Journal of Traditional and Complementary Medicine 10 (2020) 511–519

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Calcium supplementation shows a hepatoprotective effect against high-fat diet by regulating oxidative-induced inflammatory response and lipogenesis activity in male rats

Sandeep Das, Dipayan Choudhuri*

Department of Human Physiology, Tripura University (A Central University), Suryamaninagar, Agartala, Tripura, 799022, India

ARTICLE INFO

Article history:
Received 20 November 2018
Received in revised form
28 May 2019
Accepted 1 June 2019
Available online 4 June 2019

Keywords:
Obesity
NAFLD
Oxidative
Antioxidant
Inflammation

ABSTRACT

Background and aim: High-fat diet (HFD) triggers obesity-related metabolic diseases like non-alcoholic fatty liver diseases (NAFLD). Calcium supplementation is known to have an anti-obesity effect. However, the effect of calcium supplementation has not been evaluated so far in context to hepatic functions on exposure to HFD. The goal of the present study was to investigate the role of calcium supplementation on hepatic function and other physiological markers in HFD induced NAFLD rats.

Experimental procedure: 18 male Wistar rats were divided into two groups; first group considered control group (n = 6) for the entire treatment period and the second group (n = 12) fed with HFD for 6 weeks to induce NAFLD model and then sub-divided into two groups (n = 6 rats); one group received HFD and other group received 1.0 gm $CaCO_3$/100 gm HFD for 30 days. After treatment, all animals were euthanized to collect the blood and liver for biochemical, enzymatic, oxidative, anti-oxidant, western blot and histological study.

Results and conclusion: Calcium supplementation significantly improved the anthropometric parameters and decreases the level of serum cholesterol, triglyceride, FFA and hepatic enzymes. Calcium supplementation significantly down-regulated the hepatic PPAR-γ mediated FAS activity, hepatic lipid accumulation, oxidative stress and restored the activities of antioxidant enzyme which further prevented the stimulation of pro-inflammatory response. Calcium supplementation also increases the hepatic protein expression of phosphorylated AMP-activated protein kinase. So, calcium supplementation showed a hepatoprotective effect during NAFLD by downregulating the oxidative induced inflammatory response stimulated by hepatic lipogenesis activity and subsequent lipid accumulation.

1. Introduction

Obesity and its associated cardiovascular and metabolic complications including insulin resistance, diabetes, hypertension, and dyslipidemia is a major global health concern. The most common cause of obesity is excess calorie intake mostly through diet. When energy consumption exceeds the energy expenditure, an excess accumulation of triglyceride occurs not only within the adipose tissue but also in other tissues including liver.[1] Excessive accumulations of fat during obesity are often associated with the disruption of normal cellular and physiological function which leads to the pathological condition if neglected for a prolonged period of time.[2]

The liver plays a critical role in lipid metabolism by taking up serum free fatty acids (FFA) that are involved in the synthesis, storage, and transport of lipid metabolites. The accumulation of excess triacylglycerol (TG) within the liver due to the entry of excess FFA from the obese adipose tissue due to increased lipolysis leads to the development of non-alcoholic fatty liver diseases (NAFLD).[3,4] During obesity the excess accumulation of lipid within the liver leads to the fatty liver by stimulating peroxisome proliferator-activated receptor γ (PPARγ), a major metabolic transcription factor particularly associated with the induction of hepatic lipogenesis.[5] HFD can up-regulate the PPARγ activity and act

* Corresponding author.
E-mail address: dipayanchoudhuri@tripurauniv.in (D. Choudhuri).
Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2019.06.002

Abbreviations

FFA	free fatty acid
IL-6	interleukin-6
TNF-α	tumor necrosis factor-α
NAFLD	non-alcoholic fatty liver diseases
PPARγ	peroxisome proliferator-activated receptor γ
NASH	non-alcoholic steatohepatitis
HFD	high fat diet
HFD + Ca	high fat diet with calcium-supplementation
CAT	catalase
SOD	superoxide dismutase
GR	glutathione reductase
GPx	glutathione peroxidase
PCO	protein carbonyl content
NO	nitric oxide assay
TG	triglyceride
TC	total cholesterol
HDL	high-density lipoprotein cholesterol
AMPK	AMP-activated protein kinase
Alanine ALT	transaminase
AST	Aspartate transaminase
ALP	alkaline phosphatase
MDA	malonaldehyde
FAS	fatty acid synthase
CRP	c-reactive protein

like a pro steatotic factor leading to NAFLD.[6,7] The hepatic fat accumulation during NAFLD results in oxidative imbalance as a result of the decline in activity of several antioxidant enzymes, enhanced lipid peroxidation, and induction of pro-inflammatory cytokines like interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) as a result of mitochondrial dysfunction. This can have serious consequences starting from simple steatosis to liver cirrhosis.[8,9]

Emerging evidence revealed that dietary calcium plays a protective role against obesity and other metabolic complications like diabetes, dyslipidemia, and high blood pressure by preventing the excess accumulation of adipose tissue as well as improving body weight.[10,11] A diet enriched in calcium content is reported to improve the oxidative and inflammatory profile in transgenic obese mice as well as in obese individuals.[12] The increase in dietary calcium intake increases the fecal fat excretion by forming insoluble complexes between calcium and fat.[13]

AMP-activated protein kinase (AMPK), is a key regulator of hepatic lipid and carbohydrate metabolism. Its activation by phosphorylation stimulates the hepatic fatty acid oxidation and inhibits the hepatic lipogenesis pathway and prevents the development of NAFLD.[14] Calcium and VitD are found to activate AMPK through phosphorylation in different tissues. Post weaning low calcium diet decreases the AMPK activation in skeletal muscle.[15] On the other hand, HFD leads to obesity which further catalyzes the progression of NAFLD and non-alcoholic steatohepatitis (NASH).[16] HFD induced obesity not only promotes hepatic oxidative stress and triggers various inflammatory processes[17] but also reduces the activation of AMPK which further stimulates the metabolic disturbances during NAFLD.[18] So, the role of AMPK activity in response to calcium supplementation needs to be investigated in order to understand the therapeutic effect of calcium supplementation in NAFLD because AMPK acts as a vital signaling molecule by regulating several hepatic metabolic pathways including lipogenesis activity.

Dietary calcium supplementation has been shown to reduced weight gain and reduces metabolic complications associated with obesity.[19] The protective role of calcium supplementation against obesity-related metabolic complications is gaining attention in recent times worldwide due to its cost-effective application and its minimum side effects. Based on the accumulating evidence a dietary therapy with calcium supplementation was hypothesized to be a good option against HFD induced hepatic disorders. With this view, we aimed to study the effect of calcium supplementation in regulating the physiological alterations in various parameters including oxidative, inflammatory and lipogenesis activity within the liver of male Wistar rats exposed to the HFD.

2. Materials and methods

2.1. Animals and experimental design

The study included 18 healthy adult male albino rats of Wistar strain weighing 200–210 gm. Animals were collected from the government-registered animal supplier and housed in clean polypropylene cages in institutional animal house temperature at 22 ± 2 °C and a constant 12:12 light: dark schedule.

After one week of acclimatization, the rats were divided into two groups. The first group (n = 6) fed with control diet throughout the entire period of study and regarded as the control group. The second group (n = 12) was fed with HFD for 6 weeks to induce fatty liver as reported in the earlier study.[20] After the induction period, second group was further divided into two groups each group containing 6 rats out of which one received HFD and other group fed with calcium-supplemented with HFD as (HFD + Ca), which received 1.0 gm $CaCO_3$/100 gm HFD/day for another 30 days. HFD composed of fat 46%, carbohydrate 24%, proteins 20.3%, fiber 5%, salt mixture 3.7% and a vitamin mixture 1%. Composition of the control diet consists of fat 5%, carbohydrate 65%, protein 20.3%, fiber 5%, salt mixture 3.7% and a vitamin mixture 1%. All the compositions of the diets were in gm/kg.[21] The body weight of the animal was recorded weekly and food consumption was recorded daily during the whole period of treatment. The entire study protocol was approved by the Institutional Animal Ethical committee.

2.2. Collection and processing of samples

After the completion of the treatment period, all the animals were fasted overnight and euthanasied the next day following the Indian Council of Medical Research (ICMR) guidelines. The adipose tissue including epididymal, perirenal and omental fat tissues and the liver was dissected out from each rat. Tissues were weighed immediately to prevent the evaporative loss and kept at −80 °C for future analysis. Blood was collected immediately by cardiac puncture in a clotted vial and left to coagulate at 4 °C and then centrifuged at 3000×*g* for 15 min. The clear serum was collected for biochemical analysis.

2.3. Anthropometric parameter and food intake

The final body weight and body weight gain were calculated by subtracting the final body weight from initial body weight at the end of the study. Food consumption (gm/day/rat) was calculated daily by subtracting the amount of food left over in each cage for each rat from the measured amount of food provided on the previous day. Mean food consumption for each rat was calculated by

dividing the amount of food eaten in a week divided by seven. The average food consumptions were represented in gm/day/rat.

2.4. Preparation of liver tissue homogenate for antioxidant and oxidative assay

A 5% (W/V) tissue homogenate of liver tissue was prepared in ice-cold 50 mM phosphate buffer (pH = 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) using Potter-Elvehjem glass homogenizer. The homogenates were centrifuged (Sigma Laborzentrifugen, Germany) at 10,000 rpm for 20 min at 4 °C. After the centrifugation, the supernatant was collected and used for catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), reduced glutathione, protein carbonyl content (PCO) and nitric oxide assay (NO) assay. For lipid peroxidation assay a 10% (W/V) of liver tissue homogenate was prepared in 1.5% KCl.

2.5. Biochemical analysis of blood

Lipid profile including serum triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels was assayed by the enzymatic colorimetric method using standard commercial kits (Erba, Mannheim) in a semi auto-analyzer (Analytica, Biochemistry Analyzer, RMS). Non-HDL cholesterol was calculated using the formula as mentioned in the following study.[22] Serum free fatty acids (FFA) were estimated colorimetrically according to the manufacturer's protocol (Abcam, USA). Liver enzymes alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were assayed by the colorimetric method using commercial kits (Erba, Mannheim) in semi-auto-analyzer. Serum calcium level was estimated by the Arsenazo dye method using a commercial calcium kit (Erba, Mannheim). Serum C-reactive protein (CRP) level was estimated using the enzyme-linked immunosorbent assay (ELISA) kit in microplate reader according to the protocol provided with the kit (Ray Biotech, USA).

2.6. Measurement of hepatic antioxidant enzymes

Catalase *(EC: 1.11.1.6)*: 50 μl of the supernatant was added to a cuvette containing 2 ml of phosphate buffer (pH 7.0) and 1 ml of 30 mM H_2O_2. CAT activity was measured spectrophotometrically (Spectroquant Pharo 300, Merck, Germany) at 240 nm and expressed as a micromole of H_2O_2 utilized/minute/mg of protein.[23]

Superoxide Dismutase *(EC: 1.15.1.1):* The final assay mixture of 1 ml consists of 75 mM of Tris−HCl buffer (pH 8.2), 30 mM EDTA, 2 mM pyrogallol and at the end 50 μl of tissue supernatant was added as the enzyme source and the absorbance was recorded spectrophotometrically at 420 nm. The activity was expressed as unit/mg of protein.[24]

Glutathione peroxidase *(EC: 1.11.1.9)*: The final volume consists of 1 ml, 0.05 M phosphate buffer with 2 mM EDTA, (pH 7.0) 0.025 mM sodium azide, 0.15 mM reduced glutathione, and 0.25 mM nicotinamide adenine dinucleotide phosphate reduced (NADPH) and 100 μl of 5% tissue supernatant as the enzyme source. The reaction was started by the addition of 0.36 mM H_2O_2. Absorbance at 340 nm was recorded spectrophotometrically and expressed as nmol of NADPH oxidized/min/mg protein.[25]

Glutathione Reductase (*EC 1.6.4.2*): Briefly, 1 ml of 2.728 mM glutathione oxidized (GSSG) solutions and 40 μl of tissue supernatant were incubated in a water bath at 37 °C. After incubation for 5 min, the reaction was initiated by the addition of 200 μl of 1.054 mM NADPH solution. Decrease in absorbance was measured at 340 nm using a spectrophotometer and recorded every 30 s over a period of 5 min. GR activity was expressed as unit per mg protein based on the molar extinction coefficient of 6.22×10^3 L/mol/cm. One unit of GR defined as the amount of enzyme that catalyzes the oxidation of 1 nmol of NADPH per minute.[26]

2.7. Reduced glutathione content

1.0 ml of the 5% sample was mixed with 0.1 mL of 25% trichloroacetic acid (TCA). The mixture was centrifuged at 5000×*g* for 10min to remove the precipitate. Supernatant (0.1 mL) was mixed with 2 mL of 0.6 mM Ellman's reagent prepared in 0.2 M sodium phosphate buffer pH (8.0). Absorbance was read at 412 nm and expressed as nmol/mg of protein.[27]

2.8. Lipid peroxidation assay

Lipid peroxidation was determined by thiobarbituric acid (TBA) method by quantifying the formation of malonaldehyde (MDA), an end product of lipid peroxidation.[28] To 1 ml of 10% tissue homogenate, 2.5 ml of 20% TCA was added and the contents were centrifuged at 3500 g for 10 min and the precipitate was dissolved in 2.5 ml of 0.05 M sulphuric acid. To this, 3 ml of TBA was added and the samples were kept in a hot water bath for 30 min. Samples were cooled and MDA was extracted with 4 ml of butanol and the pink color developed was determined spectrophotometrically at 530 nm and expressed as the nmol of MDA/mg of protein.

2.9. Nitric oxide assay

The accumulation of nitrite in the tissue supernatant, an indicator of the production of NO, was determined with a colorimetric assay with Greiss reagent 0.1% of N-(1-Naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid].[29] Concentration of nitrite was expressed as micromole/mg of protein.

2.10. Estimation of protein carbonyl content

Briefly, 200 μl of supernatant was added to plastic tubes containing 400 μl of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). Subsequently, 500 μl of 20% TCA was added to each tube. The mixture was vortexed and centrifuged at 14,000×*g* for 3 min and the supernatant obtained was discarded. The pellet was washed with 1 ml ethanol/ethyl acetate (1:1 v/v), vortexed and centrifuged at 14,000×*g* for 3 min. The supernatant was then discarded and the pellet-suspended in 600 μl of 6 M guanidine (prepared in a 20-mM potassium phosphate solution, pH 2.3), vortexed and incubated at 60 °C for 15 min. Samples were then centrifuged at 14,000×*g* for 3 min and the supernatant used to measure absorbance at 370 nm and reported the PCO in nmol/mg protein.[30]

2.11. Tissue protein content

Protein content from liver homogenate was estimated using bovine serum albumin as the standard protein.[31]

2.12. Western blot

The hepatic tissue was homogenized into cold lysis buffer and the lysate was centrifuged and the supernatant was collected in a fresh tube supplemented with protease inhibitor cocktail (Himedia, India) and kept at −80 °C.[32] The protein samples were heat denatured and separated by SDS-PAGE followed by electrotransfer to the nitrocellulose membrane. The membranes were blocked in blocking buffer and then exposed to specific primary antibodies diluted in the blocking buffer and kept overnight at 4 °C. The primary antibodies used were rabbit polyclonal anti-phospho AMPK, anti-

AMPK, anti-IL-6 and anti-TNF-α (Abcam, USA). The next day membrane was washed and subjected to incubation for 1 h with anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Thermo Fisher Scientific, USA) diluted in the same blocking buffer. After a series of washes, they were exposed with the chemiluminescent ECL substrates (Thermo Scientific, USA) and the desired protein bands were detected by chemiluminescent gel documentation system (Bio-Rad, USA). β-Actin was used as a housekeeping protein for equal amounts of protein loaded into the gel. The protein bands were densitometry quantified by Image J, software (NIH, USA) and data presented as arbitrary units.

2.13. Determination of nuclear PPAR-γ transcriptional activity within the liver

Hepatic nuclear extracts were extracted using hypotonic lysis buffer which was again followed by high salt extraction.[33] The freshly isolated nuclear extract was used to measure the nuclear PPAR-γ transcriptional factor activity according to the protocol provided with the PPAR-gamma transcription factor assay kit (Abcam, USA).

2.14. Measurement of hepatic fatty acid synthase (FAS) activity and triglyceride level

Preparation of hepatic mitochondrial supernatant was done according to Schneider et al.[34] The freshly prepared supernatant was used for FAS activity measurement. FAS activity in the liver was measured by the malonyl CoA–dependent oxidation of NADPH at 37 °C.[35] One unit of enzyme activity represents 1 mmol of NADPH oxidized per minute at 37 °C. Results were expressed as mU/g of liver tissue. Triglyceride was extracted from the liver using chloroform: methanol (2:1) mixture and quantified according to this study.[36] Triglyceride was presented as mg of triglyceride/g of tissue.

2.15. Determination of fecal fat content

Around 0.5 gm of faces collected at 6th week of the study were soaked in deionized water for 24 h at 4 °C, followed by homogenization by vortexing at high speed for one minute. Now, lipids were extracted with methanol: chloroform (2:1, v: v) and shaken for 30 min, followed by addition of 2.5 ml of deionized water and 2.5 ml of chloroform and further shaking for 30 min. The mixture was then centrifuged at 2000 g for 15 min and the lipophilic layer from the extraction was collected and dried under vacuum. The total fat content was weighed using a laboratory balance.[37]

2.16. Histological analysis

The liver was gently washed in phosphate buffer saline (PBS) and subjected to fixation in 10% neutral buffered formalin and thereafter further processing were done which includes dehydration and infiltration followed by embedding in paraffin. Then 5 (μm) sections of paraffin block were obtained by using a microtome and taken for staining with Hematoxylin-Eosin (H & E) stain. The pictures of stained slides were obtained under a microscope (Leica DM400 BLED, Germany) at 20X magnification.

2.17. Statistical analysis

Results were presented as means ± standard error mean (SEM). All the parametric values follow the normality assumptions and it has been verified through the Kolmogorov-Smirnov test. Differences between the means of groups were analyzed by one way ANOVA followed by Tukey HSD posthoc test using SPSS (Statistical Program for the Social Sciences) 16.0 for Windows. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Effect calcium supplementation on anthropometric parameters and food intake

At the end of the study, the final body weight and the body weight gain was significantly decreased (**$p < 0.01$;** Table 1**)** in (HFD + Ca) group in comparison to the HFD group. The mass of the adipose tissue and the liver was significantly decreased (**$p < 0.01$;** Table 1**)** in (HFD + Ca) group as compared to the HFD group. There was no significant difference in the daily food intake among the three groups throughout the entire period of study.

3.2. Effect of calcium supplementation on blood biochemical parameters

The (HFD + Ca) group showed an improvement in the lipid profile by significantly decreasing (**$p < 0.01$;** Table 2) the TG, TC and non-HDL cholesterol level and significantly increasing (**$p < 0.05$;** Table 2) the HDL-cholesterol as compared to the HFD group. Serum FFA was significantly reduced (**$p < 0.01$;** Table 2) in (HFD + Ca) group as compared to the HFD group. The three hepatic enzymes ALT, AST, and ALP were significantly reduced (**$p < 0.01$;** Table 2) in (HFD + Ca) group as compared to the HFD group. No significant difference was observed in the serum calcium level among the three groups at the end of the study in Table 2.

3.3. Effect of calcium supplementation on PPAR-γ activity, FAS activity and triglyceride level within the liver

Hepatic PPAR-γ, FAS activity, and triglyceride level were significantly reduced (**$p < 0.01$;** Fig. 1-A, B & C) in the (HFD + Ca) group as compared to the HFD group.

3.4. Effect of calcium supplementation on hepatic antioxidant enzyme activities and reduced glutathione level

The hepatic activities of antioxidant enzymes like CAT, SOD, GPx, and GR were significantly increased (**$p < 0.01$;** Table 3) in the (HFD + Ca) group as compared to the HFD group. The amount of reduced glutathione level was significantly increased (**$p < 0.01$;** Table 3) in (HFD + Ca) group as compared to the HFD group.

3.5. Effect of calcium supplementation on oxidative stress parameters

Results of our study showed that calcium supplementation plays a protective role against HFD induced oxidative stress in the liver.

Table 1
Anthropometrical data and food intake.

Parameters	CONTROL	HFD	HFD + Ca
Initial body weight (gm)	203.83 ± 1.74	205.13 ± 1.77	206.12 ± 1.81
Final body weight (gm)	352.5 ± 3.77	404.26 ± 4.23	364.5 ± 3.26[a]
Body Weight gain (gm)	149.67 ± 1.37	199.13 ± 1.71	158.38 ± 1.41[a]
Adipose tissue mass (gm)	14.91 ± 0.48	22.71 ± 0.82	17.01 ± 0.56[a]
Liver Weight (gm)	11.37 ± 0.35	14.82 ± 0.43	12.59 ± 0.39[b]
Daily food intake (gm/day/rat)	21.57 ± 0.29	21.92 ± 0.49	19.95 ± 0.69

Values represent the mean ± SEM of six (6) rats in each group. [a]Significantly different from the HFD group at $p < 0.01$. [b]Significantly different from the HFD group at $p < 0.05$.

Table 2
Biochemical analysis of blood.

Blood Parameters	CONTROL	HFD	HFD + Ca
Total cholesterol (mg/dl)	92.56 ± 2.12	112.04 ± 3.12	95.6 ± 2.52[a]
Triglyceride (mg/dl)	71.82 ± 1.41	84.89 ± 1.77	74.58 ± 1.48[a]
HDL- cholesterol (mg/dl)	34.76 ± 1.02	27.51 ± 0.97	32.64 ± 0.83[b]
Non-HDL cholesterol (mg/dl)	57.80 ± 1.69	84.52 ± 1.87	63 ± 1.67[a]
Serum FFA (μmole/L)	97.33 ± 2.57	178.62 ± 3.82	115.66 ± 3.08[a]
ALT (U/L)	67.8 ± 1.96	102.87 ± 2.95	71.23 ± 2.23[a]
AST (U/L)	32.49 ± 1.34	69.29 ± 1.72	35.82 ± 1.25[a]
ALP (U/L)	167.92 ± 3.71	249.44 ± 4.16	178.41 ± 3.77[a]
Serum calcium (mmol/l)	2.38 ± 0.05	2.54 ± 0.07	2.71 ± 0.05

Values represent the mean ± SEM of six (6) rats in each group. [a]Significantly different from the HFD group at $p < 0.01$. [b]Significantly different from the HFD group at $p < 0.05$. ALT-Alanine Transaminase; AST-Aspartate Transaminase; ALP-Alkaline Phosphatase; HDL cholesterol High-density lipoprotein cholesterol.

The following oxidative stress parameters including lipid peroxidation, protein carbonylation level and NO generation measured as nitrite within the hepatic tissue were significantly reduced (**p < 0.01;** Fig. 3 A, B, C) in (HFD + Ca) group as compared to the HFD group.

3.6. Effect of calcium supplementation on hepatic p-AMPK expression and inflammatory markers within the liver and serum

The p-AMPK expression was significantly increased (**p < 0.01;** Fig. 2B) in the (HFD + Ca) group as compared to the HFD group. The (HFD + Ca) group found to have a significantly lower (**p < 0.01;** Fig. 2C) the expression of IL-6 and TNF-α as compared to the HFD group. The serum CRP level in (HFD + Ca) group was significantly decreased (**p < 0.01;** Fig. 2D) in comparison to the HFD group at the end of the study.

3.7. Effect of calcium supplementation on fecal fat excretion

The (HFD + Ca) group rats showed significantly increased (**p < 0.01,** Fig. 4) rate of fecal fat excretion during the 6th week of the experimental period as compared to the HFD group.

3.8. Effect of calcium supplementation on histopathological changes

At the end of the study, HFD seemed to increase the size of the liver organ as compared to other two groups observed in Fig. 5. For further evaluation of hepatic tissue histopathological study was

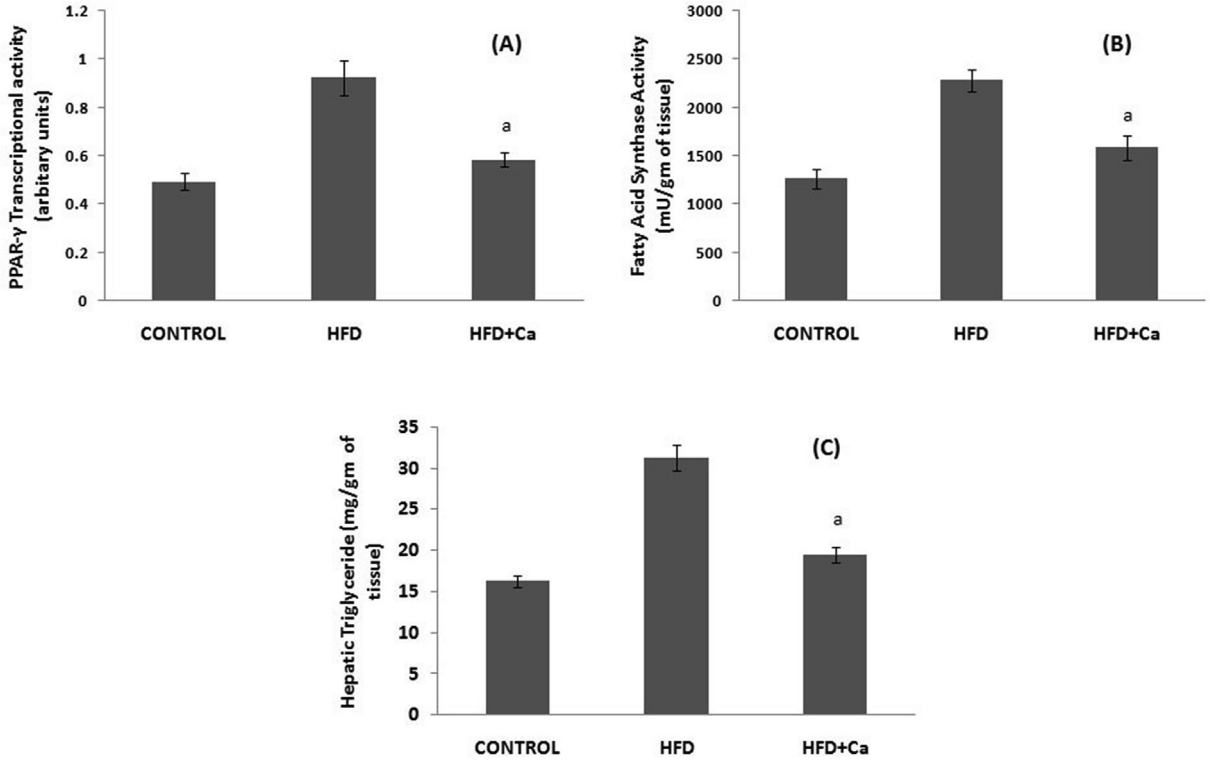

Fig. 1. Determination of hepatic PPAR-γ activity, fatty acid synthase (FAS) activity, and tissue triglyceride level. A-Hepatic PPAR-γ activity, B-Fatty acid synthase activity, and C-Hepatic triglyceride level. Values represent the mean ± SEM of six (6) rats in each group. [a]Significantly different from the HFD group at $p < 0.01$.

Table 3
Activity of antioxidant enzymes and reduced glutathione level within the liver.

Parameters	CONTROL	HFD	HFD + Ca
Catalase (μmole of H_2O_2 utilized/minute/mg of protein)	36.16 ± 1.54	27.01 ± 1.72	32.98 ± 1.27[a]
Superoxide Dismutase (Unit/mg of protein)	6.36 ± 0.28	3.43 ± 0.19	5.56 ± 0.25[a]
Glutathione Peroxidase (nmol of NADPH oxidized/min/mg protein)	23.76 ± 1.16	15.52 ± 1.01	21.27 ± 1.12[a]
Glutathione Reductase (Unit/mg of protein)	149.36 ± 2.59	126.29 ± 2.03	145.81 ± 2.56[a]
Reduced glutathione Content (nmol/mg protein)	27.71 ± 1.12	16.16 ± 0.82	24.28 ± 0.97[a]

Values represent the mean ± SEM of six (6) rats in each group. [a]Significantly different from the HFD group at $p < 0.01$.

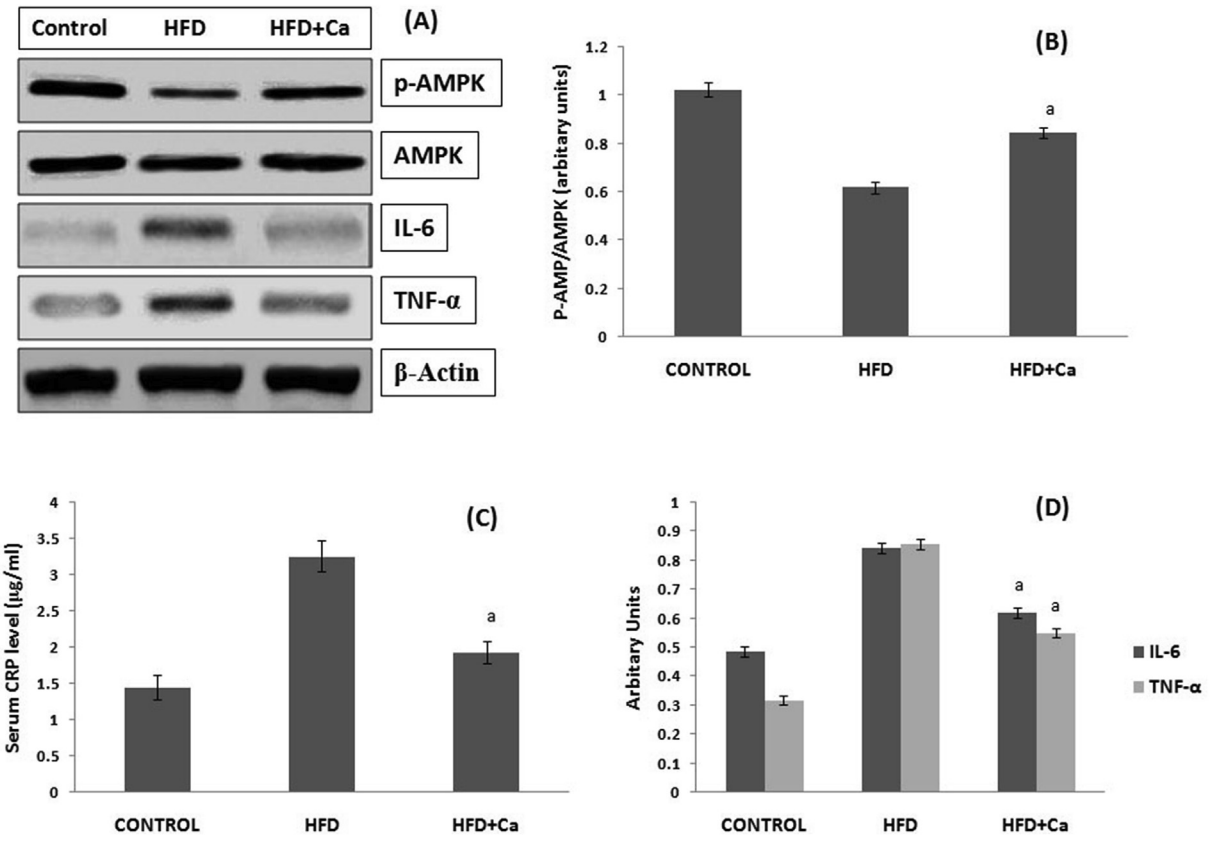

Fig. 2. Protein expression. A-Shows the images of protein expression of p-AMPK, AMPK and inflammatory markers (IL-6 and TNF-α) within the liver carried by western blot analysis. β-Actin served as the loading control. B-Protein expression of p-AMPK. C-Protein expression of inflammatory markers (IL-6 and TNF-α). All the expressions were analyzed and normalized with β-Actin and represented as arbitrary units. Values expressed as Mean ± SEM of three (3) rats in each group. C-Estimation of serum CRP level to detect the circulating inflammatory response. Values represent mean ± SEM of six (6) rats in each group for serum CRP level. [a]Significantly different from the HFD group at $p < 0.01$.

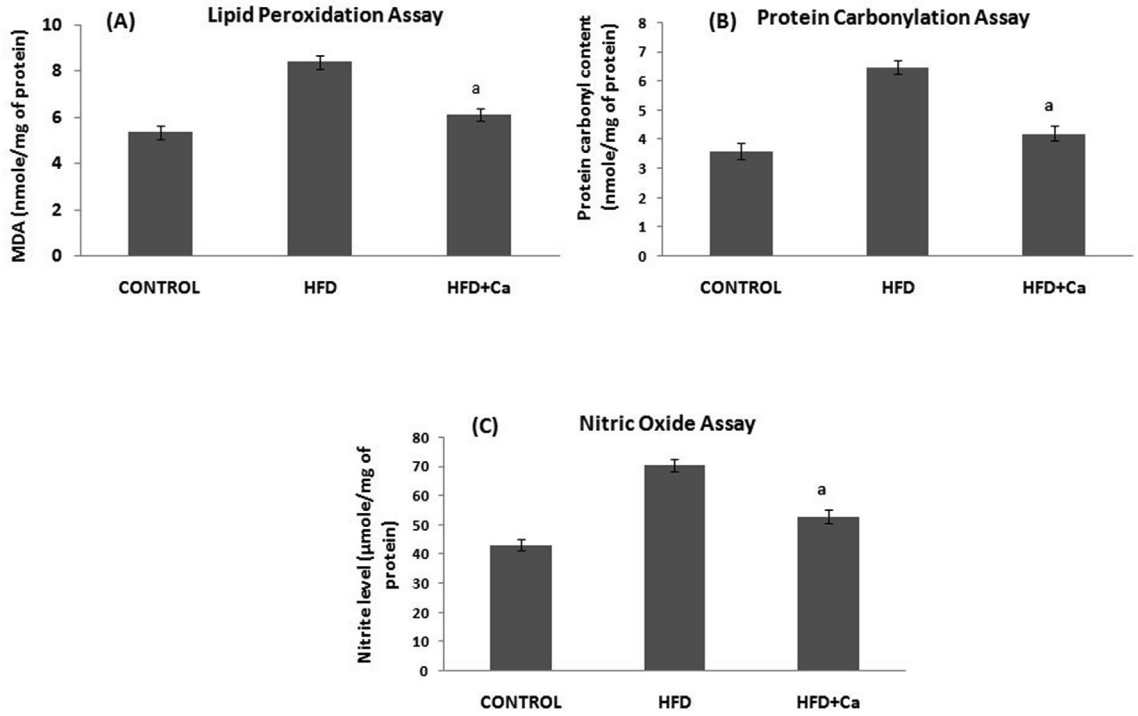

Fig. 3. Hepatic lipid peroxidation, nitric oxide, and protein carbonylation assay. A-Malonaldehyde level, B- Protein carbonylation and C- Nitrite level. Values represent the mean ± SEM of six (6) rats in each group. [a]Significantly different from the HFD group at $p < 0.01$.

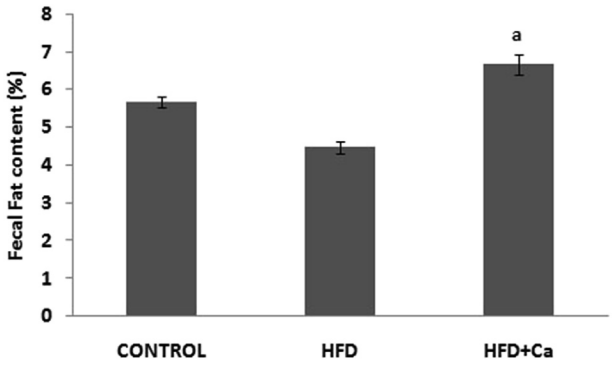

Fig. 4. Determination of fecal fat excretion. Values represent the mean ± SEM of six (6) rats in each group. [a]Significantly different from the HFD group at $p < 0.01$.

carried out by H & E staining from three groups in order to evaluate the effect of calcium supplementation on HFD induced NAFLD presented in Fig. 5. The control group showed normal histological architecture with no lipid droplet accumulation and swelling of hepatocytes. Induction of NAFLD by exposure to HFD was confirmed by the presence of lipid droplets marked by black arrows shown in Fig. 5. On the other hand formation of lipid droplets were almost absent in the HFD + Ca supplemented rats and showing similar histological architecture with those of the control group observed in Fig. 5.

4. Discussion

Current approach to inhibit NAFLD includes weight management, prevention of adipocyte dysfunction, oxidative stress reduction, improvement in insulin resistance, and lipid profile optimization. Several herbal and natural supplements have been tried recently as possible therapeutic agents against NAFLD because of their anti-oxidant, anti-inflammatory, anti-apoptotic and anti-adipogenesis effect.[38] But most of these therapies lack specificity and effectiveness owing to their poor bioavailability, poor aqueous solubility, chemical instability, and intestinal metabolism. Researchers are trying to overcome this shortcoming by combining these substances with nanoparticles for targeted drug delivery.[39] Studies suggested that several natural constituents present in food possess insulin-sensitizing, hypolipidemic, anti-adipogenic, anti-inflammatory and antioxidant effects and can have a beneficial effect against HFD induced NAFLD.[38] Earlier reports from both epidemiological as well as in-vivo studies reported that diet rich in calcium content improves the metabolic profile during the obese condition by regulating several cellular events.[10] The preventive role of a high calcium diet against excess fat accumulation and body weight gain were observed in earlier studies.[40,41] These reports supported our present observation regarding the anti-obesity effect of calcium supplementation against HFD that involved an improvement in the anthropometric parameters like decrease in the body weight gain, accumulation of excess adipose tissue and liver weight gain.

Consumption of HFD leads to dyslipidemia, a common metabolic disorder in obesity with an increase in blood triglyceride, total cholesterol, non-HDL cholesterol and a decrease in HDL-cholesterol level.[42] Calcium supplementation in our study showed a protective effect against HFD induced dyslipidemia by preventing alteration in the lipid profile. Similar to our findings, Shifdar et al. observed that calcium supplementation in overweight men reduced the risk of cardiovascular diseases by lowering the serum total cholesterol level, LDL-cholesterol and blood pressure.[19] Hence, calcium supplementation with HFD shown to have a protective role against chronic dyslipidemia which increases the risk of both cardiovascular and fatty liver diseases.

We observed that HFD stimulated hepatic oxidative stress by increasing lipid peroxidation, nitric oxide generation and oxidation of protein molecules and depletion of hepatic anti-oxidant enzymes including SOD, CAT, GPx and GR which were supposed to neutralize the toxic effect of the excess free radicals generated during oxidative stress causing hepatocellular injury. These alterations in the physiological balance between the oxidative stress and antioxidant enzymes during HFD consumption further worsen the hepatic functions leading to the development of NASH and insulin

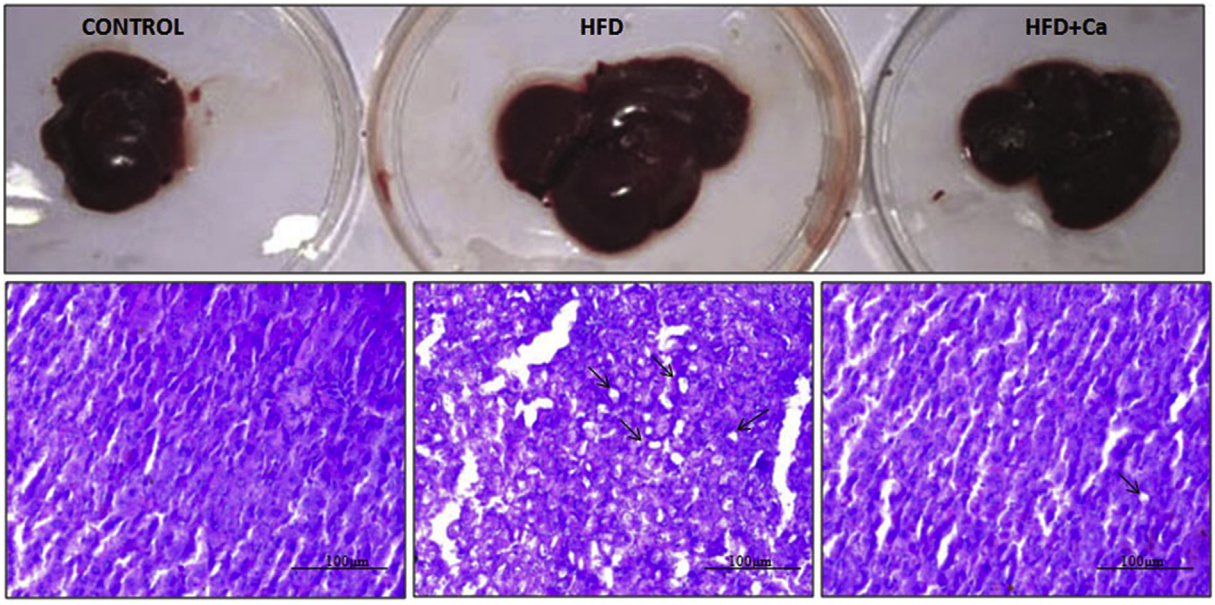

Fig. 5. Histopathological analysis was carried out from the liver of various groups. The images of fresh liver shown in Fig. 5 from the three groups. Histopathological examination of liver tissue was carried by hematoxylin and eosin (H & E) staining and the microscopic images were viewed under 20× magnification (scale bar = 100 μm). The presence of lipid droplets from the H & E stained sections were marked by black arrows indicating the features of HFD induced NAFLD.

resistance.[2,17] Various agents including some of the dietary components were reported to induce liver damage by increasing oxidative stress in hepatic cells. Substances acting against oxidative stress have been found to be beneficial in such conditions. One of the widely studied substances that prevent oxidative stress and thereby protect against hepatic damage is naturally occurring curcumin.[43] In our study, calcium supplementation showed a similar protective effect against HFD induced oxidative stress by restoring the physiological balance between oxidative stress and antioxidant enzymes. The protective role of the calcium supplementation might be due to the inhibition of the excess hepatic lipid accumulation which is considered to be a potent stimulator for inducing oxidative stress in HFD obese rats.[2] During HFD consumption it lowered the level of reduced glutathione concentration which was restored to near the normal level by calcium supplementation. A similar decline of the glutathione with exposure to HFD was observed in studies of Poudyal et al..[44] The oxidative damage within the hepatic tissue during HFD consumption is often associated with the alteration in the liver enzymes like ALP, ALT, and AST which is generally seen in the obesity-associated fatty liver due to the changes in the hepatic metabolism. Restoration of the liver enzymes to their physiological levels in the calcium-supplemented group revealed the preventive effect of calcium supplementation against hepatic oxidative stress in the HFD group.

Lipid peroxidation is associated with cellular injury which in turn releases TNF-α as well as increases the hepatic expression of IL-6 along with an increase in plasma IL-6 level in HFD induced obesity.[45] Similar to these findings, our observation also showed an increase in hepatic IL-6 and TNF-α expression in the HFD group. On the other hand, calcium supplementation showed a protective effect against the HFD induced inflammatory response within the liver by reducing both hepatic IL-6 and TNF-α expression. The decrease in hepatic inflammation further resulted in the decline of the serum CRP level in calcium-supplemented rats which generally increases during obesity.[45] The decrease in hepatic inflammation in calcium-supplemented rats might provide future protection against insulin resistance as well as hepatic steatosis associated with obesity.[1] Further, we observed that calcium supplementation increased the fecal fat excretion which might lower the circulating free fatty acids thereby preventing the uptake of FFA by the liver. During the intake of calcium-rich diet, the calcium binds to the fatty acids in the colon and prevents the fat absorption thereby eliminating the fatty acids through feces.[46] Thus, the low level of circulating FFA in our calcium supplemented rats might further prevent the activation of the inflammatory response by down-regulating the production of TNF-α which are produced by FFA induced activated Kupffer cells.[14]

The hepatic fatty acid metabolism is under the tight regulation of diverse nuclear receptors, like peroxisome proliferator-activated receptor (PPARs) and liver X receptor (LXR). These receptors are regulated by hepatic lipid accumulation which in turn alters the expression of several genes involved in fatty acid metabolism.[14] We observed a decline in PPAR-γ activity in animals of the calcium-supplemented group which might, in turn, suppressed the lipogenic activity as shown by inhibition of hepatic FAS activity and triglyceride accumulation. The entry of fatty acid through blood circulation into the liver from obese adipose tissue in the HFD group might have up-regulated the PPAR-γ activity as fatty acids within the liver act as a ligand for its activation.[47] PPAR-γ up-regulation by HFD triggers NAFLD through the stimulation of lipogenic factors, which are implicated in the fatty acid synthesis and exerting noxious effects by increasing the hepatic lipogenesis.[48] The decrease in the PPAR-γ activity in calcium-supplemented group protects from lipid accumulation as seen in liver-specific PPAR-γ knock out mice which shows protection against hepatic lipid accumulation, improved glucose tolerance and down-regulation of the lipogenic pathway.[7] This reduction in the hepatic lipid accumulation by downregulating the lipogenesis activity might have reduced the degree of lipid droplet formation as well as the degree of steatosis and prevented the progression towards NASH and as observed from the histopathological data of our study. The down-regulation of hepatic lipid accumulation prevents the hepatocytes from hepatic oxidative stress in HFD induced NAFLD rats.[49]Dietary effect of calcium supplementation seemed to prevent the progression of NAFLD towards NASH as seen by an effective dietary supplement like Naringenin, through several cellular mechanism involving the inhibition of pro-inflammatory reactions mediated by TNF-α, IL-6, TLR-4, inducible nitric oxide synthase (iNOs), cyclooxygenase-2 and NADPH oxidase-2 and also prevent the metabolic dysregulation by activating AMP-activated protein kinase (AMPK).[14]

In addition, we further examine the involvement of hepatic AMP-activated protein kinase (AMPK) activity after considering its potential role in hepatic lipid metabolism and could be a possible target for the treatment of fatty liver.[14] The down-regulation AMPK activity during HFD consumption in our study may be associated with the pathological consequences of obesity-induced fatty liver as seen in earlier reports.[50] AMPK, is a crucial metabolic sensor whole activation through phosphorylation down-regulates the hepatic lipogenesis activity and glucose production during the development of NAFLD.[51] Thus, re-activation of AMPK activity in response to calcium supplementation in our study suppressed the lipogenic pathway thereby reducing the hepatic lipid content. Not only calcium and vitamin D regulate AMPK activity in muscle and heart[15,52] but calcium supplementation individually also seemed to regulate hepatic AMPK activity as observed from our study. Further, it was also observed that activation of AMPK may down-regulate the hepatic PPAR-γ activity and subsequent lipid accumulation as in HFD induced obese mouse model.[53] Apart from regulating metabolic pathways, AMPK activation can also suppress the oxidative stress as well as nuclear kappa beta (NF-κβ) mediated inflammatory responses in HFD diet-induced obesity and its associated metabolic complications including NAFLD.[17] So the changes in the activity of hepatic AMPK through phosphorylation seemed to play a vital role against HFD induced fatty liver.

5. Conclusion

In conclusion, calcium supplementation showed a hepatoprotective effect against HFD by preventing the oxidative stress, inflammation, up-regulating the AMPK activity and down-regulating the PPAR-γ mediated lipogenesis activity. Since liver plays a vital role in the development of insulin resistance further studies regarding the effect of dietary calcium on several hepatic signaling pathways associated with glucose metabolism and diabetes are required to fully elucidate the role of calcium supplementation in the progression of metabolic disorders during HFD consumption.

Conflicts of interest

The authors declare they have no conflict of interest.

Acknowledgments

Indian Council of Medical Research (ICMR) Govt. of India, New Delhi is deeply acknowledged for this study. The instrumental facility is provided by The State Biotech Hub, Tripura University is acknowledged.

References

1. Jung UJ, Choi MS. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *Int J Mol Sci*. 2014;15:6184–6223.
2. Kakimoto PA, Kowaltowski AJ. Effects of high fat diets on rodent liver bioenergetics and oxidative imbalance. *Redox Biology*. 2016;8:216–225.
3. Musso G, Gambino R, Cassader M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Prog Lipid Res*. 2009;48:1–26.
4. Stankovic MN, Mladenovic DR, Duricic I, et al. Time-dependent changes and association between liver free fatty acids, serum lipid profile and histological features in mice model of nonalcoholic fatty liver disease. *Arch Med Res*. 2014;45:116–124.
5. Ragab SSM, Abd Elghaffar SK, El-Metwally TH, Badr G, Mahmoud MH, Omar HM. Effect of a high fat, high sucrose diet on the promotion of non-alcoholic fatty liver disease in male rats: the ameliorative role of three natural compounds. *Lipids Health Dis*. 2015;14(83):1–11.
6. Vidal-Puig A, Jimenez-Liñan M, Lowell BB, et al. Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. *Eur J Clin Investig*. 1996;97:2553–2561.
7. Morán-Salvador E, López-Parra M, García-Alonso V, et al. Role for PPARγ in obesity-induced hepatic steatosis as determined by hepatocyte-and macrophage-specific conditional knockouts. *FASEB J*. 2011;25:2538–2550.
8. Rolo AP, Teodoro JS, Palmeira CM. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radic Biol Med*. 2012;52:59–69.
9. Than NN, Newsome PN. A concise review of non-alcoholic fatty liver disease. *Atherosclerosis*. 2015;239:192–202.
10. Shrager S. Dietary calcium intake and obesity. *J Am Board Fam Pract*. 2005;18:205–210.
11. Jacqmain M, Doucet E, Després JP, Bouchard C, Tremblay A. Calcium intake, body composition, and lipoprotein-lipid concentrations in adults. *Am J Clin Nutr*. 2003;77:1448–1452.
12. Zemel MB, Sun X. Dietary calcium and dairy products modulate oxidative and inflammatory stress in mice and humans. *J Nutr*. 2008;138:1047–1052.
13. Christensen R, Lorenzen JK, Svith CR, et al. A Effect of calcium from dairy and dietary supplements on faecal fat excretion: a meta-analysis of randomized controlled trials. *Obes Rev*. 2009;10(4):475–486.
14. Zobeiri M, Belwal T, Parvizi F, et al. Naringenin and its nano-formulations for fatty liver: cellular modes of action and clinical perspective. *Curr Pharmaceut Biotechnol*. 2018;19:196–205.
15. He YH, Li ST, Wang YY, et al. Postweaning low-calcium diet promotes later-life obesity induced by a high-fat diet. *J Nutr Biochem*. 2012;23:1238–1244.
16. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic and clinical implications. *Hepatology*. 2010;51(2):679–689.
17. Kesh SB, Sarkar D, Manna K. High-fat diet-induced oxidative stress and its impact on metabolic syndrome: a review. *Asian J Pharmaceut Clin Res*. 2016;9(1):47–52.
18. Smith BK, Marcinko K, Desjardins EM, Lally JS, Ford RJ, Steinberg GR. Treatment of nonalcoholic fatty liver disease: role of AMPK. *Am J Physiol Endocrinol Metab*. 2016;311:E730–E740.
19. Shidfar F, Moghayedi M, Kerman SRJ, Hosseini S, Shidfar S. Effects of a calcium supplement on serum lipoproteins, apolipoprotein B, and blood pressure in overweight men. *Int J Endocrinol Metab*. 2010;8(4):194–200.
20. Sasidharan SR, Joseph JA, Anandakumar S, Venkatesan V, Madhavan CNA, Agarwal A. Ameliorative potential of *Tamarindus indica* on high fat diet induced nonalcoholic fatty liver disease in rats. *Sci World J*. 2014;507197:1–10.
21. Noeman SA, Hamooda HE, Baalash AA. Biochemical study of oxidative stress markers in the liver, kidney and heart of high fat diet induced obesity in rats. *Diabetol Metab Syndrome*. 2011;3(17):1–8.
22. Xavier HT, Izar MC, Faria Neto JR, et al. Diretriz Brasileria de dislipidemia e prevenção da aterosclerose. *Arq Bras Cardiol*. 2013;101:1–20.
23. Aebi H. Catalase in vitro. *Methods Enzymol*. 1984;105:121–126.
24. Marklund S, Marklund G. Involvement of superoxide anion radical in the antoxidation of pyrogallol: a convenient assay for superoxide dismutase. *Eur J Biochem*. 1974;47:469–476.
25. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med*. 1967;70:158–169.
26. Goldberg DM, Spooner RJ. Assay of glutathione reductase. In: *Methods of Enzymatic Analysis*. 1983:258–265.
27. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959;82:70–77.
28. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by the thiobarbituric acid reaction. *Anal Biochem*. 1979;95:351–358.
29. Green LC, Wagner DA, Glogowski J. Analysis of nitrate, nitrite, and [15 N] nitrate in biological fluids. *Anal Biochem*. 1982;126:131–138.
30. Reznick AZ, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol*. 1994;233:357–363.
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Follin phenol reagent. *J Biol Chem*. 1951;193:265–275.
32. Garcia PV, Seiva FRF, Carniato AP, et al. Increased toll-like receptors and p53 levels regulate apoptosis and angiogenesis in non-muscle invasive bladder cancer: mechanism of action of P-MAPA biological response modifier. *BMC Canc*. 2016;16(422):1–18.
33. Guilherme A, Tesz GJ, Guntur KVP, Czech MP. Tumor necrosis factor-α induces caspase-mediated cleavage of peroxisome proliferator-activated receptor in adipocytes. *J Biol Chem*. 2009;284(25):17082–17091.
34. Schneider WC. Intracellular distribution of Enzymes III the oxidation of octanoic acid by rat liver fractions. *J Biol Chem*. 1948;176:259–266.
35. Halestrap AP, Denton RM. Insulin and the regulation of adipose tissue acetyl CoA carboxylase. *Biochem J*. 1973;132:509–517.
36. Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem*. 1957;226(1):497–509.
37. Tsujita T, Takaichi H, Takaku T, Aoyama S, Hiraket J. Antiobesity action of epsilon-polysine, a potent inhibitor of pancreatic lipase. *J Lipid Res*. 2006;47:1852–1858.
38. Perumpail BJ, Li AA, Iqbal U, et al. Potential therapeutic benefits of herbs and supplements in patients with NAFLD. *Diseases*. 2018;6:1–19.
39. Wan S, Zhang L, Quan Y, Wei K. Resveratrol-loaded PLGA nanoparticles: enhanced stability, solubility and bioactivity of resveratrol for non-alcoholic fatty liver disease therapy. *R. Soc. open sci*. 2018;5(181457):1–15.
40. Zemel M. Regulation of adiposity and obesity risk by dietary calcium: mechanisms and implications. *J Am Coll Nutr*. 2002;21, 146S–51S.
41. Zemel M. Mechanisms of dairy modulation of adiposity. *J Nutr*. 2003;133, 252S–6S.
42. Woo MN, Bok SH, Lee MK, et al. Anti-obesity hypolipidemic effects of a proprietary herb fiber combination (S&S PWH) in rats fed high-fat diets. *J Med Food*. 2008;11:169–178.
43. Farzaei MH, Zobeiri M, Parvizi F, et al. Curcumin in liver diseases: a systematic review of the cellular mechanisms of oxidative stress and clinical perspective. *Nutrients*. 2018;10:1–28.
44. Poudyal H, Campbell F, Brown L. Olive leaf extract attenuates cardiac, hepatic, and metabolic changes in high carbohydrate–high fat–fed rats. *J Nutr*. 2010;140(5):946–953.
45. Meli R, Raso GM, Calignano A. Role of innate immune response in non-alcoholic fatty liver disease: metabolic complications and therapeutic tools. *Front Immunol*. 2014;5:1–14.
46. Soares MJ, She-Ping-Delfos WLC. Postprandial energy metabolism in the regulation of body weight: is there a mechanistic role for dietary calcium? *Nutrients*. 2010;2:586–598.
47. Gavrilova O, Haluzik M, Matsusue K, et al. Liver peroxisome proliferator-activated receptor γ contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. *J Biol Chem*. 2003;278:34268–34276.
48. Souza-Mello V. Peroxisome proliferator-activated receptors as targets to treat non-alcoholic fatty liver disease. *World J Hepatol*. 2015;7(8):1012–1019.
49. Davodi I, Rahimini R, Abdollahi M, et al. Promising effect of Rosa damascena extract on high-fat diet-induced nonalcoholic fatty liver. *J Trad Complement Med*. 2017;7:508–514.
50. Lindholm CR, Ertel RL, Bauwens JD, Schmuck EG, Mulligan JD, Saupe KW. A high-fat diet decreases AMPK activity in multiple tissues in the absence of hyperglycemia or systemic inflammation in rats. *J Physiol Biochem*. 2013;69:165–175.
51. Steinberg GR, Kemp BE. AMPK in health and disease. *Physiol Rev*. 2009;89:1025–1078.
52. Gao L, Cao JT, Liang Y, et al. Calcitriol attenuates cardiac remodeling and dysfunction in a murine model of polycystic ovary syndrome. *Endocrine*. 2015;52:363–373.
53. Park YJ, Lee GS, Cheon SY, Cha YY, An HJ. The anti-obesity effects of Tongbi-san in a high-fat diet-induced obese mouse model. *BMC Complement Altern Med*. 2019;19:1–14.