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Involvement of the toll-like receptors-2/nuclear factor-kappa B signaling pathway in atherosclerosis induced by high-fat diet and zymosan A in C57BL/6 mice

Priyanka Arya, Uma Bhandari

Abstract:

OBJECTIVE: Accumulated evidence reported a link between the immune system, microbial infection, and the development of atherosclerosis. Excess intake of high-fat diet (HFD) increases blood lipid levels and induces inflammatory pathways whereas zymosan A (Zym), a microbial component, mediates inflammatory response through the stimulation of specific ligand of toll-like receptors (TLRs) of the immune system. The current research work was aimed to evaluate the mechanism behind atherosclerosis mediated by HFD and Zym in C57BL/6 mice.

MATERIALS AND METHODS: The mice were orally fed with HFD for 30 days and Zym (80 mg/kg, single intraperitoneal injection on day 8th). On the 31st day, blood was withdrawn from overnight fasted mice by tail vein puncture and estimated for serum lipids and tumor necrosis factor-alpha (TNF-α). Animals were sacrificed, and cardiac, liver, and aortic tissues were isolated for the estimation of cardiac TLR-2, nuclear factor-kappa B (NF-κB); hepatic low-density lipoprotein receptors (LDLR); and base of aorta analyzed for histopathology.

RESULTS: It was found that HFD and Zym administration increased arterial inflammation directly through modulation of the TLR-2/NF-κB pathway, thereby upregulate serum TNF-α, cardiac TLR-2, and NF-κB levels. Further, HFD and Zym treatment significantly increased serum lipid levels and marked decrease in LDLR protein expression in the liver when compared to normal control mice. Histopathological analysis showed the formation of atherosclerotic plaque.

CONCLUSION: The study is first, to our current knowledge, to demonstrate the involvement of the TLR-2/NF-κB signaling pathway in atherosclerosis induced by HFD and Zym in C57BL/6 mice, resulting in increased degradation of LDLR protein, thereby, increasing the serum lipid levels.

Keywords:

Inflammation, low-density lipoprotein receptor, toll-like receptors, tumor necrosis factor-α

Department of Pharmacology, School of Pharmaceutical Education and Research, Jamia Hamdard (Deemed to be University), New Delhi, India

Address for correspondence:
Prof. Uma Bhandari, Department of Pharmacology, School of Pharmaceutical Education and Research, Jamia Hamdard (Deemed to be University), New Delhi - 110 062, India.
E-mail: uma_bora@hotmail.com

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Introduction

Despite the lifestyle modification and potential therapeutic approaches, the incidence of atherosclerosis is continued to rise. Atherosclerosis is an arterial inflammatory disease, described by the adherence of the lipids and fibers within an innermost layer of the vessel walls.[1-3] Initially, atherosclerosis was considered to be the result of dyslipidemia, but further investigation by researchers, it was revealed that there is a link between the immune system, microbial infection, and atherosclerosis.[2,4,5] The immune response is mainly triggered by an inflammatory response, altered endothelium functions.

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This, in turn, leads to the transmigration of inflammatory cells, such as leukocytes, adhesion molecule, chemokines and promotes the mobilization of monocytes and T-cells, which migrates inside the intima and enhances uptake of modified lipoprotein particles and which lead to the formation of foam-cell. Chronic inflammatory stimulation can cause local protein breakdown, impairment of cholesterol transport, arterial stiffness, plaque disruption, and thrombus formation, which produces atherosclerosis, stroke, and cardiac arrest.[1,2]

The previous study suggests a mechanistic link between atherosclerosis and toll-like receptors (TLRs) activation, but the mechanism is still unclear. TLRs are the principal sensor of the innate immune system which functions for specific receptors known as pattern recognition receptor which recognizes the microbial structure and mediate protection against infection. TLRs bind wide ranges of microbial origin molecules, such as Gram-positive obtained bacterial lipoproteins, lipoteichoic acid, and zymosan A (Zym).[5-7] In the current study, high-fat diet (HFD) and Zym were used as an experimental model to induce atherosclerosis in C57BL/6 mice. Zym is a fungal product, prepared from the cell wall of *Saccharomyces cerevisiae*. It is an inflammagen used as a pharmacological tool to develop animal models of various diseases include acute peritonitis, rheumatoid arthritis, and dyslipidemia. Zym induces an inflammatory response in macrophages through TLRs TLR2 and TLR6, which further transmit transmembrane signals that stimulate the inflammatory pathway.[5,8,9] The Epidemiological evidence also suggests a strong link between infection and inflammation, which causes an alteration in lipoprotein metabolism through the mechanism of intracellular disruption of low-density lipoprotein receptors (LDLR) by inflammatory cytokines.[10,11] Therefore, the present study was aimed to investigate the unclear molecular mechanism responsible for atherosclerosis mediated by HFD and Zym together in C57BL/6 mice. The HFD and Zym preclinical model will help the researcher's pharmacologists as well as cardiologists to better understand the pathogenesis of long-term metabolic disruptions like atherosclerosis and others.

Materials and Methods

Drugs and chemicals

HFD (45% kcal fat, 35% kcal carbohydrates, and 20% kcal protein) was obtained as a free sample from Ashirwad Industries, Punjab, (India). Zymosan A (Zym) were procured from Sigma Chemicals (USA).

Experimental animals

C57BL/6 mice were attained from Central Animal House Facility of Jamia Hamdard, New Delhi (India), after the approval of Institutional Animal Ethics Committee, followed regulations of Committee for Control and Supervision of Experiments on Animals (CPCSEA) (Registration no. of JHAEC: 173/GO/Re/S/2000/CPCSEA, Approved Protocol No. 1389). Animals were placed under-maintained laboratory environment at 22°C ± 2°C, relative humidity 55% ± 5% at 12 h day/night cycle with food and water *ad libitum*.

Study design

After acclimatization, C57BL/6 mice were then randomly divided into two groups, e (n = 6 per group). The experimental atherosclerosis was produced in C57BL/6 mice by feeding pellets of HFD randomly for 30 days along with Zym at the 8th day, as a single intraperitoneal (i.p.) injection. Zym (80 mg/kg) was suspended in sterile phosphate buffer saline solution (PBS) to produce a final concentration of 5 mg/ml.[8,10,12] Group I/Normal Control; Mice were fed chow diet for 30 days and the sterile PBS, Zym vehicle (5 mg/ml, i.p., single injection) at 8th day; Group II/Pathogenic Control-Mice were fed HFD for 30 days and Zym 80 mg/kg, single i.p. injection on day 8th. Mice were fasted overnight before sacrificing. On the 31st day, blood was collected through tail vein puncture and centrifuged to separate serum. Followed the collection of blood samples; animals were sacrificed using ether anesthesia and heart, the base of aorta and liver were isolated, washed, wrapped, and stored at −80°C for the estimation of biochemical and histopathological parameters.

Assessment of anthropometric parameters

Weekly body weight deviation of mice was evaluated from the difference between the final and starting weight of mice. Daily intake of food of mice was evaluated by minus the quantity of food remains in the cage of mice.[13]

Measurement of serum lipid parameters

Total cholesterol (TC), triglycerides (TGs), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein (LDL) levels were determined in serum, using Span Diagnostics Kits according to the manufacturer's instructions.[14-18]

Assessment of tumor necrosis factor-alpha toll-like receptors-2 and low-density lipoprotein receptors levels

Tumor necrosis factor-α (TNF-α) was determined in the serum sample; TLR-2 was measured in heart tissue homogenate whereas, LDLR protein was evaluated in hepatic tissue homogenate, using Genxbio Health Sciences ELISA kits, in accordance with the manufacturer's protocol.

Immunohistochemical analysis (nuclear factor-kappa B) of heart tissue

Heart tissue sections were fixed with acetone for a fixed time interval. After that, tissue sections were incubated with 0.3% hydrogen peroxide solution to remove background staining and then washed with tris-buffer solution three times. Further, the samples were incubated with anti-nuclear factor-kappa B (NF-ƙB) antibody [1:100 dilutions; Calbiochem (EMD Millipore)], overnight at 4°C. Then, we washed the tissue sections and followed 1 h incubation with a secondary antibody to form peroxidase complex. Reactive signals were visualized using diaminobenzidine. The slides were examined under the Meiji microscope by using a blinded approach. The NF-ƙB protein percentage area was estimated using ImageJ software.[19,20]

Histopathological assessment of base of aorta tissue

The base of the aorta was isolated, fixed in 10% formalin solution, and then fixed in paraffin. The tissues were dye by using hematoxylin and eosin (H and E) solutions. The slides were observed under the Meiji microscope. Microscopic atherosclerotic plaque area was quantified by the Fiji (Image J) software.[19,20]

Statistical analysis

The current data were presented as mean ± standard error of the mean (SEM) ($n = 6$). Significant differences among groups of weekly body weight were evaluated using two-way ANOVA followed by Bonferroni-post tests and other parameters by unpaired t-test. $P < 0.05$ was considered significantly. Statistical comparisons of all results were performed using GraphPad Prism software (version 3.0), San Diego, California, USA.

Results

Effect on daily food and water intake

In pathogenic control (Group II), it was observed that feeding of HFD caused an increase in the intake of food and water in C57BL/6 mice in the 1st week as compared with normal chow diet-fed mice, i.e., Group I (normal control). However, after the administration of Zym at the 8th day (80 mg/kg) single i.p. injection and feeding HFD randomly for 30 days, there was a decrease in food and water intake when compared with normal chow diet-fed mice, i.e., Group I.

Effect on weekly body weight measurement

In pathogenic control (Group II), the bodyweight of mice was increased significantly ($P < 0.001$) on feeding HFD in the 1st week. However, after the administration of Zym at 8th day (80 mg/kg) single i.p. injection and feeding HFD randomly for 30 days, it was observed that bodyweight in the II, III, and IV weeks was significantly ($P < 0.001$) reduced than the normal chow diet-fed mice group [Figure 1].

Effect on the serum lipid parameters

Treatment of mice with HFD and Zym, i.e.,. Group II showed a remarkable ($P < 0.001$) increase in TC, LDL-C, very LDL, and TGs levels than the Group I. However, a notable ($P < 0.001$) decrease in the serum HDL levels was detected in HFD and Zym administered group when compared to Group I [Table 1].

Effect of high-fat diet and zym on the levels of cardiac risk indexes

The results showed that the atherogenic index and coronary risk index were remarkably elevated in HFD and Zym treated mice than the normal chow diet-fed mice [Table 1].

High-fat diet and zym administration decreases the expression of hepatic low-density lipoprotein receptors protein

Hepatic LDLR protein levels were evaluated to verify whether HFD and Zym upregulate serum lipids by increasing LDLR degradation. LDLR protein expression in hepatic tissue was significantly ($P < 0.001$) decrease in HFD and Zym treated group compared with normal control mice [Figure 2a].

Effect on the expression of cardiac toll-like receptors-2

Treatment of mice with HFD and Zym observed a significant ($P < 0.001$) increment in TLR-2 expression as compared to the normal chow diet-fed mice [Figure 2b].

High-fat diet and Zym administration increases the expression of cardiac nuclear factor-kappa B in C57BL/6 mice

It was found that HFD and Zym treated mice, i.e., Group II produced significant ($P < 0.001$) increase

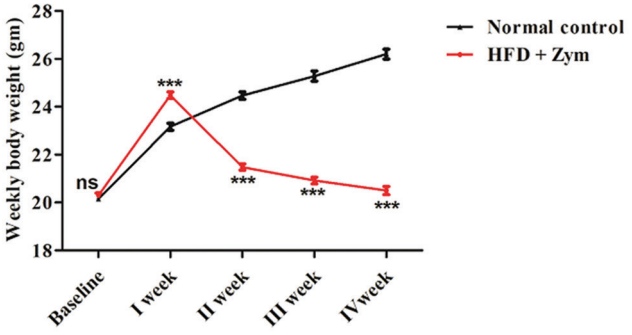

Figure 1: Effect of high-fat diet and Zym on weekly body weight in C57BL/6. Data in the graph are expressed as mean ± standard error of mean ($n = 6$ animals per group). Significance was determined by two-way ANOVA followed by Bonferroni-post tests. ***$P < 0.001$, $^{ns}P > 0.05$ (Group II vs. Group I). ns = Nonsignificant

in NF-ƙB expression (~62.8%) in heart tissue on immunohistochemical analysis as compared to heart tissue of normal control group (~8%), i.e., Group I [Figure 3].

Effect of high-fat diet and Zym on the levels of serum tumor necrosis factor-alpha in C57BL/6 mice

To investigate the effect of HFD and Zym on arterial inflammation, we evaluated the expression of pro-inflammatory cytokine (TNF-α) in serum by using an ELISA kit. The results showed that serum TNF-α levels were significantly ($P < 0.001$) rise in HFD and Zym treated group than that in the normal control group [Figure 2c].

Histopathological analysis

In the base of aortic tissue, we observed that there was remarkably increase in atheromatous percentage plaque area (~63%) in HFD and Zym administered mice than the

Table 1: Effect of high-fat diet and zymosan A on biochemical parameters in serum in C57BL/6 mice

Groups	TC (mg/dl)	TGs (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	AI	CRI
Group I (normal control)	116±0.33	69.4±0.36	47.6±0.13	54.5±0.32	13.88±0.07	1.14±0.007	2.43±0.006
Group II (HFD + Zym)	254±2.32***	263±0.37***	15.1±0.90***	186±2.34***	52.66±0.07***	12.29±0.18***	16.77±0.20***

Data are expressed as mean±SEM (n=6 animals per group). Significance was determined by unpaired t-test ***P<0.001 (Group II vs. I). HFD=High-fat diet, Zym=Zymosan A, TC=Total cholesterol, TGs=Triglycerides, HDL=High-density lipoprotein, LDL=Low-density lipoprotein, VLDL=Very LDL, AI=Atherogenic index, CRI=Coronary risk index, SEM=Standard error of the mean

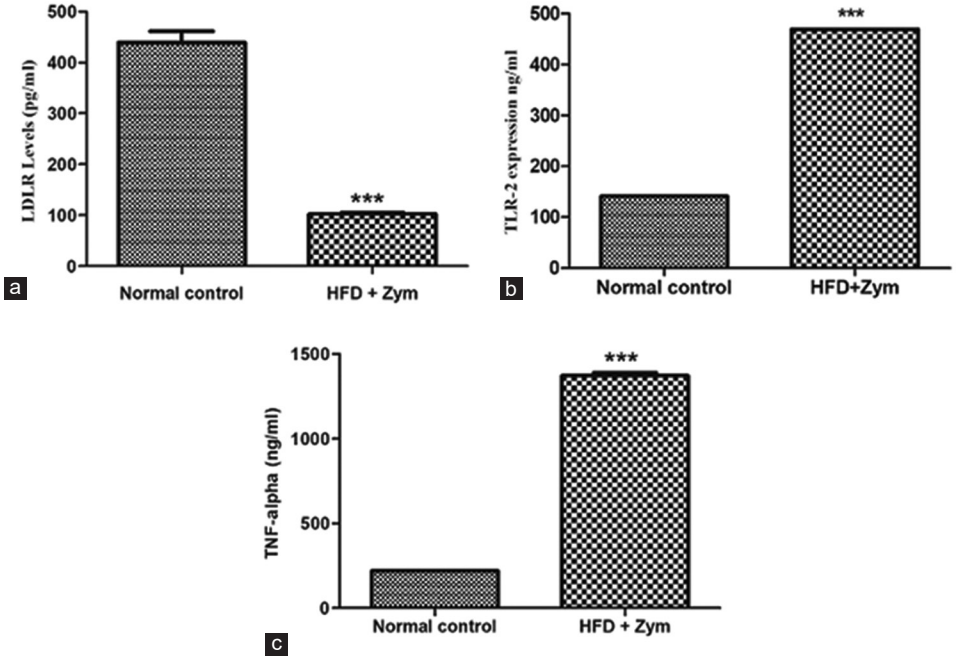

Figure 2: (a) Representative bar diagram shows the effect of high-fat diet and Zym on low density lipoprotein receptor in C57BL/6 mice. (b) Effect of high-fat diet and Zym on Toll like receptor-2 in C57BL/6 mice. (c) Effect of high-fat diet and Zym on tumor necrosis factor-alpha in C57BL/6 mice. Data in the graph are expressed as mean ± standard error of mean (n = 6 animals per group). Significance was determined by unpaired t-test. ***P < 0.001 (Group II vs. I)

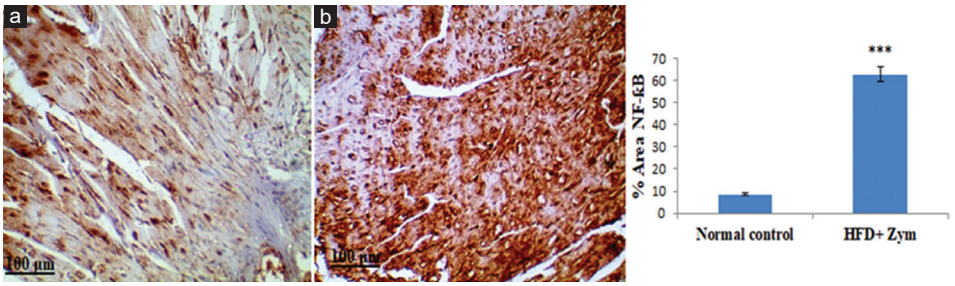

Figure 3: Immunohistochemical analysis of nuclear factor-kappa B in heart tissue of different group animals (Scale bar-100 µm): (IHC, ×40) (a) Group I/Normal control (sterile phosphate buffer saline as Zym vehicle), (b) Group II/high-fat diet (30 days) and Zym (80 mg/kg, single intraperitoneal injection at day 8th). The Fiji (Image J) software was employed for the semi-quantification of the expression of proteins. Data in the graph are expressed as mean ± standard error of mean (n = 6 animals per group). Significance was determined by unpaired t-test ***P < 0.001 (Group II vs. I)

normal chow diet-fed mice wherein the base of the aorta tissue showed normal morphology in (~4.4%) percentage plaque area [Figure 4].

Discussion

The findings of the current study highlight a few effects of HFD and Zym on atherosclerosis in C57BL/6 mice, which may be fruitful in understanding its pathogenesis. Accumulated evidence has shown that atherosclerosis is not only the result of hypercholesterolemia; other risk factors such as bacterial and viral infection, physical inactivity and HFD are important risk factors for obesity and atherosclerosis. Excess intake of lipid enriched diet increases blood cholesterol and lipids levels.[13] Epidemiological studies suggest a strong association between inflammation and arterial disease.[2,4,5,21] The inflammatory reaction in blood vessels contributes a crucial role in the progression of atherosclerosis until the precipitation of clinical symptoms such as myocardial infarction and stroke.[12] Therefore, it is very important to elucidate the molecular mechanism underlying atherosclerotic inflammation for developing potent and safe therapeutic approaches for patients with atherosclerosis.

Therefore, the current research was undertaken to elucidate the mechanism behind atherosclerosis mediated by HFD and Zym in C57BL/6 mice. The current study showed that the inflammatory signals induced by HFD and Zym together promote the development of atherogenesis.

In the current investigation, it was found that oral feeding of high caloric food intake in the form of HFD randomly in the 1st week, i.e., for first 7 days without Zym in group II mice significantly increased weekly bodyweight, daily food, and water intake as compared to normal chow diet-fed mice, i.e., Group I mice. However, Zym administration (80 mg/kg, single dose, i.p.) on 8th day along with feeding HFD for further 23 days (total 30 days of HFD feeding) induced acute vascular inflammation in mice which resulted in a drastic reduction in body weight, daily food and water intake (weekly measured) when compared with the normal chow diet-fed group. Our results have corroborated the findings of Zhang *et al*. in 2008. Wherein they reported that administration of HFD together with Zym (20 mg/kg once every 3 days, i.p., for 2 weeks) in Wistar rats for a period 9 weeks resulted in decreased activity and decrease in food and water intake for 2 consecutive weeks as compared to the normal control group. Furthermore, Malik *et al*. in 2011 reported that the administration of Zym in C57BL/6 mice induced acute inflammation, which resulted in catabolic state with 8% lower food consumption, 5%–7% decrease in body weight, and a 40% reduction in fecal mass in the first 24 h.[8,22]

Experimental evidence suggests that inflammation contributes to an essential role in the development of atherosclerosis.[12] The TLR-2/NF-κB pathway has a pivotal contribution to atherosclerosis because it modulates the arterial inflammatory response. TLR-2 is a pattern-recognition receptor found in cell surface protein, which acts as a principal sensor of the innate immune system. TLR-2 activates the transcription factor, NF-κB which is linked to the various pro-inflammatory genes, including cytokines, adhesion molecules, free radicals, and chemokines, etc.[23-25] Schoneveld *et al*. reported that exogenous TLR-2 activation increases atherosclerotic plaque formation in *APOE*−/−atherosclerotic mice.[7] In the present study, we found that the administration of HFD and Zym dramatically upregulate the vascular inflammation through significantly ($P < 0.001$), increasing the levels of cardiac TLR-2, NF-κB which are consistent with previous findings.

Collected evidence suggests that the release of inflammatory cytokine TNF-α has a direct link with atherosclerosis. Previous literature reported that TNF-α deficient mice reduce the burden of atherosclerosis by decreasing the aortic plaque area than that of *ApoE*-deficient mice.[23] The findings of our study indicate that the administration of HFD and Zym for 30 days

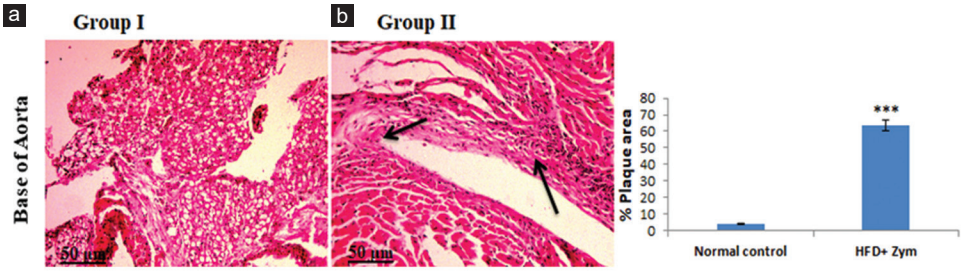

Figure 4: Photomicrographs (H and E, ×10) (Scale bar-50 µm) of the base of aorta section of different groups. Representative sections from base of aorta section showing atherosclerotic plaque (arrow). Group I/Normal control (sterile phosphate buffer saline as Zym vehicle), (b) Group II/high-fat diet (30 days) and Zym (80 mg/kg, single intraperitoneal injection at day 8th).The Fiji (Image J) software was employed for the semi-quantification of the percentage area of plaque. Data in the graph are expressed as mean ± standard error of mean (n = 6 animals per group). Significance was determined by unpaired t-test ***$P < 0.001$ (Group II vs. I)

showed an abnormal upsurge in the levels of TNF-α. Thus, we conclude that HFD, together with Zym, may play a vascular inflammatory role in the progression of atherosclerosis.

Accumulated evidence suggests that inflammation modulates the cholesterol homeostasis through the mechanism of intracellular disruption of LDLR by inflammatory cytokines.[10,11] The major novel findings of the present research work were that the administration of HFD and Zym for 30 days showed a decrease in the expression of hepatic LDLR, which led to drastic changes in the levels of serum lipoproteins and development of atherosclerotic plaque. Further, the results showed a rise in serum lipid levels and a decline in the serum HDL-C levels than the normal control, indicating that the HFD and Zym treated mice had a higher risk of developing atherosclerosis. The AI and CRI were significantly increased in HFD and Zym administered group than the control group. AI and CRI may be believed as important markers for predicting the risk of atherosclerotic disease. The study is first, to our current knowledge, to provide the evidence that hepatic LDLR degradation mediated by HFD and Zym together led to impairment of lipid homeostasis, resulting in atherosclerosis. In the present study, histological H and E staining of a base of aorta section clearly show the formation of plaque (~63%) in HFD and Zym administered group than the normal control group (~4.4%).

The result of the current investigation revealed that the treatment of HFD together with Zym exert vascular inflammatory reaction by stimulation of the TLR-2/NF-ƙB signaling pathway, which initiates the release of the proinflammatory cytokine, i.e., TNF-α, which, in turn, leads to the degradation of hepatic LDLR expression due to which impairment of lipid homeostasis takes place, which causes atherosclerosis.

Conclusion

The study provided strong evidence that HFD and Zym could accelerate the arterial inflammatory reactions through activating the TLR-2/NF-κB signaling pathway, which further accompanied the release of cytokines (TNF-α) and degradation of hepatic LDLR resulting in lipid impairment which is not reported earlier. It further suggests that it may be a better experimental model for studying the mechanism involved in atherosclerosis in humans and also for testing new potent drugs for treating patients with chronic atherosclerosis.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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