Anti-atherosclerotic Potentials of Montelukast in Chronic Ovalbumin Challenged Asthmatic Guinea Pigs Fed High Fat Diet: A Comparative Study with Fluticasone

Sally Abdallah[1], Ahmed Abdel Salam El-Melegy[1], Olfat Ahmed Hasan[1], Amany Helmy Hasanin[1*], Ghada Farouk Mohamed[2], Sawsan Aboul-Fotouh[1],

[1]Department of Pharmacology, [2]Department of Histology. Faculty of Medicine, Ain Shams University, Cairo, Egypt.
helmy_amany@yahoo.com

Abstract: Background: Recent evidence links the pathophysiology of asthma to cardiovascular diseases. A pro-atherogenic role of leukotrienes has been reported which is suggestive of a potential anti-atherosclerotic effect for leukotriene antagonists. Retrospective observations suggest that inhaled corticosteroids may reduce atherothrombotic mortality by altering systemic inflammation. **Aim:** the present study was designed to investigate the impact of the leukotriene antagonist montelukast on vascular dysfunctions in guinea pigs exposed to chronic ovalbumin (OVA) challenge and fed high fat diet (HFD). The potential anti-atherosclerotic effects of montelukast were compared to those of the inhaled corticosteroid fluticasone. **Method:** Forty-eight male guinea pigs were divided into: control non-asthmatic-chow fed (n=6), non-asthmatic-dyslipidemic; fed HFD (n=6), asthmatic-chow fed (n=18), and asthmatic-dyslipidemic groups (n=18), the last 2 groups were further subdivided into 3 groups (n=6 each): vehicle treated, montelukast- treated (10mg/kg orally) and fluticasone- treated (100µg/2ml of 0.1%ethanol PBS by inhalation) groups. Anti-asthma and Anti-atherosclerotic effects of the tested drugs were assessed. **Results:** Chronic OVA challenge induced atherogenic vascular changes, compared to control and potentiated the vascular dysfunctions induced by HFD in asthmatic dyslipidemic animals. Conversely, feeding animals a HFD resulted in airway inflammation and remodeling with hyper-responsiveness. Montelukast, exhibited anti-atherosclerotic effects (significant increase in % Ach-induced relaxation and phenylephrine (PhE) EC50 with reduction of PhE E-max in isolated aortic rings) of asthmatic dyslipidemic animals. These changes were associated with reduction of aortic mast cell infiltration and intima/media ratio together with a reduction in airway remodeling and mast cell infiltration. Fluticasone induced a lesser reduction in mast cell infiltration in vascular tissue as well as in the airways with lack of anti-atherosclerotic effects and reduced effects on airway remodeling. **Conclusion**: The anti-atherosclerotic potentials of montelukast observed implicate leukotrienes released from mast cells as possible inflammatory mediators linking asthma, obesity and atherosclerosis. The effects of montelukast may be the result of a direct effect on the vasculature as well as an indirect effect on airway remodeling through a crosstalk (involving mast cells and leukotrienes) between the airway and vascular tissue. Targeting excess leukotrienes by leukotrienes modifiers rather than by corticosteroids in dyslipidemic asthmatics might provide better control of their airway disorder as well as a reduction in the risk of atherosclerosis.

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1.Introduction

An increasing body of literature suggests a connection between asthma, on one hand, and obesity and atherosclerosis on the other. Multiple logistic regression models revealed that asthmatic patients were more likely to have heart diseases than non-asthmatics **(Dorga et al., 2007)**. Indeed, asthma has been reported in several studies to contribute to enhanced risk for atherosclerosis **(Micheal et al., 2005)**. A possible explanation for the association of asthma, obesity and atherosclerosis may be the fact that all these disorders share a common underlying pathology: inflammation **(Shore, 2008)**.

Mast cells are inflammatory cells widely distributed throughout the body. Although they are best known for their actions in allergic reactions they are involved in many physiological processes of the body **(Rao and Brown, 2008)**. They appear to play a pathophysiological role in different conditions, such as asthma, atherosclerosis and obesity **(Liu et al., 2009)**.

A potential link between leukotrienes (one of the mast cells mediators) and atherosclerosis has been proposed. The expression of leukotriene biosynthetic enzymes and leukotriene receptors has been identified in coronary atherosclerotic plaques and the levels of biosynthetic enzymes have been correlated with the clinical symptoms of unstable plaques **(Whatling et**

al., 2007). Within atherosclerotic lesions in humans, the 5-lipoxygenase enzyme was found in macrophages, dendritic cells, mast cells and neutrophil granules **(Spanbroek et al., 2003).** Polymorphisms in the 5-lipoxygenase and 5-lipoxygenase–activating protein genes, 2 key genes in the regulation of leukotriene synthesis, predict a high risk for atherosclerosis **(Helgadottir et al., 2005)**.

In support of a role of increased production of leukotrienes for the observed association of asthma with atherosclerotic disease are the reports showing that asthmatic patients receiving 5-lipoxygenase pathway modifiers have lower blood risk factors including inflammatory biomarkers and lipid levels associated with cardiovascular disease **(Hooman et al., 2007).**

Although cortisol is involved in the development of coronary artery disease **(Bhallaccharyya et al., 2008),** and corticosteroids appear to elevate all lipoprotein cholesterol levels **(Henkin et al., 1992)**, much debate has been raised concerning the role of corticosteroids in atherosclerotic cardiac diseases **(Girod and Brotman, 2004).** Asthma patients on steroids have been reported to have fewer atherosclerotic changes in their carotid arteries compared to control non asthmatics **(Otsukiet et al., 2010)**. Moreover, retrospective observations suggest that inhaled corticosteroids may reduce atherothrombotic mortality by altering systemic inflammation**,** very low doses of inhaled corticosteroids may be associated with a reduction in the risk of acute myocardial infarction **(Fimognari et al., 2008).**

Given this newly generated interest relating the pathophysiology of asthma to cardiovascular diseases together with suggested pro-atherogenic role of leukotrienes**,** the present study was designed to investigate the impact of the leukotriene antagonist montelukast on vascular dysfunctions in guinea pigs exposed to chronic ovalbumin (OVA) challenge and fed the high fat diet (HFD).The potential anti-atherosclerotic effects of montelukast were compared to those of the inhaled corticosteroid fluticasone**.**

2. Materials and Methods

2.1. Drugs and chemicals

Montelukast sodium (Merk Sharp & Dhome) powder was dissolved in distilled water. Fluticasone propionate (Glaxo-Smith Kline) powder was dissolved in 0.1% ethanol/phosphate buffered saline (PBS). OVA (Albumin chicken egg, grade III) was dissolved in 0.9% saline, Aluminum Hydroxide powders was dissolved in 0.9% saline, Acetyl-beta methylcholine bromide (Methacholine), L-Phenylephrine hydrochloride and acetylcholine hydrochloride powders were purchased from (Sigma-Aldrich chemicals Co., USA) and were dissolved in distilled water. Cholesterol (Winlab) powder was added to diet.

2.2. Animals

Male guinea pigs weighing 250-300 g were used in this study. They were housed in standard laboratory conditions under a 12 h light/dark cycle and controlled temperature of 22 ± 3°C, with free access to food and water. Experimental procedures were approved by the ethical committee of the Faculty of Medicine, Ain Shams University, Cairo, Egypt.

2.3. Experimental design

Forty-eight animals were divided into 4 main groups. Group I; control non-asthmatic-chow fed (n=6), Group II; non-asthmatic-dyslipidemic fed a high fat diet (n=6), Group III; asthmatic-chow fed (n=18), and Group IV; asthmatic-dyslipidemic groups (n=18) each group of the last two groups were subdivided into 3 groups (n=6 each): vehicle-treated, montelukast-treated (10mg/kg orally) and fluticasone-treated (100µg/2ml of 0.1%ethanol PBS by inhalation) groups. Chronic asthma was induced by chronic Ovalbumin (OVA) challenge for 6 weeks while dyslipidemia was induced by feeding animals a high fat diet for 9 weeks. Drugs were administered from the 4^{th} week till the end of the study (9^{th} week).

2.4. Chronic Asthma Protocol

Guinea pigs were actively sensitized according to the method described by **Schuling et al. (1998)**, an allergen solution containing 100µg OVA and 100 mg Aluminum Hydroxide per ml saline was used. The mixture was gently rotated for 60 minutes to obtain an alum-gel of which 0.5 ml was injected intraperitoneally, while another 0.5 ml was divided equally over seven intradermal injection sites in the proximity of lymph nodes in the paws, lumbar regions, and the neck on day (0). Animals were challenged with aerosolized 2% OVA solution for 10 minutes, twice/ week for 6 weeks starting from the 4^{th} week to the 9^{th} week (end of the study) **(Ohki et al., 2002).**

2.5. High Fat Diet Feeding Protocol

The high fat diet is composed of powdered chow supplemented by 1% cholesterol and 15% lard **(Kunitomo et al., 1983; Emme et al., 1992)**. The whole cocktail was mixed together using water then pellets were made and left to dry. Guinea pigs were fed this diet for 9 weeks starting day (0) to the end of the study.

2.6. Outcome measures

2.6.1 Measurement of Airway Reactivity to Methacholine

Twenty-four hours after the last OVA challenge, conscious guinea pigs were placed in the double chambers plethysmograph (Hugo Sachs Elektronik-Harvard Apparatus, Germany) for measurement of specific airway resistance (RxV) in Cm H2O X sec. The method used for recording RxV was previously described by **Pennock et al. (1979)**,

which is based on measuring the time delay between thoracic and nasal air flow or respiration i.e. the increasing phase displacement (Plugsys Module calculated it by specific serial equations) accompanying the increased airway resistance. The RxV was calculated as airway resistance multiplied by the thoracic gas volume (Cm H_2O x sec). RxV provides an indirect index of obstruction in the lower airways **(Finney and Forsberg, 1994).** Animals were exposed to consecutive doubling concentrations of aerosolized methacholine (0.0156-1mg/ml) until specific airway resistance "RxV" was approximately increased to 100% of its base line value for each individual animal **(Duan et al., 2003)**.

2.6.2. Body weight

Body weights of animals were recorded at the beginning of the study to be sure that there was no significant difference between different animal groups and weekly thereafter. Body weights at the end of 9th week were measured and expressed as mean ±SD gram. The mean percent change in comparison to initial body weight was recorded.

2.6.3. Biochemical measurements

Blood samples were collected retro-orbitally from fasting animals (for 12 hours) in test tubes and centrifuged for 15 minutes 5000 rpm. Serum was collected and stored at -80 ^{o}C until assayed for t**otal cholesterol** (mg/dl) by enzymatic colorimetric method according to **Allain et al. (1974),** "CHOLESTEROL CHOD-PAP Detection Kit" (Greiner Diagnostic Gmbh, Germany), triglycerides (mg/dl) according to **Fossati and Prencipe (1982),** "TRIGLYCERIDES GPO-PAP Detection Kit" (Greiner Diagnostic Gmbh, Germany), HDL (mg/dl) according to **Fruchart (1982),** "HDL-CHOLESTEROL HDL-CHOL Precipitation Reagent" (Greiner Diagnostic Gmbh, Germany) and LDL was calculated using the Friedewald formula: LDL = Total cholesterol – (HDL + Triglyceride/5) **(Friedewald et al., 1972).**

2.6.4. Vascular reactivity in isolated rat's aortic ring

After taking blood samples from anesthetized guinea pigs, the descending thoracic aorta was dissected and cleaned. Aortic rings (5-6 mm width) were mounted in a 20 ml organ bath containing Krebs-Henseleit solution at 37° **(Hattori et al., 2000).** Rings were then allowed to equilibrate for 1-1.5 hours. Isometric responses were measured with a force transducer (K30, Hugo Sacks Electronics, Freiburg, Germany) connected to a bridge coupler type 570 and the trace was displayed on a two-channel recorder (Lineacorder, HSE, WR 3310). Aortic rings were sensitized by 100 µM of phenylephrine (PhE) until two reproducible contractions were obtained, and then a cumulative dose-response curve was constructed by cumulative addition of PhE (10 nM- 200 µM) to the bath. Finally, EC50 and Emax were determined for each curve. After reaching the plateau of PhE–submaximal contraction, the rings were relaxed by exposure to a stepwise increase in acetylcholine (Ach) concentration (100 nM – 1 mM) then, percent relaxation of aortic ring obtained for the different animal groups was computed.

2.6.5. Histopathological studies:

Samples of the lungs and the abdominal aorta was taken and fixed in 10% formalin. For determination of structural changes (remodeling) and inflammatory cellular infiltration (mast cell-neutrophils-eosinophils), Hematoxylin-Eosin (Hx&E) and Toluidine blue staining were performed on 5µm thick sections for light microscopic examination. For quantitative measurement of aortic intima/media ratio, aortic slides were analyzed with an image analysis system (Video Pro 32; Leading Edge Pty Ltd).

2.7. Statistical Analysis

The results were expressed as means±SD. Statistical analysis was performed using "Graphpad prism", USA, version 4.0. (2005). Statistical difference among groups was determined using one way ANOVA followed by Bonferroni's Multiple Comparisons Test. Differences were considered statistically significant at $p<0.05$. In the experiment of phenylephrine induced contraction, all doses were transformed into Log Molar concentration and the contractile response for each concentration was expressed as a percentage from the maximum response. Then, the effective concentration 50 (EC50) was determined using nonlinear regression sigmoid dose response curve.

3. Results

3.1. Airway Reactivity to Methacholine:

Airway hyper-responsiveness was determined by using methacholine [0.0156-1 mg/ml (65µM-4mM)]. It is clear from table (1), that animals sensitized with OVA or fed the high fat diet or both exhibited airway hyper-responsiveness as evident by a significant ($p<0.05$) decrease of PC100 of methacholine compared to control chow fed animals. Treatment with montelukast led to a significant ($p <0.05$) increase in PC100 of methacholine in both asthmatic and asthmatic dyslipidemic groups compared to their respective untreated groups. Although treatment of asthmatic group with fluticasone led to a significant ($p <0.05$) increase in PC100 of methacholine compared to asthmatic untreated group, yet it failed to induce a significant difference in asthmatic dyslipidemic group compared to the asthmatic dyslipidemic untreated group.

3.2. Atherogenic Metabolic Changes

a. Changes in Body Weight:

Chronic OVA challenge in chow fed animals induced insignificant (**p** >0.05) difference in the body weight compared to the control chow fed animals.

Similarly, chronic OVA challenge in high fat diet animals did not induce any more change in the body weight than those induced by the high fat diet as evidenced by the insignificant ($p>0.05$) difference in the asthmatic dyslipidemic animals compared to dyslipidemic animals. Treatment of asthmatic and asthmatic dyslipidemic groups with montelukast or fluticasone did not lead to any significant difference regarding the body weights of the animals in comparison to their corresponding control groups (Table-1).

Table (1): The effect of test drugs on PC100 of methacholine and body weight in asthmatic and asthmatic dyslipidemic guinea pigs.

Animal group (n=6)	PC_{100} of methacholine (mg/ml)	Body weight at the end (gm)	Mean % change in body weight
I. Control	0.52 ±0.42	388.0 ±13.51	42.59
II. Asthmatic	0.06±0.01*	398.5±29.82	41.5
III. dyslipidemic	0.09±0.02*	516.4±14.06*	82.5
IV. Asthmatic Dyslipidemic	0.04±0.01*	501.7±13.66*	78.3
V. Asthmatic +Montelukast	0.20±0.10♦	409±19.91	43.46
VI. Asthmatic+ Fluticasone	0.22±0.08♦	388.6±22.86	45
VII. Asthmatic Dyslipidemic +Montelukast	0.15±0.08 †	505.0±22.36	75.9
VIII. Asthmatic Dyslipidemic +Fluticasone	0.09±0.03	499.2±18.00	80.9

Data are mean±SD, n= number of animals. One way ANOVA followed by Bonferroni's multiple comparisons test: *$p<0.05$, compared to control group. ♦$p<0.05$ compared to asthmatic group, †$p<0.05$ compared to asthmatic dyslipidemic group.

b. Changes in Lipid Profile:

Table (2) shows that chronic OVA challenge in chow fed animals did not induce any metabolic changes regarding the lipid profile as evidenced by the insignificant ($p>0.05$) difference compared to control chow fed animals. Similarly, chronic OVA challenge in high fat diet animals did not induce any more metabolic changes regarding the lipid profile than those induced by the high fat diet as evidenced by the insignificant ($p>0.05$) difference in the asthmatic dyslipidemic animals compared to dyslipidemic animals. Treatment of asthmatic and the asthmatic dyslipidemic groups with montelukast or fluticasone did not induce any metabolic changes regarding the lipid profile as evidenced by the insignificant ($p>0.05$) difference between asthmatic and asthmatic dyslipidemic groups versus the respective groups treated with montelukast or fluticasone.

Table (2): The effect of test drugs on lipid profile in asthmatic and asthmatic dyslipidemic guinea pigs.

Animal group (n=6)	Total cholesterol (mg/dl)	TG (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
I. Control	37.67± 5.69	49.67±6.11	20.07±5.80	7.67±2.08
II. Asthmatic	35.33± 3.51	44.57±4.87	17.75±2.56	8.67±0.58
III. dyslipidemic	142.3±9.61*	137.3±7.09*	109.9±10.13*	5.0±1.73
IV. Asthmatic Dyslipidemic	136.3±9.61*	125.0±4.00*	105.7±8.80*	5.67±0.58
V. Asthmatic +Montelukast	37.0±3.61	47.33±6.66	17.53±2.23	10.0±1.0
VI. Asthmatic+ Fluticasone	40.33±4.73	50.4±2.12	21.92±2.60	8.33±2.08
VII. Asthmatic Dyslipidemic +Montelukast	131.0±2.65	131.0±6.08	99.47±2.00	5.33±1.53
VIII. Asthmatic Dyslipidemic +Fluticasone	135.0±6.24	138.7±5.69	103.6±5.38	3.67±1.15

Data are mean±SD, n= number of animals, TG= Triglycerides, LDL=low density lipoprotein, HDL= high density lipoprotein. One way ANOVA followed by Bonferroni's multiple comparisons test: *$p<0.05$, compared to control group.

3.3. Atherogenic Vascular Changes (isolated aortic tissue)

a. Vascular Reactivity to Phenylepherine and Acetylcholine:

Table (3) and figures (1&2) show that chronic OVA challenge in chow fed animals induced a significant ($p<0.05$) decrease in the mean effective concentrations 50 (EC50) of phenylephrine, a significant ($p<0.05$) increase in its maximal contractile response (Emax) and a significant ($p<0.05$) decrease in the mean % relaxation induced by acetylcholine compared to control chow fed animals. Chronic OVA challenge in animals fed a high fat diet augmented the endothelial dysfunction induced by the high fat diet alone as there was a significant ($p<0.05$) increase in the Emax in the asthmatic dyslipidemic group compared to the dyslipidemic group and augmented the decrease in the mean % relaxation induced by the high fat diet in the dyslipidemic group (36.50±7.56 vs 48.80±5.23 %),

but this did not reach a significant level. Treatment of asthmatic group with montelukast led to a significant ($p < 0.05$) increase in the EC50 of phenylephrine and a significant ($p < 0.05$) decrease in its E-max compared to the asthmatic untreated group. Also treatment of asthmatic dyslipidemic group with montelukast led to a significant ($p < 0.05$) increase in the EC50 and a significant ($p < 0.05$) decrease in E-max compared to asthmatic dyslipidemic untreated group with a significant ($p < 0.05$) increase in the mean % relaxation induced by acetylcholine in both asthmatic and asthmatic dyslipidemic groups compared to the respective untreated groups. Treatment with fluticasone resulted in insignificant change regarding EC50, E-max of phenylephrine and the mean % relaxation of Ach in both asthmatic and asthmatic dyslipidemic groups compared to the respective untreated groups.

Table (3): The effect of test drugs on the vascular reactivity to phenylephrine and to acetylcholine of isolated aortic ring pre-contracted by phenylephrine in asthmatic and asthmatic dyslipidemic groups of guinea pigs.

Animal group (n=5)	EC_{50} for Phenylephrine (µM)	E_{max} for Phenylephrine (gm)	Acetylcholine Relaxation (%)
I. Control	10.34±2.63	0.69±0.13	83.79±8.23
II. Asthmatic	1.11±0.60*	1.08±0.09*	49.70±12.38*
III. Dyslipidemic	0.57±0.18*	1.39±0.10*	48.80±5.23*
IV. Asthmatic Dyslipidemic	0.57±0.20*	1.68±0.12$^{*\delta}$	36.50±7.56*
V. Asthmatic + Montelukast	12.16±5.15$^{\blacklozenge}$ (995.5%)^{a}	0.83±0.06$^{\blacklozenge}$ (-23.1%)^{a}	71.63±7.76$^{\blacklozenge}$ (44.1%)^{a}
VI. Asthmatic + Fluticasone	0.40±0.14	1.17±0.04	52.38±7.0
VII. Asthmatic Dyslipidemic + Montelukast	6.93±2.37† (1115.8%)^{b}	1.34±0.07† (-20.2%)^{b}	58.31±8.22† (59.7%)^{b}
VIII. Asthmatic Dyslipidemic + Fluticasone	3.34±1.87	1.74±0.15	38.80±12.60

Data are mean±SD, n= number of animals. One way ANOVA followed by Bonferroni's Multiple Comparisons test, $^{*}p<0.05$ compared to control group, $^{\blacklozenge}p<0.05$, compared to asthmatic group, $^{\delta}p< 0.05$ compared to dyslipidemic group, $^{\dagger}p<0.05$ compared to asthmatic dyslipidemic group. a = mean% change from asthmatic group, b = mean % change from asthmatic dyslipidemic group.

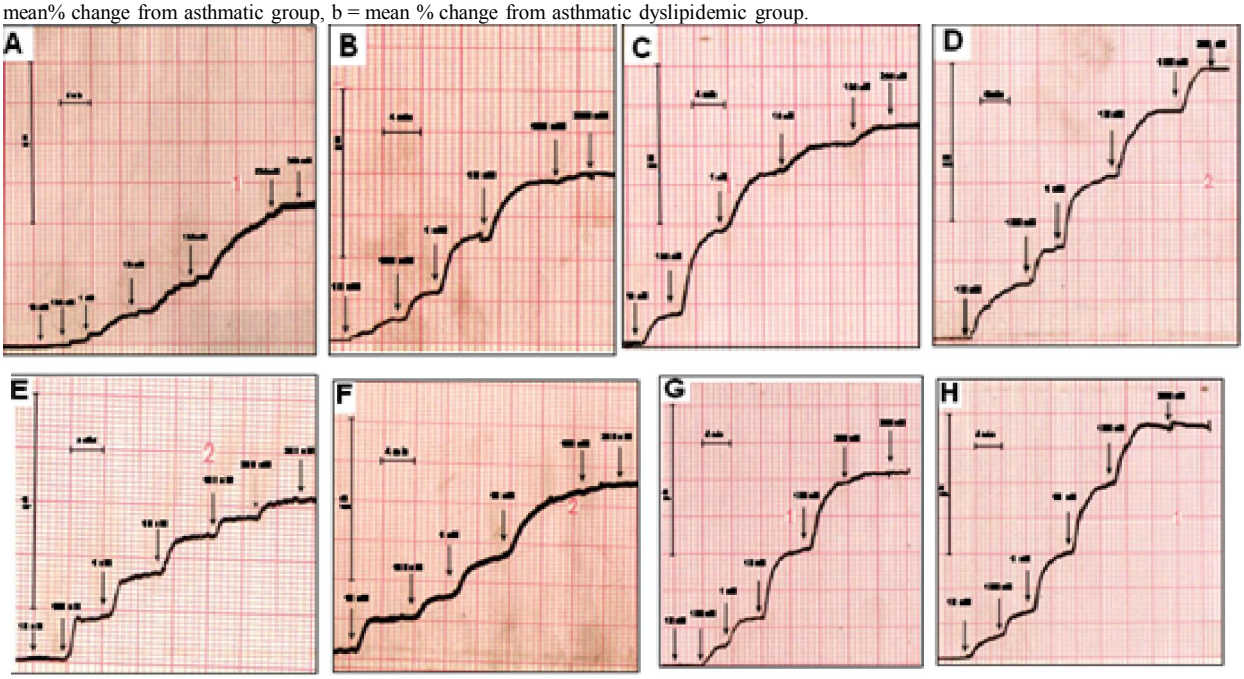

Figure (1, A-H): The cumulative dose response curves for phenylephrine (10nM-200µM) in guinea pigs aortic rings isolated from:
(A)Control group
(B) Asthmatic group
(C) Dyslipidemic group
(D) Asthmatic dyslipidemic group.
(E) Asthmatic group treated with montelukast
(F) Asthmatic group treated with fluticasone
(G) Asthmatic dyslipidemic group treated with montelukast
(H) Asthmatic dyslipidemic group treated with fluticasone
Chart speed = 2.5mm/min, **Tension**= 2g

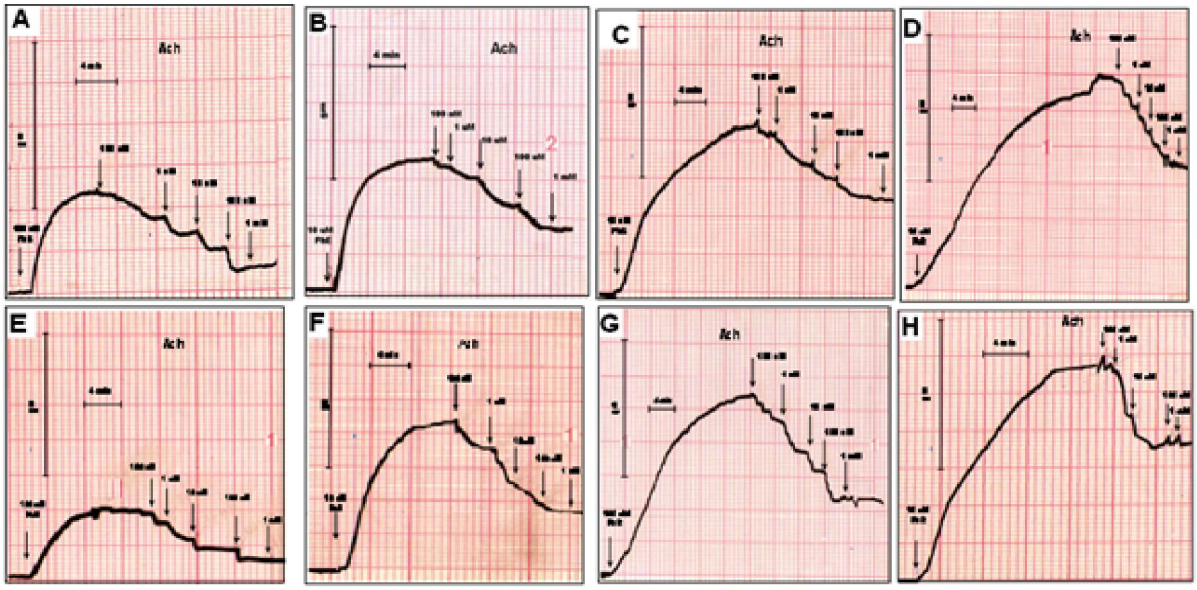

Figure (2, A-H) Acetylcholine induced relaxation (100nM-1mM) in aortic rings prepartions precontracted by sub maximal dose of phenylephrine isolated from:
(A)Control group
(B) Asthmatic group
(C) Dyslipidemic group
(D) Asthmatic dyslipidemic group.
(E) Asthmatic group treated with montelukast
(F) Asthmatic group treated with fluticasone
(G) Asthmatic dyslipidemic group treated with montelukast
(H) Asthmatic dyslipidemic group treated with fluticasone
Chart speed = 2.5mm/min, **Tension**= 2g

b. Aorta Histopathological Changes: Intima/Media Ratio- Mast Cells- Neutrophils Content:

Histopathological sections of the aorta from animals exposed to chronic OVA challenge demonstrated some structural changes compared to control chow fed animals with mild mast cell and neutrophilic infiltrations. Chronic OVA challenge to animals fed high fat diet led to a significant ($p<0.05$) increase in the aortic intima/media ratio and an observed increase in mast cell and neutrophilic infiltration, changes that were much more pronounced than those seen in the dyslipidemic group. Animals treated with montelukast exhibited marked improvement in aortic structural changes and a significant ($p <0.05$) decrease in aortic intima/media ratio, as well as in neutrophils and mast cells infiltration in both asthmatic and asthmatic dyslipidemic groups compared to the respective untreated groups. On the other hand, fluticasone induced only significant decrease in the intima/media ratio in asthmatic animals with insignificant change in aortic intima/media ratio and minimal effect on aortic structural changes and neutrophilic and mast cells infiltration in asthmatic dyslipidemic group (Table-4, Figures 3&4:).

Table (4): The effect of test drugs on histopathological changes in aortic sections of asthmatic and asthmatic dyslipidemic groups of guinea pigs.

Animal group (n=6)	Aortic intima/media ratio Mean±SD	Mast cells	Neutrophils
I. Control	0.06±0.012	-	-
II. Asthmatic	0.09±0.010	++	+
III.Dyslipidemic	0.36±0.040*	+++	++
IV. Asthmatic Dyslipidemic	0.46±0.036$^{*\delta}$	+++++	+++
V. Asthmatic + Montelukast	0.06±0.011$^{\blacklozenge}$	-	-
VI. Asthmatic + Fluticasone	0.07±0.010$^{\blacklozenge}$	+	+
VII. Asthmatic Dyslipidemic + Montelukast	0.28± 0.070†	++	+
VIII. Asthmatic Dyslipidemic + Fluticasone	0.41± 0.065	++++	+++

n = number of animals. (-) = hardly seen, (+) → (+++++) = gradually increasing in intensity. One way ANOVA followed by Bonferroni's Multiple Comparisons tests . *p<0.05 compared to control group, ♦p<0.05 compared to asthmatic group, δp< 0.05 compared to dyslipidemic group, †p<0.05 compared to asthmatic dyslipidemic group.

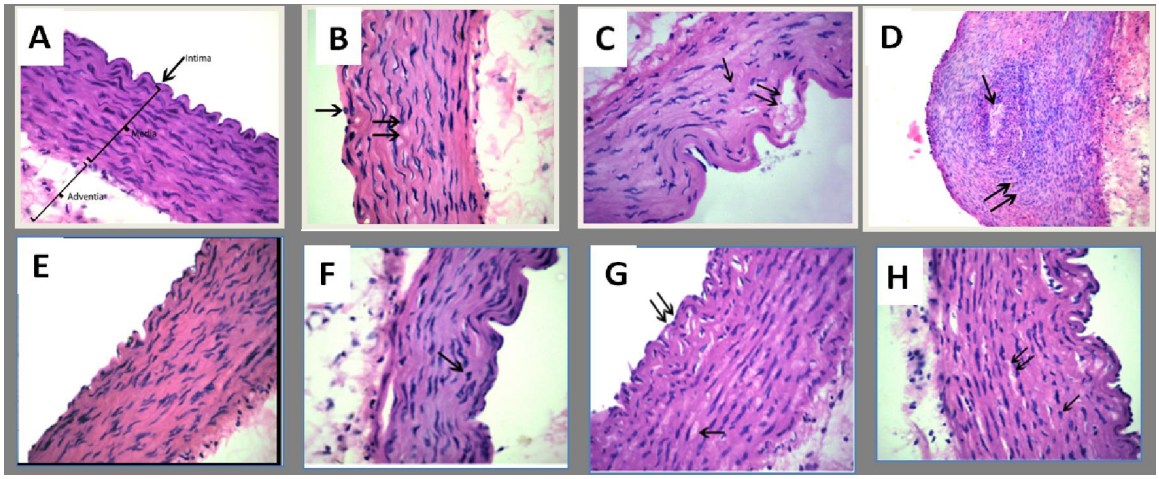

Figure (3, A-H): Photomicrographs of aortic sections showing the effect of test drugs on the changes of the aortic structure using Hx& E.

- **A.** Control group: **Normal contour and thickness. The wall is formed of intima, media and adventitia, with normal endothelial corrugation of the intima.** Hx& E X 640
- **B.** Asthmatic group: **Area of irregularity of the wall of the aorta. Loss of corrugation of the endothelium with apparent splitting between elastic laminae (↑↑). Notice presence of neutrophils (↑).** Hx&E X 640
- **C.** Dyslipidemic group: **irregularity of wall of the aorta with increased wall thickness. Loss of the normal corrugation and discontinuity of the endothelium, large areas of vaculation (↑↑) are seen underneath the intima with apparent increase in the intima/media ratio. Notice the presence of neutrophils (↑).** Hx&E X 640
- **D.** Asthmatic Dyslipidemic group**: large atheromatous patch (↑) with marked increase in the wall thickness of the aorta. The atheromatous patch is formed occupying the intima and most of the media formed of cellular infiltrate areas of vaculation (↑↑) and central necrosis (↑).** Hx&E X 250
- **E.** Asthmatic + Montelukast group**: the wall is formed of intima, media and adventitia with normal proportions and normal endothelial corrugation.** Hx&E X 640
- **F.** Asthmatic + Fluticasone group**: persistent loss of normal endothelia corrugation of the intima neutrophils (↑) can be identified.** Hx& E X 640
- **G.** Asthmatic Dyslipidemic group + Montelukast: **Moderate restoration of the corrugation of the intima. Notice the areas of vaculation (↑) are seen with marked decrease in the cellular infiltration (↑↑).** Hx& E X 640
- **H.** Asthmatic Dyslipidemic + Fluticasone group: **loss of the endothelial corrugation presence of areas of vaculation (↑↑) and areas of mononuclear infiltration (↑)** Hx& E X 640

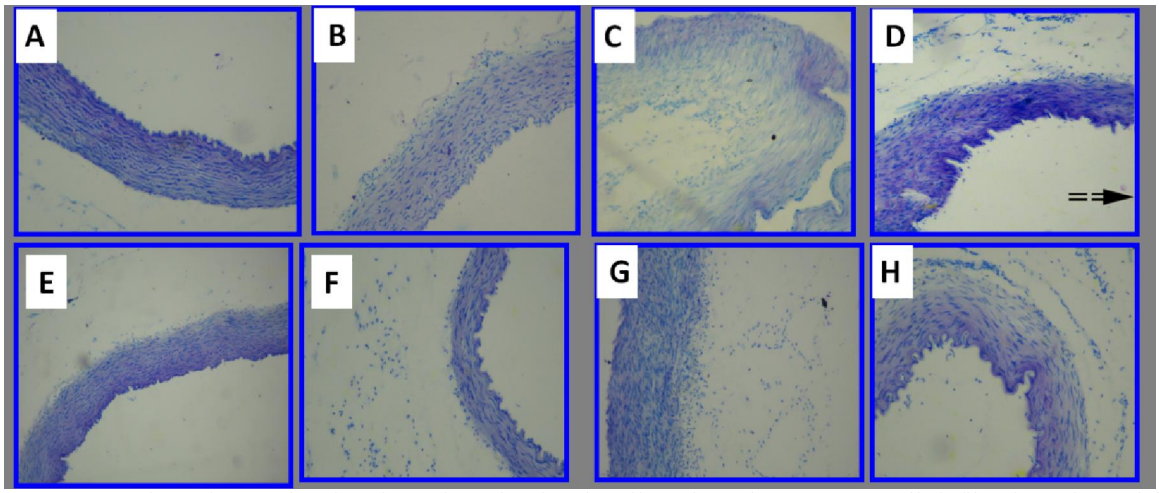

Figure (4, A-H): Photomicrographs of aortic sections showing the effect of test drugs on mast cells infiltration using Toluidine blue.

A): Control group: **Hardly seen mast cells. Toluidine blue X 250**
B): Asthmatic group: **Mild increase in mast cells in the adventitia. Toluidine blue X250**
C): Dyslipidemic group: **Moderate increase in mast cells. Toluidine blue X 250**
D): Asthmatic Dyslipidemic group: **Marked increase in mast cells. Toluidine blue X 250**
E): Asthmatic + Montelukast group**: Marked decrease in mast cells compared to asthmatic group. Toluidine blue X 640**
F): Asthmatic + Fluticasone group**: Mild decrease in mast cells compared to asthmatic group. Toluidine blue X 640**
G): Asthmatic Dyslipidemic group + Montelukast: **Marked decrease in mast cells compared to asthmatic dyslipidemic group. Toluidine blue X 640**
H): Asthmatic Dyslipidemic + Fluticasone group: Mild decrease in mast cells compared to asthmatic dyslipidemic group**.** Toluidine blue X 640.

3.4. Airway Histopathological Changes:

As shown in figures (5) and table (5) animals fed a HFD exhibited mild airway remodeling (epithelial desquamation, inflammatory cellular infiltration and increased smooth muscle thickness around bronchial passages) compared to the control chow fed-animals. Feeding asthmatic animals a HFD augmented airway remodeling induced by chronic OVA challenge with presence of more areas of interstitial hemorrhage and emphysema in asthmatic-HFD animals compared to asthmatic-chow fed animals. That animals treated with montelukast exhibited marked improvement in airway remodeling, with decrease in smooth muscle thickness around bronchioles in both asthmatic-chow fed and the asthmatic-HFD groups compared to their respective untreated groups. Fluticasone induced mild effect on airway remodeling in both asthmatic-chow fed and the asthmatic-HFD groups compared to their respective untreated groups.

It is clear from figure (6) and table (5) that animals fed a HFD exhibited mild inflammatory cellular infiltration into airways and lung parenchyma. Mild mast cell infiltration compared to control chow fed animals was also noted. Feeding asthmatic animals a HFD augmented the inflammatory cellular infiltration induced by chronic OVA challenge. Mast cells infiltration was also augmented in the asthmatic-HFD group versus the asthmatic-chow fed group. Lung tissues isolated from animals treated with montelukast showed marked decrease in mast cell infiltration in both asthmatic-chow fed and asthmatic-HFD groups compared to their respective untreated groups. A decrease in neutrophils was noticed in asthmatic-HFD animals. Fluticasone exerted a mild decrease in mast cell infiltration both in asthmatic-chow fed and asthmatic-HFD groups compared to their respective untreated groups. No change in neutrophils was noticed in either group.

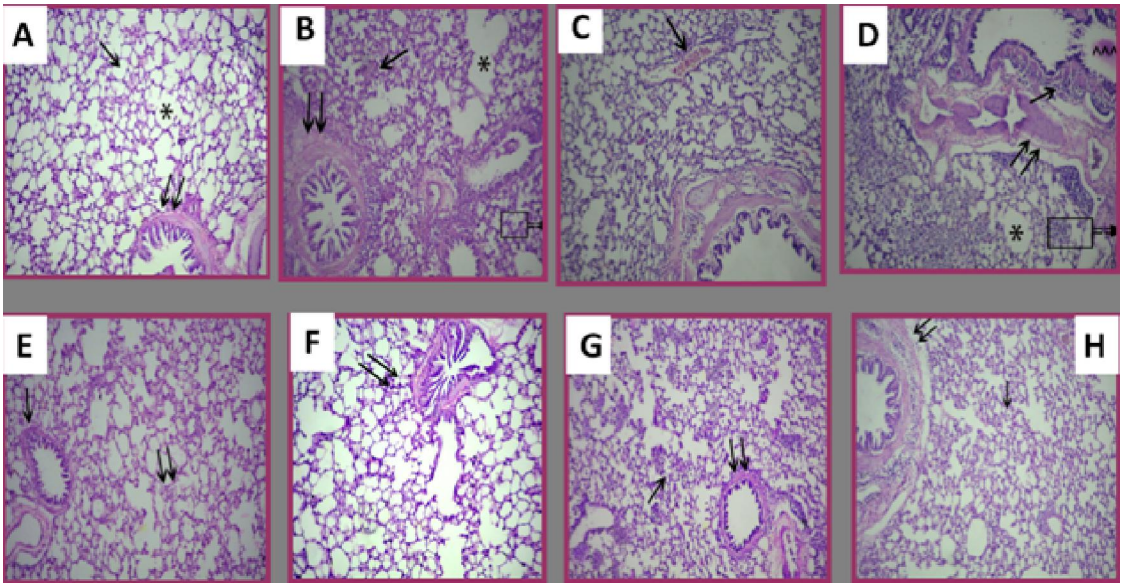

Figure (5, A-H): Photomicrographs of lung sections in different animal groups **(Hx&E X 250)**
A): Control group: Normal lung architecture with alveoli (↑), alveolar sacs (*) and bronchioles, notice thin interalveolar septum and smooth muscles around the bronchioles (↑↑).
B): Asthmatic-chow fed group: Obliteration of the alveoli with cellular infiltration (↑), thickening of the interalveolar septum and apparent increase in muscle thickness around the bronchioles (↑↑), notice emphysematous areas(*).
C): HFD-group: Mild cellular infiltration inside the alveolar lumen and interstitial hemorrhage (↑).
D): Asthmatic-HFD group: Marked cellular infiltration in the alveolar lumen, areas of mononuclear cellular infiltration around bronchioles(↑), areas of emphysema(*) and marked remodeling with increase smooth muscle thickening around bronchioles (↑↑), notice cellular deposits inside the lumen of the bronchi(^^^).
E): Asthmatic-chow fed + Montelukast group: More or less lung architecture regarding smooth muscle thickness around bronchioles (↑), normal appearance of alveoli except for small areas of cellular infiltration (↑↑).
F): Asthmatic-chow fed + Futicasone group: Minimal cellular infiltration with persistent increased smooth muscle thickness around bronchioles (↑↑).
G): Asthmatic-HFD + Montelukast group: Marked improvement in the remodeling around the bronchioles (↑↑) with decreased cellular infiltration (↑) as compared to Asthmatic Dyslipidemic group
H): Asthmatic-HFD + Futicasone group: Decreased cellular infiltration (↑) inside the lumen of the alveoli as compared to the Asthmatic Dyslipidemic group with persistence of the remodeling of the smooth muscle around the bronchioles (↑↑).
Montelukast (oral 10mg/kg/day), Fluticasone (inhaled 100µg/2ml of 0.1% ethanol PBS).

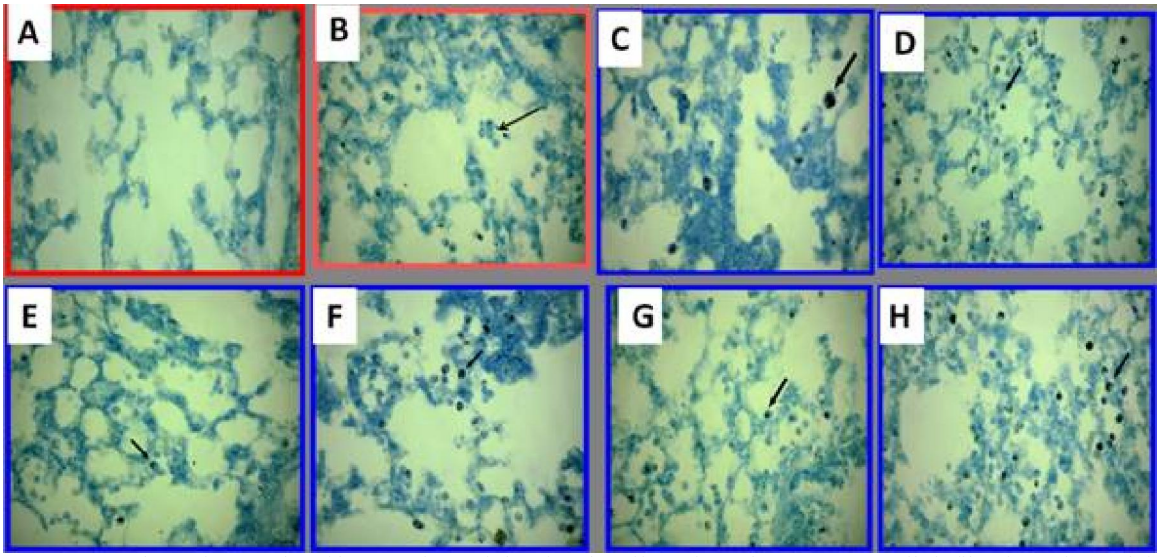

Figure (6, A-H): Photomicrographs of lung sections of showing the effect of test drugs on mast cells infiltration using Toluidine blueX640

A): Control group: Hardly seen mast cells.
B): Asthmatic group: Moderate increase in mast cells in the alveolar lumen and inter alveolar septum.
C): Dyslipidemic group: Mild increase in mast cells.
D): Asthmatic Dyslipidemic group: Marked increase in mast cells.
E): Asthmatic + Montelukast group: Marked decrease in mast cells in comparison to asthmatic group.
F): Asthmatic + Futicasone group: Mild decrease in mast cells compared to asthmatic group.
G): Asthmatic Dyslipidemic + Montelukast group: Marked decrease in the mast cells compared to Asthmatic Dyslipidemic group.
H): Asthmatic Dyslipidemic + Futicasone group: Mild decreased in mast cells compared to Asthmatic Dyslipidemic group.

Table (5): The effect of test drugs on airway remodeling and inflammatory cell infiltration in lung tissues of asthmatic and asthmatic dyslipidemic groups of guinea pigs.

	Airway Remodeling				Inflammatory Cellular Infiltration			
Animal Group (n=5)	**S M Thickness**	**Inflam Cells Infil.**	**Areas of hg.**	**Areas of Emphy.**	**Inflam Cells Infil.**	**Eosino.**	**Neutro.**	**Mast Cells**
I.Control	+	-	-	-	-	-	-	-
II.Asthmatic-	+++	++++	++	++	++++	+++	+	+++
III. Dyslipidemic group	+	+	+	-	+	+	+	+
IV. Asthmatic-Dyslipidemic	+++++	+++++	+++	++++	+++++	++++	++	+++++
V.Asthmatic-+Montelukast	+	+++	-	+	+++	++	+	+
VI.Asthmatic- + Fluticasone	++	++	+	++	++	+	+	++
VII. Asthmatic-Dyslipidemic +Montelukast	++	++++	+	++	++++	+++	+	++
VIII. Asthmatic-Dyslipidemic + Fluticasone	++++	++	++	+++	++	++	++	++++

n = number of animals, S M = smooth muscle, Inflam.= inflammation, Infil.= infiltration, hg= hemorrhage, Emphy= emphysema, eosino.= eosinophils, Neutro.= neutrophils.
(-) = hardly seen (+) → (+++++) = gradually increasing in intensity
Montelukast (oral 10mg/kg/day), Fluticasone (inhaled 100µg/2ml of 0.1% ethanol PBS)

4. Discussion

In the current work, animals fed a HFD for 9 weeks exhibited airway hyper-responsiveness and remodeling with infiltration of mast cells. Several explanations could be formulated to explain the airway dysfunctional changes associated with HFD. One such explanation may be related to the dyslipidemia induced by the HFD. Significant increases in total cholesterol, and triglycerides, has been shown to increase exhaled nitric oxide(NO) in healthy subjects, suggesting that a high-fat diet may contribute to chronic inflammatory diseases of the airway and lung **(Rosenkranz et al., 2010).** It has been suggested that dietary cholesterol might enhance pulmonary allergic inflammation, possibly through non-specific inflammatory processes as well as lymphocyte activities. Indeed the broncho-alveolar lavage fluid of cholesterol fed C57BL6 mice challenged with ovalbumin, were found to contain higher numbers of eosinophils and elevated levels of IL-5, PGE2, and MCP-1. Lymphocytes isolated from the lungs were also found to exhibit elevated production of IL-4 and IFN- gamma. These inflammatory indicators were all significantly correlated with serum cholesterol levels **(Yeh and Huang 2004)**. Hypercholesterolemia has also been reported to induce lung remodeling with airway hyper-responsiveness by promoting chronic inflammation in the circulation through TNF–α-mediated processes **(Naura et al., 2009).**

The reduction in high density lipoprotein (HDL) noted in the present work (though statistically insignificant) might have been also responsible for the airway functional disturbances observed. Indeed, HDL has been shown to play an important and likely specific anti-inflammatory role in the airways. A 2-fold increase in HDL by genetic deletion of endothelial lipase was shown to decrease airway hyper-responsiveness and pulmonary inflammation in OVA-sensitized mice **(Wang et al., 2010).**

The increase in body weight induced by HFD might have also contributed to the airway dysfunctional changes. Indeed, there is a growing body of literature that relates the possible role of adipose tissue in modulating asthma susceptibility and symptoms. For example: compared to lean controls, there are altered T cell responses and mast cell numbers in the trachea of obese mice sensitized to ovalbumin. Furthermore, increased airway hyper-responsiveness and inflammation to ozone have been noted in obese mice **(Johnston et al., 2006)**. Obesity might also lead to more accelerated airway remodeling with each asthma exacerbation **(Shore and Fredberg., 2005).**

Conversely, chow fed animals that were chronically challenged with OVA exhibited dysfunctional vascular changes in aortic rings (reduced endothelium-dependent relaxation to Ach and increased contractile responses to phenylephrine together with increase neutrophils and mast cell infiltration). Chronic OVA challenge in animals fed a high fat diet resulted in augmentation of the atherogenic vascular changes induced by the HFD with more airway remodeling and infiltration of mast cells in both airways and aortic wall.

The findings of the present study add further proof to the reported association of asthma and atherosclerosis both clinically (**Micheal et al., 2005**) and experimentally. OVA challenge was reported to significantly reduce the endothelium-dependent relaxant responses to acetylcholine and histamine, and enhance the contractile effect of phenylephrine of pulmonary arteries from sensitized guinea-pigs. Incubation of the sensitized arteries with the mast cell stabilizer, disodium cromoglycate protected the reduced responsiveness to endothelium-dependent vasodilators following challenge, suggesting that OVA challenge causes an abnormality in endothelial cell reactivity of pulmonary vasculature **(Uydes-Dogan et al., 1995).** Similar results were reported in a study in brown Norway rats fed on an atherogenic diet and immunized with ovalbumin. Severe aortic lesions were noted and intimal thickness was positively correlated with the level of chymase (an indicator of mast cell degranulation), supporting the hypothesis that mast cells play a role in the early stage of atherosclerosis **(Nishizono et al., 1999).**

There is growing evidence that mast cells participate in the development of atherosclerosis, coronary inflammation and cardiac ischemia. Mast cells have been shown to accumulate in the shoulder region of human coronary atheromas, especially in association with plaque rupture and myocardial infarction **(Laine et al., 1999)**. Activated mast cells secrete large amounts of cytokines, and mediators such as LTs in addition to histamine, tryptase, and chymase which may mediate or modulate atherogenesis **(Okayama et al., 2007; Pejler et al., 2007)**. In addition, activated mast cells nonspecifically bind to low-density lipoproteins (LDL), which can be phagocytosed by macrophages to form foam cells, a major cellular component of advanced human atherosclerotic lesions **(Dwyer et al., 2004).**

In the current work, montelukast treatment significantly reduced airway hyper-responsiveness and remodeling as well as the atherogenic vascular changes in asthmatic–dyslipidemic animals. Montelukast also reduced the increase in mast cells in lung as well as vascular tissue. In contrast, fluticasone treatment induced a lesser reduction in mast cells and airway remodeling and failed to exhibit anti-atherosclerotic effects.

The anti-atherosclerotic potentials of montelukast were reported in apolipoprotein E/LDL receptor-double knockout mice but its effects were less pronounced, compared to 5-lipoxygenase activating protein inhibitors **(Jawien et al., 2008; Jawien, 2009).** The inhibitory effects of montelukast on mast cells reported in the current work were also observed in other studies. Direct examination of airway tissue of asthmatic adult subjects confirmed that montelukast decreased the number of mast cells in asthma **(Ramsay et al., 2009)**. The ability of montelukast to decrease the number of mast cells in the airways and vasculature, and to simultaneously ameliorate the vascular as well as the airway dysfunctional changes induced by OVA challenge and the HFD are suggestive of an involvement of mast cell and leukotrienes released from it in the observed association between the airway dysfunction and the vascular atherogenic changes. Indeed a major role for the LT pathway in vascular disease was suggested by identification of enzymes and receptors in the 5-Lipoxygenase pathway expressed in human atherosclerotic plaques **(Spanbroek et al., 2003)** and in subjects with atherosclerosis as measured by an increased intima-media thickness **(Dwyer et al., 2004)**. LTs through the activation of specific LTB and CysLT receptors induce pro-inflammatory signaling and have been implicated in the early lipid retention and the accumulation of foam cells, the development of intimal hyperplasia, and advanced atherosclerotic lesions. LTs have also been reported to play an important role in the rupture of atherosclerotic plaque and in the development of ischemic injury (**Mocatta et al., 2007; Khan et al., 2007).**

The excess LTs reported to be associated with obesity (**Giouleka et al., 2011**) together with the reports indicating that corticosteroids fail to reduce LTs synthesis in vivo (**Dworski et al., 1994**) might explain the lack of anti-atherosclerotic potentials of fluticasone in the asthmatic animals fed the HFD.

Involvement of mast cells and leukotrienes in a crosstalk between the airway and vascular tissue might also explain the differences in response between montelukast and fluticasone noted in the current work. A possible explanation for the association of asthmatic airway changes and atherogenic vascular changes could be that increased airway remodeling following chronic OVA challenge might have resulted in increased mast cell infiltration with excessive production of LTs. Since, airways come into contact with circulatory elements in a continuous manner; it could be possible that a spillover of mast cells and LTs from the airways into the systemic circulation could have subsequently contributed to an inflammatory reaction in the vascular wall with subsequent induction of atherosclerotic changes **(Chung and Barnes, 1999; Kony et al., 2004)**. Accordingly, it could be postulated that the anti-atherosclerotic potential of montelukast might have resulted not only through a reduction in mast cells infiltration and antagonism of leukotriene in vascular tissues but might have also been linked to its effects on the airways. The marked reduction in remodeling and mast cell infiltration in the airways by montelukast observed in the current work could have resulted in a decrease in the spillover of mast cells and leukotrienes from the lungs to the vascular tissues with subsequent potentiation of its anti- atherogenic effects. In contrast, the lesser reduction in mast cell number in the airways induced by fluticasone coupled to its reduced efficacy against airway remodeling could have resulted in a greater spillover of inflammatory cells and LTs to the vascular tissue, further contributing to the insignificant anti-atherosclerotic effects of the drug.

Conclusion

The current work highlights the reciprocal relationship between airway inflammation and atherosclerotic vascular changes. The anti-atherosclerotic potentials of montelukast observed in the present work implicate leukotrienes released from mast cells as possible inflammatory mediators linking asthma, obesity and atherosclerosis. The effects of montelukast may be the result of a direct effect on the vasculature as well as an indirect effect on airway remodeling through a crosstalk (involving mast cells and leukotrienes) between the airway and vascular tissue. An effect on mast cells and inflammatory leukotrienes may be the possible link between the antiasthma efficacy of montelukast and its anti-atherosclerotic potentials. The reduced effect of steroids on these mediators, in both lungs and vasculature, might explain the lack of its anti-atherosclerotic effects. The findings of the current work are clinically relevant to asthmatic patients with the metabolic syndrome. Targeting excess LT by LT modifiers rather than by corticosteroids in this subset of asthmatics may provide not only better control of their airway disorder but might also reduce the risk of atherosclerotic cardiovascular diseases.

Conflict of Interest

The authors declare that there were no conflicts of interest

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