CHAPTER III

MATERIALS AND METHODS

Chemical substances

Aloe gel 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
Ether
Phosphate buffer solution

Animal preparations

A total of 48 male Wistar rats weighing 200 to 250 grams were used in this study. The animals were divided into four groups of 12 animals each as the following:

Control group

The animals were anesthetized with ether. 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.

Burn wound group

The animals were anesthetized with ether. After shaving and depilating, the animals in this group were produced the partial thickness burn injury, called secondary degree burn. By using the model of Zawacki (1974), the hot plate (3.5 x 4.6 cm (wide x length)) 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 as shown in figure 3.1. This group did not receive any treatments. After burning, the burned area of each animal was copied onto translucence paper for measurement of size. Then these animals were housed with free access to water and standard laboratory chow until the day of performing chamber implant experiment.

Burn wound with normal saline treatment group

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). After receiving NSS, the burned area of each animal was copied for measurement of size. The same amount of NSS was applied to this group once a day until the day of performing chamber implanted experiment.

Burn wound with aloe vera treatment group

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 gel powder at dose of 300 mg/kg body wt in sterile water (10 ml/kg body wt) (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 (Mukdavan Saizukk, 1991; Nonglak Prayoonrak, 1995; Meadows, 1983). After receiving aloe gel powder, the burned area of each animal was copied for measurement of size. And the same amount of topical aloe gel powder were applied to this group once a day until the day of performing chamber implanted experiment.

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

In order 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 the seventh day and fourteenth day. The procedures of animal preparation were concluded in diagram as shown in figure 3.2.

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Methods

On the day of dorsal skinfold chamber implant experiment, the animal was anesthetized with intraperitoneal injection (ip) of 50 mg/kg body wt of sodium pentobarbital. A constant level of anesthesia was maintained throughout the experiment by intraperitoneal injection of supplement dose (20 % of original dose) of the anesthetic agent every 30-45 minutes (Koller and Kaley, 1990). Then the burned area of each animal was copied.

Under spontaneous respiration, the trachea was cannulated to facilitate respiration. Then a fine polyethylene catheter (PE 10, inner diameter 0.28 mm) was inserted into the right common carotid artery until it reached the aortic arch. The common carotid arterial pressure (CAP) was recorded via this catheter by using pressure transducer (Nikon model RM 6000). The other catheter was placed into jugular vein for injection of fluorescent-labeled macromolecule or fluorescent-labeled leukocyte into the blood stream. Both catheters were filled with heparinized saline.

For implantation of chamber, a chamber frame weighing 3.2 g shown in figure 3.2 was built from 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°. 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 by retaining ring.

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 skin 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 incoporated with one half of the aluminum frame. The bolts were put at the three holes in the shoulder of chamber to make the skin protrude and then the skin was cut to be 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 aluminium frames were implanted so as to sandwich the extended double layer of the skin.

After completion of the implantation, the dermal microvascular changes was studied with intravital fluorescence microscopy. The animal was placed on the microscope stage of fluorescent microscope equipped with

transillumination and epiillumination 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 20x long working distance objective (CF Achromat) and eyepiece, 10X and 20X, 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-X311) for playback analysis using a videocassette recorder. A videotape connected to a video timer (UTG 33) for time later recorder. During the experiment, microvessel images could be printed by using video graphic printer (Sony, UP-890 CE). All instruments used for quantitative studies of hemodynamic and the morphologic parameters in the microcirculation were shown in figure 3.4

4. Quantitation of diameter changes in arterioles

To measure diameter of second-and third-order arterioles, a fluorescent plasma marker (0.2 ml of FITC-labeled dextran, MW. 150,000, 5mg/100 μl of physiological saline solution) was injected into jugular vein to provide immediate contrast enhancement within intravascular space (Lehr, 1993). Then the second-and third-order arterioles were recorded on videotape for further analysis of diameter.

Videotapes of each experiment were played back frame by frame and then the frame of second-and third-order arterioles was paused for further digital into x, y arrays of 1024x1024 pixels by using the software called “Global Lab Image”. The diameter of each selected arteriole was assessed by the software indicated by number of pixels(n). Then the software could convert number of pixels to micrometer. Figure 3.5 shows measurement of

distance between two points (B to C) by using the position of the reference point in order to define points A to B.

Determination of the permeability of postcapillary venules

Under pathologic condition, release of endogenous factors selectively enhanced the permeability of postcapillary venule (10-60 um) more than that of capillaries (4-9 um) (Galey and Wayland, 1978; Lehr et al., 1991; Majno and Palade, 1961; Majno et al., 1961) and the postcapillary venule displayed the primary site of neutrophil exudation and plasma leakage (Simionescu et al., 1975, 1978). Therefore these vessel segments were chosen for investigation. In this experiment, postcapillary venules between 20-40 um in diameter were selected for the study.

After the arteriolar diameter recording, the field of postcapillary venule was chosen and recorded for further digital image analysis of intensity within inside (I in) and outside (I out) vessels. The ratio of I out/I in was calculated at zero time and thirty minutes after the intravenous injection of FITC-dx-150.

Videotapes of each experiment was played back frame by frame. The frame of selected postcapillary venule was paused. The position of reference point (point A) was defined and then the gray level of pixels inside (point B) and out side (point C) vessel as shown in figure 3.6 was assessed using the software called "Global lab Image".

Studies of leukocyte-endothelium interaction in postcapillary venule

For visualization of the leukocyte, the fluorescent marker acridine orange was infused intravenously (0.5 mg/kg/min) for 5 minutes (Lehr et al., 1991, 1993). During experiment, leukocytes were recorded on videotape for further observation of leukocyte adhesion.

Videotape of each experiment was played back and then adherent leukocytes were counted by visual observation. Adhesive leukocytes were defined as cells which did not move or detach from the endothelial lining within the entire observation for a period equal to or greater than 30 seconds whereas rolling leukocytes was defined as nonadherent leukocyte passing through the observed vessel segment within the observation period (Gaboury and Kubes, 1994).

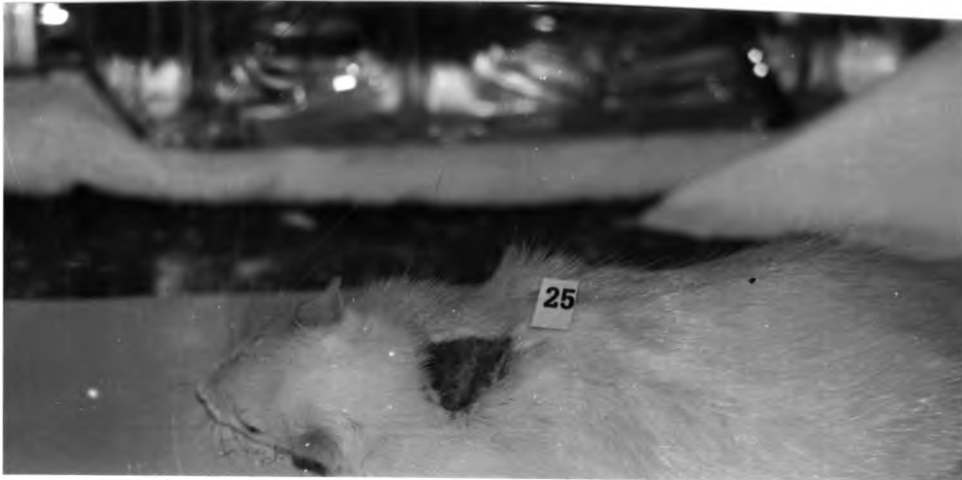


Figure 3.1 Photograph of partial thickness burn injury performed in this investigation. This model is a model of second degree burn model modified by Zavacki (1974).

Statistical analysis

Data are shown as means ± standard error. One-way analysis of variance (ANOVA) was performed to examine the difference of each parameter between Burn wound-rats, Normal saline-treated burn wound-rats and Aloe vera-treated burn wound-rats.

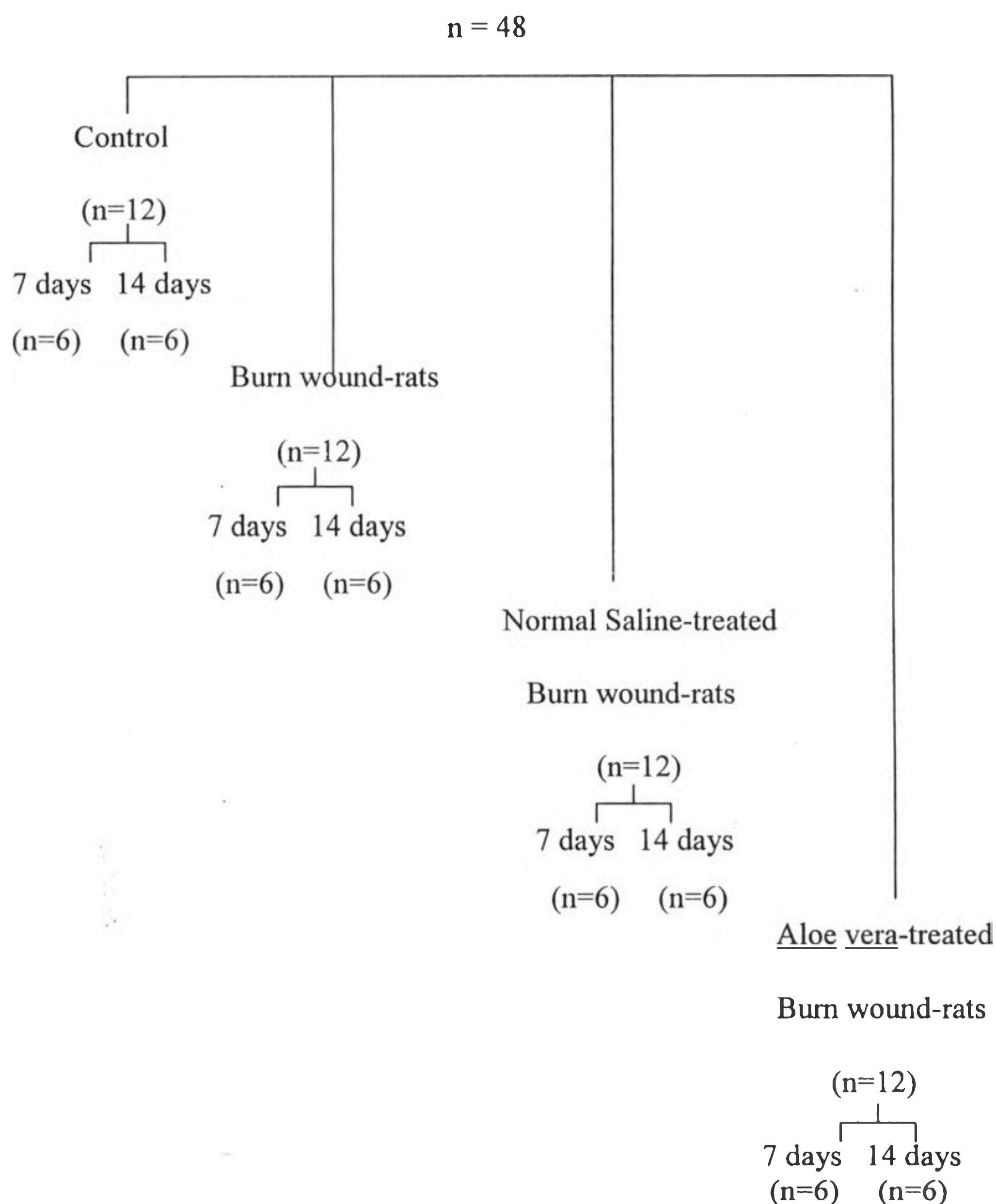


Figure 3.2 Diagram of experimental animal group.

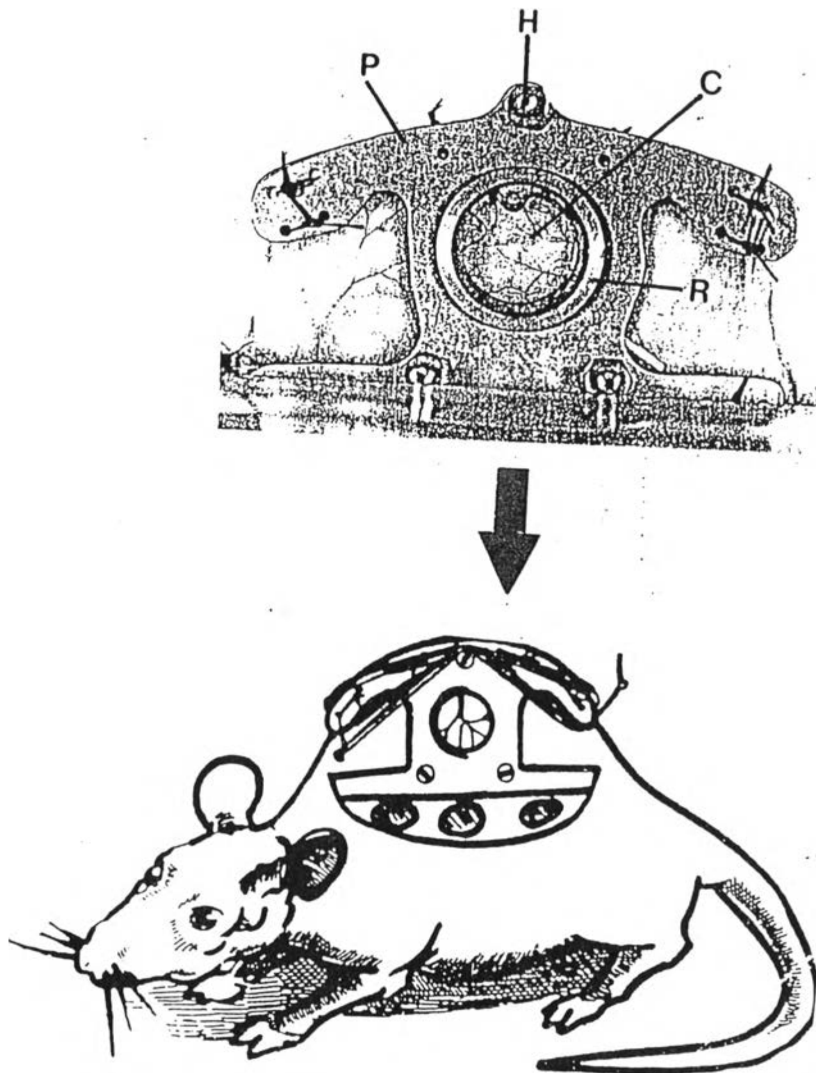


Figure 3.3 Photograph of aluminum chamber which inserted into the dorsal skinfold: P = plate, H = hole, C = cover glass, R = retaining ring.

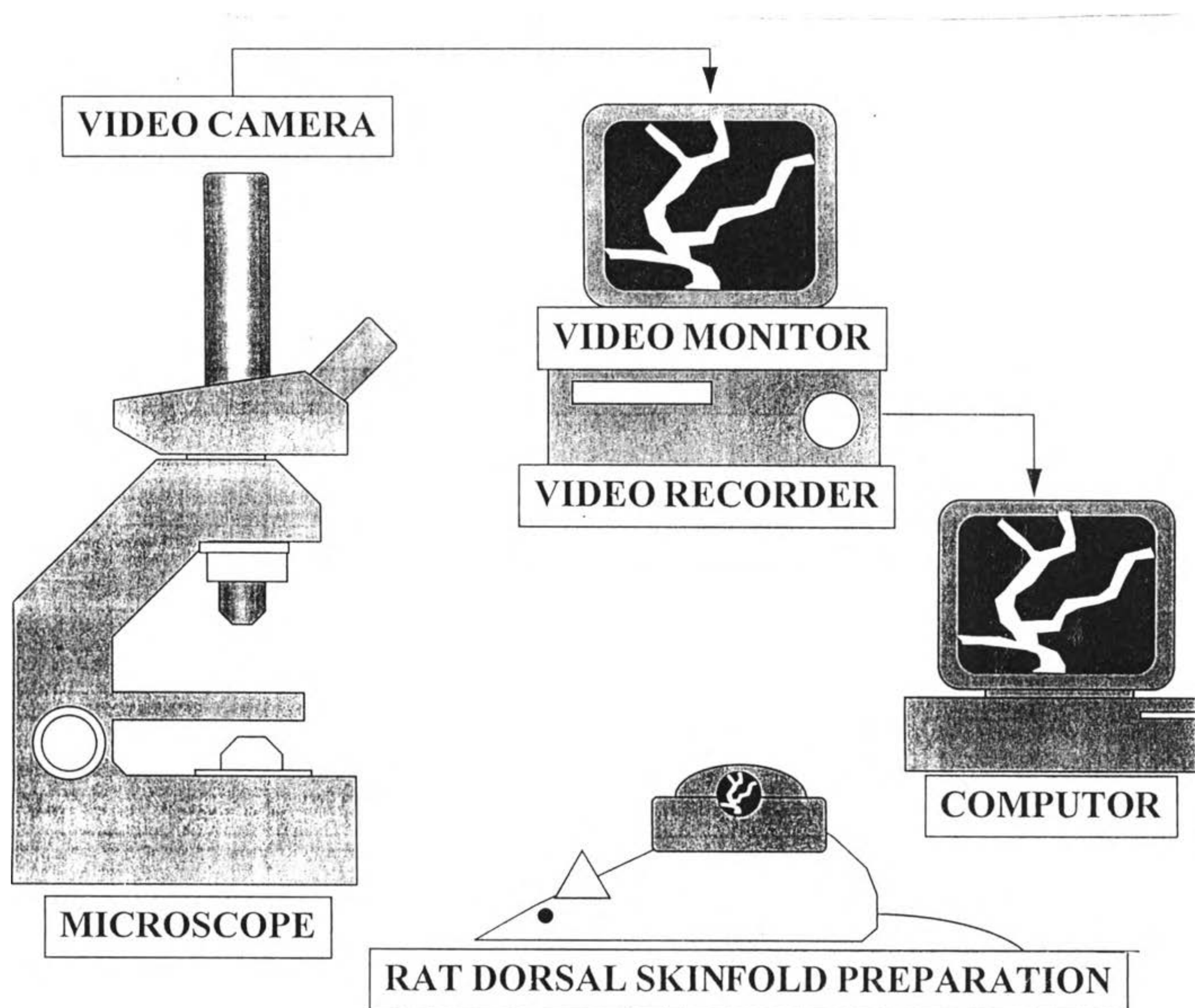


Figure3.4 Intravital fluorescence microscopy and instruments used for Quantitative studies of hemodynamic and morphologic microvasculature.

Arteriole

FITC -dx- 150
(5 mg/kg in 100 μl NSS)

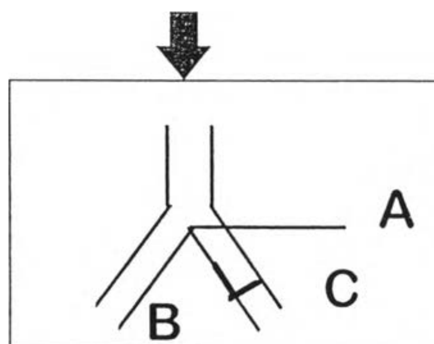
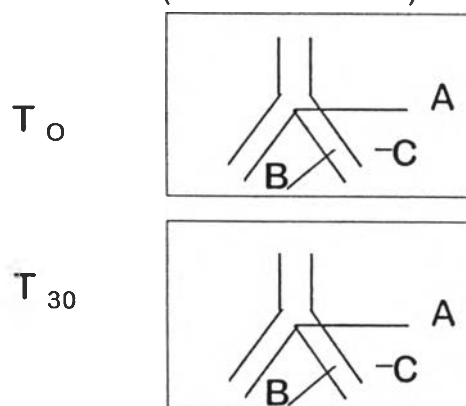


Figure 3.5 Schematic of arteriole showed the reference point (A) and defined point (B,C). The diameter of arteriole was measured as the length of B,C.

Postcapillary venular permeability
(FITC -dx- 150)



Global Lab Image Analysis

$$\frac{I_{out}}{I_{in}}_{30} - \frac{I_{out}}{I_{in}}_{0} = \text{Intensity ratio}$$

Figure 3.6 Schematic of postcapillary venule showed the reference point (A) and defined point (B,C). The postcapillary venule macromolecular extravasation was measured as a intensity ratio of I in and I out (I in = gray level of point B, I out = gray level of point C).