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The Effect of Estrogen on Endothelial Permeability of Aorta and the Level of Serum Nitrite Concentration in Cholesterol-Fed Ovariectomized Rabbit

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ABSTRACT

Estrogen Replacement Therapy (ERT) in postmenopausal women may decrease the risk of Coronary Artery Diseases (CAD). We hypothesized that Nitric Oxide (NO) releasing due to ERT may be the essential factor by which endothelial permeability decreases. Four groups of ovariectomized rabbits were under investigation for five weeks. Groups 1 & 4 received high cholesterol diet and other two groups (2 & 3) had normal diet. Estradiol valerate (5 mg) was injected weekly in groups 1 & 2. Blood samples were taken before and after the experiment. Finally, the animals were sacrificed for endothelial permeability determination and pathological investigation of aortae. After five weeks, the total cholesterol, triglycerides, HDL and LDL were significantly different between high cholesterol-fed and normal diet groups ($P<0.05$). In cholesterol-fed groups, triglycerides concentration was also different significantly ($P<0.05$). Nitrite concentration was increased significantly in group 1, and it was different from other groups ($P<0.05$). A considerable decrease of aorta permeability was obtained in group 1 but it was not significantly different from group 4 ($P<0.1$). The considerable existence of fatty streaks was observed in the animals aortae of group 4, and it was significantly different from group 1 ($P<0.05$). It suggests that prevention of intimal collection of foam cells and fatty streak in aorta by estrogen may be exerted by NO production. ***Iran. Biomed.*** *J. 6 (2 & 3): 77-82, 2002*

Keywords: Estrogen, Nitric Oxide, Permeability, Aorta

INTRODUCTION

Estrogen Replacement Therapy (ERT) in postmenopausal women may decrease the risk of coronary artery disease (CAD). Estrogen may regulate nitrite production [1], and it also directly regulates vascular tone through the release of NO [2]. Tamura *et al.* [3] reported that ovariectomy increased the level of inducible endothelial NO synthase in rat, but treatment with estrogen inhibited the induction of inducible NO synthase in aorta. On the other hand, it has been found that NO synthase activity in ovariectomized rat arteries with ERT (ERT) is more than ovariectomized group [4]. A high NO production in the luminal endothelium of the arterial wall precedes a low cholesterol accumulation in rabbit with hypercholesterolemia [5]. The reduction in plaque size by estrogen is always increased by endothelial NO production [6]. The basal NO release was increased in estrogen therapy rabbits after four weeks, and circulating nitrite was increased by estrogen [7]. Estrogen treatment significantly increases the endothelial NO generation in ovariectomized animal [8]. A link was reported between the number of estrogen receptor and basal release of endothelial NO in aorta of mice [9]. The aorta of female rabbit released a greater amount of NO than did that of ovariectomized female and male rabbit [10]. Akishita *et al.* [11] reported that serum nitrite level was significantly lower in cholesterol diet rabbit than in normal diet, but a non significant trend towards higher nitrite level after estrogen replacement. Atherosclerosis process begins with existence of fatty dots and fatty streaks, and this process is characterized by increase of endothelial permeability of aorta [12]. Therefore we hypothesized that NO releasing due to ERT may be the essential factor for decrease endothelial

*Corresponding Author; Abbreviations: ERT, Estrogen Replacement Therapy; CAD, Coronary Artery Diseases; NO, Nitric Oxide; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein; EB,: Evan Blue; SE, Standard Error.

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permeability and fatty streaks in hypercholesterolemic condition in rabbits.

MATERIALS AND METHODS

Thirty-eight white female ovariectomized rabbits weighing 2-3 kg (2.4 ± 0.05) were obtained from the Pasteur Institute of IRAN. After three days of habituation to the laboratory and an over night fasting, blood samples were taken. Sodium and potassium were measured with flame photometer C. (ACE model F20, Italy). Lipids and lipoproteins were measured with Elan Eppendorf auto-analyzer (Germany), using Pars Azmoon kit (Iran). The animals were randomized in four groups. Cholesterol rich diet was prepared by addition of 1 g cholesterol in 4 ml olive oil to 0.1 kg of commercial rabbit chow. To avoid the effect of olive oil, normal diet was obtained by addition of 4 ml olive oil to 0.1 kg of rabbit chow. The groups were treated for five weeks as follows:

Group 1 (n = 10): Cholesterol rich diet + weekly intramuscular injection of 0.5 ml (5 mg) estradiol valerate (Abureihan Co. IRAN); Group 2 (n = 9): Normal diet + weekly intramuscular injection of 5 mg estradiol valerate; Group 3 (n = 9): Normal diet + weekly intramuscular injection of 0.5 ml distilled water and Group 4 (n = 10): Cholesterol rich diet + weekly intramuscular injection of 0.5 ml distilled water.

After five weeks, the animals of each group were randomly divided into two subgroups (A & B). Subgroup A was subjected for direct blood pressure measurement, plasma lipids and lipoproteins, electrolytes, and nitrite determination in addition of pathological studies of aortae. Subgroup B was subjected for direct blood pressure, plasma lipid, lipoproteins, electrolytes and nitrite determination in addition to endothelial permeability measurement of aortae.

Pathological studies of aorta. After an overnight fasting, the animal was anesthetized with 40 mg/kg sodium of pentobarbital (Sigma) and injected into ear vein. Femoral artery was cannulated for direct blood pressure measurement with a pressure transducer. Blood samples were obtained and the animals were sacrificed. The abdominal aorta was removed for pathological investigation via pathological procedures. Existence of fatty streak or fatty dots-an intimal collection of foam cells- was grading between zero (no fatty streak) to 4 (existence of fatty streak in all part of aorta).

Endothelial permeability determination of aorta. Endothelial permeability of aorta was measured by Evan Blue (EB) method that is described elsewhere [13]. Briefly, after an overnight fasting, the animal was injected with 10 mg/kg of EB solution. After three hours, the anesthetization and femoral cannulation were performed for direct blood pressure measurement. Blood sample was taken and the animal was sacrificed. The whole aorta was removed, opened and washed in saline for two hours. Excess water was removed and the aorta wet weight was determined. In order to extract the EB from the tissue, the aorta was kept in 5 ml of formamide at 80°C for two hours. After standing overnight, the concentration of EB (μg/g weight of tissue) in formamide solution was determined photometrically at a wavelength of 623 nm using standard curves.

Plasma nitrite determination. All blood samples centrifuged for 20 minutes (3500 c/s). The supernatant poured in lab tubes and kept in -70°C. The nitrite concentration was determined by Griess reagent Kit (Promega Corp; U.S.A., cat # G2930) photometrically at wavelength of 520 nm using standard curves.

Statistical analysis. The results are reported as mean ± SE. All groups were statistically compared with one way analysis of variance, using Duncan test for determining of any significant differences between the groups. Student's *t*-test was used for comparison between two groups. Statistical values of less than 0.05 were considered as significant.

RESULTS

The data of plasma lipids and lipoproteins are given in Table 1. No significant differences existed between the groups before treatment. After five weeks of treatment, the total cholesterol, triglycerides, HDL, and LDL in groups 1 & 4 were significantly different from normal diet groups ($P<0.05$). These parameters didn't indicate any significant differences between groups 2 & 3. Plasma triglycerides concentration in group 4 was also different from group 1 ($P<0.05$). The data of mean arterial, systolic and diastolic pressures are shown in Table 2 and there was not any significant differences between the groups. The data of electrolytes and protein concentrations are given in Table 3. Sodium concentration showed a significant difference in groups 1 & 4 from other two groups

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Table 1. The plasma lipid and lipoprotein concentration in four groups of experimental animal.

Parameter (mg/dl)	Group	Before	After
cholesterol	1	85.60 ± 10.36	515.15 ± 32.89
	2	98.00 ± 7.06	162.44 ± 20.11
	3	98.11 ± 8.64	152.16 ± 18.21
	4	94.20 ± 6.25	595.80 ± 75.60
HDL	1	33.30 ± 2.37	42.45 ± 4.80
	2	29.00 ± 1.23	34.33 ± 1.81
	3	31.55 ± 2.00	39.72 ± 2.16
	4	30.20 ± 2.99	55.30 ± 5.38
LDL	1	32.60 ± 10.95	403.00 ± 27.74
	2	48.00 ± 8.78	113.16 ± 22.29
	3	42.00 ± 8.84	95.33 ± 18.00
	4	36.40 ± 6.22	373.90 ± 33.96
triglycerides	1	93.10 ± 20.82	247.60 ± 25.4
	2	105.44 ± 13.87	67.27 ± 5.55
	3	122.44 ± 25.02	85.44 ± 6.59
	4	136.10 ± 19.18	454.10 ± 81.12

p values of before were not significant.; *p* values of after all were <0.05 (groups 1 & 4 are different from other two groups).

Table 2. Mean arterial, systolic and diastolic pressures after five weeks of treatment.

Group	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	Mean arterial pressure, (mmHg)
1	66.21 ± 2.15	48.00 ± 2.63	54.07 ± 2.15
2	70.75 ± 2.91	51.25 ± 2.33	57.75 ± 2.50
3	76.80 ± 2.38	50.40 ± 1.19	59.20 ± 1.52
4	66.57 ± 5.28	46.71 ± 5.36	55.33 ± 5.17

p value of all were not significant.

(*P*<0.05), but the sodium level in all groups are actually normal. The endothelial permeability of aorta in groups 1 to 4 are 4.80 ± 0.84, 7.10 ± 0.96, 4.82 ± 1.03, and 7.00 ± 1.06 microgram per gram tissue respectively. There is a considerable difference between groups 1 & 4 and groups 2 & 3 (*P*<0.1). The result of nitrite concentration is given in Table 4. A significant increase of nitrite content achieved in group 1, and it was significantly different from other groups (*P*<0.05). The results for pathological studies are shown in Figure 1. The considerable existence of fatty streaks was observed in group 4. The grade for fatty dots or fatty streaks in the aorta of group 1 was considerably less than group 4 (1.6 ± 0.67 vs 0.2 ± 0.19; *P*<0.05). There were no pathological changes in the aortae of groups 2 & 3.

DISCUSSION

The effects of estrogen on plasma nitrite concentration and endothelial permeability of aorta were the main objectives of this study. After five weeks of treatment, the plasma cholesterol, triglyceride, HDL and LDL levels of groups 1 & 4 were statistically different from the other two groups. It is obvious that these differences are directly related to animal regimen [13, 14]. According to these findings, it seems that estrogen does not attenuate plasma cholesterol, HDL, and LDL in high cholesterol fed rabbits. The data obtained for triglycerides of group 4 also showed significant difference from other groups. There is a possibility that the estrogen attenuates the level of triglycerides in group 1. The epidemiological data indicate that plasma cholesterol is not different from postmenopausal estrogen user and non-user

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Table 3. The serum electrolytes and protein concentrations in four groups of experimental animal before and after treatment.

Parameter	Group	Before	After
sodium	1	147.80 ± 1.20	144.30 ± 1.23
(meq/l)	2	143.22 ± 2.89	145.16 ± 1.13
	3	146.00 ± 0.86	147.88 ± 1.22
	4	148.30 ± 1.12	137.65 ± 4.29
potassium	1	4.56 ± 0.19	4.19 ± 0.24
(meq/l)	2	4.64 ± 0.24	3.61 ± 0.15
	3	5.63 ± 0.14	5.37 ± 0.85
	4	5.67 ± 0.35	4.89 ± 0.84
protein	1	6.81 ± 0.12	7.27 ± 0.16
(g/dl)	2	6.2 1± 0.18	6.87 ± 0.34
	3	6.38 ± 0.16	7.25 ± 0.32
	4	6.95 ± 0.16	7.46 ± 0.37

p values of before were not significant; *p* values of after only sodium was <0.05 (groups 1&4 are different from other groups), and others were not significant.

[15, 16]. The increase of HDL and triglycerides and decrease of LDL also were found in postmenopausal estrogen user [15-17]. On the other hand, experimental data in male cholesterol-fed rabbits did not confirm these epidemiological data completely [14]. These data were also different from epidemiological findings [15-17]. Nitrite concentration in group 1 was statistically different from other groups. Estrogen replacement in ovariectomized rabbit may increase NO synthase activity [4], and endothelial NO generation [8]. Akishita *et. Al.* [11] reported that serum nitrite level was decreased in cholesterol-fed, but increased by ERT [11]. Even though there are controversial data [7, 8, 11], it appears that both estrogen and cholesterol increase the plasma nitrite concentration. From the point of pathological view, in comparison between groups 1 & 4, considerable reduction of fatty streaks was seen in group 1, and more intimal collection of foam cells and fatty streaks was observed in group 4. There is a relation between endothelial permeability and existence of fatty streaks in animal aorta due to high level of plasma cholesterol [13, 14]. Finally, the considerable reduction of permeability in group 1 than group 4, is related to the estrogen. As a conclusion, estrogen has protective effect to prevent the formation of fatty streaks in aorta, and the reduction of fatty streaks is related to the increase of the serum nitrite concentration. More study is suggested for pathological changes of aorta endothelial findings by electron microscopy.

Table 4. Nitrite concentration (micromole/liter) in four groups of experimental animal before and after five weeks of treatment.

Group	Before	After
1	1.98 ± 1.37	138.20 ± 60.08*
2	5.19 ± 0.89	20.54 ± 3.91
3	8.23 ± 1.70	19.64 ± 4.30
4	4.34 ± 1.24	45.11 ± 13.79

* Significant difference from other groups (P<0.05).

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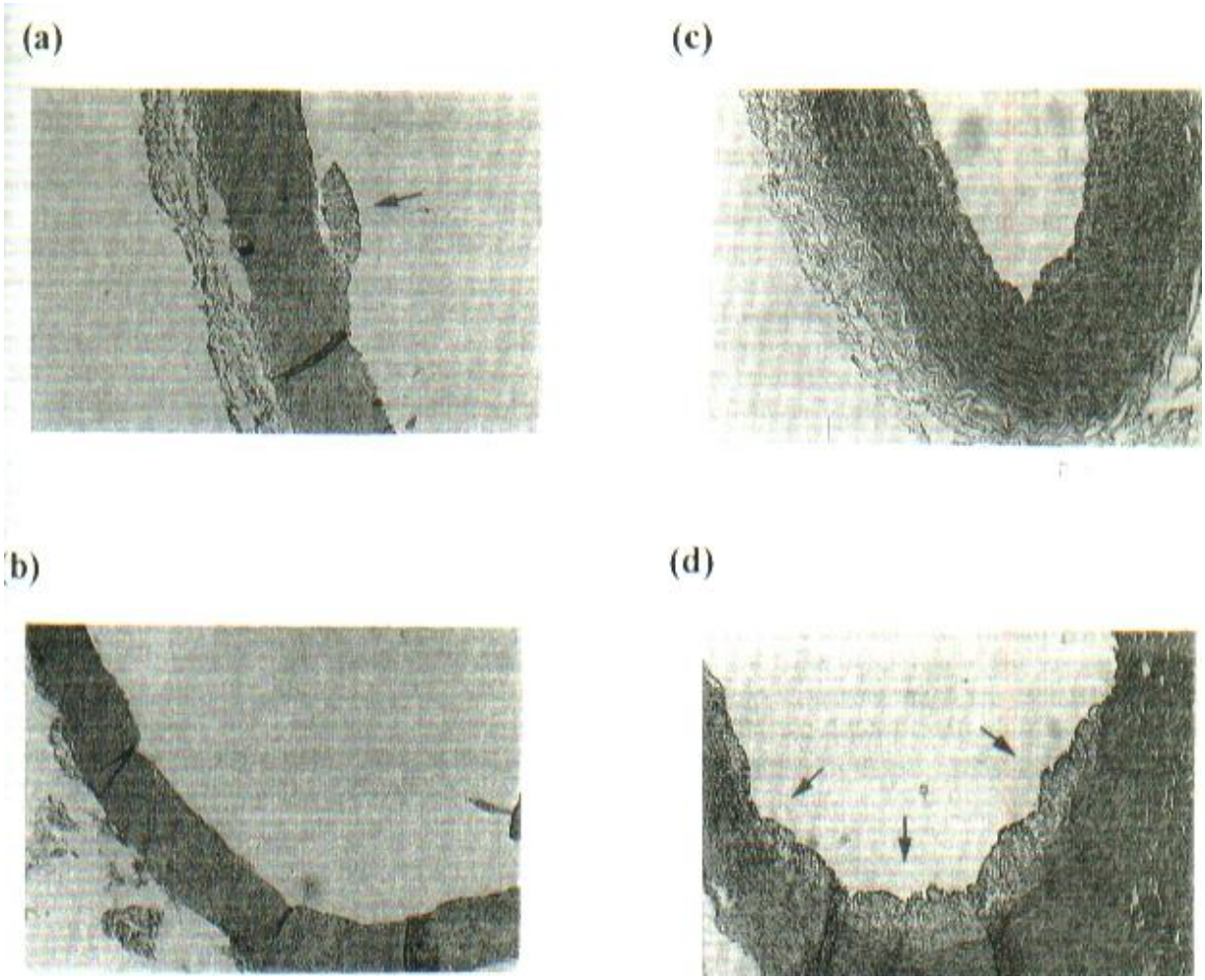

Fig. 1. Existence of Fatty Streak in sample animals of four groups of experiment. **(a)** From group 1 (100 x), existence of fatty streak in one point of intima (grade: 1); **(b)** From group 2 (100 x), no fatty streak (grade: 0); **(c)** From group 3 (200 x), no fatty streak (grade: 0); **(d)** From group 4 (200 x), existence of fatty streak in all part of intima (grade: 4)

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