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Alpha amyrin attenuates high fructose diet-induced metabolic syndrome in rats

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Abstract
This study investigated the effect of alpha-amyrrn (a pentacyclic triterpene) on high-fructose diet (HFD)-induced metabolic syndrome in rats. Male Wistar rats were randomly distributed into different groups. Control group was fed normal rat chow diet. HFD group was fed HFD (60%; w/w) for 42 days. Pioglitazone (10 mg/kg, p.o. once daily) was used as a standard drug. Alpha amyrrn was administered in three doses (50, 100 and 200 mg/kg, orally; once daily along with HFD). Plasma glucose, total cholesterol, triglycerides and HDL-C were estimated. Changes in blood pressure, oral glucose tolerance and insulin tolerance were measured. Hepatic oxidative stress as well as mRNA and protein levels of PPAR-α were analyzed. A significant increase in systolic blood pressure, plasma glucose, total cholesterol, plasma triglycerides and a significant decrease in HDL-C was observed in HFD rats as compared to control rats. Glucose tolerance and insulin tolerance were also significantly impaired with HFD. Alpha amyrrn prevented these changes in a dose dependent manner. Hepatic oxidative stress as well as micro- and macrovesicular fatty changes in hepatocytes caused by HFD were also attenuated by alpha amyrrn. Alpha amyrrn preserved the hepatic mRNA and protein levels of PPAR-α which was reduced in HFD group. This study thus demonstrates that alpha amyrrn attenuates HFD-induced metabolic syndrome in rats.

KEY WORDS: Alpha amyrrn, Metabolic syndrome, High-fructose diet, Oxidative stress, PPAR-α
Introduction

Metabolic syndrome (MetS) is a cluster of conditions – obesity, hyperglycemia, dyslipidemia, insulin resistance and hypertension that predisposes individuals to the development of type 2 diabetes and cardiovascular diseases (Tran et al. 2009a; Handelsman 2009). A high fructose intake causes adverse metabolic changes, the spectacular changes being an increase in plasma triglycerides, hepatic insulin resistance and hepatic steatosis (Tappy et al. 2010). Fructose consumption has increased dramatically in the last 100 years (El-Bassossy and Shaltout 2015). It is widely used as sweetener in fruit juices, jams, candies, high fructose corn syrup and soft drinks throughout the world (Fan et al. 2014) and is selectively metabolised by liver. Fructose causes metabolic syndrome through deregulation of metabolic pathways (Khitan and Kim 2013). Moreover, it is postulated that oxidative stress could be responsible for the development of insulin resistance and progression of MetS in rats caused by fructose consumption (Bloch-Damti and Bashan 2005; Mahmoud and Elshazly 2014). Additionally, increased oxidative stress either by excess generation of reactive oxygen species (ROS) or depletion of antioxidant enzymes has been associated with metabolic alteration including fatty changes in liver (Kunde et al. 2011; Nomura and Yamanouchi 2012).

Peroxisome proliferator-activated receptors (PPARs) are ligand activated nuclear hormone receptors. They regulate genes which play important role in lipid and glucose homeostasis (Grygiel-Gorniak 2014). Peroxisome proliferator-activated receptors alpha (PPAR-α), an isoform of PPARs superfamily, is expressed in tissues with high capacity for fatty acid oxidation like liver, heart and skeletal muscle. PPAR-α has been reported to regulate lipid and glucose metabolism (Grygiel-Gorniak 2014). Fructose consumption has been associated with decreased level of mRNA and protein of PPAR-α (Monsalve et al. 2013). Interestingly, PPAR-α agonists improved not only hyperlipidemia but also insulin sensitivity in high-
fructose fed rats (Nagai et al. 2002). Taken together, PPAR-α may act as a potential therapeutic target in the management of MetS and its related risk factors, especially cardiovascular complications. Taking into account the fact that plants are rich source of ligand for PPARs (Huang et al. 2005), this study was designed to evaluate the effect of alpha amyrin against HFD-induced MetS in rats. Pioglitazone, a thiazolidinedione was used as standard drug in this study. It improves insulin sensitivity as well as lipid profile. This drug is used for the treatment of type-2 diabetes mellitus and MetS (Tjokroprawiro 2006).

Alpha amyrin, a pentacyclic triterpene, is a phytoconstituent present in various plants like *Ficus racemosa*, *Protium heptaphyllum*, *Protium kleinii*, *Alstonia boonei*, etc (Narender et al. 2009; Singh et al. 2015; Okoye et al. 2014). *Ficus racemosa* (Roxb.) is commonly known as ‘Gular’ in north India and is used for the treatment of diabetes in Indian system of medicine (Ahmed et al. 2011). Alpha amyrin has been reported to possess anti-diabetic (Santos et al. 2012, Singh et al. 2009), anti-dyslipidemic (Santos et al. 2012), antioxidant (Holanda et al. 2008), hepatoprotective (Oliveira et al. 2005) and anti-inflammatory (Holanda et al. 2008) properties. This phytoconstituent also reduced atherogenic risk factors and improved glucose tolerance in mice possibly by its antioxidant effects (Holanda et al. 2008). Therefore, the present study was designed to investigate the effect of alpha amyrin in HFD-induced MetS in rats. The hepatic mRNA and protein levels of PPAR-α was also studied.

**Materials and methods**

The protocol was approved by the Institutional Animal Ethics Committee, All India Institute of Medical Sciences (AIIMS), New Delhi, India (532/IAEC/09). All experimental protocols were performed in compliance with the National Institute of Health (NIH) Guidelines for the Care and Use of the Laboratory Animals (NIH Publication no. 85723, revised 1996).
Experimental animals

Male Wistar rats (150–200 g, 10–12 weeks) were maintained under standard laboratory conditions (temperature: 25 ± 2 °C, relative humidity: 50 ± 15% and natural dark/light cycle). Food and water were provided ad libitum. Body weight was measured twice weekly with the help of an electronic balance (Oras Tech, India). A mean of three consecutive readings was taken.

Drugs and chemicals

Alpha amyrin and all chemicals used for experiments were of analytical grade and were purchased from Sigma –Aldrich Co., USA.

Experimental design

Rats were randomly assigned to seven experimental groups as follows (n=6):

2. HFD: fed with high fructose diet (60%, w/w).
3. Pio+HFD: fed with pioglitazone (10 mg/kg, orally, once daily) along with high fructose diet.
4. AA50+HFD: administered alpha amyrin (50 mg/kg, orally, once daily) along with high fructose diet.
5. AA100+HFD: administered alpha amyrin (100 mg/kg, orally, once daily) along with high fructose diet.
6. AA200+HFD: administered alpha amyrin (200 mg/kg, orally, once daily) along with high fructose diet.
7. AA per se: administered alpha amyrin (200 mg/kg, orally, once daily) along with standard rat chow diet.
The high fructose diet (60% w/w) was prepared in our laboratory. Normal rat chow diet (Ashirwad, India) containing, protein: 24% w/w, fat: 5% w/w, fiber: 4% w/w, carbohydrates: 55% w/w, calcium: 0.6% w/w, phosphorus: 0.3% w/w was ground to powder. Fructose was added then to achieve final concentration 60% w/w. This mixture was made into a wet mass by adding sufficient water to prepare diet pellets which was then air dried. This diet was given to the rats in place of the normal rat chow diet in all the groups except control and alpha amyrin per se groups. High fructose diet and drugs (alpha amyrin in all the doses and pioglitazone) were administered for 42 days. Blood was withdrawn at baseline and on day 42 for the estimation of plasma glucose, plasma triglycerides, plasma total cholesterol and plasma HDL-cholesterol, except, glucose tolerance and insulin tolerance test.

Rats were sacrificed at the end of the study. A portion of liver (50% of the largest hepatic lobe) was rapidly excised, dipped in liquid nitrogen and stored at -80°C for biochemical and PPAR-α mRNA & protein studies. The remaining 50% of liver was kept in 10% formalin for histopathological study.

**Fasting plasma glucose, total cholesterol, triglycerides and HDL-cholesterol levels**

After overnight (12 ± 1 h) fast, blood sample was collected and was centrifuged at 3000 rpm for 10 min at 4°C for separation of plasma. The fasting plasma glucose, total cholesterol, triglycerides and HDL-C were estimated as per standard protocol given by the manufacturers of the kits (Vital Diagnostics Pvt. Ltd., India) using a semi-autoanalyzer (Minitecno, India).

**Blood pressure (BP)**

The indirect tail cuff method was used for measurement of blood pressure (Biopac, USA) as described earlier (Prabhakar et al. 2014). Briefly, tail of rats were preheated in a chamber at 37°C for 10 min and then placed in plastic restrainers. A cuff with a pneumatic pulse sensor
was attached to the tail. Rats were allowed to habituate to this procedure for 7 days before actual measurement was performed. Mean of three consecutive readings was taken as the systolic and diastolic blood pressures of each rat.

**Oral glucose tolerance test (OGTT)**

OGTT was done to assess the glucose induced insulin secretion and glycemic control. After overnight (12 ± 1 h) fast, each rat was fed glucose through a gastric gavage in a dose of 2 g/kg body weight after blood glucose measurement at baseline (0 min). Blood was collected at 0, 30, 60 and 120 min for blood glucose estimation. The oral glucose tolerance test was evaluated by the total area under blood glucose curve using the trapezoidal method (Collino et al. 2010).

**Insulin tolerance test**

Insulin tolerance test was performed for evaluation of insulin sensitivity. Rats were administered regular insulin (0.75 U/kg, Torrent Pharmaceuticals Ltd, India) (Lehnen et al. 2010) subcutaneously after baseline (0 min) collection of blood sample from the tip of tail (de Moura et al. 2009; Ghezzi et al. 2012). Subsequent blood samples were collected at 30 and 60 min after injection of insulin for estimation of blood glucose (Narasimhan et al. 2015).

**Oxidative stress parameters**

Liver tissue samples were thawed once and homogenized in 10% w/v ice-cold 0.1 M phosphate buffer (pH 7.4). The homogenates were used for estimation of thiobarbituric acid reactive substances (TBARS) as a marker of lipid peroxidation and endogenous antioxidants like reduced glutathione (GSH) level, superoxide dismutase (SOD) and catalase activities. Protein was estimated using Bradford’s reagent.
**Thiobarbituric acid reactive substances**

Thiobarbituric acid reactive substances (TBARS) were estimated as described by Ohkawa et al. (1979). Briefly, liver tissue was homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (7.4). The reagents, 1.5 ml acetic acid (20%) pH-3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium lauryl sulphate (8.1%) were added to 0.1 ml of processed tissue sample. The mixture was heated at 95°C for 60 min. The mixture was cooled. Then 5 ml of n-butanol : pyridine (15:1) and 1 ml distilled water was added. After centrifugation at 5000 rpm for 10 min at room temperature, the organic layer was separated and absorbance was measured at 532 nm using a spectrophotometer (Systronic 119, India).

**Reduced glutathione**

Reduced glutathione (GSH) was estimated by the method as described by Ellman (1959). Briefly, liver tissue was homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). This homogenate was centrifuged with 5% trichloroacetic acid to extrude out proteins. To this homogenate, 2 ml phosphate buffer (pH-8.4), 0.5 ml 5’5 dithiobis (2-nitrobenzoic acid) (DTNB) and 0.4 ml of double distilled water was added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min.

**Superoxide dismutase**

Superoxide dismutase (SOD) was estimated as described earlier (Kakkar et al. 1984). Briefly, liver tissue was homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). The reagents sodium pyrophosphate buffer 1.2 ml (0.052 M, pH-8.3), 0.1 ml phenazine methosulphate (186 µM), 0.3 ml nitro blue tetrazolium (300 µM) and 0.2 ml NADH (780 µM) was added to 0.1 ml of processed sample. The mixture was incubated for 90 s at 30°C. Then 4 ml of n-butanol and 1 ml of acetic acid was added. After centrifugation at 4000 rpm
for 10 min, the organic layer was separated and absorbance was measured at 560 nm using spectrophotometer.

**Catalase**

Catalase was estimated as described earlier (Aebi 1974). Briefly, liver tissue was homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). Sample (0.05 ml) was added to 2 ml of 50 mM phosphate buffer and at last 1 ml hydrogen peroxide (H₂O₂) was added. Extinction was read at 240 nm at intervals of 15 s for a total of 30 s.

**Hepatic PPAR-α mRNA level**

The mRNA level of hepatic PPAR-α was examined using real time PCR detection system (CFX96, Bio-Rad, Richmond, CA, USA) as described earlier (Moran-Salvador et al. 2011). Briefly, total RNA from liver tissue was isolated using pureZol reagent (Bio-Rad, USA). Isolated RNA concentration was assessed in a UV spectrophotometer (Nanodrop Technologies ND-1000 V3.6.0, Wilmington, USA), and its integrity was determined on 1.4% agarose gel electrophoresis. Subsequently, equal amount of RNA was taken for cDNA synthesis using RevertAid First Strand cDNA synthesis kit (Thermo Scientific, USA). The quality of the cDNA produced was confirmed by PCR amplification using beta-actin primers as a housekeeping gene. The primers used for real time PCR were designed from the individual mRNA transcripts using Primer 3 Input (version 0.4.0) and synthesized from Sigma Aldrich, USA (Table 1). Real-time quantitative PCR was performed using SYBR Green PCR Master Mix (Bio-Rad, USA) and protocol was followed as prescribed by supplier (Bio-Rad, USA). The PCR cycling conditions were: 95°C for 3 min (hold), followed by 40 cycles of 95°C for 10 s (denaturation), 60°C for 30 s (annealing) and 72°C for 30 s (extension). We used β-actin as a housekeeping gene as a standard procedure.
reported earlier (Gao et al. 2012). The expression of the housekeeping gene, β-actin, was used to normalise the expression of target genes. β-actin has been used earlier in our laboratory and has also been used by other authors for real time PCR analysis (Castro et al. 2012; Gao et al. 2012; Hsieh et al. 2013; Prabhakar et al. 2015).

Threshold cycle (Ct) values were obtained, and comparative \(2^{-\Delta\Delta Ct}\) method was used for relative quantification of gene where, \(\Delta\Delta Ct = \) is the cycle threshold normalized first with the endogenous control β-actin (Ct sample – Ct β-actin = ΔCt) and then with the control (ΔCt Sample - ΔCt Control = ΔΔCt) (Alwahsh et al. 2014).

**Hepatic PPAR-α protein level**

Hepatic tissue was homogenized in lysis buffer as described by Moran-Salvador et al. 2011. Briefly, total of 40 µg of nuclear proteins from liver tissue (determined by the Bradford protein assay) was resuspended in SDS-containing Laemmli sample buffer, heated for 3 min at 95°C, and resolved on a 10% SDS-PAGE (from 30% acrylamide/bis; Bio-Rad, Hercules, CA, USA). Proteins were transferred electrophoretically for 60 min at 100 V at 4°C onto nitrocellulose membranes, and the efficiency of the transfer was visualized by Ponceau S staining. Membranes were blocked for 45 min at room temperature in 5 % (w/v) skimmed milk. Blots were washed 3 times for 5 min each with 0.1% phosphate buffered saline solution with the detergent Tween20 (PBS-T) and subsequently treated overnight at 4°C with primary antibody (dilution 1:1000; Abcam, UK). Immunoblots were obtained using primary antibodies against rabbit anti-mouse PPAR-α and Lamin. After washing 3 times for 5 min each with 0.1% PBS-T, blots were incubated with horseradish-peroxidase-linked goat anti-rabbit secondary antibody (dilution 1:2000) for 2 h at room temperature. Bands were visualized by enhanced chemiluminescence (Luminata, Millipore, USA) detection system (Cell Biosciences, USA).
Histopathological analysis

Liver tissue samples were fixed in 10% formalin and embedded in paraffin. Each specimen was cut in 5 µm thick paraffin sections from the paraffin-embedded tissue blocks and stained. Briefly, each paraffin sections were deparaffinized by immersing in xylene and rehydrated through a series of graded alcohols (100%, 95% and 75%), for 15 min each. The slides were stained with hematoxylin & eosin for evaluation for necrosis. Another set of slides was stained with picrosirius red for evaluation of fibrosis (i.e. collagen content). The slides were examined under light microscope by two researchers who were blinded to the study groups. Images were taken at magnification x 40 (Maulik et al. 2012). The fatty changes in the liver was histologically scored according to the published method of histological scoring system for nonalcoholic fatty liver disease (Kleiner et al. 2005).

Statistical analysis

Data were analysed using SPSS (version 20.0) statistical package (IBM, Armonk, NY). The results are expressed as mean ± standard error of mean. The area under the curve for glucose tolerance and insulin tolerance tests were calculated since these were measured at 0, 30, 60 and 120 minutes. The difference in mean values of all the other parameters among the groups were compared using one way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post hoc test. The number of multiple comparissions done was six comparisions [control vs HFD, HFD vs pioglitazone, HFD vs different doses of alpha amyrin (3 comparisons) and control vs alpha amyrin 200 mg/kg per se]. A value of $p < 0.05$ was considered statistically significant.
Results

Effect of alpha amyrin on fasting plasma glucose levels

At baseline, no significant difference was observed in plasma glucose level amongst the groups. There was a significant increase in fasting plasma glucose levels in HFD rats as compared to control group at the end of 42 days (8.32 ± 0.1 mmol/L vs 5.30 ± 0.3 mmol/L; \( p < 0.001 \)). Alpha amyrin (100 and 200 mg/kg) significantly decreased the fasting plasma glucose level as compared to HFD on 42 day (7.50 ± 0.1 mmol/L; \( p < 0.05 \) and 7.39 ± 0.1 mmol/L; \( p < 0.01 \) vs 8.32 ± 0.2 mmol/L, respectively). Alpha amyrin per se did not cause any significant change on fasting plasma glucose levels (Fig. 1).

Effect of alpha amyrin on oral glucose tolerance test

Area under curve (AUC) of glucose in HFD rats was significantly higher as compared to control group (3600 ± 55 mmol/L x 120 min vs 2540 ± 74 mmol/L x 120 min; \( p < 0.001 \)) implying an impaired oral glucose tolerance in HFD group at the end of 42 days. Alpha amyrin (100 mg/kg and 200 mg/kg) significantly decreased the area under curve of glucose tolerance as compared to HFD group in a dose dependent manner (\( p < 0.05 \) and \( p < 0.01 \) respectively). Alpha amyrin per se did not cause significant change in AUC of glucose tolerance (Fig. 2).

Effect of alpha amyrin on insulin tolerance test

In the insulin tolerance test, AUC was significantly higher in HFD group (1250 ± 43 mmol/L x 60 min vs 586 ± 42 mmol/L x 60 min, \( p < 0.001 \)) as compared to the control group at the end of 42 days which implicates impaired insulin tolerance with HFD. Pioglitazone (755 ± 21 mmol/L x 60 min) caused significant insulin sensitivity at the end of 42 days as compared to HFD. Alpha amyrin caused a mild but statistically insignificant improvement in insulin
sensitivity as compared to HFD. Alpha amyrin *per se* had no significant effect on insulin sensitivity as compared to control (Fig. 3).

**Effect of alpha amyrin on lipid profile**

As compared to the control group, a significantly higher levels of plasma triglycerides (1.2 ± 0.02 mmol/L vs 0.61 ± 0.01 mmol/L; *p* < 0.001), plasma total cholesterol (1.91 ± 0.2 mmol/L vs. 1.29 ± 0.1 mmol/L; *p* < 0.01) and lower level of plasma HDL-cholesterol (0.31 ± 0.04 mmol/L vs 0.68 ± 0.03 mmol/L; *p* < 0.001) was observed in the HFD group. Alpha amyrin significantly decreased the plasma triglycerides [50 mg/kg, (0.82 ± 0.01 mmol/L; *p* < 0.01); 100 mg/kg, (0.72 ± 0.03 mmol/L; *p* < 0.001) and 200 mg/kg, (0.64 ± 0.03 mmol/L; *p* < 0.001) vs 1.2 ± 0.02 mmol/L in HFD] and plasma total cholesterol (200 mg/kg; 1.44 ± 0.07 mmol/L vs 1.91 ± 0.2 mmol/L; *p* < 0.05) as well as increased the plasma HDL-cholesterol [100 mg/kg; 0.48 ± 0.02 mmol/L (*p* < 0.01) and 200 mg/kg; 0.52 ± 0.03 mmol/L (*p* < 0.001) vs 0.31 ± 0.04 mmol/L in HFD] levels as compared to HFD. Similar protection was observed with pioglitazone treated rats. Alpha amyrin treatment *per se* did not cause any significant change in any lipid parameters during the study as compared to control (Fig. 4 A - C).

**Effect of alpha amyrin on blood pressure**

There was a significant increase in systolic blood pressure at 42 days in HFD rats as compared to the control group (146 ± 5 mmHg vs 115 ± 4 mmHg; *p* < 0.001). Alpha amyrin, 100 and 200 mg/kg significantly decreased the systolic blood pressure at 42 days as compared to the HFD group (135 ± 2 mmHg; *p* < 0.01 and 130 ± 3.6 mmHg; *p* < 0.01 respectively) (Fig.5). There were no significant differences in the diastolic blood pressure among the different groups. Alpha amyrin *per se* did not cause any significant change in systolic as well as diastolic blood pressures.
Effect of alpha amyrin on body weight

No significant differences in the body weight were observed between the groups at any point of time.

Effect of alpha amyrin on hepatic oxidative stress parameters

A significant increase in hepatic TBARS level (144.6 ± 4.3 nmol/g wet tissue vs 57.08 ± 9.2 nmol/g wet tissue, *p* < 0.001) and a significant decrease in hepatic GSH (14.1 ± 1.7 mg/g wet tissue vs 34.5 ± 1.0 nmol/g wet tissue, *p* < 0.001) level, hepatic SOD (64.9 ± 2.0 U/mg protein vs 109.9 ± 6.4 U/mg tissue, *p* < 0.001) as well as hepatic catalase (0.50 ± 0.07 U/mg protein vs 2.01 ± 0.1 U/mg protein, *p* < 0.001) activities were observed in HFD rats as compared to the control. Administration of alpha amyrin, 100 mg/kg (121.54 ± 4.8 nmol/g wet tissue; *p* < 0.05) and 200 mg/kg (97.08 ± 7.7 nmol/g wet tissue; *p* < 0.01) significantly prevented the HFD-induced increase in TBARS levels (Fig. 6A). Alpha amyrin, 100 mg/kg (23.5 ± 1.5 mg/g wet tissue; *p* < 0.05) and 200 mg/kg, (28.91 ± 1.5; *p* < 0.01) also increased the hepatic GSH level as compared to HFD group (Fig. 6B). Alpha amyrin, 200 mg/kg (101.70 ± 6.1 U/mg protein; *p* < 0.05) significantly prevented the decreased hepatic SOD activity in HFD rats (Fig. 6C). Alpha amyrin, 100 mg/kg (1.07 ± 0.1 U/mg protein; *p* < 0.05) and 200 mg/kg (1.41 ± 0.5 U/mg protein; *p* < 0.01) significantly ameliorated the decrease in hepatic catalase activity as compared to the HFD group (Fig. 6D). Pioglitazone significantly decreased hepatic oxidative stress as shown by the decreased hepatic TBARS levels and the increased hepatic GSH, hepatic catalase and hepatic SOD levels as compared to HFD rats. Alpha amyrin *per se* did not cause significant change in any of the oxidative stress parameters.
Effect of alpha amyrin on mRNA level of PPAR-α

There was significant decrease in hepatic PPAR-α mRNA level ($p < 0.001$) in HFD fed rats as compared to control group. Alpha amyrin (200 mg/kg) administration significantly increased PPAR-α mRNA level as compared to HFD rats (Fig. 7). Alpha amyrin per se did not cause any significant change in the mRNA of PPAR-α in normal rat.

Effect of alpha amyrin on protein level of PPAR-α

There was significant decrease in protein level of hepatic PPAR-α in HFD group as compared to control. Alpha amyrin (200 mg/kg) increased the protein level of PPAR-α as compared to HFD (Fig. 8). Alpha amyrin per se had no significant effect on hepatic protein level of PPAR-α.

Effect of alpha amyrin on hepatic histopathology studies

Histopathological studies of liver demonstrated that there were micro- and macrovesicular fatty changes of the hepatocytes in HFD group (Fig. 9B). No infiltration of inflammatory cells, necrosis or fibrosis was observed in the HFD group. Treatment with alpha amyrin led to protection of micro- and macrovesicular fatty changes of hepatocytes as compared to HFD group in a dose dependent manner. Alpha amyrin per se did not cause any change on the hepatocytes. The score of fatty changes in different groups of animal as shown in Fig. 9 is as follows: A. Control – 0, B. HFD – 3, C. Pio +HFD – 1, D. AA 50+HFD – 3, E. AA 100+HFD-2 and F. AA 200+HFD-1.

Discussion

In the present study, HFD caused MetS in rats as evidenced by increased plasma glucose level, deranged lipid profile, elevated systolic blood pressure, insulin resistance and impaired
glucose tolerance. Our findings are in agreement with some earlier studies wherein these changes were observed with HFD (Ibrahim et al. 2014; Fan et al. 2014). MetS in rats was associated with increased hepatic oxidative stress, as indicated by increased hepatic TBARS level and decreased endogenous antioxidant (reduced glutathione, superoxide dismutase and catalase) levels. Additionally, micro- and macrovesicular fatty changes in liver tissue were observed in high-fructose fed rats similar to human disease i.e. nonalcoholic fatty liver disease (Chung et al. 2014). Moreover, HFD decreased the levels of hepatic mRNA and protein level of PPAR-α in liver. Alpha amyrin prevented these metabolic and hepatic changes in terms of oxidative stress, microscopic changes, and decrease in hepatic mRNA as well as hepatic protein level caused by HFD.

It has been found that diets with high concentration of simple carbohydrates reduce antioxidant levels (de Castro et al. 2012). Oxidative stress is an important characteristic of diet-induced MetS in animals (Pakdeechote et al. 2014) as well as humans (Hokayem et al. 2013). Moreover, there is some evidence that oxidative stress helps in progression of MetS (Roberts and Sindhu 2009; Yubero-Serrano et al. 2013; Giris et al. 2014). Further, liver is the primary organ responsible for fructose metabolism (Basciano et al. 2005) and metabolism of fructose facilitates oxidative damage and lipid peroxidation (Alwahsh et al. 2014; Kunde et al. 2011). In our study, MetS was associated with increased hepatic oxidative stress as evidenced by increased hepatic TBARS and decrease in hepatic GSH, catalase as well as SOD levels in HFD group. This was prevented by alpha amyrin probably due to its reported scavenger activities for reactive oxygen species (Oliveira et al. 2005; Holanda et al. 2008). In addition, oxidative stress disrupts the insulin-signaling cascade by activation of cellular stress sensitive pathways which interferes with the normal transcription of specific proteins, leading to insulin resistance (Bloch and Bashan. 2005). Treatment with antioxidants have been found to improve glucose utilization and insulin resistance in T2DM patients (Bloch and Bashan...
Pioglitazone also reduced the hepatic lipid peroxidation and prevented decrease in hepatic reduced GSH, catalase and SOD levels in our study. Free radical scavenging effect of pioglitazone has also been reported earlier in renal oxidative stress (Gumieniczek 2005).

PPARα acts as a sensor of fatty acid load. Elevation in the circulating levels of free fatty acids or their metabolites, results in activation of PPARα. This, in turn, upregulates the expression of critical catabolic enzymes that are involved in mitochondrial and peroxisomal β-oxidation and microsomal ω-oxidation. As a result, there is increased hepatic fatty acid catabolism and prevention of increased deposition of lipids in liver (Azhar 2010). Interestingly, PPAR-α also regulates glucose homeostasis along with hepatic lipid metabolism (Lefebvre et al. 2006). PPAR-α ligand improved symptoms of the MetS (visceral obesity, insulin resistance and dyslipidemia) (Monsalve et al. 2013; Lefebvre et al. 2006), thus suggesting that PPAR-α might be a possible target for the prevention or treatment of MetS. Ours is the first study which demonstrates that alpha amyrin prevents the reduction in level of mRNA and protein of hepatic PPAR-α induced by HFD in rats. Thus, alpha amyrin might be a potential agent in the management of MetS.

Alpha amyrin prevented the increase in triglycerides and total cholesterol as well as decrease in HDL-cholesterol caused by HFD. Supporting this finding, beneficial effect of alpha amyrin against dyslipidemia has been reported earlier in experimental conditions like streptozotocin-induced diabetes in mice (Santos et al. 2012). In the present study, pioglitazone also prevented the increase in triglycerides, total cholesterol and decrease in HDL-cholesterol caused by HFD. Pioglitazone has earlier been reported to be effective against increase in triglycerides in fructose-induced insulin resistance (Suzuki et al. 1997).

In our study, impaired glucose tolerance and altered insulin sensitivity were observed in HFD fed rats. Alpha amyrin prevented the increase in fasting plasma glucose, impaired glucose tolerance and alteration in insulin sensitivity in HFD fed rats. Although, antihyperglycemic
effect of alpha amyrin has been reported earlier in streptozotocin-induced diabetic mice (Santos et al. 2012), this study is the first study to report anti-hyperglycemic effect of alpha amyrin in rat model of MetS. It is important to note that alpha amyrin *per se* did not cause any effect on plasma glucose level. The exact mechanism of action of alpha amyrin against HFD-induced impaired glucose tolerance and insulin resistance is not clearly known.

In this study, HFD fed rats did not show a significant increase in body weight as has been reported earlier (de Castro et al. 2013; de Moura et al. 2009) and no significant change was observed in alpha amyrin treated group. Though, MetS is characterized by hyperglycemia, dyslipidemia, insulin resistance, obesity and hypertension in humans, there is wide variability in the criteria of metabolic syndrome in rats. These differences in rats may be due to the variability in the diet composition, study protocols, form of fructose administration (diet or water); duration of dietary administration, use of different rodent models etc. (de Castro et al. 2013).

In our study, HFD caused an elevation in systolic blood pressure which has also been reported earlier (Mostarda et al. 2012). In clinical practice, hypertension is a component of MetS, the exact mechanism of which is not well established. Certain factors have been implicated for fructose-induced hypertension such as increased sympathetic activity (Penicaud et al. 1998), increased level of catecholamines in circulation (Tran et al. 2009b), raised renin-angiotensin system activity and angiotensin II levels (Kobayashi et al. 1993), altered sodium reabsorption (DeFronzo 1981), impaired endothelium-dependent relaxation (Katakam et al. 1998), etc. These factors might have contributed to an increased vascular tone and endothelial dysfunction leading to elevated systolic blood pressure. Moreover, it has been reported that hypertriglyceridemia, insulin resistance or both, could contribute to the development of hypertension in rats (Dimo et al. 2001). In our study, alpha amyrin prevented the increase in systolic blood pressure caused by HFD which might be secondary to its
protective effect on development of MetS. Moreover, diets rich in pentacyclic triterpenes have been reported to improve endothelial dysfunction in spontaneously hypertensive rats (Rodriguez-Rodriguez et al. 2007; Jager et al. 2009). Alpha amyrin per se did not cause any significant change in blood pressure.

**Conclusion**

The present study demonstrated that administration of HFD lead to MetS in rats which was associated with increased hepatic oxidative stress as well as hepatic micro- and macrovesicular fatty changes. Alpha amyrin attenuated HFD induced metabolic changes, altered glucose tolerance, impaired insulin tolerance, hepatic oxidative stress, micro- and macrovesicular fatty changes, reduction in mRNA as well as protein level of hepatic PPAR-α. This study suggests a preventive role of alpha amyrin in HFD-induced MetS which has not been reported in earlier.

**Conflict of interest**

On behalf of all authors, the corresponding author states that there is no conflict of interest.

**Acknowledgement**

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References


Castro, M.C., Francini, F., Schinella, G., Caldiz, C.I., Zubiria, M.G., Gagliardino, J.J. et al. 2012. Apocynin administration prevents the changes induced by a fructose-rich diet on rat


Table 1. Primers used for real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (FP)</th>
<th>Reverse Primer (RP)</th>
<th>Annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR-α</td>
<td>TAATTTGCTGTGGAGATCGGC</td>
<td>TTGAAGGAGTTTTGGGAAGAG</td>
<td>60</td>
</tr>
<tr>
<td>β-actin</td>
<td>TGAAGATCAAGATCATTGCTCCTC</td>
<td>TCATCGTACTCCTGCTTGCTGA</td>
<td>60</td>
</tr>
</tbody>
</table>

Legends to figures

Fig. 1. Effect of alpha amyrin on plasma glucose levels. Values are expressed as mean ± SEM (n=6). *p < 0.05, **p < 0.01, ***p < 0.001; a - as compared to control, b - as compared to HFD.

Fig. 2. Effect of alpha amyrin on oral glucose tolerance test. Values are mean ± S.E.M (n=6). *p < 0.05, **p < 0.01, ***p < 0.001; a- as compared to control, b- as compared to HFD.

Fig. 3. Effect of alpha amyrin on insulin resistance test. Values are mean ± S.E.M (n=6). ***p < 0.001; a- as compared to control, b- as compared to HFD.

Fig. 4. (A) Effect of alpha amyrin on plasma triglycerides levels. (B) Effect of alpha amyrin on plasma total cholesterol levels on HFD-induced MetS. (C) Effect of alpha amyrin on plasma HDL-cholesterol levels on HFD-induced MetS. Values are expressed as mean ± SEM (n=6). *p < 0.05, **p < 0.01, ***p < 0.001; a - as compared to control, b - as compared to HFD.

Fig. 5. Effect of alpha amyrin on systolic blood pressure. Values are expressed as mean ± SEM (n=6). **p < 0.01, ***p < 0.001; a - as compared to control, b - as compared to HFD.
Fig. 6. (A) Effect of alpha amyrin on hepatic TBARS. (B) Effect of alpha amyrin on hepatic reduced glutathione. (C) Effect of alpha amyrin on hepatic superoxide dismutase. (D) Effect of alpha amyrin on hepatic catalase activity. Values are expressed as mean ± SEM (n=6). *p < 0.05, **p < 0.01, ***p < 0.001; a - as compared to control, b - as compared to HFD.

Fig. 7. Effect of alpha amyrin on hepatic mRNA level of PPAR-α. ***p < 0.001; a - as compared to control, b - as compared to HFD.

Fig. 8. Effect of alpha amyrin on hepatic protein level of PPAR-α. *p < 0.05, **p < 0.01, ***p < 0.001; a - as compared to control, b - as compared to HFD.

Fig. 9. Histopathological studies of hepatic tissue. (A) Control hepatic tissue (B) High fructose diet tissue (C) Pioglitazone treated tissue (D-F) Alpha amyrin (50, 100 and 200 mg/kg, respectively) treated tissues.
86x30mm (300 x 300 DPI)
76x45mm (300 x 300 DPI)
Relative level of hepatic PPAR-α mRNA

Control  HFD  HFD+Pio  HFD+AA  AA per se

76x46mm (300 x 300 DPI)