



Effects of *Pueraria Mirifica* on Vascular Function of Ovariectomized Rabbits

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Estrogen stimulates endothelial nitric oxide (NO) production and attenuates endothelial dysfunction in ischemia/reperfusion and menopause. Recent studies have shown that phytoestrogens from dietary sources improve endothelial function and reduce cardiovascular risks. The Thai medicinal plant *Pueraria mirifica* (PM) contains many potent phytoestrogens including miroestrol and deoxymiroestrol but no study on vascular function has been established. Ground powder of PM was orally given to ovariectomized White New Zealand rabbits (OVX + PM group) ($n = 4$) weighing 3.2-4.0 kg at the dose of 100 mg/kg for 90 days. Saline-treated ovariectomized rabbits were assigned as a control group (OVX group) ($n = 5$). At the end of treatment thoracic aorta was isolated for functional evaluation. Maximal relaxant response to acetylcholine (ACh) was significantly increased (24%) with 3.5-fold decrease in EC₅₀ while no change in relaxant response to sodium nitroprusside was observed. Minimal and maximal responses to 17 β -estradiol (E2) were increased in the OVX + PM group and L-NAME (100 mM) attenuated E_{max} of E2. PM significantly decreased maximal contractile responses to norepinephrine (NE), but no change in EC₅₀ was observed. In addition to vascular study, the authors found no significant alteration in serum cholesterol, LDL, triglyceride, HDL, ALT, AST, alkaline phosphatase, and lipid peroxidation in OVX + PM rabbits. These data demonstrate that PM (100 mg/kg/d) improved endothelial function through NO-dependent pathway and increased response to E2 while sensitivity to NE was reduced. In addition, it had no impact on lipid profile, liver enzymes, and ALP activities. PM is a potential source of phytoestrogens for postmenopausal women to improve cardiovascular function or reduce cardiovascular risks.

Keywords: *Pueraria mirifica*, Ovariectomized rabbits, Endothelial function, Nitric oxide

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Estrogen plays a significant protective role in the cardiovascular system. Postmenopausal women experience a markedly increased risk of cardiovascular disorders, and some studies suggest that hormone replacement therapy reduces the mortality rate due to cardiovascular disease^(1,2). Accumulating evidence indicates that estrogen improves the vascular endothelial function via nitric oxide (NO)-dependent

pathway, which may contribute to its cardiovascular protective effects⁽³⁻⁵⁾. Moreover, low-dose hormone replacement treatment (HRT) improves lipid profiles and brachial artery endothelial function in women at risk for coronary heart disease⁽⁶⁾. However, several studies suggest that estrogens may increase the risk of developing breast and endometrial cancer⁽⁷⁻¹⁰⁾. Recently, selective estrogen receptor modulators (SERMs) have been introduced for postmenopausal new therapy. SERMs are compounds that interact with the estrogen receptors and have tissue-specific effects distinct from those of estradiol that they are estrogen agonists in certain tissues and antagonists

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in others. The main potential benefit of SERMs is that they selectively interact with specific receptors, coactivators and corepressors in different organ systems proving a better risk:benefit profile relative to standard hormone replacement therapy. While many attempts and several clinical trials have been conducted toward the development of ideal SERMs⁽¹¹⁻¹³⁾, benefits from the use of phytoestrogens in menopause has increasingly become evident⁽¹⁴⁻¹⁸⁾. Several phytoestrogens has been identified such as, genistein, daidzein, formononetin, equol, biochanin A while a search for phyto-SERMs is on the way. For instance, a soy-derived product (DT56a (Tofupill/Femarelle) and an extract from *Cimicifuga racemosa* (BNO 1055) possess SERM characteristics and may be used as alternative HRT^(19,20).

Recent studies show that phytoestrogens from dietary sources, such as soybeans, improve the impaired endothelial-dependent relaxation⁽²¹⁻²³⁾. In Thailand, the rhizome of *Pueraria mirifica* (iKwao Kreu) has long been used as a tonic for women in folk medicine. It contains several known compounds, including isoflavones, coumestans, and deoxymiroestrol^(24,25). The phytoestrogenic effects were demonstrated in recombinant yeast, MCF-7 cell proliferation and HepG2 cell transient transfection assay^(26,27). However, its effect on endothelial function has not been investigated. Here, the authors use the experiment model of ovariectomized female rabbits treated with *P. mirifica* to determine its effect on vascular reactivity in vitro. In addition, its effects on blood lipid profile, lipid peroxidation and liver enzyme function were also determined.

Material and Method

Animals

Female mature New Zealand White rabbits (3.2-4.0 Kg) were subjected to bilateral ovariectomy and they were divided into two groups; control (OVX, n = 5), and *Pueraria mirifica* (PM) treated group (OVX + PM, n = 4). Four weeks after surgery, control rabbits (OVX) received saline and OVX + PM rabbits received ground powder of PM (100 mg/kg/day) orally for 90 days. The PM powder was kindly provided by Assoc. Prof. Chaiyo Chaichantipyuth, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Plant material was standardized as a commercial product.

Vascular reactivity studies

At the end of the treatment period, rabbits

were euthanized with an overdose of sodium pentobarbital. Vascular study was performed as previously described⁽²⁸⁾. Briefly, thoracic aortas were removed and placed in cold Krebs solution. Aortic rings (5 mm in length) were then placed under 1 g of tension in an organ bath containing 10 ml Krebs solutions at 37°C and bubbled with 95% O₂ and 5% CO₂. The vascular reactivity studies included contractile response to norepinephrine (NE), relaxation response to acetylcholine (Ach) (Sigma), 17- β -estradiol (Sigma) in the presence or absence of L-NG-Nitroarginine methyl ester, hydrochloride (L-NAME) (Sigma), and sodium nitroprusside (SNP) (Sigma).

Serum lipid profile and blood chemistry

Blood was drawn from the ear vein and serum/plasma samples were corrected at week 0, 4, 8 and 12 of PM treatment. The analysis of cholesterol, LDL, triglyceride, HDL, ALT (SGOT), AST (SGPT) and alkaline phosphatase was determined by automatic analysis technique using commercially available reagents and performed at the Laboratory of Faculty of Allied Health Sciences, Chulalongkorn University.

Serum lipid peroxidation

The extent of cellular lipid peroxidation was determined by measuring the concentrations of thiobarbituric acid-reactive substances (TBARS) as described by Menendez et al⁽²⁹⁾ with minor modification. Briefly, sample oxidation was terminated by adding butylated hydroxytoluene (Sigma) to the final concentration of 10 μ M. Malondialdehyde (MDA) (Sigma) solutions (0 to 10 μ M) were used to generate standard curve. Two hundred microliters of standards or samples were incubated with thiobarbituric acid solution containing 0.5% thiobarbituric acid (Sigma), 6% trichloroacetic acid (Merck), and 1 mM EDTA (Sigma), at 95 °C for 90 min. The reaction mixtures were then centrifuged at 9,000 x g for 10 min. The supernatants were removed and measured the absorbance at 532 nm (Shimadzu UV-1601). Total protein content in serum was determined by Bradford assay using bovine serum albumin as protein standard (Biorad). TBARS values were expressed as MDA equivalences (nmol MDA/mg protein).

Data analysis

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using *t* test or one-way ANOVA with repeated measurement and with Bonferroni post hoc test. A value of *p* < 0.05 was



considered significant.

Results

Changes in vascular reactivity

Isolated vascular contractile responses are shown in Table 1 and Fig. 1. Significant alteration in acetylcholine-induced relaxation was observed (Fig. 1A). A statistically significant 3.5-fold reduction in EC₅₀ (522 ± 131 vs 150 ± 44 nM, OVX vs OVX + PM) was observed, with significant change (24% increase) in maximal relaxant response ($E_{\max} 62.5 \pm 0.6\%$ vs $82.4 \pm 0.8\%$). In contrast to diminished acetylcholine responses, no significant change in vasorelaxant response to the endothelium independent and spontaneous NO donor sodium nitroprusside was observed between treatments (Table 1 and Fig. 1B). Significant increases in minimal and maximal relaxation responses to 17β -estradiol were observed ($E_{\min} 5.11 \pm 1.62\%$ vs $29.80 \pm 9.04\%$; $E_{\max} 63.76 \pm 0.55\%$ vs $76.45 \pm 0.49\%$, OVX vs OVX + PM, $p < 0.05$). In addition,

the EC₅₀ of 17β -estradiol in PM treated rabbits increased by 3.8-fold (Table 1 and Fig. 1C). In the presence of a nitric oxide synthase inhibitor L-NAME, significant reduction in $E_{\max}$ was detected whereas no modification in EC₅₀ was found in aortic rings from OVX + PM rabbits (Table 1 and Fig 1D).

In addition to alteration in vasorelaxant effect, maximal vascular contractile response to α -receptor agonist phenylephrine was significantly decreased in the PM-treated group (OVX + PM vs OVX, $p < 0.05$) without change in EC₅₀ (Table 1, Fig 1E).

Serum lipid profile, liver enzyme, and alkaline phosphatase activities

Shown in Table 2 are serum lipid profiles of ovariectomized rabbits at week 0, 4, 8, and 12 following saline (OVX) and 100 mg/kg/day PM (OVX + PM) treatments. There was no significant change in total cholesterol, LDL, and HDL both when

Table 1. Vascular reactivities of aorta from ovariectomized rabbits

Tested agent	MAXIMUM RESPONSE ($E_{\max}$)		EC ₅₀ (M)	
	OVX	OVX + PM	OVX	OVX+PM
NE	1.73 ± 0.003 g	1.53 ± 0.02 g*	$2.69 \pm 0.825E-7$	$3.79 \pm 1.58E-7$
ACh	$62.43 \pm 0.63\%$	$82.10 \pm 0.76\%*$	$5.22 \pm 1.31E-7$	$1.50 \pm 0.44E-7*$
SNP	$94.80 \pm 1.12\%$	$96.14 \pm 0.53\%$	$8.39 \pm 6.80E-7$	$2.77 \pm 1.95E-6$
17β -estradiol	$63.76 \pm 0.54\%$	$76.45 \pm 0.49\%*$	$1.10 \pm 0.14E-6$	$4.19 \pm 1.23E-6*$
L-NAME+ 17β -estradiol	$80.94 \pm 1.58\%$	$66.75 \pm 1.408\%*$	$4.48 \pm 2.91E-6$	$4.00 \pm 1.22E-7$

* $p < 0.05$ vs OVX

Table 2. Serum lipid profile and some enzyme activities of ovariectomized rabbits

Parameter	OVX				OVX + PM			
	week 0	week 4	week 8	week 12	week 0	week 4	week 8	week 12
Total cholesterol (mg/dL)	57.8 ± 12.3	51.2 ± 9.4	76.0 ± 24.4	65.0 ± 10.0	98.0 ± 31.1	60.6 ± 9.0	59.8 ± 10.1	50.8 ± 7.6
Triglyceride (mg/dL)*	72.0 ± 11.14	68.2 ± 8.7	67.0 ± 5.2	80.0 ± 23.1	51.8 ± 9.23	47.8 ± 5.9	50.2 ± 2.6	61.6 ± 2.7
LDL cholesterol (mg/dL)	17.2 ± 5.9	12.2 ± 3.8	11.8 ± 2.5	11.2 ± 3.0	53.2 ± 24.1	12.8 ± 6.5	14.8 ± 3.4	12.5 ± 4.4
HDL (mg/dL)	43.0 ± 11.7	43.4 ± 7.1	46.5 ± 5.5	45.5 ± 8.0	49.0 ± 13.1	46.8 ± 5.2	37.9 ± 3.6	42.4 ± 7.0
ALT (SGOT) (IU/L)	23.4 ± 3.2	26.0 ± 2.2	19.8 ± 2.2	$36.4 \pm 10.6**$	17.8 ± 4.5	21.8 ± 4.8	16.4 ± 3.9	$37.6 \pm 9.1**$
AST (SGPT) (IU/L)	47.0 ± 11.9	38.4 ± 3.1	32.5 ± 5.0	44.6 ± 6.5	36.8 ± 8.4	43.6 ± 8.0	32.6 ± 5.8	50.6 ± 10.8
Alkaline Phosphatase (IU/L)	51.7 ± 13.6	49.4 ± 6.5	48.3 ± 11.0	51.0 ± 10.6	45.3 ± 7.6	52.0 ± 5.5	43.2 ± 4.0	42.6 ± 4.2

* $p < 0.05$, statistical different between OVX vs OVX + PM after allowing for effects of differences in weeks (one way ANOVA with repeated measurements); ** $p < 0.05$, statistical different when compared to baseline (week 0)

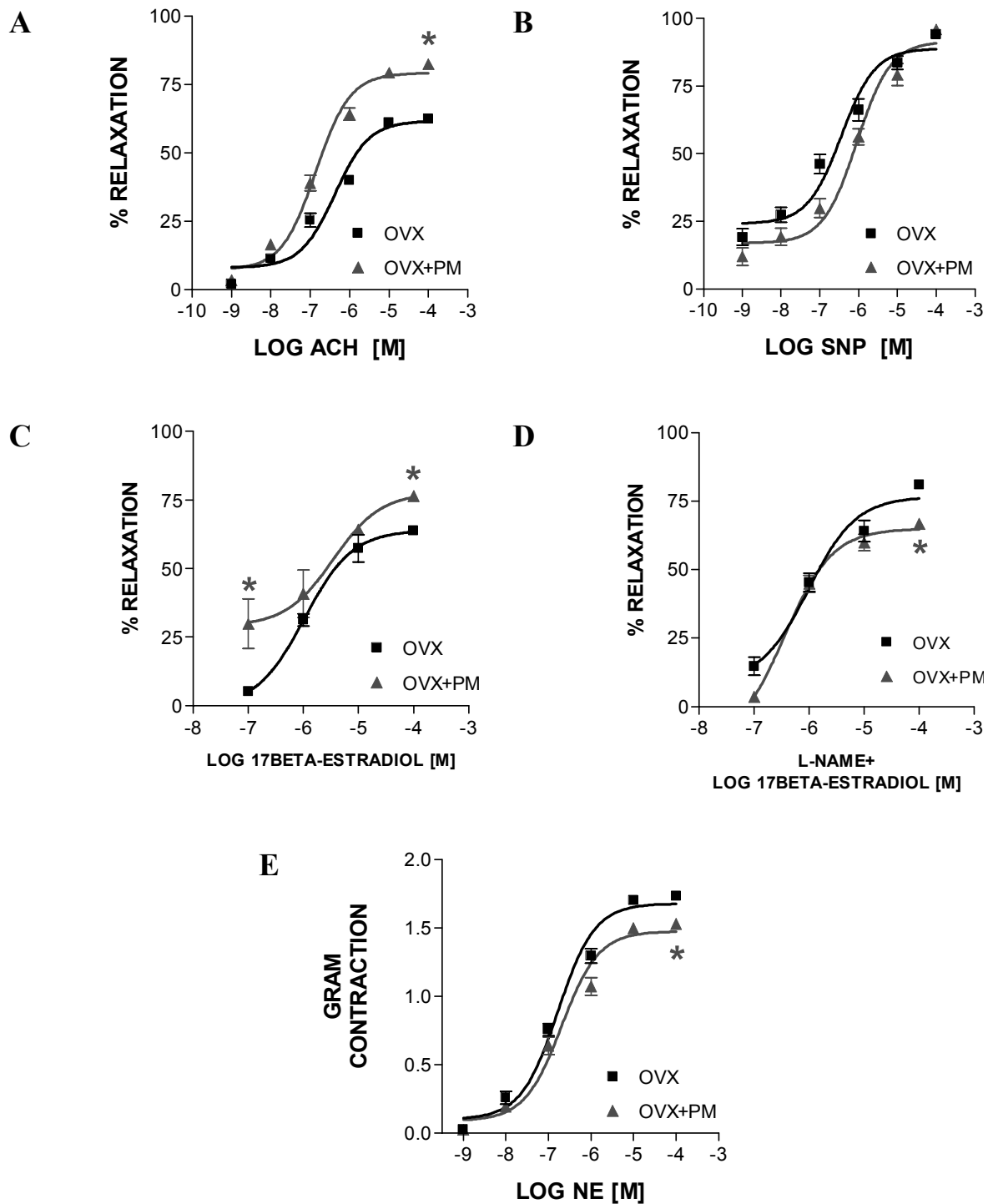


Fig. 1 Vascular responses in isolated aortic rabbit rings. **A:** concentration-response relationships for phenylephrine (1 nM to 1 μ M) in vessels obtained from OVX (squares) and *P. mirifica*-treated rats (triangles). Vasorelaxation response to acetylcholine (ACH), sodium niroprusside (SNP), 17 β -estradiol, and L-NAME (100 mM) + 17 β -estradiol are shown in **B**, **C**, **D**, and **E**, respectively. *, $p < 0.05$ vs OVX

compared between groups and weeks. The levels of triglyceride (TG) were significantly higher in the OVX group. This appears to have been caused by two animals in the group that had baseline and week 4 serum TG ranging from 93 to 103 mg/dL. The TG levels of these animals were decreased afterwards.

Liver function of the animals was evaluated by monitoring ALT and AST activities. There were significant increases in serum ALT levels of animals in both groups at week 12 ($p < 0.05$ vs week 0) while no change was observed in AST levels (Table 2).

Alkaline phosphatase (ALP) is an enzyme found in all tissues but present at high concentrations particularly in liver, bile ducts, placenta, and bone. Tissue injury or certain pathological conditions show increased enzyme levels in blood. In the present study, no significant alteration in ALP levels was observed either between groups and weeks.

Serum lipid peroxidation

Quantification of lipid peroxidation is essential to assess the role of oxidative injury. Lipid peroxidation is monitored by measuring the lipid peroxide product malondialdehyde (MDA). Since the authors' aim was to evaluate the effect of PM on serum lipid peroxidation in ovariectomized rabbits the authors show here only the levels MDA at the end of the treatment course (week 12). Although slight decrease in serum lipid peroxidation was observed the statistical analysis indicated no significant

difference (OVX 3.39 ± 0.78 and OVX + PM 2.82 ± 0.29 nmol/mg protein) (Fig. 2).

Discussion

Menopause is associated with impairment in endothelium-dependent vasodilation in normotensive and essential hypertension and estrogens provide protective effect on endothelial function⁽³⁰⁾. Endothelial plays a pivotal role in maintaining vascular tone through its release of endothelial derived relaxing factors (EDRFs), particularly nitric oxide (NO). Under normal physiological conditions the release of NO is activated by shear stress or stimulants such as acetylcholine and carbachol. Endothelial dysfunction is related to decreased activity of vasorelaxation through NO-dependent pathway and is evident in a wide array of cardiovascular risk factors, including hypercholesterolemia, thrombosis, atherosclerosis and hypertension^(31,32). Thus, lowering the cardiovascular risk factors may positively improve cardiovascular health and prevent disease progression.

In the present study, the authors used a well documented model of ovariectomized rabbits to evaluate the effect of *Pueraria mirifica* (PM) treatment on vascular function. The authors have demonstrated that long-term treatment of PM in ovariectomized rabbits improved nitric oxide (NO)-dependent endothelial function while no significant change in sodium nitroprusside (NO-independent pathway) response

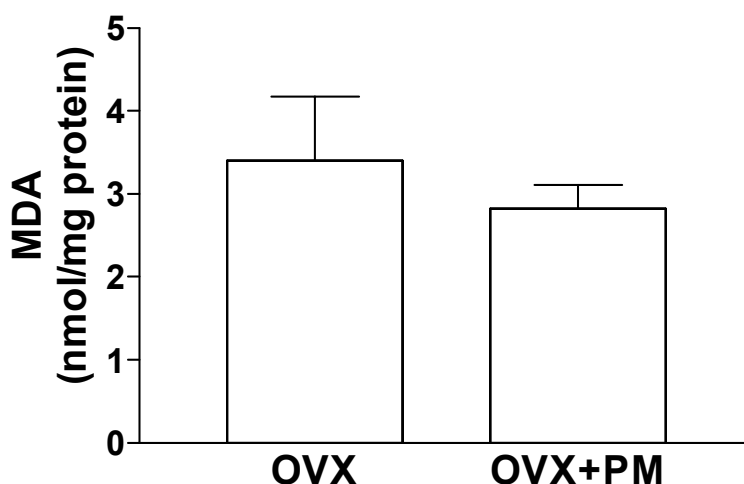


Fig. 2 Effect of *P. mirifica* treatment on serum lipid peroxidation. Sera of control ovariectomized rabbits (OVX) and *P. mirifica*-treated ovariectomized rabbits (OVX + PM) at 12-wk were analyzed for liperoxidation stable product malondialdehyde (MDA) as described in Material and Method. No significant decrease in serum MDA was observed in the OVX + PM group



was observed. This finding was consistent with previous studies that phytoestrogens improved the endothelial NO-dependent relaxation response both in an experimental model of ovariectomized rats⁽²¹⁾ and postmenopausal women⁽²²⁾.

It is well established that estrogen caused vasorelaxation is in part due to its stimulation of endothelial NO production and treatment with hormone replacement therapy attenuates endothelial dysfunction. Estrogen increases the translocation of endothelial NO synthase (eNOS or NOS III) in a Ca^{2+} -dependent manner⁽³³⁾ and upregulates eNOS expression in endothelial cells through the activation of eNOS promoters⁽³⁴⁻³⁶⁾. In the present study, the effect of PM on NO-dependent endothelial function was evaluated using 17β -estradiol (E2)-induced vasorelaxation. The present results provide evidence that PM modulates the characteristics of E2 response by increasing its minimal and maximal responses. However, elevation of EC50 observed may not agree with increase in sensitivity. It needs to be interpreted cautiously since the EC50 is calculated based on the following equation:

$$Y = \frac{\text{BOTTOM} + (\text{TOP} - \text{BOTTOM})}{1 + 10^{(\text{LogEC50} - X) \cdot \text{Hillslope}}}$$

The variable HILLSLOPE controls the slope of the curve. When HILLSLOPE is less than 1.0, the curve is more shallow. When HILLSLOPE is greater than 1.0, the curve is steeper. BOTTOM is the Y value at the bottom plateau; TOP is the Y value at the top plateau, and LogEC50 is the X value when the response is halfway between BOTTOM and TOP. Thus, when BOTTOM was relatively very high as appeared in dose-response curve generated from E2-induced vasorelaxation in aortic rings of PM-treated rabbits (OVX + PM, compared to vehicle-treated rabbits, OVX) the EC50 values are raised in parallel to BOTTOM.

Additionally, aortic rings from PM-treated rabbits had reduction in maximal response to E2 when preincubated with 100 mM L-NAME (an NO synthase inhibitor) while no change in EC50 was observed. The Emax and EC50 of E2 in the presence of L-NAME in aortic rings from OVX + PM were changed to the levels that were comparable to those of OVX rabbits. The results suggest that PM improves endothelial impairment in ovariectomized rabbits in part due to the enhanced production of endothelial NO. There are conflicting results in publications

regarding the mechanism by which E2 induces vasodilation. Some studies suggested that NOS inhibitors abrogate the vasorelaxant effect of E2^(37,38) while others observed that the direct vasodilatory effect of E2 is independent of the endothelium^(39,40). The inconsistent data found may be contributed by several factors such as animal species, models, type of vascular bed, and dose/duration of E2 treatment.

In addition to endothelial dysfunction, abnormal vascular responsiveness to vasoconstrictors may play an important role in pathoethiology of cardiovascular disease. The increased contractile potency of the alpha-adrenergic agonist NE has been demonstrated in vessels from portal hypertensive animals⁽⁴¹⁾ and heart failure rats⁽⁴²⁾ which resulted from decreased basal nitric oxide release. Therefore, sensitivity to vasoconstrictors may be another parameter useful in the assessment of vascular function. Consistent with increased in NO production observed in PM treated rabbits, PM also decreased maximum response to NE while no change in EC50 was found. The present data suggest that the increased vascular responsiveness to NO-dependent stimulator as well as reduction in sensitivity to vasoconstrictor may have beneficial role in cardio-protective effect.

Since dyslipidemia is an important cardiovascular risk factor, the authors evaluate the modification of lipid profile of ovariectomized rabbits as a consequence of long-term PM treatment. In addition, the authors evaluated its impact on liver enzyme function (ALT and AST) and alkaline phosphatase activity (ALP). There was no change in lipid profile of ovariectomized rabbits in both groups except that the basal levels of triglyceride in OVX were higher than that of the OVX + PM group. This was due to two outliers in the group. No significant moderation in liver enzyme activities was shown in the present study except that the increase in the liver enzyme ALT was found at the end of the treatment but the patterns were similar in both groups. Similarly, PM had no effect on ALP activity. Increased levels of serum ALP may indicate tissue injury but to specify the location of injury each tissue need to be evaluated separately. In addition, total serum ALP is commonly used as a marker of bone formation. However, ALP lacks sensitivity and specificity because it is produced not only by the osteoblasts during bone formation but also by the liver and intestinal tract. Thus, more specific isoenzymes should be used to determine tissue specificity.



In conclusion, the present study suggests that PM at 100 mg/kg/day improved vascular endothelial function and had no impact on lipid profile, liver enzymes, and ALP activities. PM is a potential source of phytoestrogens for postmenopausal women to improve cardiovascular function or reduce cardiovascular risks.

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ผลของกาวเครือขาวต่อการทำงานของหลอดเลือดในกระต่ายถูกตัดรังไข่

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เอสโตรเจนกระตุ้นการสร้างไนตริกออกไซด์ และช่วยลดความสูญเสียการทำงานของเซลล์เยื่อหลอดเลือด ในภาวะ ischemia/reperfusion และในหญิงวัยหมดประจำเดือน การศึกษาที่ผ่านมาแสดงให้เห็นว่า phytoestrogens จากแหล่งอาหารทำให้การทำงานของเซลล์เยื่อหลอดเลือดดีขึ้นและลดความเสี่ยงต่อการเกิดโรคหัวใจและหลอดเลือด ในประเทศไทยมีสมุนไพรกาวเครือขาว ซึ่งประกอบด้วย phytoestrogens ที่มีฤทธิ์แรง ได้แก่ miroestrol และ deoxymiroestrol แต่ยังไม่มีการศึกษาถึงผลของสมุนไพรชนิดนี้ต่อการทำงานของหลอดเลือด การศึกษานี้ใช้กระต่ายพันธุ์นิวซีแลนด์ที่ถูกตัดรังไข่โดยแบ่งกระต่ายออกเป็น 2 กลุ่ม กลุ่มหนึ่ง 4 ตัว ได้รับผงกาวเครือขาวในขนาด 100 มก./กก./วัน เป็นเวลา 90 วัน อีกกลุ่มหนึ่ง 5 ตัว ได้รับน้ำเกลือ จากนั้นจึงตัดหลอดเลือดแดงเอออด้าออกมาเพื่อศึกษาการทำงาน พบว่ากระต่ายกลุ่มที่ได้รับกาวเครือขาว มีค่า Emax ต่อ acetylcholine เพิ่มขึ้น 24% และมี EC50 ลดลง 3.5 เท่า แต่ไม่มีการเปลี่ยนแปลงต่อการตอบสนองต่อ sodium nitroprusside นอกจากนี้พบว่า Emin และ Emax ของการตอบสนองต่อ 17 β -estradiol (E2) มีค่าเพิ่มขึ้น และ L-NAME (100 mM) ทำให้ Emax ของการตอบสนองต่อ E2 มีค่าลดลง นอกจากนี้กาวเครือขาวทำให้ Emax ของการตอบสนองต่อ norepinephrine (NE) ลดลง และ พบว่าระดับ cholesterol, LDL, triglyceride, HDL, ALT, AST, alkaline phosphatase และ lipid peroxidation ในซีรัมไม่มีการเปลี่ยนแปลงหลังได้รับกาวเครือขาว สามารถสรุปได้ว่ากาวเครือขาวในขนาด 100 มก./กก./วัน ทำให้การทำหน้าที่ของเซลล์เยื่อหลอดเลือดดีขึ้นโดยผ่านกลไกของไนตริกออกไซด์ และยังเพิ่มการตอบสนองต่อ E2 และลดความไวต่อการกระตุ้นของ NE นอกจากนี้ยังไม่พบผลต่อระดับไขมันในเลือดและการทำงานของเอนไซม์ตับ และ alkaline phosphatase กาวเครือขาวจึงอาจเป็นแหล่งของ phytoestrogens สำหรับหญิงวัยหมดประจำเดือน เพื่อเพิ่มประสิทธิภาพของการทำงานในระบบหัวใจและหลอดเลือด หรือเพื่อลดปัจจัยเสี่ยงต่อการเกิดโรคหัวใจและหลอดเลือด

