Antihyperlipidemic, Antioxidant and Anti-Inflammatory Effects of Ginkgo Biloba in High Cholesterol Fed Rabbits.

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Abstract

Introduction: Atherosclerosis is a complex multifactorial process in which dyslipidemia, lipid peroxidation and inflammation are involved. Ginkgo biloba has been widely used as adjutant therapy in cardiovascular and cerebrovascular disorders due to its multiple pharmacological activities. The aim of study was to evaluate the effect of ginkgo biloba on atherosclerosis progression by interfering with inflammatory and oxidative pathways and improving lipid profile.

Method: 24 male rabbits were randomly divided into three groups; Control group, animals were fed normal chow diet, High cholesterol diet group, animals were fed 1% cholesterol enriched diet and Ginkgo biloba group, animals were fed with 1% cholesterol enriched diet together with ginkgo biloba given as 10 mg/kg/day. Serum cholestrol (TC), triacylglycerol (TAG) and high density lipoprotein (HDL-C) were measured at zero time and every 6 weeks. Inflammatory markers including serum high sensitive C - reactive protein (hsCRP) and serum intracellular adhesion molecule-1 (ICAM-1) were determined at zero time and after 12 weeks. At end of the study, the aorta was removed and used to assess tissue malondialdehyde (MDA) and tissue reduced glutathione (GSH) as well as histopathological alterations.

Results: Ginkgo biloba treatment significantly improved lipid profile parameters compared to high cholesterol diet group. The increments in inflammatory markers levels induced by high cholesterol diet were suppressed by gingko biloba as evident by significant reduction in serum hsCRP and ICAM-1 levels. Additionally, gingko biloba caused significant lowering in MDA levels and elevation in GSH levels of aortic tissue. As well as the development of atherosclerotic lesion was significantly attenuated by ginkgo biloba in comparison with induced untreated group.

Conclusions: Ginkgo biloba prevented atherosclerosis progression via modulating of lipid profile and suppressing inflammatory response and oxidative stress

Keywords: atherosclerosis, ginkgo biloba, inflammatory parameters, oxidative stress.

INTRODUCTION

Atherosclerosis is the leading cause of cardiovascular morbidity and mortality globally. It is a complex multifactorial process, possibly caused by high-fat diet and sedentary lifestyle. Hypercholesterolemia can be considered as the most important risk factors in progression and subsequent manifestation of cardiovascular diseases due to atherosclerosis [1]. Additionally, oxidative stress, lipid peroxidation and vascular inflammation are implicated in all stages of atherogenesis from initiation and progression as well as formation of atherosclerotic plaque [2, 3]. Oxidative modification of LDL is a lipid peroxidation process which result in increasing in level of lipid peroxide by products such as MDA and depletion of vascular antioxidant status such as GSH [3].The inflammatory reactions are also involved in pathogeneses of atherosclerosis[4]. CRP is an acute phase protein and consider as prototypic inflammatory marker. It has been reported that CRP directly implicated in first phase of atherosclerosis as evident by deposition in the intima, as well as by directly impact Nitric oxide bioavailability thus causing increased oxidative stress, endothelial dysfunction, and hyperplasia of intima [4, 5]. Additionally, increased expression of vascular adhesion molecules such as VCAM-1 play a critical role in the pathogenesis of atherosclerotic diseases [6]. Therefore, interfering with oxidative stress and inflammation is considered as the therapeutic potential for drugs and herbs in evaluating their anti-atherosclerotic activity [5, 6].

Ginkgo biloba (GB) is a plant that belong to tree species. The extract of ginkgo biloba (GBE) is obtained from dried leaves and it has been used to treat peripheral arterial diseases and neurological disorders since many decades.

The main constituents of the extract are flavonoid glycosides and terpen lactones. Flavonoid glycosides are thought to be responsible for its free radical scavenging abilities. The GBE antioxidant effect have been proposed to be responsible for its useful effects in many diseases [7, 8]. GBE decreases atherosclerotic nanoplaque formation and size in patients with high cardiovascular risk and can also reduce the generation of reactive oxygen species intracellularly, nuclear factor κB (NF-κB) and adhesion molecule expression on the blood vessels and thereby block cytokine-stimulated endothelial adhesiveness [9, 10]. Also GBE ameliorate cardiac ischemia-reperfusion injury and endothelial dysfunction stimulated by reactive oxygen species [11]. Although the exact mechanism is not fully understood, collecting in vitro and in vivo evidences reveal the ameliorating effects of GBE in cardiovascular and cerebrovascular diseases [12]. The current study was constructed to evaluate the ginkgo biloba ameliorating effects on atherosclerosis progression through contracting inflammatory and oxidative pathways and improving lipid profile.

MATERIALS AND METHODS

Animals

Twenty-four male rabbits (weighing 1.4-2kg) were used in this study. Rabbits were placed in the animal house of Al Kufa Faculty of Pharmacy. They were reserved in cages in air conditioned room with 25°C ±2 temperature, 60–65% humidity and a 12hr light:12hr dark cycle. There was acclimatization period of 1 week before the experiment. The study was conducted according to the national guidelines for the Care and Use of Laboratory Animals. The study protocol was approved by the High Committee

for Review and Approval of Research Proposals of the Faculty of pharmacy of the University of Kufa.

Study design

The animals were randomly grouped into three groups:

I. **Control group (n=8)**: rabbits were fed normal chow diet and tap water for 12 weeks.

II. **High cholesterol diet group (n=8)**; rabbits were fed high cholesterol diet (a 1% cholesterol) and tap water for 12 weeks

III. **Ginkgo biloba group (n=8):** All rabbits of this group were fed same high cholesterol diet in group II plus ginkgo biloba (10 mg/kg) once daily at morning for 12 week.

Serum and tissue preparations

About 4 milliliters of blood were withdrawn from the central ear artery of the rabbit after an 12 hrs fasting at zero time and then every six weeks of the experiment. Samples of blood were kept for clotting at 37 C and serum was obtained by centrifugation at 3000 rpm for 10 min centrifuged and used to determine serum TC, triacylgcerols, HDL- C, hsCRP and ICAM-1 level. At the end of the study, aorta was removed after scarification of rabbits and divided into two parts. First part used to prepare tissue homogenate which was carried out in media of pH 7.4 phosphate- buffered saline (0.1 M) that contain 1% Triton-100 and protease inhibitor cocktail and processed using high intensity ultrasonic liquid processor. The homogenates were centrifuged at 4 C^o and supernatants were used for determination of MDA and GSH in aortic tissue. The other part used histopathological study using light microscopy (Olympus CX31) to assess the degree of atherosclerosis lesions per American Heart Association classification of atherosclerosis phases [13].

Statistical analysis

SPSS 21.0 for windows lnc. was utilized for statistical analysis. Data were expressed as mean ± SEM.; paired t-test was used to compare between the means at different time for the same group. Multiple comparisons among all groups were carried out by Analysis of Variance (ANOVA). LSD method was used for post-hoc tests. Chi-square test was also used to compare histopathological changes in various groups. Level of significance was P< 0.05 for statistical decision.

RESULTS

Effect of ginkgo biloba on serum lipid profile

Lipid profile levels at zero time were with no significant difference among all groups of study. Cholesterol-enriched diet caused significant increment in levels of lipid profile in groups (II &III) after 6 weeks and at end of experiment as compared with zero time (Table 1). In addition, lipid profile of the groups on high cholesterol diet (II and III) were significantly higher (P<0.01) than control group (I). However, ginkgo biloba treatment in group (II) caused significant (P<0.01) lowering in TC and TAG and elevation in HDL-C in comparison to induced untreated group (II) (Table 1).

Effect of gingko biloba on inflammatory markers

There was significant (P<0.01) increase in serum level of inflammatorily markers hs-CRP and ICAM-1 in cholesterol fed groups (II &III) at the end of experiment compared to base line value and to normal group (Table 2). Gingko biloba treatment for 12 weeks caused significant (P<0.05) lowering in serum level hs-CRP and ICAM-1 compared to induced untreated group (II) (Table 2).

Table 1: Changes in the serum lipid profile of the study groups.

Group		TC(mg/dl)	TAG(mg/dl)	HDL-C(mg/dl)
Control group (n=8)	at Zero time	80±1.9	36.3±4.6	13.7±3.0
	6 weeks	90±2.6	37.4±7.4	14.4±2.9
	12 weeks	85±4.3	35.61±4.22	14.52±1.82
High cholesterol diet Group (n=8)	at zero time	95.1±1.8	35.2±3.71	14.2±1.92
	6 weeks	843±21.6^{ab}	243±20.2^{ab}	37.1±2.6^{ab}
	12 weeks	995±31^{ab}	348±16.3^{ab}	39±5.4^{ab}
Ginkgo biloba group (n=8)	at Zero time	89±4.7	36.8±6.1	14.4±4.6
	6 weeks	640±7.89^{ac}	141±8.7^{ac}	35.8±2.6^{ac}
	12 weeks	757±11.72^{a}	206±11.9^{ac}	40.1±3.7^{ac}

TC, total cholesterol; TAG, triacylgycerol; HDL-C, high density lipoprotein cholesterol. Data expressed as mean ±SEM. ^{a} p<0.05 in comparison to zero time; ^{b} p<0.05 for (induced untreated vs Control group); ^{c}p<0.05 for (ginkobiloba vs induced untreated group)

Table 2: Changes in serum inflammatory markers of the all experimental groups.

Study groups	Hs-CRP mg/ml		ICAMpg/ml	
	At zero time	After 12 weeks	At zero time	After 12 weeks
Control group	1.3±0.7	1.6±0.9	7.6±1.09	7.5±0.6
Induced untreated group	1.1±0.5	23.6±3.4^{ab}	7.7±0.9	26.7±1.9^{ab}
Gingko biloba treated group	1.6±0.7	9.7±1.7^{ac}	8.2±0.17	18.1±1.2^{ac}

Hs-CRP, high sensitive C- reactive protein; ICAM-1, intercellular adhesion molecule. Data expressed as mean ±SEM. ^{a}p<0.05 compared to zero time in each group; ^{b}p<0.05 for (induced untreated vs control group);^{cp}<0.05 for (ginko biloba vs induced untreated group)

**Table 3: The oxidative stress parameters at the end of the study.**

Group	MDA μmole/gm	GSHnmole/gm
Normal control	2.1 ± 0.24	37.3± 2.6
Induced untreated	10.2 ± 0.51[b]	19.8± 1.5[b]
Ginkgo biloba 0.5 mg/kg	3.91± 0.62[c]	34.4± 1.2[c]

MDA, cardiac tissue malondialdehyde; GHS, reduced glutathione. Data expressed as means ±SEM. [b]p<0.05 for (induced untreated group versus control group); [c]p<0.05 for (ginko biloba group versus induced untreated group)

Table 4: Degree aortic atherosclerotic lesions in the three-experimental group.

Animal groups	Normal	Initial lesion	Intermediate lesion	Advance lesion
Control group (n.= 8)	100%	0	0	0
Induced untreated group (n.= 8)	0	12.5%	37.5%	50%
Gingko biloba group (n.= 8)	0	62.5%	25%	12.5%

Data expressed as percentage of lesion degree in each group and analyzed by Chi-square test
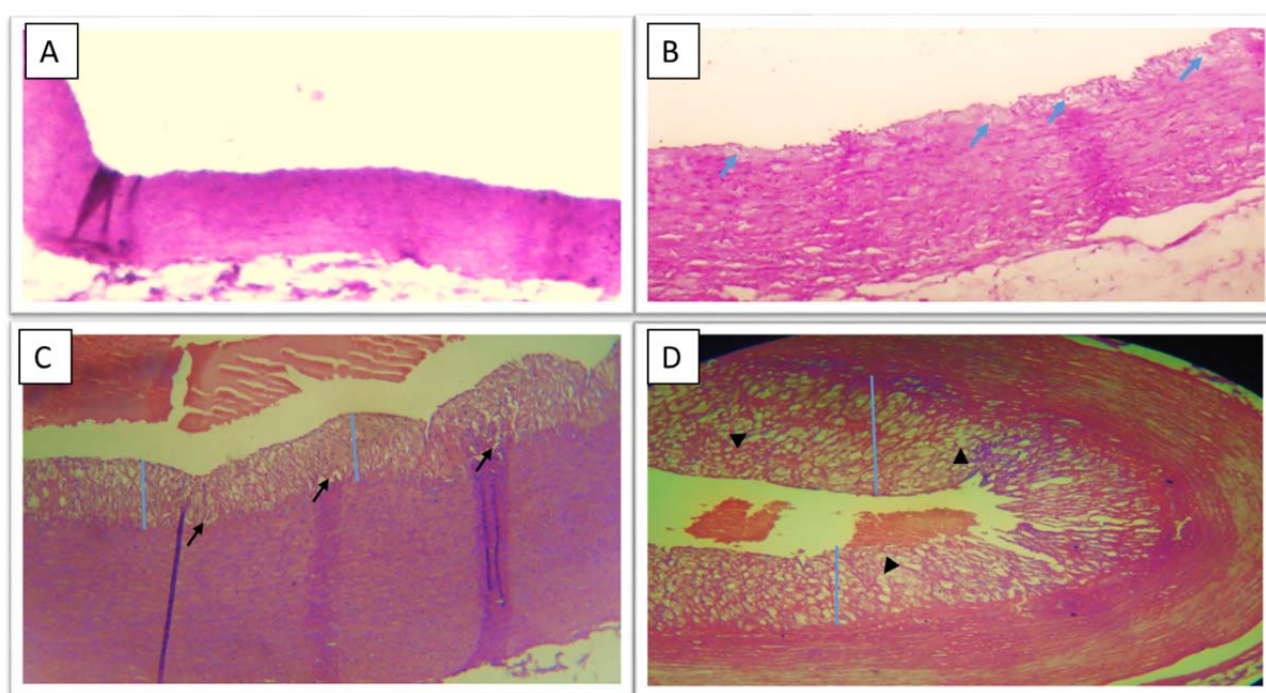
Figure 1: Photomicrographs of rabbit aorta section taken 100X magnification.
(A) showed normal arterial wall; (B) initial atherosclerotic lesion showed many lipid laden macrophages (Foam cells;blue arrow) forming fatty streak; (C) Intermediary lesion showed intimal thickening (blue line) with incompletely coalesced extracellular lipid (black arrow) and (D) advance lesion showed diffuse intima thickening (blue line) narrowing of lumen and confluence of lipid collections (black arrow head). Scale bar 20 um.

Effect of gingko biloba on oxidative stress
At end of experiment, high cholesterol diet caused highly significant (P<0.01) increment in aortic MDA level and highly significant (P<0.01) decrement in aortic GSH level in group II compared to control group (I) (Table 3). Interestingly these changes were reversed by gingko biloba treatment as evident by significantly (P<0.01) lower aortic MDA level and higher aortic GSH level compared to induced untreated group (II) (Table 3).

Histopathological study
A cross section of rabbit's aorta in control group (I) showed the normal appearance of all three arterial wall layers (intima, media and adventitia) while high cholesterol fed rabbits in induced untreated group showed 100% association of aorta with different severity of atherosclerotic lesion at the end of the study (after 12 weeks) and these changes were significantly (P<0.05) attenuated by gingko biloba treated rabbits (Table 4).

DISCUSSION
In the present study, feeding of a high-cholesterol diet to enrolled rabbits for 12 weeks resulted in marked increase in lipid profile parameters compared to base line and control group. Interestingly, this study demonstrated that ginkgo biloba treatment was significantly lowered TC and TAG while increased HDL-C compared to induced untreated group. The advantageous effect of ginkgo biloba on lipid parameters had been also demonstrated by reducing TC, TAG and LDL-C levels in animal study indicating potential

anti-hyperlipidemic effect [14]. Also, the results of current study showed that high cholesterol diet induced low grade inflammatory response characterized by significant elevation in the serum level of hsCRP in comparison with that of control group. This is in agreement with that of Sun et al (2005) who showed, for the first time, that C-reactive protein level was significantly raised in hypercholesterolemic rabbits compared to normal groups and the increase in C-reactive protein levels were correlated with the magnitude of hypercholesterolemia and atherosclerosis in rabbits [15]. In addition to that. Our result also showed that vascular inflammatory response and expression of adhesion molecule has been initiated by high cholesterol diet as evident by remarkable elevation in the serum of VACM-1 of induced untreated group compared to initial value of that of control group. Interestingly, these inflammatory responses induced by high cholesterol diet suppressed by ginkgo biloba treatment to notable extent as reflected by lowering serum level of hsCRP and VACM-1 than that in induced untreated groups. To the best of our knowledge there is no previous study regarding the effect of gingko biloba on hsCRP and VACM-1 in hypercholesteremic rabbit. However, suppressing effects of gingko biloba on proinflammatory cytokine has been demonstrated by reducing level of interleukin-1beta and tumornecrosis factor- alpha [10, 16]. Additionally, ginkgo biloba can inhibit cytokine-induced adhesion molecule expression in aortic endothelial cells by preventing ROS formation and down regulating of NF-κB [9, 10].

Additionally, the results of current study showed that diet with high cholesterol caused increment in oxidative stress and lipid peroxidation, which was manifested by pronounced elevations in aortic MDA level and notable reduction in aortic GSH level. These effects were expected since the previous studies in rabbits have demonstrated that high cholesterol diet associated with increased lipid peroxidation and depletion of antioxidant molecules [17, 18]. In many studies, Gingko biloba has been reported to reduce the level of lipid peroxide products such as MDA and preserved the antioxidant status by increasing GSH level [19, 20]. Consistently, in our study, gingko biloba treatment remarkably reduced lipid peroxidation induced by high cholesterol diet and completely restored the antioxidant molecules as manifested by lowering aortic MDA and elevating aortic GSH level respectively in comparison with induced untreated group. Therefore, ginkgo biloba extract has cryoprotective properties in cardiovascular and cerebrovascular diseases associated with increased free radical generation. The flavonoid components of ginkgo biloba responsible for its powerful antioxidant by scavenging the reactive oxygen species such as superoxide anion and hydroxyl radical [7, 19].

The current study revealed that animals fed by atherogenic diet developed severe hypercholesterolemia and lesions of atherosclerosis characterized by fatty streaks, atheroma and fibrous plaques. This progression of atherosclerotic lesions were significantly reduced by gingko biloba treatment to remarkable degree so that advance atherosclerotic lesions were observed in only one rabbit (12.5% of the rabbits in gingko biloba group) compared to four rabbits (50% of the rabbits in induced untreated group). Consistently, the antiatherosclerosis effect of gingko biloba has been previously reported by Liu F et al who showed that the extract of ginkgo biloba prevented homocysteine-evoked thickening of intima of abdominal aorta after balloon injury in rabbit [21]. Also, gingko biloba treatment reduced neointimal formation and prevented plaque development in diabetic rats after preparing balloon injury to the carotid artery [22]. Oxidative stress involves in several processes of atherogenesis, including endothelial cell injury, LDL oxidation and expression of adhesion molecules [23]. Also, inflammation plays an essential role in atherogenesis and mediating all levels of this disease from initiation to progression and, finally, the thrombotic complications of atherosclerosis [4]. Collectively, the findings of our study showed that gingko biloba treatment prevented development of atherosclerosis via modulating lipid profile, suppressing the inflammatory response and inhibiting expression of adhesion molecule as well as preventing lipid peroxidation. All these pharmacological effects of gingko biloba including antihyperlipidemic, antioxidant and anti-inflammatory effects might provide possible explanation for its antiatherosclerosis effect and hence its cardiovascular protective effects when used as adjuvant therapy.

CONCLUSIONS
Ginkgo biloba treatment effectively prevents atherosclerosis progression through improving lipid profile and interfering with inflammation and oxidative stress.

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