

고지방식 가토에서 nitric oxide와 대동맥 동맥경화증에 대한 에스트로겐의 영향

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=Abstract=

Effects of Estrogen on Nitric Oxide and Aortic Atherosclerosis in Cholesterol-Fed Rabbits

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Background: Although estrogen is known to retard atherosclerosis, the mechanisms of which remain unclear. To examine the effect of estrogen on blood nitric oxide production and aortic atherosclerosis, we did an experiment with rabbits. **Material and Method:** Forty male New Zealand White rabbits aging 5 weeks were randomly divided as 10 normal diet(ND), 10 normal diet(ND) + transdermal estradiol-2 patches(END; 0.02 μ g/d), 10 hypercholesterol diet(CD ; 0.5% of diet; w/w), and 10 CD + transdermal estradiol-2 patches(ECD) group, respectively. After 12 weeks, we measured the serum estradiol-2, nitric oxide and cholesterol and examined the atherosclerotic lesion of thoracic and abdominal aorta with light and scanning electron microscopy. **Result:** The concentration of estradiol-2 was increased in both ED and ECD groups($p < 0.001$). The serum nitric oxide production of END, CD, and ECD groups decreased significantly than that of ND group($p < 0.001$). The total, HDL and LDL cholesterol concentrations were increase in both CD and ECD groups($p < 0.001$). Aortic lesion was observed only in CD and ECD groups, without any difference. On light microscopic examination, the lesions were consists mainly of accumulation of lipid-laden macrophages (foam cell). On scanning electron microscopic examinations, CD group showed scattered areas of endothelial damage. However, estradiol-2 administration to the cholesterol-fed animals did not significantly change these pathologic findings. **Conclusion:** The results shows hyperlipidemia brings a decreased production of nitric oxide, probably due to endothelial dysfunction. Estradiol-2 patch administration decreased nitric oxide level in male cholesterol-fed rabbits with unknown mechanism. The transdermal estradiol-2 patch did not show any effect on blood cholesterol level. The estrogen did not cause any change on atherogenesis on both thoracic and abdominal aorta. The gender difference and administration

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method may offset the beneficial effect of estrogen on atherogenesis. To gain a complete understanding for the action of estrogen on atherogenesis, further detailed studies for both genders are needed.

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Key words : 1. Estrogen
2. Atherosclerosis

INTRODUCTION

The incidence of cardiovascular disease, the leading cause of mortality in western societies, is higher in men than in premenopausal women but the difference is disappeared in postmenopausal women. Many epidemiological data support a role of estrogens in this antiatherogenic effect, prompting recommendations for their widespread use in postmenopausal estrogen replacement therapy. The Nurses' Health Study reported about 50% reduction in cardiovascular risk in postmenopausal women currently on estrogen therapy compared with women who had never used estrogens¹⁾. The beneficial effect of estrogen therapy in postmenopausal women may in part result from increases in HDL cholesterol and reduction in LDL cholesterol to a more favorable ratio, retarding atherogenesis^{2,3)}. However, the mechanism by which this protection is mediated remains largely unresolved, because beneficial effects of estrogen on the blood lipid profile account for only 20~30% of the overall protection⁴⁾.

There are many studies about the other beneficial action of estrogen on atherogenesis. Estrogen increases cardiac output and decrease vascular resistance, systolic and diastolic blood pressure^{5,6)}. Estrogen alternates hemostatic factor and inhibits platelet aggregation⁷⁾ and cholesterol deposition in the arterial wall⁸⁾. In addition, a number of animal studies strongly suggest a direct effect on the vascular wall^{9,10)}. However, the mechanisms of these artery wall effect remain obscure.

Estrogens have been reported to increase endothelial nitric oxide synthase expression in experimental animals^{11~13)}. Increased nitric oxide in blood would promote vasodilation, inhibit proliferation of the adjacent vascular smooth muscle, reduce platelet aggregation, and inhibit monocyte adhesion to the endothelium and the inflammatory reaction induced by cytokines, all key contributors in the development of atherosclerosis.

To examine the effect of estrogen on nitric oxide production and atherogenesis, we undertook the present study to determine

whether the early atherosclerotic lesion development and nitric oxide production are effected after administration of 17-beta estradiol sub 2 to cholesterol-fed rabbits. The production of NO, which is a very unstable molecule, was assessed by monitoring serum levels of nitrite and nitrate, the two stable metabolite of NO metabolism. In addition, we measured the serum total, HDL and LDL cholesterol, and triacylglycerol concentrations.

MATERIAL AND METHOD

Experimental design

Forty young male New Zealand White Rabbits aging 5 weeks were randomly divided into 4 groups: (1) 10 on normal diet (ND; Purina pellet for rabbit, Korea), (2) 10 on ND + transdermal 17-beta estradiol sub 2 patches (END; 0.02 μ g/d, Estraderm MX; CIBA-GEIGY Limited, Basel, Switzerland), (3) 10 on hypercholesterol diet (CD; 0.5% of diet; w/w), and (4) 10 on a CD + transdermal estradiol sub 2 patches (ECD).

Cholesterol was purchased from Sigma Chemical Company (USA). Rabbits were fed the respective diets for 12 weeks. Blood samples were monitored when sacrifice the animal for 5 each group at fourth and 12th weeks, respectively. Each two sections from the thoracic and abdominal aorta were prepared for light and electron microscopy.

Measurement of serum estradiol levels

Serum 17 beta-estradiol sub 2 levels were measured in samples of heart blood. Five animals from each group underwent sampling at 4 weeks and 12 weeks of the experiment, respectively. Estradiol levels were determined with Biochemical Immuno Systems(Estradiol Maia Switzerland) and gamma counter(Cobra II by Packard, USA) by a commercial laboratory (SCL, Seoul, Korea; CAP accredited Lab. No 66755-01).

Measurement of serum nitric oxide

Serum levels of nitrite and nitrate were assessed as nitric

oxide production. Serum(150 μ l) was ultra-filtered by centrifugation at 12,000 rpm for 30 min, using 10 Kd molecular weight filters(Ultrafree-MC, Millipore). NO assay was performed in a standard flat bottomed 96-well polystyrene microtitre plate containing 50 μ l/well of standard or sample. The assay was blanked against phosphate buffered solution. Fifty μ l of nitrate reductase and β -NADPH were added to each well giving final concentration of 300U/L and 25 μ mol/L, respectively. The plate was incubated at room temperature for 3 hours. Excess β -NADPH was consumed by addition of 10 μ l of phosphate buffered saline containing Lactic dehydrogenase, pyruvic acid (final concentrations 10 mg/l, 10 Mmol/L) followed by a 5 min incubation at 37°C. The nitrite concentration was then measured by the addition of 50 μ l each of Griess reagents 1 and 2, and the absorbance read at 540nm using a plate reader after a 10 min incubation at room temperature.

Measurement of serum lipoprotein

Serum total, LDL and HDL cholesterol, and triacylglycerol were determined by a commercial laboratory(SCL, Seoul, Korea; CAP accredited Lab. No 66755-01). In brief, enzymetric method has performed with Hitachi 7150(Hitachi, Japan) spectrometer.

Evaluation of intimal atherosclerotic lesion

Neointimal thickening was evaluated by average of the scale after elastic staining. The scale was decided as 0 : no lesion, 1 : lesion involved below 1/3 of the circumference of the aorta, 2 : lesion involved between 1/3 and 2/3, and 3 : lesion involved over 2/3.

Scanning electron microscopy

Tissue specimens for scanning electron microscopy were rinsed in Millonig's phosphate buffer(pH 7.2) and fixed in 4oC 2.5% glutaraldehyde for 2.5 hours, and postfixed with osmium tetroxide solution. After epon embedding, thin sectioned and stained with uranyl acetate and lead citrate and the sections were examined by use of Hitachi S-2500 scanning electron microscope.

Statistical methods

Data from serum 17 beta-estradiol sub 2 levels, nitric oxide production, cholesterol, and lesion scale from each group were analyzed. Statistical significance was assessed by Duncan's comparison test using one-way ANOVA. Values of P<0.05 were

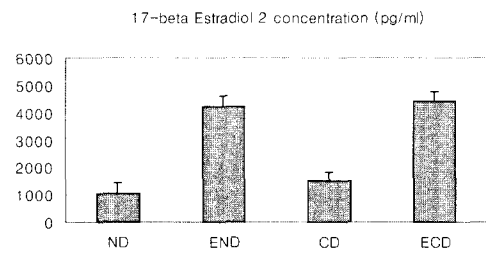


Fig. 1. Serum 17-beta estradiol sub 2 concentration(pg/ml). Two 17-beta estradiol sub 2 administrated groups show significant increase of the concentration(p<0.001). Data are mean \pm SEM.

ND, Normal Diet; END, Normal Diet + transdermal 17-beta estradiol sub 2 patch; CD, Cholesterol Diet; ECD, Cholesterol Diet + transdermal 17-beta estradiol sub 2 patch.

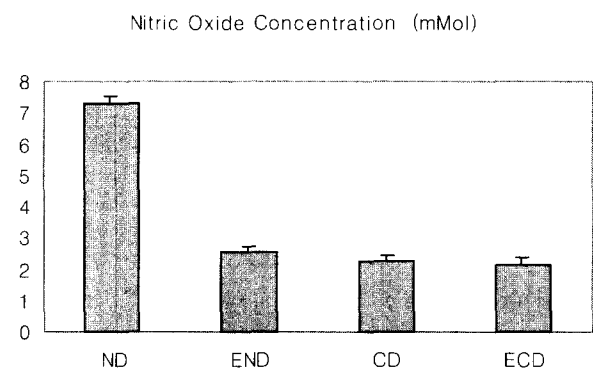


Fig. 2. Serum nitric oxide concentration. Serum levels of END, CD, and ECD groups decreased significantly than that of ND group (p<0.001). Data are mean \pm SEM.

ND, Normal Diet; END, Normal Diet + transdermal 17-beta estradiol sub 2 patch; CD, Cholesterol Diet; ECD, Cholesterol Diet + transdermal 17-beta estradiol sub 2 patch.

considered statistically significant.

RESULTS

Serum estradiol level

Radioimmunoassay of serum from each experimental group showed that 17 beta-estradiol sub 2 was increased both ED and CED groups than ND or CD groups(Fig. 1).

Nitric oxide production

Nitric oxide production was significantly decreased in END,

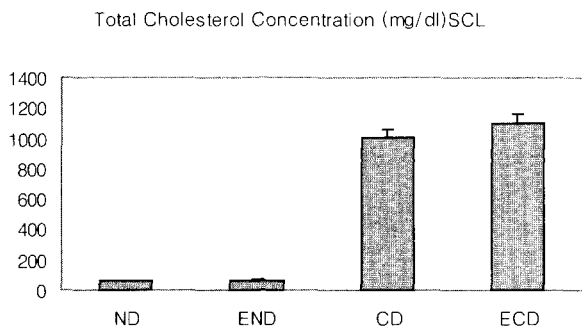


Fig. 3A.

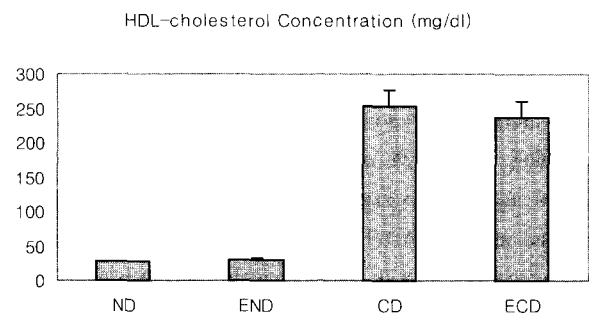


Fig. 3C.

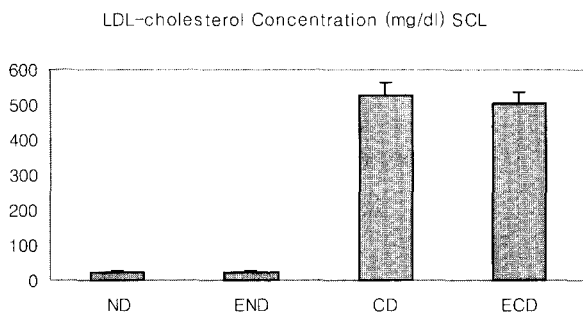


Fig. 3B.

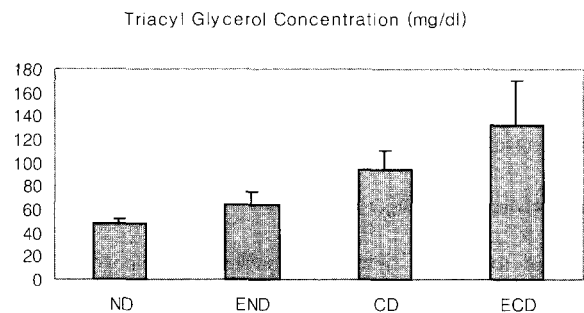


Fig. 3D.

Fig. 3(a,b,c,d). Serum cholesterol and triacylglycerol concentration. The total, HDL and LDL cholesterol concentrations were increase in both CD and ECD groups ($p < 0.001$). The triacylglycerol concentration was also increased in both CD and ECD groups but, there was no significance ($P > 0.05$). Data are mean \pm SEM. ND, Normal Diet; END, Normal Diet + transdermal 17-beta estradiol sub 2 patch; CD, Cholesterol Diet; ECD, Cholesterol Diet + transdermal 17-beta estradiol sub 2 patch.

CD, and ECD groups than that of ND group(Fig 2).

Serum lipoprotein concentration

Total and LDL-cholesterol concentration were significantly increased in both CD and ECD groups. However, the concentration was not effected by 17-beta estradiol sub 2 administration(Fig. 3).

Light and scanning electron microscopic examination

No gross and microscopic atherosclerotic lesion can be demonstrated from all thoracic and abdominal aortas of ND and END groups. However, cholesterol-fed animals had a certain degree of lesions on the aortic endothelium. On light microscopic examination, the lesions were consists mainly of accumulation of lipid-laden macrophages(foam cell) at the subintimal area at 4 weeks. The lesion was thickened at 12

weeks, but any fibrous plaque lesion was not observed. The severity of the lesion was almost same in CD and ECD group on thoracic aorta, but the degree was decreased slightly on abdominal aorta at ECD group(Fig. 4).

On scanning electron microscopic examinations, luminal surfaces in ND group revealed a regular, parallel arrangement of the endothelial cells. No monocytic or platelet adhesions were observed(Fig. 5a). The END group also showed similar appearances with ND group. Animals from CD group showed scattered areas of endothelial damage or adhesion of monocytes and platelets(Fig. 5b). Such findings were observed both 4 weeks and 12 weeks-animals but were increased the severity with time(Fig. 5c). 17-beta estradiol administration to the cholesterol-fed animals did not change these pathologic findings significantly(Fig. 5d).

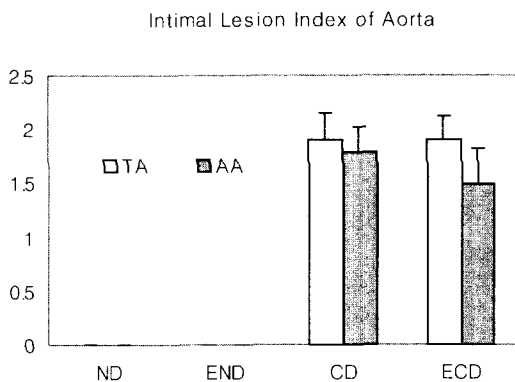


Fig. 4. The lesion scale of aorta. Aortic lesion was observed only in CD and ECD groups. There was no significance between these two groups($p>0.05$). The abdominal aorta(AA) reveals mild decrease of the lesion scale than thoracic aorta (TA), but no significance was demonstrated($p>0.05$). Data are mean \pm SEM.

DISCUSSION

Present studies indicate that cholesterol diet decrease nitric oxide production, increase serum cholesterol level, and raise early atherosclerotic lesion in rabbits. The transdermal 17-beta estradiol sub 2 administration increased serum 17-beta estradiol sub 2 level, but decreased production of nitric oxide. The estrogen did not show any effect on serum cholesterol level and lesion development.

We speculated that the decreased nitric oxide production by cholesterol diet is due to chemical and/or mechanical injury to the endothelium by hyperlipidemia. Other study also revealed significant lowering of serum nitrite/nitrate levels in cholesterol-fed rabbit¹⁴. Hyperlipidemia brings increased oxidation of cholesterol by endothelium and macrophages. The oxidized LDL is a key component in endothelial injury^{15,16}. The oxidized LDL has a cytotoxic effect to the endothelium and can recruit blood monocytes or lymphocytes. In addition, oxidized LDL inhibited basal NO release to a greater extent in coronary arteries from pigs¹⁷. We could see the damages by pathologic examination of thoracic and abdominal aortas. Ultrastructural findings of the endothelium show variable but consistent damage. Besides these morphological changes, the endothelium may has functional derangement by the hyperlipidemia. Similar mechanisms may operate in humans as well.

In this study, the reason why nitric oxide is decreased in estrogen plus normal diet(END) group is mystery. Previous studies showed increased nitric oxide level by estrogen treatment^{12,18}. Estrogen may increase the transport of L-arginine into the endothelial cell by induction of a transport carrier protein or may induce the NOS enzyme responsible for generation of nitric oxide¹⁶. We can guess circulating nitric oxide metabolites could be made from variable cells, which have nitric oxide synthase. Among these cells, only endothelial and neuronal cells have constitutive nitric oxide synthase. Since estrogen stimulates the basal release of NO from endothelium, it is reasonable that the circulating NO are solely endothelium-derived. This discordance may be sex-related differences in the impact of these conditions on endothelial function, because there is significant gender difference in NO production^{19,20}. The present study used male rabbit instead of female. We hypothesized estrogen may be beneficial for antiatherogenesis not only female but also in male. In fact, Hutchison et al. suggested that high concentration of estradiol causes vasodilation principally by endothelium independent mechanisms in a gender independent fashion²¹. The concentration of estrogen in our study was about 4 times higher than the normal control. Although the exact mechanism is unknown, we speculated that the gender difference can not be a complete explanation in this experiment.

The hyperlipidemic diet increased total cholesterol about fifteen times higher than normal diet animals. The LDL cholesterol is increased about twenty two times, but HDL cholesterol is increased about eight times. There is no change by estrogen administration. Rabbit has somewhat different cholesterol metabolism from that of humans²⁷. The cholesterol increases in rabbits is mainly due to an increase in the VLDL + IDL fraction. Although we did not measure VLDL and IDL cholesterol, LDL and HDL cholesterol and triacylglycerol were increased significantly. Estrogen did not show any effect on these cholesterol levels. Comparison of the estrogenic effect on lipoprotein of the present and previous studies revealed that transdermally administrated 17-beta-estradiol has no effect on lipoprotein levels, suggesting that the hepatic effects of estrogen absorbed through the gut are important for changes in lipoprotein levels²³.

The aortas revealed atherosclerotic lesion development consistently by the hyperlipidemic diet. We evaluated both thoracic and abdominal aortas, and abdominal aorta showed much development of the early lesion than that of thoracic aorta.

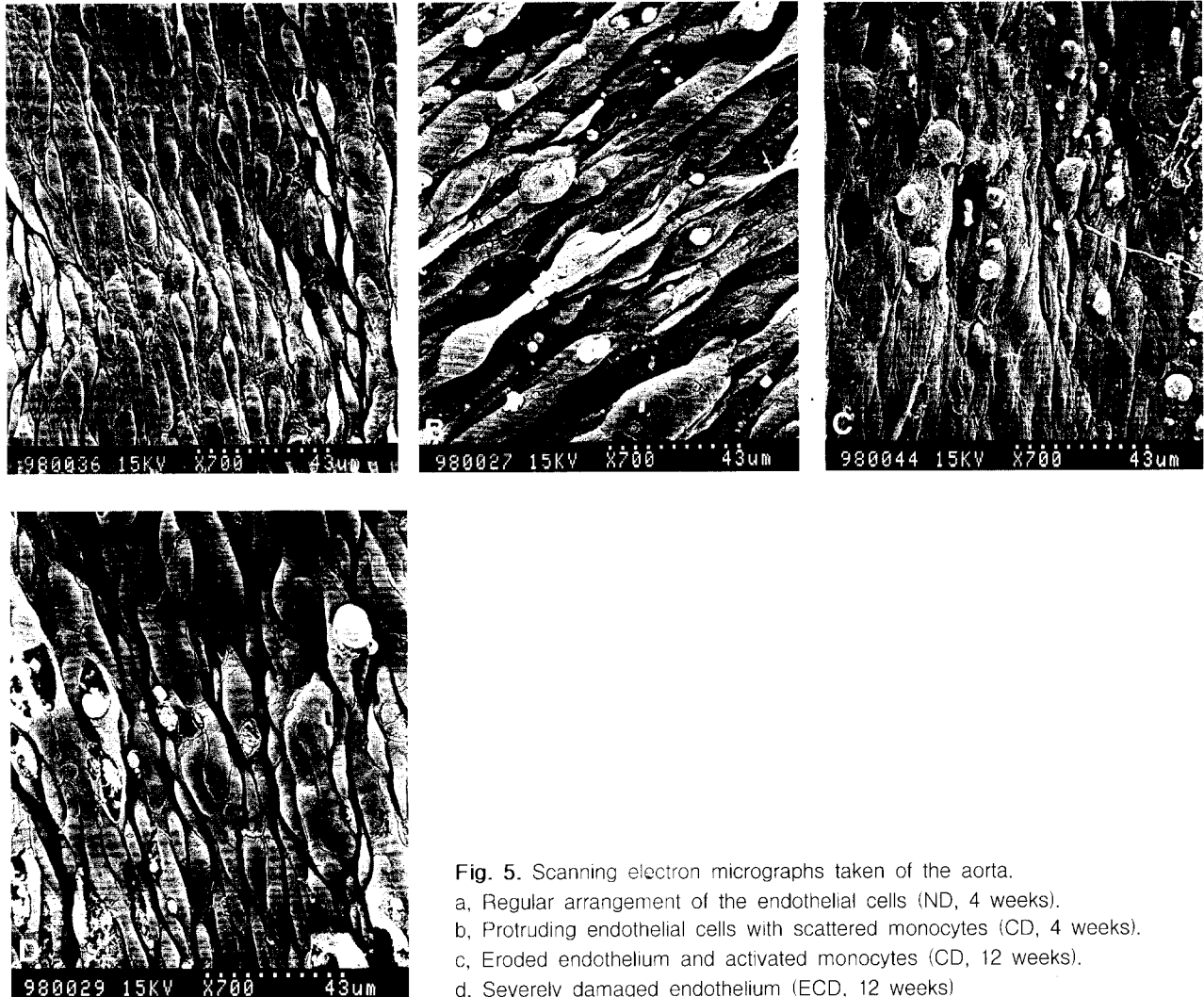


Fig. 5. Scanning electron micrographs taken of the aorta.
a, Regular arrangement of the endothelial cells (ND, 4 weeks).
b, Protruding endothelial cells with scattered monocytes (CD, 4 weeks).
c, Eroded endothelium and activated monocytes (CD, 12 weeks).
d, Severely damaged endothelium (ECD, 12 weeks)

The lesion mainly consists of accumulation of lipid-laden macrophages in subintimal area, suggesting fatty streak in humans. Any advanced lesion showing necrosis or fibrosis can not be observed. Fatty streak is thought to an early atherosclerotic lesion, and it progresses into fibrous plaque.

In this study, although abdominal aortic lesion has decreased slightly by estrogen administration, there was no significance. We speculate the gender difference offset the antiathrogenic effect of estrogen in the early stage of atherosclerosis, since serum estradiol-2 level in estrogen treated group was four times higher than other groups.

This study clearly shows hyperlipidemia brings a decreased production of nitric oxide, probably due to endothelial dysfunction. The 17-beta estradiol sub 2 administration decreased nitric oxide level in male cholesterol-fed rabbit with unknown

mechanism. The transdermal estrogen did not show any effect on blood cholesterol level. To gain a complete understanding for the action of estrogen on atherosclerosis, we planned a more detailed study for both genders.

CONCLUSION

To examine the effect of estrogen on blood nitric oxide production and aortic atherosclerosis, we did an experiment with cholesterol-fed rabbits for 12 weeks. Forty animals were randomly divided as 10 normal diet(ND), 10 ND + transdermal 17-beta estradiol sub 2 patches(END), 10 hypercholesterol diet (CD;0.5% of diet; w/w), and 10 CD + transdermal E sub 2 patches(ECD) group, respectively.

The results suggest that hyperlipidemic diet decrease the

production of nitric oxide possibly due to endothelial dysfunction by oxidized LDL. A gender difference and administration method may be a possible explanation for the reason why nitric oxide was decreased in END group. Transdermal 17-beta estradiol sub 2 patch treatment can not effect on serum total, LDL and HDL cholesterol, and triacylglycerol level. The estrogen did not effect on atherogenesis on both thoracic and abdominal aorta. In order to understand the action of estrogen on atherogenesis, further detailed study for both genders are needed.

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=국문초록=

배경: 에스트로겐이 동맥경화증의 진행을 억제하고 예방하는데 중요한 역할을 하는 것으로 알려져 있으나 그 기전은 아직 명확하지 않다. 본연구에서는 에스트로겐이 혈관의 확장에 중요한 역할을 하고 있는 nitric oxide와 동맥경화에 어떠한 영향을 미치는지를 알아보고자 하였다. **대상 및 방법:** 뉴질랜드종 웅성 가토 40 마리를 실험동물로 하여 정상식이군(ND), 정상식이와 0.02 ig 의 estradiol-2 patch 투여군 (END), 0.5% 콜레스테롤 투여 군(CD), 0.5% 콜레스테롤 투여와 0.02 μ g 의estradiol-2 patch 투여 군 (ECD)의 네 군으로 나누어12 주간 실험을 하여 혈중 estradiol-2, nitric oxide, cholesterol 등을 측정하고 흉부동맥과 복부동맥의 동맥경화 병변을 관찰하였다. **결과:** 혈중 estradiol-2의 농도는 ED , ECD 군에서 ND, CD 군보다 의의 있게 높았다 ($P<0.0001$). 혈중 nitric oxide 농도는 정상 대조 군 (ND)을 제외한 나머지 세 군(END,CD, ECD) 에서 모두 의 의 있게 감소하였다($P<0.001$). 혈중 콜레스테롤은 총콜레스테롤, LDL콜레스테롤, HDL 콜레스테롤은 CD, ECD의 두 군에서 모두 증가하였으나 의의는 없었다($P>0.05$). 혈중 콜레스테롤과 중성지방의 농도는 에스트로겐에 의한 영향이 관찰되지 않았다. 흉부 및 복부동맥의 동맥경화 병변은 ND군과END군에서는 발생하지 않았으나 콜레스테롤을 투여한 CD군과 ECD군의 두 군에서 현저하게 발생하였으며 혈관별 발생정도의 차이는 없었다. 에스트로겐에 의한 병변의 발생 차이는 관찰되지 않았다. 광학현미경상 동맥경화병변은 거의 지방을 탐식한 대식구의 증식으로 이루어져 있었으며, 괴사나 섬유화 병변은 관찰되지 않았다. 전자현미경상에서는 내피세포의 광범위한 손상이 관찰되었다. 병리학적으로 에스트로겐에 의한 영향은 인정하기 어려웠다. **결론:** 고지방식은 혈중 nitric oxide의 생산을 크게 저하 시키며, 그 원인으로는 지방의 과산화에 의한 내피세포의 손상때문인 것으로 추정된다. 또한 estradiol-2 patch 처치에 의해서 nitric oxide가 감소된 것은 그 이유를 설명하기가 곤란하나 성차이 및 에스트로겐의 투여방법에 의한 어떤 영향이 있었을 것으로 추측된다. 피부를 통한 에스트로겐의 투여는 혈중 콜레스테롤이나 중성지방의 농도에 영향을 주지 못했으며, 동맥경화의 진행에도 영향이 없었다. 에스트로겐의 동맥경화에 주는 영향에 대해서는 지속적이고 광범위한 연구가 더 필요할 것으로 사료된다.

중심 단어: 에스트로겐, 동맥경화, nitric oxide