Antiatherosclerotic potential of aspirin: Antioxidant and anti-inflammatory approaches

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الخلاصة

أجريت هذه الدراسة لتقيم تأثير الاسبرين على تقدم تصلب الشرايين. تم استخدام 42 ذكر من الأرانب في هذه الدراسة.وقسمت هذه الحيوانات بشكل عشوائي إلى 6 مجموعات (7 أرانب في كل مجموعة)واعطيت الحيوانات في المجموعة الأولى غذاء طبيعي قياسي واعتبرت مجموعة سيطرة في حين الأرانب في المجموعات الثلاثة الباقية أعطيت غذاء عالي الدهون (2% كوليستيرول) و عولجت كالتالي لمدة 8 أسابيع.

1. مجموعة السيطرة (عالية الدهون) لم تعطى أي علاج
2. مجموعة المذيب (الايثانول) 10%
3. مجموعة عقار الاسبرين أعطيت اسبرين 10 ملغم لكل كغم يوميا.

في نهاية ألثمان أسابيع تم التضحية بكل الحيوانات وتم جمع عينات من الدم لقياس المؤشرات التالية: صورة الدهون, مؤشرات الأكسدة (المدي أي و الجي أس أج) و مؤشر الالتهاب السي ا ربي عالي التحسس. كما أخذت عينة نسيجية من الشريان الابهر لمعرفة مدى درجة تصلب الشرايين حسب تصنيف الجمعية الأمريكية لأمراض القلب بالإضافة إلى ذلك جرى فحص مؤشرات الالتهاب في النسيج حيث تم فحص مدى ظهور كل من في كام -1, ام سي بي -1, تي ا ناف الفا في طبقات الشريان الابهر.

مقارنة بمجموعة السيطرة عالية الدهون,لم يكن للاسبرين تأثير مميز على صورة الدهون في الدم مقارنة بمجموعة السيطرة عالية الدهون, بينما أظهرت التحاليل أن كل من الاسبرين يقللان بصورة واضحة مؤشر الالتهاب في الدم (السي ا ربي عالي التحسس)كما أظهرت النتائج أن الاسبرين تقلل بصورة واضحة مؤشرات الأكسدة (ألجي أر أس و والمدي أي), كما أظهرت الدراسة أن كل من الاسبرين والكلوبيدوكريل والخليط من الاسبرين والكلوبيدوكريل يقللان بصورة واضحة علامات الالتهاب في النسيج (في كام -1, التي ا ناف ألفا, ا مسي بي _1).واخيرا اظهرت الدراسات ان الاسبرين يقلل بصورة واضحة من تقدم تصلب الشرايين .مماسبق يمكن ان نستنتج ان بغض النظر عن عدم تأثير الاسبرين على مستوى الدهون فانه يقلل من تقدم تصلب الشرايين بصورة واضحة.

Abstract

Objective: this study was undertaken to evaluate the effect of aspirin on the progression of atherosclerosis.

Materials and methods: A total of 28 local 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).

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Blood samples were collected at the end of experiment (8 weeks) for measurement of serum triglycerides (TG), total cholesterol (TC), HDL-C, plasma high sensitive C-reactive protein (hsCRP), plasma malondialdehyde (MDA) and plasma reduced glutathione (GSH). Immunohistochemical analysis (VCAM-1, MCP-1, and TNF-α) 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 ($p< 0.05$). Immunohistochemical analysis showed that aortic expression of VCAM-1, MCP-1, and TNF-α 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 aspirin don't have significant effect on lipid profile. Aspirin causes statistically significant reduction in hsCRP and MDA ($p<0.05$). Aspirin treatment causes significantly increase the level of GSH. Aspirin treatment significantly reduced aortic expression of VCAM-1, MCP-1, and TNF-α ($p<0.05$). Histopathologic examination of aortic arch showed that aspirin significantly reduced atherosclerotic lesion ($p<0.05$).

Conclusions: It thus can conclude that aspirin reduces lipid peroxidation, systemic inflammation and aortic expression of inflammatory markers used in this study and hence reduce the progression of atherosclerosis.

Key words: atherosclerosis, aspirin, oxidative stress

Introduction

Atherosclerosis is an inflammatory disease characterized by endothelial activation and dysfunction, lipid accumulation, monocyte infiltration and differentiation, T-cell infiltration and activation, foam cell formation, and fibrosis in the lesion area [(1)]. The proposed initial step in atherogenesis is endothelial dysfunction leading to a number of compensatory responses that alter the normal vascular homeostatic properties [(2)]. Pro-

inflammatory 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, E-selectin, ICAM-1 and VCAM-1 which mediate the attachment of circulating monocytes and lymphocyte[3,4]. Atherosclerotic lesions develop as a result of inflammatory stimuli, subsequent release of various cytokines, proliferation of smooth muscle cells, synthesis of connective tissue matrix, and accumulation of macrophage and lipid. Atherosclerosis is likely initiated when endothelial cells over-express adhesion molecules in response to turbulent flow in the setting of an unfavorable serum lipid profile. Animals fed a pro-atherogenic diet rapidly over express vascular cell adhesion molecule-1 (VCAM-1) [5]. VCAM-1 expression increases recruitment of monocytes and T-cells to sites of endothelial injury; subsequent release of monocyte chemo-attractant protein-1 (MCP-1) by leukocytes magnifies the inflammatory cascade by recruiting additional leukocytes, activating leukocytes in the media, and causing recruitment and proliferation of smooth muscle cells [6] . However in response to signals generated within the early plaque, monocytes adhere to the endothelium and then migrate through the endothelium and basement membrane by elaborating enzymes, including locally activated matrix metalloproteinase (MMP) that degrade the connective tissue matrix . Recruited macrophages both release additional cytokines and begin to migrate through the endothelial surface into media of the vessel. This process is further enhanced by the local release of monocytes-colony stimulating factor (M-CSF), which causes monocytic proliferation; local activation of monocytes leads to both cytokine-mediated progression of atherosclerosis, and oxidation of low-density lipoprotein (LDL) [7]. Platelets serve major functions in three key aspects of atherosclerosis: atherogenesis, inflammation, and atherothrombosis [8]. Therefor the present study was undertaken to evaluate the effect of aspirin on the progression of atherosclerosis.

Materials and methods

Animals

A total of 28 local domestic rabbits, weighing (1.1-1.5) kg, were used in this study. All experiments were conducted in the Department of Pharmacology, College of Medicine, Qadaysia University, according to the guidelines 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±2◦C) and ambient humidity. Lights were maintained on a 12-h light/dark cycle. The animals had free access to water *ad libitum*.

Drugs

Aspirin was used 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 according to the body weight once daily by stomach tube [10].

Animal model of atherosclerosis.

Induction of atherosclerosis was carried out by feeding the rabbit an atherogenic diet [2% cholesterol (BDH Chemicals Ltd Poole England, prod 43011) enriched rabbit chow] made by addition of cholesterol powder to chow pellets for 8 weeks [11, 12].

Experimental Protocol

After 2 weeks of acclimatization period, the animals randomized into 4 groups (of 7 rabbits each): Normal diet control group (NC, group I), high-cholesterol diet group which 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 duration of treatment was 8 weeks. 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 [13]. The chest was opened by thoractomy, blood sample was collected directly from the heart and aorta was separated before following investigations were performed:

- Lipid profile including total serum cholesterol (TC), low density lipoprotein (LDL), and high density lipoprotein (HDL).
- Immunohisatochemistray for assessment of VCAM, TNFα, and MCP1.
- Oxidation parameter including MDA and GSH.
- Systemic inflammatory marker hsCRP
- Histopathological examination of the aorta for assessment of atherosclerosis.

All specimens were immediately fixed in 10% formaldehyde solution for subsequent processing.

Biochemical Procedures

Serum lipid profile, including total cholesterol and TG, were determined by enzymatic methods using an automatic analyzer (Abbott, Alcyon 300, and USA). Plasma GSH levels was determined using methods 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 CRP ELISA kit supplied by (KAMIYA BIOMEDICAL COMPANY; Cat. No. KT-097).The measurement was carried out according to the manufacturer's instructions.

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) [15].

Immunohistochemistry

Immunohistochemistry was performed with polyclonal goat antibodies, raised against rabbitVCAM-1, TNFα, 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 [16] (Figure1).

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.

Effects of aspirin treatment

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

Immunohistochemistry

The result of immunohistochemical analysis for rabbit's aortic arch of VCAM-1, MCP-1, and TNF-alpha were significantly different between all the 4 study groups. The median intensity of these markers was highest in AC group (very strong for all markers) and lowest in NC group (normal for all markers). Aspirin treated group was associated with a median stain intensity of moderate for VCAM-1 and weak for MCP-1 and TNF-alpha that is significantly lower than the atherogenic control.

Histopathological findings

The atherosclerotic lesions of aortic arch were graded as normal, initial, intermediated, advance and complicated lesions (figure2). The median histopathological grade of atherosclerotic changes was significantly different between all the 4 study groups. The median was highest in atherogenic control (advance) and lowest in the normal diet control (no abnormality). Aspirin treated group was associated with a median aortic change (initial) that is significantly lower than the atherogenic control.

Table (1): Change in serum lipid profile in the normal control (NC), atherogenic control (AC), vehicle control (VC) and aspirin treated groups.

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

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

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.

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

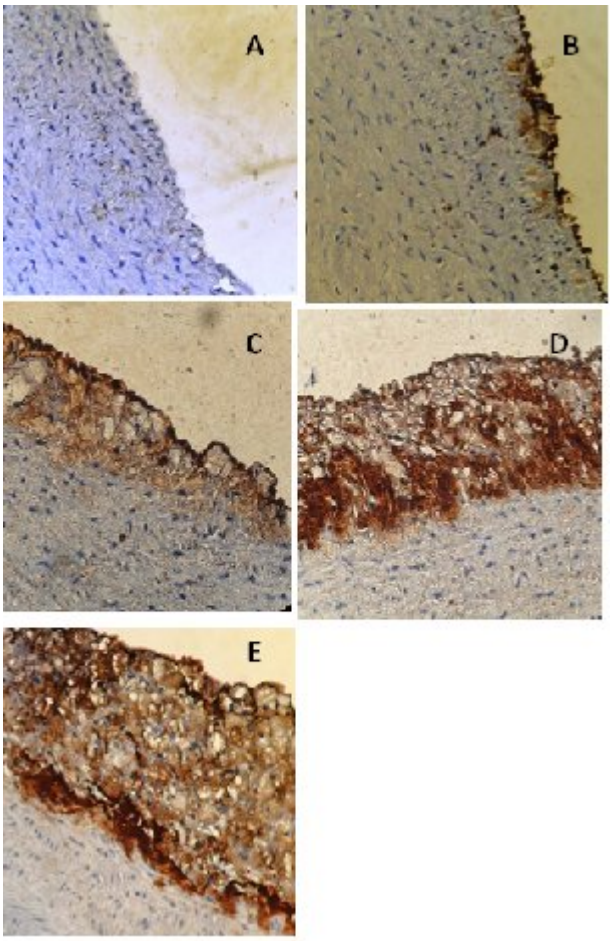
Results are expressed as mean ± SEM.
*$p < 0.05$, as compare to NC group, **$p < 0.05$, as compare to AC group.
^{N} not significant as compare to AC group

Table (3) the difference in median tissue (VCAM-1, MCP-1, and TNF alpha) immunostain intensity between the 4 study groups.

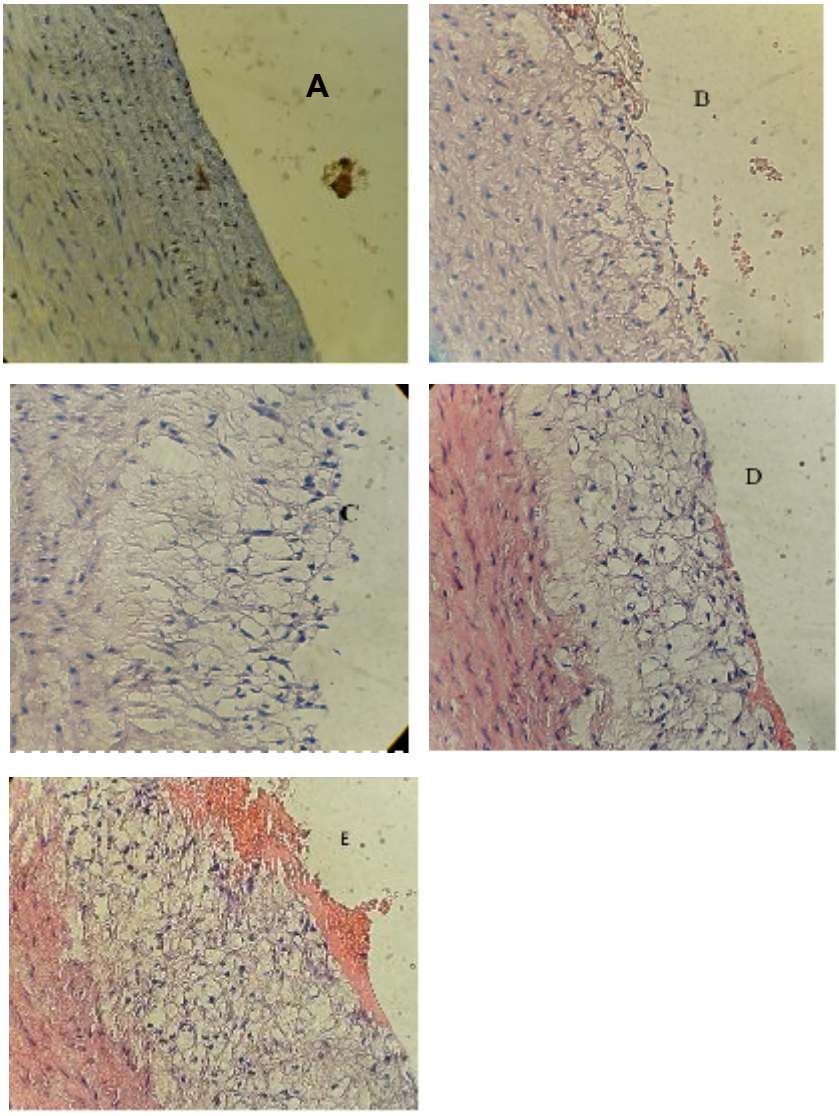
Groups				
Aspirin treated	AC	NC	VC	**Markers**
Moderate**	Very strong *	Negative	Very strong *	VCAM-1
Moderate**	Very strong*	Negative	Very strong *	MCP-1
Moderate**	Very strong*	Negative	Very strong *	TNFα

*p < 0.05, as compare to NC group.

**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.

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