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ZOFENOPRIL DOWN REGULATES INFLAMMATORY BIOMARKERS AND AMELIORATES ATHEROSCLEROSIS IN HYPERLIPIDEMIC MALE RABBITS

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ABSTRACT

The objective of this study is to assess the effects of Zofenopril on atherosclerosis via interfering with inflammatory and oxidative pathways. Twenty four local domestic rabbits were assigned to three groups (eight rabbits in each group): Group I is the control; group II, rabbit fed 1% cholesterol-diet (induced untreated group) and group III, 1% cholesterol-diet + zofenopril (0.5 mg/kg daily orally). Blood samples were collected at baseline, after 6 and after 12 weeks on experimental diets for measurement of serum lipids, serum high sensitive C-Reactive Protein (hsCRP) , serum interleukin-6 (IL-6) and tumor necrotic factor-alpha (TNF-a). Histopathological and histomorphometrical examination of the aorta was done at the end of 12 weeks to assess the atherosclerotic changes and aortic intima-media thickness respectively. Aortic malondialdehyde (MDA) and reduced glutathione (GSH) was also measured. Results showed a significant improvement in the levels of lipid parameters, atherogenic index in group III compared to group II at ($P < 0.05$). Zofenopril counteract the change in hsCRP, IL-6, TNF-a, GSH and MDA in compare with induced untreated group ($P < 0.05$). Morphologic analysis revealed that zofenopril markedly reduced ($P< 0.05$) the severity of atherosclerotic lesion in the aorta compared with rabbits on a high-fat diet alone

and decreased aortic intima-media thickness in histomorphometric measurements. In conclusion, the current study illustrates the protective role of zofenopril against atherosclerosis progression in hypercholesterolemic rabbit via inhibition of inflammatory and oxidative pathways.

Key words: Zofenopril, histomorphometric measurements, atherosclerosis.

INTRODUCTION

Atherosclerosis is a chronic inflammatory, fibroproliferative disease of large and medium-sized arteries filled by lipids, it is mostly associated with hyperlipidemia and other several risk factors[1].Among the many cardiovascular risk factors, elevated plasma cholesterol level is probably unique in being sufficient to drive the development of atherosclerosis, even in the absence of other known risk factors . If all adults had plasma cholesterol levels <150 mg/dl, symptomatic disease would be rare. The other risk factors, such as hypertension, diabetes, smoking, male gender, and possibly inflammatory markers (e.g., C-reactive protein, cytokines, and so on), appear to accelerate a disease driven by atherogenic lipoproteins, the first of which being low-density lipoprotein (LDL)[2].Multiple cytokines and acute phase reactants have been studied having possible parts to predict cardiovascular events in healthy men as well as for risk stratification in established cases of CAD[3]. Among the most important inflammatory marker are acute-phase reactants such as C-reactive protein (CRP), serum amyloid A, and fibrinogen provide an indirect measure of cytokine-dependent arterial inflammatory process [4]The treatment of atherosclerosis relies heavily on the reduction of risk factors particularly elevated serum lipids level, hypertension, diabetes and cigarette smoking. The incidence of cardiac and cerebral infarction can be diminished considerably by controlling risk factors. Lifestyle factors are also of greatest importance in the treatment of atherosclerosis [5,6]. The most important and commonly used medications prescribed in the treatment of atherosclerosis include anti-hypertensives, cholesterol reducing drugs and antioxidant medications.

Zofenopril (also known as Zofenoprilum [Latin]) is an angiotensin converting enzyme (ACE) inhibitor with cardioprotective properties indicated for the treatment of hypertension [7]. Previous studies showed that zofenopril appeared significantly more effective than the older antihypertensives atenolol and enalapril, and was associated with less adverse effects [8,9]. Zofenopril is a highly lipophilic ACE inhibitor characterized by long-lasting tissue penetration and sustained cardiac ACE inhibition. As shown in preclinical studies, zofenopril

possess cardioprotective properties and attenuates ventricular remodelling in animal models of myocardial injury. Furthermore, zofenopril has antioxidant activity in vitro and in vivo, which may contribute to the anti-ischemic and antiatherogenic effects observed in experimental models. Orally administered zofenopril is rapidly converted into the active moiety zofenoprilat and is excreted through hepatic and renal routes [10,11]. It's clinical safety and efficacy has been demonstrated in patients with hypertension and in patients with acute myocardial infarction (AMI). The Survival of Myocardial Infarction Long-term Evaluation project provided valuable information regarding the safety of early onset ACE inhibition with zofenopril after AMI and a greater perception of the early and late benefits.

Aim of the Study

This study was undertaken to evaluate the effects of Zofenopril on progression of atherosclerosis and whether the suppression of atherosclerosis is associated with a decrease in inflammation and whether the suppression of atherosclerosis is associated with a decrease in the oxidative stress.

MATERIALS AND METHODS

Animals and study design

All experimental procedures and animal uses related to this study were approved by the Scientific and Ethical Committee of the College of Medicine –Kufa University. Twenty four local domestic rabbits (their genus and species are Oryctolagus Cunuiculus)[12,13] were used in this study. Their weight ranged between 1.4-1.7kg, they were housed in the animal house of AlKufa College of medicine. They were kept in cages under a 12-h light :12-h dark cycle at room temperature 25°C, and humidity was kept at 60–65%. , and they were allowed to drink tap water and given standard chow diet ad libitum. During 1 week of adaptation; alfalfa and other grasses or vegetables were given but with small amounts until the end of this week when they were prevented completely to avoid their influence on hypercholesterolemic atherosclerosis protocol. After the 1st week of acclimatization the rabbits were randomized into three groups as follow:

Normal control group (Group I): This group consists of 8 rabbits; all rabbits of this group were kept on standard chow diet and tap water throughout the duration of study (12 weeks).

High cholesterol diet control group (Group II): (induced untreated group): This group consists of 8 rabbits; all rabbits of this group were kept on atherogenic diet (a 1% cholesterol-enriched diet) and tap water throughout the duration of study (12 weeks).

Zofenopril treated group (Group III): This group consists of 8 rabbits. All rabbits in this group were kept on atherogenic diet (1% cholesterol enriched diet) and tap water for 6weeks, then they were treated with Zofenopril 0.5 mg./kg. body weight / day orally [14] in a single dose for the next 6 weeks. The treatment continued together with the atherogenic diet.

Blood sampling

From each rabbit about 3 ml of blood was collected from the central ear artery after an overnight fasting. The blood sampling was done firstly at the start of the study, i.e. at zero time, 6 weeks of the induction period, i.e.: before the start of treatment with zofenopril then at the end of the treatment course. The blood samples were allowed to clot at 37°C and centrifuged at 3000 rpm for 15 min. Sera were removed and analyzed for determination of serum total cholesterol (TCh), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C), tumor necrotic factor alpha (TNF-alpha), interleukin 6 (IL-6) and C-Reactive protein (CRP).

Tissue preparation for oxidative stress measurement

Unless immediately used, some of aortic tissue about 1gm (aorta) are taken and stored in deep freezer (-80) and then the aorta homoginized or sonicated after washing with phosphate buffer and during sonicating also use cold phosphate buffer and added Na_2EDTA then sonicating in the ice field and using the centrifuge for 6000 rpm for 30 minutes and the supernatant was drawn and analyzed for oxidative stress (GSH and MDA) i.e: 20% homogenates of aortic tissue were prepared in phosphate buffer[15] at pH 7.5 containing 1 mmol/l Na_2EDTA.The homogenates were centrifuged at 6000 rpm at 4°C for 30 min and the supernatants were used for measurements of GSH &MDA level.

Tissue sampling for histopathology

At the end of the experiment (12 weeks on their respective diets), rabbits were sacrificed with high dose of Phenobarbital sodium (200mg, intravenously). The rabbits were dissected through the chest wall to make the aorta accessible for resection. The aortic arch was exteriorized, cleaned of adherent fat and connective tissue excised. All specimens were immediately fixed in 10% formaldehyde solution. After fixation they were processed in usual manner. The sections were examined by microscope under magnification power of (×10 and ×40) [16,17]

Histopathological procedure

The aortic arch with about 1 cm of the descending aorta was fixed in 10% formalin for 24 hour, then washed and dehydrated in ascending series of ethanol solution (70,80,90,95%) for 2hr. in each concentration and 4hr divided on two changes for 100% (v/v) , cleared in two changes of xylen, 15 minutes for each, then embedded in paraffin wax. Thin section about (6mm) was dewaxed in xylen for 6 minutes, hydrated in descending series of ethanol (2 min. for each 2 change in 100% ethanol, then 2 min, in each of 95, 90, 80 and 70% v/v of ethanol solution, then transferred to distilled water for 2 minutes. The sections were stained in haematoxylin for 2-5 min, washed in tap water for 2-3 min, discolored in 0.5-1% HCL in 70% alcohol for few seconds, washed in tap water for at least 5 min and stained in 1% aqueous eosin for 1-2 min., then the section washed in tap water and dehydrated in ascending series of ethanol (70,80,90,and 100% v/v) for the same period of hydration, then cleared in xylen and mounted in DPX [18].

Statistical analysis

Statistical analyses were performed using SPSS 14.0 for windows.lnc. Data were expressed as mean ± SEM.; paired t-test was used to compare the mean values within each group at different time. Analysis of Variance (ANOVA) was used for the multiple comparison among all groups followed by post-hoc tests using LSD method. In all tests, $P< 0.05$ was considered to be statistically significant.

RESULTS

Serum lipids and atherogenic index showed a significant improvement in week 12 in the zofenopril treated group compared to week 6 measurements at $p\leq0.05$. No similar relation was identified in the normal control group, or in the high cholesterol diet control group (Table 1).

Table (1): Changes of serum lipids and atherogenic index among the three groups. The data expressed as Mean ±SEM (N=8in each group)

Parameters	Groups	Baseline	6 Weeks	12 Weeks
# Total Cholesterol (mg/dl)	Group I	47.8±0.9	48.8±0.79	49.5±1.14
	Group II	49±0.98	616±27*	883±45**
	Group III	47±0.73	550±18*	400±18**
# Triglycerides	Group I	40±0.6	39±1.05	39±0.76
	Group	40±0.96	150±1.82*	225±9**

(mg/dl)	**II**			
	Group III	40±0.85	148±3.3*	110±6.14**
# High density lipoprotein cholesterol (mg/dl)	**Group I**	17±0.42	17±0.49	18±0.47
	Group II	19±0.42	14±0.23*	12±0.36**
	Group III	18±0.42	13±0.47*	16±0.3**
# Low density lipoprotein cholesterol (mg/dl)	**Group I**	23±0.47	23±0.57	23±0.47
	Group II	23±0.88	500±27*	650±22**
	Group III	23±0.42	485±22*	375±21**
# Very low density lipoprotein cholesterol (mg/dl)	**Group I**	8±0.36	8±0.42	9±0.66
	Group II	8±0.25	30±0.36*	45±1.8**
	Group III	8±0.8	28±0.47*	20±0.65**
# Atherogenic Index of Plasma (AIP)	**Group I**	1.75±4.2	1.78±4.7	1.8±4.2
	Group II	1.73±6.14	42±2.9*	73±6**
	Group III	1.78±4.7	37±.84*	27±1.3**

*p≤0.05, significant difference between week 6 and baseline

**p≤0.05, significant difference between week 12 and week 6

# p<0.05, significant difference between Group II & Group III at week 12

Group I: Normal control group; Group II: High cholesterol diet control group; Group III: Zofenopril treated group

In addition, there was a significant reduction in serum lipids and AIP in group III compared to Group II at week 6 (Figure 1) as seen below:

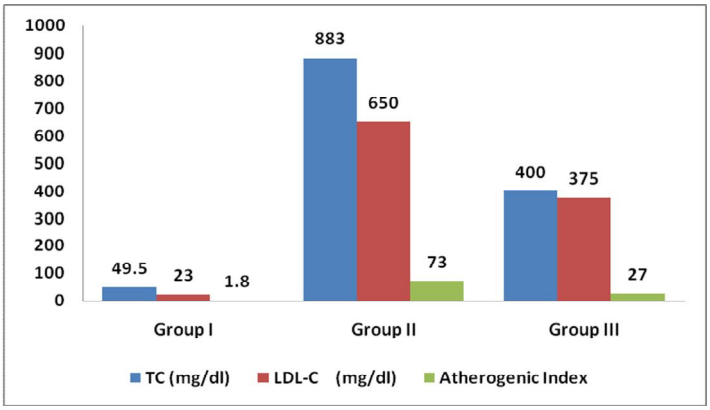

Figure 1. Significant reduction in serum TC, LDL-C & Atherogenic Index in Group III compared to Group II after 12 weeks of study at (p<0.05).

TC: Total Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol; Group I: Normal control group; Group II: High cholesterol diet control group; Group III: Zofenopril treated group

This was associated with a significant improvement in the inflammatory markers as expressed by the hsCRP, IL-6 and TNF-a in week 12 of group III compared to the earlier measurements at p≤0.05. This was not the case in group II and group III (Table 2 and Figure 2).

Table (2): Changes of inflammatory markers among the three groups. The data expressed as Mean ±SEM (N=8in each group)

Parameters	Groups	Baseline	6 Weeks	12 Weeks
# High sensitive C-reactive protein (mg/L)	Group I	2.9±0.27	2.7±0.3	3±0.23
	Group II	2.6±0.24	39±0.88*	53±0.95**
	Group III	2.75±0.2	40±0.57*	24±0.8**
# Interleukin-6 (pg/ml)	Group I	8±0.47	8.5±0.4	8.16±0.4
	Group II	8±0.47	20±0.36*	28±0.47**
	Group III	8.16±0.4	21±0.55*	12±0.4**
# Tumor necrotic factor alpha (pg/ml)	Group I	1.3±0.66	1.35±0.28	1.4±0.77
	Group II	1.33±0.94	7±0.36*	12±0.3**
	Group III	1.35±0.28	7.5±0.4*	4±0.36**

*p≤0.05, significant difference between week 6 and baseline

**p≤0.05, significant difference between week 12 and week 6

# p<0.05, significant difference between Group II & Group III at week 12

Group I: Normal control group; Group II: High cholesterol diet control group; Group III: Zofenopril treated group

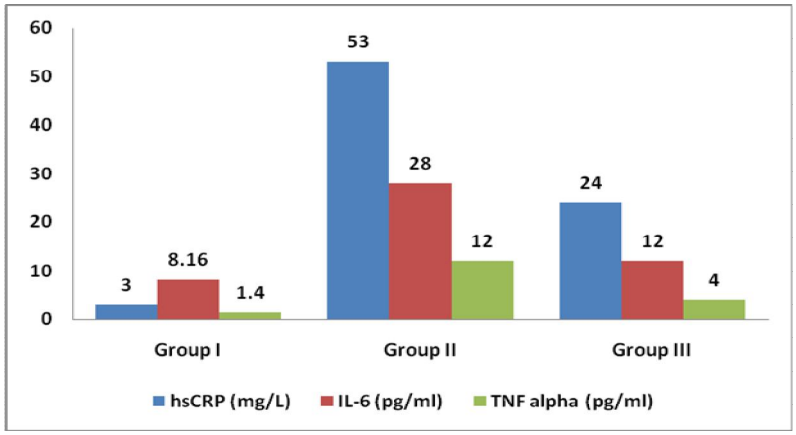

Figure 2. Significant reduction in serum inflammatory markers was seen in Group III compared to Group II at 12 weeks of the study (p<0.05)

hsCRP: High Sensitivity C Reactive Protein; ; IL-6: Interleukin 6; TNF-alpha: Tumor Necrotic Factor Alpha; Group I: Normal control group; Group II: High cholesterol diet control group; Group III: Zofenopril treated group

In addition, there was a significantly reduction in MDA (1.50±0.45) in group III compared to group II. This was associated with a significant increase in reduced glutathione (30.33±0.67) in the same group at p≤0.05. The aortic intima thickness was significantly reduced in group III (212.5±12) compared to group II at p≤0.05 (Table 3).

Table (3): Changes of oxidative stress markers and aortic intima thickness among the three groups. The data expressed as Mean ±SEM (N=8in each group)

Parameters	Group I	Group II	Group III
Malondialdehyde (umol/L)	1.283±0.5	2.2±0.4*	1.50±0.45**
Reduced glutathione (mmol/L)	37.16±0.67	24.5±0.67*	30.33±0.67**
Aortic Intima thickness (um)	87.33±12	325.83±12*	212.5±12**

*p≤0.05, significant difference between group II and group I

**p≤0.05, significant difference between group III and group II

Group I: Normal control group; Group II: High cholesterol diet control group; Group III: Zofenopril treated group

Histologically all induced-untreated rabbit showed significant atherosclerosis lesions ($P < 0.05$). Morphologic analysis revealed that Zofenopril markedly reduced ($P < 0.05$) the severity of atherosclerotic lesion in the aorta compared with rabbits on a high-fat diet alone and decreased aortic intima-media thickness in histomorphometric measurements (Figure 3):

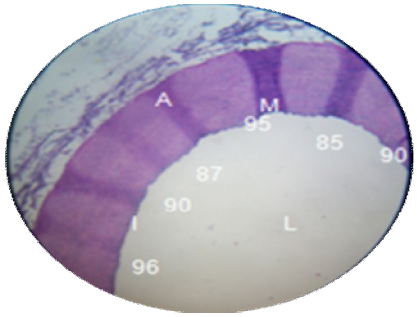

Figure 3 A. Photomicrograph of histophotometric section in aortic arch of rabbit fed on normal diet for 12 wks (normal control) show the normal intimal thickness and intact continuous endothelium. Stained with haematoxylin and Eosin (x10), where, I: intima of the aorta, M: media of the aorta, A: adventitia of the aorta and L: the lumen of the aorta.

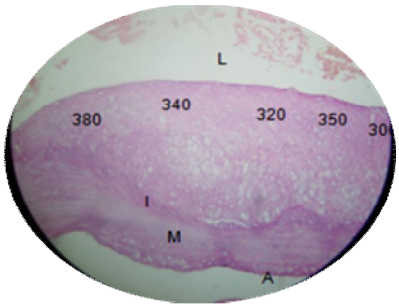

Figure 3 B. Photomicrograph of histomorphometric section in aortic arch of rabbits on atherogenic diet for 12 wks (induced untreated show diffuse intima thickening and in completely coalesced extracellular lipid underneath a layers of macrophages and smooth muscle cells. The section stained with haematoxylin and eosin(x10). Where, I: intima of the aorta, M: media of the aorta, A: adventitia of the aorta and L: the lumen of the aorta.

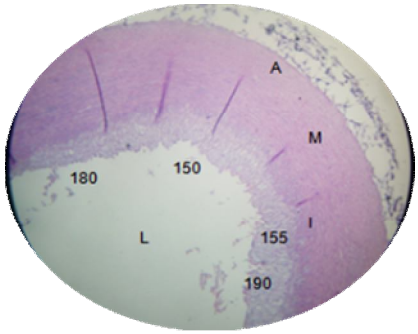

Figure 3 C. Photomicrograph of histomorphometric section in aortic arch of zofenopril treated hyperlipidemic rabbits. The section stained with haematoxylin and eosin (x10). where, I: intima of the aorta, M: media of the aorta, A: adventitia of the aorta and L: the lumen of the aorta.

DISCUSSION

In the present study, Zofenopril treatment reduced lipid profile, atherogenic index and increased HDL level and these results are consistent with that reported by (Napoli ..et al 1999)[19] who studied the effects of angiotensin-converting enzyme (ACEI)-inhibition by zofenopril on the development of atherosclerosis and low-density lipoprotein (LDL) in hyperlipidemic rabbits. After 6 weeks of treatment, zofenopril reduced lipid profile and the aortic cumulative lesion area by 34% in the zofenopril-treated group compared with control group. Also in our study, treatment with zofenopril significantly reduced hs-CRP level which agrees with (Prasad K..et al ; 2006) [20] who showed that zofenopril has potential capability of controlling the initiation and progression of atherosclerotic lesions beyond its antihypertensive effect. The result of this study demonstrated a significant effect of zofenopril on inflammatory acute phase protein by inhibiting the elevation of hs-CRP in rabbit on high fat diet that suggesting a potent anti-inflammatory activity of zofenopril on the vascular inflammatory responses induced by high fat diet. In the present study, treatment of hyperlipidemic rabbits with zofenopril results in reducing level of TNF-a compared with control untreated rabbits, these results are supported by (Cominacini..et al 2002) [21]. Who compared the effects of two different angiotensin converting enzyme (ACE) inhibitors, one possessing an active sulfhydryl group (zofenopril) and one lacking this group (enalapril) on the cellular redox state. Zofenoprilat, the active form of zofenopril, significantly and dose dependently reduced tumor necrosis factor-alpha (TNF-alpha). Enalaprilat, the active form of enalapril, was ineffective. Zofenoprilat but not enalaprilat also decreased the consumption of the intracellular GSH induced by ox-LDL and decreased TNF-alpha. Also, zofenoprilat significantly and dose dependently reduced the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and E-selectin induced by ox-LDL because, the sulfhydryl (SH)-containing ACE inhibitors may be useful in inhibiting foam cell formation and thus, slow the development of atherosclerosis by decreasing TNF-a level .Also, in our study, Zofenopril treatment causes significant reduction in IL-6 level compared with that of control untreated rabbits and these results are in line with (Walter..et al 2000) [22] who found that treatment with Zofenopril associated with decreasing IL-6.Also, (Prasad K..et al ; 2006) [20]who showed that zofenopril have potential capability of controlling the initiation and progression of atherosclerotic lesions beyond its antihypertensive effect. The result of this study demonstrated a significant effect of zofenopril on inflammatory acute phase protein by inhibiting the elevation of hsCRP. Also, inhibits IL-6 in rabbit on high fat diet that suggesting a potent anti-inflammatory activity of

zofenopril on the vascular inflammatory responses induced by high fat diet. Therefore, both CRP, and IL-6 levels were markedly lowered by zofenopril.

In our study, rabbits treated with zofenopril and compared with control untreated rabbits showed significant reduction in oxidative stress parameters and these results are in line with (Cominacini..et al 2002) [21] who studied the effects of two different angiotensin converting enzyme inhibitors, one possessing an active sulfhydryl group (Zofenopril) and one lacking this group (enalapril) on the cellular redox state (monitored by measuring intracellular reactive oxygen species and thiol status), expression of adhesion molecules, and activation of NF-kappaB in human umbilical vein endothelial cells (HUVECs). Zofenoprilat, the active form of Zofenopril, significantly and dose dependently reduced the intracellular reactive oxygen species (ROS) and superoxide formation induced by oxidized low-density lipoprotein (ox-LDL) .Enalaprilat, the active form of enalapril, was ineffective. Zofenoprilat but not enalaprilat also decreased the consumption of the intracellular GSH induced by ox-LDL . Therefore, the sulfhydryl (SH)-containing ACE inhibitors may be useful in inhibiting oxidative stress and foam cell formation and thus, slow the development of atherosclerosis. Zofenopril, a new potent sulphydryl angiotensin-converting enzyme (ACE) inhibitor, was characterized by high lipophilicity, selective cardiac ACE inhibition, and antioxidant and tissue protective activities. In vitro and in vivo experiments suggest that zofenopril exerts antioxidant properties at clinically achievable tissue concentrations. In endothelial cells, Zofenopril enhances nitric oxide production, attenuates atherosclerotic lesion development and inhibits adhesion molecule expression by reducing reactive oxygen species. These peculiar characteristics are reflected in the drug's cardioprotective activity, which has been shown to be greater than that of non-sulphydryl ACE inhibitors.These results are reported by (Evangelista S. et al., 2005)[23].

Also, in the present study, Zofenopril treatment reduce the aortic intimal thickening compared with control group. This result supported by a study reported by (Napoli C. et al, 2008) [24] who studied the effects of angiotensin-converting enzyme inhibitors, zofenopril and enalapril, on aortic and carotid IMT and found that zofenopril treatment resulted in significant lowering in the progression of aortic and carotid IMT than occurred with enalapril and the cause might be due to that Zofenopril has anti-atherosclerotic, anti-inflammatory and anti-oxidant effects. Also, in our study, the effect of treatment with the new sulfhydryl angiotensin-converting enzyme (ACE)-inhibitor, zofenopril, on aortic intema-

media thickness was supported by a study obtained by (Niqris et al 2001) [25] who studied the comparison of Zofenopril with the classical sulfhydryl ACE-inhibitor captopril or enalapril on the development of atherosclerosis. After treatment with ACEIS, Zofenopril reduced the aortic cumulative lesion area by 78% at 0.05 mg/kg/day and by 89% at 1 mg/ml/day of zofenopril compared to that of thecontrol group. Captopril reduced by 52% aortic lesions compared with control group. Enalapril did not reduced aortic lesions.Thus, chronic treatment with the new sulfhydryl ACE-inhibitor zofenopril has antiatherosclerotic and antioxidant effects. This protection was significantly higher than that reached with captopril and at lower doses of the drug. Treatment with 0.5 mg/kg/day of enalapril did not provide any protective effect. Therefore, Zofenopril was considered to be superior in reducing aortic IMT comparing with other ACEIs.

Conflict of interest: The authors declare that they have no conflict of interest

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