

# Anti-inflammatory Effect of Ursolic Acid on Male Wistar Rats with High-carbohydrate High-fat Diet-induced Metabolic Syndrome

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## ABSTRACT

**Background:** Metabolic syndrome (MS) comprises a cluster of cardiometabolic risk factors. The presence of these risk factors predisposes an individual to other medical complications. Increased cellular inflammation produces increased levels of biomarkers that are correlated with both insulin resistance and low-grade inflammation, leading to the progression of MS and increased risk for diabetes and CVD. This study investigated the mechanism by which ursolic acid (UA) exerts its anti-inflammatory effect in high-carbohydrate high-fat diet (HCHFD)-induced MS in male Wistar rats. **Methods:** Twenty male Wistar rats were randomly divided into 4 groups (n=5) and fed daily for 20 weeks. Group I received normal diet (ND) and distilled water (DW); group II received ND and UA; group III received HCHFD and DW; group IV received HCHFD and UA. The HCHFD was formulated in-house and the drinking water was augmented with 20% fructose. A dose of 250 mg/kg body weight of UA was administered orally to UA-treated groups starting 12 weeks after initiation of the HCHFD for a further 8 weeks. Body mass index (BMI) was measured. Blood samples were obtained for serum insulin, lipid profile, TNF- $\alpha$ , IL-6 and adiponectin assessment. Adipose tissue PPAR- $\gamma$  mRNA was evaluated using real-time polymerase chain reaction technique. Histology of liver and adipose tissue was performed. All significant results were reported at P<0.05. **Results:** The percentage increase in BMI was lower in the HCHFD+UA-fed animals compared to the HCHFD+DW-fed animals. In the HCHFD+UA-fed animals, serum insulin, cholesterol, triglyceride and LDL-C decreased while HDL-C increased compared to the HCHFD+DW-fed animals respectively. The HCHFD+UA group had decreased serum TNF- $\alpha$  and IL-6 and increased adiponectin level compared to the HCHFD+DW-fed animals. Adipose tissue PPAR- $\gamma$  mRNA level was upregulated in the HCHFD+UA animals compared to HCHFD+DW group. The architecture of the liver parenchyma was

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restored to near normal by UA. **Conclusion:** UA has anti-inflammatory effect on male Wistar rats with HCHFD-induced MS by decreasing serum TNF- $\alpha$  and IL-6, increasing adiponectin and upregulating adipose tissue PPAR- $\gamma$  mRNA levels, respectively.

**Keywords:** Metabolic Syndrome, High-carbohydrate high-fat diet, 20 weeks, Ursolic acid, Anti-inflammation, PPAR- $\gamma$ .

## INTRODUCTION

Metabolic syndrome (MS) comprises of risk factors of cardiovascular disease (CVD) and type 2 diabetes (T2D). These risk factors, which are diagnostic criteria for MS, are central obesity, dyslipidemia, high triglycerides and/or low high-density lipoprotein cholesterol, hypertension, and impaired fasting glucose<sup>[1,2]</sup>.

Obesity, a component of MS, is associated with inflammation. Chronic low-grade tissue inflammation has been identified as a major sign of obesity and a key contributing factor to T2D and CVD<sup>[3, 4]</sup>. According to recent data, more than 1 billion people in the world are obese, including 650 million adults, 340 million adolescents, and 39 million children, which will cause about 167 million people to become ill by 2025<sup>[5]</sup>. Obesity-induced inflammation is mainly mediated by tissue immune cells such as macrophages. Macrophages have been identified as important non-adipocyte cells contributing to adipose tissue production of inflammatory factors which contribute significantly to the systemic inflammation and insulin resistance associated with obesity<sup>[6]</sup>. These inflammatory factors such as TNF- $\alpha$  and IL-6 impair human fat cell differentiation<sup>[7]</sup> and induce inflammatory events in 3T3-L1 adipocytes by activating the nuclear factor kappa B (NF- $\kappa$ B) pathway<sup>[6]</sup>. Excess adipose tissue is hypothesized to contribute to a state of chronic inflammation which promotes development of insulin resistance as well as other metabolic complications by stimulating NF- $\kappa$ B and Jun N-terminal kinase (JNK) pathways in adipocytes and the liver<sup>[8]</sup>. High energy diet has been shown to cause increased serum level of IL-1, IL-6, TNF- $\alpha$ , increased NF- $\kappa$ B

and reduced peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ) expression<sup>[9]</sup>, confirming the inflammatory responses associated with weight gain. One major pathogenic feature of obesity is adipose tissue inflammation. The inflammation is contributed to by adipokines, cytokines, and chemokines which are secreted from dysfunctional adipocytes and infiltrating immune cells<sup>[10]</sup>. Obesity-associated comorbidities such as chronic kidney disease (CKD) and non-alcoholic fatty liver disease (NAFLD) also contribute to CVD<sup>[11, 12]</sup>. Furthermore, obesity-associated, low-grade chronic inflammation in metabolic tissues (e.g., adipose tissues and liver) alters the expression of adipocytokines and lipoproteins such as adiponectin and high-density lipoproteins (HDLs), which influences energy metabolism and causes endothelial dysfunction to increase the risk of CVD<sup>[13]</sup>.

Ursolic acid (UA), which is a carboxylic acid and a naturally occurring pentacyclic triterpenoid, is highly concentrated in many plants including apples, and has become an integral part of the human diet<sup>[14]</sup>. Several studies, both in vitro and in vivo, have revealed that UA has diverse biological roles, including anti-inflammatory<sup>[15]</sup>, anti-oxidative<sup>[16]</sup>, anti-carcinogenic<sup>[17]</sup>, anti-obesity<sup>[18]</sup>, and anti-diabetic activities<sup>[19]</sup>. We have shown that UA prevents the development of high-carbohydrate high-fat diet (HCHFD)-induced MS<sup>[20]</sup>. However, the mechanism by which UA exert its anti-inflammatory effect in HCHFD-induced MS in male Wistar rats is still debatable. Therefore, this study investigated the mechanism by which UA exerts its anti-inflammatory effect in HCHFD-

induced MS in male Wistar rats.

## MATERIALS AND METHODS

### Experimental Animals

A total of twenty (20) male Wistar rats, 8-9 weeks old and weighing 120 - 170 grams were sourced from the animal house, Department of Human Physiology, Ahmadu Bello University, Zaria. The rats were housed in well-aerated plastic cages and allowed to acclimatize having free access to commercial grower mash feed and water ad libitum. After two weeks of acclimatization, the cages' beddings were changed from shaving sawdust to aluminum beddings. This allowed us to quantify spill over food and measure total daily food consumption.

### Formulation of high-carbohydrate high-fat diet (HCHFD):

The high-carbohydrate, high-fat diet (HCHFD) was formulated in-house following the method of Panchal *et al.* [21] and Wong *et al.* [22] with little modification. The high-carbohydrate, high-fat diet consists of condensed milk (39.5%), fructose (17.5%), thermally oxidized palm oil (20%), Powdered rat food (15.5%), Hubble, Mendel & Wakeman (HMW) salt mixture (2.5%), and water (5%) together with 20% fructose in drinking water [20]. Proximate analysis of the diets was carried out by the method of AOAC [23] and the results are presented in table 1 below.

Table 1: Macronutrients in a normal diet and HCHF diet

Macronutrient	Normal diet	HCHF diet
Total carbohydrate (g/100g)	57.24	53.82
Total fat (g/100g)	4.78	17.29
Protein (g/100g)	25.76	14.05
Crude fibre (g/100g)	2.68	1.44
Ash (g/100g)	5.62	3.40
Moisture (g/100g)	4.06	10.02

Note: Drinking water in the HCHFD-fed rats was augmented with 20% fructose.

### Preparation of thermally oxidized palm oil

Fresh palm oil was thermally oxidized as described previously [24]. Briefly, fresh palm oil was subjected to heat at 150°C in a stainless-steel pot. The heating was for five rounds, and each round lasted 20 minutes. After each round, the oil was allowed to

cool for 5 hours. The obtained thermally oxidized palm oil was then used in the formulation of the HCHFD.

### Experimental Design

After two weeks of acclimatization, animals were randomly divided into 4 groups and treated as follows:

- (i) Group 1: Normal diet-fed rats + Distilled water (ND+DW;  $n = 5$ ),
- (ii) Group 2: ND + Ursolic acid (ND+UA;  $n = 5$ ),
- (iii) Group 3: High-carbohydrate high-fat diet-fed rats (HCHFD) + Distilled water (DW) (HCHFD + DW,  $n = 5$ ),
- (iv) Group 4: HCHFD + Ursolic acid (HCHFD+UA,  $n = 5$ ).

### Feeding of animals and administration of ursolic acid

The animals were fed their respective diets daily for 20 weeks. The ND-fed rats were provided with normal tap water while drinking water in the HCHFD-fed rats was augmented with 20% fructose. A dose of 250 mg/kg body weight of ursolic acid [25] was adopted. Ursolic acid was dissolved in an equal volume of distilled water and 50% DMSO, and administered orally through oral gavage to both ND+UA and HCHFD+UA groups starting 12 weeks after initiation of the HCHFD for a further 8 weeks period.

### Determination of percentage change in body mass index

All rats were monitored daily for food and water intake. The body weight of rats was measured every 4 weeks from week 0 until week 12 and measured weekly from week 12 until week 20 using a standard weighing scale. While body length (nose to anus) and abdominal circumference were measured using a standard measuring tape every 4 weeks. The body mass index (BMI) of each rat was calculated as body weight (in grams) / [body length (in centimeters)]<sup>2</sup> [21]. The difference between BMI values at weeks 0 and 20 were determined and used

to calculate the percentage change (increase).

#### Calculation of percentage change or increase

Change = values at week 20 – values at week 0.

If the change is positive, it means there is an increase and if negative, it means there is a decrease. In this study, all changes were positive, meaning there were increases. Therefore, in this study, the percentage increase was calculated as follows:

Percentage increase (%) = increase/values at week 0 x 100.

#### Animal sacrifice and collection of blood samples

At the end of 20 weeks, animals were fasted overnight for 12 hours and anesthetized using a combination of 75 mg/kg body weight of ketamine and 5 mg/kg body weight of diazepam injection, and blood samples were collected via cardiac puncture<sup>[26]</sup>. The blood samples, collected in plain bottles, were centrifuged for 10 minutes at 6000RPM to harvest the serum. The serum was collected and stored at -70°C for further biochemical analyses.

#### Biochemical analyses

The lipid profile was analyzed by measuring serum levels of total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), and triglycerides (TG) using commercial colorimetric assay kits (Agape®, Switzerland).

Serum insulin concentration was measured using a Fine Test Rat Insulin ELISA kit (Coon Koon Biotech®, Shanghai, China) according to the manufacturer's instructions. Serum adiponectin, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were measured using the respective rat ELISA kit (Coon Koon Biotech®, Shanghai, China) according to the manufacturer's instructions.

#### Real-time polymerase chain reaction

Retroperitoneal adipose tissue was harvested and used to determine the levels of expression of

PPAR-γ mRNA by the real-time PCR method. Primer centrifuging was done using a micro refrigerated centrifuge (MTX-150) at 15,000 RPM. The primers were then reconstituted by using 150 μl of PCR graded water. Primer sequences were designed using the National Center for Biotechnology Information Primer Design tool. All primers were purchased from BIONEER®, USA. The synthesized sequences of PCR primers are: PPAR-γ – forward: 5'-

AACCGGAACAAATGCCAGTA-3' and reverse: 5'-TGGCAGCAGTGGAAGAATCG-3'. β-actin – forward:

5'TTGTAACCAACTGGGACGATATGG 3' and reverse:

5'GGATCGTCGTGGTAGTTCTAGTTCTAG 3'

<sup>[27]</sup>. β-actin primer was used as a housekeeping gene primer to amplify the endogenous control product<sup>[28]</sup>.

The RNA extraction was carried out under ice rack. Samples were homogenized with a homogenizer and 500 μl of RB Buffer was added. The lysate was centrifuged at 13,000 RPM for 3 minutes and the supernatant was transferred into a new microcentrifuge tube. 200 μl of 100% ethanol was added and mix immediately using pipette. The sample was then transferred to a binding column in a 2 ml collection tube, the lid was closed and centrifuged at 14,000 RPM for 20 seconds. 700 μl of RWA1 was added without wetting the rim and centrifuged at 14,000 RPM for 20 seconds and supernatant was discarded. The sample was then transferred into another collection tube and 500 μl of RWA2 Buffer was added without wetting the rim and centrifuged at 14,000 RPM for 20 seconds and supernatant also discarded. The sample was transferred into another collection tube and 500 μl of RWA2 Buffer was added without wetting the rim and centrifuged at 14,000 RPM for 4 minutes (2 minutes per orientation) and supernatant discarded. Ethanol was completely removed by centrifuging once more at 14,000 RPM for 1 minute. The binding column was then transferred to a new 1.5 ml tube for elution by adding 50 μl of ER Buffer and after waiting for 5 minutes at room temperature (25°C), it was then centrifuged at 10,000 RPM for 1

minute to elute. After total RNA extraction, cDNA was synthesized from total RNA using the 'high capacity' cDNA Reverse Transcription kit (BIONEER, USA). A 3 µl of each primer and 17 µl of each RNA, making 20 µl, was added to each reaction in the reverse transcription premix tube. The tubes were loaded in the PCR machine at 42°C for 1 hour. Quantitative real-time polymerase chain reaction (PCR) was performed using the Real-Time PCR Detection System (Applied Biosystem GeneAmp®). Primers and cDNA were thawed. A 20-µL reaction mixture containing 10-µL of real-time PCR reagent (AccuPower® 2X GreenStar™ qPCR Master Mix, BIONEER, USA), 4-µL of gene-specific primers (2-µL for forward primer and 2-µL for reverse primer) plus 4-µL of cDNA template, together with appropriate concentration of PCR graded water and ROX dye in PCR tubes. The mixture was mixed by vortexing and the tubes were sealed using optical adhesive film for real-time PCR. Centrifuging was done at 3,000 RPM for 2 minutes and the tubes were loaded into the Real-Time PCR Detection System. The reactions were performed under the following conditions:

### Histological evaluation of liver and adipose tissues

Liver and adipose tissues were harvested and prepared using routine tissue processing techniques outlined by Bancroft [30]. The prepared tissues were placed immediately into the fixative (10 % formal saline). After proper fixing for about 48 hours, the tissues were dehydrated through ascending grades of alcohol from 70% alcohol to 90% alcohol and absolute (100%) alcohol for 16 hours. The tissues were then cleared in toluene for 2 hours after which they were impregnated in molten paraffin for 4 hours. Thereafter, the tissues were then embedded in paraffin wax and sectioned using a rotary microtome at 5 µ thickness. The sections were then stained using the hematoxylin and eosin (H&E) staining technique. The stained sections were examined using a light microscope and relevant photomicrographs were taken using an Amscope® digital camera for microscope (DCM500), 5M pixels (Japan).

### Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA), followed by

**Table 2: PCR conditions**

Step	Temperature	Time	Cycles
Pre denaturation	94 °C	5 min	1 cycle
Denaturation	94 °C	30 sec	40
Annealing	54 °C	30 sec	Cycles
Extension	72 °C	30 sec	
Final extension	72 °C	5 min	

Thereafter, mRNA expression level was determined as fold change (FC) as follows:

$$FC = 2^{(-\Delta\Delta Ct)} [29]$$

the Tukey post-hoc test. Values at  $P < 0.05$  was considered statistically significant. The SPSS version 23 (IBM, Armonk, NY, USA) was used for statistical analysis.

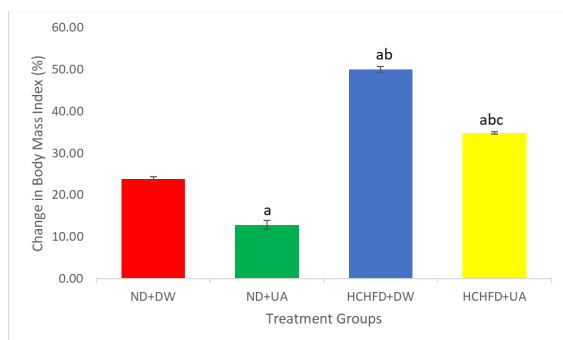
Ethical approval for the study was obtained from the Ahmadu Bello University Committee on Animal Use and Care (Approval No: ABUCAUC/2020/37). The experiment was conducted in accordance with the laboratory care and policy on animal research of the Ahmadu Bello University, Zaria.

State your ethical considerations including the ethical clearance certificate number from your INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) or its equivalent.

## RESULTS

### Effect of ursolic acid on body mass index in rats fed a high-carbohydrate high-fat diet

Figure 1 shows the effects of HCHFD and UA treatment on the percentage increase in BMI of all the groups. The ND+UA-fed animals had a significantly ( $P < 0.05$ ) lower BMI increase ( $12.81 \pm 1.07$ ) compared to the ND+DW group ( $23.84 \pm 0.57$ ). The HCHFD+DW group had a significantly ( $P < 0.05$ ) higher BMI increase

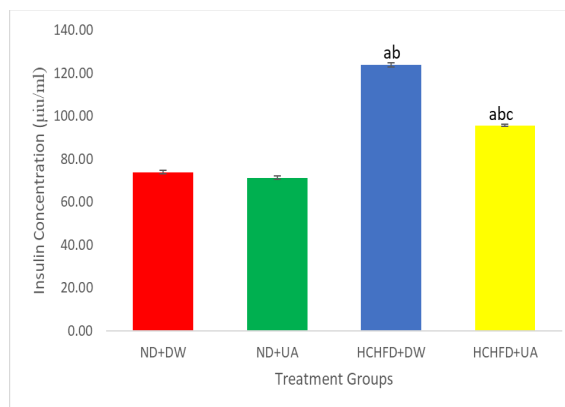


**Figure 1: Percentage change in body mass index of male Wistar rats fed HCHFD and UA.** Data were expressed as Mean $\pm$ SEM (n=5). a = significant vs ND+DW at  $P < 0.05$ ; b = significant vs ND+UA at  $P < 0.05$ ; c = significant vs HCHFD+DW at  $P < 0.05$ . ND+DW: normal diet + distilled water group; ND+UA: normal diet + ursolic acid group; HCHFD+DW: high-carbohydrate high-fat diet + distilled water group; HCHFD+UA: high-carbohydrate high-fat diet + ursolic acid group.

( $50.0 \pm 0.69$ ) compared to the ND+DW group ( $23.84 \pm 0.57$ ). While the HCHFD+UA-fed rats had a significantly ( $P < 0.05$ ) lower BMI increase ( $34.78 \pm 0.30$ ) compared to the HCHFD+DW group ( $50.0 \pm 0.69$ ).

### Effect of ursolic acid on fasting serum insulin in rats fed a high-carbohydrate high-fat diet

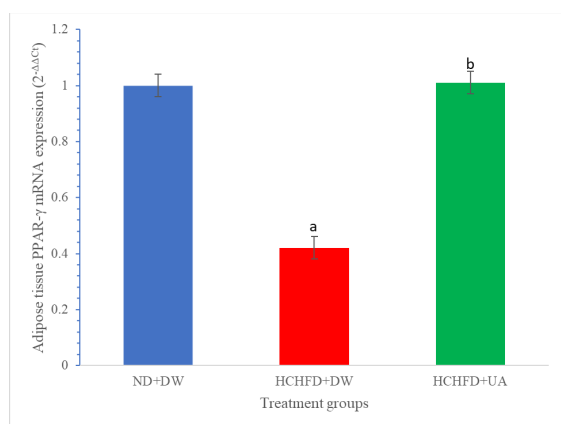
Figure 2 shows levels of serum insulin in all the groups. The level of insulin was similar among ND-fed groups. There was a significant increase ( $P < 0.05$ ) in fasting insulin level in the HCHFD+DW group ( $123.76 \pm 0.95$ ) compared to the ND+DW group ( $73.84 \pm 0.72$ ). The HCHFD+UA-fed animals had a significantly lower ( $P < 0.05$ ) insulin level ( $95.64 \pm 0.48$ ) compared to the HCHFD+DW group ( $123.76 \pm 0.95$ ).



**Figure 2: Serum insulin concentration of male Wistar rats fed HCHFD and UA.** Data were expressed as Mean $\pm$ SEM (n=5). a = significant vs ND+DW at  $P < 0.05$ ; b = significant vs ND+UA at  $P < 0.05$ ; c = significant vs HCHFD+DW at  $P < 0.05$ . ND+DW: normal diet + distilled water group; ND+UA: normal diet + ursolic acid group; HCHFD+DW: high-carbohydrate high-fat diet + distilled water group; HCHFD+UA: high-carbohydrate high-fat diet + ursolic acid group.

### Effect of ursolic acid on lipid profile in rats fed a high-carbohydrate high-fat diet

Table 3 shows the effects of HCHFD and UA on serum levels of lipid profile in all the groups. Levels of TC, TG, and LDL-C were similar in ND-fed groups, whereas the level of HDL-C was significantly higher ( $P < 0.05$ ) in ND+UA-fed rats ( $38.40 \pm 0.99$ ) compared to the ND+DW ( $28.30 \pm 0.91$ ) group. In the HCHFD+DW group, levels of TC ( $126.18 \pm 1.70$ ), TG ( $118.18 \pm 1.25$ ), and LDL-C ( $72.86 \pm 1.05$ ), respectively were significantly higher ( $P < 0.05$ ) compared to the ND+DW group - TC ( $65.10 \pm 1.75$ ), TG ( $57.94 \pm 1.22$ ) and LDL-C ( $43.52 \pm 1.30$ ), respectively. While HDL-C was similar in both groups. In HCHFD+UA-fed animals, levels of TC ( $105.62 \pm 1.84$ ), TG ( $83.38 \pm 1.19$ ), and LDL-C ( $54.74 \pm 1.03$ ), respectively were significantly lower ( $P < 0.05$ ) compared to the HCHFD+DW group - TC ( $126.18 \pm 1.70$ ), TG ( $118.18 \pm 1.25$ ) and LDL-C ( $72.86 \pm 1.05$ ), respectively. Whereas in HCHFD+UA fed rats, HDL-C ( $78.86 \pm 0.97$ ) was significantly higher ( $P < 0.05$ ) compared to the HCHFD+DW group ( $36.12 \pm 1.91$ ).



**Figure 3: Adipose tissue PPAR-γ mRNA expression in HCHFD and UA-treated male Wistar rats.** Data were expressed as Mean±SEM (n = 5). a = significant vs ND+DW at  $P < 0.05$ ; b = significant vs HCHFD+DW at  $P < 0.05$ . ND+DW: normal diet + distilled water group; HCHFD+DW: high-carbohydrate high-fat diet + distilled water group; HCHFD+UA: high-carbohydrate high-fat diet + ursolic acid group.

Data were expressed as Mean±SEM (n = 5). Values with different superscripts within rows are significantly different ( $P < 0.05$ ). ND+DW: normal diet + distilled water group; ND+UA: normal diet + ursolic acid group; HCHFD+DW: high-carbohydrate high-fat diet + distilled water group; HCHFD+UA: high-carbohydrate high-fat diet + ursolic acid group.

HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

### Effects of ursolic acid on inflammatory biomarkers in rats fed a high-carbohydrate high-fat diet

Table 4 shows the effects of HCHFD and UA on serum levels of adiponectin, TNF-α and IL-6 in all the groups. Level of adiponectin ( $30.72 \pm 0.28$ ) significantly ( $P < 0.05$ ) increased in ND+UA-fed animals compared to the ND+DW group ( $28.30 \pm 0.30$ ), whereas there was significant ( $P < 0.05$ ) decrease in the level of adiponectin ( $13.44 \pm 0.32$ ) in HCHFD+DW-fed animals compared to the ND+DW group ( $28.30 \pm 0.30$ ). Also, level of adiponectin ( $23.60 \pm 0.33$ ) was significantly ( $P < 0.05$ ) higher in HCHFD+UA-fed animals compared to the HCHFD+DW group ( $13.44 \pm 0.32$ ). Level of TNF-α ( $69.70 \pm 0.55$ ) significantly ( $P < 0.05$ ) decreased in ND+UA-fed animals compared to ND+DW group ( $76.48 \pm 0.32$ ), whereas level of TNF-α ( $221.50 \pm 0.37$ ) in HCHFD+DW-fed animals significantly ( $P < 0.05$ ) increased compared to the ND+DW group ( $76.48 \pm 0.32$ ). Also, level of TNF-α ( $98.14 \pm 0.41$ ) in HCHFD+UA-fed animals was significantly ( $P < 0.05$ ) lower compared to the HCHFD+DW group ( $221.50 \pm 0.37$ ). Level of IL-6 ( $73.36 \pm 0.48$ ) in ND+UA-fed animals significantly ( $P < 0.05$ ) decreased compared to ND+DW group ( $81.84 \pm 0.45$ ), whereas level of IL-6 ( $131.44 \pm 0.38$ ) in HCHFD+DW-fed animals was significantly ( $P < 0.05$ ) higher compared to the ND+DW group ( $81.84 \pm 0.45$ ). Also, there was significant ( $P < 0.05$ ) decrease in the level of IL-6 ( $86.12 \pm 0.33$ ) in HCHFD+UA-fed animals compared to the HCHFD+DW group ( $131.44 \pm 0.38$ ).

**Table 3: Levels of serum lipids following high-carbohydrate high-fat diet feeding and treatment with ursolic acid in male Wistar rats.**

Lipid Profile	ND+DW	ND+UA	HCHFD+DW	HCHFD+UA
<b>Total Cholesterol (mg/dl)</b>	65.10±1.75 <sup>a</sup>	65.20±1.85 <sup>a</sup>	126.18±1.70 <sup>c</sup>	105.62±1.84 <sup>b</sup>
<b>Triglyceride (mg/dl)</b>	57.94±1.22 <sup>a</sup>	58.04±0.96 <sup>a</sup>	118.18±1.25 <sup>c</sup>	83.38±1.19 <sup>b</sup>
<b>HDL-C (mg/dl)</b>	28.30±0.91 <sup>a</sup>	38.40±0.99 <sup>a</sup>	36.12±1.91 <sup>a</sup>	78.86±0.97 <sup>b</sup>
<b>LDL-C (mg/dl)</b>	43.52±1.30 <sup>a</sup>	43.64±1.37 <sup>a</sup>	72.86±1.05 <sup>c</sup>	54.74±1.03 <sup>b</sup>

Data were expressed as Mean±SEM (n = 5). Values with different superscripts within rows are significantly different (P<0.05). ND+DW: normal diet + distilled water group; ND+UA: normal diet + ursolic acid group; HCHFD+DW: high-carbohydrate high-fat diet + distilled water group; HCHFD+UA: high-carbohydrate high-fat diet + ursolic acid group.

HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

**Table 4: Levels of serum inflammatory biomarkers following high-carbohydrate high-fat diet feeding and treatment with ursolic acid in male Wistar rats.**

	ND+DW	ND+UA	HCHFD+DW	HCHFD+UA
<b>Adiponectin (pg/ml)</b>	28.30±0.30 <sup>c</sup>	30.72±0.28 <sup>d</sup>	13.44±0.32 <sup>a</sup>	23.60±0.33 <sup>b</sup>
<b>TNF-α (pg/ml)</b>	76.48±0.32 <sup>b</sup>	69.70±0.55 <sup>a</sup>	221.50±0.37 <sup>d</sup>	98.14±0.41 <sup>c</sup>
<b>IL-6 (pg/ml)</b>	81.84±0.45 <sup>b</sup>	73.36±0.48 <sup>a</sup>	131.44±0.38 <sup>d</sup>	86.12±0.33 <sup>c</sup>

Data were expressed as Mean±SEM (n = 5). Values with different superscripts within rows are significantly different (P<0.05). ND+DW: normal diet + distilled water group; ND+UA: normal diet + ursolic acid group; HCHFD+DW: high-carbohydrate high-fat diet + distilled water group; HCHFD+UA: high-carbohydrate high-fat diet + ursolic acid group.

TNF-α: Tumor necrosis factor - alpha; IL-6: Interleukin-6.

Data were expressed as Mean±SEM (n = 5). Values with different superscripts within rows are significantly different (P<0.05). ND+DW: normal diet + distilled water group; ND+UA: normal diet + ursolic acid group; HCHFD+DW: high-carbohydrate high-fat diet + distilled water group; HCHFD+UA: high-carbohydrate high-fat diet + ursolic acid group.

TNF-α: Tumor necrosis factor - alpha; IL-6: Interleukin-6.

#### Effects of ursolic acid on relative expression of peroxisome proliferator-activated receptor-gamma mRNA in the adipose tissues of rats fed a high-carbohydrate high-fat diet

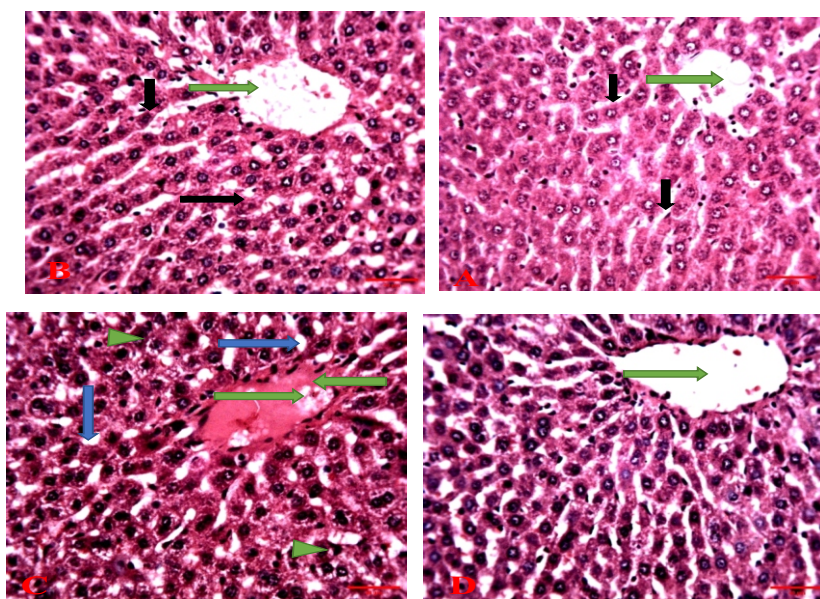
Figure 3 shows the effects of HCHFD and UA supplementation on the expression of PPAR-γ mRNA in the adipose tissue (AT) of HCHFD+DW and HCHFD+UA- fed rats relative to the ND+DW-fed rats (normal control). The PPAR-γ mRNA level

was significantly (P<0.05) downregulated in the HCHFD+DW-fed rats (0.42±0.05) compared to normal control (1.00±0.04). The UA significantly (P<0.05) upregulated AT PPAR-γ mRNA level in the HCHFD+UA- fed rats (1.01±0.07) compared to HCHFD+DW-fed rats (0.42±0.05).

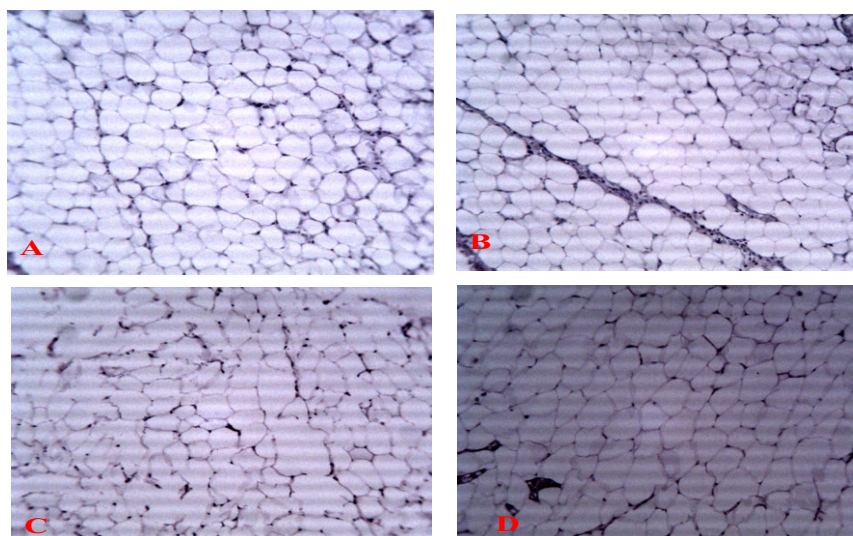
#### Effect of ursolic acid on the histology of liver and adipose tissues in rats fed a high-carbohydrate high-fat diet

Plate 1A and 1B show the normal histological structure of the liver in rats fed with a normal diet indicated by a clear central vein as shown with green arrows and sinusoids as shown with black arrows. Plate 1C shows the damaged histological structure of the liver in rats fed only HCHFD, showing steatosis as indicated by blue arrows; vesicular fatty change as indicated by yellow arrowheads; cellular infiltration as indicated by green arrowheads; and congested central vein with





**Plate I: Photomicrograph of liver tissues of HCHF+fed and UA-treated male Wistar rats (H&E; X 250).** **A:** ND+DW group; **B:** ND+UA group; **C:** HCHF+DW group; and **D:** HCHF+UA group. ND+DW: normal diet + distilled water group; ND+UA: normal diet + ursolic acid group; HCHF+DW: high-carbohydrate high-fat diet + distilled water group; HCHF+UA: high-carbohydrate high-fat diet + ursolic acid group.



**Plate II: Photomicrograph of adipose tissues of HCHF+fed and UA-treated male Wistar rats (H&E; X 250).** **A:** ND+DW group; **B:** ND+UA group; **C:** HCHF+DW group; and **D:** HCHF+UA group. ND+DW: normal diet + distilled water group; ND+UA: normal diet + ursolic acid group; HCHF+DW: high-carbohydrate high-fat diet + distilled water group; HCHF+UA: high-carbohydrate high-fat diet + ursolic acid group.

thickening of its wall as indicated by green arrows. Plate 1D shows a near-normal architecture of the liver parenchyma in rats fed an HCHF+UA, showing a clear central vein as indicated by the

green arrow.

Plate IIA and IIB show small sized adipocytes in rats fed a normal diet. In Plate IIC histology shows enlarged adipocytes in HCHF+DW-fed rats. Plate IID shows moderate sized adipocytes in the

HCHFD+UA-fed group.

## DISCUSSION

In this study, we reported HCHFD-induced obesity as indicated by increased BMI and 8 weeks supplementation with UA mitigated the obesity. This anti-obesity effect of UA occurs possibly via increased Akt phosphorylation and improved skeletal muscle glucose uptake<sup>[25,31,32]</sup>, inhibition of lipogenesis in adipose and hepatic tissues, and enhanced lipolysis in adipose cells<sup>[31]</sup>. These conditions have been found to increase the release of free fatty acid (FFA) into circulation<sup>[33, 34]</sup>. Studies have shown that UA supplementation does not induce an elevation of circulating FFA level, and conversely, significantly decreases its concentration in diet-induced obese mice<sup>[35,36]</sup>. This suggests that UA mitigates HCHFD-induced obesity, as shown by our findings, through enhanced adipocyte lipolysis, and increased FFA utilization, likely by the skeletal muscle.

We observed HCHFD-induced hyperinsulinemia and dyslipidemia in this study as indicated respectively by increased fasting serum insulin and increased TG, TC, LDL-C and decreased HDL-C. We have reported insulin resistance and decreased rate of glucose clearance in HCHFD-fed rats<sup>[20]</sup>. Excess dietary fructose<sup>[37]</sup>, fat<sup>[38]</sup>, or a combination of fructose and fat<sup>[39]</sup> has been widely reported to contribute to insulin insensitivity and dyslipidemia. High fructose feeding increases lipogenesis through the upregulation of SREBP-1c<sup>[40]</sup>. Endogenous peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) ligands are associated with activated SREBP-1c and this, consequently, increases adipogenesis/lipogenesis<sup>[41, 42]</sup>. Also, our findings showed that HCHFD caused a state of pro-inflammation by significantly decreasing adiponectin and adipocyte PPAR- $\gamma$  mRNA levels, and by significantly increasing TNF- $\alpha$  and IL-6 levels. Several inflammatory biomarkers have been identified in various disease conditions such as hypertension, type-2 diabetes, arteriosclerosis, and obesity<sup>[43]</sup>.

However, fasting hyperinsulinemia was

significantly alleviated by UA, suggestive of the insulin-sensitizing effect of UA. Also, our present study showed that UA significantly decreased TG, TC, and LDL-C while HDL-C was increased. These suggest that UA has hypolipidemic and anti-hyperlipidemic effects. Prevention of insulin resistance and improved glucose tolerance by UA as reported<sup>[20]</sup>, (due both to its lipolytic and FFA utilization effects) may be responsible for the amelioration of dyslipidemia.

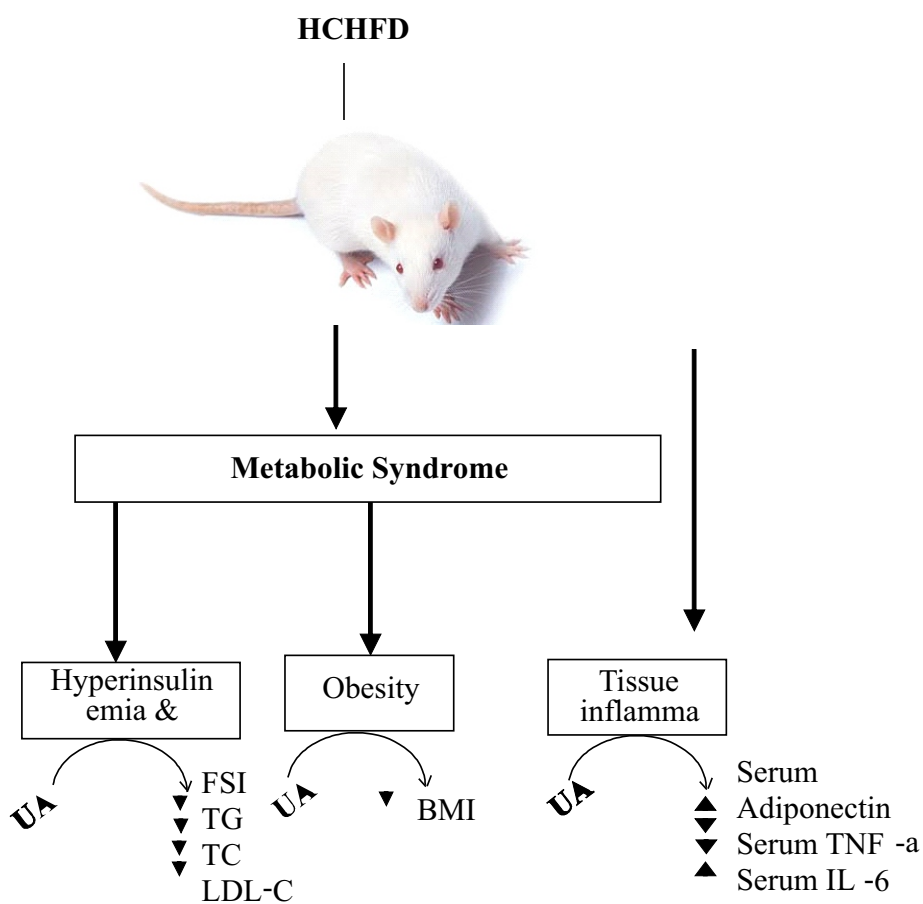
Our present study also showed that UA has anti-inflammatory effects by significantly increasing adiponectin concentration and PPAR- $\gamma$  mRNA expression, and by significantly decreasing TNF- $\alpha$  and IL-6 concentrations. It has been demonstrated that adiponectin increases fat oxidation via activation of AMP-activated protein kinase in rat skeletal muscle and C2C12 myocytes<sup>[44, 45]</sup>. Adiponectin stimulates the proliferation of skeletal muscle tissue through p38-MAPK pathway<sup>[46]</sup>. Increase in adiponectin concentration may increase skeletal muscle glucose uptake. It's likely the insulin resistance reported<sup>[20]</sup> was induced by inflammatory cytokines. The HCHFD-induced hepatic steatosis and cellular infiltration observed in this study (as shown in our histology of liver tissue) is associated with insulin resistance which is linked to hyperinsulinemia and the secretion of proinflammatory cytokines<sup>[47, 48]</sup>. However, UA exerts its insulin-sensitizing effect through the anti-inflammatory action of adiponectin which represses secretion of proinflammatory cytokines including IL-6 and TNF- $\alpha$ . It has been shown that TNF- $\alpha$  inhibits the differentiation of adipocyte precursor cells and 3T3-L1 cells via inhibition of a master regulator of adipocyte differentiation, i.e. PPAR- $\gamma$ , at the mRNA level<sup>[49, 50]</sup>. IL-6 may also inhibit adipocyte differentiation<sup>[51]</sup>. This results in the enlargement of remaining fat cells (as shown in our histology of adipocytes) and, consequently, a reduced adipose tissue lipid buffering capacity and further impairment of adipokine secretion, leading to insulin resistance. Decreased TNF- $\alpha$  and IL-6 concentrations by UA suggests that UA prevents enlargement of fat cells by enhancing

differentiation of adipocyte precursor cells through the activation of PPAR- $\gamma$  resulting in formation of small adipocytes.

**CONCLUSION**

Findings from this study showed that UA has anti-inflammatory effect by ameliorating proinflammatory biomarkers in male Wistar rats fed a HCHFD for 20 weeks. The mechanism by which UA exert its anti-inflammatory effect is

believed to be through the downregulation of adipose tissue PPAR- $\gamma$ . Further study is recommended to unravel the mechanistic link between the PPAR- $\gamma$  in adipose tissue and other metabolic tissues, such as the liver, in high energy diet-related inflammatory response.



**Figure 4: Summary of proposed mechanism by which ursolic acid mitigate HCHFD-induced metabolic syndrome and pro-inflammation**



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