CHAPTER III

MATERIALS AND METHODS

CHEMICAL SUBSTANCES

Aloe powder [Lipo Chemical(USA.)] 99.95%
Fluorescein isothiocyanate-labeled dextran, MW. 150,000
(FITC-dx-150) (Sigma Chemical Co.)
Fluorescein marker acridine orange (Sigma Chemical Co.)
Heparin
Sodium pentobarbital

EXPERIMENTAL ANIMALS

Wistar Furth rats were supplied by the National Laboratory Animal Center of Mahidol University Salaya Campus. A total of 72 adult male Wistar Furth rats weighing 200-250 grams were used in this study. The animals were housed five per cage in stainless-steel bottom cages. They were kept in a well-ventilated room in which the temperature was 28-32 °C with an automatic lighting schedule, which provided darkness from 7.00 PM to 6.00 AM. All animals were allowed access of food (Purina Laboratory Chow, Premium Quality Feed, Zuelig Gold Coin Mills Pte., Singapore) and tap water. All animals were accustomed to daily handing for at least 5 days before experimentation.

ANIMAL PREPARATIONS

In this study the animals were equally divided into four groups as follows:-

1. Control group (CON)

The animals were anesthetized by intraperitoneal administration of 60mg/kg BW. of sodium pentobarbital. As a sham group, the back of animal between the lower part of both scapulas was shaved and depilated without any treatments. Then these animals were housed with free access to water and standard laboratory chow until the day of performing chamber implant experiment.

2. Burn wound group (BURN)

The animals were anesthetized by intraperitoneal administration of 60 mg/kg BW. of sodium pentobarbital. After shaving and depilating, the animals in this group were produced the partial thickness burn injury, called second degree burn. By using the model of Zawacki (1974), the hot plate of 3.5×4.6 cm^2 (in size) with temperature maintained at 75 °C was put on the prepared area for 10 seconds. This burned area was equal to 10 percent of total body surface area. This group did not receive any treatments. Then these animals were housed with free access to water and standard laboratory chow until the day of performing chamber implant experiment.

3. Burn wound with normal saline treatment group (BURN-NSS)

In order to make the second degree burn, the same protocol was performed as the burn wound group. But after removal of the hot plate, the

animals were immediately applied topically with one ml of normal saline (NSS). The same amount of NSS was applied to this group once a day until the day of performing chamber implant experiment.

4. Burn wound with Aloe vera treatment group (BURN-ALOE)

In order to make the second degree burn, the same protocol was performed as the burn wound group. But after the removal of the hot plate, the animals were then immediately applied topically with Aloe vera gel powder at dose of 300 mg/kg BW. in sterile water (10 ml/kg BW.) (Davis et al., 1990). Aloe vera used in this study was "Lyophilized Aloe Vera Gel", prepared by the Lipo Chemical Co., USA. Lyophilization was a process for the preparation of dried Aloe vera gel with unchanged properties (Saizukk, 1991., Prayoonrak, 1995., Meadows, 1983). The same amount of topical aloe gel powder was applied to this group once a day until the day of performing chamber implant experiment.

The animals in each group were then equally subdivided into 3 subgroups for the study on the 3, 7 and 14 pastburn days.

METHODS

On the day of experiment, before the examination of dermal microvascular changes, the burned area of each animal was taken picture again in order to compare with previous image for further evaluation of healing area.

MEASUREMENT OF BURN-WOUND AREA

The digital camera (Olympus) with a constant working distance was used to take a photograph of burned area of each animal. The digital datafile

of burned area was taken immediately after burning and also on the day of each experiments (3, 7 and 14 days).

The digital datafile of each burned area was then defined for its area by using software called "Vascular Measuring Tool" supported by Deparment of Computer Engineering, Faculty of Engineering, Chulalongkorn University.

TISSUE PERFUSION MEASUREMENT

The tissue perfusion was measured using a Laser Doppler Flowmetry with the fiber optic needle probe (wavelength 780 nm) (Model ALF 21, Advance Co. Ltd., Japan). The needle probe was fixed perpendicularly to and above the skin about 1 mm (Figure 3.2). Five different measurements (at the center and four corners) were performed at each time and the mean was used for calculation.

Principles of Laser Doppler Flowmetry

Laser Doppler Flowmetry (LDF) is an established technique for the real-time measurement of microvascular red blood cell (or erythrocyte) perfusion in tissue. Perfusion is sometimes also referred to as microvascular blood flow or red blood cell flux.

LDF works by illuminating the tissue under observation with low power laser light from a probe containing optic fibre light guides. Laser light from one fibre is scattered within the tissue and some is scattered back to the probe. Another optical fibre collects the backscattered light from the tissue and returns it to the motor (Figure 3.1).

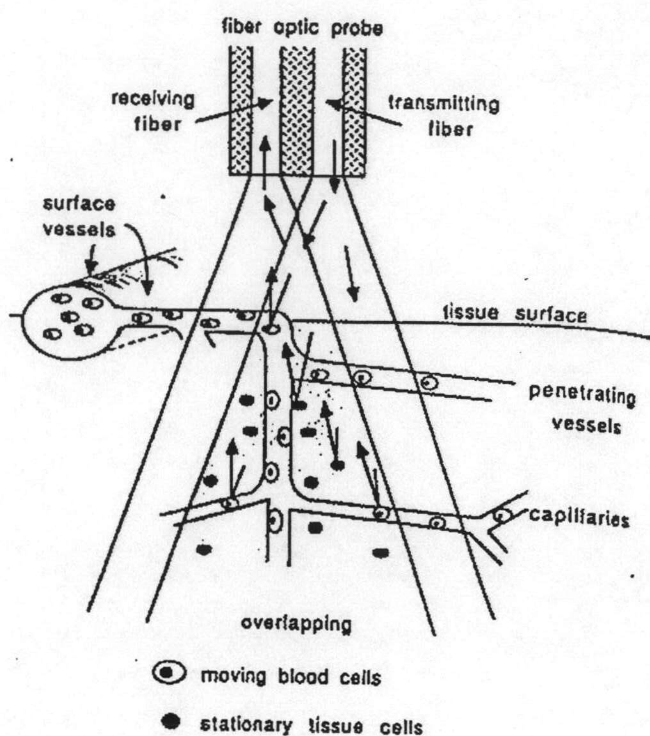


Figure 3.1 *Schematic diagram of Laser Doppler probe, skin surface, and skin microcirculation.*

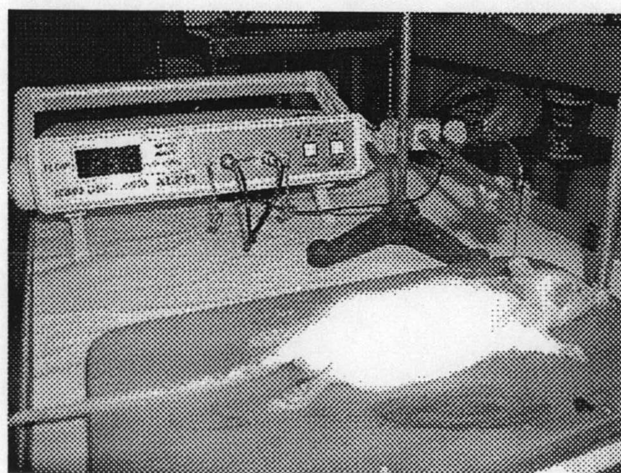


Figure 3.2 *The method of measuring the tissue perfusion in the burn wound rat.*

INTRAVITAL MICROSCOPIC STUDY

To examine the dermal microvascular changes, dorsal skinfold chamber preparation (Papenfuss et al., 1979;Endrich et al., 1980;Lehr et al., 1993) and intravital microscopic technique (Menger and Lehr, 1993) were performed on day 3, 7 and 14 of experimental period . The procedures of animal preparation were shown in Figure 3.4.

The dorsal skinfold chamber is a chamber frame weighing 3.2 g shown in Figure 3.3 designed by Workshop of the Institute for Surgical Research, Munich, Germany. The identical base plate (part a) is made of a 0.64 mm thick aluminum flat sheet. The round bottom part of the plate called the shoulder is bent by 45 °C. The shoulder is used to prevent the implanted chamber from tilting aside together with the rat's dorsal skinfold. The three big holes (part b) in the shoulder reduce the chamber weight. The one cm diameter glass window was pressed against the inner conical shoulder of the collar called the retaining ring.

On the day of dorsal skinfold chamber implant experiment, the animals were anesthetized by intraperitoneal administration of 60 mg/kg of sodium pentobarbital. A constant level of anesthesia was maintained through the experiment by intraperitoneal injection of supplement dose (10 mg/kg BW) of the anesthetic agent every 30-45minutes (Koller and Kaley, 1990). Then the picture of burned area of each animal was taken by using digital camera.

The surgical procedure was done by placing the animal on the surgical stage. The back of each animal was drawn a circular outlining of approximately 15 mm in diameter. This circular area was cut by scissors, thus producing skin flap. Then the skin of the dorsum was gently pulled from the back by sutures strung between the edge of the skinfold and two stands. The

skin flap was then removed using a scalpel with an effort to follow the hypodermis superior to the fascia. The area was then trimmed and manicured with a pair of fine forceps and iris scissors. All but two of the fascia layers were removed from one side of the skinfold, and the remaining layer, consisting of epidermis, subcutaneous tissue and the striated muscle.

During the surgery, the area was kept by allowing drops of the warmed normal saline to fall on the incision. The body temperature of the animals was kept constant at 36-37 °C by means of heating pad.

After completion of the surgery, the tissue was covered by a microcover glass incorporated with one half of the aluminum frame. The bolts were put at the tree holes in the shoulder of chamber to make the skin protrude and then the skin was cut to make a hole. Then the bolts were introduced through the hole. During the implanted chamber procedure, meticulous care was taken to prevent microhemorrhages into the chamber tissue. The other side of the chamber was inserted for matching and the mechanical connection of the two chambers was fixed by bolts and spacers. Consequently, two aluminum frames were implanted so as to sandwich the extended double layer of the skin.

Under spontaneous respiration, the trachea was cannulated to facilitate respiration. Then a fine polyethylene catheter (PE 10, inner diameter of 0.28 mm) was inserted into the right jugular vein for injection of fluorescent-labeled macromolecule or fluorescent-labeled leukocyte into the blood stream.

After completion of the implantation, the dermal microvascular changed was studied with intravital fluorescence microscopy were showed in figure 3.4. The animal was placed on the microscope stage of fluorescent

microscope equipped with transillumination and epiilumination optics (Nikon Optiphot-2). After intravenous application of fluorescence, epiillumination was achieved with a 50 W, mercury lamp with a 488 nm attached to excitation filter and 515 emission barrier filter. An intravital microscope with a ×20 long working distance objective (CF Achromat) and eyepiece, ×10 and ×20 , were used to observe microvessels in the chamber. A video camera mounted on the microscope projected the image onto a black-white monitor. The images of microvessels were stored on videotape (Sony, SLV-×311) for playback analysis using a videocassette recorder. A videotape was connected to a video timer (UTG 33) for real-time record. During the experiment, microvessel images could be also printed by using video printer (Sony, Video Graphic Printer UP-890 CE).

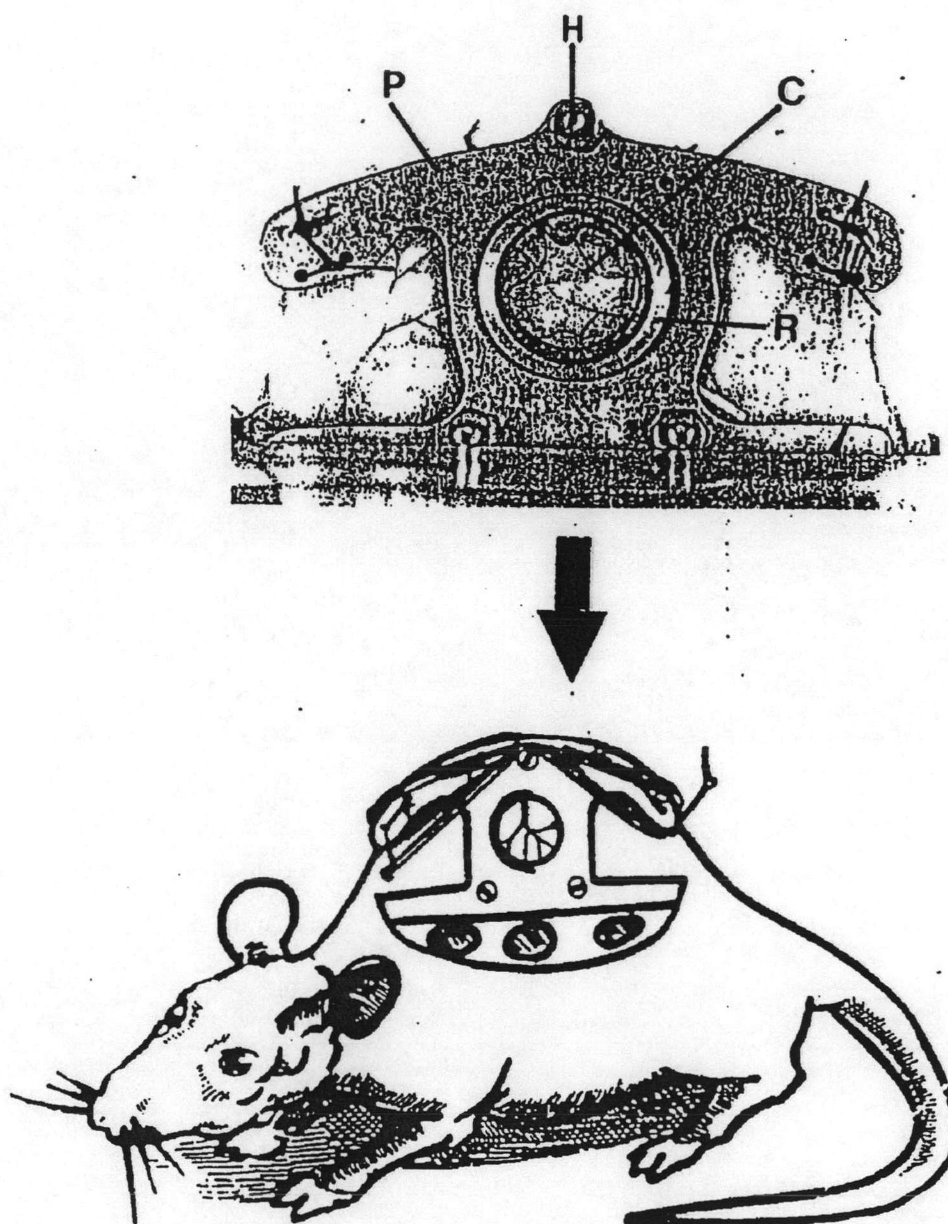


Figure 3.3 *Photograph of aluminum chamber which was inserted into the dorsal skinfold :P= plate, H= hole, C= cover glass, R=retaining ring*

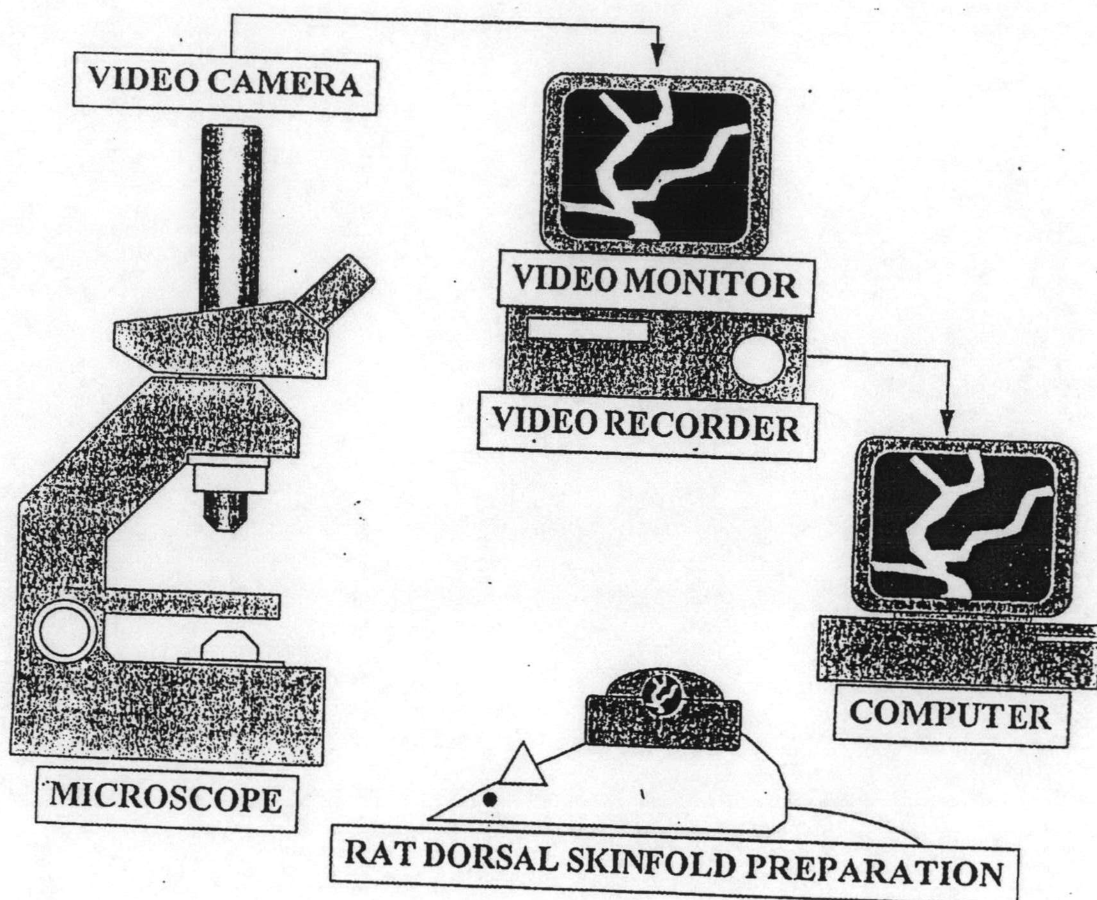


Figure 3.4 *Intravital fluorescence microscopy and instruments used for quantitative studies of hemodynamic and morphologic microvasculature.*

Measurement of arteriolar diameter

To measure diameter of arterioles, a fluorescent plasma marker (0.2 ml of FITC-labeled dextran, MW. 1500,000, 5mg/100 µl of physiological saline solution) was injected into jugular vein to provide immediate contrast enhancement within intravascular space (Lehr, 1993). The selected area of the second- and third-order arterioles was recorded on videotape for further off-line analysis of diameter changes.

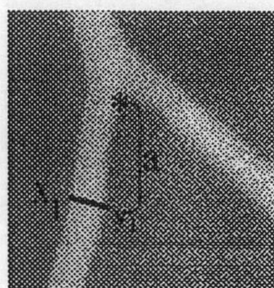
Videotapes of each experiment were played back frame by frame and then the frames arterioles of norminal diameter ranging from 15 to 40 µm were paused for measurement with a software called Global Lab Image II (Datatranslation Co. USA). The arteriolar diameter in micrometer (µm) was calculated as the mean of triple measurements from three video frames by using the same reference point (*) as a marker for the consistent position of measurement of each vessel in each frame as shown in Figure 3.5.

Leukocyte imaging

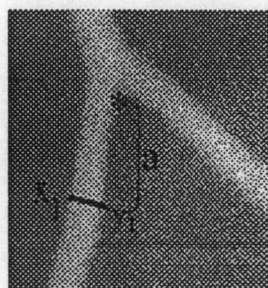
For visualization of the circulating and adhering leukocytes, the fluorescent marker acridine orange was infused intravenously (0.5 mg/kg/min) for 5 minutes (Lehr et al., 1991, 1993). During the experiment, the selected area of postcapillary venules with the adhesive leukocytes was real time recorded on videotape for further assessing of the leukocyte adhesion.

The leukocyte that was counted as adherent one has to remain stationary for not less than 30 seconds, the number of adherent cells were totally expressed as percentage per 100-µm length of the postcapillary venule

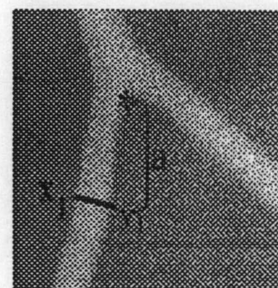
(diameter 10-30 μm). However, if there are many cells of leukocytes that adhere to endothelial wall as a group, it will be ignored and not be counted.



Frame 1:t=1 min
after recording



Frame 2:t=2 min
after recording



Frame 3:t=3 min
after recording

$$\text{mean arteriolar diameter} = \frac{x_1y_1 + x_2y_2 + x_3y_3}{3}$$

First is to define the (x,y) position of the selected reference point (*) by Global Lab II software and then plus the "y" value with a constance "a" in order to get position of y_1. x_1y_1 straight line was drawn perpendicularly to the vessel that will be represented as its diameter.

Figure 3.5 *Method for measurement of arteriolar diameter using Global Lab Image II*

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Blood samples of each rat were collected after the intravital fluorescent procedures. Then blood sample was centrifuged at 1,500 g. Serum was stored at -70 °C until the day of analysis.

TNF-α assay

Serum levels of TNF-α were determined by using the enzyme-linked immunosorbent assay (ELISA) kit of Endogen, Inc. (Woburn, MA, USA).

The experimental procedures were performed as following:-

- Add 50 μl of standard or sample. Incubate the covered plate at room temperature (20-25 ° C) for 1 hour.
- Wash the plate three time.
- Add 50 μl for prepared Biotinylated Antibody Reagent to each well being utilized.
- Incubate the covered plate at room temperature(20-25 ° C) for 2 hour.
- Wash the plate three time.
- Add 100 μl of prepared Streptaavidin-HRP Solution to each well.
- Incubate the covered plate at room temperature (20-25 ° C) for 30 minutes.
- Wash the plate three time.
- Add 100 μl of Premixed TMB Substrate Solution to each well.
- Develop the plate in the dark at room temperature (20-25 ° C) for minutes.
- Stop reaction by adding 100 μl of the Stop Solution provided to each well.
- Read the absorbance of the plate reader set at 450 nm by using Spectophotometer (DRGANON TEKNIKA microwell system).

IL-6 assay

Serum levels of IL-6 were determined by using the enzyme-linked immunosorbent assay (ELISA) kit of Endogen, Inc. (Woburn, MA, USA). The experimental protocol used for IL-6 assay is similar to that of TNF-α assay. However, the incubation times and reagent concentration were different.

The standard curves for TNF-α and IL-6 were showed in Figure 3.6. The measured OD (450 nm) of each unknown was then converted to its corresponding concentration by using these standard curves.

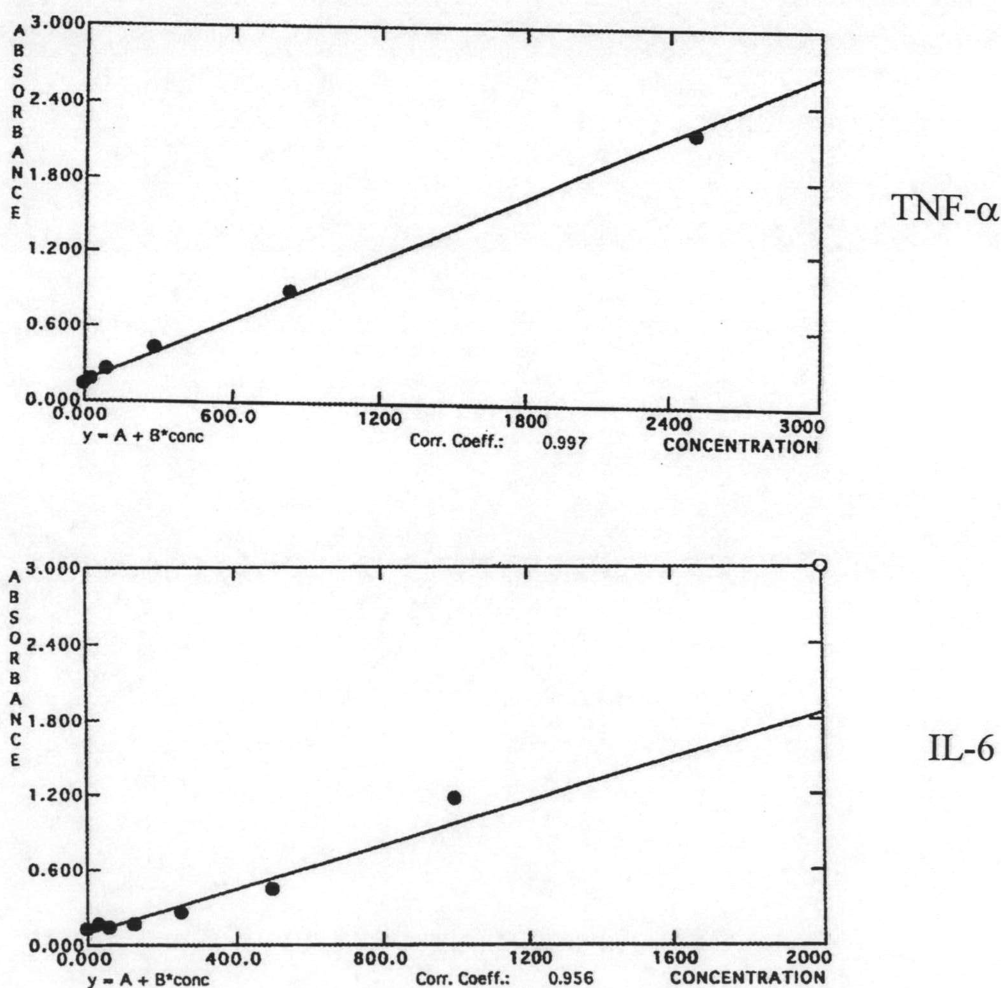


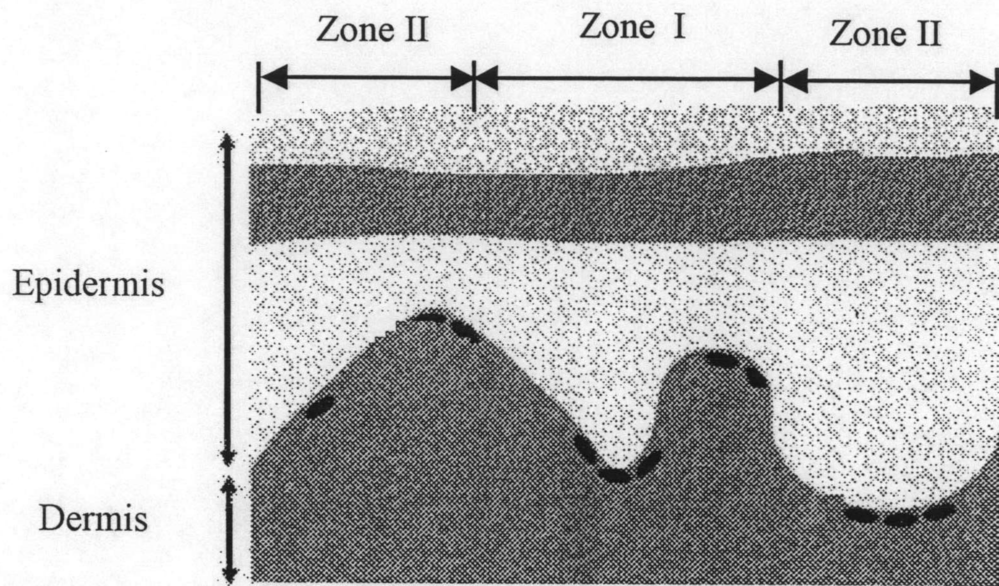
Figure 3.6 *The standard curves of TNF-α and IL-6.*

HISTOLOGIC STUDY

The specimen of elliptical biopsies of skin,0.5 cm × o.5 cm in size, was taken from the middle of burn area or called "zone I" as shown in Figure 3.7. All of them were fixed in buffered formalin. Every section was stained with hematoxylin and eosin. The light microscope (Olympus BX50F) was used with the ×20 and ×40 objective lens.

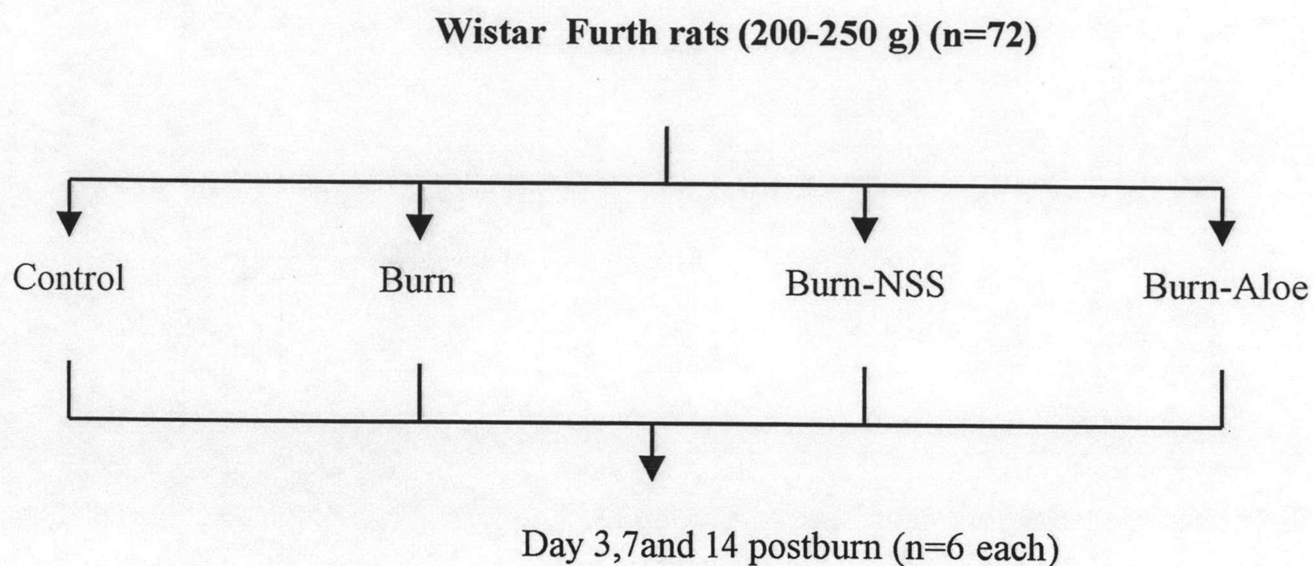
DATA ANALYSIS

All data were expressed as mean ± standard deviation (SD). Statistical analysis of the results was done using two-way analysis of variance followed by student's t-test. A probability value (P) of less than 0.05 was considered to be statistically significant.



Tissue below skin: muscle, fat, bone, etc.

Figure 3.7 *Area of burn wound section*



Day 3,7and 14 postburn (n=6 each)

- Measurement of burn wound healing area
- Skin blood flow measurement by Laser doppler flowmetry
- Fluorescent microscopic study for arteriolar diameter and leukocyte adhesion
- IL-6 and TNF-α assay

Figure 3.8 Diagram of experimental animal groups