CHAPTER IV

RESULTS

This chapter is composed of five major parts of results which were : 1) the effects of genistein on bone mineral contents, 2) effects of genistein on serum estrogen levels and uterine weight, 3) effects of genistein on bone biochemical markers, TNF-α and IL-6, 4) effects of genistein on serum VEGF and bone capillary density, and 5). the relationship between serum VEGF , bone capillary density, and bone mineral content.

4.1. The effects of genistein on bone mineral contents

To determine the effects of genistein on bone mineral content, the ovariectomized rats and the same dose of genistein, 0.25 mg/kgBW/day, that could prevent endothelial dysfunction reported by Khemapech *et al.* (2003) were used. Animals were divided into three groups; $Sham_{veh}$, OVX_{veh}, and OVXgen.

Table 4.1.1 showed that in OVX_{veh}, bone mineral content of left tibia was significantly decreased at 6 weeks after ovariectomy compared to $Sham_{veh}$ ($p<0.05$). Genistein significantly increased bone mineral content of the tibia at both 3- and 6-weeks periods compared to OVX_{veh}.

Table 4.1.1 Means ± SD of ash/dry matter (%) which represented tibial bone mineral content after 3 and 6 weeks of ovariectomy.

Group	Ash/Dry matter (%)	
	3 weeks	6 weeks
$Sham_{veh}$ (n=6)	99.11±0.06	99.43±0.25
OVX_{veh} (n=6)	98.68 ± 0.26^{NS}	$98.69\pm0.24^{*}$
OVX_{gen} (n=6)	$99.48\pm0.05^{\#}$	$99.35\pm0.03^{\#}$

NS = non-significantly different as compared to $Sham_{veh}$.

* $p< 0.05$ significantly different as compared to $Sham_{veh}$.

# $p< 0.05$ significantly different as compared to OVX_{veh}

This study demonstrated that tibia bone mineral content was significantly decreased at 6-wk after ovariectomy, which **was existed later than the finding of endothelial dysfunction** as reported previously by Khemapech *et al.*, 2003. Moreover, the result also showed that genistein at the dose of 0.25 mg/kgBW/day could retard this tibial bone mineral content lost.

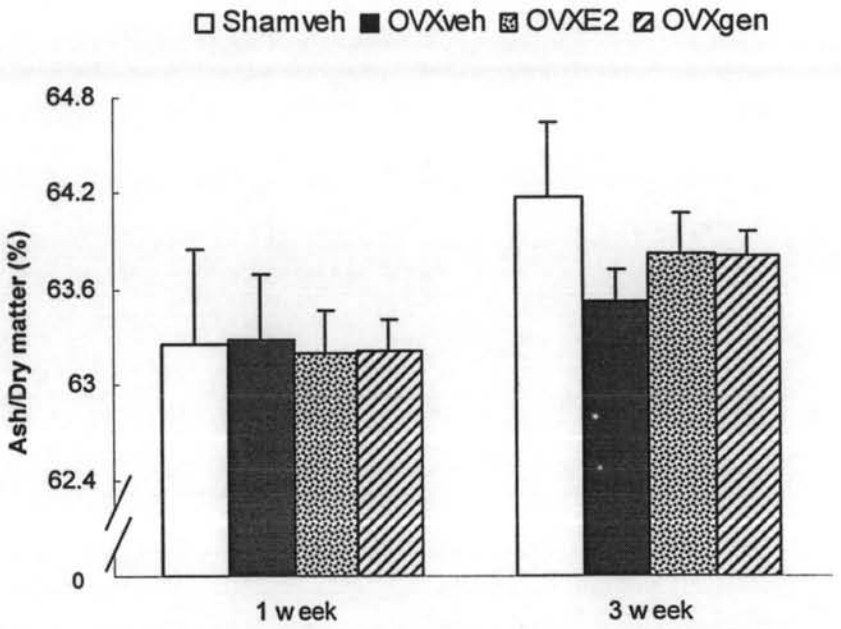
From this fiding, therefore, in the next experimental protocols, the early phase of E_2 depletion, 1 and 3-week after ovariectomy were then performed in order to further examine the effects of genistein on bone vascularization and bone remodeling. *The idea was that genistein might be able to protect bone loss due to it can preserve bone microcirculation since the early state of E_2 depletion.*

In Table 4.1.2 and Figure 4.1, the results showed that means of femur bone mineral content were not different among groups of $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1-wk and 3-wk.

Table 4.1.2 Means ± SD of bone mineral content (ash/dry matter (%)) were obtained from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy (n=8).

Group	Ash/Dry matter (%)	
	1 week	3 weeks
$Sham_{veh}$ (n=8)	63.26±1.32	64.18±0.47
OVX_{veh} (n=8)	63.29±0.83	63.53±0.19
OVX_{E2} (n=8)	63.20±0.59	63.82±0.26
OVX_{gen} (n=8)	63.20±0.47	63.81±0.16

Figure 4.1 Means ± SD of bone mineral content (ash/dry matter (%)) were obtained from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy (n=8).



4.2. Effects of genistein on serum E_2 levels and uterine weight

Serum estrogen levels were significantly reduced at 1-wk and 3-wk of OVX_{veh} when compared to $Sham_{veh}$ group (Table 4.2 and Figure 4.2.1). The genistein administration showed no significant effects, however, 17β-estradiol replacement markedly increase serum estrogen to the levels resembling those observed in $Sham_{veh}$ at 1-wk and 3-wk after ovariectomy (Table 4.2 and Figure 4.2.1).

Uterine weights were significantly decreased at 1-wk and 3-wk in OVX_{veh} when compared to $Sham_{veh}$ and genistein supplementation showed no effect as well (Table 4.2 and Figure 4.2.2). The rats treated with 17β-estradiol, OVX_{E2}, had a significant greater uterine weight than those values of OVX_{veh} and OVX_{gen} rats (Table 4.2 and Figure 4.2.2).

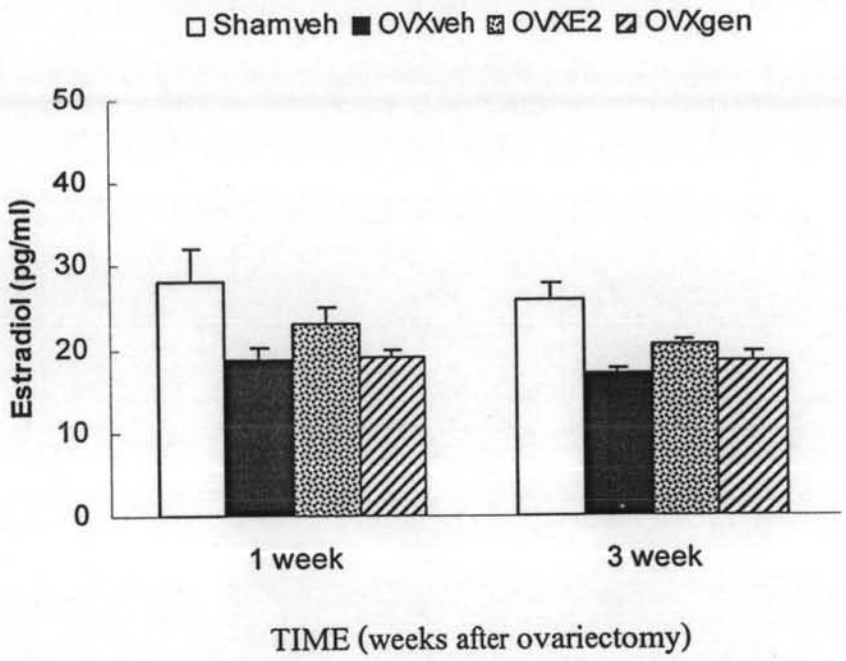
Table 4.2 Means ± SD on serum E_2 levels (pg/ml) and uterine weight (g/100gBW) were obtained from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy (n=10).

Group	Estradiol (pg/ml)		Uterine weight (g/100g BW)	
	1 week	3 weeks	1 week	3 weeks
$Sham_{veh}$(n=10)	28.09±10.83	25.86±5.10	0.18±0.06	0.16±0.05
OVX_{veh}(n=10)	18.61±3.26*	16.97±1.94***	0.08±0.01*	0.04±0.01***
OVX_{E2}(n=10)	23.00±5.06	20.45±1.15	0.16±0.04#	0.17±0.02###
OVX_{gen}(n=10)	19.03±2.09*	18.35±3.53**	0.08±0.01*	0.06±0.01***

* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ significantly different as compared to $Sham_{veh}$.

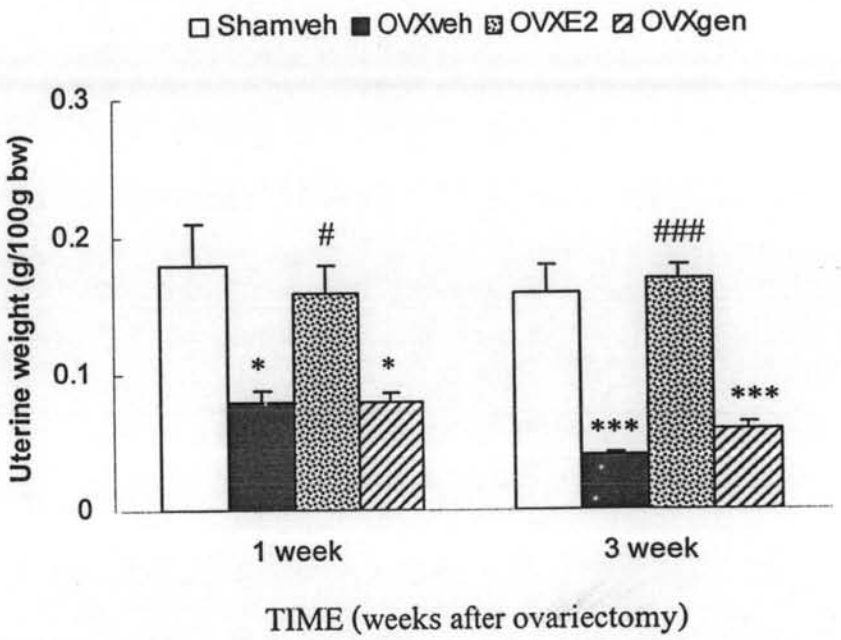
# $p< 0.05$, ### $p< 0.001$ significantly different as compared to OVX_{veh} and OVX_{gen}.

Figure 4.2.1 Serum E_2 levels (pg/ml) were determined from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy (n=10). Values were expressed as means ± SD.



* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ significantly different as compared to $Sham_{veh}$.

Figure 4.2.2 Means ± SD of uterine weight (g/100g BW) were determined from each group : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy (n = 10).



$p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ significantly different as compared to $Sham_{veh}$.

# $p< 0.05$, ### $p< 0.001$ significantly different as compared to OVX_{veh}.

4.3. Effects of genistein on bone biochemical markers and serum TNF-α and IL-6

In Table 4.3.1 and Figure 4.3.1-4.3.2, the results showed that bone biochemical markers, serum osteocalcin and alkaline phosphatase activity, were significantly increased at 3-wk OVX_{veh} as compared to 3-wk $Sham_{veh}$ ($p<0.001$ and $p<0.05$). The 17β-estradiol treatment was associated with reduced serum osteocalcin and alkaline phosphatase activity at both 1-wk and 3-wk as compared to ovariectomized rats. However, the results of genistein treated groups demonstrated the significant difference ($p< 0.01$) of both markers only at 3-wk, not for 1-wk (Table 4.3.1 and Figure 4.3.1 - 4.3.2).

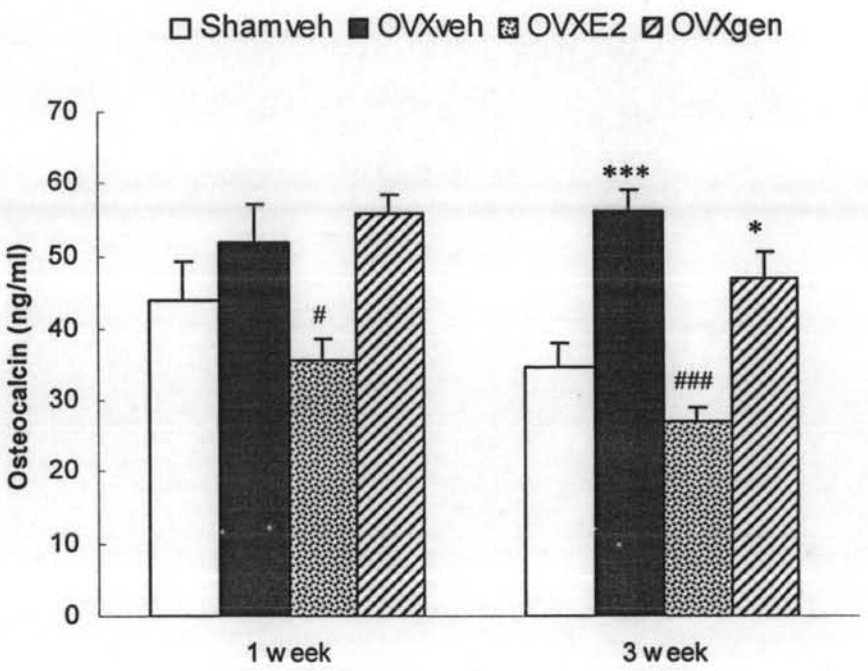
Table 4.3.1 Means ± SD of bone biochemical markers, serum osteocalcin and alkaline phosphatase activity, were determined from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy. (n = 10)

Group	Osteocalcin (ng/ml)		Alkaline phosphatase (U/L)	
	1 week	3 weeks	1 week	3 weeks
$Sham_{veh}$(n=10)	44.01±13.06	34.75±9.58	45.25±8.84	35.00±4.19
OVX_{veh}(n=10)	51.93±12.32	56.17±7.37***	49.33±7.37	51.75±8.37*
OVX_{E2} (n=10)	35.70±7.14$^{\#}$	27.16±5.12$^{\#\#\#}$	28.83±2.66$^{\#}$	29.37±3.79$^{\#\#}$
OVX_{gen}(n=10)	55.67±5.85	46.76±8.57*,$^{\#}$	54.75±7.46	36.75±1.56$^{\#}$

* $p< 0.05$, *** $p< 0.001$ significantly different as compared to $Sham_{veh}$.

$^{\#}$ $p< 0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ significantly different as compared to OVX_{veh}

Figure 4.3.1 Means ± SD of serum osteocalcin (ng/ml) were determined from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy. (n = 10)

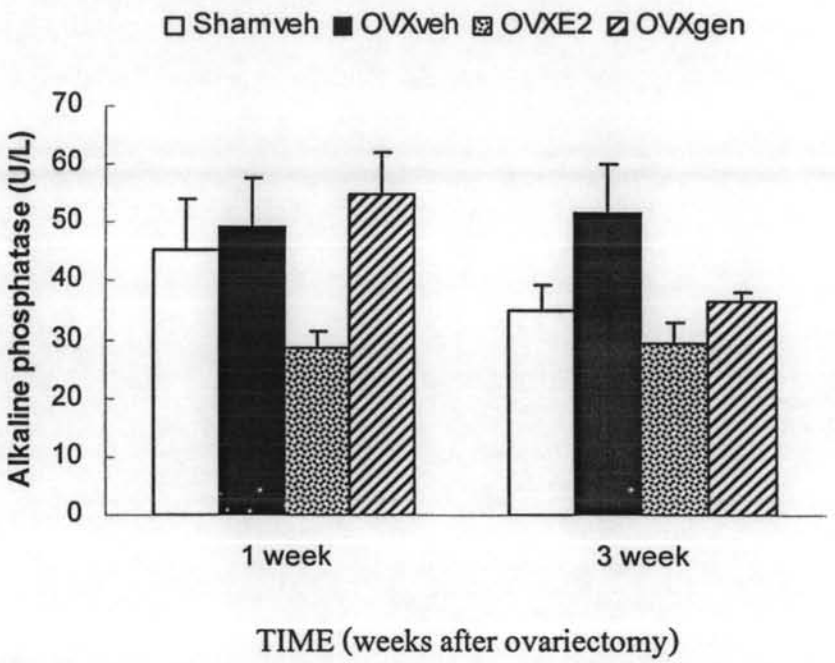


TIME (weeks after ovariectomy)

* $p< 0.05$, *** $p< 0.001$ significantly different as compared to $Sham_{veh}$.

# $p< 0.05$, ### $p< 0.001$ significantly different as compared to OVX_{veh}.

Figure 4.3.2 Means ± SD of serum alkaline phosphatase (U/L) were determined from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy. (n = 10)



* $p< 0.05$ significantly different as compared to $Sham_{veh}$.

\# $p< 0.05$, ## $p< 0.01$ significantly different as compared to OVX_{veh}.

In table 4.3.2 and Figure 4.3.3 showed that both 1-wk and 3-wk OVX_{veh} TNF-α levels were significantly produced more than those of 1-wk and 3-wk $Sham_{veh}$. However, after 3-wk treatments with 17β-estradiol or genistein, the increased production of TNF-α was significantly attenuated as compared to 3-wk OVX_{veh} ($p<0.05$). The levels of cytokine IL-6 was no significant difference for all groups, except for 1-wk OVX_{E2} the serum level of IL-6 was higher significantly than $Sham_{veh}$ ($p< 0.05$) (Table 4.3.2 and Figure 4.3.4).

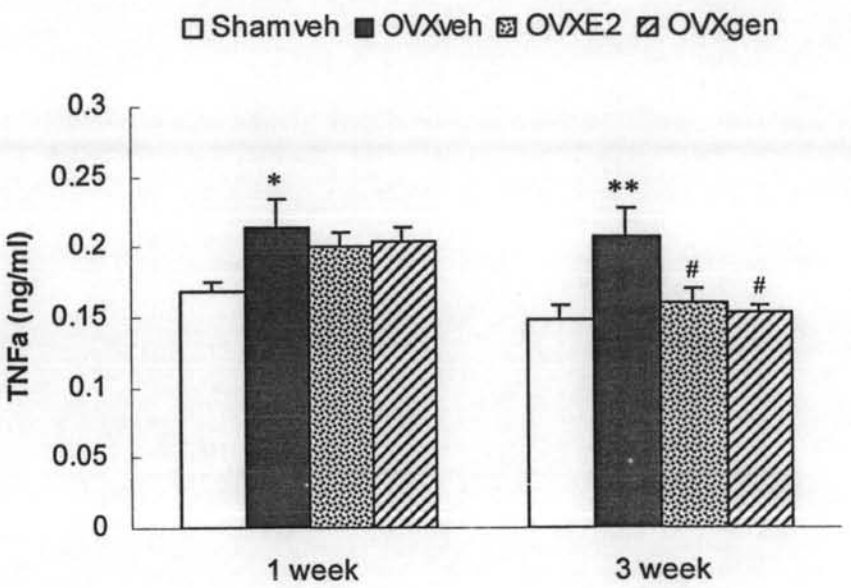
Table 4.3.2 Serum TNF-α (ng/ml) and IL-6 (ng/ml) levels were determined from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy. Values were expressed as means ± SD. (n = 8)

Group	TNF-α (ng/ml)		IL-6 (ng/ml)	
	1 week	3 weeks	1 week	3 weeks
$Sham_{veh}$(n=8)	0.168±0.01	0.148±0.04	0.306±0.06	0.420±0.10
OVX_{veh}(n=8)	0.289±0.18*	0.207±0.05**	0.315±0.02	0.421±0.04
OVX_{E2} (n=8)	0.272±0.09	0.160±0.05$^{\#}$	0.414±0.22*	0.398±0.08
OVX_{gen}(n=8)	0.204±0.03	0.154±0.01$^{\#}$	0.303±0.06	0.440±0.09

* $p< 0.05$, ** $p< 0.01$ significantly different as compared to $Sham_{veh}$.

$^{\#}$ $p< 0.05$ significantly different as compared to OVX_{veh}

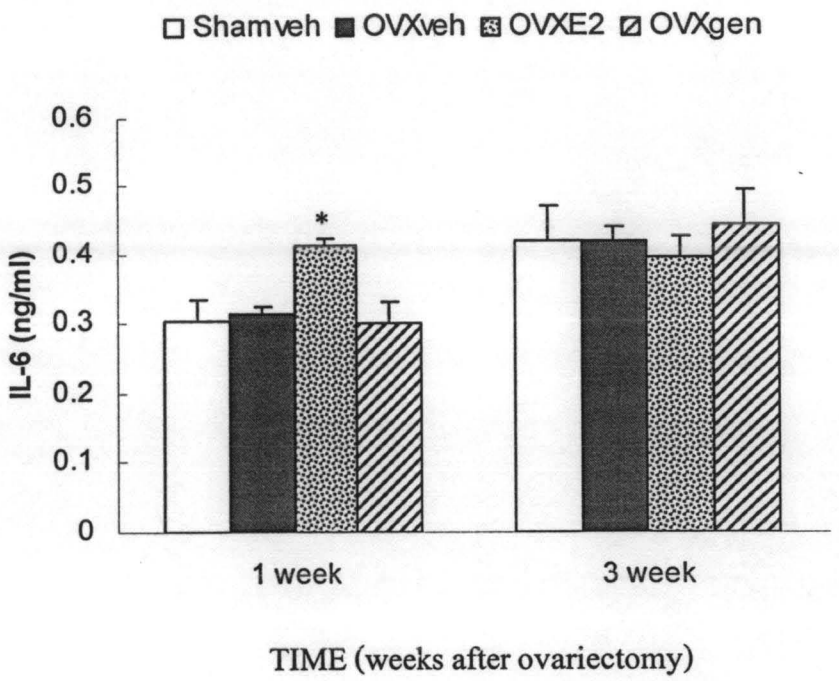
Figure 4.3.3 Means ± SD of serum TNF-α levels (ng/ml) were determined from $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy. (n = 8)



* $p < 0.05$, ** $p < 0.01$, significantly different as compared to $Sham_{veh}$.

# $p < 0.05$, significantly different as compared to OVX_{veh}.

Figure 4.3.4 Means±SD of serum IL-6 levels (ng/ml) were determined from: $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy. (n = 8)



* $p < 0.05$ significantly different as compared to $Sham_{veh}$.

4.4. Effect of genistein on serum VEGF and bone capillary density

4.4.1 Effect of genistein on serum VEGF

At 1-wk and 3-wk, the serum VEGF levels of OVX_{veh} were significantly different when compared to $Sham_{veh}$ (Table 4.4 and Figure 4.4.1). Interestingly, the depletion of serum VEGF in ovariectomized rat was significantly improved by treatments of 17β-estradiol or genistein (Table 4.4 and Figure 4.4.1).

4.4.2. Effect of genistein on bone capillary density

By using Confocal laser microscopy, the ovariectomy caused the changes of bone microvascularization was demonstrated as shown in Figure 4.4.2 and 4.4.3 The result showed that there was a marked decreased in femur bone capillary microvessels ($d \leq 15$ μm) since 1-wk after ovariectomy (Figure 4.4.3B). In addition, the results indicated that lost femur bone microvessels seem to progressive decreased at 3-wk after ovariectomy (Figure 4.4.4 B). However, number of bone capillary microvessels seem to maintain by 17β-estradiol or genistein supplementation at both experimental periods (Figure 4.4.3D, Figure 4.4.4 C and D).

By using the digital image analysis software as described previously (Chapter III : Experiment), the number of bone capillary density were calculated and summarized as shown in Table 4.4 and Figure 4.4.2. The significant decrease in bone capillary density in OVX_{veh} at both 1-wk and 3-wk after ovariectomy was confirmed (Table4.4). Interestingly, both 17β-estradiol and genistein treatments showed significant effects on preventing loss of bone capillary density (Table 4.4).

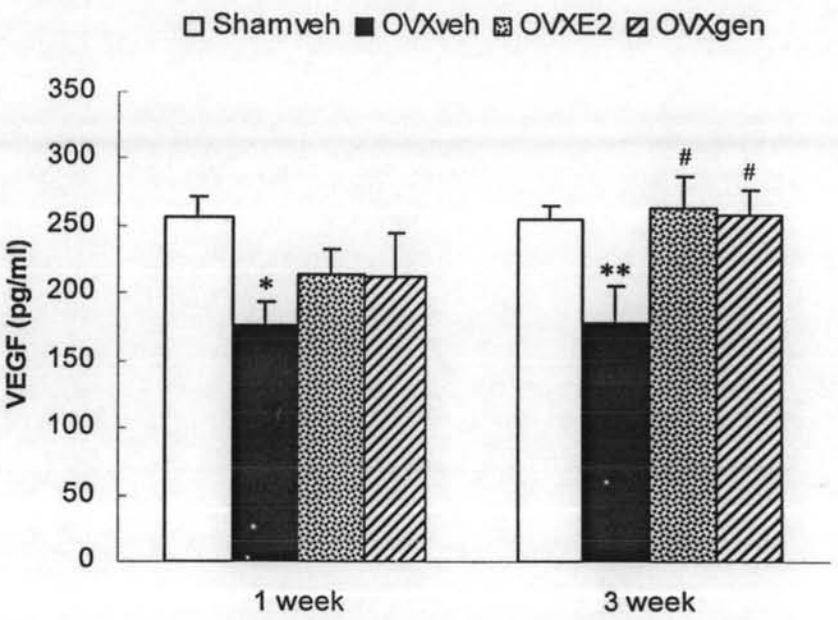
Table 4.4 Serum VEGF levels (pg/ml) and bone capillary density (%) were determined from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy. (n = 8) Values were expressed as means ± SD.

Group	VEGF (pg/ml)		Capillary density (%)	
	1 week	3 weeks	1 week	3 weeks
$Sham_{veh}$ (n=8)	256.20±15.67	254.40±10.48	23.85±0.52	25.36±0.71
OVX_{veh} (n=8)	176.00±17.49*	177.65±27.93**	20.40±1.00*	18.37±0.67***
OVX_{E2} (n=8)	214.70±18.65	262.60±24.22$^{\#}$	24.70±2.04	22.08±1.10*,##
OVX_{gen} (n=8)	212.10±32.05	258.15±37.36$^{\#}$	24.76±1.60$^{\#}$	21.36±1.16**,#

* $p< 0.05$, ** $p< 0.01$, ** $p< 0.001$ significantly different as compared to $Sham_{veh}$.

$^{\#}$ $p< 0.05$, $^{\#\#}$ $p< 0.01$ significantly different as compared to OVX_{veh}

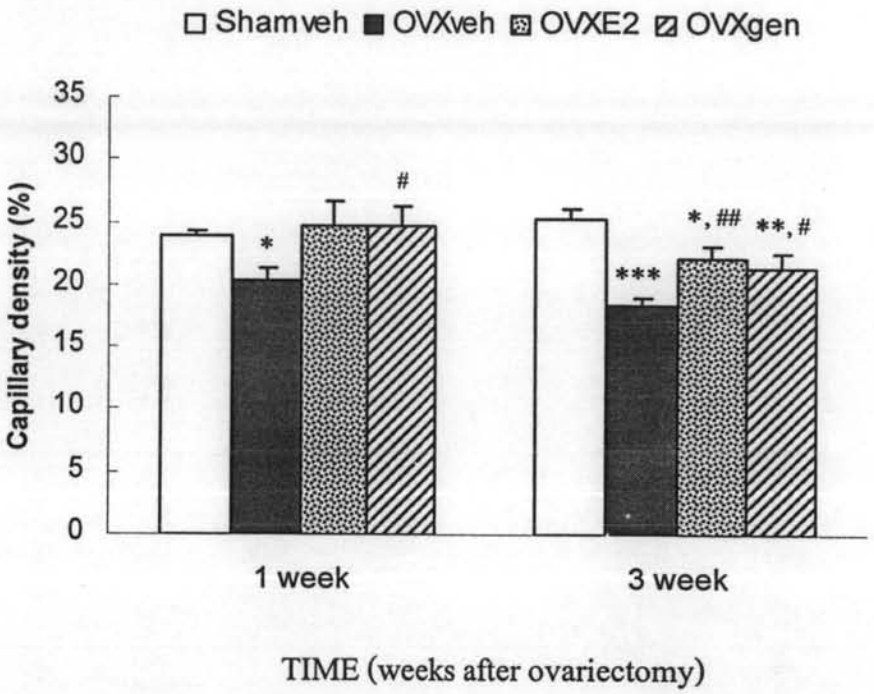
Figure 4.4.1 Serum VEGF levels (pg/ml) were determined from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy. (n = 8) Values were expressed as means ± SD.



* $p< 0.05$, ** $p< 0.05$ significantly different as compared to $Sham_{veh}$.

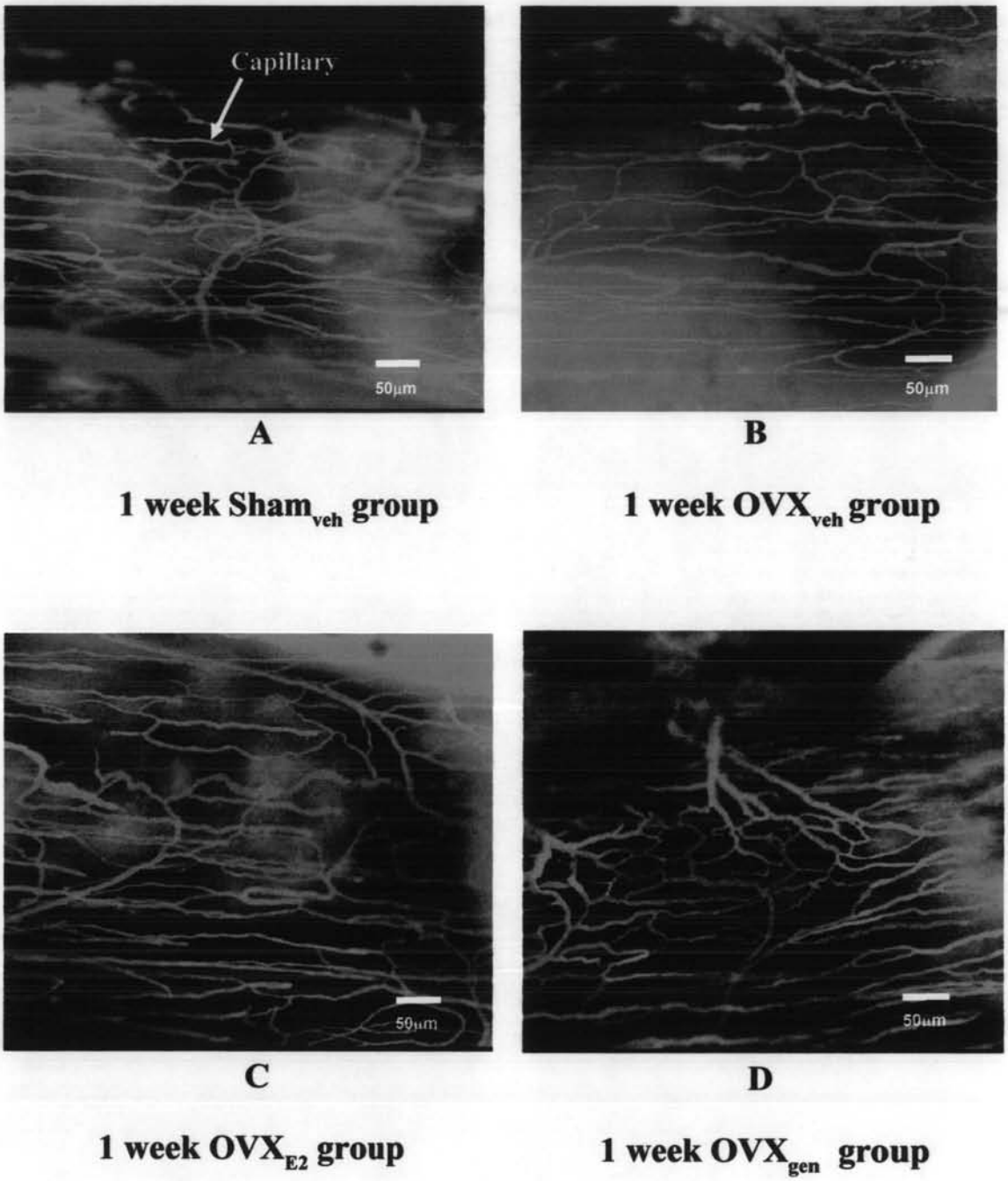
# $p< 0.05$ significantly different as compared to OVX_{veh}.

Figure 4.4.2 Capillary densities (%) were determined from : $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1 and 3 weeks after ovariectomy. (n = 8) Values were expressed as means ± SD.



* $p < 0.05$ significantly different as compared to $Sham_{veh}$.

# $p < 0.05$, ## $p < 0.01$ significantly different as compared to OVX_{veh}.



A

1 week Sham$_{veh}$ group

B

1 week OVX$_{veh}$ group

C

1 week OVX$_{E2}$ group

D

1 week OVX$_{gen}$ group

Figure. 4.4.3 Confocal laser images of microvasculature in femur bones were taken from: Sham$_{veh}$ (A), OVX$_{veh}$ (B), OVX$_{E2}$ (C) and OVX $_{gen}$ (D) at 1-wk after ovariectomy. (10X).

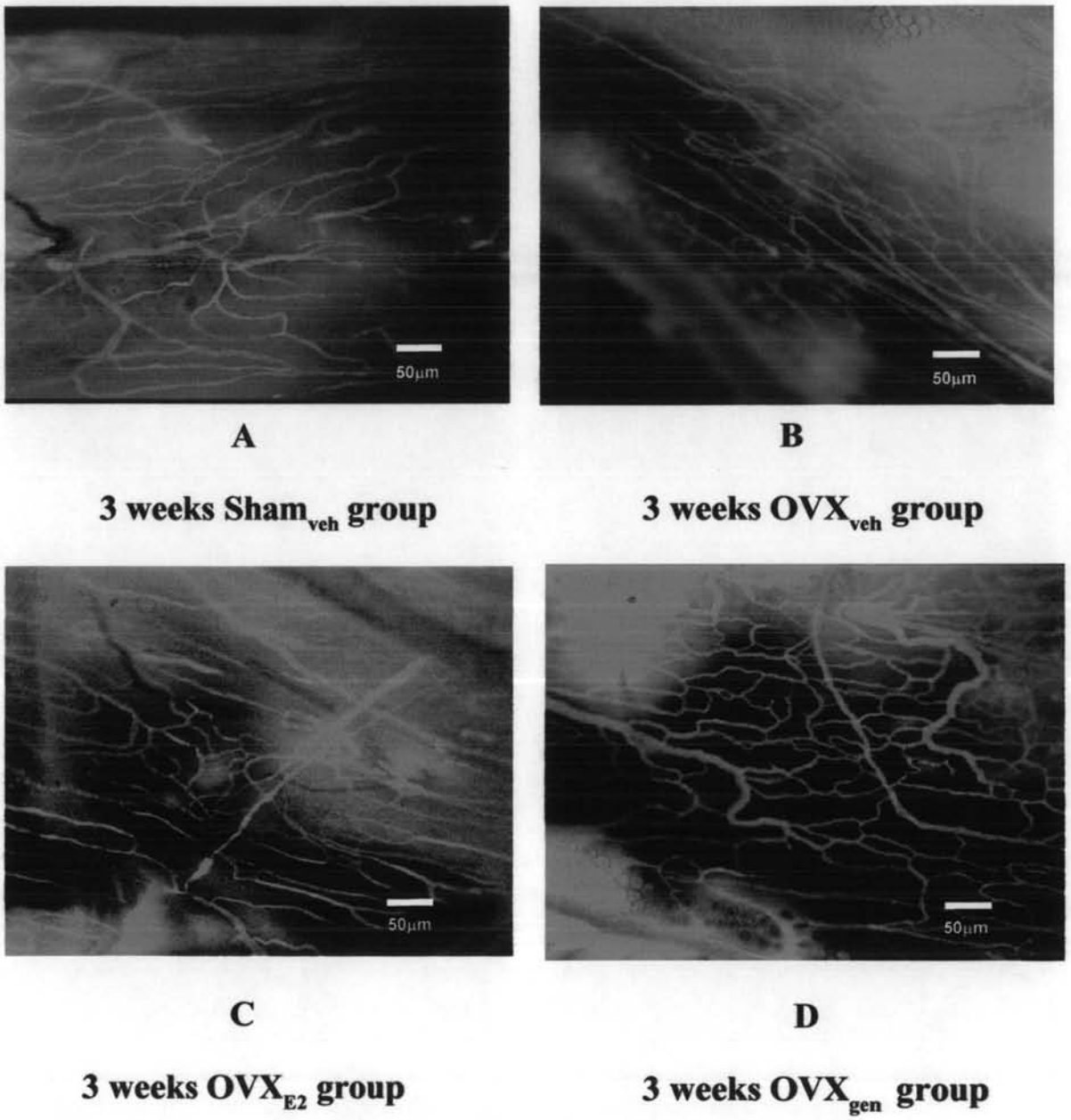


Figure. 4.4.4 Confocal laser images of microvasculature in femur bones were taken from: Sham$_{veh}$ (A), OVX$_{veh}$ (B), OVX$_{E2}$ (C) and OVX $_{gen}$ (D) at 3-wk after ovariectomy. (10X).

4.5. The relationships between serum VEGF, bone capillary density, and bone mineral content

The relationships between serum VEGF, capillary density, and bone mineral content of $Sham_{veh}$, OVX_{veh}, OVX_{E2} and OVX_{gen} at 1-wk or 3-wk were analysis. Figure 4.5.1 demonstrated the relationship between the serum VEGF concentration and capillary density at 1-wk after ovariectomy. No significant correlation was found between serum VEGF concentration and capillary density ($r = 0.142, p = NS$).

Figure 4.5.2 demonstrated that there was no significant relationship between the values of serum VEGF concentration and bone mineral content at 1-wk after ovariectomy ($r=0.0001, p = NS$).

Figure 4.5.3 demonstrated that there was no significant relationship between the values of capillary density and bone mineral content ($r = 0.003, p = NS$).

However, after 3-wk of ovariectomy the relationship between serum VEGF concentration and capillary density become significantly but weakly correlated ($r = 0.308, p < 0.05$)(Figure 4.5.4). In addition, at 3-wk after ovariectomy the relationship between serum VEGF concentration and bone mineral content shown in Figure 4.5.5 ($r =0.314, p < 0.01$) as well as the relationship between capillary density and bone mineral content was significantly but weakly correlation shown in Figure 4.5.6 ($r =0.382, p < 0.01$)

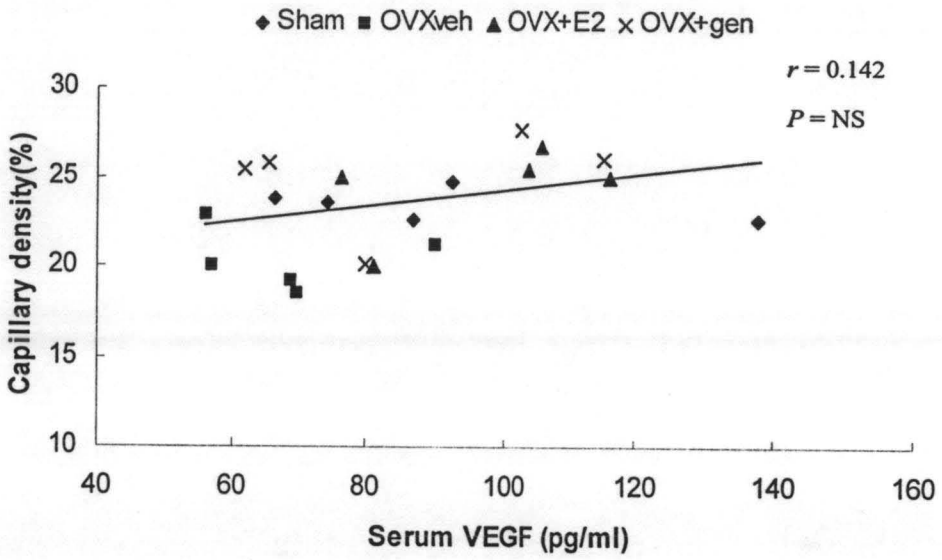


Figure 4.5.1 Relationship between serum VEGF and capillary density at 1-wk after treated with genistein.

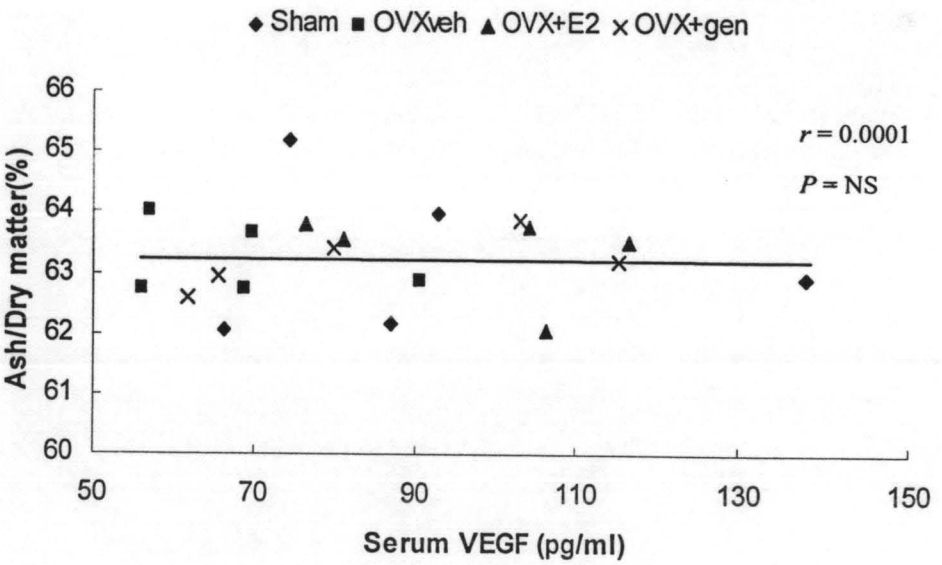


Figure 4.5.2 Relationship between serum VEGF and bone mineral content at 1-wk after treated with genistein.

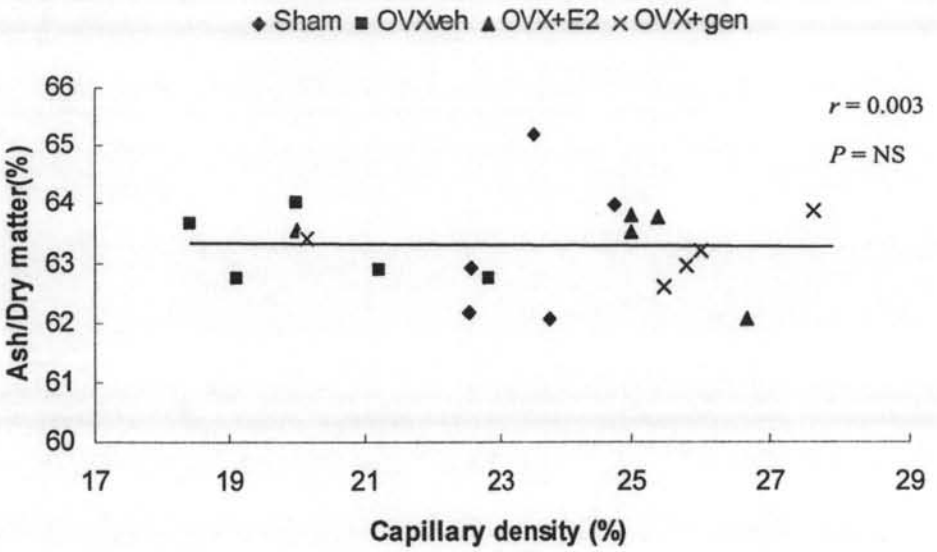


Figure 4.5.3 Relationship between capillary density and bone mineral content at 1-wk after treated with genistein.

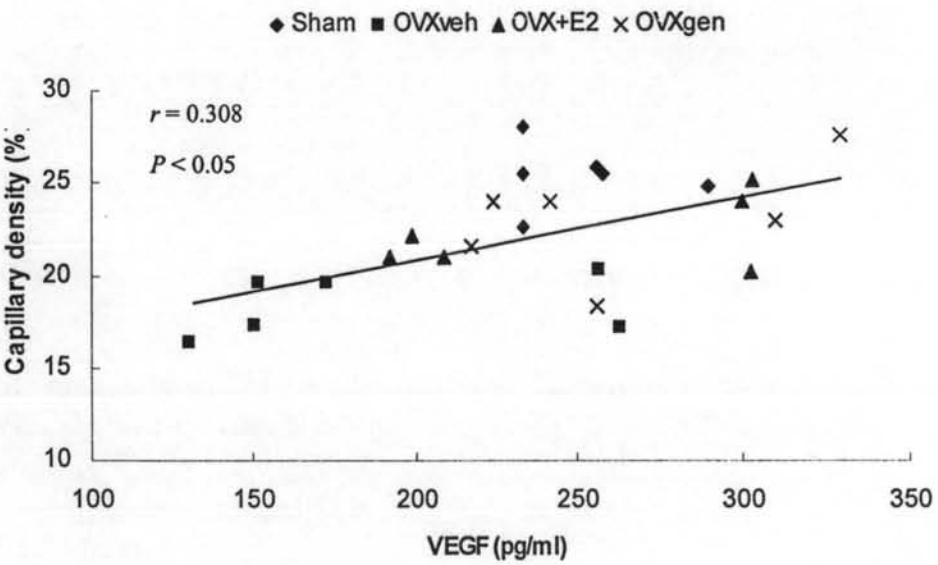


Figure 4.5.4 Relationship between serum VEGF and capillary density at 3-wk after treated with genistein.

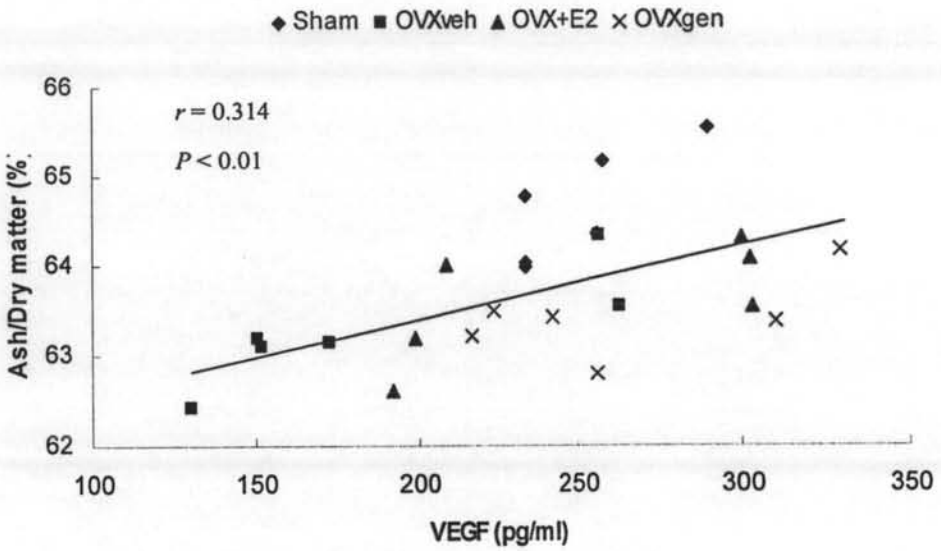


Figure 4.5.5 Relationship between serum VEGF and capillary density at 3-wk after treated with genistein.

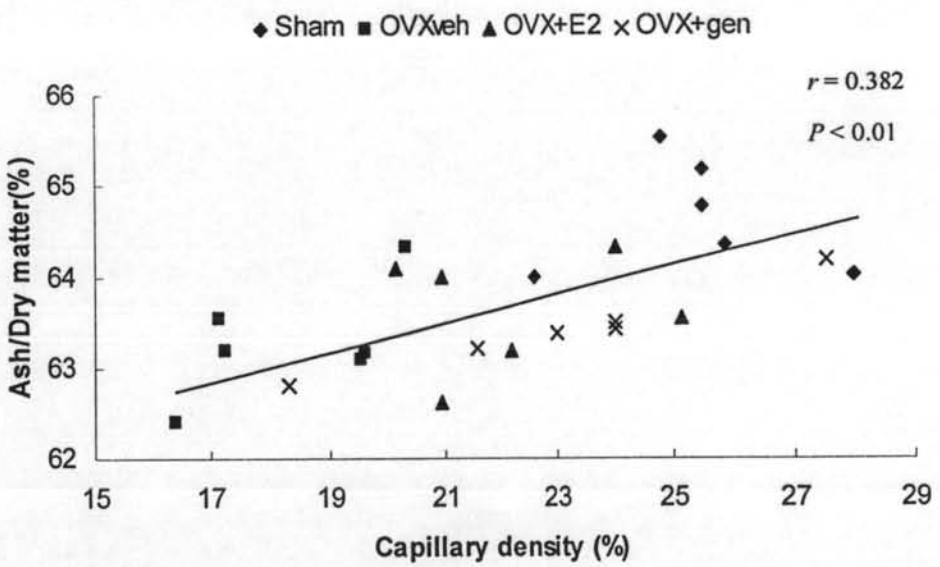


Figure 4.5.6 Relationship between capillary density and bone mineral content at 3-wkk after treated with genistein.