

## Aspirin retards the progression of atherosclerosis in hypercholesterolemia rabbits via down regulation of oxidative and inflammatory pathways

Najah R. Hadi<sup>1\*</sup>, Bassim I Mohammad<sup>2</sup>, Heider S Qassam<sup>1</sup>, Fadhil G. Al-Amran<sup>3</sup>, and Hussam H Sahib<sup>1</sup>

**Running title:** Role of aspirin in hypercholesterolemia

### Abstract

This study was designed to evaluate the effect of aspirin on the progression of atherosclerosis, twenty eight native domestic rabbits were assigned to four groups: group I (normal control), group II (atherogenic control), group III (vehicle control), group IV (aspirin 10 mg/kg daily). Blood samples were collected at the end of experiment (8 weeks) for measuring of cholesterol triglycerides (TG), total cholesterol (TC), HDL-C, plasma high sensitive cholesterol (hsCRP), plasma malondialdehyde (MDA) and plasma reduced glutathione (GSH). Immunohistochemical analysis (VCAM-1, MCP-1, and TNF- $\alpha$ , IL-17A) and histopathologic assessment of aortic atherosclerotic changes were also performed. Compared to NC, levels of lipid profile, atherogenic index, hsCRP, and MDA are increased where as GSH were reduced in animals on atherogenic diet ( $P<0.05$ ). Immunohistochemical analysis showed that aortic expression of VCAM-1, MCP-1, TNF- $\alpha$  and IL-17A were considerably 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, aspirin don't have significant effect on lipid profile. Aspirin causes statistically significant reduction in hsCRP and MDA ( $P<0.05$ ). Aspirin treatment causes considerably increase the level of GSH. Aspirin treatment considerably reduced aortic expression of VCAM-1, MCP-1, TNF- $\alpha$  and IL-17A ( $P<0.05$ ). Histopathologic examination of aortic arch showed that aspirin considerably reduced atherosclerotic lesion ( $P<0.05$ ). It thus will conclude that aspirin reduces lipid peroxidation, systemic inflammation and aortic expression of inflammatory markers utilized in this study and therefore reduce the progression of cholesterol.

**Key words:** Atherosclerosis, Aspirin, Oxidative stress, Rabbits

---

\*Corresponding Author: Najah R. Hadi [drnajahhadi@yahoo.com](mailto:drnajahhadi@yahoo.com)

<sup>1</sup> Department of pharmacology & therapeutics, Faculty of medicine, University of Kufa, Iraq

<sup>2</sup> College of Pharmacy, University of Al Qadisiyah, Al Diwaniyah, Iraq

<sup>3</sup> Department of cardiothoracic surgery, Faculty of medicine, University of Kufa, Iraq

Received 11 October 2013; accepted 12 February 2014

Copyright © 2014 NH. *et al.* This is an open access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

---

## Introduction

Atherosclerosis is a complicated vascular inflammatory disease characterized by endothelial activation and dysfunction, monocyte infiltration and differentiation, lipid accumulation, T-cell infiltration and activation, and fibrosis within the lesion space [1]. The adhesion of leukocytes to the vascular epithelium, mediated by endothelial cell adhesion molecules including intercellular adhesion molecule-1, vascular adhesion molecule-1, and E-selectin, is the early event in atherogenesis [2]. The initial step in pathology is endothelial dysfunction this may result in many responses that alter the normal vascular homeostatic properties [3]. High fat diet, hyperglycemia, insulin resistance, hypercholesterolemia trigger the endothelial expression of adhesion molecules like, E-selectin, P-selectin, ICAM-1 and VCAM-1 that mediate the attachment of circulating monocytes and lymphocyte [4, 5].

In response to turbulent flow within the setting of an unfavorable serum lipid profile, endothelial cells over-express adhesion molecules like VCAM-1; this will increase monocytes and T-cells enlisting to sites of endothelial injury and so release of monocyte chemo-attractant protein-1 (MCP-1) by leukocytes will increase the inflammatory cascade by inducing more leukocytes, activating leukocytes within the media, and leading to enlisting and proliferation of smooth muscle cells [6,7].

Mast cells, T-cells macrophages, and T-cells infiltrate atherosclerotic plaques and platelets play a fundamental role releasing inflammatory mediators that stimulate plaque progression. aspirin is wide elementary treatment of stroke and infarction and has anti-inflammatory properties that include different mechanisms of action, by block of platelet activation and platelet-leukocyte interactions [8]. So this study was designed to address the effect of aspirin on the progression of atherosclerosis.

## Materials

A complete of twenty eight native domestic rabbits, weighing (1.1-1.5) kg, were utilized in this study. All experiments were conducted within the Department of pharmacology, school of medicine, Qadaysia University, consistent with the rules for the Care and Use of Laboratory Animals in scientific research. The animals were placed in an animal house, in a group caging system, at controlled temperature ( $25\pm 2^{\circ}\text{C}$ ) and close wetness. Lights were maintained on a 12-h light/dark cycle, and the animals had free access to water spontaneously. Aspirin was utilized in a dose of 10 mg/ kg orally [9]. (Aspin 100mg, SDI, BN.7M810. Iraq) was dissolved in ethanol (10%) and given to the rabbit per the body weight once daily by stomach tube [10].

## Animal model of atherosclerosis

Induction of atherosclerosis was applied by feeding the rabbit an atherogenic diet [2% cholesterol (BDH Chemicals Ltd Poole England, prod 43011) enriched rabbit chow] created by addition of cholesterol powder to chow pellets for eight weeks [11, 12].

## Experimental Protocol

After two weeks of adaptation period, the animals randomized into four groups (of seven rabbits each): traditional diet control group (NC, group I), high-cholesterol diet group that served as atherogenic control (AC, Group II), high-cholesterol diet with ethanol group (Group III) and high-cholesterol diet with aspirin group (Group IIII) .The NC group was fed normal rabbit chow, whereas the high cholesterol diet groups were fed a 2% high-cholesterol (atherogenic) diet. The period of treatment was eight weeks. At the end of the experiment, food was withheld for 16-18 hour and animals were anaesthetized by ketamine (HIKMA pharmaceuticals B.N 3310) at 66 mg/kg and xylazine (alfasan B.N 1004111-07) at 6 mg/kg intramuscular [13]. The chest was opened by thoracotomy, blood sample was collected directly from the heart and aorta was separated before following investigations were performed:

- Immunohistochemistry for assessment of VCAM, TNF $\alpha$ , MCP1 and IL-17A.
- Lipid profile including total serum cholesterol (TC), low density lipoprotein (LDL), and high density lipoprotein (HDL).
- Oxidation parameter including MDA and GSH.
- Systemic inflammatory marker hsCRP
- Histopathological examination of the aorta for assessment of atherosclerosis.

All specimens were directly fixed in 100 percent formaldehyde solution for subsequent processing.

### **Biochemical Procedures**

Serum lipid profile, including total cholesterol and TG, were determined by enzymatic methods using an automatic analyser (Abbott, Alcyon 300, and USA). Plasma GSH levels was determined using strategies of Beutler [14]. Plasma MDA level was determined by using competitive inhibition enzyme immunoassay technique (cusabio; Catalog No. CSB-E13712Rb). While, determination of hsCRP was done by using rabbit high-sensitive ELISA enzyme-linked-immunosorbent serologic assay kit provided by (KAMIYA biomedical COMPANY). The measurement was carried out consistent with the manufacturer's directions.

### **Histological examination of the aorta**

For histological analysis of atherosclerosis, the specimens were processed, and embedded in paraffin and cut into 5  $\mu$ m thick sections. The sections of tissue were stained with hematoxylin and eosin. The evaluation of atherosclerotic changes was performed consistent with 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 advanced lesion) and type VI (complicated lesion) [15].

### **Immunohistochemistry**

Immunohistochemistry was performed with polyclonal goat antibodies, raised against rabbit VCAM-1, TNF $\alpha$ , MCP-1 and IL-17A. Staining procedure was administered consistent with the manufacturer's directions (Santa Cruz Biotechnology, Inc). The intensity of stain was scored to 0: Indicated no staining, 1: Weak, 2: Moderate, 3: Strong, 4: very strong stain intensity [16] (fig.1).

### **Statistical analysis**

Statistical analyses were performed using SPSS 12.0 version. Information were expressed as mean  $\pm$  SEM. Paired t-test was accustomed compare the mean values within every group at completely different time. Analysis of Variance (ANOVA) was used for the multiple comparison among all groups. The histopathological scoring was assessed by Mann-Whitney test.  $P < 0.05$  was regarded to be statistically significant in all tests.

## Results

### Effect of high cholesterol diet

Compared to NC group, rabbits consumed cholesterol-enriched diet showed significant changes in serum lipid profile, oxidation and inflammatory markers. serum levels of TG, TC and LDL-C as well as plasma level of MDA and hs-CRP were considerably ( $P < 0.001$ ) raised. additionally plasma levels of GSH were considerably ( $P < 0.001$ ) lower in rabbits consumed cholesterol-enriched diet as compared to animals on normal diet.

### Effects of aspirin treatment

Compared to atherogenic control, treating hyperlipidemic rabbits with aspirin resulted in considerably ( $P < 0.001$ ) lower levels of plasma hs-CRP and MDA. However, aspirin treatment caused no significant ( $P > 0.05$ ) alteration within the serum lipids and GSH levels.

### Immunohistochemistry

The results of immunohistochemical analysis for rabbit's aortic arch of VCAM-1, MCP-1, TNF-alpha and IL-17A were considerably totally different between all the 4 study groups. The median intensity of those markers was highest in AC group (very strong for all markers) and lowest in NC group (normal for all markers). aspirin treated group was related to a median stain intensity of moderate for VCAM-1, MCP-1, TNF-alpha and IL-17A that is considerably below the atherogenic control.

### Histopathological findings

The atherosclerotic lesions of aortic arch were graded as normal, initial, intermediated, advance and complicated lesions (fig. 2). The median histopathological grade of atherosclerotic changes was considerably totally different between all the 4 study groups, where The median was highest in atherogenic control (advance) and lowest within the normal diet control (no abnormality). aspirin treated group was related to a median aortic change (initial) that is considerably lower than the atherogenic control.

| Groups                    |                           |           |                           | Parameters   |
|---------------------------|---------------------------|-----------|---------------------------|--------------|
| Aspirin treated           | AC                        | NC        | VC                        |              |
| 1008.4±65.31 <sup>N</sup> | 1017.1±64.94 <sup>*</sup> | 46.3±0.99 | 1116.4±42.91 <sup>N</sup> | TC (mg/dl)   |
| 327.9±40.07 <sup>N</sup>  | 337.1±40.87 <sup>*</sup>  | 60±3.47   | 357±35.18 <sup>N</sup>    | TG(mg/dl)    |
| 24.9±1.28 <sup>N</sup>    | 24.1±1.86 <sup>*</sup>    | 15.7±1.46 | 22.1±0.77 <sup>N</sup>    | HDL(mg/dl)   |
| 918±64.47 <sup>N</sup>    | 925.6±63.93 <sup>*</sup>  | 18.6±1.46 | 1022.9±38.77 <sup>N</sup> | LDL(mg/dl)   |
| 65.6±8.01 <sup>N</sup>    | 67.4±8.17 <sup>*</sup>    | 12±0.69   | 71.4±7.04 <sup>N</sup>    | VLDL (mg/dl) |

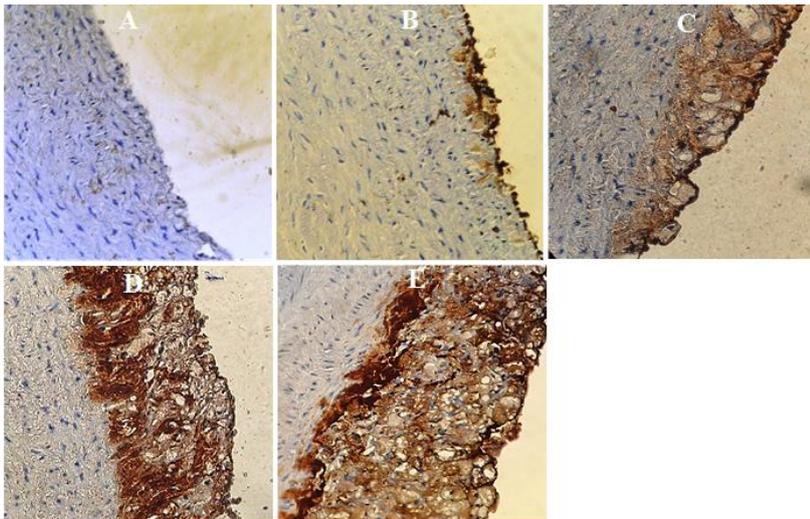
**Table 1.** Change in serum lipid profile in the normal control (NC), atherogenic control (AC), vehicle control (VC) and aspirin treated groups. Results are expressed as mean ± SEM; <sup>\*</sup>P < 0.05, as compare to NC group. <sup>N</sup> not significant as compare to AC group.

| Groups                     |                          |              |                           | parameters          |
|----------------------------|--------------------------|--------------|---------------------------|---------------------|
| Aspirin treated            | AC                       | NC           | VC                        |                     |
| 0.783±0.0312 <sup>**</sup> | 0.568±0.024 <sup>*</sup> | 1.114±0.0338 | 0.527±0.0137 <sup>N</sup> | Plasma GSH (mmol/L) |
| 0.312±0.0212 <sup>**</sup> | 0.51±0.0136 <sup>*</sup> | 0.129±0.0066 | 0.511±0.0142 <sup>N</sup> | Plasma MDA(μmol/L)  |
| 87.9±1.79 <sup>**</sup>    | 134.1±1.2 <sup>*</sup>   | 33.3±0.78    | 135.7±2.09 <sup>N</sup>   | Plasma hsCRP (μg/L) |

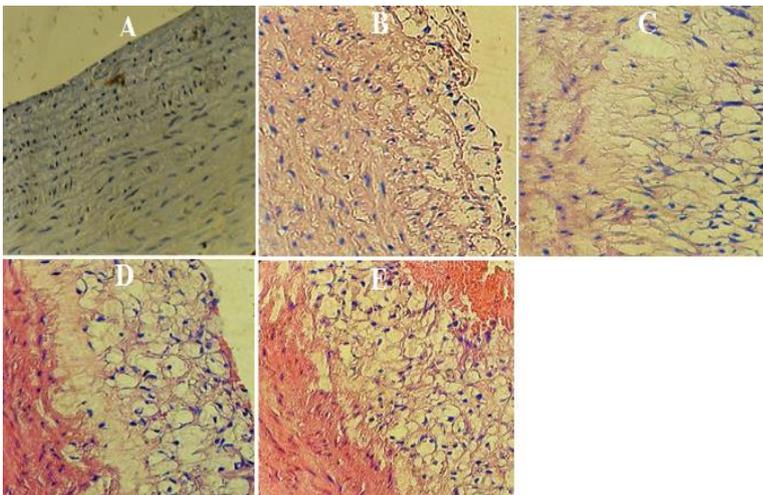
**Table 2.** Change in mean plasma levels of hs-CRP, MDA and GSH in the normal control (NC), atherogenic control (AC), vehicle control (VC) and aspirin treated groups. Results are expressed as mean ± SEM; <sup>\*</sup>P < 0.05, as compare to NC group; <sup>\*\*</sup>P < 0.05, as compare to AC group. <sup>N</sup> not significant as compare to AC group.

| Groups                 |                          |          |                          | Markers |
|------------------------|--------------------------|----------|--------------------------|---------|
| Aspirin treated        | AC                       | NC       | VC                       |         |
| Moderate <sup>**</sup> | Very strong <sup>*</sup> | Negative | Very strong <sup>*</sup> | VCAM-1  |
| Moderate <sup>**</sup> | Very strong <sup>*</sup> | Negative | Very strong <sup>*</sup> | MCP-1   |
| Moderate <sup>**</sup> | Very strong <sup>*</sup> | Negative | Very strong <sup>*</sup> | TNFα    |
| Moderate <sup>**</sup> | Very strong <sup>*</sup> | Negative | Very strong <sup>*</sup> | IL-17 A |

**Table 3.** The difference in median tissue (VCAM-1, MCP-1, and TNF alpha) immunostain intensity between the 4 study groups; <sup>\*</sup>P < 0.05, as compare to NC group; <sup>\*\*</sup>P < 0.05, as compare to AC group.



**Figure 1.**  
Immunohistochemical staining intensity (x40).  
A: Negative  
B: Weak stain intensity  
C: Moderate stain intensity  
D: Strong stain intensity  
E: Very strong stain intensity



**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.

## Discussion

In this study we have a tendency to found that aspirin had little and no important effects on lipid profile in comparison with induced untreated group. This can be in agreement with GUYI et al (2006) [17]. In the present study, we found that aspirin had significant effect on plasma MDA and GSH levels where aspirin inhibited the increase of plasma MDA induced in high cholesterol-fed and it increased the plasma level of GSH, these suggesting decrease in Oxyradicals and subsequent lipid peroxidation. Moreover the results of our study showed found that aspirin had significant effect on plasma hs-CRP level where aspirin inhibited the increase of plasma hs-CRP induced in high cholesterol-fed. This results suggest that aspirin

have antioxidant and anti-inflammatory effects on the development of hypercholesterolemic atherosclerosis. These assumptions are in consistent with other researchers [18,19]. During this study it's been shown that aspirin considerably reduced the elevation of inflammatory markers (VCAM-1, MCP-1, TNF- $\alpha$ , IL-17) in atherosclerosis model of hypercholesterolemic rabbit.

The immunohistochemistry results associated with decrease of (VCAM-1, MCP-1, TNF- $\alpha$ ) as compared with evoked untreated group. This result was also supported by Tillmann and his colleagues [20]. Generally, the therapeutic effect of aspirin is as a result of its platelet inhibitory function and their effect on inflammatory markers may well be secondary to the antiplatelet and antiatherosclerotic effects of the drug. it's documented that aspirin, after interference COX activity, allows this enzyme to provide potent anti-inflammatory mediators like lipoxins, that activate their own receptor. apparently, the activation of those receptors is related to inhibition of cytokines at the transcriptional level [21]. This could be another mechanism effect on vascular inflammation. To the best our information there's no knowledge offered for the effect of aspirin on aortic IL-17 expression in hypercholesterolemic rabbits, but it plays proinflammatory role in atherogenesis by enhancing monocyte/ scavenger cell recruitment into the aortic wall [22]. The present study demonstrated that aspirin treatment significantly suppress atherosclerotic lesion induced by atherogenic diet in rabbits as compared with induced untreated group. The similar observations were reported by Hong et al (2010) [23], Kouraklis et al (2004) [24].

#### **Authors' contributions**

All coauthors are shared equally to prepared this study. Authors read and approved the final manuscript.

#### **Competing interests**

Authors declares that they have no financial or personal relationship(s) that may have inappropriately influenced him in this study.

#### **References**

1. Berenson GS, Srinivasan SR, Bao W,William P, Richard E, Wendy A et al. Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. *The New England Journal of Medicine* 1998; **338**: 1650-1656.
2. Chen YH, Lin SJ, Chen YL, Liu PL, Chen JW. Anti-inflammatory effects of different drugs/agents with antioxidant property on endothelial expression of adhesion molecules. *Cardiovasc Hematol Disord Drug Targets* 2006; **6**(4): 279-304.

3. Pyorala K, Laakso M, and Uusitupa M. Diabetes and atherosclerosis: an epidemiologic view. *Diabetes Metab Rev* 1987; **3**: 463–524.
4. Fan J, Teruo W. Inflammatory Reactions in pathogenesis of atherosclerosis. *J atheroscler thromb* 2004; **10**(2): 122.
5. Packard RS, Libby P. Inflammation in Atherosclerosis: From Vascular Biology to Biomarker Discovery and Risk Prediction. *Clin Chem* 2008; **54**: 24-38.
6. Li H, Cybulsky MI, Gimbrone MA, Jr., Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *ArteriosclerThromb* 1993; **13**(2): 197–204.
7. Libby P. Inflammation in atherosclerosis. *Nature*. 2002; **420**(6917):868–874.
8. Arazi HC, Badimon JJ. Anti-inflammatory effects of anti-platelet treatment in atherosclerosis. *Curr Pharm Des*. 2012; **18**(28): 4311-25.
9. Majid Khazaei , Mehdi Nematbakhsh, Hossein Ahmadi. The effect of aspirin on serum vascular endothelial growth factor and nitric oxide concentration in high-cholesterol fed rabbits. *ARYA Atherosclerosis Journal* 2007; **2**(4): 133-142.
10. Achhrish goel, Ruchi gupta, Anubhav goswami, Madhu soodan sharma, Yogesh sharma et al. pharmacokinetic solubility and dissolution profile of non-steriodal anti-inflammatory drugs. *International Journal Of Pharma Professional's Research* **2011**; 2: 3-12.
11. Sasani M, Burak Yazgan, Irfan Celebi, Nurgul Aytan, Betul Catalgol, Tunc Oktenoglu et al. Hypercholesterolemia increases vasospasm resulting from basilar artery subarachnoid hemorrhage in rabbits which is attenuated by Vitamin E. *Surg Neurol Int* 2011; **14**(2): 29.
12. Alan Daugherty, Ben S. Zweifel & Gustav Schonfeld. Probucol attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Br. J. Pharmacol* 1989; **98**: 612-618.
13. Toshio Hayashi, Jon M. Fukuto, Louis J. Ignarro and Gautam Chaudhuri. Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: Implications for atherosclerosis. *Proc. Nad. Acad Sci. USA* 1992; **89**: 11259-11263.
14. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. *J Lab Clin Med* 1963; **61**: 882-888.
15. Sonai L, Mumford. Laboratory procedure manual. Second edition, USFWS, Oilympia Fish Health Center. Olympia Washington 2004.
16. Yung-Hsiang Chen, Shing-Jong Lin, Jaw-Wen Chen, Hung-Hai Ku and Yuh-Lien Chen. Magnolol attenuates VCAM-1 expression in vitro in TNF- $\alpha$  treated human aortic endothelial cells and in vivo in the aorta of cholesterol-fed rabbits. *British Journal of Pharmacology* 2002; **135**: 37-47.

17. Guo Yi, Wang Qi-zhang, Tang Bing-shan, Zuo Yan-fang, Fang-ming Li, Jiang Xin et al. Effects of aspirin on atherosclerosis and the cyclooxygenase-2 expression in atherosclerotic rabbits. *Chin Med J* 2006; **119**(21): 1808-1814 .
18. Kailash Prasad and Paul Lee. Suppression of oxidative stress as a mechanism of reduction of hypercholesterolemic atherosclerosis by aspirin. *J Cardiovasc Pharmacol Ther* 2003; 8 (1): 61-69.
19. Yang XY, Wang L, Zhou N, Zeng HS. Antiatherosclerotic mechanism of aspirin: experiment with rabbits. *Chin Med J* 2007; **87**(46): 3298-3301.
20. Tillmann Cyrus, Syuan Sung, Lei Zhao, Colin D. Funk, Syun Tang and Domenico Praticò et al. Effect of low-dose aspirin on vascular inflammation, plaque stability, and atherogenesis in low-density lipoprotein receptor-deficient mice. *Circulation* 2002; **106**: 1282-1287.
21. Serhan C. Lipoxins and novel aspirin-triggered 15-epi-lipoxins (ATL): a jungle of cell-cell interaction or a therapeutic opportunity? *Prostaglandins* 1997; **53**: 107–137.
22. Smith E, Prasad KM, Butcher M, Dobrian A, Kolls JK et al. Blockade of interleukin-17A results in reduced atherosclerosis in apolipoprotein E-deficient. *Circulation* 2010; **121**(15): 1746-55.
23. Hong Liu, Deqian Jiang, Shebing Zhang and Baiqing Ou. Aspirin inhibits fractalkine expression in atherosclerotic plaques and reduces atherosclerosis in apoe gene knockout mice. *Cardiovascular Drugs and Therapy* 2010; **24**(1): 17-24.
24. Kouraklis G, Patapis P, Misiakos E, Glinavou A, Sioka C, Karayiannakos PE et al. Effects of acetylsalicylic acid on experimental atherogenesis induced in rabbits. *Int Angiol* 2004; **23**(2): 139-143.