Journal of Clinical & Experimental Cardiology
ISSN: 2155-9880

Hadi et al., J Clin Exp Cardiolog 2013, 4:6
DOI: 10.4172/2155-9880.1000250

Research Article Open Access

Monteleucast and Zileuton Retard the Progression of Atherosclerosis via Down Regulation of the Inflammatory and Oxidative Pathways

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Abstract

Background: Atherosclerosis and its thrombotic complications are responsible for remarkably high numbers of deaths. Leukotrines are involved in different stages of atherosclerosis. Therefore this study was undertaken to evaluate the effect of montelukast and zileuton on the progression of atherosclerosis.

Materials and methods: Thirty-five male rabbits were used in this study. These animals randomized into 5 groups (7 rabbits each). Rabbits in first group were maintained on normal rabbit chow diet and used as normal diet control group (NC). While the rabbits in other four groups were fed on atherogenic diet (2% cholesterol) for 8 weeks. The second group, Atherogenic Control Group (AC) rabbits received atherogenic diet alone. Third group, Positive Control Group (PC) rabbits received atherogenic diet and ethanol as vehicle. Forth group, Montelukast Treated Group (MT) rabbits received montelukast 1.5 mg per kg daily and the fifth group, Zileuton Treated Group (ZT) rabbits received zileuton 150 mg per kg daily. At the end of 8th weeks animals were sacrificed, blood sample was collected to measure the following parameters: lipid profile, plasma GSH, MDA, and hsCRP. Immunohistochemical analysis (VCAM-1, MCP-1, TNF-α, and IL17) and histopathologic assessment of aortic atherosclerotic changes were also performed.

Results: Compared to NC, levels of lipid profile, atherogenic index, hsCRP, and MDA are increased while GSH were decreased in animals on atherogenic diet ($p<0.001$). There was statistically insignificant difference in the study parameters between positive control groups, when compared with those on atherogenic diet. Immunohistochemical analysis showed that expression of aortic VCAM-1, MCP-1, TNF-α and IL17 were significantly increased in AC group compared to NC group ($p<0.001$). Histopathologic finding showed that animals on atherogenic diet have significant atherosclerotic lesion compared to NC group. Compared to AC group both montelukast and zileuton do not have significant effect on lipid profile. Montelukast and zileuton cause statistically significant reduction in hsCRP and MDA, ($p<0.001$). Montelukast and zileuton treatment caused statistically significant, increase in plasma levels of GSH and reduced plasma MDA level ($p<0.001$). Both montelukast and zileuton treatment significantly reduced the expression of aortic VCAM-1, MCP-1, TNF-α and IL17 ($p<0.001$). Histopathologic examination of aortic arch showed that both montelukast and zileuton significantly reduced atherosclerotic lesion ($p<0.001$).

Conclusion: Both montelukast and zileuton reduce lipid peroxidation, systemic inflammation and aortic expressions of inflammatory markers used in this study and reduced the progression of atherosclerosis.

Keywords: Atherosclerosis; VCAM-1; MCP-1; TNF-α; IL17

Introduction

Atherosclerosis is a disease of large and medium-sized muscular arteries and is characterized by endothelial dysfunction, vascular inflammation, and the buildup of lipids, cholesterol, calcium, and cellular debris within the intima of the vessel wall, this buildup results in plaque formation, vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow and diminished oxygen supply to target organs [1]. Hypercholesterolemia enhances the response to vasoconstrictor agonists and attenuates endothelium-dependent relaxation in isolated vessels and in vivo. EDNO is now recognized to inhibit several pathologic processes that are critical to the development of atherosclerosis. These include monocyte adherence and chemotaxis, platelet adherence and aggregation, and vascular smooth muscle proliferation [2].

In metabolic disorders associated with atherosclerosis as hypercholesterolemia, hypertension, and diabetes mellitus, a reduced endothelium-mediated, NO-dependent vasodilation is observed, which may contribute to the initiation and progression of atherosclerosis associated with these disorders [3].

Endothelial cells (ECs) normally resist leukocyte adhesion. Proinflammatory stimuli, including a diet high in saturated fat, hypercholesterolemia, obesity, hyperglycemia, insulin resistance, hypertension, and smoking, trigger the endothelial expression of adhesion molecules such as P-selectin and vascular cell adhesion molecule-1 (VCAM-1), which mediate the attachment of circulating monocytes and lymphocytes [4-6]. The adhesion of monocytes to the vascular endothelium and their subsequent recruitment into the artery wall are key features in the pathogenesis of atherosclerosis. VCAM-1, an adhesion molecule expressed on the endothelial cell surface, may be partly responsible for the recruitment of monocytes during

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Received May 07, 2013; **Accepted** May 24, 2013; **Published** May 26, 2013

Citation: Hadi NR, Mohammad BI, Almudhafer A, Yousif N, Sultan AM (2013) Monteleucast and Zileuton Retard the Progression of Atherosclerosis via Down Regulation of the Inflammatory and Oxidative Pathways. J Clin Exp Cardiolog 4: 250. doi:10.4172/2155-9880.1000250

atherogenesis. VCAM-1 expression has been demonstrated in human coronary atherosclerotic plaques, and this is consistent with the belief that this adhesion molecule plays a role in the disease [7].

Morphologic studies have established that, once adherent to the endothelial cell, leukocytes enter the intima by diapedesis between endothelial cells at their junctions. Investigators have defined families of chemoattractant cytokines (chemokines) capable of recruiting leukocytes into the arterial intima. For example, monocyte chemoattractant protein-1 (MCP-1), overexpressed in human and experimental atheroma, can recruit the mononuclear phagocytes that characteristically accumulate in the nascent atheroma. IL-8 may have a similar role as a leukocyte chemoattractant during atherogenesis [8]. Atheroma overexpresses other chemokines that may contribute to lymphocyte recruitment, including a trio of CXC chemokines induced by interferon-γ (IFN-γ) [9]. Chemoattraction of mast cells found in atheroma may depend on eotaxin, a CC chemokine also overexpressed in these lesions [10]. Within the intima, monocytes mature into macrophages under the influence of macrophage colony stimulating factor, which is overexpressed in the inflamed intima [11]. Although the use of statins may decrease both systemic and local inflammation in atherosclerosis, specific anti-inflammatory agents targeting key immune reactions in the atherosclerosis process could anticipate a potential further benefit. Ever since the concept of inflammation and atherosclerosis was raised, a number of inflammatory mediators have been explored as potential therapeutic targets in this disease [12]. The inflammatory response induced through leukotrine (LT) signaling may be of particular interest since several drugs targeting this pathway are either available or under development [13]. Studies of genetic polymorphisms have established significant associations for the LT pathway with early signs of atherosclerosis [14,15]. Furthermore, mechanistic studies have implicated the LT pathway at several different stages of the atherosclerosis process [16]. The present study was undertaken to assess the p[ossible antiatherosclerotic potential of Zileuton and Monteleucast via interference with oxidative and inflammatory pathways. Zileuton, is a 5-lipooxygenase inhibitor and thus inhibits leukotrines(LTB4,LTC4,LTD4 and LTE4) formation while Monteleucast is a selective leukotrine receptor antagonist that specifically inhibits the cysteinyl leukotrine CysT1 receptor.

Materials and Methods

Preparation of animals

A total number of 35 Male Rabbits weighing 2-2.5 kg were used in this study. All experiments were conducted in the Department of Pharmacology, College of Medicine, Al Qadisiyah University, according to the guidelines for the care and use of laboratory animals in scientific research. The animals were maintained under 25 C, 45 ± 5% humidity and 12:12 h light: dark cycle. They were supplied with standard laboratory chow and water ad libitum and left to acclimatize for 2 week before the experiments.

The study design

After 2 weeks of acclimatization period, animals were randomized into 5 groups, each group contains 7 rabbits and as follow:

Normal control group: rabbits of this group were kept on standard chow diet and tap water throughout 8 weeks study period.

Atherogenic control group: (induced untreated group): rabbits of this group were kept on atherogenic diet (2% cholesterol-enriched diet) and tap water throughout 8 weeks study period.

Vehicle group: (positive control): rabbits of this group were kept on atherogenic diet (2% cholesterol enriched diet) and tap water. The administration of the vehicle (ethanol 10%) as solvent for the tested drugs continued together with the atherogenic diet throughout 8 weeks study period.

Montelukast treated group: rabbits of this group were kept on atherogenic diet (2% cholesterol enriched diet) and tap water. The treatment continued together with the atherogenic diet throughout 8 weeks study period.

Zileuton treated group: rabbits of this group were kept on atherogenic diet (2% cholesterol enriched diet) and tap water. The treatment continued together with the atherogenic diet during 8 weeks study period.

Animal model of atherosclerosis

Induction of hyperlipidemia and subsequent development of atherosclerosis were carried out by feeding the rabbits an atherogenic diet (2% cholesterol, BDH Chemicals Ltd Poole England, prod 43011)-enriched diet made by addition of cholesterol powder to chow pellets) for 8 weeks [17].

Preparation of drugs

Montelukast: Montelukast (MSD B.N 302048) was dissolved in ethanol [18]; ten tablets of this drug were dissolved in ethanol to prepare a fresh solution, and used in a dose of 1.5 mg/kg/day [19]. This drug was administered once daily to the animal according to body weight by oral route through stomach tube.

Zileuton: Zileuton (CORNERSTONE THERAPEUTICS INC. B.N 3083072) was dissolved in ethanol [18]; ten tablets of this dug were dissolved in ethanol to prepare a fresh solution and used in a dose of 150 mg/kg/day [20]. This drug was administered once daily orally according to body weight through stomach tube.

Preparation and collection of sample: At the end of the experiment, food was withheld for 16-18 hour and animals were anesthetized by ketamine (HIKMA pharmaceuticals B.N 3310) at 66 mg/kg and xylazine (alfasan B.N 1004111-07) at 6 mg/kg intramuscular [21]. The chest was opened by thoracotomy, blood sample was collected directly from the heart and aorta was separated before the following investigations were performed:

1. Lipid profile including total serum cholesterol (TC), serum triglyceride, LDL-C, VLDL-C and HDL.
2. Immunohistochemistry for assessment of VCAM-1, TNF-α, IL17, and MCP-1.
3. Oxidative stress parameter including MDA and GSH. Systemic inflammatory marker hsCRP.
4. Histopathological examination of the aorta for assessment of atherosclerosis.

Preparation of blood sample

From each rabbit, 7.5 ml of blood were collected from the heart directly, by using disposable syringe and divided in to the following:

2.5 ml placed in serum tube and left to stand for 30 minutes and then centrifugated then 1 ml collected and used for determination of lipid profile, and five ml placed in tube contain EDTA as anticoagulant, the plasma was prepared by centrifugation at 4000 rpm for 10

minutes, 2 ml of plasma frozen at (-20 C°) and subsequently used for determination of oxidation parameter (MDA and GSH) and hsCRP.

Preparation of tissue sample: The aortic arches were exteriorized, cleaned of adherent fat and connective tissues and excised. All specimens were immediately placed in a fixative solution (10% formaldehyde solution) for 24 hours; dehydration was achieved by ascending series of ethyl alcohols concentration. Clearing was done by using two changes of xylene for 1 hour for each change. Infiltration with two changes of molten paraffin in oven set at 60 °C for 3 hrs. After that paraffin blocks made using embedding boxes. The blocks then were left to cold. Sectioning was done by using microtome of 5 µm thickness [22, 23].

Histological examination of the aorta: For histological evaluation of atherosclerosis, the specimens were processed in usual manner, and embedded in paraffin and cut into 5 µm thick sections. The tissue sections were stained with hematoxylin and eosin. The assessment of atherosclerotic changes was performed according to the American Heart Association classification of atherosclerosis; Type I and Type II lesions (early lesions), Type III lesions (intermediate lesions or preatheroma), Type IV lesions (atheroma), Type V lesions (fibro-atheroma or advance lesion) and Type VI (complicated lesion) [24].

Immunohistochemistry

Immunohistochemistry was performed with polyclonal goat antibodies, raised against rabbit VCAM-1, TNFα, IL17 and MCP-1 Staining procedure was carried out according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc). The stain intensity was scored to 0: Indicated no staining, 1: Weak, 2: Moderate, 3: Strong, 4: Very strong stain intensity [25] (Figure 1).

Statistical analysis

Statistical analyses were performed using SPSS 12.0 version. 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. The histopathological grading was assessed by Mann-Whitney test. In all tests, $P<0.05$ was considered to be statistically significant.

Results

Effect of high cholesterol diet

Compared to NC group, rabbits fed on cholesterol-enriched diet showed significant changes in serum lipid profile, oxidation and inflammatory markers. Serum levels of TC, TG and LDL-C as well as plasma level of MDA and hs-CRP were significantly ($p<0.001$) increased. In addition plasma levels of GSH were significantly ($p<0.001$) lower in rabbits fed on cholesterol-enriched diet in comparison to animals on normal diet (Tables 1 and 2).

Effects of treatment

Compared to atherogenic control, treating hyperlipidemic rabbits with montelukast and zileuton resulted in significantly ($p<0.001$) lower levels of plasma hs-CRP and MDA with significantly ($p<0.001$) higher levels of plasma GSH levels. However, montelukast and zileuton treatment caused no significant ($p>0.05$) alteration in the serum lipids (Tables 1 and 2).

Immunohistochemistry

The result of median intensity of immunohistochemical analysis

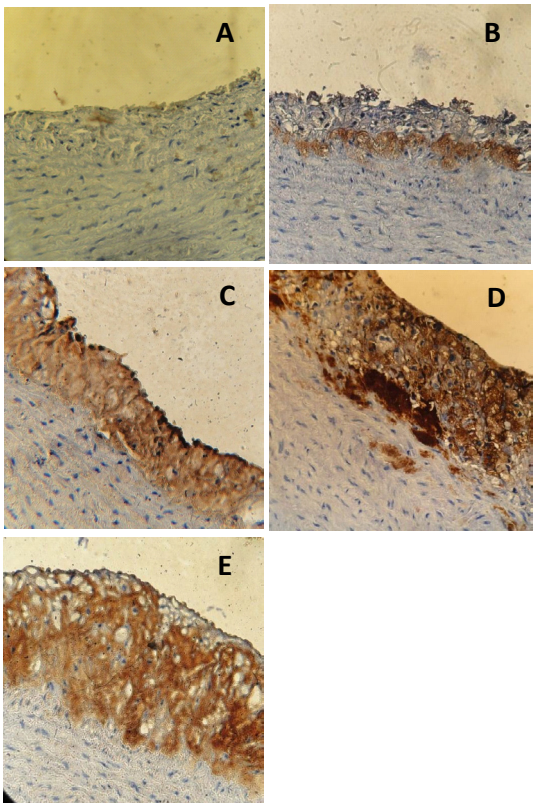

Figure 1: Immunohistochemical staining in aortic arch from cholesterol fed rabbits(x40) A: negative, B: weak stain intensity, C: moderate stain intensity, D: strong stain intensity,E: very strong stain intensity.

for rabbit's aortic arch of VCAM-1, MCP-1, IL17 and TNF-α were significantly highest in AC group (very strong for all markers) and lowest in NC group (normal for all markers). There is no statistically significant difference in median intensity of these markers between PC and AC control groups on atherogenic diet. Montelukast and zileuton treated groups were associated with a median stain intensity of moderate for VCAM-1, MCP-1, IL17 and TNF-alpha that is significantly lower than the atherogenic control (Table 3).

Histopathological findings

The atherosclerotic lesions of aortic arch were graded as normal, initial, intermediate, advance and complicated lesions (Figure 2). The median was highest in atherogenic control (advance) and vehicle control (PC) whereas lowest in the normal diet control (no abnormality). Montelukast and zileuton treated groups were associated with a median aortic change (initial) that is significantly lower than the atherogenic control.

Discussion

Effect of montelukast

In comparison to atherogenic control, the present study demonstrated that montelukast treatment had no effect on lipid profile. These findings were in agreement with Song Ge et al. [27]. Further, this study revealed that the values for the lipid peroxidation marker (MDA) were significantly reduced by montelukast treatment. GSH levels were significantly higher in montelukast treated animals. These finding suggested that montelukast markedly reduce oxidative stress. Go¨ksel

Groups					Parameters
Zileuton treated	Montelukast treated	PC	AC	NC	
1170.7 ± 39.96^{N}	1126.4 ± 34.38^{N}	1116.4 ± 42.9	1121.4 ± 41.6*	45.1 ± 0.94	TC (mg/dl)
358.7 ± 42.77^{N}	363.7 ± 37.74^{N}	357 ± 35.18	357.7 ± 41.3*	56 ± 1.13	TG(mg/dl)
22.7 ± 1.17^{N}	21.9 ± 0.8^{N}	22.1 ± 0.77	23 ± 1.35^{N}	18.1 ± 0.99	HDL(mg/dl)
1076.3 ± 41.91^{N}	1031.8 ± 33.89^{N}	1022.9 ± 38.7	1026.9 ± 37.61*	15.8 ± 1.71	LDL(mg/dl)
71.7 ± 8.55^{N}	72.7 ± 7.55^{N}	71.4 ± 7.04	71.5 ± 8.27*	11.2 ± 0.23	VLDL (mg/dl)

Results are expressed as mean ± SEM.
*p < 0.05, as compare to NC group, ^{N} not significant as compare to PC group

Table 1: Change in serum lipid profile among the five study groups.

Groups					Parameters
Zileuton treated	Montelukast treated	PC	AC	NC	
0.798 ± 0.0164**	0.752 ± 0.0164**	0.52 ± 0.013	0.568 ± 0.024*	1.102 ± 0.0258	Plasma GSH (mmol/L)
0.229 ± 0.0117**	0.254 ± 0.0107**	0.51 ± 0.0142	0.51 ± 0.0145*	0.133 ± 0.005	Plasma MDA(µmol/L)
62.3 ± 1.69**	67.7 ± 1.94**	135.9 ± 2.09	135.3 ± 1.4*	32.9 ± 0.88	Plasma hsCRP (µg/L)

*p < 0.05, as compare to NC group, **p < 0.05, as compare to AC group

Table 2: Change in mean plasma levels of hs-CRP, MDA and GSH among the five study groups.

Groups					Markers
Zileuton treated	Montelukast treated	Pc	AC	NC	
Moderate**	Moderate**	Very strong *	Very strong *	Negative	VCAM-1
Moderate **	Moderate **	Very strong*	Very strong*	Negative	MCP-1
Moderate **	Moderate **	Very strong*	Very strong*	Negative	TNFα
Moderate **	Moderate **	Very strong*	Very strong*	Negative	IL-17

*p < 0.05, as compare to NC group. **p < 0.05, as compare to PC group

Table 3: The difference in median tissue (VCAM-1, MCP-1,IL17 and TNF alfa)immunostain intensity among the five study groups.

et al. investigated the possible protective effect of montelukast against oxidative damage in a rat model of chronic renal failure (CRF), induced by 5/6 reduction of renal mass. CRF caused significant decreases in tissue GSH and which were accompanied with significant increases in MDA levels.They concluded that montelukast treatment associated with increased GSH level and decreased MDA levels [27]. Hulya et al. tested the effect of montelukast on histologic damage induced by testicular torsion-detorsion in rats and showed that Montelukast treatment significantly decreased the I-R induced elevation in testes tissue MDA and glutathione levels were found to be preserved [28].

Concerning hsCRP this study showed that montelukast treatment significantly reduces plasma hsCRP compared to AC group. Hooman et al. (2007) revealed that montelukast treatment to asthmatic patients associated with significantly lower serum CRP compared to placebo [29]. Other findings of this study revealed that montelukast significantly reduces the expression of aortic inflammatory marker (MCP-1, TNF-α, VCAM-1, and IL17). Regarding to MCP-1, this finding was in agreement with Song Ge et al. [26]. Regarding to TNF-α, this finding was in agreement with Ali et al. [30]. Regarding to VCAM-1, this finding was in agreement with A. Y. Wu et al. [31].

Although they do not produce CysLTs, neutrophils do possess receptors for LTC4 and LTD4, activation of which triggers relatively modest pro-inflammatory responses in these cells [32]. Interference with neutrophil activation by CysLTs released from other cell types, such as monocytes/macrophages, mast cells or eosinophils, may therefore underlie the neutrophil-directed therapeutic efficacy of montelukast. Alternatively, montelukast may possess secondary anti-inflammatory properties that are distinct from conventional antagonism of CysLT receptors. These include interference with activation of the transcription factor, nuclear factor $_{k}$B in immune and inflammatory cells, promotion of sustained production of interleukin-10 or by inhibition of signaling pathways triggered by P2Y receptors [33,34]. However, the contribution of these mechanisms to the possible neutrophil-targeted anti-inflammatory activity of montelukast is unclear. In addition, montelukast antagonizes the pro-inflammatory activities of neutrophils by another mechanism involving inhibition of cyclic nucleotide phosphodiesterases (PDE) and cAMP mediated attenuation of Ca2+ influx [32].

The beneficial effect of montelukast on the progression of atherosclerosis may be due to their favourable effect on oxidative stress, systemic inflammation and aortic inflammatory marker (VCAM-1, MCP-1 IL17 and TNF-α).

Effect of zileuton

In comparison to atherogenic control, the present study demonstrated that zileuton treatment had no effect on lipid profile. Further, this study revealed that the values for the lipid peroxidation marker (MDA) were significantly reduced by zileuton treatment. GSH levels were significantly higher in zileuton treated animals. These finding suggested that Zileuton markedly reduce oxidative stress. Xian-kun Tu et al. demonstrated that zileuton reduced MDA content in rats. The selective 5-LOX inhibitor zileuton inhibited the activation of NF-κB and reduced the expression and activation of iNOS , In addition, NF-κB has been shown to regulate the expression of iNOS and other inflammatory mediators, attenuation of iNOS expression and NO production has been demonstrated to have protective role [35].

Concerning hsCRP this study showed that zileuton treatment significantly reduces plasma hsCRP compared to AC group. Jean et al. showed that treatment with 5-Lipoxygenase inhibitor VIA-2291 (Atreleuton) in patients with recent acute coronary syndrome caused a significant reduction in hs-CRP [36]. Other findings of this study revealed that zileuton significantly reduces the expression of

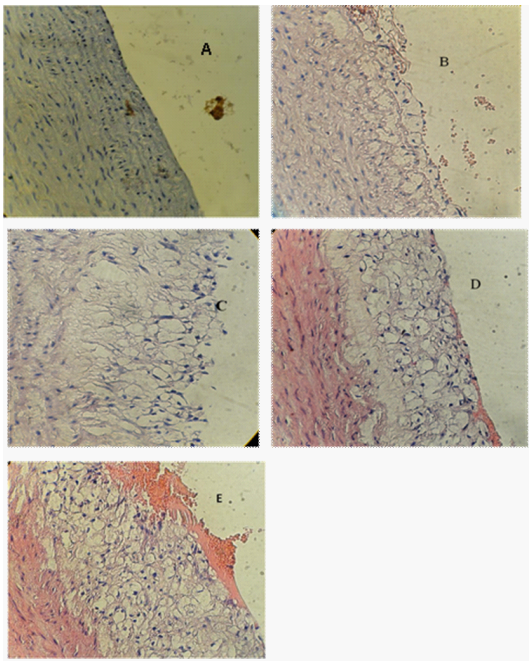

Figure 2: A cross section of aortic arch from hypercholesterolemic rabbit represented atherosclerosis progression (x40). **A**: Normal arterial appearance, **B**: Initial atherosclerotic lesion characterized by lipid laden macrophage (foam cells), **C**: Intermediate atherosclerotic lesion characterized by extracellular lipid pool. **D:** Advance atherosclerotic lesion characterized by core of extracellular lipid and. **E**: Complicated atherosclerotic lesion characterized by haemorrhagic thrombus.

aortic inflammatory marker (MCP-1, TNF-α, VCAM-1, and IL17). Regarding the effect on MCP-1, this finding was in agreement with Li Huang et al., they found that LTB4 strongly induces MCP-1 production in primary human monocytes. The LTB4-induced MCP-1 in human Monocytes may play a critical role in the atherogenicity of LTB4 [37]. Concerning the effect on TNF-α , this finding was in agreement with Marco et al. they found that Pharmacological inhibition of leukotrienes by zileuton in an animal model of bleomycin-induced acute lung injury resulted in a marked reduction in TNF-α immunostaining in lungs in Zileuton treated group [38]. The mechanisms of TNF-α pro-inflammatory activity are likely to involve both direct effects of TNF-α, itself on regulation of adhesion molecule expression and induction of other cytokines and growth factors capable of mediating leukocyte chemotaxis and survival [39]. Regarding to VCAM-1, Salvatore et al. (2005) revealed that 5-Lipoxygenase modulates colitis through the regulation of adhesion molecule expression and neutrophil migration; they concluded that the upregulation of P-selectin, E-selectin, ICAM-1, and VCAM-1 in the lung was largely attenuated with Zileuton treatment [40]. Mehrabian et al. reported that heterozygotes for the 5-Lipoxygenase gene on the LDLR-/- background had considerably reduced aortic lesions despite hypercholesterolemia as compared with the advanced lesions of LDLR-/- mice [41]. Dwyer et al. showed that variant alleles of 5-Lipoxygenase genes were associated with a significant increase of carotid intima thickness [14]. Helgadottir et al. demonstrated a significant association between the gene encoding 5-Lipoxygenase activating protein (FLAP) and myocardial infarction by analysis of single-nucleotide polymorphism haplotype in humans [42]. The beneficial effect of zileuton on the progression of atherosclerosis may be due to their favourable effect on oxidative stress, systemic inflammation and aortic inflammatory marker (VCAM-1, MCP-1 IL17 and TNF-α) via inhibition of 5-lipoxygenase enzyme that catalyzes the formation of leukotrines.

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