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Effect of febuxostat on biochemical parameters of high fat diet-induced hyperlipidemia in rabbits

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Abstract

Objective—To test the potential anti-oxidant and anti-inflammatory effects of febuxostat and/or stopping high-fat diet on the biochemical parameters of high fat diet-induced hyperlipidemic rabbits.

Background—Febuxostat, a highly potent xanthine oxidase inhibitor with antioxidant effect, inhibits elevated xanthine oxidase, leading to reduction of reactive oxygen species (ROS) and oxidative stress, the main causes of vascular inflammation in hyperlipidemia.

Methods— Male New Zealand rabbits were distributed in three groups: normal control (NC) fed standard chow for 12 weeks; the other two groups fed high-fat diet (HFD) with 1% cholesterol for 8 weeks, and then shifted to standard chow for 4 weeks. During the last 4 weeks; the (HFD) group received vehicle 0.5% carboxymethyl cellulose (CMC), while the treated (FX) group received febuxostat in doses (2 mg/kg/day p.o.).

Results—Febuxostat significantly lowered "bad" cholesterol. Febuxostat also displayed a potent anti-inflammatory and antioxidant activity by decreasing serum levels of lipid peroxidation index, pro-inflammatory cytokines and enhancing antioxidant-enzyme activity. Stopping hyperlipidemic diet in HFD group didn't show improvement.

Conclusions— These findings indicate antioxidant and anti-inflammatory effects of febuxostat that may be common mechanisms of the anti-hyperlipidemic effect of this drug. Stopping hyperlipidemic diet without treatment is not sufficient once injury occurred.

Keywords: Hyperlipidemia; Febuxostat; Inflammation; Oxidative stress; reactive oxygen species; TNF- α .

1. Introduction

Hyperlipidemia is considered an influential risk factor for most cardiovascular diseases (CVDs). The high-fat diet (HFD) plays a crucial role in hyperlipidemia, which increases the predominance of CVDs ([Watts and Burke 1996](#)). The link here is when plasma concentration of oxidized low-density lipoprotein (Ox-LDL) is elevated; it induces vascular inflammation, ending with vascular intimal thickening, thrombogenesis and promote atherosclerosis ([Matsumoto et al. 2014](#)).

The oxidative stress state that results from oxidation of accumulated LDL-cholesterol; greatly enhances the recruitment of the inflammatory cells and massive release of pro-inflammatory cytokines ([Münzel et al. 2010](#)). These pro-inflammatory cytokines up-regulate the expression of endothelial cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), in the endothelium of arteries to mediate and regulate the advanced and firm adherence of monocytes and macrophages to endothelial cells ([Bhatia and Moochhala 2004](#); [Seneviratne et al. 2012](#)). Thus, these pro-inflammatory cytokines and adhesion molecules are considered potent clinical biomarkers highly associated with initiation and development of vascular inflammation.

Febuxostat is a highly potent non-purine selective xanthine oxidase inhibitor (XO I) ([Khosravan et al. 2006](#)). Xanthine oxidase (XO) is an enzyme that catalyzes the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid, the process that produces superoxide radicals ($O_2^{\bullet-}$) ([Nishino et al. 2008](#)). Thus, xanthine oxidase (XO) is a crucial source of reactive oxygen species (ROS) and is involved in the development of inflammatory diseases ([Kelley et al. 2010](#)). It has been reported that increased xanthine oxidase enzyme expression and activity is linked to increased oxidative stress ([Schröder et al. 2006](#); [Bravard et al. 2011](#)). Therefore, it is

obvious that inflammatory stimuli such as hyperlipidemia may lead to elevated xanthine oxidase (XO) levels ([Schröder et al. 2006](#)), which means increased xanthine oxidase-derived ROS formation, ending with oxidative stress-induced alterations in the vascular function. On the other hand, xanthine oxidase (XO) was found to be involved in the uptake of oxidized-LDL into macrophages and consequently in the release of the inflammatory cytokines ([Kushiyama et al. 2012](#); [Nomura et al., 2013](#)).

Some recent studies have shown that xanthine oxidase inhibitors, such as febuxostat, may attenuate the increased oxidative stress, preventing the consequences of hyperlipidemia ([Kushiyama et al. 2012](#); [Omori et al. 2012](#); [Tsuda et al. 2012](#)). Therefore, the current study was designed to evaluate the impact of febuxostat on the biochemical measurements of high fat diet-induced hyperlipidemia in male rabbits. Our study confirmed that inhibition of xanthine oxidase by febuxostat alters the inflammatory response and oxidative stress induced by high fat diet.

2. Materials and methods

2.1. Experimental procedures

2.1.1. Drugs and Chemicals

Cholesterol and Carboxymethyl cellulose (CMC) were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Febuxostat, as powder was obtained from Hikma Pharmaceuticals (6th of October City, Egypt). Other chemicals used in the study were of highest analytical grade.

2.1.2. Animal feed

A) Standard diet; as pellets was purchased from Foudaco group, Al Sharqia, Egypt. Composed of; 7% Corn, 9.3% barley, 30% wheat bran, 18% Alfalfa, 19% Soybean, 0.1% anti-coccidials, 1.2% calcium carbonate, 0.3% sodium chloride, 0.001% yeast, 1858.8 kcal/kg energy value. **B) High fat diet;** of the same composition as standard diet with addition of: cholesterol (1%), sunflower oil (4%) (Wilson et al. 1990), cholic acid (0.5%), casein (20%) (Shivali et al. 2010) and egg yolk (22.5%) (Tappayuthpijarn et al. 1989), 2925.6 kcal/kg energy value (Fig. 1). After homogenous mixing, the mixture was compressed into pellets having the same shape as standard diet, and packed in special package with red label.

2.1.3. Animals:

Adult male New Zealand White (NZW) rabbits (8 weeks old; $1.81 \text{ kg} \pm 0.05 \text{ kg}$ weight each, $n = 18$) were purchased from Faculty of Agriculture; (Mansoura University, Egypt), and housed individually in steel cages under temperature-controlled conditions about $22 \pm 2^\circ \text{C}$, humidity at $60\% \pm 15\%$ and regular 12 h light/ 12 h dark cycle (light on at 8:00 am). During the 2-weeks period of adaptation, the rabbits were restricted to 100 g/day of standard rabbit chow (at

8:00 am daily) and free access to tap water at all times by automatic watering system. Animal care and handling were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH) (publication No. 85-23, revised 1996) and under the regulation of the committee of animals' experimentation of Mansoura University. After stabilization the rabbits were divided into 3 groups, each group containing 6 rabbits, receiving 100g of the assigned diet/day according to our plan described in the "Experimental design".

2.2. Experimental design:

18 Rabbits were randomly distributed in three groups (six each) as the following: **Group I:** Normal control (NC), fed with standard rabbit chow for 12 weeks; **Group II:** high fat diet (HFD) positive control group, fed with 1% cholesterol enriched chow for 8 weeks, and then shifted to standard diet chow for further 4 weeks. During those 4 weeks, each rabbit received 1ml of 0.5% w/v carboxymethyl cellulose per 2Kg body weight/day ± 0.1 ml/200 g excess body weight/day. **Group III (FX):** fed with 1% cholesterol enriched chow for 8 weeks. Then was shifted to standard diet chow, with oral administration of febuxostat with doses 2 mg/kg/day for further 4 weeks. The test drug febuxostat was dissolved in 0.5% CMC for oral administration using oral gavage via a curved feeding needle. All preparations were freshly prepared daily. Food intake was recorded daily by weighing the residual amount of food.

2.2.1. Body weight monitoring

Body weights were monitored weekly. Each rabbit was weighed at the end of each week and its growth rate curve was established. Each animal had its own profile showing its week-by-week growth chart. Those who did not show satisfying growth rate during adaptation: were rejected and replaced by others that did. Before beginning in induction process; all animals had

satisfying growth rate, with no significant difference between them. At the end of the experiment the mean body weight for each group was determined and expressed as mean $\pm$ standard error of mean (SEM).

2.2.2. Blood Sampling

Blood samples were drawn from marginal ear vein, after an overnight fast (12 hours), before (0 time), after 8 weeks, and at the end of experiment (after 12 weeks). Blood samples left to clot for 60 min., then centrifuged at 4000 rpm ($1790 \times g$) for 15 min at 4 °C to get the clear non-hemolyzed serum. Serum samples were aliquoted in 1.5 mL tubes and stored at -80 °C until the moment of analysis.

At the end of the 12 weeks, rabbits were fasted for 16 hours before euthanized, to avoid the effect of food intake on serum level of biochemical parameters. Besides, euthanize was 48 h after the last drug dose, to exclude the acute effect of the drug. Rabbits were euthanized by injection of an overdose of sodium thiopental solution (20 mg/kg body weight) ([Sarrafzadeh-Rezaei et al. 2010](#)).

2.3. Biochemical measurements

2.3.1. Assessment of serum lipid profile

Total cholesterol (TC), triglycerides (TGs) and high-density lipoprotein cholesterol (HDL-C) were determined using enzymatic colorimetric method (Spinreact, S.A. Ctra. Santa Coloma, Spain) according to the method described by ([Allain et al. 1974](#); [Bucolo and David 1973](#)) and ([Grove 1979](#)) respectively. Low-density lipoprotein cholesterol (LDL-C) was determined using enzymatic colorimetric method (Biodiagnostics, Giza, Egypt) as described by

(Wieland and Seidel 1983). Very low-density lipoprotein (VLDL) concentration was estimated using Friedewald formula (Friedewald et al. 1972).

2.3.2. Assessment of serum ALT/AST

Serum alanine transaminase (ALT) and aspartate transaminase (AST) activities were assayed by IFCC method (Kinetic-UV) without pyridoxal phosphate (Bergmeyer et al. 1986) at 340 nm using ELITech kit (ELITech Clinical Systems SAS - Zone industrielle 61500 SEES, France) and expressed as (IU/L).

2.4. Assessment of oxidative stress biomarkers

2.4.1. Determination of lipid peroxidation

Lipid peroxidation, a biomarker for membrane damage, was determined by measuring malondialdehyde (MDA), as thiobarbituric acid reactive substances (TBARS) in the sample, depending on the thiobarbituric acid (TBA) method as described by (Satoh 1978; Ohkawa et al. 1979) and (Erdinçler et al. 1997), using Biodiagnostic kit No. MD 25 29 (Biodiagnostic Co., Giza, Egypt). Briefly, thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95 °C for 30 min to form thiobarbituric acid reactive product. The absorbance of the resultant pink product can be measured at 534 nm and expressed as (nmol/ml) malondialdehyde (MDA) formed.

2.4.2. Determination of Antioxidant Enzymes activities

2.4.2.1. Determination of superoxide dismutase activity (SOD)

SOD activity was determined in serum using colorimetric method as described by (Nishikimi et al. 1972) and (Sun et al. 1988) using Biodiagnostic kit No. SD 25 21 (Biodiagnostic Co., Giza, Egypt). This assay based on the ability of SOD enzyme to inhibit the

phenazine methosulphate-mediated reduction of nitro blue tetrazolium dye. One unit of SOD enzyme is defined as the amount of the enzyme required to cause 50% inhibition of the nitro-blue tetrazolium (NBT) photo-reduction rate, in the form of change in sample absorbance (ΔA) within time (Heikkila et al. 1976) and (Durak et al. 1993). SOD activity was expressed as units/ml serum.

Briefly, 0.1 ml sample was mixed with 1 ml phosphate buffer (pH 8.5), 0.1 ml nitro blue tetrazolium (NBT) and 0.1 ml NADH. The reaction was initiated by adding 0.1 ml phenazine methosulphate (PMS), and then absorbance was read at 560 nm for 5 min. The change in absorbance at 560 nm over 5 min was measured.

2.4.2.2. Determination of glutathione reduced (GSH)

Levels of GSH were detected spectrophotometrically in serum as described by (Ellman 1959) and (Beutler et al. 1963) using Biodiagnostic kit No. GR 25 11 (Biodiagnostic Co., Giza, Egypt). The principle is based on the reduction of Ellman's reagent DTNB [5,5'-Dithiobis(2-nitrobenzoic acid)] by SH groups (in glutathione) to form nitro-mercaptobenzoic acid, has an intense yellow color. The absorbance measured at 405 nm and the concentration expressed as (mg/ml).

2.4.2.3. Determination of catalase activity (CAT)

CAT activity was determined in serum using Biodiagnostic kit No. CA 25 17 (Biodiagnostic Co., Giza, Egypt) based on the spectrophotometric method of (Aebi 1984). The method is based on the decomposition of H_2O_2 by catalase. Briefly, catalase (from the sample) reacts with a known concentration of H_2O_2 . The reaction is stopped after exactly 1 min. with catalase inhibitor. The remaining amount of H_2O_2 in the reaction mixture is then determined by

the oxidative coupling reaction with 3,5-Dichloro -2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (4-aminoantipyrine, AAP) in the presence of horseradish peroxidase (HRP) to form a quinone-imine chromophoric dye with a color intensity inversely proportional to the amount of catalase in the sample. The resulting quinone-imine dye was measured at 510 nm, the enzyme activity was expressed as units/L serum.

2.5. Determination of serum nitric oxide (NO) level

Nitric oxide formation was measured in serum by assaying nitrite level (NO_2^-), the last metabolite of nitric oxide (NO), using spectrophotometric method of (Montgomery and Dymock 1961) that involves the Griess reagent (1% sulphanilamide, 0.1% N-1-naphthyl ethylene diamine dihydrochloride NEDA, and 2% phosphoric acid) (Moshage et al. 1995), using Biodiagnostic kit No. NO 25 33 (Biodiagnostic Co., Giza, Egypt). Briefly, (NO) was determined in acid medium and in the presence of nitrite; the formed diazonium ion is coupled with N-(1- naphthyl) ethylenediamine to form a chromophoric azo compound. This azo dye has a deep reddish purple color, which can be measured at 540 nm. The total NO_2^- levels were expressed as ($\mu\text{mol/l}$).

2.6. Determination of Pro-inflammatory Cytokines

2.6.1. Serum pro-inflammatory Cytokines

Serum interleukin-1 beta ($\text{IL-1}\beta$) and tumor necrosis factor- α ($\text{TNF-}\alpha$) concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit (CUSABIO® Biotech; Wuhan, Hubei Province, China) specific for rabbit. Manufacturer's instructions were followed. Absorbance was determined using a standard automated microwell plate ELISA reader (Molecular Devices, Orleans, CA, USA) at 450 nm, and concentrations were computed by the standard curve run on each assay plate. The results were expressed as (pg/ml).

2.6.2. Tissue pro-inflammatory Cytokines

Tissue homogenate preparation

100 mg aortic tissue was rinsed with $1\times$ cold PBS, homogenized in 1 ml of $1\times$ cold PBS using Glass Potter-Elvehjem tissue homogenizer and stored overnight at $-20\text{ }^{\circ}\text{C}$. After performing two freeze-thaw cycles (to break the cell membranes): the homogenates were centrifuged for 5 minutes at $5000\times g$, $4\text{ }^{\circ}\text{C}$. The supernatant was collected and assayed immediately using specific ELISA kit for tissue homogenate (CUSABIO® Biotech; Wuhan, Hubei Province, China). Tissue aliquots were homogenized as described in (Folch et al. 1957). The pro-inflammatory cytokines (IL- 1β and TNF- α) were expressed as (pg/mg. aortic tissue).

2.7. Determination of serum Pro-atherogenic molecules (CAMs)

The collected serum samples were assayed by ELISA kit (CUSABIO® Biotech; Wuhan, Hubei Province, China) to detect the level of Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1). Both expressed as (ng/ml).

2.8. Statistical method

Data analysis was carried out using GraphPad Prism 6 (GraphPad Software, Inc.). All data are expressed as means ($\bar{x}$) $\pm$ standard error of mean (SEM). Statistical analysis was performed by analysis of variance (One-Way ANOVA) followed by Tukey-Kramer post hoc test with correction for multiple comparisons, to identify the statistical differences among groups. Changes in body weight in various groups during weeks were compared by two-way ANOVA followed by Tukey's multiple comparisons test. The difference was defined to be statistically significant when a P -value was less than 0.05.

3. Results

3.1. Effect of febuxostat on body weight

The growth rate of the HFD group was 19% significantly less than normal control (NC) rate ($P < 0.05$). The growth rate of normal control (NC) through the 8 weeks has increased 1.4 fold (38%), while the other groups did not exceed 1.2 fold (17%) increase in body weight. The (HFD) group showed a significant decrease ($P < 0.001$) in body weight through the last 4 weeks by about 1 fold (0.6%), compared to their main weight recorded at the end of the 8 weeks of induction of hyperlipidemia. Administration of febuxostat resulted in significant regain of growth rate by 1.2 fold (18%) with doses 2 mg/kg/day (Fig. 2).

Food intake by all groups was almost the same (≈ 100 g/day) up to week 4, when both groups fed on HFD started to consume less food ($\approx 75.4 \pm 6.9$ g/day) and became more idle showing a notable decrease in movement and activity. Febuxostat treated group showed improvement in daily food intake ($\approx 87.25 \pm 6.7$ g /day) and activity, compared to untreated group (Fig. 3).

Note; rabbits treated with febuxostat started with slightly lower body weight compared to NC and HFD ($\approx 1.8 \pm 0.19$, 1.9 ± 0.17 and 1.9 ± 0.18 Kg, respectively). This difference was statistically non-significant and it was purely a coincidence, because the distribution into the three groups happened randomly after the 8 weeks of induction of hyperlipidemia. Therefore, this did not influence the outcome of the study.

3.2. Biochemical measurements

3.2.1. Serum lipid profile

HFD induced a significant ($P < 0.0001$, $n = 6$) increase in TC, TGs, LDL-C and VLDL-C during the 8 weeks of induction of hyperlipidemia by 1200, 300, 6000 and 300%, respectively, compared to NC group. The lipid profile was further significantly ($P < 0.01$) increased in (HFD) group during the last 4 weeks by 5, 49, 3 and 49% for TC, TGs, LDL-C and VLDL-C, respectively. Treatment with febuxostat significantly ($P < 0.001$, $n = 6$) decreased the HFD-induced levels of TC, TGs, LDL-C and VLDL-C in 4 weeks by 56, 62, 59 and 62%, respectively.

The protective HDL-C was significantly ($P < 0.0001$, $n = 6$) decreased by HFD during the 8 weeks by 37%. It was further significantly ($P < 0.0001$) decreased in (HFD) group during the last 4 weeks by 36%. It was significantly ($P < 0.0001$) enhanced with febuxostat treatment by 34% (Fig. 4).

3.2.2. Liver function

Through 8 weeks; the HFD significantly ($P < 0.0001$, $n = 6$) increased the sGPT (ALT) and sGOT (AST) by 1000 and 1400%, respectively compared to normal level. During the four-week treatment, the treated (FX) group showed liver function improvement by lowering ($P < 0.0001$, $n = 6$) the sGPT (ALT) and sGOT (AST) by 4 fold (77% and 75%, respectively). On the other hand, the untreated group (HFD) showed a significant continued rise ($P < 0.001$, $n = 6$) in the sGPT (ALT) and sGOT (AST) by about 1 fold (15% and 10%, respectively) compared to the level recorded at the end of the 8 weeks of induction of hyperlipidemia (Fig. 5).

3.3. Oxidative stress biomarkers

Through 8 weeks; the HFD significantly ($P < 0.0001$, $n = 6$) increased serum MDA and serum CAT by 990 and 88%, respectively. While there was a significant ($P < 0.0001$) reduction

in serum SOD and GSH levels by 26 and 43%, respectively. During the following 4 weeks; the (HFD) group showed a significant ($P < 0.001$, $P < 0.0001$) continued rise in serum MDA and CAT by 25 and 26% respectively, and a significant ($P < 0.0001$) continued reduction in serum SOD and GSH levels by 52 and 51% respectively. Febuxostat treatment significantly ($P < 0.0001$) reduced MDA levels by 67% and the CAT levels by 42%. In the meanwhile, it significantly increased SOD levels ($P < 0.0001$) by 124%, and GSH levels ($P < 0.0001$) by 52% (Fig. 6).

3.4. Serum NOx

HFD feeding for 8 weeks significantly ($P < 0.0001$) increased serum nitrite (NO_2^-) by 466% compared to NC group. The (HFD) group had further significant ($P < 0.0001$) increase by 4.5% through 4 weeks. Febuxostat treatment significantly ($P < 0.0001$) decreased NOx by 69% (Fig. 6).

3.5. Pro-inflammatory Cytokines (IL-1 β and TNF- α):

3.5.1. Serum pro-inflammatory cytokines

Through the 8 weeks, the HFD feeding significantly ($P < 0.0001$) increased serum IL-1 β and TNF- α by 77 and 100%, respectively. The (HFD) group showed a significant ($P < 0.0001$) continued increase by 36 and 17% respectively, through 4 weeks. Febuxostat significantly ($P < 0.0001$) decreased the IL-1 β and TNF- α levels through 4 weeks by 25 and 43%, respectively (Fig. 7).

3.5.2. Tissue pro-inflammatory cytokines

Through 8 weeks of induction of hyperlipidemia by high-fat diet: the aortic tissue IL-1 β and TNF- α in all groups fed on high fat diet was significantly elevated ($P < 0.0001$) about 100%

and 70%, respectively compared to normal level. Treatment with FX significantly ($P < 0.0001$) decreased the IL-1 β and TNF- α levels by 41% and 47%, respectively. The untreated (HFD) group showed a significant continued rise ($P < 0.0001$) in the aortic tissue IL-1 β and TNF- α levels by about 47% and 22%, respectively, compared to the level recorded at the end of the 8 weeks of induction of hyperlipidemia (Fig. 8).

3.6. Serum pro-atherogenic cell adhesion molecules

The HFD significantly ($P < 0.0001$, $n = 6$) increased serum ICAM-1 and VCAM-1 during 8 weeks by 225 and 200% respectively, compared with NC group. The (HFD) group showed a significant ($P < 0.0001$) continued increase by 53 and 43% respectively, through 4 weeks. Febuxostat significantly ($P < 0.0001$) decreased ICAM-1 and VCAM-1 levels through 4 weeks by 61 and 63%, respectively (Fig. 7).

4. Discussion

Our study was primarily designed to evaluate effect of febuxostat, a xanthine oxidase inhibitor, on oxidative stress and vascular inflammation resulted from high fat diet in rabbits. The fundamental findings revealed the positive effect of febuxostat as an effective inhibitor of progression of high fat diet-induced hyperlipidemia, by attenuating oxidative stress and vascular inflammation parameters.

4.1. Effect of Febuxostat on growth rate of hypercholesterolemic rabbits

During the first 5 weeks, both rabbits feeding on high fat diet (HFD) and standard diet did not seem to be different with respect to their feeding habits and growth rate. On the 6th week, the HFD groups started to show a decrease in their appetite (consumed less than 100 g/day) and a significant decrease in their growth rate after, compared with normal control. These results are

consistent with previous studies (Brattsand 1975; Ismail et al. 2014; Lee et al. 2017) etc. We believe this is due to liver injury, induced by high-fat diet that resulted in lipid peroxidation and decrease cellular antioxidants. The lipid peroxidation results in accumulation of by-products (probably aldehydes) in the liver, causing liver injury. Besides, oxidative stress may either cause direct cell toxicity or up-regulate the expression of pro-inflammatory cytokines, causing greater oxidative load to the liver. This leads to hepatic dysfunction associated with the progression from steatosis to cirrhosis. (Polavarapu et al. 1998; Barbuio et al. 2007; McArdle et al. 2013). To make sure of that, we have made further study on liver parameters, including oxidative stress, pro-inflammatory cytokines and liver histology (liver histology and analysis of pro-inflammatory cytokines in liver are shown in the supplementaries for revision). The positive oxidative stress biomarkers, elevated pro-inflammatory cytokines, swollen hepatic cells, inflammation, biliary stasis, hydropic degeneration and different patterns of steatosis, which we observed are considered a concrete evidence for our view and might give a mechanistic explanation of the observed effect of febuxostat. Administration of Febuxostat revealed a positive effect on their appetite and growth rate in general.

4.2. Biochemical measurements

4.2.1. Febuxostat effects on serum lipid profile

Rabbits fed on HFD showed abnormal serum lipid levels, with a significant decrease in HDL-C and significant increase in TC, TGs, LDL-C and VLDL, indicating that these groups had a higher risk on the liver and cardiovascular system (Carmena et al. 2004). These results are compatible with other previous studies (Bolayirli et al. 2007; Norris et al. 2015; Zhang et al. 2017). The significant difference between before and after stopping HFD indicates that stopping the HFD without treatment does not lead to any improvement. This is likely due to vascular

inflammation, which is an immune-related issue, so it is a cascade process. Once the cause of injury exists, the disease will progress (Hansson and Hermansson 2011; Sage and Mallat 2017). Administration of febuxostat led to a significant improvement; reducing risk factors TC, TGs, LDL, VLDL and increasing the protective HDL, suggesting antioxidant and anti-inflammatory properties of febuxostat.

4.3. Febuxostat anti-oxidant effect protects against HFD-induced oxidative stress

Hyperlipidemic groups showed a significant increase in lipid peroxidation (MDA), reactive nitrogen species (NO_2^-) and (CAT enzyme) levels along with a significant reduction in protective SOD and GSH activity. These results are consistent with (Öhrvall et al. 1993; Koneru et al. 1995; Decsi et al. 1997; Wu et al. 2016).

The increased levels of MDA indicates lipid peroxidation, which is a component of high-fat diet-induced oxidative stress (Olusi 2002; Amirkhizi et al. 2010). Treatment with febuxostat significantly reduced the HFD-induced lipid peroxidation, which suggest an anti-peroxidative effect of febuxostat.

Reduction in glutathione reduced (GSH) and superoxide dismutase (SOD) in HFD-fed groups is consistent with previous studies (Ming et al. 2009; Wu et al. 2016). This reduction may result from its consumption during the process of scavenging of the reactive oxygen species (ROS) associated with oxidative stress and hypercholesterolemia (Prasad 2008). Decrease in GSH levels would result in elevation of H_2O_2 which in turn would deplete SOD leading to a decrease in SOD activity (Mantha et al. 1993). Treatment with febuxostat showed remarkable antioxidant effect via significantly increasing GSH and SOD levels compared with HFD group.

In our study, the CAT levels have been elevated in the HFD-fed groups. This is in agreement with (Mantha et al. 1993; Noeman et al. 2011; Rindler et al. 2013). This could be explained as a “cholesterol-induced rise” (Mantha et al. 1993). When H_2O_2 is increased (induced by HFD), it is countered by the induction of CAT by upregulation mechanism (Rindler et al. 2013). Moreover, the CAT is not the primary antioxidant defense enzyme. SOD and GSH are the primary involved antioxidant defense enzymes (Boccio et al. 1990). GSH has been reported to be more efficient than CAT in scavenging free-radicals and preventing the oxidative stress consequences (Suttorp et al. 1986; Raes et al. 1987; Michiels and Remacle 1988). The vascular oxidant burden (resulted from hypercholesterolemia) favors GSH depletion because of enhanced oxidation of the tripeptide or its consumption by electrophilic compounds like lipo-peroxidation aldehydes (Wolin 2000). This is consistent with our results that showed that GSH has been influenced 49.3% more than SOD, suggesting that GSH is the first antioxidant defense enzyme involved against free radicals, then comes the SOD, and finally the CAT. This difference makes CAT ideal for consuming toxic levels of H_2O_2 while allowing the GSH and SOD to manipulate the lower sub-toxic amounts of H_2O_2 . Besides, the decrease occurred in the GSH would lower the body capacity to detoxify hydrogen peroxide H_2O_2 and possibly led to an elevation in the CAT activity (Mantha et al. 1993).

4.4. Effect of febuxostat on serum NOx

The HFD-fed groups showed a significant induced (NO_2^-) production (which is an indicator of NO). This is consistent with previous literatures (Shin et al. 2013; Stanimirovic et al. 2016). The over-accumulation of lipids in hepatic cells alters the liver ability to metabolize lipids, which causes oxidative stress (Reddy and Rao 2006). This leads to activation of inflammatory responses and increase expression of inducible nitric oxide synthase (iNOS)

resulting in elevation of NO production ([Stanimirovic et al. 2015](#)). Provoking inflammatory response upregulates inflammatory cytokines, and leads to release of NO from macrophages as a defense response ([Reddy and Rao 2006](#)). This continuous release of NO leads to inflammatory diseases ([Shin et al. 2013](#)). Treatment with febuxostat significantly decreased NO production, reporting potential anti-inflammatory effect of febuxostat in treating chronic inflammatory diseases.

4.5. Febuxostat anti-inflammatory and anti-atherogenic properties

Animals fed with HFD showed significant increase in the circulating pro-inflammatory cytokines (IL-1 β and TNF- α) in serum. This is in line with ([Zhang et al. 2007](#); [Kim et al. 2014](#)). The pro-inflammatory cytokines are released in high levels as a response to inflammatory stimulation by the reactive oxygen species (ROS) associated with oxidative stress. These pro-inflammatory cytokines in return regulate the continued and advanced accumulation and migration of leukocytes, thus induce further inflammation ([Li et al. 2012](#)).

Serum pro-atherogenic adhesion molecules ICAM-1 and VCAM-1 were significantly elevated in HFD groups. Our results are consistent with previous reports ([Iiyama et al. 1999](#); [Fang et al. 2007](#)). ICAM-1 and VCAM-1 are cytokine-inducible molecules ([Nakashima et al. 1998](#)). Pro-inflammatory cytokines activate the expression genes of endothelial cell adhesion molecules (ICAM-1 and VCAM-1) ([Collins et al. 1995](#)). These molecules promote the adhesion of monocytes and then the proliferation and migration to endothelium, promoting the development of atherosclerotic plaques.

The (HFD) group showed increased production of pro-inflammatory cytokines and pro-atherogenic adhesion molecules during the last 4 weeks. The underlying cause may be the continuous release of the pro-inflammatory cytokines as a response to the inflammation and

injury occurred in the endothelial cells. Therefore, treatment must include disruption of the pro-inflammatory cytokines release, to achieve endothelial recovery and stop development of inflammation (Krasinski et al. 2001; Ohta et al. 2005), not just stopping HFD once injury occurred.

Febuxostat significantly reduced the pro-inflammatory cytokines and decreased the expression of the pro-atherogenic adhesion molecules. It is therefore possible that febuxostat has an anti-inflammatory and anti-atherogenic effect. Supporting this notion, febuxostat has been reported to attenuate the expression of pro-inflammatory cytokine genes in aortas of atherosclerotic mice (Nomura et al. 2014) and in ischemia–reperfusion injured rat kidneys (Tsuda et al. 2012). It has recently been shown that febuxostat reduces HFD-induced liver inflammation and fibrosis in mice (Nakatsu et al. 2015). Febuxostat-mediated anti-inflammatory effects are potentially related to its ability to limit oxidative stress, so reduce the production of inflammatory cytokines and down-regulate the pro-atherogenic adhesion molecules.

Conclusion

Our study reveals an anti-hyperlipidemic effect of febuxostat, a highly potent xanthine oxidase inhibitor, due to its antioxidant and anti-inflammatory capacity. Changing lifestyle (by stopping high-fat diet) without treatment that stops internal inflammation; would not stop the progression of atherosclerosis.

Conflict of interest:

Authors declare that they have no conflict of interest with any institution. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Figure Captions List:

Figure 1: Nutrition facts. Ingredients and nutrient content of each meal (100 g) provided for each rabbit daily, calculated based on composition mentioned on the chow label.

Figure 2: Body weight monitoring. Body weight progress during induction of hyperlipidemia (weeks 1-8) and during the 4-week treatment (weeks 9-12). NC: Normal control, HFD: high-fat diet for 8 weeks followed by standard chow for 4 weeks with vehicle 0.5% CMC, FX: high-fat diet for 8 weeks followed by standard chow for 4 weeks with febuxostat dose 2 mg/kg/day.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to NC group.

Figure 3: Food intake (g/day) during 12 weeks. Each animal received 100g diet/day. Food intake was recorded daily by weighing the residual amount of food. NC: Normal control, HFD: high-fat diet for 8 weeks followed by standard chow for 4 weeks with vehicle 0.5% CMC, FX: high-fat diet for 8 weeks followed by standard chow for 4 weeks with febuxostat dose 2 mg/kg/day.

Figure 4: Effect of febuxostat treatment (2 mg/kg/day p.o.) for 4 weeks on serum total cholesterol (TC, panel A), triglycerides (TGs, panel B), low-density lipoprotein-cholesterol (LDL-C, panel C), very low-density lipoprotein (VLDL, panel D), high-density lipoprotein-cholesterol (HDL-C, panel E) in high fat diet fed (8 weeks) hyperlipidemic male rabbits.

Values represent means $\pm$ SEM ($n = 6$).

t_1 : after diet-induced hypercholesterolemia (before treatment), t_2 : after four-week treatment. NC: Normal control, HFD: high-fat diet for 8 weeks followed by standard chow for 4 weeks with vehicle 0.5% CMC, FX: high-fat diet for 8 weeks followed by standard chow for 4 weeks with febuxostat dose 2 mg/kg/day.

$\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.0001$ as compared to t_1 HFD group (before treatment).

$*** P < 0.001$, $**** P < 0.0001$ as compared to t_1 FX group (before treatment).

$#### P < 0.0001$ as compared to HFD group.

Figure 5: Effect of febuxostat treatment (2 mg/kg/day p.o.) for 4 weeks on serum glutamic-pyruvic transaminase (ALT; IU/L, panel A) and serum glutamic-oxaloacetic transaminase (AST; IU/L, panel B) in high fat diet fed (8 weeks) hyperlipidemic male rabbits. Values represent means $\pm$ SEM ($n = 6$).

$\dagger\dagger\dagger P < 0.001$ as compared to t_1 HFD group (before treatment).

$**** P < 0.0001$ as compared to t_1 FX group (before treatment).

$#### P < 0.0001$ as compared to HFD group.

Figure 6: Effect of febuxostat treatment (2 mg/kg/day p.o.) for 4 weeks on serum malondialdehyde (MDA; nmol/ml, panel A), serum catalase (CAT; U/ml, panel B), serum superoxide dismutase (SOD; U/ml, panel C), serum glutathione reduced (GSH; mg/ml,

panel D), serum nitrite (NO_2^- ; $\mu\text{mol/L}$, panel E) in high fat diet fed (8 weeks) hyperlipidemic male rabbits. Values represent means $\pm$ SEM. ($n = 6$).

††† $P < 0.001$, †††† $P < 0.0001$ as compared to t_1 HFD group (before treatment).

**** $P < 0.0001$ as compared to t_1 FX group (before treatment).

$P < 0.0001$ as compared to HFD group.

Figure 7: Effect of febuxostat treatment (2 mg/kg/day p.o.) for 4 weeks, on serum IL-1 β (pg/ml, panel A), TNF- α (pg/ml; panel B), ICAM-1 (ng/ml; panel C), VCAM-1 (ng/ml; panel D) in high fat diet fed (8 weeks) hyperlipidemic male rabbits. Values represent means $\pm$ SEM. ($n = 6$).

†††† $P < 0.0001$ as compared to t_1 HFD group (before treatment).

**** $P < 0.0001$ as compared to t_1 FX group (before treatment).

$P < 0.0001$ as compared to HFD group.

Figure 8: Effect of febuxostat treatment (2 mg/kg/day p.o.) for 4 weeks, on aortic tissue pro-inflammatory cytokines: IL-1 β (pg/mg.Aorta, panel A) and TNF- α (pg/mg.Aorta; panel B) in high fat diet fed (8 weeks) hyperlipidemic male rabbits. Values represent means $\pm$ SEM. ($n = 6$).

†††† $P < 0.0001$ as compared to HFD 8 group (before treatment).

Figure 1:

Nutrition Facts		
	Standard chow	High fat
Amount Per Serving (100g)		
Calories	185.9 kcal	292.6 kcal
Total Fat	5.3 g	15.3 g
Saturated Fat	0.8 g	3.3 g
Monounsaturated Fat	1.1 g	6.1 g
Polyunsaturated Fat	2.9 g	5.0 g
Cholesterol	0.0 g	1.0 g
Sodium	0.3 g	0.3 g
Total Carbohydrate	29.4 g	30.3 g
Dietary Fiber	10.8 g	10.8 g
Sugars	1.8 g	1.9 g
Protein	12.05 g	15.6 g
<div> <div>Fats 10%</div> <div>Carb 78%</div> <div>Protn 12%</div> </div> <div> <div>Fats 36%</div> <div>Carb 49%</div> <div>Protn 15%</div> </div>		

Figure 2:

Figure 3:

(A) serum TC (mg/dl)

Group	t ₁	t ₂
NC	~50	~50
HFD	~700	~750 ^{††}
FX	~720	~300 ^{****}

(B) serum TGs (mg/dl)

Group	t ₁	t ₂
NC	~60	~60
HFD	~260	~390 ^{†††}
FX	~260	~90 ^{****}

(C) serum LDL (mg/dl)

Group	t ₁	t ₂
NC	~10	~10
HFD	~640	~660 ^{††}
FX	~660	~260 ^{****}

(D) serum VLDL (mg/dl)

Group	t ₁	t ₂
NC	~8	~8
HFD	~38	~56 ^{†††}
FX	~38	~14 ^{****}

(E) serum HDL (mg/dl)

Group	t ₁	t ₂
NC	~31	~31
HFD	~21	~13 ^{†††}
FX	~19	~25 ^{***}

Figure 5:

Figure 6:

Figure 7:

Figure 8: