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Phytoestrogen α-Zearalanol Inhibits Atherogenesis and Improves Lipid Profile in Ovariectomized Cholesterol-Fed Rabbits

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Although favorable effects of estrogen replacement therapy on atherosclerosis have been recognized, the benefit versus risk of estrogen replacement on overall cardiovascular health remains controversial. The main adverse effect jeopardizing the clinical usage of estrogen is the increased risk of breast and endometrial cancer. Zearalenone (ZEN) is a universal endogenous hormone possessing estrogen-like effects and facilitating plant growth. α-Zearalanol (α-ZAL), a new phytoestrogen, is a reductive product of ZEN. Our preliminary evidence suggested that α-ZAL is anti-atherosclerotic. The aim of this study was to examine the effect of α-ZAL on atherosclerotic formation and serum lipid profile. Adult female nulliparous rabbits were ovariectomized or sham-operated and fed a high-cholesterol diet with different doses of α-ZAL or 17β-estradiol for 12 wk. The aortic intimal atherosclerotic plaque was significantly larger in the cholesterol-fed group compared to control and sham groups. α-ZAL and 17β-estradiol treatments significantly reduced plaque formation and improved serum profile of lipid (TC, TG, HDL-C, and LDL-C) and lipoprotein (ApoAl and ApoB). Both α-ZAL and 17β-estradiol reconciled ovariectomy-induced uterine atrophy, although α-ZAL was significantly less potent than 17β-estradiol in stimulating uterine growth. Our findings indicate that the phytoestrogen α-ZAL has an important anti-atherogenic property, analogous to that of estrogen.

Key Words: Phytoestrogen; estrogen; zearalenone; atherosclerosis; lipid profile.

Introduction

Atherosclerosis is among the leading causes of increased cardiovascular and cerebral vascular morbidity and mortality *(1,2)*. Premenopausal women have a lower risk of cardiovascular diseases, including atherosclerosis, than men or postmenopausal women. Clinical and experimental evidence has shown that estrogen replacement therapy (ERT) may benefit cardiac contractile action, blood lipid profile, vascular resistance, and oxidative stress, thus reducing the morbidity and mortality in postmenopausal women *(3–5)*. The cardiovascular protection derived from estrogen has been attributed largely to its favorable actions on lipid profiles *(6)*, although it was suggested that the lipid regulatory effect of estrogen may only account for a moderate portion of its clinical benefit *(7)*. Meta-analysis of estrogen studies revealed an estimated relative risk of coronary events of 0.65 for women with ERT *(8)*. However, recent clinical trials such as the Heart and Estrogen/Progestin Replacement Study (HERS) have brought us some unpredicted or even surprising findings *(9,10)*. For example, ERT did not elicit significant beneficial effects on cardiac function in women with coronary heart diseases, thus making the clinical application of ERT to improve the overall cardiovascular health somewhat controversial *(9,10)*. With the life span in women prolonged from 60 to 80 yr, an average woman will likely spend more than one third of her life in an "estrogen-less" state, making the proper use of ERT to improve the quality of life a practical issue. However, estrogen monotherapy has been found to increase the risk of breast and endometrial cancer *(11–13)*. Although combined estrogen–progesterone–testosterone therapy may reduce the incidence of endometrial carcinoma, breast cancer remains increased *(14)*, inclusion of progesterone in the combined therapy did not efficiently alleviate the tumor-promoting property of estrogen while it significantly antagonized the anti-atherosclerotic and anti-Alzheimer effects of estrogen. These suboptimal therapeutic and adverse effects of progesterone and progesterone combined therapy have added to the already complex and controversial ERT clinical application *(15)*. Therefore,

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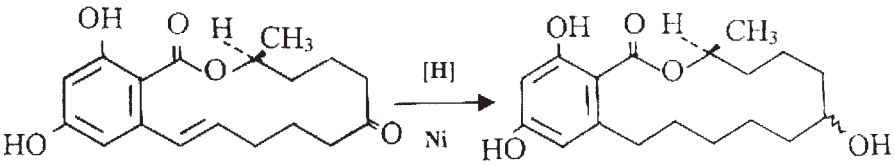

Fig. 1. Chemical structure of zearalenone (ZEN, left panel) and α-zearalanol (α-ZAL, right panel).

the search for efficacious preventive and therapeutic strategies is in high demand to reconcile the estrogen deficiency–related cardiovascular diseases in postmenopausal women.

Recently, attention has been drawn to the value of plant-derived phytoestrogen as a potential replacement for estrogen. α-Zearalanol (ZAL) *(16)*, a reductive product of the *Gibberella zeae* metabolite zearalenone (ZEN) (Fig. 1), was isolated from culture medium of *Gibberella zeae*. ZEN is a member of β-resorcylates, which is a family of natural products, and has been shown to facilitate animal growth without known adverse effect *(17,18)*. The biological activity of α-ZAL is three times that of ZEN with a lower toxicity. Low doses of α-ZAL were shown to promote protein synthesis and increase the lean meat ratio, in a manner similar to the growth-promoting effect of estrogen, however, without tissue proliferation *(17–20)*. α-ZAL is rapidly metabolized in the body with few residues left in organs such as muscle, heart, liver, pancreas, kidney, and blood *(21,22)*. Mounting evidence has shown that α-ZAL is highly efficient and safe for use in animal husbandry in the United States and Canada *(22)*.

In 1980, Li and colleagues reported a zearalenone-like substance in wheat shoot apices possessing a potential role in wheat development *(23)*. It was later identified as ZEN and considered as a universal endogenous hormone for plant growth *(24)*. We later hypothesized that the natural plant phytoestrogen α-ZAL may possess certain physiological properties of estrogen such as therapeutic effects on atherosclerosis, Alzheimer's disease, and osteoporosis, with less adverse effects especially on the uterine and mammary glands. The present study was designed to evaluate the effect of α-ZAL on the progression of atherosclerosis. Since the ovariectomized, cholesterol-fed rabbit model of atherosclerosis has been shown to be suitable for the studies of estrogen and atherosclerosis *(25)*, we investigated the effect of α-ZAL on this in vivo animal model.

Results

Aortic Intimal Lipid Plaque Deposition and Uterine Weight

Lipid plaque deposition in rabbit aorta exhibited as ratio of aortic atherosclerotic plaque to total area of aortic intima was significantly enhanced after high-cholesterol diet feeding (Sham + CHO group). Bilateral ovariectomy significantly exacerbated the progression of lipid plaque formation compared to the Sham group when cholesterol diet was present. Low dosage of α-ZAL (0.1 mg/kg/d) did not significantly alter the cholesterol-induced lipid deposition, whereas medium (0.5 mg/kg/d) and high doses (2.5 mg/kg/d) of α-ZAL significantly reduced aortic lipid plaque area. The maximal effect was achieved at the medium dose of α-ZAL (0.5 mg/kg/d). Interestingly, the α-ZAL-induced reduction of lipid plaque was similar to that achieved by a similar dose (0.5 mg/kg/d) of 17β-estradiol (Fig. 2).

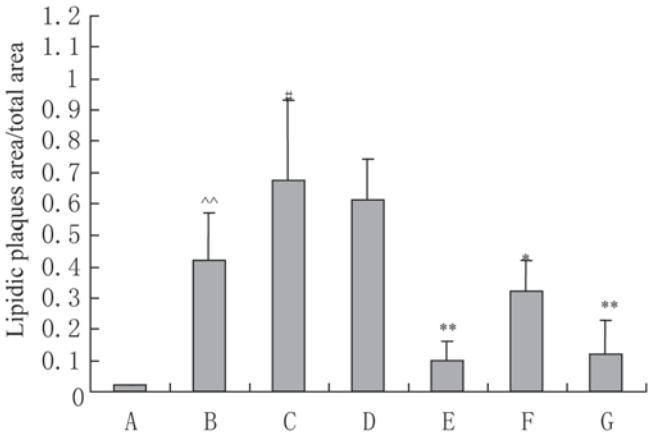

Fig. 2. Ratio of area of aortic intimal lipid plaques to total area of aortic intima of rabbits in seven groups following a 12-week cholesterol (CHO)-feeding. A: Control ($n = 6$ rabbits); B: Sham + CHO ($n = 7$); C: OVX + CHO ($n = 7$); D: OVX + CHO + ZAL-low ($n = 7$); E: OVX + CHO + ZAL-medium ($n = 7$); F: OVX + CHO + ZAL-high ($n = 7$); G: OVX + CHO + 17β-estradiol ($n = 7$). Mean ± SEM, $^{\wedge\wedge}p < 0.01$ vs group A; $^{\#}p < 0.05$ vs group B; $^{*}p < 0.05$, $^{**}p < 0.01$ vs group C.

To evaluate the impact of ovariectomy, cholesterol, α-ZAL, and 17β-estradiol dietary feeding on uterine growth, we measured the uterine weight following the 12-wk feeding period. The uterine weights were 1.96 ± 0.49 g/kg body weight in group A (Control); 1.84 ± 0.74 g/kg in group B (Sham + CHO); 0.41 ± 0.14 g/kg in group C (OVX + CHO); 0.52 ± 0.25 g/kg in group D (OVX + CHO + ZAL-low); 1.07 ± 0.29 g/kg in group E (OVX + CHO + ZAL-medium); 2.14 ± 1.05 g/kg in group F (OVX + CHO + ZAL-high); and 3.72 ± 0.62 in group G (OVX + CHO + 17β-estradiol). These results revealed that cholesterol feeding itself did not alter the uterine weight, while the ovariectomy procedure

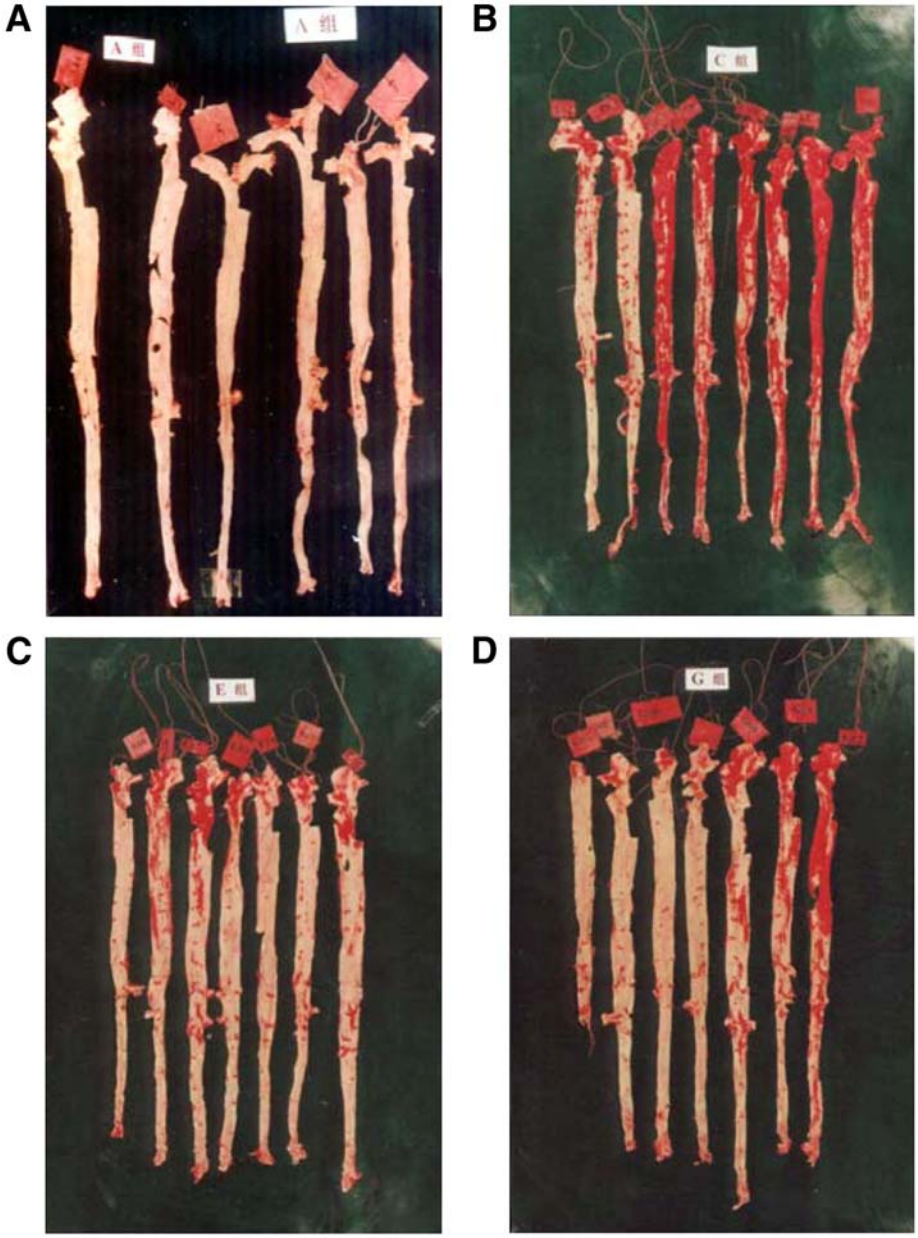

Fig. 3. Aorta morphology. (**A**) Aorta obtained from group A (control) where no lipid plaques were found; (**B**) Aorta obtained from group C (OVX + CHO) where lipid deposition was severe and wide spread from ascending aorta to ilial artery bifurcation; (**C**) Aorta obtained from group E (OVX + CHO + ZAL-medium), where lipid deposition was significantly ameliorated and there were only a few scattered plaques, mostly located in the aortic arch; (**D**) Aorta obtained from group G (OVX + CHO + 17β-estradiol), where lipid deposition was significantly ameliorated and there were only a few scattered plaques, mostly limited to the aortic arch but occasionally in the descending aorta.

significantly triggered uterine atrophy ($p < 0.05$ between group C and groups A and B). Low dose of α-ZAL did not affect the ovariectomy-induced uterine atrophy, whereas medium or high dose of α-ZAL reconciled ovariectomy-induced uterine atrophy ($p < 0.05$ between group C and groups E and F). Interestingly, similar dose of 17β-estradiol induced significant overgrowth of uterine compared to α-ZAL ($p < 0.05$ between group G and group E). These results indicated that α-ZAL is significantly less potent in stimulating uterine growth than estrogen.

Morphological and Pathological Characterization of Aortic Lipid Plaque

Morphological characterization showed severe lipid plaque deposition in aortic arch, ascending and descending aorta intima from OVX groups consuming cholesterol diet. Addition of either α-ZAL or 17β-estradiol significantly ameliorated the cholesterol-induced lipid deposition (Fig. 3). The microscopic characterization revealed obvious endothelial cell pathology such as outward shifting of the intimal elastic layer, accumulation of foam cells and lipid plaque, liquid-

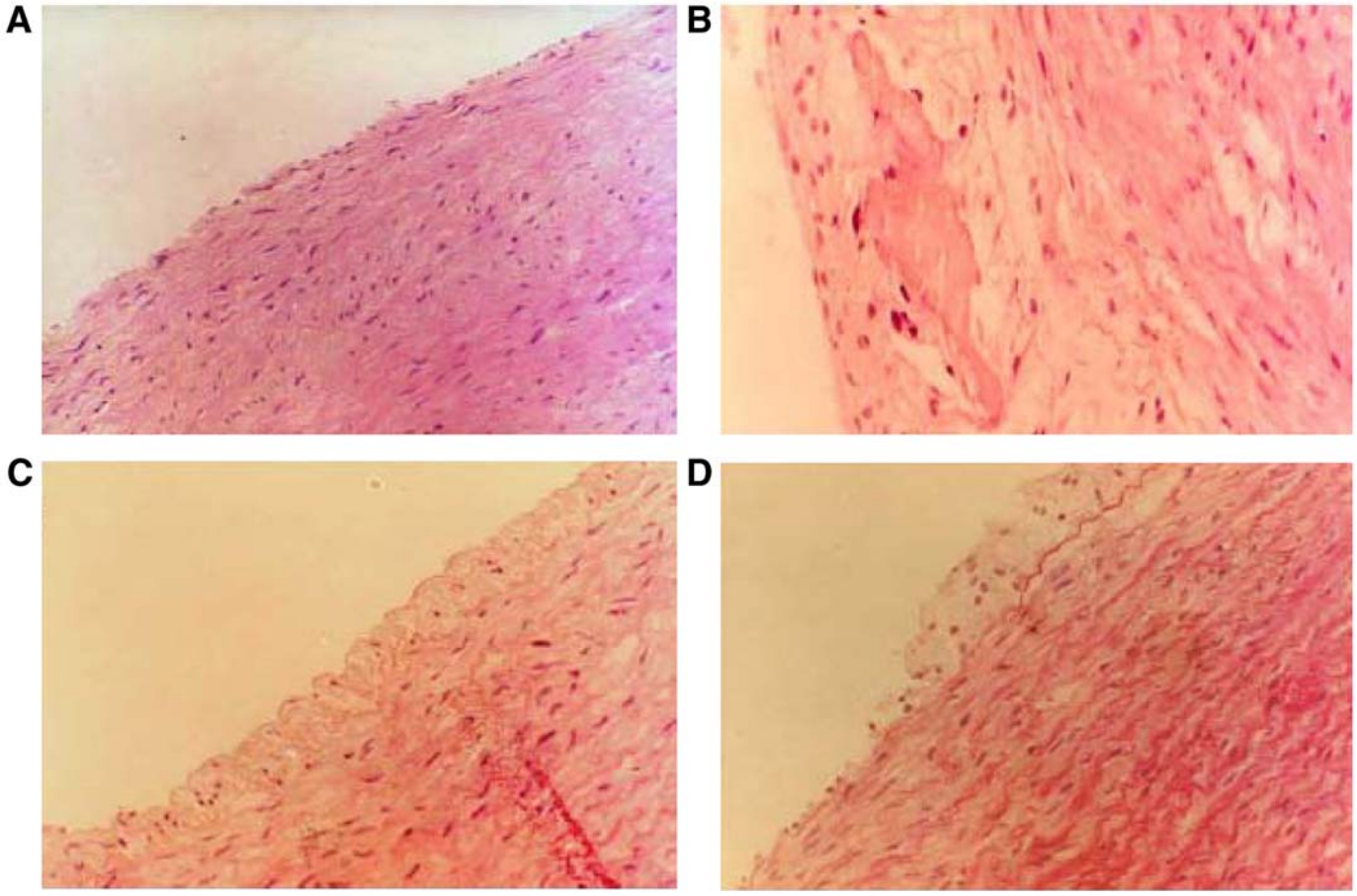

Fig. 4. Microscopic pathological characterization of aortic wall. (**A**) Normal vascular walls, intact endothelial cells and intimal elastic layer in control group (group A); (**B**) Severe endothelial cell damage and loss, obvious outward shifting of intimal elastic layer, large of accumulation of foam cells and lipid plaque, necrosis and calcification in OVX + CHO group (group C); (**C**) Aforementioned pathological alterations were significantly attenuated, basically intact endothelial cells, slight outward shifting of intimal elastic layer and accumulation of foam cells and lipid plaque in OVX + CHO + ZAL-medium group (group E); (**D**) These alterations were similar in the OVX + CHO + 17β-estradiol group (group G), compared to group E.

like necrosis, and calcification in OVX group consuming cholesterol. These pathological alterations were significantly attenuated by α-ZAL or 17β-estradiol (Fig. 4).

Serum Lipids, Lipoproteins, and Estrogen

Changes of serum lipid (TC, TG, HDL-C, LDL-C) and lipoprotein (ApoA and ApoB) levels during the 12-wk feeding period were measured (Tables 1–6). All indices except ApoA demonstrated a marked increase following 4 wk of cholesterol feeding. The rise continued with prolonged cholesterol feeding through 12 wk. With the exception of ApoA and HDL-C, all other experimental parameters showed various degrees of decline at wk 4 (TC) or wk 8 (LDL-C, TG, and ApoB) in both treatment groups (α-ZAL and 17β-estradiol) compared with the OVX + CHO group. α-ZAL exhibited a weak inhibitory effect at low dosage (0.1 mg/kg/d) and elicited strong protection at doses higher than 0.5 mg/kg/d, suggesting that the inhibitory effect on lipids and lipoprotein accumulation may be best achieved in the intermediate- to high-dose range (Tables 1–6). Serum estrogen (17β-estradiol) levels determined by radioimmunoassay were 169.58 ± 76.86 pg/mL in control group (group A), 28.75 ± 16.79 pg/mL in OVX + CHO group (group C), 372.11 ± 162.84 pg/mL in OVX + CHO + 17β-estradiol group (group G), and 119.89 ± 48.32 pg/mL in OVX + CHO + ZAL-medium group (group E).

Discussion

Our results demonstrated for the first time that the plant phytoestrogen α-ZAL possesses protective actions against experimental atherosclerosis and improves lipid profile. The cholesterol-induced atherosclerosis in the present study was severe and typical with obvious aortic lipid plaque deposition *(26–28)*. α-ZAL, at the dosages tested, showed various degrees of protection, with the intermediate dose (0.5 mg/kg/d) eliciting the maximal effect (aortic lipid plaque area decreased by approx 85%). The effect was similar to that of 17β-estradiol in alleviating the pathological alteration in the vascular intima of the aorta. More important, α-ZAL

Table 1
Changes of Serum Total Cholesterol (TC) Levels in Seven Experimental Groups (m*M*)

Groups	Week 0	Week 4	Week 8	Week 12
A (n = 6)	1.28 ± 0.16	1.33 ± 0.11	1.42 ± 0.17	1.37 ± 0.17
B (n = 8)	1.47 ± 0.22	15.9 ± 3.17^^	16.33 ± 2.18^^	26.51 ± 1.83^^
C (n = 10)	1.33 ± 0.15	29.95 ± 2.45##	37.12 ± 2.46##	49.07 ± 4.64##
D (n = 8)	1.27 ± 0.11	18.64 ± 2.28*	25.32 ± 2.31*	25.32 ± 1.71**
E (n = 10)	1.27 ± 0.15	18.09 ± 2.27*	18.48 ± 3.55**	28.20 ± 2.80**
F (n = 8)	1.20 ± 0.15	17.70 ± 3.24*	17.47 ± 2.12**	21.83 ± 2.14**
G (n = 10)	1.34 ± 0.11	18.96 ± 2.25*	17.02 ± 2.64**	25.83 ± 2.49**

^^$p < 0.01$ vs group A; ##$p < 0.01$ vs group B; *$p < 0.05$ and **$p < 0.01$ vs group C; all values expressed as mean ± SEM.

Table 2
Changes of Serum HDL-C Levels in Seven Experimental Groups (m*M*)

Groups	Week 0	Week 4	Week 8	Week 12
A (n = 6)	0.42 ± 0.06	0.41 ± 0.06	0.45 ± 0.06	0.51 ± 0.08
B (n = 8)	0.54 ± 0.06	2.05 ± 0.73^^	2.01 ± 0.20^^	3.24 ± 0.45^^
C (n = 10)	0.48 ± 0.07	2.00 ± 0.26	2.62 ± 0.12	3.23 ± 0.24
D (n = 8)	0.42 ± 0.05	2.74 ± 0.17	3.30 ± 0.35	4.32 ± 0.27
E (n = 10)	0.38 ± 0.06	2.53 ± 0.41	2.48 ± 0.31	3.45 ± 0.47
F (n = 8)	0.43 ± 0.05	3.16 ± 0.35	2.44 ± 0.18	3.72 ± 0.27
G (n = 10)	0.44 ± 0.06	2.42 ± 0.32	2.56 ± 0.33	2.90 ± 0.33

^^$p < 0.01$ versus group A, all values express as mean ± SEM.

Table 3
Changes of Serum LDL-C Levels in Seven Experimental Groups (m*M*)

Groups	Week 0	Week 4	Week 8	Week 12
A (n = 6)	0.45 ± 0.09	0.42 ± 0.09	0.38 ± 0.31	0.48 ± 0.06
B (n = 8)	0.44 ± 0.14	7.25 ± 1.42^^	7.51 ± 0.76^^	8.23 ± 0.68^^
C (n = 10)	0.45 ± 0.06	8.48 ± 0.95	13.42 ± 0.92##	16.27 ± 1.42##
D (n = 8)	0.41 ± 0.07	8.41 ± 1.06	10.37 ± 0.85*	10.29 ± 0.68**
E (n = 10)	0.41 ± 0.08	7.90 ± 0.96	8.49 ± 0.84**	9.16 ± 0.77**
F (n = 8)	0.34 ± 0.07	8.72 ± 1.69	7.08 ± 1.08**	8.79 ± 1.04**
G (n = 10)	0.36 ± 0.04	7.97 ± 0.98	8.96 ± 0.96**	10.84 ± 1.03**

^^$p < 0.01$ vs group A; ##$p < 0.01$ vs group B; *$p < 0.05$ and **$p < 0.01$ vs group C, all values express as mean ± SEM.

Table 4
Changes of Serum Triglycerides Levels in Seven Experimental Groups (m*M*)

Groups	Week 0	Week 4	Week 8	Week 12
A (n = 6)	1.04 ± 0.27	1.09 ± 0.18	1.13 ± 0.22	1.03 ± 0.21
B (n = 8)	0.84 ± 0.13	1.37 ± 0.14	2.39 ± 0.29^^	2.95 ± 0.59^^
C (n = 10)	1.06 ± 0.10	2.27 ± 5.25	4.10 ± 0.46#	6.16 ± 1.33#
D (n = 8)	0.87 ± 0.09	2.18 ± 0.35	3.34 ± 0.06	3.35 ± 0.29
E (n = 10)	1.04 ± 0.15	1.23 ± 0.21	2.06 ± 0.36*	2.82 ± .32*
F (n = 8)	0.80 ± 0.07	1.06 ± 0.17	1.77 ± 0.30**	1.83 ± 0.33**
G (n = 10)	0.91 ± 0.13	0.93 ± 0.11	1.52 ± 0.46**	1.81 ± 0.22**

^^$p < 0.01$ vs group A; #$p < 0.05$ vs group B; *$p < 0.05$ and **$p < 0.01$ vs group C; all values express as mean ± SEM.

Table 5
Changes of Serum ApoAl Levels in Seven Experimental Groups (mg/dL)

Groups	Week 0	Week 4	Week 8	Week 12
A (n = 6)	25.7 ± 2.61	22.5 ± 1.63	27.8 ± 2.65	26.3 ± 3.10
B (n = 8)	28.3 ± 0.53	32.6 ± 2.16^^	29.0 ± 2.02	39.7 ± 5.44^
C (n = 10)	31.8 ± 4.59	38.4 ± 2.43	37.5 ± 1.83	36.0 ± 1.64
D (n = 8)	25.0 ± 2.26	34.1 ± 3.18	37.6 ± 2.40	38.0 ± 1.70
E (n = 10)	28.7 ± 2.18	36.0 ± 3.54	34.0 ± 2.25	34.8 ± 2.09
F (n = 8)	23.4 ± 1.73	31.3 ± 2.23	32.9 ± 1.87	35.7 ± 2.19
G (n = 10)	30.9 ± 2.09	32.4 ± 1.99	31.2 ± 1.61	29.7 ± 2.37

^$p < 0.05$, ^^$p < 0.01$ vs group A; all values express as mean ± SEM.

Table 6
Changes of Serum ApoB Levels in Seven Experimental Groups (mg/dL)

Groups	Week 0	Week 4	Week 8	Week 12
A (n = 6)	4.8 ± 1.06	5.1 ± 1.47	4.5 ± 1.22	3.8 ± 1.06
B (n = 8)	5.1 ± 1.38	26.4 ± 3.78^^	26.3 ± 2.83^	32.1 ± 7.18^^
C (n = 10)	4.8 ± 0.57	27.7 ± 3.48	55.8 ± 4.93$^{\#\#}$	86.0 ± 10.8$^{\#\#}$
D (n = 8)	4.4 ± 1.10	29.9 ± 3.43	41.8 ± 3.89	30.0 ± 3.54**
E (n = 10)	4.7 ± 0.92	27.3 ± 3.38	36.0 ± 5.63*	36.1 ± 4.55**
F (n = 8)	3.3 ± 0.57	22.8 ± 4.38	29.8 ± 4.03**	25.5 ± 4.31**
G (n = 10)	3.6 ± 0.57	22.1 ± 2.78	31.0 ± 5.82**	28.4 ± 5.85**

^^$p < 0.01$ vs group A; $^{\#\#}p < 0.01$ vs group B; *$p < 0.05$ and **$p < 0.01$ vs group C, all values express as mean ± SEM.

restored the ovariectomy-induced uterine weight loss but did not induce uterine overgrowth elicited by 17β-estradiol, indicating that α-ZAL does not have the uterine tissue growth-promoting property of 17β-estradiol.

Longitudinal monitoring of serum lipids and lipoproteins revealed that all experimental parameters except ApoAl exhibited a marked increase following prolonged cholesterol feeding. The rise of ApoAl was mild and delayed without reaching statistical significance, suggesting a possible difference in the timing of various serum lipoprotein increases. The rise of ApoAl was delayed significantly, consistent with the clinical observation *(29)*. However, since ApoB started to rise at wk 4, the ratio of ApoA/ApoB declined significantly at wk 4. Similarly, the rise in LDL-C was far more significant than that of HDL-C, making the ratio of HDL-C/LDL-C inevitably low at wk 4, and it continued to decline even further throughout the remaining duration of cholesterol feeding. It has been suggested that the ratio of ApoAl/ApoB, and HDL-C/LDL-C reflect hyperlipidemia more accurately. It was found that the levels of LDL-C were significantly elevated whereas that of HDL-C remained unchanged in postmenopausal women *(29)*, consistent with our present experimental data in the ovariectomized rabbits.

Except ApoAl and HDL-C, all other indices including TC, LDL-C, TG, ApoB showed various degrees of decline at wk 4 (TC) or wk 8 (LDL-C, TG and ApoB) in all treatment groups compared with the OVX + CHO group. α-ZAL exhibited a weak inhibitory effect at low dosage and elicited strong protection at medium dosage, suggesting efficacious inhibition on aortic lipid plaque formation and lipoprotein accumulation at medium to high doses. It is worth mentioning that the extent of serum lipid improvement was not paralleled with the aortic lipid plaque area. Our data showed that the high dose of α-ZAL provided greater improvement on lipid profile, whereas the medium dose of α-ZAL demonstrated greater inhibition on aortic lipid plaque deposition. In addition, the inhibitory effect on aortic plaque formation was more obvious than lipoprotein derangement. Although short-term treatment of 17β-estradiol and α-ZAL improved lipid derangement after cholesterol feeding, the hyperlipidemic status was by no means abrogated. The fact that overall aortic lipid plaque deposition was significantly lessened by α-ZAL and 17β-estradiol suggests that lipid derangement may be only one of the mechanisms in atherogenesis. Investigations on the anti-atherosclerotic property of estrogen have been focused on its effects on plasma lipid profile *(3,6,12)*. However, recent evidence revealed that the direct vascular protective effect of estrogen may be more important *(6)*, which may be the case for α-ZAL. Atherogenesis is essentially proliferation of endothelium and vascular smooth muscle cells in response to hyperreactive inflammation due to various damages. Not surprisingly, this is consistent with the fact that estrogen protects endothelial

cells and inhibits vascular smooth muscle cell proliferation and migration. Our preliminary study demonstrated that α-ZAL and 17β-estradiol may inhibit rabbit aortic vascular smooth muscle cell proliferation, DNA synthesis, c-myc mRNA, and MCP-1 mRNA expression, and oxidized LDL-induced increases in cytosolic Ca2 + (Lu, Duan, Dai et al., unpublished data). This may explain why aortic serum lipid plaque may be improved significantly in the presence of hyperlipidemia observed in the present study. It is possible that α-ZAL and 17β-estradiol effectively protect the vasculature from lipid plaque deposition in the intima even under a hyperlipidemic environment. It is also possible that an optimal dose may exist for the α-ZAL-induced inhibition of intimal plaque formation.

Another interesting observation was the comparison between 17β-estradiol and various doses of α-ZAL on ovariectomized rabbit uteri. As a plant phytoestrogen, α-ZAL increased the OVX uterine weight but to a significantly lesser extent than that of 17β-estradiol. The uterine weight increase induced by α-ZAL (Group E) was only approx 29% of the uterine weight gain induced by the same dose of 17β-estradiol (Group G). Pathological observation and receptor binding showed a similar trend between α-ZAL and 17β-estradiol (Duan, Dai et al., unpublished result). Although there are striking similarities between the binding of α-ZAL and 17β-estradiol to hepatic estrogen receptor in vitro *(30)*, the affinity of binding to the estrogen receptor for α-ZAL in the uterus and cardiovascular systems is estimated to be only one-tenth of that for 17β-estradiol (Duan, Dai et al., unpublished data), consistent with the uterine weight gain elicited by the two compounds. The stimulatory effect of ZAL-medium on the mammary gland was also significantly less than that of 17β-estradiol (Duan, Dai et al., unpublished data). Further study is warranted to elucidate the pharmacology and pharmacokinetics of α-ZAL and its interaction with estrogen receptor subtypes (α- and β-estrogen receptors) or its own α-ZAL receptor. Last but not least, although our study did not reveal apparent organ pathological alterations as a result of α-ZAL treatment, it is worth mentioning that adverse effects of α-ZAL do exist and certain caution has to be taken. Prenatal or postnatal α-ZAL exposure has been shown to induce severe testicular abnormalities, decreased levels of interferon-γ and associated immune response, and symptoms of hyperestrogenism, especially the reproductive and developmental disorders *(31–33)*. Zearalenone (ZEN), the parent compound of α-ZAL, has also been shown to elicit a number of estrogenic effects such as decreased fertility, increased embryolethal resorption, reduced fetal size, changed weight of adrenal, thyroid, and pituitary glands, and alteration in serum levels of progesterone, as well as teratogenic effects *(34)*.

Mounting evidence indicates that ZEN is present in many plants and vegetables such as wheat, cotton, corn, celery, carrots, and beets. As an endogenous hormone, ZEN is believed to play a significant role in herbal development and growth *(35–37)*. Toxicological studies revealed that the ZEN reductive metabolite α-ZAL is safer than 17β-estradiol *(19,22)*. As a stimulator for animal growth, reports have been seen regarding the effect of ZEN on animal growth, including organ and gland systems *(38,39)*. During the last 10 years, hormones from natural sources have drawn much attention, especially on their ability to prevent or reduce cardiovascular morbidity and mortality *(16,40)*. Our results have shed some light onto the use of α-ZAL in the treatment of atherosclerosis. Future research on ZEN and its derivative α-ZAL should be focused on drug pharmacology, potential adverse effects, comparison with natural estrogens, and their molecular mechanism of action.

Methods

Experimental Animals and Dietary Feeding Regimes

The experimental protocols described in this study have been approved by Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, P.R. China). In brief, 60 healthy sexually mature nulliparous female rabbits of New Zealand strain (average body weight: 2.5 kg) were obtained from the Department of Experimental Animals, Chinese Pharmaceutical and Biological Institute (Beijing). All rabbits were fed a standard rabbit chow, and housed in approved animal facilities at room temperature with a 12/12-hour light–dark cycle. After an acclimation period, bilateral ovariectomy or sham-operation performed under anesthesia (pentobarbital) and sterile conditions. Sham operation consisted of anesthesia, visualization of the ovaries through incisions into the abdominal cavity, and closure of the wounds. Animals completely recovered 1 wk after surgery. The rabbits were assigned to one of the following seven groups with equivalent total cholesterol values and body weights.

Group A: Control (6 rabbits), standard rabbit chow;
Group B: Sham + CHO (8 rabbits), CHO was initiated 10 d after sham operation;
Group C: OVX + CHO (10 rabbits), CHO was initiated 10 d after bilateral ovariectomy;
Group D: OVX + CHO + ZAL-low (8 rabbits), CHO diet supplemented with low dose of α-ZAL (0.1 mg/kg/d) was initiated 10 d after bilateral ovariectomy;
Group E: OVX + CHO + ZAL-medium (10 rabbits), CHO diet supplemented with medium dose of α-ZAL (0.5 mg/kg/d) was initiated 10 d after bilateral ovariectomy;
Group F: OVX + CHO + ZAL-high (8 rabbits), CHO diet supplemented with high dose of α-ZAL (2.5 mg/kg/d) was initiated 10 d after bilateral ovariectomy;
Group G: OVX + CHO + 17β-estradiol (10 rabbits), CHO diet supplemented with 17β-estradiol (0.5 mg/kg/d) was initiated 10 d after bilateral ovariectomy.

To induce atherosclerosis, the rabbits were fed a diet containing cholesterol (CHO, 0.2 g/kg/d, Beijing Chemical Reagent Co., Beijing, China) for 12 wk. 17β-Estradiol

(Shanghai the 9th Pharmaceutical Co., Shanghai, China) and α-ZAL purified from *Fusarium roseum graminearum* (gift from Prof. Jilun Li at Chinese University of Agriculture in Beijing, P.R. China) were dissolved in olive oil (0.5 mL/kg/d) and were fed to rabbits using the gavage method. Control rabbits received 0.5 mL/kg/d olive oil only. For cholesterol diet, cholesterol was first mixed with the regular rabbit chow to make a cholesterol-containing diet (1%, w/w). The cholesterol-containing diet was then fed to experimental rabbits using the gavage method at 0.2 g/kg/d each morning before the animals received any regular rabbit chow (non-gavage feeding manner). All animals were maintained on these diets for 12 wk.

Uterine Weight, Aortic Specimen Preparation, and Morphological Observation

At the end of the 12-wk feeding period, the rabbits were euthanized with intravenous injections of 10% pentobarbital solution. Uterine tissue was surgically dissected and weighed using a standard laboratory scale. The descending, ascending aorta, and aortic arch were isolated, dissected free and opened longitudinally. The vessels were fixed with 10% formaldehyde. Some aortic tissues were stained with regular oil red O (Fluka Co., Switzerland), to be used for photographic and image scanning analysis. Some tissues were saved for other analysis. Atherosclerotic plaques were identified using positive stain scanning by our institutional Key State Laboratory Scanning System. Image analysis was performed with a Guant image analytical program.

Determination of Serum Lipoprotein

Blood samples were obtained from the central ear artery and serum was collected prior to drug treatment and at wk 4, 8, and 12, and stored at –20°C. The lipids were extracted with chloroform and methanol and lipids and proteins were separated. Total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), Apo-protein A (ApoA), and Apo-protein B (ApoB) levels were determined by a Biochemical Analyzer (Olympus AU1000, Japan) from Peking Union Medical College Hospital (Beijing, P.R. China).

Statistical Analysis

Data were presented as mean ± SEM. Statistical significance ($p < 0.05$) for each variable was estimated by analysis of variance (ANOVA) using the SPSS software.

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