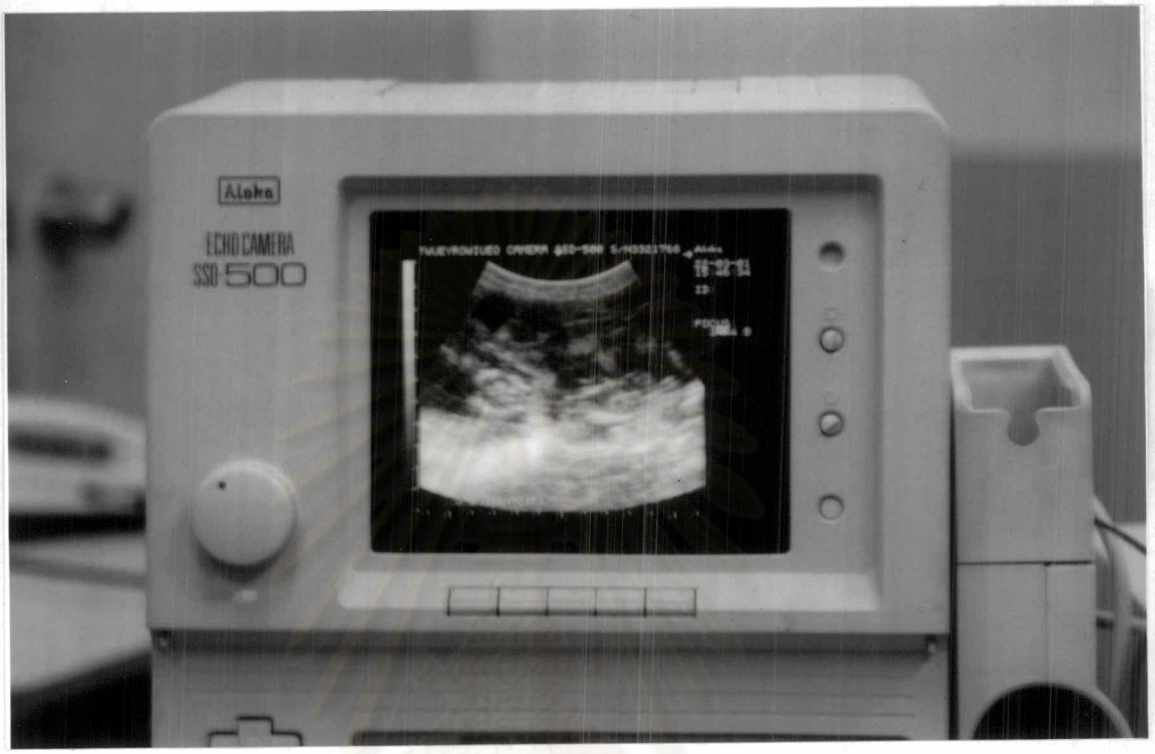
4. Human – sheep model transplantation procedure

4.1 The recipients were sheep fetuses of timed pregnant sheep that was acclimatized for 7 days before transplantation at the Faculty of Veterinary, Chulalongkorn University.

4.2 The volume of 1 ml CD34 + donor cell was injected into peritoneal cavity with a 22-gage 3.5 inch (9 cm) spinal needle under real time

ultrasound guidance.

4.3 Two ultrasound guided intraperitoneal transplants were done spared 1 week apart.



Ultrasound machine (Aloka SSD 2000) Department Obstertrics and Gynecology, Faculty of Medicine, Chulalongkorn University.

Cleaning of abdominal sheep pregnancy before in utero transplantation.

Ultrasound guided intraperitoneal transplantation

Following the transplants, no special monitoring was required until the age of fetal viability at which time the fetus should be followed by serial ultrasound to assess growth and look of evidence of possible GVHD. At birth, signs of cutaneous or visualized GVHD would be recovered.

5. Detection of donor cell engraftment

The presence of human cells in newborn lamb PB was analyzed after birth. Lamb was analyzed at 10 days and 1 month by a combination methods including Flow Cytometric analysis and fluorescence *in situ* hybridization (FISH) as described previously (see method of Flow Cytometry and FISH analysis).

Statistical analysis

1. The number of human lymphocytes in mixed dilutions between human and sheep lymphocytes measured by both methods (Flow Cytometry and FISH analysis) were compared using paired – T test from the Statistical Packages for the Social Program (SPSS). The P value of less than 0.05 was considered to be statistically significant.

2. The comparison between the detection of donor cells (human lymphocytes) taken from chimeric lambs at the time point 10 days and 1 month after birth were determined by a unpaired- t test from SPSS. The P value of less than 0.05 was considered to be statistically significant.

3. The coefficients of variation (CV) was calculated according to the following formula. That was shown below :

$$\% CV = SD / \bar{x} \times 100$$

$$\bar{x} = \sum x_i / n$$

$$SD = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$