Effect of montelukast on progression of atherosclerosis

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الخلاصة

أجريت هذه الدراسة لتقيم تأثير المونتيلوكاست على تقدم تصلب الشرايين. واستخدم 28 ذكر من الأرانب في هذه الدراسة. قسمت هذه الحيوانات بشكل عشوائي إلى 4 مجموعات (7 أرانب في كل مجموعة)، أعطيت الحيوانات في المجموعة الأولى غذاء طبيعي قياسي واعتبرت مجموعة سيطرة في حين أعطيت الأرانب في المجموعات الثلاثة الباقية 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 منتيلوكاست أعطيت بجرعة 1.5 ملغم لكل كغم يوميا.

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

مقارنة بمجموعة السيطرة عالية الدهون، لم يكن للمونتيلوكاست تأثير مميز على صورة الدهون في الدم بينما أظهرت التحاليل أن المونتيلوكاست يقلل بصورة واضحة مؤشر الالتهاب في الدم (السي اربي عالي التحسس)كما أظهرت النتائج أن المونتيلوكاست يقلل بصورة واضحة مؤشرات الأكسدة (الام دي أي و ألجي أر أس)، كما أظهرت الدراسة أن المونتيلوكاست يقلل بصورة واضحة علامات الالتهاب في النسيج (في كام -1، التي ان اف ألفا، و ال ام سي بي -1). وأخيرا أظهرت الدراسة أن المونتيلوكاست يقلل بصورة واضحة من تقدم تصلب الشرايين. مما سبق يمكن أن نستنتج أن بغض النظر عن عدم تأثير المونتيلوكاست على مستوى الدهون فإنه يقلل تقدم تصلب الشرايين بصورة واضحة.

Abstract

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

Materials and methods: A total of 28 local domestic rabbits were assigned into four groups: Group I (normal control), Group II (atherogenic control), Group III (vehicle control),Group IV (montelukast 1.5 mg\kg daily). 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.

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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 montelukast don't have significant effect on lipid profile. Montelukast causes statistically significant reduction in hsCRP and MDA ($p<0.05$). Montelukast treatment causes significantly increase the level of GSH. Montelukast treatment significantly reduced aortic expression of VCAM-1, MCP-1, and TNF-α ($p<0.005$). Histopathologic examination of aortic arch showed that montelukast significantly reduced atherosclerotic lesion ($p<0.005$).

Conclusions: It thus can conclude that montelukast 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, montelukast, oxidative stress

Introduction

Atherosclerosis is a disease of large and medium-sized arteries and is characterized by endothelial dysfunction, vascular inflammation, and the build up of lipids, cholesterol, calcium, and cellular debris within the intima of the vessel wall [(1)]. Atherosclerotic plaques can grow large enough to significantly reduce the blood flow, but most of the damage occurs when they become fragile and rupture. Plaques that rupture cause emboli that can block downstream intracranial cerebral blood vessels originating from the carotid artery, commonly causing transient ischemic attacks, stroke, disability, retinal infarctions, or death [(2)]. 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 [(3)]. 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 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 [(4)]. 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 [5]. . Accumulation of cholesteryl esters in the cytoplasm converts macrophages into foam cells, i.e., lipid-laden macrophages characteristic of early-stage atherosclerosis. In parallel, macrophages proliferate and amplify the inflammatory response through the secretion of numerous growth factors and cytokines, including tumor necrosis factor α and interleukin-1β [6]. The atherosclerotic lesion contains cytokines that promote a T-helper 1 response, inducing activated T cells to differentiate into T-helper 1 effector cells [7]. These cells amplify the local inflammatory activity by producing proinflammatory cytokines such as IFN-γ and CD40 ligand (CD40L, CD154), which contribute importantly to plaque progression [8]. TNF-α induces inflammatory responses in the vascular endothelium by enhancing the expression of adhesion molecules and the secretion of inflammatory mediators, upon stimulation by various stimuli including TNF-α and interleukin (IL-1β), MCP-1 and IL-8 are expressed by the endothelium [9]. CRP directly participates in the process of atherogenesis by modulating endothelial function and its concentration known to predict cardiovascular events [10]. CRP is one of the substances present in the atherosclerotic lesion, more specifically in the vascular intima, where it co-localizes with monocytes, monocyte-derived macrophages and lipoproteins, this localization makes a direct contribution to the atherosclerotic process possible [11].

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

Montelukast (MSD B.N 302048) was dissolved in ethanol [12]; 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 [13]. This drug was administered once daily to the animal according to body weight by oral route through stomach tube.

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 [14].

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 group with ethanol 10 % as vehicle served as positive control group(PC, Group III) and high-cholesterol diet with montelukast group (Group IV) 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 withhold 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 [15]. 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-1, 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, USA). Plasma GSH levels was determined using methods of Beutler [16] .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) [17].

Immunohistochemistry

Immunohistochemistry was performed with polyclonal goat antibodies, raised against rabbit VCAM-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 [18] (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 montelukast treatment

Compared to atherogenic control, treating hyperlipidemic rabbits with montelukast 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 treatment caused no significant ($p>0.05$) alteration in the serum lipids.

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). There is no statistically significant difference in median intensity of these markers between PC and AC control groups on atherogenic diet. Montelukast treated

group was associated with a median stain intensity of moderate for VCAM-1, 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 was highest in atherogenic control (advance) and vehicle control (PC) whereas lowest in the normal diet control (no abnormality). Montelukast 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 among the four study groups.

Groups				Parameters
Montelukast treated	PC	AC	NC	
1126.4±34.38^{N}	1116.4±42.9	1121.4±41.6*	45.1±0.94	TC (mg/dl)
363.7±37.74^{N}	357±35.18	357.7±41.3*	56±1.13	TG(mg/dl)
21.9±0.8^{N}	22.1±0.77	23±1.35^{N}	18.1±0.99	HDL(mg/dl)
1031.8±33.89^{N}	1022.9±38.7	1026.9±37.61*	15.8±1.71	LDL(mg/dl)
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 (2): Change in mean plasma levels of hs-CRP, MDA and GSH among the four study groups.

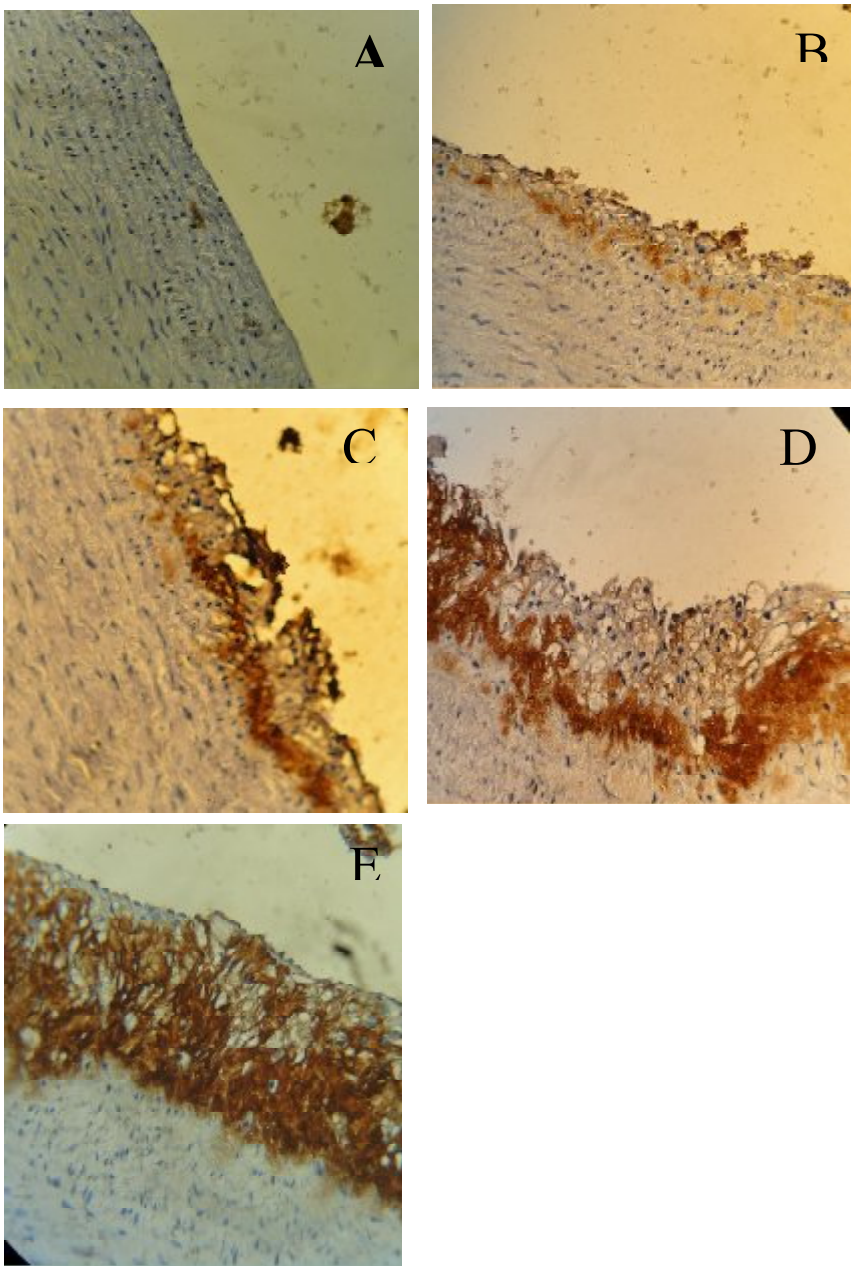
Groups				
Montelukast treated	PC	AC	NC	**Parameters**
0.752±0.0164**	0.52±0.013	0.568±0.024*	1.102±0.0258	Plasma GSH (mmol/L)
0.254±0.0107**	0.51±0.0142	0.51±0.0145*	0.133±0.005	Plasma MDA(µmol/L)
67.7±1.94**	135.9±2.09	135.3±1.4*	32.9±0.88	Plasma hsCRP (µg/L)

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

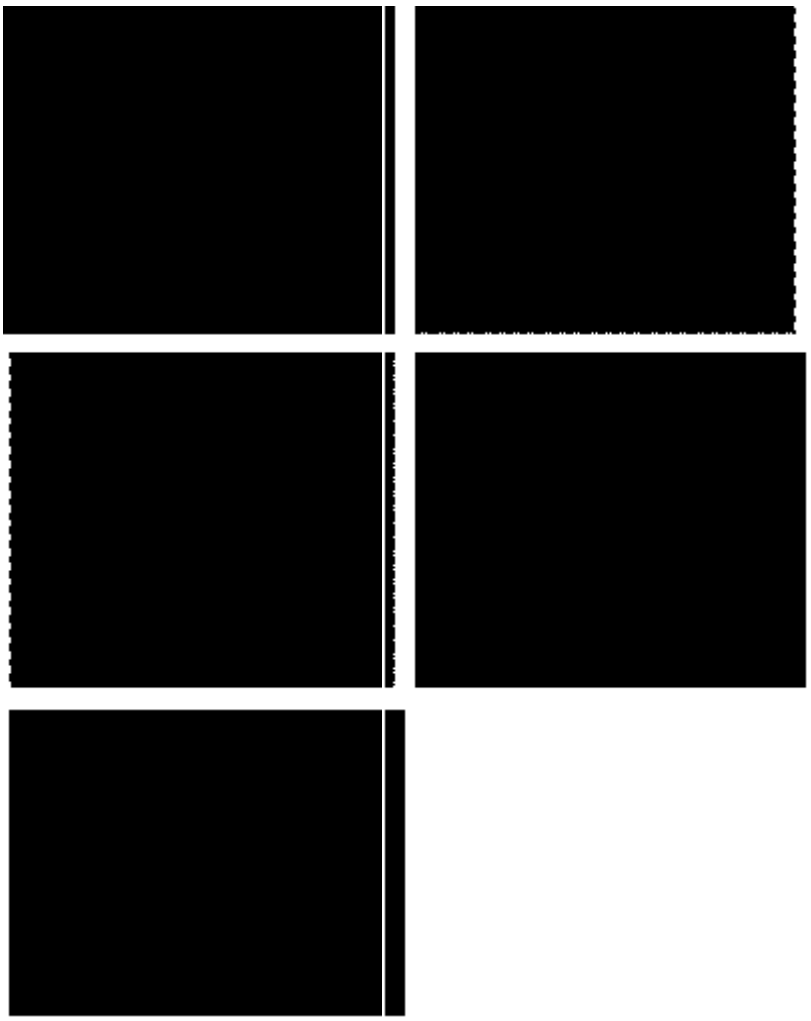
Table (3): The difference in median tissue (VCAM-1, MCP-1 and TNF alpha) immunostain intensity among the four study groups.

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

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



(Figure 1): Immunohistochemical staining in aortas 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.



(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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