

Allopurinol Ameliorates High Fructose Diet-Induced Metabolic Syndrome via up-regulation of Adiponectin Receptors and Heme Oxygenase-1 Expressions in Rats

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ABSTRACT

Objective: to explore allopurinol action on the metabolic syndrome (MS) components induced by high fructose diet (HFD). Material & methods: Twenty-one rats were classified randomly into 2 groups; group A (7 rats; normal control) and group B (14 rats; received a high fructose diet (HFD). Meanwhile, group B is further classified into 2 subgroups: B1 received no treatment and B2 in which rats received allopurinol (4mg/kg/d for 4 weeks). Results: Allopurinol significantly decreased body weight (BW), normalized kidneys and heart weight, blood pressure (BP) and insulin level with normalized both of fasting glucose level and insulin resistance (IR). Furthermore, triglycerides (TGs) and low-density lipoprotein cholesterol (LDL-c) were significantly decreased with normalized high-density lipoprotein cholesterol (HDL-c), total cholesterol, creatinine, blood urea nitrogen (BUN), and serum uric acid (SUA) levels. Surprisingly, allopurinol significantly up regulate adiponectin receptor one and two (adipo R1/R2) and heme oxygenase-1 (HO-1) in heart, liver and kidneys pancreas associated with up regulation of endothelial nitric oxide synthase (eNOS) expression in liver, kidneys, heart only associating with amelioration of the fibrotic changes in different tissue studied. Moreover, it normalized IR, pancreatic AdipoR2, and HO-1 expression. Conclusion: allopurinol could be considered an ideal agent for an amelioration of MS components possibly through up regulation of adipo R1/R2, HO-1 and eNOS in different tissues; however more experimental and clinical studies are needed to weight the expected allopurinol benefit against its long term use related side effects.


Keywords: Allopurinol, metabolic syndrome, Adiponectin R1/R2, Heme oxygenase-1.

INTRODUCTION

The metabolic syndrome (MS) is a cluster of dyslipidemia, elevated blood pressure, IR and

abdominal obesity ¹. There is a strong relationship between MS components and hyperuricemia ². Allopurinol is a drug that suppresses xanthine



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oxidase activity resulting in decreased uric acid and xanthine oxidase-mediated free radical formation ³.

Adiponectin is a hormone produced by both white and brown adipose tissues. It modulates a series of metabolic processes such as blood glucose regulation and fatty acids oxidations ⁴. It is considered as a key protein that regulates insulin activity and decreases tissue inflammatory process. Meanwhile, it reduces systemic insulin resistance (IR) and overall predicts cardiovascular disease ⁵. Adiponectin action is mediated mainly through adiponectin receptor one and two (AdipoR1/R2).

HO-1 is an enzyme that is if induced it degrades heme to ferrous iron, biliverdin and carbon monoxide (CO) in an equal molar amount ⁶. Following stress, The HO-1 is induced resulting in an important role in the protection of cell against injury ⁷.

Nitric oxide (NO) generated by endothelial cells from L-arginine is present in three different synthases forms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS), among which NO generated by eNOS plays an important protective role through preserving hepatic blood flow, prevention of leukocyte adhesion, decrease platelet aggregation, and free radicals eradication ⁸.

The current study was to evaluate the action of allopurinol on the MS produced by HFD stressing on the possible impact of AdipoR1/R2, HO-1 and eNOS in different tissues.

MATERIAL AND METHODS

Drugs and chemicals

Allopurinol: Zyloric tablets, 100mg (GSK, *Egypt*). All tablets were crushed in distilled water and allowed for complete dissolution.

Fructose: Uni-fructose powder purchased from Universal Industrial Pharmaceutical Co. (UNIPHARMA) - *Egypt*.

Animals

Twenty-one male Sprague Dawley rats were included (140-155gm weight) and handled following local ethical committee guidelines that agree with that of Helenski, the animal then put in metal cages with 12 hours dark and 12 hours light

every day, allowed one week free before starting the experiment for acclimatization. The protocol of this work was accepted by Beni Suef medical college ethical committee, *Egypt*.

A standard diet composed of 9.2% fat, 52.8% carbohydrates, and 38% protein were used to fed rats, with water free access, classified into two groups.

Group A (normal control group): Seven rats were received a standard chow diet and vehicle (water, 1 ml/kg).

Group B: Fourteen rats received HFD consisting of fructose and normal chow diet in a proportion of 6:4 for 8 weeks (till the end of the experiment) ⁹. Group B was furtherly divided into two groups, seven rats in each one:

Group B1: received HFD only and distilled water 1 ml/kg orally.

Group B2: received allopurinol (5 mg/kg) orally.

Allopurinol and distilled water were given daily starting from the 4th weeks and lasting for four weeks after.

By the end of the 8th week, BW, and blood pressure (mean (MBP), systolic (SBP) and diastolic (DBP)) of all rat were recorded digitally by a non-invasive blood pressure analysis using a tail-cuff sphygmomanometer method (Biosynthesis Biotechnology TSE-system, Homburg, Germany). Blood pressure was measured thrice and the mean of this measurement was obtained, then all rats were sacrificed, the weight of kidneys, liver, heart, spleen and pancreas of each rat were recorded prior to PCR and histopathology.

Biochemical examination

Under inhalation anesthesia, a 5ml blood sample was drawn after at least 6-8 fasting hours (fasted overnight), centrifuged at 804.96 g for 20 min. and the serum was stored at "20 °C in clean vials. The levels of insulin, glucose (Monobind Inc., USA), serum uric acid (SUA), low density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c), triglycerides (TGs), creatinine, blood urea nitrogen (BUN) (Biodiagnostic, *Egypt*) were estimated using specific kits following the guidelines of its manufacturers.

Calculation of insulin resistance Homeostasis (HOMA-IR; IR index)

homeostasis of IR (HOMA-IR) was calculated according to Matthews *et al.* 1985 using the following equation: $HOMA-IR = \text{fasting glucose (mg/dL)} \times \text{fasting insulin } (\mu\text{U/L})/405^{10}$.

Adiponectin receptor one and two (adipo R1/R2) (ADIPO R1/2), heme oxygenase-1 (HO-1) and endothelial nitric oxide synthase (eNOS) expression assay in pancreatic, liver, kidney and heart tissues:

RNA extraction

Total RNA was extracted from pancreas, liver, kidneys and heart tissues of all studied groups according to the manufacturer instructions, using QIamp RNA kit provided by Qiagen Inc, USA. To assess purification, the RNA extracted was quantitated by spectrophotometry at 260 nm.

Real time PCR (RT-PCR)

RT-PCR experiments were done for detecting HO-1, eNOS, adiponectin receptor1 (AdipR1) and adiponectin receptor2 (AdipR2) using the corresponding primer sequences as shown in (Table 1). cDNA was synthesized from 1000ng of the extracted RNA with 1 μL (20 pmol) antisense primer and 0.8 mL superscript avian myeloblastosis virus (AMV) reverse transcriptase for around 60 minutes at 37°C. Fast Start Universal Syber Green Master mix (Thermo Scientific) was used in Applied Biosystem Instrument with software version 2.1 (StepOne™,

USA). Briefly, 20 μL was prepared, of which 5 μL of synthesized cDNA was included for PCR, a final concentration of 0.5mM of each forward and reverse primer and 12.5 μL of Syber Green Master mix was used. Cycling conditions involve enzyme activation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 60 seconds. Targets that are amplified successfully are expressed in Ct values or the cycle at which the target amplicon is initially detected above background fluorescence levels as determined by the instrument software. Each RT-PCR sample was performed at least triplicate. The PCR data sheet includes Ct values of assessed genes and the house keeping gene (β -actin). The relative quantization of target genes was normalized by the

Histopathological examination

Light microscopy

At room temperature; samples from liver, heart, and kidneys were collected and fixed in 10% buffered formalin for 72 hours. Trimming of samples was done for a size of one cubic centimeter. Routine histological procedures (dehydration, clearing and paraffin embedding) were carried out. 4-6 μm thickness sections were stained with routine hematoxylin and eosin and Masson's trichrome stains for collagen identification¹¹.

Morphometry

Morphometrical analysis of stained Masson's trichrome sections was carried for liver, heart and kidney specimens. The main parameter

Table 1: Oligonucleotide primers sequences used for qRT-PCR

Gene Symbol	Primer Sequence	Gene Bank Accession Number
HO-1	sense 52 -CTGTTGGCGACCGTGGCAGT-32 antisense 52 -CTGGGCTCAGAACAGCCGCC-32	NM_012580.2
eNOS	sense 52 -GAAGGCGTTTGACCCCCGGG-32 antisense 52 -CCACCGCTCGAGCAAAGGCA-32	NM_021838.2
AdipoR1	sense 52 -GAGGAGAAGGGTAAGCG-32 antisense 52 -CAGAGGAAGGGTCAGCAC-32	NM_207587.1
AdipoR2	sense 52 -CTCCTACAAGCCCATCATG-32 antisense 52 -CAATCTGGCACCACATCA-32	XM_006237186.3
Actin	sense 52 -ACCAGTTCGCCATGGATGACGAT-32 antisense52 -CCTAGGGCGGCCACGATGG-32	NM_031144.2

for morphometrical analysis was area percentage measurements of the collagen contents. The measurements were carried out in thirty non-overlapping of each rat at x100 (for liver specimens) and x200 (for heart and kidney specimens) magnification.

Statistical analysis

Using SPSS 16 for windows (SanDiego, CA, USA); data were collected, tabulated, analyzed using one-way analysis of variance (ANOVA) followed by the LSD (Least Significant Difference) as a post-hoc test to compare the means from different groups. The results expressed as Mean \pm standard deviation (SD), *P* value is significant if <0.05 .

RESULTS

Effect on body and organ weights

Administration of HFD for eight weeks in group B1 resulted in a significant increase in BW, heart, kidney, pancreas and insignificant increase in spleen and liver weight compared to normal control group (A). Administration of allopurinol in group B2 significantly ameliorated the increased BW, kidney, heart and spleen weight compared to HFD group. Moreover, allopurinol normalized the kidney and heart weight (Table2).

Effect on Blood pressure

Administration of HFD for eight weeks in group B1 resulted in a significant increase in systolic, diastolic and the mean blood pressure compared to the normal control group (A). Allopurinol in group B2 significantly decreases the systolic, diastolic and the mean blood pressure compared to HFD group (B1) (Table 3).

Effect on blood glucose, Insulin and Insulin Resistance

HFD in group B1 resulted in significant

increase in fasting blood glucose, insulin level, and IR compared to the normal control group (A). Allopurinol in group B2 significantly decreases serum blood glucose, insulin, and IR compared to HFD. Moreover, allopurinol normalizes glucose level and IR (Table 3, Figure 1).

Effect on the uric acid level and Renal Function

Administration of HFD for 8 weeks in group B1 significantly increased serum uric acid (SUA), BUN and creatinine as compared to the group A. Allopurinol normalized serum creatinine, BUN and urea level (Table 3).

Effect on lipid profile

HFD significantly increased serum total cholesterol, TG, LDL-c and decreased HDL-c compared to the normal control group (A). Administration of allopurinol in the group (B2) significantly ameliorated serum total cholesterol, TG, LDL-c and decrease HDL-c compared to HFD group (B1). Surprisingly, allopurinol administration normalized cholesterol and HDL-c level (Table 3).

Effect on Adipo R1/2, HO-1 and eNOS expression levels

HFD significantly decrease AdipoR1/2, HO-1 and eNOS expression level in the liver, heart, kidneys and pancreatic tissues compared to tissues of normal control group (A). Administration of allopurinol for four weeks significantly increase AdipoR1/2, the HO-1 expression level in all examined tissues as well as NOS expression in liver, heart and kidney tissues only compared to HFD group. Moreover, allopurinol normalizes the pancreatic AdipoR2 and HO-1 expression level (Fig. 2).

Histopathology of the heart, liver, kidneys

Histopathologically, variable degrees of collagen fibers proliferation could be detected in routinely HE-stained specimens in different groups.

Table 2: Body and organ weights of the studied animal groups

	Total Body Weight	Liver Weight	Kidney Weight	Heart Weight	Pancreases Weight
Control Group (A)	148.3 \pm 6.06	6.8 \pm .82	1.05 \pm .08	.56 \pm .04	.33 \pm .012
HFD Group (B1)	299.8 \pm 8.5*	6.9 \pm .52	1.6 \pm .19*	.85 \pm .3*	1.3 \pm .34*
Allopurinol Group (B2)	251.7 \pm 8.8"	5.9 \pm 1.4	1.3 \pm .26" #	.62 \pm .09" #	.65 \pm .38"

Consequently, Masson's trichrome-stained sections revealed the presence of collagen fibers proliferation with green color in livers (Fig. 3a-c), hearts (Fig. 3e-g) and kidneys (Fig. 3i-k). The highest level of collagen fibers proliferation could be detected in HFD group (B1) (Fig. 3b, f, j). On the other hand, a normal distribution of collagen fibers could be seen in the normal control group (A) (Fig. 3a, e, i) in livers, hearts, and kidneys, respectively. The

Allopurinol-administered group (B2) exhibited mild to moderate proliferation of collagen fibers in different organs including, livers, hearts, and kidneys (Fig. 3c, g, k). Statistical comparison of collagen fibers area percentages in livers, hearts, and kidneys of rats revealed the high significant difference between HFD group (B1) and other two groups ($P<0.05$). Meanwhile, no significant difference could be found between the normal control group (A) and Allopurinol

Table 3: Blood pressure measurements and biochemical parameters of the studied animal groups

	Normal Control group (A)	HFD Group (B1)	Allopurinol Group (B2)
Systolic Bp (mmHg)	119.3±1.5	173.4±9.6*	145.7±7.8 "
Diastolic (mmHg)	72.1±6.4	103.6±13*	95.6±3.2
Mean BP (mmHg)	87.2±4.9	124±4.6 *	112.1±4.3 "
Glucose (mg/dl)	90.4±10.1	190.7±32.15*	94.14±8.26" #
Insulin (mIU/ml)	1.34±0.58	15.9±3.11*	4.03±1.5 "
Uric acid (mg/dl)	1.41±0.33	4.21±1.19*	1.39±0.39 " #
Creatinine (mg/dl)	0.75±0.06	2.26±0.61*	0.59±0.13 " #
BUN (mg/dl)	22.1±3.3	100.3±16.03*	31.3±7.6 " #
Triglycerides (mg/dl)	65.3±12.6	137.29±25.27 *	97.43±25.32 "
Cholesterol (mg/dl)	78.6±6.2	95.14±11.3 *	72.86±10.02 " #
HDL (mg/dl)	30.14±3.53	20.29±1.79 *	27.71±3.35 " #
LDL (mg/dl)	25±2.71	40±4.6 *	31.9±5.4 "

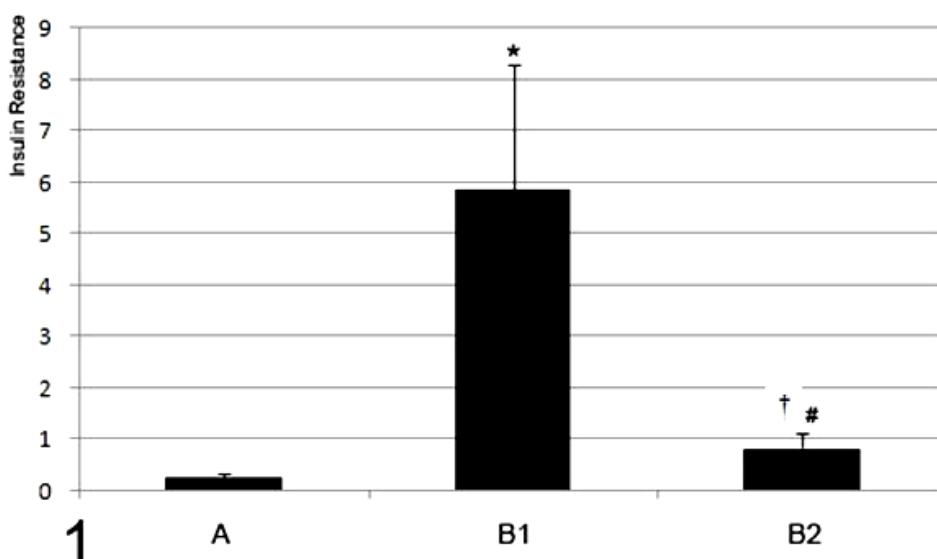


Fig. 1: Insulin resistance among investigated groups

group (B2) in livers, hearts and kidneys specimen ($P=0.469, 0.341, 0.074$ respectively) i.e. allopurinol hindered the histopathological changes associated with MS in livers, hearts and kidneys tissues.

DISCUSSION

In the current study, HFD induced MS as evident by significant increase of BW, BP (MAP, SBP and DBP), BUN, creatinine, insulin, serum glucose, IR, UA, total cholesterol, TGs, LDL-c level, with significant decrease of serum HDL-c level compared to vehicle group (A), these results confirm the previous report ¹².

Treatment with allopurinol ameliorated BW induced by HFD. Previous results stated that SUA levels are correlated with muscle and fat masses ¹³. The beneficial effect of allopurinol in the reduction of body weight (BW) may be through the anti-hyperuricemia effect ¹⁴.

HFD results in induction of hypertension which is successively reversed by allopurinol. The present result is in accordance with other experimental ¹⁵ and clinical study as well ¹⁶.

Hyperuricemia can predispose to hypertension through Ang II production stimulation and oxidative stress mediated by AT1 ¹⁷ or through increasing the sensitivity of the proximal tubule to the circulating Ang II ¹⁸.

Endothelial nitric-oxide synthase (eNOS) produced NO which is a potent vasodilator. It enhances the action of acetylcholine, bradykinin, and substance P in vascular beds by induction of smooth muscle relaxation ¹⁹. In the current work, hypertension in HFD group was associated with significant decrease in eNOS level which is successively reversed by allopurinol in most tissues.

SUA plays an important role in the exacerbation of IR ²⁰. It may increase the hepatic gluconeogenesis production through stimulation of Adenine mono phosphate deaminase (AMPD) and inhibition of AMP-activated protein kinase (AMPK) enzyme activity. AMPD stimulates hepatic gluconeogenesis ²¹. It reduced the production of eNOS, which is essential for insulin stimulated glucose uptake ²².

AdipoR1 and R2 play important roles in *in vivo* regulation of glucose metabolism and insulin

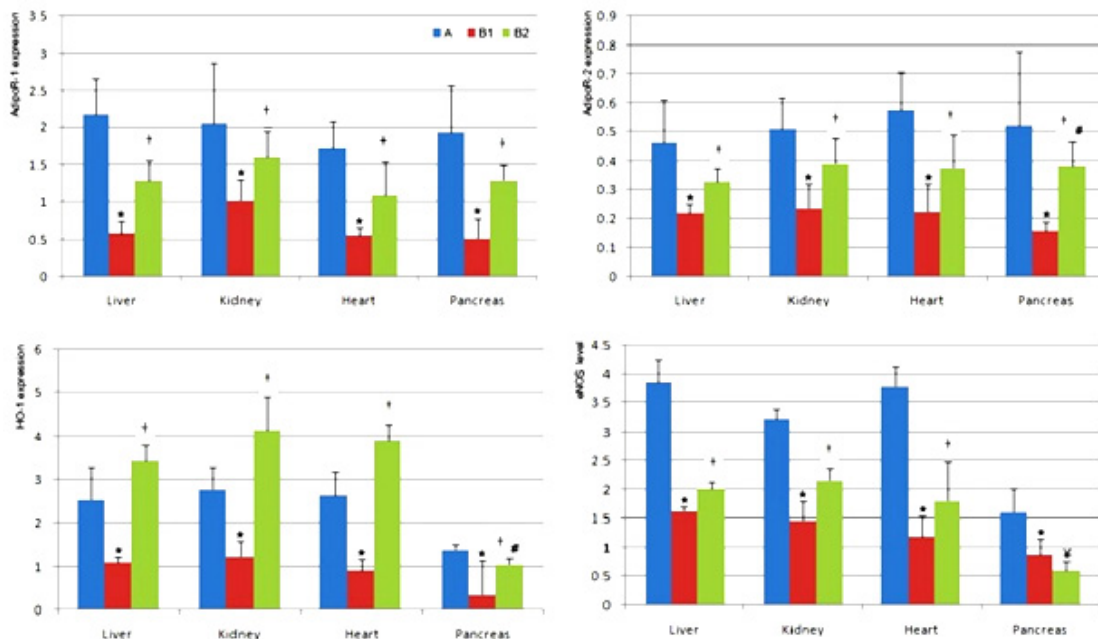


Fig. 2: AdipoR1/2, HO-1 and eNOS expression in liver, kidneys, heart and pancreatic tissues

sensitivity²³. Adiponectin binding to its receptors leads to the glucose transporter 4 translocation to the cell membrane that results in increased glucose transport and promotes glycogen synthesis²⁴ that results in IR improvement.

In the present study, HFD significantly increased blood glucose, insulin and IR concomitant with decreased AdipoR1/R2 expression levels in all examined tissues (liver, kidneys, heart, and pancreas) which are correlated with a significant

decrease in eNOS in liver, kidney and heart tissues of HFD group (B1) compared to group A.

Down regulation of AdipoR1/R2 and eNOS signaling result in a decrease of adiponectin efficacy leading to IR²⁵. Also, upregulation of AdipoR1 or AdipoR2 in the liver of db/db mice ameliorated diabetes significantly²³. Based on that opinion, using AdipoR1/R2 agonists or any agents that increase AdipoR1/R2 expressions may provide a new treatment for IR and diabetes type 2.

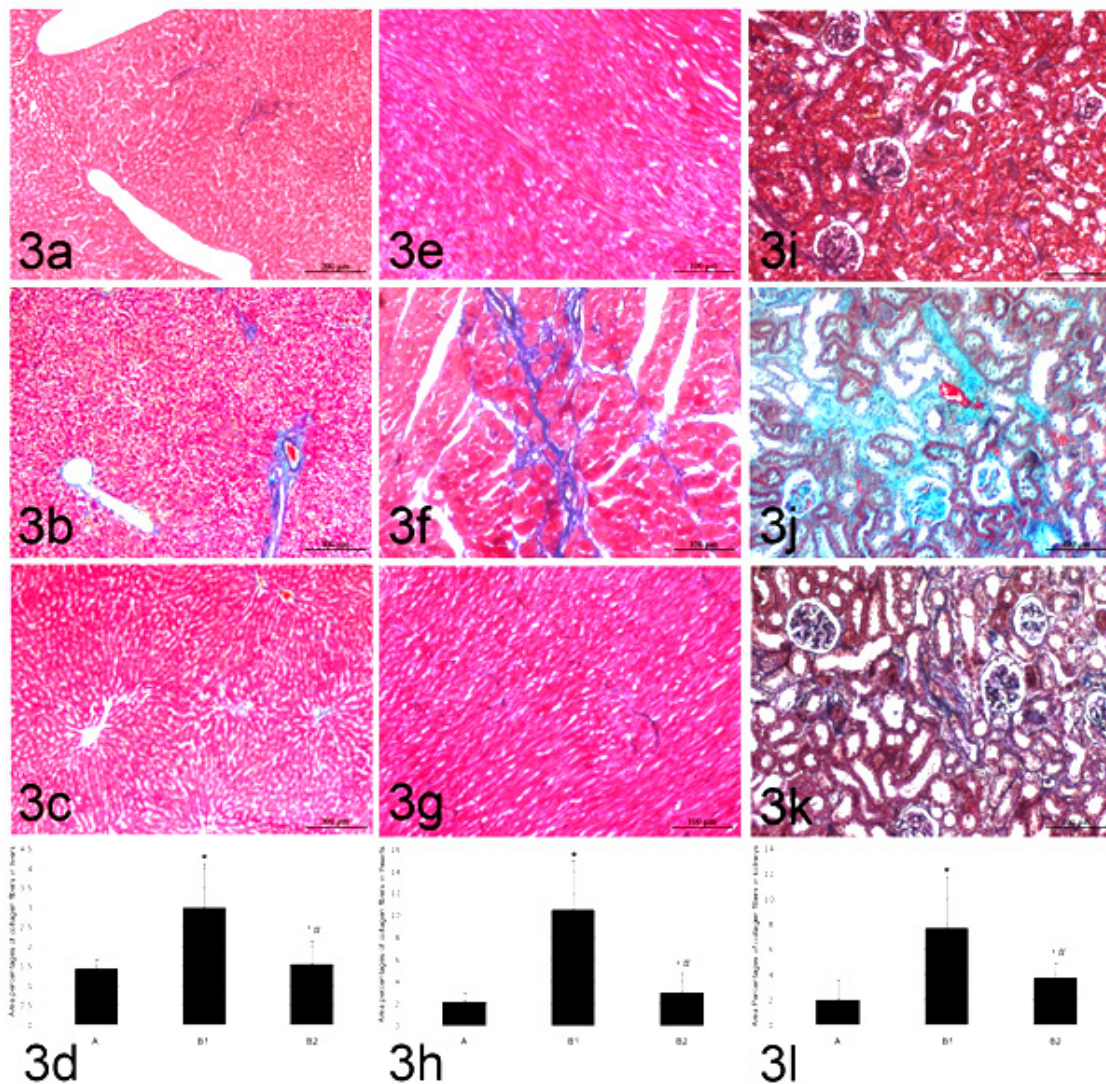


Fig. 3: Masson's trichrome staining of liver, heart and kidneys sections measuring variable collagen fibers

In the current study, allopurinol significantly decreased insulin level associated with upregulation of pancreatic AdipoR1/R2; moreover, Allopurinol normalizes pancreatic AdipoR2 signaling while insignificant differences in eNOS signaling compared to HFD group indicating that allopurinol may regulate insulin secretion mainly due to up regulation of AdipoR1/R2.

The fasting glucose reflects mainly hepatic glucose production²⁶, the glucose and HOMA-IR reflect mainly hepatic insulin resistance. In the present work, allopurinol decrease blood glucose and IR compared to HFD group with the restoration of normal hepatic architecture, this is run along with an increase hepatic AdipoR1/R2 and eNOS expression. Surprisingly, allopurinol normalizes IR, glucose level, indicating that allopurinol may normalize glucose level through combined up regulation of AdipoR1/R2 and eNOS expression in liver tissues.

Allopurinol significantly decreased creatinine and BUN levels compared to HFD group, whereas there are insignificant differences compared to vehicle group i.e. it normalized these parameters.

Allopurinol mediated a renal protection through increased eNOS and HO-1 expression compared to HFD group, this is associated with marked improvement of collagen contents of corresponding histological examination or indirectly through improvement of glucose and IR status.

HO-1 is an inducible enzyme that degrades heme to form an equal molar amount of carbon monoxide (CO), ferrous iron and biliverdin⁶. Following stress, The HO-1 is induced resulting in an important role in the protection of cell against injury⁷. HO-1 protects against vascular disease through promoting endothelial cell viability and function²⁷ via CO generation that possesses potent anti-apoptotic properties or through the generation of biliverdin and bilirubin from the catabolism of toxic free heme that possesses potent antioxidant activities²⁸.

Allopurinol significantly improves total cholesterol, TGs, HDL-c and LDL-c levels compared to that in HFD group, these results run with another study in fructose-fed rats²⁹.

Allopurinol improvement of dyslipidemia is correlated with up regulation of AdipoR1/R2 or as a consequence to eNOS expressions.

AdipoR1/R2 regulate lipid metabolism via increase the activities of the AMP-activated kinase (AMPK) and PPARs. Adiponectin binding to AdipoR1/R2 results in apoptosis protection; while adiponectin – through unidentified adiponectin receptor activate NF-kB leading to increases macrophages IL-6²³.

In the present study, a detection of collagen fibers proliferation or fibrosis was carried out by the use of the Masson's Trichrome (MT)-stained paraffin-embedded sections. A severe degree of fibrosis could be in different organs of HFD treated group (B1) including livers, kidneys, and hearts with the highest area percentages of collagen in comparison to other groups. Such finding is supported by other studies, which mentioned that diet rich in fructose promoted oxidative stress and ROS to up regulate CD36, and subsequently provoked NLRP3 inflammasome in a TLR4/6-IRAK4/1-dependent manner leading to promotion of fibrosis and inflammation in rats suffering from metabolic syndrome³⁰. Additionally, no significant difference of fibrosis could be detected between vehicle and Allopurinol treated group. Our results supported by Kang *et al.* who proved the cardio protective effect of allopurinol against HFD induced inflammation and fibrosis³⁰.

CONCLUSION

Allopurinol may represent a good agent for the amelioration of MS components possibly through up regulation of adipo R1/R2, HO-1 and eNOS- in different tissues; however more experimental and clinical studies are needed to weight the expected allopurinol benefit against long term use related side effects.

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