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Effect of Bosentan in Experimentally Induced Hyperlipidemic Mice

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ABSTRACT

Aim of study is to investigate the possible effect of Bosentan as anti-hyperlipidemic agent in mice. The. Thirty-two male albino mice were fed a high cholesterol diet for 28 days to construct hyperlipidemic models. The anti-hyperlipidemic activity Bosentan against hyperlipidemia induced was evaluated in mice. Atorvastatin was used as a standard. Total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol levels were measured. Compared with normal mice, hyperlipidemic mice possessed significantly higher lipid and liver enzymes profile outcomes. After treatment Bosentan, lipid levels and liver enzymatic activities in hyperlipidemic mice significantly decreased. Besides that, Bosentan treated group showed significant improvement in levels of tissue MDA and GPx in hyperlipidemic mice.

Keywords: *Anti-hyperlipidemic, High-fat diet, bosentan, antioxidant effects, statin, HFD*

INTRODUCTION

The major risk factor for the development of atherosclerosis and heart disease, hyperlipidemia is brought on by an excess of lipids or fatty substances in the blood. Depending on the underlying reasons, hyperlipidemia can be classified as either primary or secondary. Changes in lipids, including those found in cholesterol, triglycerides, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and intermediate density lipoproteins (IDL), may lead to consequences in humans include acute pancreatitis, blood vessel blockage, and cholesterol gallstones (1).

Although drugs therapies available for the treatment of hyperlipidemia includes use of drugs like niacin, fibrates, HMG-CoA reductase inhibitors, bile acid binding resins, Omega-3 Polyunsaturated Fatty Acids (PUFA) and PCSK9 inhibitors but associated with lots of side effects. Therefore, herbal treatment for hyperlipidemia has been appreciated because of fewer side effects, less cost and easy availability (2). The dual endothelin receptor antagonist, bosentan, is an orally active therapy, which has been proved to be effective in the treatment of pulmonary arterial hypertension (PAH).

This review critically addresses and highlights pharmacological aspects of bosentan such as safety, tolerability and drug interactions (3).

Experimental animals

The study was conducted from March 2022 through September 2022 at the department of pharmacology–College of Medicine /AL Nahrain University. The experiments were approved by the Ethical Committee at the College of Medicine /AL Nahrain University.

Thirty-two apparently healthy, albino male mice 2-3 months old, weight about 20-30g, were obtained from the National center for drug control and researches. The animals were acclimatized in standard environmental conditions and fed with food and water ad libtum for a week before commencement of the experiment.

Induction of Hyperlipidemia

Hyperlipidemia was induced in mice by addition of High Fat Diet (2% cholesterol and 1% peanut butter) along with the standard for 28 days (4).

TABLE 1: standard and high fat diets composition

Standard diet	**High Fat Diet**
Seeds (sunflower, groundnut)	Seeds (sunflower, groundnut)
Cereals	Cereals
Fruits (grapes, apple)	Fruits (grapes, apple)
Vegetables	Vegetables
Vitamin A	Vitamin A
Vitamin D3	Vitamin D3
Vitamin E	Vitamin E
	Cholesterol powder
	Peanut butter

Experimental design

The mice were divided into 4 groups, 8 mice each group:

Group 1 (normal): standard diet for 28 days .
Group 2 (induced): High Fat Diet (HFD) for 28 days.
Group 3 (treated): HFD for 28 days then atorvastatin 10 mg/kg for further 28 days.
Group 4: HFD for 28 days then Bosentan 100 mg/kg for further 28 days.

Blood collection

The animals were fasted for 12 hours prior blood collection. Blood was collected by piercing the facial vein with a lancet. The blood samples were collected in plain glass tubes and allowed to clot for 20 minutes at room temperature and centrifuged at 3000 RPM for 20 minutes .

The serum obtained was kept at 0°C until analyzed. Serum was used for the estimation of the serum lipid profile and liver function test.

Biochemical analysis

Serum lipid total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), very low-density lipoprotein (vLDL), high density lipoprotein (HDL), aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP) levels of mice were detected with a biochemical auto-analyzer (Shimadzu ,Japan) and respective commercial test kits (Abbott diagnostic, USA) according to the manual instructions.

Measurement of oxidative stress

The liver was homogenized for malondialdehyde (MDA) and glutathione peroxidase (GPx) investigation. The liver was rinsed in ice-cold PBS (0.02mol/L, pH 7.2-7.4). Remove excess blood thoroughly and weighed before homogenization. The tissues were sliced into small pieces and homogenized them in a certain amount of Phosphate-buffered saline (PBS)

(Usually 10mg tissue to 100µl PBS) with a glass homogenizer on ice. The resulting suspension was subjected to two freeze-thaw cycles to further break the cell membranes. After that, centrifugate homogenates for 15 minutes 5000 rpm.

Histopathological examination

The liver obtained from each animal after sacrificed and fixed in 10% formalin solution, then processed by the paraffin technique. Sections of 5µm thickness were cut and stained by haematoxylin and eosin (H&E) for histological examination. The sections were analyzed using an Olympus light microscope with an attached photograph machine (5).

Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for social Science) version (17), and Microsoft Excel Worksheet 2010. Crude data was analyzed to obtain mean and standard deviation (SD). Student t- test was used to compare between two groups. ANOVA test was used to compare between different groups. P-value of ≤ 0.05 considered being significant and P-value of ≤ 0.001 considered as highly significant.

RESULTS

Serum lipid profile

From the data presented in table 2 it is observed that the administration of high fat diet induced hyperlipidemia in mice (Group 2). Concurrent administration of bosentan at 100mg/kg body weight (Group IV) showed a highly significant reduction in the levels of serum total cholesterol, LDL, VLDL as well as triglycerides. In comparison with atorvastatin treated group, group treated with bosentan showed significant increase in serum HDL level.

Liver enzymes activity

In this study, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities were significantly high in high-cholesterol fed diet than in normal mice. On the other hands, the bosentan revealed a highly significant reduction in AST and ALP levels. In comparison with atorvastatin treated group, group treated with bosentan showed a highly insignificant in serum ALP and AST; table 3.

Antioxidant activities

The MDA were significantly increased in induced (non-treated) group and Bosentan in comparison with healthy group. Meanwhile, the glutathione peroxidase level in induced (non-treated) group in comparison with healthy group decreased highly significant while it increased highly significant in Bosentan treated group; table 2.

In comparison with atorvastatin treated group, group treated with Bosentan showed a highly significant increment in MDA and statistically insignificant in glutathione peroxidase levels; table 3.

TABLE 2: Comparison between hyperlipidemic induced (non-treated) group and induced (hyperlipidemic) group Bosentan in relation to different parameters.

Group	Induced group Mean ±SD	Normal group Mean ±SD	Bosentan (100mg/kg) Mean ±SD
TC (mg/dl)	270.62 ±9.69	113.25 ±12.04^{a**}	188.87 ±17.80 ^{a**}
TG (mg/dl)	269.50 ±20.33	108.75 ±9.03 ^{a**}	120.12 ±6.61 ^{a**}
HDL (mg/dl)	47.25 ±1.39	54.87 ±2.54 ^{aNS}	75.62 ±6.64 ^{a**}
LDL (mg/dl)	246.37 ±12 .64	85.50 ±1.48 ^{a**}	67.37 ±2.12 ^{a**}
vLDL (mg/dl)	64.62 ±6.54	33.62 ±1.60 ^{a**}	29.75 ±2.88 ^{a**}
AST (U/l)	216.62 ±29.77	20.37 ±2.54 ^{a**}	117.00 ±7.54 ^{a**}
ALT (U/l)	161.25 ±16.06	26.62 ±3.64 ^{a**}	138.62 ±15.79 ^{aNS}

ALP (U/I)	222.50 ±17.12	83.75 ±7.52 [a**]	163.25 ±2.25 [a**]
MDA (ng/ml)	101.56 ±4.40	20.34 ±2.19 [a**]	26.04 ±0.99 [a**]
GPx (ng/ml)	0.601 ±0.03	2.67 ±0.07 [a**]	1.253 ±0.27 [a**]

a: Comparison with induced group, NS: not statistically significant (p>0.05), **: Highly statistically significant (p≤0.001), TC: total cholesterol, TG: triglycerides, HDL: high density lipoprotein, LDL: low density lipoprotein, vLDL: very low-density lipoprotein, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, MDA: Malondialdehyde, GPx: glutathione peroxidase.

TABLE 3: Comparison of group treated with Bosentan with induced (non-treated) and Atorvastatin treated group in relation to different parameters.

Group	Induced (non-treated) group Mean ±SD	Atorvastatin treated group (10mg/kg) Mean ±SD	Bosentan (100mg/kg) Mean ±SD
TC (mg/dl)	270.62 ±9.69	178.62 ±27.98 [a**]	188.87 ±17.80 [a**, bNS]
TG (mg/dl)	269.50 ±20.33	115.37 ±6.25 [a**]	120.12 ±6.61 [a**, bNS]
HDL (mg/dl)	47.25 ±1.39	47.37 ±1.52 [aNS]	75.62 ±6.64 [a**, b**]
LDL (mg/dl)	246.37 ±12 .64	82.50 ±2.07 [a**]	67.37 ±2.12 [a**, bNS]
vLDL (mg/dl)	64.62 ±6.54	22.75 ±1.27 [a**]	29.75 ±2.88 [a**, bNS]
AST (U/l)	216.62 ±29.77	110.00 ±8.10 [a**]	117.00 ±7.54 [a**, bNS]
ALT (U/l)	161.25 ±16.06	84.75 ±7.98 [a**]	138.62 ±15.79 [aNS, b**]
ALP (U/I)	222.50 ±17.12	183.12 ±10.30 [a**]	163.25 ±2.25 [a**, bNS]
MDA (ng/ml)	101.56 ±4.40	25.43 ±1.93 [a**]	26.04 ±0.99 [a**, bNS]
GPx (ng/ml)	0.601 ±0.03	1.672 ±0.18 [a**]	1.253 ±0.27 [a**, bNS]

a: Comparison with induced group, b: comparison with atorvastatin group, NS: not statistically significant (p>0.05), **: Highly statistically significant (p≤0.001), TC: total cholesterol, TG: triglycerides, HDL: high density lipoprotein, LDL: low density lipoprotein, vLDL: very low-density lipoprotein, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, MDA: Malondialdehyde, GPx: glutathione peroxidase.

Histopathological examination of the liver

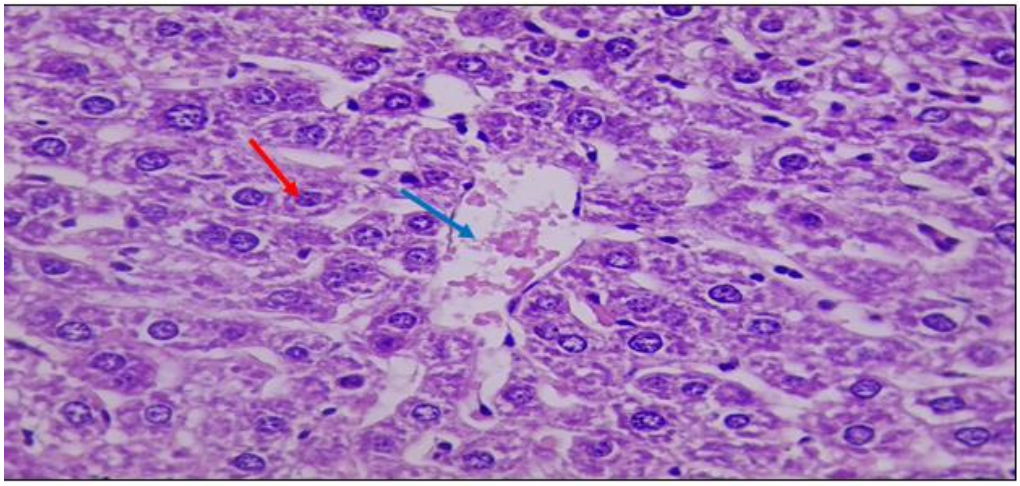

FIGURE 1: Histological section of liver tissue for normal group showing normal structure of hepatocytes (red arrow) and central vein (blue arrow). (H&E stain, 40X)

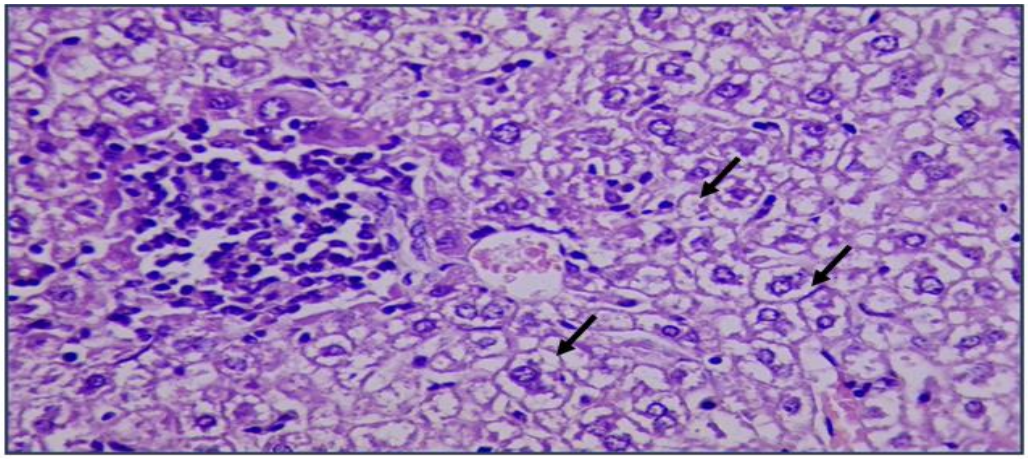

FIGURE 2: Histological section of liver tissue for hyperlipidemic group showing sever and diffuse cytoplasmic fatty infiltration (microvesicular steatosis). (H&E stain, 40X)

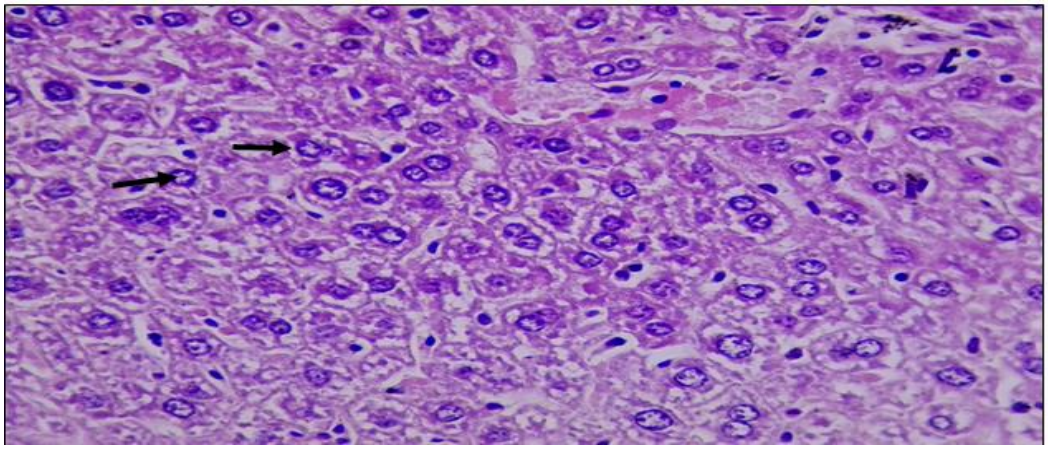

FIGURE 3: Histological section of liver for hyperlipidemic group treated with atorvastatin showing mild & focal microvesicular steatosis. (H&E stain, 40X)

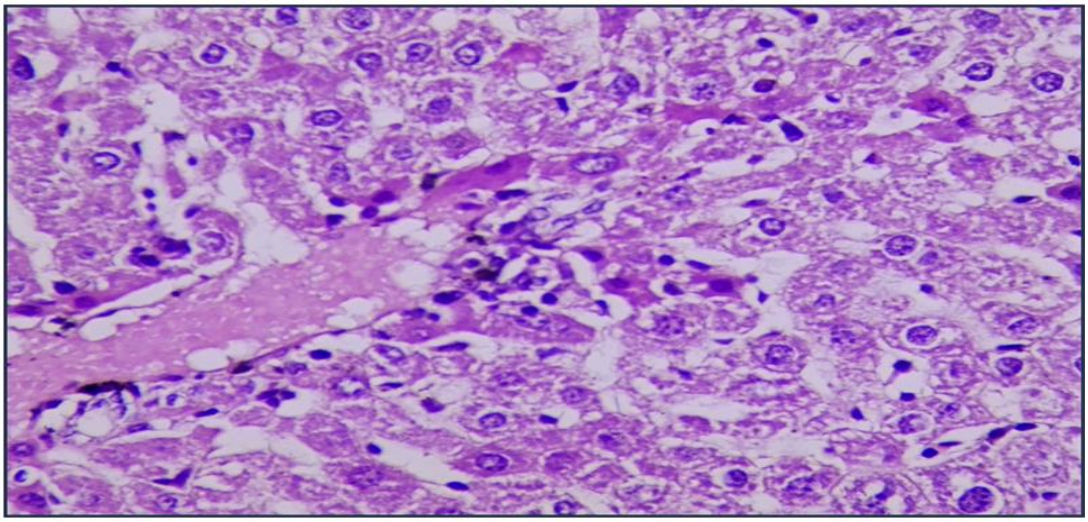

FIGURE 4: Histological section of liver for hyperlipidemic group treated with Bosentan showing diffused and moderate microvesicular steatosis. (H&E stain, 40X).

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DISCUSSION

Hyperlipidemia is characterized by increase in serum lipid profile namely, triglycerides (TG), total cholesterol (TC) and low-density lipoprotein- cholesterol (LDL-C) and thus is considered one of the primary risk factors leading to CVDs and myocardial infarction. Hyperlipidemia is directly linked with a prominent metabolic dysregulation in the affected patients (6). The above-mentioned changes in association with declining (HDL-C) serum level eventually result in hyperlipidemia, causing advanced cardiac pathological conditions (7). Moreover, the interfering harmful effect of HFD with the process of lipid metabolism in the liver is the primary factor responsible for the development of nonalcoholic fatty liver disease (8). Hyperlipidemia, in particular raised low-density lipoproteins (LDL hypercholesterolemia), is one of the primary risk factors contributing to the evolution of atherosclerotic cardiovascular disease (6). Practical strategies to treat hyperlipidemia, include the decrease lipids synthesis and their gut absorption using synthetic therapeutic agents as fibrates, statins and bile acid sequestrants. The use of these agents might be associated with series of side effects most notably myopathy, rhabdomyolysis and increase risk of gallstone formation. Hence, developing a novel and effective anti-hyperlipidemic therapeutic agents with minimal side effects is urgently required (9).

In the present study, feeding the mice high fat diet (HFD) for 4 weeks led to highly significant increase in serum total TC, TG, LDL and VLDL in induced (hyperlipidemic) mice as compared to normolipidemic group fed normal standard diet. These changes observed in hyperlipidemic group may be due to that HFD induced hyperlipidemia by demodulating lipid metabolism, mainly by decreasing β-oxidation and increasing cholesterol synthesis and oxidative stress by decreasing free radical scavenger enzyme gene expression (10). Also, Rui-Li (2008) reported that HFD induced abnormal increases in lipid peroxidation, serum concentrations of total cholesterol, triacylglycerol, and low-density lipoprotein cholesterol in addition to decreased lipoprotein lipase activity, accompanied by a depressed antioxidant defense system. Oxidative stress has been documented to play a pivotal role in the patho-physiology and progression of diverse human diseases including CVD, CVI and DM (11). Moreover, according to (12) study suggested that elevated cholesterol in the plasma can stimulate ET-1 production. During the course of atherosclerotic progression, plasma atherogenic lipoproteins, such as LDLs, accumulate in the arterial intima and subsequent oxidative modification of LDLs may then occur. Since oxidation of LDLs can take place within the vascular wall, it is possible that oxLDLs may influence EC-derived ET-1 production and secretion.

The serum ALT, AST and ALP levels were extensively elevated in high-cholesterol fed diet than in normal mice. This may due to the disturbance of lipid metabolism because of high fat intake, resulting in accumulation of TG in liver and an increased increment of the liver index, and hepatic steatosis occurred (13) since the liver has a crucial role in regulating plasma lipid level all the way through LDL clearance and HDL cholesterol recruitment (14). Moreover, the elevation in liver enzymes may also due to excess reactive oxygen species (ROS) production in the mitochondria as a result of lipid overload. The surplus ROS generation exhausted the endogenous antioxidants (15).

The increased ET-1 in the arterial wall may play multiple functional roles in the lesion development. For example, excess ET-1 may facilitate local monocyte migration, and through a local paracrine system, ET-1 may initiate SMC proliferation and migration. With the advancement of the lesions, accumulated macrophages take up lipids, are transformed into foam cells, and constitute the major parts of the fatty streak. In return, these foam cells may become the major source of ET-1 to maintain the progression of the lesions. In coronary arterial atherosclerosis, macrophage-derived ET-1 may contribute to hyper-vasoconstriction, which leads to abnormal coronary vasomotion in patients with unstable angina. This may also explain why vasospastic events occur preferentially at the site of atherosclerotic lesions (16).

Atorvastatin as a standard cholesterol-lowering drugs is used in present study which has been

associated with a high significant reduction in lipid profile (TC, TG, LDL and VLDL) along with Bosentan (100mg/kg; p.o) treated group. It has been associated with hepatoprotective effect through high significant reduction in liver enzymes (ALT, AST and ALP). In other animal study, atorvastatin and bosentan treatment has been associated with a broad spectrum of hepatic adverse effects. The most common is usually transient elevation of serum aminotransferase levels (Argo et al., 2008)(17). Although the underlying mechanism remains unclear, it may result from changes in the lipid components of the hepatocyte membrane, leading to an increase in its permeability with a subsequent leakage of liver enzymes (Bhardwaj and Chalasan, 2007).

In respect to lipid profile of hyperlipidemic-induced mice group treated with Bosentan (100mg/kg; p.o) were highly significant reduced except for HDL levels which was increased markedly in comparison with hyperlipidemic induced mice group. The elevation of HDL level may because of the inhibition the secretion of ET-1(12) while the reason behind the reduction of other of lipid profile may due to restoring NO-mediated endothelial functions (12).

Tissue homogenate (MDA) of atorvastatin (10mg/kg/day; p.o.) treated group were highly significant reduction in comparison with induced group and displayed statically insignificancy in comparison with Bosentan (100mg/kg; p.o) treated group. This indicates that the atorvastatin and Bosentan possess a hepatoprotective properties throughout antioxidant action of increase the function of the endogenous antioxidants and reduce lipid peroxidation by a high significant increase Gpx level since its evolved specific defenses against these ROS in eukaryotic cells whose function is to reduce the cumulative load of ROS within the cell, or intracellular space (18). Histopathological examination of liver showed improvement and these support anti-hyperlipidemic of bosentan. Bosentan is reported to decreases steatosis and restores the microvascular architecture in high-fat diet feeding rats (19.20.21).

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