CHAPTER III

EXPERIMENT

The diagram shown in Figure 3.1, indicated the conceptual framework of this study. The idea of this conceptual framework was based on two target organs including bone and bone microvascular were considered during E_2 depletion.

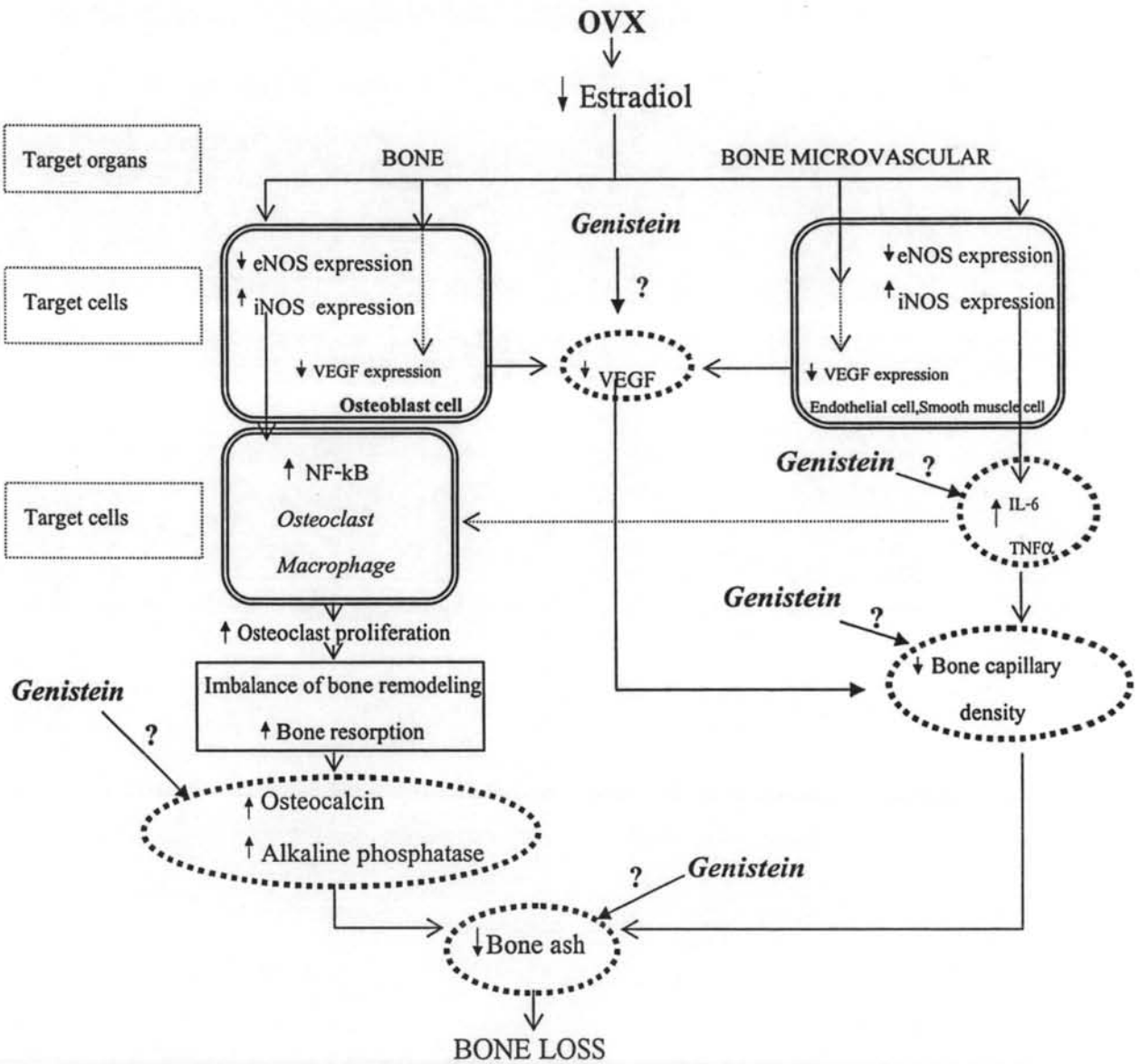


Figure 3.1 The conceptual framework of the present study showed when E_2 depletion two target organs, bone and microcirculation, were effected by direct and indirect mechanisms mediated through increased proinflammatory cytokines and decreased VEGF, then producing bone loss. Therefore, the hypothesis of this study is that genistein can prevent the alterations of bone vascularization and bone remodeling in ovariectomized rats by inhibiting the increased proinflammatory cytokines and decreased VEGF.

Chemicals

The list of chemical substances used in this study were given below;

Chemical	Company
Dimethyl sulfoxide	Sigma, USA.
Fluorescein isothiocyanate-dextran	Sigma, USA.
Genistein	Sigma, USA.
Normal saline	Thainakornpatana Co.,Ltd., Thailand
Pentobarbiturate sodium (Nembutal R)	Sanofi, Thailand
17β-estradiol	Sigma, USA

Animal Preparation

Mature female Wistar rats weighing 220-280 g with aged about 12 weeks [*] were used for in this study. These animals were obtained from National Laboratory Animal Center of Salaya Campus, Mahidol University, and were maintained on normal rat food and tap water *ad libitum* under controlled environmental conditions of 12-hr light/dark period.

NOTE [*] The rats were used to study of ovariectomy-induced bone loss have to age at least 12-16 week old at which age the adult female rats were characterized similar to that of humans (Miep and Stuart, 2003).

Ovariectomized Preparation

Before starting the operation, (solid) food was withdrawn from the animal's diet for 12 hours, with no limitation on water supply. For ovariectomized procedure, rats were anesthetized by intraperitoneal (i.p.) injection of sodium

pentobarbital at a dose of 45 mg/kg body weight. They were subjected to bilateral ovariectomy (OVX rats) (Waynforth, 2001). For the $Sham_{veh}$ group, all procedures were repeated except ovaries were not cut out ($Sham_{veh}$ rats).

Experimental design

According to our previous study performed by Khemapetch *et al.*, 2003, the results showed that subcutaneously injection with 0.25 mg/kg BW/day genistein could prevent endothelial dysfunction in 7-wk OVX rats. Moreover, their study also indicated that endothelial dysfunction had existed already at **3-wk** after ovariectomy. Therefore, in our experimental design, two experimental protocols were performed.

Experimental protocol 1, we would like to examine whether bone loss will occur at the same period of 3-wk or not. Moreover, the preventive effects of genistein on bone mineral content at 6-wk after ovariectomy was evaluated.

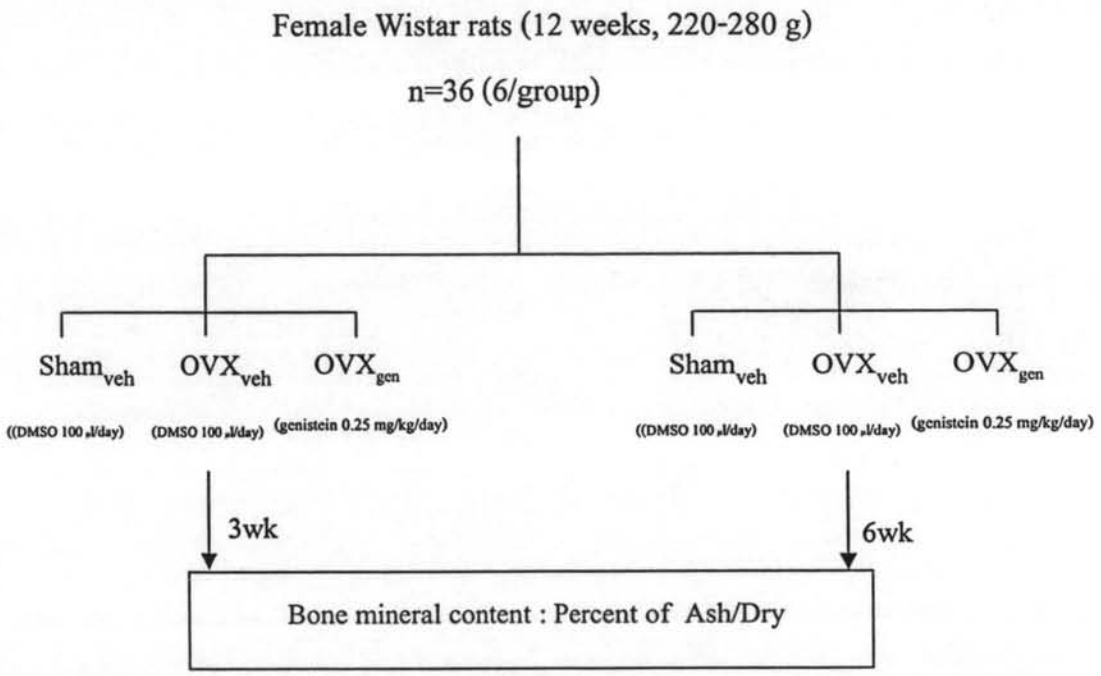
Experimental protocol 2, the mechanisms of genistein supplementation in early phased E_2 depletion on bone vascularization and bone remodeling were then examined. The experimental design for protocol 2 was based on the hypothesis and the conceptual framework (Figure 3.1) of the present study as shown previously.

Protocol 1. To study the effect of genistein on bone mineral content

Animals were divided into three major groups: 1) $Sham_{veh}$ used as controls and treated with vehicle (10% DMSO 100 µl/day sc; $Sham_{veh}$), 2) OVX-treated with vehicle (10 % DMSO 100 µl/day sc; OVX_{veh}), 3) OVX-treated with genistein (0.25 mg /kg BW/day sc ; OVX_{gen})(0.25 mg of genistein dissolved in 10% DMSO). The treatment with DMSO or genistein was started immediately after surgery. Before the treatments all animals were weighted and randomly assigned to receive genistein (0.25 mg /kg BW/day sc ; OVX_{gen}) or vehicle (DMSO 100 µl, sc.; OVX_{veh}) daily and continued for 3-wk and 6-wk.

At the end of each study, the animals were terminated by over-dose intraperitoneal injection of sodium pentobarbital. Left tibial bone [*] was removed and cleaned from surrounding tissue for determining of bone mineral content.

Note [*] Tibial bone was used because this bone is rich in cancellous bone and it have the greatest magnitude to inform of bone mineral content changing during early phase in OVX as mostly reported by several authors (Miep and Stuart, 2003; Chow *et al.*, 1992 and Mekraldi *et al.*, 2003)



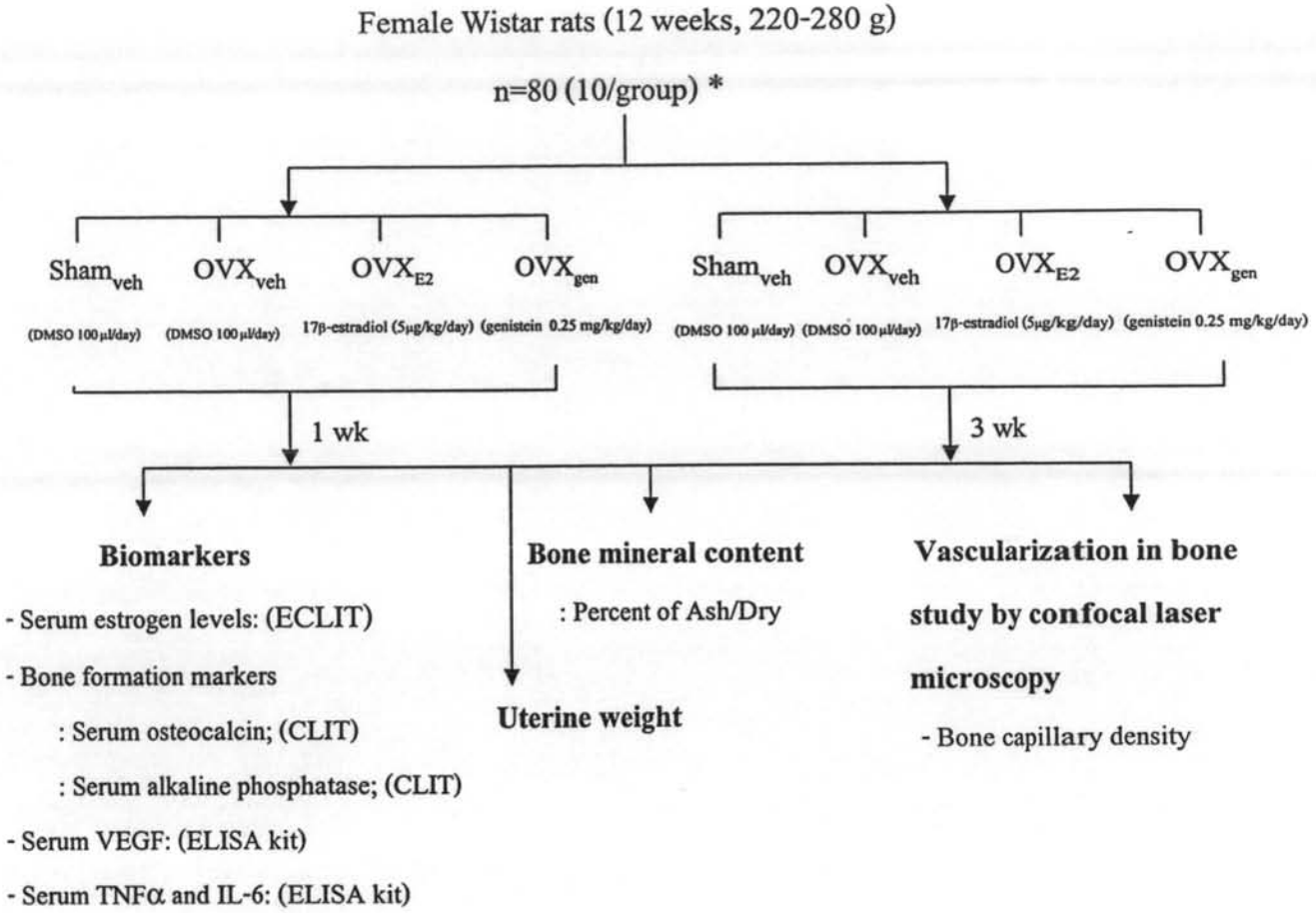
Bone mineral content: Percent of Ash/Dry matter

Left tibial or femur bone was placed in 70 % alcohol for degassing. Bone was dried at 100°C for 48 hours, weighed and ashed in cover crucible at 600°C for 16 hours. After cooling, then ashed bone was weighed again to determine the percent mineral content. (Farzad *et al.*, 2003)

Protocol 2 To study the mechanism(s) of genistein in early phase of E_2 depletion on bone vascularization and bone remodeling.

To determine the mechanism(s) of genistein, VEGF, TNF-α, IL-6, and bone capillary density were determined from each group : 1) $Sham_{veh}$ used as controls and treated with vehicle (10% DMSO 100 µl/day sc; **$Sham_{veh}$**), 2) OVX-treated with vehicle (10% DMSO 100 µl/day sc; **OVX_{veh}**), 3) OVX-treated with 17β-estradiol (5 µg/kg BW/day sc; **OVX_{E2}**), 4) OVX-treated with genistein (0.25 mg /kg BW/day sc; **OVX_{gen}**). All types of treatments; DMSO, 17β-estradiol, or genistein, were started immediately after surgery and continued for 1-wk or 3-wk duration **[*]**.

NOTE **[*]** 1-wk or 3-wk duration were used because the previous experimental results showed that the endothelial dysfunction was already existed since 3 weeks after ovariectomy. Therefore, our idea was to investigate whether genistein could prevent this endothelial dysfunction or not and if it could it might be able to prevent bone loss at the same time.



***NOTE : Sample size calculation**

The calculation of the sample size in this study is based on the pilot study. This pilot study demonstrated the effect of genistein in tibial bone mineral content at 3-wk after ovariectomy, 6 animals in each group. The results have shown that bone mineral content of in $Sham_{veh}$ and OVX_{gen} were 99.11±0.06 and 99.48±0.05 (%), respectively. Sample size calculation in the case of comparison of two independent means are as follows:

$$n / group = (Z_\alpha + Z_\beta)^2 \, ó_2 / (X1-X2)^2$$

Where α = 0.05 *

β = 0.1**

Z_α = 1.96

Z_β = 1.28

S_1 = standard deviation of group 1 = 0.06

S_2 = standard deviation of group 1 = 0.05

X1 = Mean of group1 = 99.11

X2 = Mean of group 2 = 99.48

$ó_2$ = pooled variance

$= (n1-1)S_1^2+(n2-1)S_2^2 / (n1+n2)-2$

$= (6-1)0.06^2+ (6-1)0.05^2 / (6+6)-2$

= 0.003

n / group = $(1.96+1.28)^2(0.003) / (99.11-99.48)^2$

= 0.22

* Probability of incorrectly rejecting the null hypothesis that there is no difference in the average values. An Alpha of 5% corresponds to a 95% Confidence Interval.

** Probability of incorrectly failing to reject the null hypothesis that there is NO difference in the average values -- assuming no difference when a real difference exists. A Beta of 50% is used in most simple calculations of sampling error.

(Roger, 2000; Tarnopolsky and MacLennan, 2000).

Femur and femur chamber preparation

On the day of experiment, the rats were anesthetized with intraperitoneal injection of 45 mg/kg bw of sodium pentobarbital. After the tracheostomy, polyethylene catheter (PE 20) was inserted into the left external jugular vein for intravenous drug administration. A 150 mm longitudinal skin incision was made approaching the femur from lateral. In order to get the good precision of study area, the femur was carefully exposed by blunt dissection at the position between the caput vertebralis and caput pelvis muscles to visualize femur bone microvasculature (Figure 3.2A). The femur chamber (Figure 3.2B) was positioned exactly on the study area for enhanced deeper focus (Figure 3.2C).

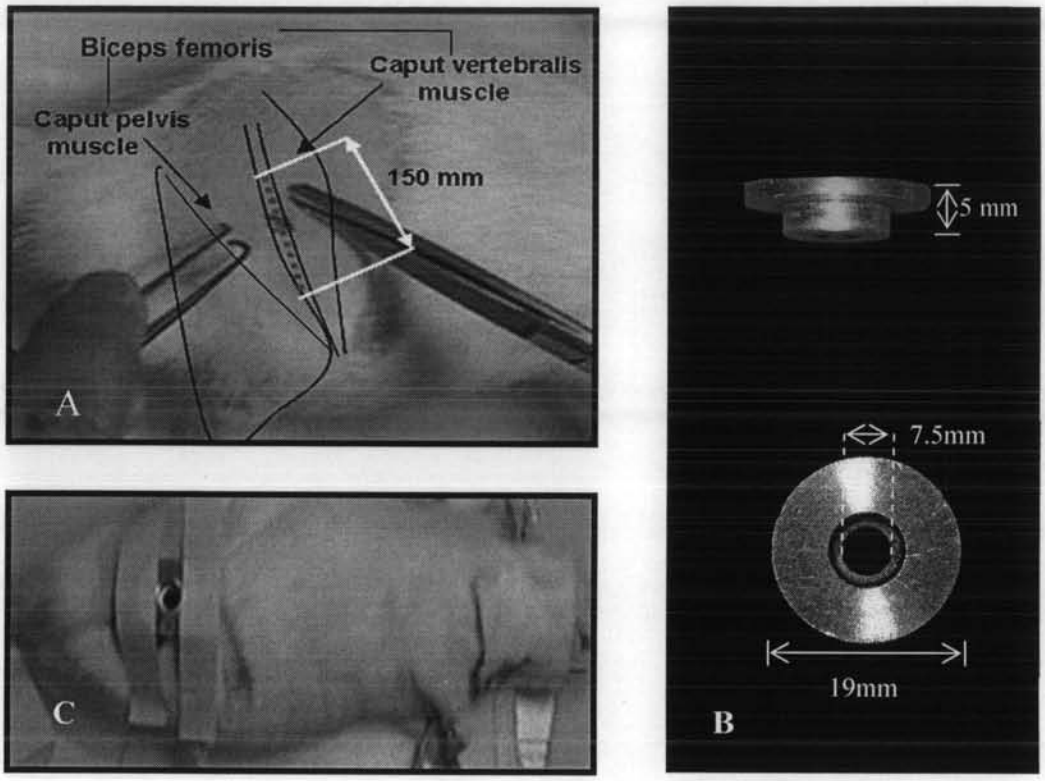


Figure 3.2 (A) The landmark used for placed the chamber on was at the position between the caput pelvis and caput vertebralis of biceps femoris muscles. (B) Femur chamber and (C) chamber attached to rat femur for enhanced focusing.

The bone microvasculature was observed under Confocal laser microscope (Nikon, Japan) (Figure 3.3). An objective lens of 10x was used. 50 µl of 0.5 % Fluoresein isothiocyanate-dextran (FITC-dextran) MW 250,000 (Sigma Chemical, USA) was injected intravenously to visualize the intralumen of microvessels. Three images of proximal, middle, and distal of femur chamber were recorded and for further analyses of bone capillary density using a digital image processing software Image Pro (Plus Software Media Cybernatics, Inc, USA).

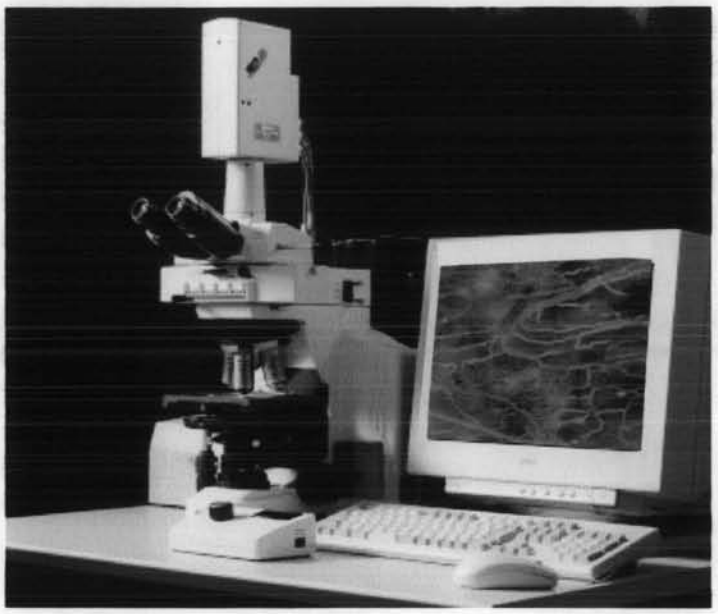


Figure 3.3 Nikon EZ-C1 Confocal microscope was used in the experimental protocol of determining bone capillary density.

Assessment of bone capillary density

1. The confocal-images of bone microvessels taken from each rat were obtained from 3 positions, proximal(1), middle(2) and distal(3) within each femur chamber.

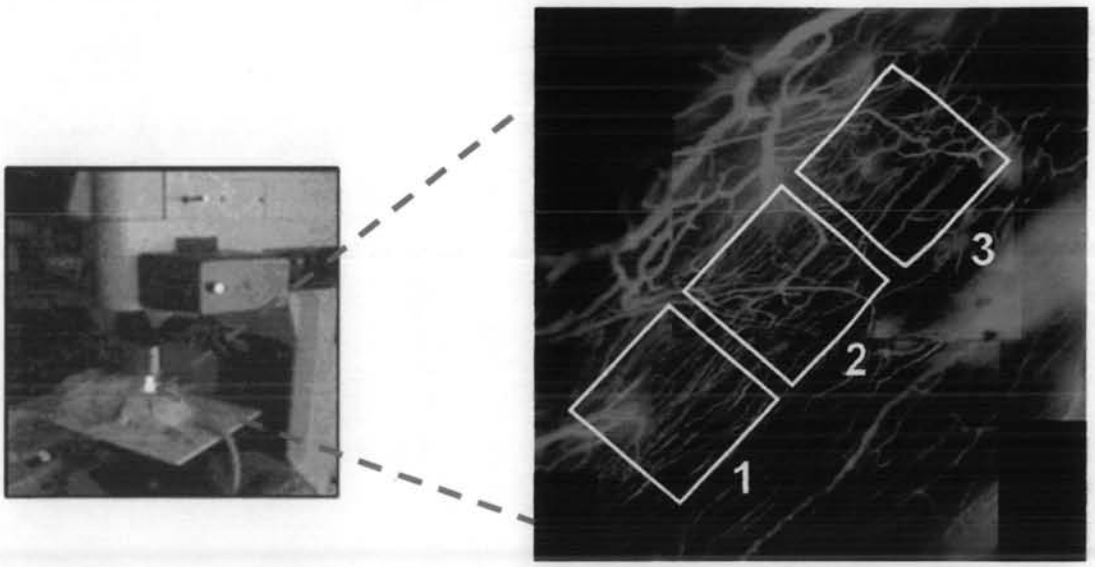


Figure 3.4 Collection bone vessels images by using Confocal fluorescence microscope. (proximal(1), middle(2) and distal(3) of femur chamber)

2. From each taken confocal images (Figure 3.5A), the window frame, 350x350 μm^2 was super-imposed on the clear selected area of bone capillary network (diameter $\leq$ 15 μm) as shown in Figure 3.5B. The bone capillary area was then analyzed by using a digital image processing software Image Pro (Plus Software Inc., USA.

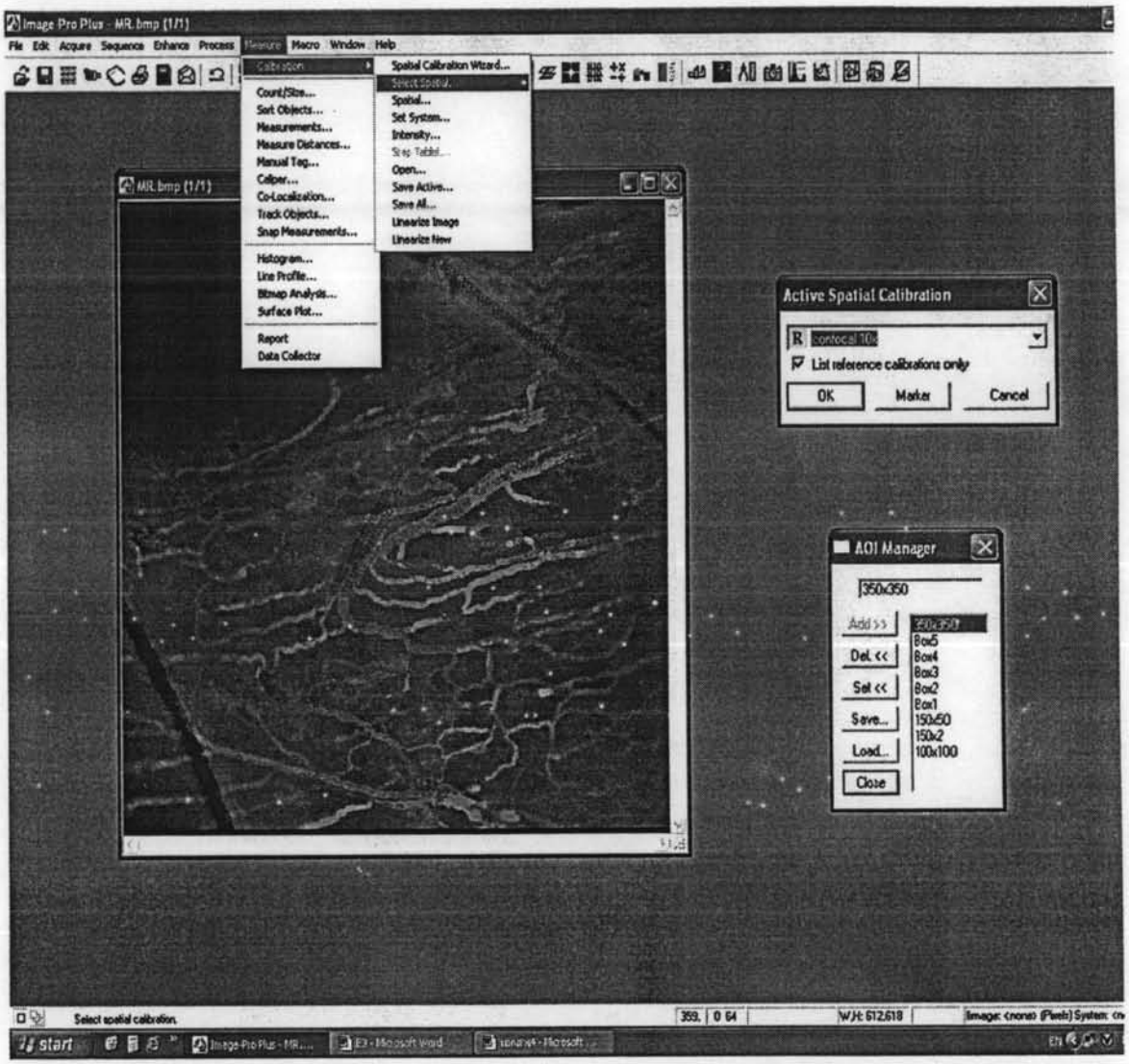


Figure 3.5 The image of femur bone capillary network of each experiment.

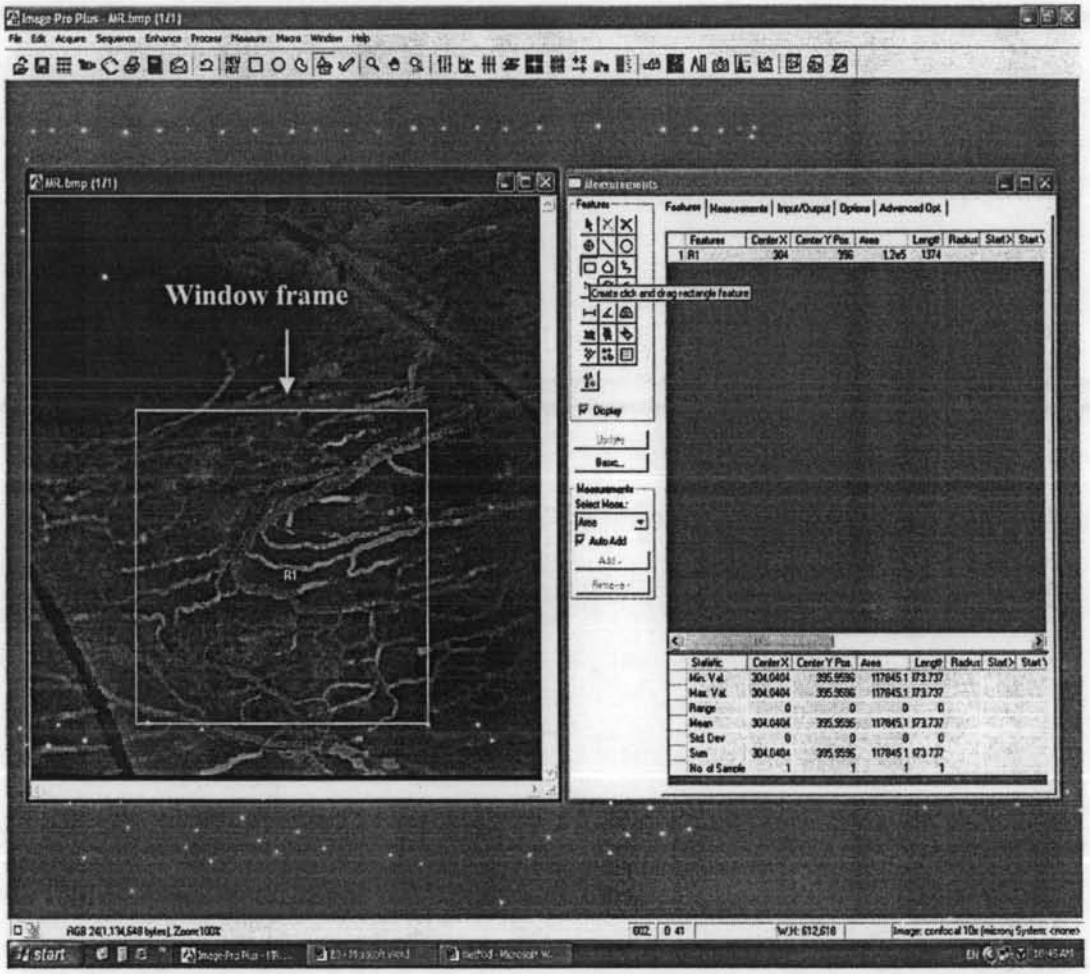


Figure 3.6 The window frame 350x350 μm^2, was posted on the selected studied area.

3. The area of each "segment" (from branch-to-branch) of capillary was determined by using computer assist as shown in Figure 3.6. Then the total area of every segmental areas were sum up. (Komai Y *et al.*, 2005).

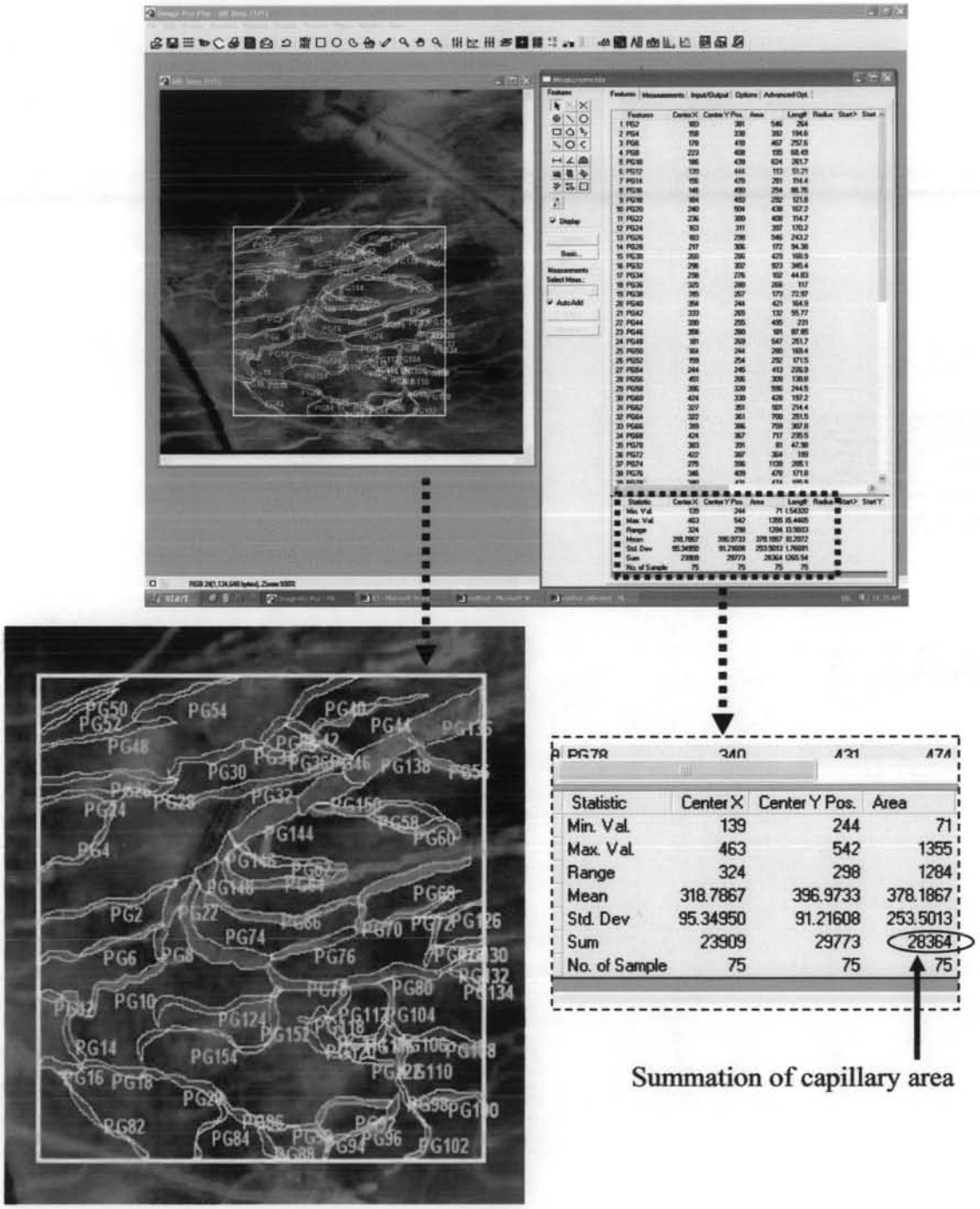


Figure 3.7 The "Measurement" functional tool was used to calculated the capillary area of each capillary segment, from branch-to-branch.

4. The percentage of bone capillary density **(CD)** was expressed by the sum of capillary area divided by the total area of window frame 350x350 μm^2, then multiplied by 100 as shown in the following equation:

% Capillary density (CD) = (Sum of capillary area/Total area) x 100

5. Bone capillary density of each rat is an average value of **% CD** at (1) proximal, (2) middle, and (3) distal.

CD of each rat = [CD (1) +CD (2) +CD (3)] / 3

6. Bone capillary density of each group was expressed by mean $\pm$ SD. [Please see the NOTE].

At the end of the experiment, blood sample was collected from abdominal aorta . Uterus was taken, cleaned, and weighted.

NOTE: The blind test was carried out in order to test the accuracy of this %CD interpretation. The blind test was performed by well-trained person who had no idea about the group difference.

Serum E_2 determination

At the end of experiment, blood samples (2 ml) were collected in polypropylene tubes, stored at room temperature for 15 minute and then centrifugation at 3000 rpm at 4°C for 20 minutes, each sample was stored at -20°C until analysis. Serum E_2 levels were determined by electrochemiluminescence immunoassay (ECLIT) with a commercial available kit. Functional sensitivity assay is 44 pmol/l (12 pg / ml) and reproducible measured with an inter-assay coefficient of variation of ≤ 20 %.

Bone biochemical markers assay

In order to evaluate serum osteocalcin and serum alkaline phosphatase levels, blood samples (2 ml) were collected in polypropylene tubes, store at room temperature for 1 hour and then centrifugation at 3000 rpm at 4°C for 20 minutes, each sample was stored at -20°C until analysis. Serum levels of osteocalcin and serum alkaline phosphatase which are an index of bone formation were determined by chemiluminescence immunoassay (CLIT) with a commercial available kit.

Enzyme-Linked Immunosorbent assay (ELISA) for serum TNF-α, IL-6, and VEGF

In order to evaluate serum TNF-α, IL-6 and VEGF concentrations, blood samples (2 ml) were collected in polypropylene tubes, store at room temperature for 2 hour and then centrifugation at 3000 rpm at 4°C for 20 minutes, each sample was stored at -20°C until analysis. To measurement of TNF-α, IL-6 and VEGF in serum, commercially available ELISA-Kit (R&D Systems, USA) were used. Results were calculated from a standard curve and expressed the levels of TNF-α and IL-6 in nanograms per milliliter (ng/ml) and VEGF concentration in picograms per milliliter (pg/ml) of serum respectively.

Data Analysis

Results were shown as means ± SD. One–way ANOVA was used to determine the difference of means. Tukey Post-Hoc test was used for multiple comparisons among groups. The relationships between VEGF and bone capillary density, VEGF and bone mineral content, and bone capillary density and bone mineral content were made by Pearson correlation (p-value) and Model fit curve (r). The statistical differences were considered at the probability level (p-value) of lower than 0.05.