CHAPTER IV

RESULTS

1. Physiological and biochemical measurements

1.1 Body weight

The changes in body weight of all rats during the experimental period are presented as the increasing percentages of body weight compared with body weight at the beginning of the study (week 0) in each group. (Table 4.1 and Figure 4.1)

Throughout the experiments, the percentages of body weight of all groups were increase progressively. The increase of the percentages of body weight in OVX + *P. mirifica* was significant lower than those in OVX group (week 1-6) and Sham group (week 3-6), but not different from the OVX + Estrogen.

Table 4.1 The percentage increase of body weight compared with body weight at the beginning of the experiment (week 0) in study rats.

Groups	Time period (weeks)						
	0	1	2	3	4	5	6
OVX + *P. mirifica*	100.00	100.86±0.06*	102.17±0.17*	103.20±0.16*$^{+}$	104.05±0.21*$^{+}$	105.04±0.28*$^{+}$	106.18±0.38*$^{+}$
OVX + Estrogen	100.00	100.77± 0.10*	101.78± 0.13*	102.69± 0.17*$^{+}$	103.61± 0.21*$^{+}$	104.57± 0.34*$^{+}$	105.35± 0.32*$^{+}$
OVX	100.00	104.01± 1.09	107.01± 1.04	108.63± 0.84	111.20± 1.00	112.73± 1.14	115.23± 1.36
Sham	100.00	101.30± 0.14*	102.61± 0.16*	104.10± 0.23*	105.62± 0.30*	107.11± 0.35*	108.97± 0.46*

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

$^{+}$ $p < 0.05$, compared with Sham group

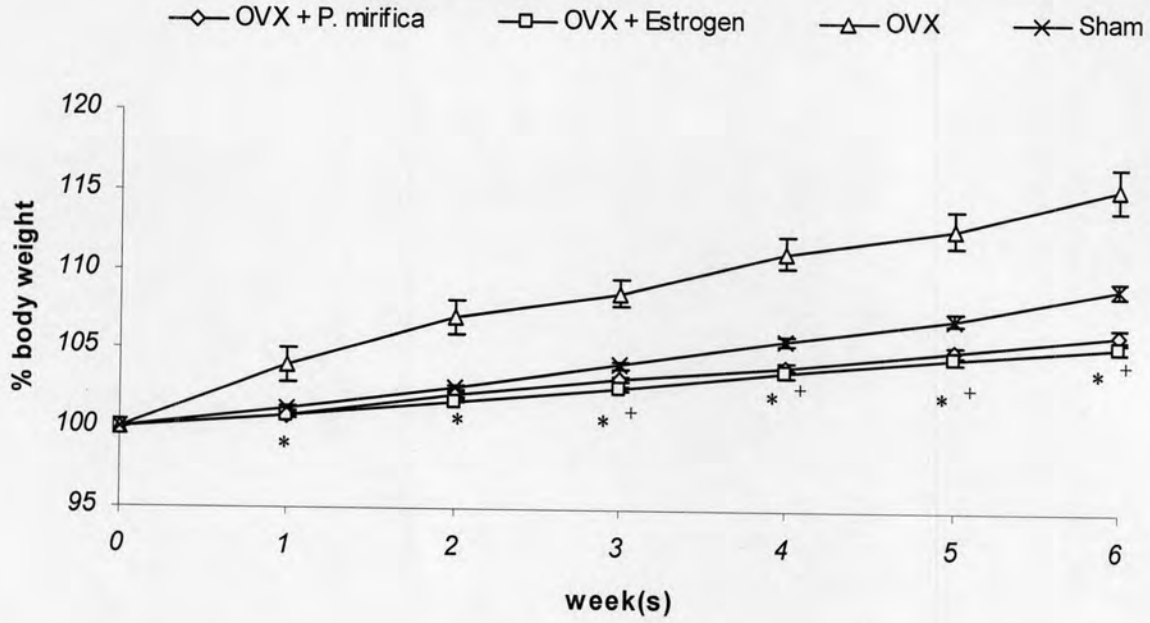

Figure 4.1 **The percentage increase of body weight compared with body weight at the beginning of the experiment (week 0) in study rats.**

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

+ $p < 0.05$, compared with Sham group

1.2 Blood biochemistry parameters

1.2.1 Determination of nitric oxide (NO) production

The results of nitric oxide (NO) production are presented in Table 4.2 and figure 4.2. The plasma level of nitric oxide in OVX + *P. mirifica*, OVX + Estrogen and Sham group were not significantly different from each other. However, the plasma NO levels of these three groups were significant higher than the OVX group.

Table 4.2 Plasma nitric oxide (NO) production of study rats.

Groups	Plasma NO production (uM)
OVX + *P. mirifica*	3.13 ± 0.14 *
OVX + Estrogen	2.71 ± 0.06 *
OVX	2.30 ± 0.03
Sham	2.85 ± 0.10 *

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

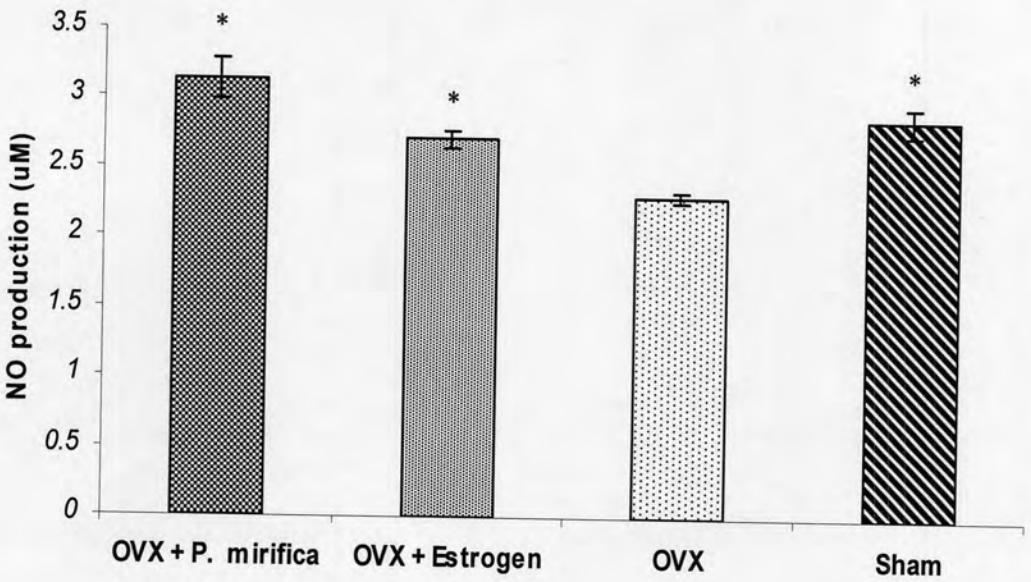

Figure 4.2 Plasma nitric oxide (NO) production of study rats.

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

1.2.2 Determination of alkaline phosphatase (ALP) level

Table 4.3 and Figure 4.3 show the results of alkaline phosphatase (ALP) level. The plasma ALP level of OVX group was significant higher than those in other groups. Whereas, the plasma ALP level of three groups (OVX + *P. mirifica*, OVX + Estrogen and Sham group) were not significantly different from each other.

Table 4.3 Plasma alkaline phosphatase (ALP) level of each group in the experiments.

Groups	Plasma ALP level (IU)
OVX + *P. mirifica*	99.94 ± 19.35 *
OVX + Estrogen	90.65 ± 14.44 *
OVX	133.60 ± 11.71
Sham	80.13 ± 7.18 *

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

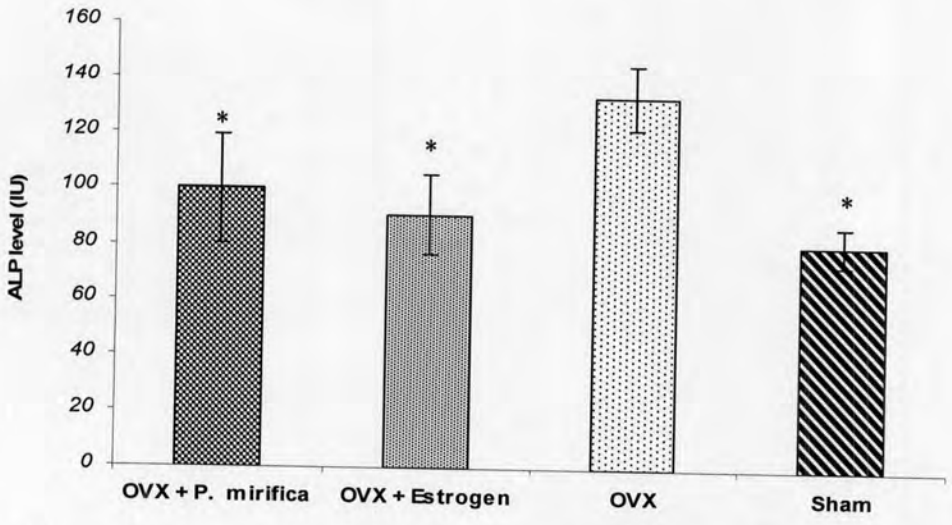

Figure 4.3 Plasma alkaline phosphatase (ALP) level of each group in the experiments.

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

1.2.3 Determination of lipid profiles

The results of plasma lipid levels of all experimental rats are summarized in Table 4.4 and Figure 4.4. The OVX group showed a considerable rise in all lipid parameters compared with that in the sham group. The total cholesterol and LDL-cholesterol level of ovariectomized rats were significantly higher.

The plasma triglyceride level in OVX + *P. mirifica* was lower than that in OVX group, but not significant different. The other lipid parameters of OVX + *P. mirifica* (total cholesterol, HDL-cholesterol and LDL-cholesterol) were not significant different comparing with the OVX group.

To compare with the OVX group, all lipid parameters in OVX + Estrogen were higher and significant differences in HDL-cholesterol, LDL-cholesterol and triglyceride were showed.

The atherogenic index, (total cholesterol - HDL-cholesterol) / HDL-cholesterol ratio, of OVX + *P. mirifica* and OVX + Estrogen group were lower than the OVX group but not significant different as demonstrated in Table 4.5 and Figure 4.5.

Table 4.4 Plasma level of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride of each group in the experiments.

Groups	Plasma lipid parameters (mg/dl)			
	Total cholesterol	HDL-cholesterol	LDL-cholesterol	Triglyceride
OVX + *P. mirifica*	78.75 ± 2.82	48.88 ± 1.67	27.15 ± 1.81	54.25 ± 8.23
OVX + Estrogen	84.88 ± 3.50	53.38 ± 1.92 $^{+}$	33.75 ± 2.14 $^{+}$	135.50 ± 12.06 $^{+}$
OVX	77.88 ± 4.32 *	45.63 ± 1.40	22.83 ± 3.10 *	67.63 ± 5.92
Sham	65.63 ± 2.49	43.38 ± 2.58	13.23 ± 1.63	57.25 ± 7.10

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with Sham group

$^{+}$ $p < 0.05$, compared with OVX group

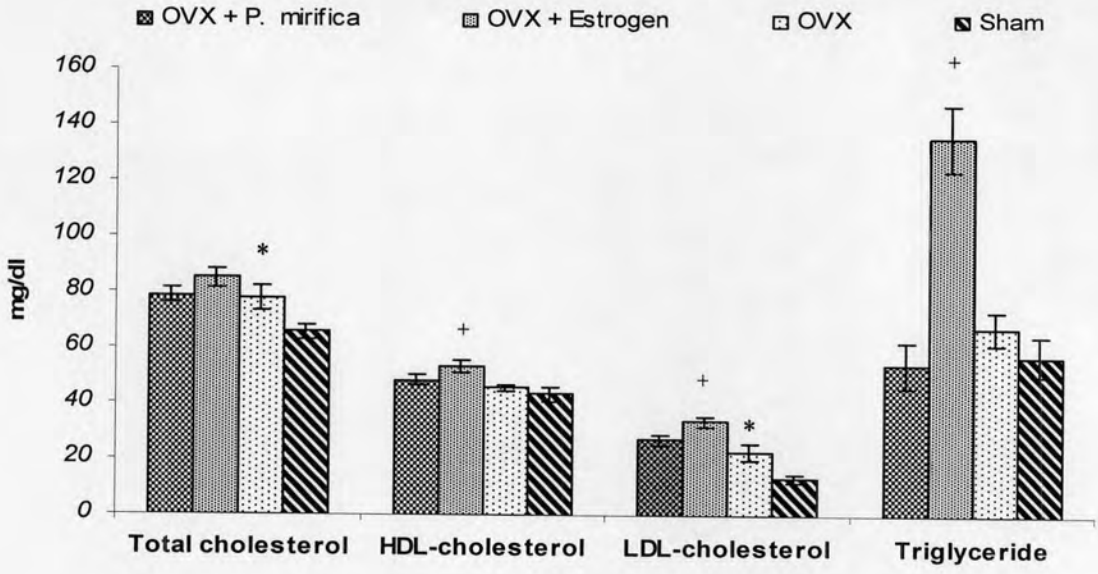

Figure 4.4 **Plasma level of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride of each group in the experiments.**

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with Sham group

+ $p < 0.05$, compared with OVX group

Table 4.5 **Atherogenic index, (total cholesterol - HDL-C) / HDL-C ratio, of each group in the experiments.**

Groups	Atherogenic index
OVX + *P. mirifica*	0.636 ± 0.110
OVX + Estrogen	0.594 ± 0.056
OVX	0.700 ± 0.056
Sham	0.528 ± 0.046

n = 8, Data are presented as mean ± S.E.M.

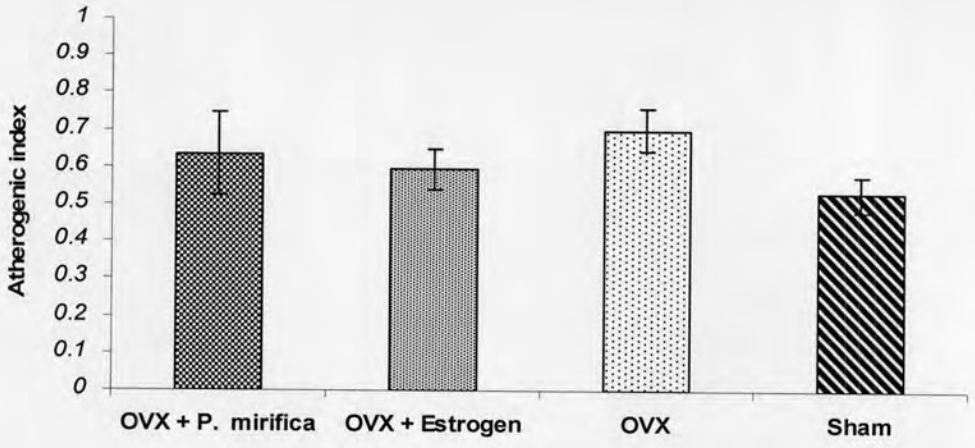

Figure 4.5 **Atherogenic index, (total cholesterol - HDL-C) / HDL-C ratio, of each group in the experiments.**

n = 8, Data are presented as mean ± S.E.M.

2. Effects of *Pueraria mirifica* on vascular functions and pathogenicities of thoracic aortas

2.1 Pharmacological measurements of vascular functions

2.1.1 Determination of noradrenaline-induced aortic contraction

The results of aortic contraction due to noradrenaline (NA) stimulation are presented in Table 4.6 and concentration response curves to noradrenaline are presented in Figure 4.6.

The cumulative addition of NA (10^{-9} M - 10^{-5} M) caused a concentration-dependent contraction on the rat thoracic aorta and a maximal contraction was obtained following the addition of 10^{-5} M NA. There were no significant different in the sensitivity and maximal response between the groups.

However, it was found that the contraction induced by higher concentration of NA was significant lower in the OVX group.

Table 4.6 **The percentages of aortic contraction induced by noradrenaline (NA) in isolated rat thoracic aorta.**

Groups	Percentages of aortic contraction					
	NA 10^{-9} M	NA 10^{-8} M	NA 10^{-7} M	NA 10^{-6} M	NA 10^{-5} M	NA 10^{-4} M
OVX + *P. mirifica*	5.02 ± 1.52	8.16 ± 1.59	32.30 ± 3.46	73.45 ± 5.74	100.00	89.91 ± 2.96*
OVX + Estrogen	7.30 ± 2.05	11.73 ± 2.22	31.74 ± 3.90	67.75 ± 4.96	100.00	90.03 ± 3.10*
OVX	3.56 ± 1.16	10.43 ± 3.71	34.01 ± 6.17	65.12 ± 5.51	100.00	77.32 ± 3.06
Sham	7.03 ± 2.16	15.10 ± 5.04	41.61 ± 4.63	78.90 ± 2.40	100.00	91.39 ± 1.79*

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

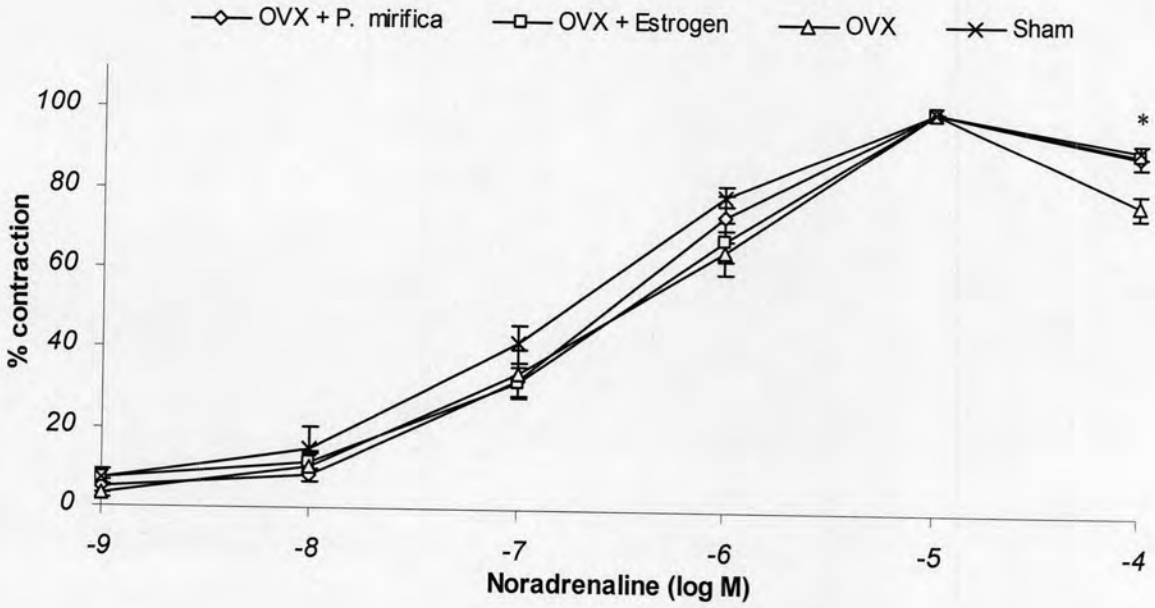

Figure 4.6 **Concentration response curves to noradrenaline (NA) of isolated rat thoracic aortas from non-OVX control, OVX, OVX+Estrogen and OVX+ *P. mirifica* groups.**

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

2.1.2 Determination of acetylcholine-induced aortic relaxation (Endothelium-dependent vascular relaxation)

The percentages of endothelium-dependent relaxation induced by Ach in the NA-precontracted isolated rat thoracic aortas are summarized in Table 4.7 and concentration response curves to Ach in NA-precontracted isolated rat thoracic aortas are presented in Figure 4.7.

The percentages of vascular relaxation in OVX + *P. mirifica* were significant higher than those in OVX group at 10^{-7} to 10^{-4} M of acetylcholine concentration.

Moreover, the vascular relaxations induced by Ach of OVX + *P. mirifica* were not significant different from the ovariectomized rats receiving estrogen treatment and sham operated non-ovariectomized rats.

Table 4.7 The percentages of endothelium-dependent relaxation induced by Ach in the NA-precontracted isolated rat thoracic aortas.

Groups	Percentages of aortic relaxation					
	Ach 10^{-9} M	Ach 10^{-8} M	Ach 10^{-7} M	Ach 10^{-6} M	Ach 10^{-5} M	Ach 10^{-4} M
OVX + *P. mirifica*	18.05 ± 5.12	44.29 ± 9.32	71.75 ± 12.39*	120.02 ± 21.80*	145.93 ± 23.71*	170.77 ± 28.24*
OVX + Estrogen	20.73 ± 4.29	39.20 ± 6.89	72.61 ± 13.57*	116.45 ± 24.08*	147.85 ± 26.81*	176.33 ± 33.62*
OVX	10.81 ± 3.32	28.54 ± 5.09	43.01 ± 6.54	66.88 ± 8.62	83.95 ± 10.89	103.05 ± 9.35
Sham	27.38 ± 8.86	53.27 ± 16.13	83.55 ± 19.78*	134.22 ± 33.61*	178.99 ± 47.02*	225.97 ± 64.75*

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

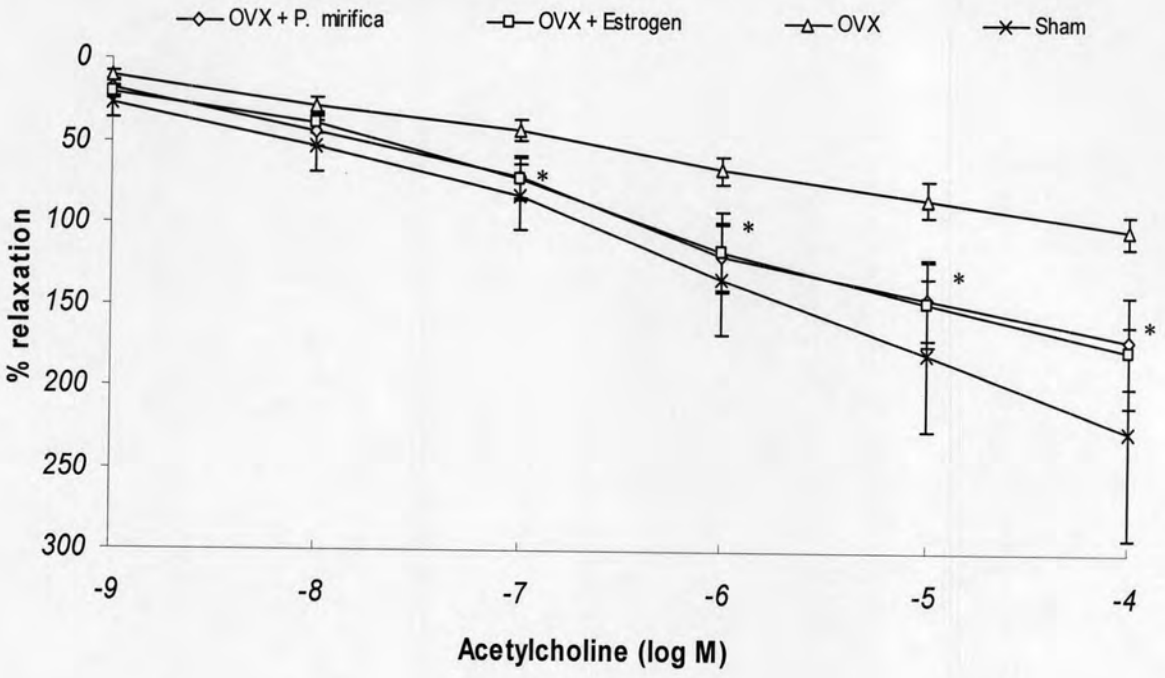

Figure 4.7 **Concentration response curves to acetylcholine (Ach) in noradrenaline-precontracted isolated rat thoracic aortas.**

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

2.1.3 Determination of sodium nitroprusside-induced aortic relaxation (Endothelium-independent vascular relaxation)

The percentages of endothelium-independent relaxation induced by SNP in the NA-precontracted isolated rat thoracic aortas are summarized in Table 4.8 and concentration response curves to SNP in NA-precontracted isolated rat thoracic aortas are presented in Figure 4.8.

No significant differences identified in vascular relaxation among the groups.

Table 4.8 The percentages of endothelium-independent relaxation induced by SNP in the NA-precontracted isolated rat thoracic aortas.

Groups	Percentages of aortic relaxation					
	SNP 10^{-9} M	SNP 10^{-8} M	SNP 10^{-7} M	SNP 10^{-6} M	SNP 10^{-5} M	SNP 10^{-4} M
OVX + *P. mirifica*	17.95 ± 3.24	70.36 ± 8.26	138.09 ±10.02	176.08 ± 17.86	193.13 ± 21.47	200.44 ± 22.32
OVX + Estrogen	29.97 ± 4.34	70.98 ± 8.45	152.19 ± 18.49	197.20 ± 26.99	206.34 ± 29.11	216.96 ± 29.75
OVX	27.64 ± 4.82	65.63 ± 10.94	115.65 ± 19.89	157.15 ± 19.86	170.90± 21.90	185.32 ± 23.45
Sham	32.58 ± 2.20	87.22 ± 16.84	143.90 ± 24.92	182.19 ± 23.42	194.07 ± 22.36	209.55 ± 18.79

n = 8, Data are presented as mean ± S.E.M.

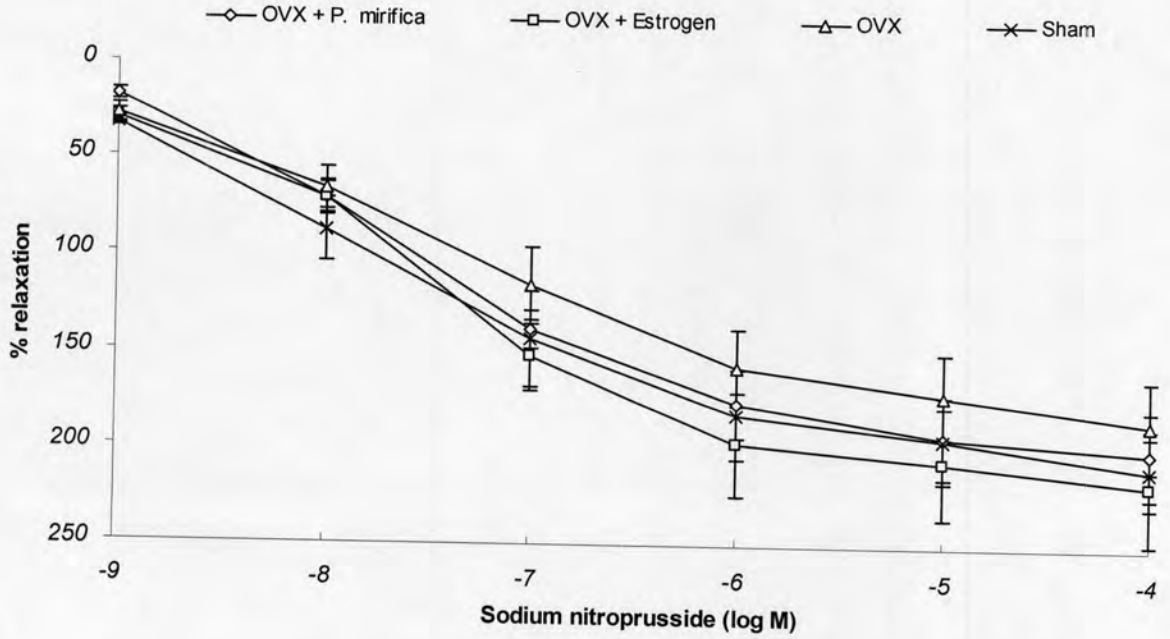

Figure 4.8 Concentration response curve to sodium nitroprusside (SNP) in noradrenaline-precontracted isolated rat thoracic aorta.

n = 8, Data are presented as mean ± S.E.M.

2.2 Pathogenicities of thoracic aortas

The pathogenicities of descending thoracic aortas were classified into 4 grades upon the following criterias.

Grade 0 : non-remarkable lesions

Grade +1 (mild) : focal medial smooth muscle cells and endothelial cells degeneration

: focal mononuclear cells adhesion and infiltration

Grade +2 (moderate): multifocal medial smooth muscle cells degeneration and disorientation

: multifocal endothelial cells degeneration

: focal internal elastic lamellae fragmentation

: multifocal mononuclear cells adhesion and infiltration

Grade +3 (severe) : diffuse medial smooth muscle cells degeneration and disorientation

: diffuse endothelial cells degeneration and erosion

: multifocal to diffuse internal elastic lamellae fragmentation and dissolution

: diffuse mononuclear cells or foam cells adhesion and infiltration

: Irregular lining of internal elastic lamina and irregular thickness of aortic wall

Microscopic studies of descending thoracic aortas in non-ovariectomized rats (Sham group) revealed mild degree of medial smooth muscle cells disorientation and vacuolation. Regular thickness and dense tunica adventitia were exhibited. Slightly mononuclear cells adhesion and invasion into subendothelium were also remarked. Most of endothelial cells and medial smooth muscle cells showed their integrity. The pathogenicities of descending thoracic aortas in Sham group were classified into mild degree of pathogenic lesions, grade 0 – (+1).

In untreated ovariectomized rats (OVX group) revealed that the aortic wall was irregular thickened and internal elastic lamina were arranged in irregular pattern. Severe diffuse disorientation and vacuolation of medial smooth muscle cells were marked. In addition, internal elastic lamellae were fragmented and dissoluted. Moderate to severe degree of endothelial cells necrosis and erosion were found. Moreover, mononuclear cells adhesion to luminal surface was found and some mononuclear cells were infiltrating into subendothelium. Loose tunica adventitia was also exhibited. The pathogenicities of descending thoracic aortas in OVX group were classified into severe degree of pathogenic lesions, grade +3.

In estrogen treated ovariectomized rats (OVX + Estrogen), moderate degree of medial smooth muscle cells disorientation and vacuolation were noted. Evidences of medial smooth muscle cells migration into subintima were found. Mild degree of endothelial cells degeneration and necrosis were also remarked. The pathogenicities of descending thoracic aortas in OVX + Estrogen were classified into moderate degree of pathogenic lesions, grade +2.

In *P. mirifica* treated ovariectomized rats (OVX + *P. mirifica*) revealed mild to moderate degree of medial smooth muscle cells disorientation and vacuolation. Slightly endothelial cells necrosis and erosion were demonstrated as well as slightly thickened of the aortic wall. The pathogenicities of descending thoracic aortas in OVX + *P. mirifica* were classified into moderate degree of pathogenic lesions, grade +2.

The histopathological figures of descending thoracic aortas of each group in the experiments were demonstrated in Figure 4.9.

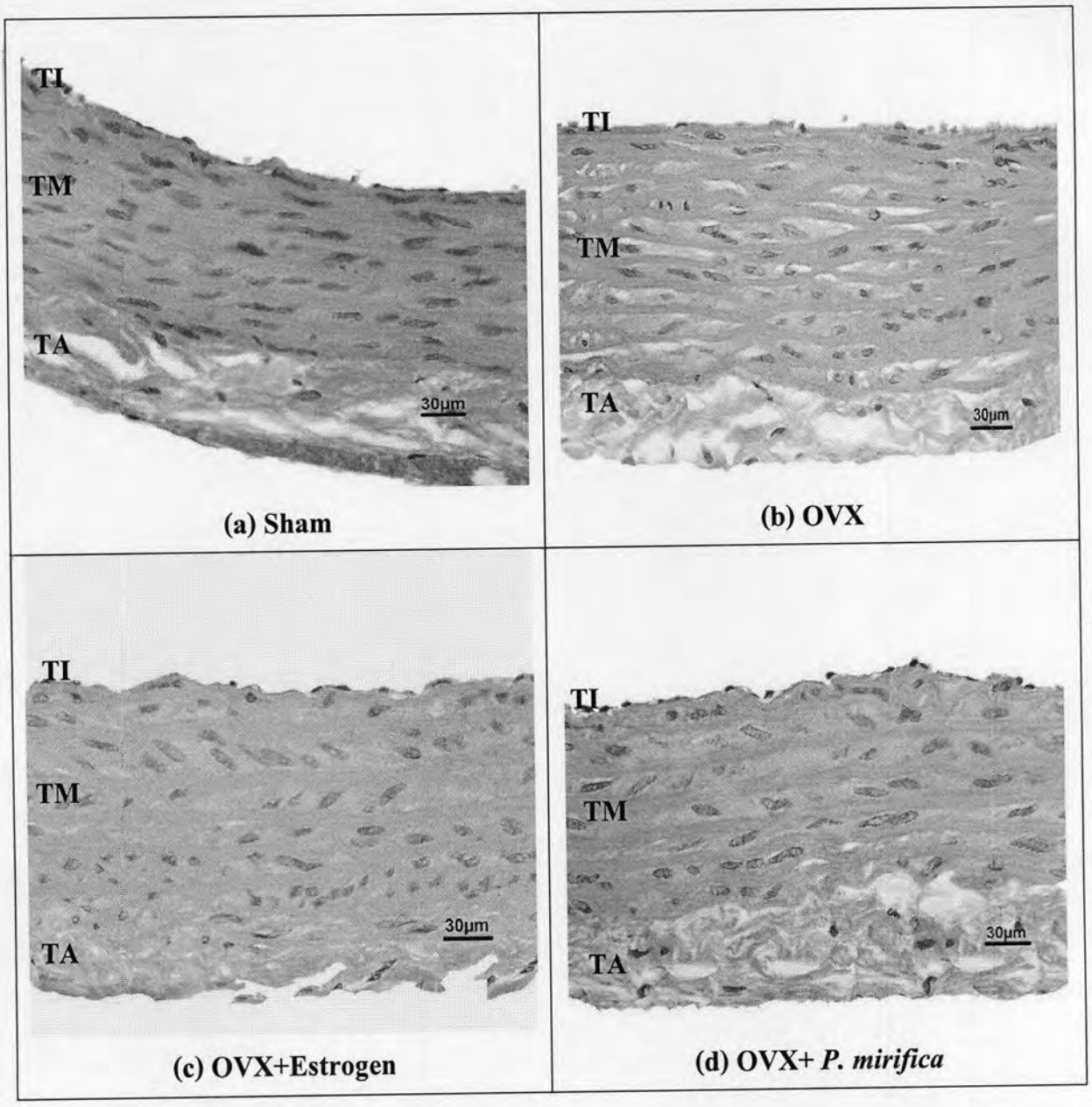

Figure 4.9 **Histopathological figures of descending thoracic aortas of each group in the experiments (H&E, X 400).**

TI: tunica intima, **TM**: tunica media, **TA**: tunica adventitia

(a) non-ovariectomized rats (Sham), **(b)** untreated ovariectomized rats (OVX), **(c)** estrogen treated ovariectomized rats (OVX+Estrogen), **(d)** *P. mirifica* treated ovariectomized rats (OVX+ *P. mirifica*)

3. Effects of *Pueraria mirifica* on bone mass and pathogenicities of femoral bones

3.1 Measurements of relative bone weight and relative ash weight

The results of relative bone weight and relative ash weight are summarized in Table 4.9 and Figure 4.10. The relative bone and ash weight of OVX group were significant lower when compared with that in the sham group.

The relative bone and ash weight of OVX + *P. mirifica* were significant higher than that in OVX group, and no significant differences found comparing with OVX + Estrogen and Sham group.

Table 4.9 The relative bone weight and relative ash weight of each group in the experiments.

Groups	Relative bone weight (%)	Relative ash weight (%)
OVX + *P. mirifica*	0.2170 ± 0.0049 *	0.1322 ± 0.0030 *
OVX + Estrogen	0.2163 ± 0.0043 *	0.1352 ± 0.0028 *
OVX	0.1989 ± 0.0041 $^{+}$	0.1222 ± 0.0021 $^{+}$
Sham	0.2285 ± 0.0068	0.1419 ± 0.0044

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

$^{+}$ $p < 0.05$, compared with Sham group

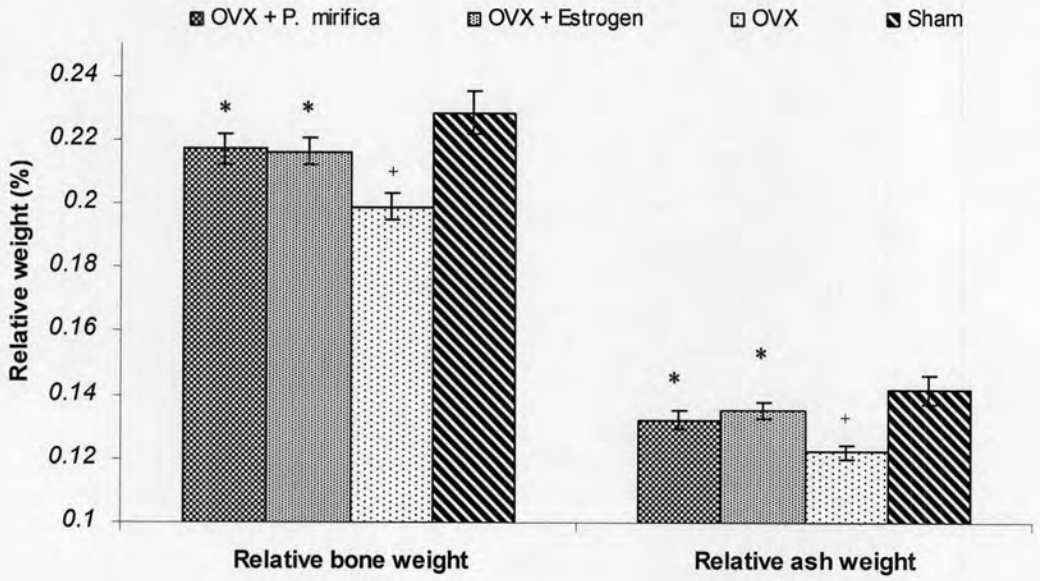

Figure 4.10 The relative bone weight and relative ash weight of each group in the experiments.

n = 8, Data are presented as mean ± S.E.M.

* $p < 0.05$, compared with OVX group

+ $p < 0.05$, compared with Sham group

3.2 Bone calcium determination

The results of bone calcium levels are presented in Table 4.10 and Figure 4.11. The bone calcium level of OVX + *P. mirifica* was considerably higher than that in OVX group, but still lower than OVX + Estrogen and Sham group. However, there were no significant differences of the bone calcium level between the groups.

Table 4.10 The bone calcium levels of each group in the experiments.

Groups	Bone calcium level (mg/g Ash)
OVX + *P. mirifica*	306.06 ± 6.33
OVX + Estrogen	306.37 ± 7.91
OVX	304.74 ± 2.98
Sham	307.01 ± 6.67

n = 8, Data are presented as mean ± S.E.M.

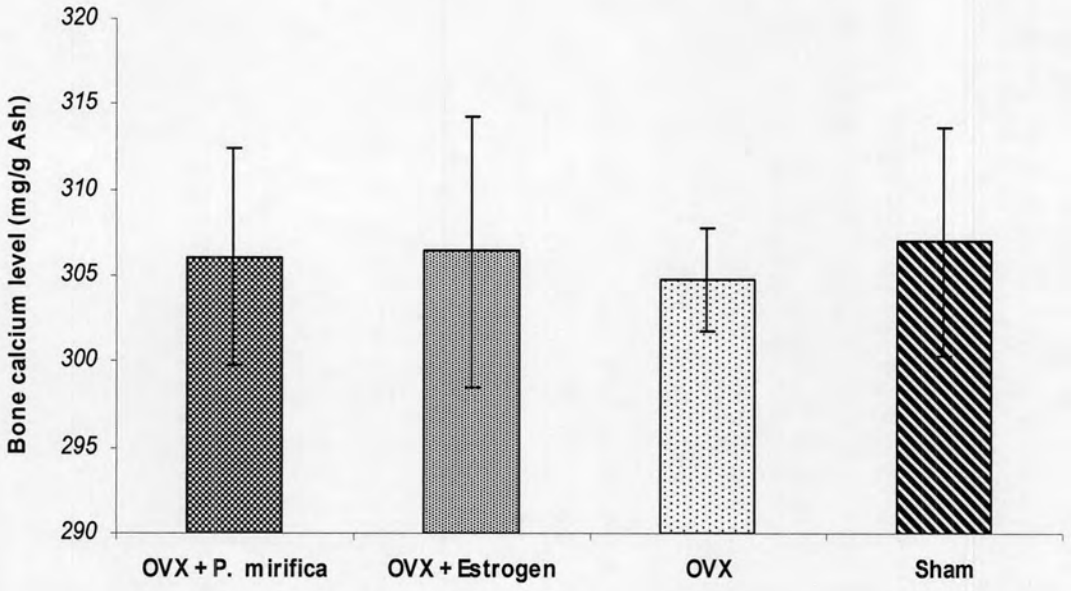

Figure 4.11 The bone calcium levels of the experimental rats.

n = 8, Data are presented as mean ± S.E.M.

3.3 Pathogenicities of femoral bones

The pathogenicities of femoral bones were classified into 4 grades upon these following criterias.

Grade 0 : non-remarkable lesions

Grade +1 (mild) : focal bone porous
: focal bone degeneration and necrosis

Grade +2 (moderate): multifocal bone porous
: multifocal bone degeneration and necrosis
: mild decrease and irregular distribution of osteocytes

Grade +3 (severe) : diffuse bone porous
: diffuse bone degeneration and necrosis
: moderate to severe decrease and irregular distribution of osteocytes
: thinning of bone shaft

Microscopic studies of femoral bones in non-ovariectomized rats (Sham group) revealed dense matrix and regular thickness of bone shaft. The amounts of osteocytes were normal and the distribution was regular. Therefore, the pathogenicities of femoral bones in Sham group were classified into grade 0 of pathogenic lesions.

In untreated ovariectomized rats (OVX group), the bone shaft revealed thinner when compared with Sham group. The decreasing in number and irregular distribution of osteocytes were exhibited. Diffuse bone porous, bone degeneration and necrosis

were also found. The pathogenicities of femoral bones in OVX group were classified into severe degree of pathogenic lesions, grade +3.

In estrogen treated ovariectomized rats (OVX + Estrogen) focal bone porous, bone degeneration and necrosis were found. Moderately decrease and irregular distribution of osteocytes were exhibited. The thickness of bone shafts between this group and Sham group were approximately equal. The pathogenicities of femoral bones in OVX + Estrogen were classified into moderate degree of pathogenic lesions, grade +2.

In *P. mirifica* treated ovariectomized rats (OVX + *P. mirifica*) moderate decreasing number and irregular distributions of osteocytes were marked. Focal bone porous, bone degeneration and necrosis were also exhibited. Moreover, an increasing in thickness of bone shaft was found, when compared with OVX group. The pathogenicities of femoral bones in OVX + *P. mirifica* were classified into moderate degree of pathogenic lesions, grade +2.

The histopathological figures of femoral bones were demonstrated in Figure 4.12.

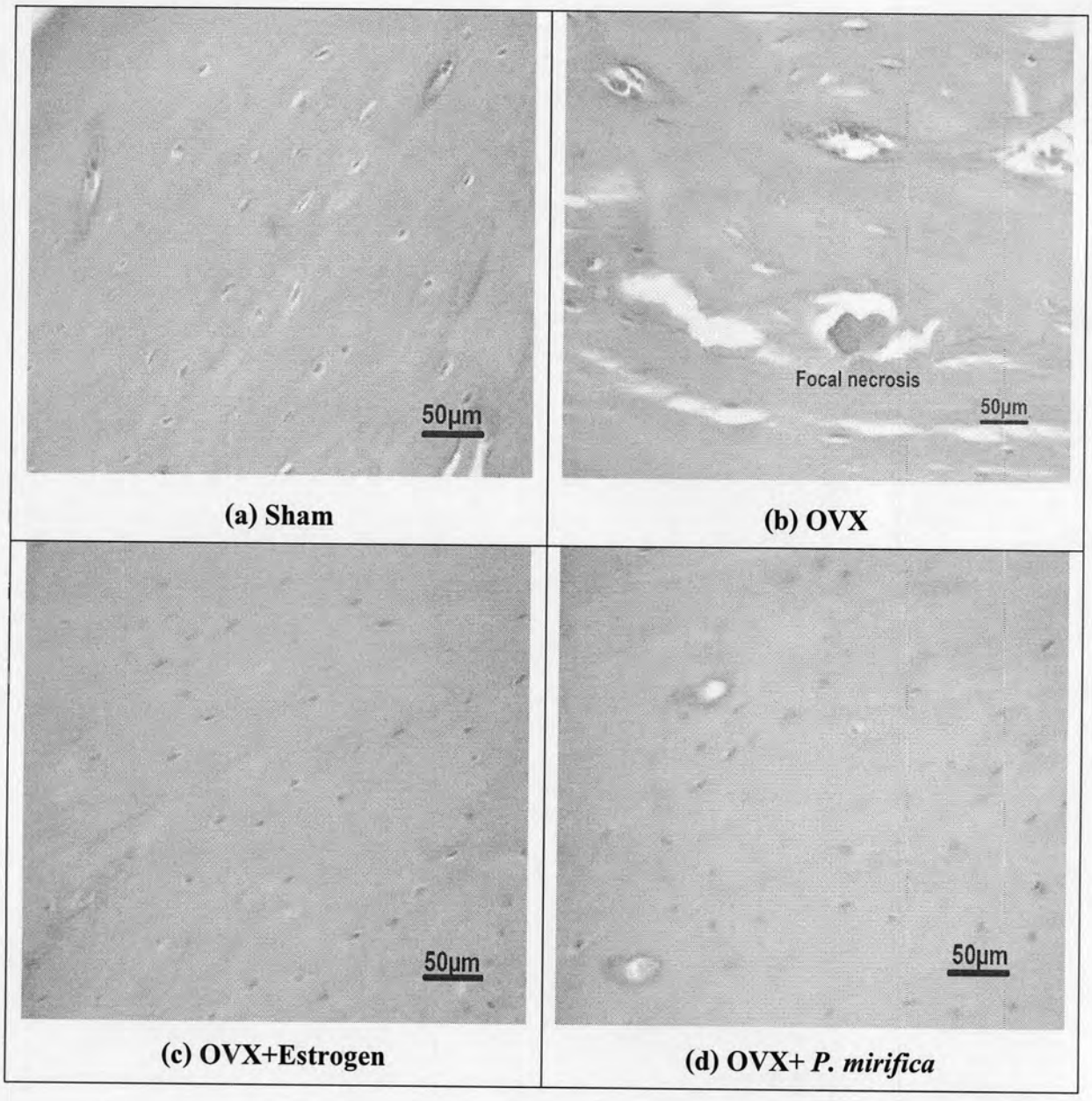

Figure 4.12 **Histopathological figures of femoral bones (H&E, X 400).**

(a) non-ovariectomized rats (Sham), **(b)** untreated ovariectomized rats (OVX), **(c)** estrogen treated ovariectomized rats (OVX+Estrogen), **(d)** *P. mirifica* treated ovariectomized rats (OVX+ *P. mirifica*)