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# Alternate day fasting ameliorates renal damage in rats received high fat and fructose diet through enhancement of renal autophagy, decreasing renal fibrosis and apoptosis.

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## Keywords

- Alternate day fasting
- High-fat diet/fructose
- Autophagy
- Oxidative stress
- Metabolic syndrome
- Inflammation
- Antioxidants
- Immunohistochemistry
- Kidney

## Abstract

**Background:** A high-fat fructose diet (HFFD) is known to increase one's risk for developing obesity, the metabolic syndrome, and lipotoxicity, all of which contribute to the development of chronic kidney disease. **Aim of the work:** The goal of the current study is to clarify the biochemical and histological impacts of alternate-day fasting on renal disease brought on by a high-fat and fructose diet. **Methods:** 18 adult albino male rats weighing 200–250 grams were randomly assigned to three groups of six: Group 1 had a conventional diet, Group 2 received a high fat and fructose diet, and Group 3 received a high fat and fructose diet as well as alternate days of fasting. Fasting for 24 hours was applied in HFFD rats alternatively from the 9th week to the $12^{th}$ week respectively. All rat groups were subjected to a histopathological study of the kidney to assess collagen deposition, LC3, α-SMA, and caspase 3 immuno-expression besides biochemical investigations to identify metabolic syndrome parameters as lipid metabolism, inflammatory markers, and oxidant-antioxidant status in the kidney tissues of all groups. **Results:** HFFD rats subjected to alternate-day fasting showed an improvement in adiposity (body weights, kidney weights, and body mass index), lipid metabolism, and cellular oxidative and inflammatory markers. Also histopathological examination confirmed the valuable effect of fasting as evidenced by enhancement of renal autophagy, decreasing renal fibrosis and apoptosis. **Conclusion:** Our study's findings reveal that ADF from the $9^{th}$ to $12^{th}$ week improved metabolic disturbances and renal injury induced by HFFD. Fasting also significantly reduced the inflammation and oxidative stress on both biochemical and histopathological levels.

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## Introduction

High-fat diet (HFD) was linked to several multi-organ impairments, including the liver, heart, muscles, brain, pancreas and alimentary canal[1, 2]. It was reported that diets containing high fat and sugar content result in the progression of metabolic disorders, disturb glucose tolerance, dyslipidemia, and lead to loss of muscle coordination [3].High fat and fructose diet (HFFD) proved to be strongly correlated with the development of chronic noncommunicable illnesses in particular obesity, hypertension, and chronic renal disease (CKD) [4].Patients have type 2 diabetes and obesity, with intake of HFFD leads to the deposition of more lipids in renal tissues which in turn results in glomerular lesions and the formation of a tubulointerstitial disorder[5].

Previous animal studies showed that HFFD is a major cause of renal damage by promoting both hemodynamic and morphological alternation with subsequent albuminuria, proteinuria, interstitial fibrosis, glomerulosclerosis, and end-stage kidney disease [6].HFFD consumption increases the risk of the development of insulin resistance and obesity that aggravate renal damage[7]. In children, severe obesity eventually terminates with end-stage kidney disease [8].However, prior experimental studies have shown that HFFD consumption causes an increase in cellular lipid accumulation and disruption of its metabolism (lipotoxicity), as well as an increase in renal inflammatory mediators like interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF) andinterleukin-1 (IL-1), which is ultimately associated with the progression of kidney injury [9–11].Reactive oxygen species (ROS) levels rise because of the overproduction of these mediators, which sets off a vicious cycle of inflammation and oxidative stress [12, 13]. Renal failure and chronic kidney disease (CKD) are the results of this process.Also,insulin resistanceand excessive production of intracellular RO modulate $Bcl_2$ family protein production and enhanced caspase cascade, with more renal cell damage[14].

Therapeutic fasting is an alternative to medication that, in addition to promoting weight reduction and metabolic improvement, promotes autophagy [15] and activates the pathways of the cellular stress response that guard against cellular and tissue damage [16]. The heart, brain, liver, and kidney are only a few of the organs that can be protected against ischemia assaults by caloric restriction and/or intermittent fasting [17].

There are three different fasting protocols listed in the full regimen: periodic fasting (consecutive fasting periods lasting more than 24 hours), alternate-day fasting, and intermittent fasting (IF)[18]. In intermittent fasting (IF), cycles of eating and fasting alternate (usually 16 hours of total food deprivation, not including water, and 8 hours of regular food consumption). The positive impacts on health of IF were said to be the reason for its popularity [19]. Several cardiovascular issues, including hypertension and post-infarction damage, were also improved by IF [20]. Fasting before surgery protects the kidney against redox and mitochondrial changes reported in a rat model of ischemia-reperfusion injury. Fasting has also been shown to provide sustained protection against fibrosis formation [21]. All of these findings prompted us to look into the protective effects of alternate-day fasting against high-fat, fructose-containing diet-induced kidney damage. We did this by demonstrating how fasting affects kidney autophagy, fibrogenesis, and apoptosis as well as by assessing oxidative stress, inflammatory markers, and kidney function tests to determine the extent of the fasting's protective effects[22].

## Material and Methods

### 1.1. Experimental Design:

The study used a total of 18 mature male albino rats that are clear of pathogens and weigh 200–250 grams. All rats were kept in metal cages with soft wood chips for bedding and fed on a commercial basal ad libitum diet and water for 2 weeks prior to the

experiment for acclimation and to guarantee normal development and behavior, as per the ethical regulations approved by the research Ethics Committee at the Faculty of Pharmacy, Delta University for Science and Technology (FPDU-REC) and holds the approval number FPDU10/2023. Animals were divided randomly into 3 equal groups (6 rats each):

- Group 1 (Basal diet group): rats fed on basal diet for 12 weeks.
- Group 2 (High fat and fructose diet group): The rats were given a diet rich in fat and fructose for a twelve-week period.
- Group 3 (HFFD & ADF group): Rats were given a diet rich in fat and fructose for a period of twelve weeks. Additionally, from the start of the ninth week to the end of the experiment, the animals were committed to the fasting protocol.

Cervical dislocation was done to sacrifice the animals. The blood sample was taken in tubes containing heparin by cardiac puncture. All animals had been sacrificed at the end of the twelfth week; Serum was then drawn from each group for biochemical evaluation. The kidney was cut in half, with one half being preserved in RNA (10 L for a single milligram of tissue specimen) (Qiagen, Hilden, German), the other half of kidney being kept frozen nightly at 2-7$^{o}$ C, in the presence of liquid nitrogen.

### 1.2. Fasting protocol:

Animals were subjected to alternate day fasting (ADF) protocol every 24 h from the 9$^{th}$ week to the 12$^{th}$week respectively.

### 1.3. Basal and high fat and high fructose diets:

The temperature was kept constant while the animals were housed in a controlled habitat with 12-hour cycles of darkness and light. The animals in the control group were fed a typical baseline meal throughout the trial that contained roughly 60,0 % carbohydrates, 21,1 % protein, 7,9 % minerals, 5,1 % fat, 3,9 % fiber, and 2,0 % vitamins, as well as water available at all times. Additionally, rats in the test group were fed a diet rich in fat, which included a 20g fat/100g meal made up of hydrogenated vegetable oils and soybean oil [23], as well as 15% fructose in drinking water.

### 1.4. Assessments of body and organ weight:

Animal body weights were gauged and noted at the beginning and conclusion of the investigation. The lengths of the rats were measured on the experiment's last day, and the pound of the kidneys had been assessed immediately following the sacrifice. Body mass index (BMI) measurements were also computed using the formulae based on body weight (gram)/naso-anal length 2 (cm2) and the formula Lee's index (g/cm) = cube root of body weight/length [24].

- ✓ Weight gain (final body weight - initial body weight).
- ✓ Body mass index (BMI) = body weight (g)/naso-anal length$^2$ (cm$^2$).
- ✓ Lee index =Lee index (g/cm) =cube root of body weight/length.
- ✓ Relative organ weight (%) =Organ weight×100/Final body weight.

### 1.5. Biochemical analysis:

The acquired serum samples from centrifuging blood samples at 4000 rpm for 10 min were kept at 20 °C until analysis.

#### 1.5.1. Assessment of lipid, glucose, and kidney function:

While blood samples were used for the estimation of blood urea nitrogen and albumin by using kit method (Agape, India) in accordance with the manufacturer's instructions, serum was used for the assessment of renal function test as creatinine and urea, lipid profile, and glucose. The Synergy H1 multimode reader (BioTek,

USA) was used for all spectrophotometric measurements.

**1.5.2. Assessment of oxidant-antioxidant status:**

The previously published methods were employed to measure the antioxidant levels in renal homogenate, including reduced glutathione (GSH) and superoxide dismutase (SOD), with a few minor modifications. To evaluate the catalase activity, the decrease in absorbance at 240 nm brought on by the oxidation of H2O2 was quantified using a UV recording spectrophotometer. As previously stated,[25], quantities of Thio barbituric acid-reactive components were evaluated in order to determine the quantity of the fatty acid peroxidation product that produced MDA.

**1.5.3. Assessment of cellular inflammation:**

Tumor necrosis factor (TNF), Interleukin 1 beta (IL-1b), and interleukin 6 (IL-6), levels were assessed in kidney homogenates using an enzyme-linked immunosorbent test (E.L.I.S.A.) (Pepro Tech., Rocky Hill, NJ, U.S.A.).

**1.5.4. Determination of Tumor Necrosis Factor-α (TNF-α), Interleukin-1B (IL-1β), Gene Expression by Real-Time PCR (RT-qPCR):**

From tissue samples of each rat in the study groups' kidney, total cellular RNA was extracted using the QIAzol reagent (Qiagen, Germany), following the manufacturer's instructions. Tissues were collected and homogenized by strokes of liquid nitrogen. Thermo Scientific NanoDrop 2000 (USA) measured the concentration and purity of the RNA yield using the 260/280 and 260/230 ratios and the absorbance at 260 nm, respectively. Reverse transcription of 1ug of RNA was performed using the Sensi. FASTTM cDNA Synthesis Kit from Bioline and the Applied Biosystems 2720 Thermal Cycler from Thermo Fisher Scientific using the following protocol: ten minutes at twenty-five degrees Celsius for a primer the annealing fifteen minutes at forty-two degrees Celsius for the process of reverse transcription, and five minutes at a temperature of 85 for inactivation.

Applied Biosystems 7500 real-time PCR equipment was used to amp up the cDNA templates. For the amplification procedure, the following components were mixed in a 20 l total volume: 6 l of nuclease-free water, 2 l of 10 pmol gene primer, and 2 l of cDNA template are required. HERA SYBR green PCR Master Mix (10 l) (Willowfort, UK). 30 seconds at 60 degrees, followed by 40 cycles of 2 minutes at 95 degrees. The sequences of the primer pairs that were employed are presented in (Table 1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Using Primer 3 software (version 4.1.0), the primer sets were found. Primer specificity was calculated using [http://primer3.ut.ee] and the Primer-BLAST program [https://www.ncbi.nlm.nih.gov/tools/primer-blast/].The specificity of the PCR products was also measured by examining melting curves. The primer sets were made by Vivantis (Vivantis Technologies, Malaysia).

The relative levels of gene expression were expressed as Ct = Ct target gene-Ct housekeeping gene, and the fold change of a gene's expression was calculated using the 2CT method [26].

**Table 1**: Real-time PCR primer sequences for IL1B, TNF-α, and caspase-3 mRNA expression.

| Gene name | Product length | Reference sequence | Forward primer | Reverse primer |
|---|---|---|---|---|
| **IL-1β** | 136 | NM_031512.2 | GCTATGGCAACTGTCCCTGA | CATCTGGACAGCCCAAGTCA |
| **TNF-α** | 133 | NM_012675.3 | GGCGTGTTCATCCGTTCTCT | CCCAGAGCCACAATTCCCTT |
| **Caspase-3** | 67 | U49930 | AATTCAAGGGACGGGTCATG | GCTTGTGCGCGTACAGTTTC (R-) |
| **B-actin** | 291 | NM_031144 | ATC TGG CAC CAC ACC TTC | AGC CAG GTC CAG ACG CA |

**1.6. Histological Examination of kidney tissues:**

Kidney samples were prepared by fixing them in 10% neutral buffered formalin, cleaning them in xylene, dehydrating them in progressively higher grades of ethyl alcohol, and then embedding them in paraffin. For general histological examination, tissue slices that were five millimeters thick were cut, fixed on glass slides, deparaffinized in xylene, and stained with both hematoxylin and eosin (H&E) stain.Masson trichrome stain to detectcollagen fibersin thekidney tissues and immunohistochemical stains for localization of LC3 as a marker of autophagy, alpha SMA antibody as a marker offibrosis and caspase 3 for detection of apoptosis in kidney tissues using streptavidin-biotin-peroxidase complex (ABC) techniques.

- **Immunohistochemistry:**

To use the streptavidin-biotin technique to inhibit endogenous peroxidase, paraffin slices were deparaffinized in xylene, rehydrated, rinsed in tap water, and then embedded in 3% hydrogen peroxide in phosphate buffer solution (PBS) for 10 min at room temperature.The sections were heated for 10 min at 95°C in a 10 mM citrate solution for antigen retrieval, and then allowed to cool for 1 h. A polyclonal rabbit anti-active caspase-3 (Clone No. C92-05, Catalog No. 55955, Phar.Mingen, San Diego, CA at 1:500 dilution) was employed to detect caspase-3, while a mouse monoclonal antibody for ASMA was used as a fibrogenic marker.Slices of kidney were incubated with each primary antibody for an entire night. Following that, the sections were exposed to a secondary goat anti-rabbit biotinylated antibody from Vector Laboratories in Burlingame, California to aid in their identification. To track the reaction, 3,3'-diaminobenzidine in H2O2 (DAB kit, Vector, CA) was added. A dark, insoluble substance was found in the cytoplasm. Sections were mounted after sections were counterstained with hematoxylin. Negative control sections were made using the same staining techniques; however, they were not treated with the primary antibodies.

**Morphometric Assessment of Histopathological Results:**

Using Image J software version 1.52a [27]and the Fiji Image j program [28], the area% of collagen (in Masson trichrome collected sections x200) and the area% of LC3, SMA, and caspase 3 immunopositive area fractions (in immunostained sections x400) per high power field were calculated. A representative sample of 24 random, straightforward microscopic fields—2 fields per section of 2 sections per mouse for each animal in the six groups of mice—was assessed in the preparations.

**Statistical Assessment:**

Standard deviations (SD) were used to represent all data as mean ± Standard Deviations (SD). The data were statistically analyzed using GraphPad Prism (version 7). Following the one-way ANOVA test, the significance between groups was evaluated using Tukey's post hoc analysis. The statistical significance was established at a 0.05 p-value.

## 2. Results

### 2.1. Effect of alternate day fasting on metabolic and adiposity parameters induced by HFFD:

In this study, body weight, weight growth, body mass index, lee index, kidney weights, and belly fat all dramatically raised in both HFFD and HFFD+ADF groups when compared to rats fed with normal basal diet and HFFD+ADF showed a significant decrease in all these parameters as compared with HFFD group (table 2). In addition, metabolic parameters such as serum glucose, insulin levels, HOMA index, total cholesterol, triglyceride, Low DL-c, and High DL-c were found higher in HFFD rats in comparison to those fed on a normal diet (ND) (Table 3). The data obtained demonstrate that long-term HFFD consumption for 12 weeks led to obesity and insulin resistance. When fasting was applied as an alternative medicine significant improvement was achieved in

both metabolic and adiposity parameters of HFFD-rats compared to HHFF-non-treated rats as well. In comparison to the untreated HFFD group, the ADF + HFFD treated rats showed a substantial decrease in body weights, body mass index, kidney weights, and abdominal fat. They also had higher levels of both glucose and lipid markers.

**Table 2: Comparison of Initial body weight, Final body weight, Weight gain, Naso-anal length (cm), BMI (g/cm$^2$), lee index(g/cm)& kidney weight (gm) between Control , HFFD, and ADF+ HFFD groups:**

| Parameters | G1 (ND; n=6) | G2 (HFD; n=6) | G3 (ADF+HFFD; n=6) | P |
|---|---|---|---|---|
| Initial body weight | **220.8±8.61** | **222.5±9.35** | **220.8±7.36** | **0.92** |
| Final body weight | **289.20±5.85** | **421.70±10.80[a]** | **365.00±8.94 [a,b]** | **<0.001*** |
| Weight gain | **68.33±5.16** | **199.17±6.65 [a]** | **144.17±5.85[a, b]** | **<0.001*** |
| Naso-anal length (cm) | **21.00±.894** | **21.33±0.51** | **20.67±0.51** | **0.34** |
| BMI (g/cm$^2$) | **0.657±0.047** | **0.922±0.033 [a]** | **0.866 ±0.0393[a,b]** | **< 0.001*** |
| Lee index (g/cm) | **0.315±.01** | **0.350±0.007** | **0.345±0.007** | **<0.001*** |
| kidney weight (gm) | **1.498±0.059** | **1.702±0.038[a]** | **1.588±0.033[a,b]** | **<0.001*** |
| Kidney /body weight ratio | **0.516±0.0137** | **0.401±0.012[a]** | **0.434±0.005[a,b]** | **<0.001*** |
| Abdominal fat/body wt | **0.011 ± 0.005** | **0.087 ± 0.004 [a]** | **0.056 ± 0.007[a,b]** | |

***Data expressed as mean & SDP: Probability *: significance≤0.05***
***P: significance between Control, HFFD, and HFFD+ ADF groups(One way ANOVA followed by post-hoc tukey's)([a] p< 0.001 HFFD vs. ND, [b]p< 0.01 ADF + HFFD vs. HFFD).***
***Significant increase in body weight, weight gain, body mass index, lee index, kidney weight, abdominal fat weight in HFFD andADF + HFFD group when compared with control one. However all these parameters showed significant decrease in ADF + HFFD when compared with HFFD group.***

**Table 3: Effect of Alternate day fasting on metabolic parameters after12 weeks of high fat fructose diet:**

| Parameters | G1 (ND; n=6) | G2 (HFFD; n=6) | G3 (ADF+HFFD; n=6) |
|---|---|---|---|
| glucose (mg/dL) | 85.3 ± 3.76 | 115.9± 8.6[a] | 96.4 ± 5.7[b] |
| Insulin (ng/mL) | 1.70 ± 0.45 | 4.90 ± 0.67[a] | 3.50 ± 0.89[b] |
| HOMA index | 11.04 ± 1.45 | 18.05 ± 1.63[a] | 13.07 ± 1.3[b] |
| Total cholesterol (mg/dl) | 38.65 ± 2.51 | 62.5 ± 4.6[a] | 41.4 ± 2.7[b] |
| LDL-C (mg/dl) | 42.4 ± 0.45 | 89.7 ± 0.36[a] | 69.0 ± 0.46[b] |
| HDL-C (mg/dl) | 52.4 ± 5.3 | 124.5 ± 6.4[a] | 98.5 ± 3.9[b] |
| Triglycerides (mg/dl) | 118.4 ± 21.5 | 225.6 ± 22.3[a] | 186.5 ± 11.7[b] |

***The data are presented as mean ± SD (n = 6/group). ND, Normal diet fed; HFFD, High fat fructose diet fed; ADF, alternate day fasting. [a] p< 0.01 HFFD vs. ND, [b] p < 0.01 Fasting vs. HFFD.***

### 2.2. Effect of alternate day Fasting on kidney function:

In HFFD rats for 12 weeks, the levels of serum creatinine, BUN, and urine albuminwere significantly increased, on the other hand urine albumin and24-hour urine volume were significantly reduced when compared to rats fed with a normal diet. All renal function parameters in ADF, HFFD-rats were still altered when compared to control rats, but these parameters significantly improved when compared to untreated HFFD rats, with significant decreases in serum creatinine, BUN, and urine albumin levels and increases in urine creatinine and 24-hour urine volume (table 4). The findings showed that fasting on alternate days from the ninth to the twelfth week considerably enhanced kidney function.

### 2.3. Effect of alternate day Fasting on renal oxidative stress markers:

In the current research, kidney tissues showed the impact of HFFD on cellular oxidant-antioxidant balance (Table 5). The tissue homogenate from HFFD rats exhibited considerably reduced levels of the antioxidant enzymes SOD, CAT, and GSH and significantly greater amounts of thiobarbituric acid reactive substances (TBARS) as compared to control ND rats. It was discovered that fasting had a considerable positive impact on the tissue

antioxidant state of HFFD rats,. The results revealed that after alternate-day fasting from the ninth to the twelfth weeks, respectively, TBARS greatly decreased and SOD, CAT, and GSH significantly improved.

**Table 4:** Effect of fasting on renal function parameters of the rats fed with normal and high fat /fructose diet for 12 weeks:

| Parameters | G1 (ND; n=6) | G2 (HFFD; n=6) | G3 (ADF+HFFD; n=6) |
|---|---|---|---|
| Serum creatinine (mg/dl) | 0.35 ± 0.015 | 0.89 ± 0.013 [a] | 0.59 ± 0.012 [b] |
| BUN (mg/dl) | 26.7 ± 3.5 | 68.7 ± 12.7[a] | 42.3 ± 15.3[b] |
| Urine creatinine level (mg/dl) | 78.283± 0.402 | 45.449± 0.449[a] | 66.267± 0.602[a,b] |
| Urine albumin level (µg/dl) | 10.650± 0.606 | 48.00± 0.537[a] | 34.150± 0.586[a,b] |
| 24-hr urine volume (mL/day | 14.3 ± 3.56 | 8.3± 3.2[a] | 12.4 ± 5.1[b] |

***The data are presented as mean ± SD (n = 6/group). ND, Normal diet fed; HFFD, High fat fructose diet; ADF alternate day fasting. [a] p< 0.01 HFD vs. ND, bp < 0.01 Fasting vs. HFFD.***

**Table 5: Effect of fasting on oxidant-antioxidant parameters in the renal homogenate of the rats fed with normal and high fat /fructose diet for after12 weeks:**

| Parameters | G1 (ND; n=6) | G2 (HFFD; n=6) | G3 (ADF+HFFD; n=6) |
|---|---|---|---|
| TBARS (nM/mg tissue) | 2.7 ± 0.15 | 4.8 ± 0.48 [a] | 3.5 ± 0.12 [b] |
| SOD (U/mg protein) | 7.82±0.89 | 3.4 ±0.28[a] | 5.2±0.75 [b] |
| Catalase (mlU/mg protein) | 18.5±1.5 | 6.9±2.7[a] | 11.6±3.8[b] |
| GSH (µM/mg protein) | 2.86 ± 0.09 | 0.65 ± 0.04 [a] | 1.7 ± 0.06 [b] |

***The data are presented as mean ± SD (n = 6/group). ND, Normal diet fed; HFFD, High fat fructose diet; ADF, alternate day fasting. [a] p< 0.01 HFFD vs. ND, bp < 0.01 Fasting vs. HFFD.***

## 2.4. Alternate day fasting improves renal inflammation:

All the rats in this study had their kidney tissues sampled for levels of the renal inflammatory markers IL-1, IL-6, and TNF (figure 1). The findings demonstrated that, in comparison to untreated controls, HFFD-treated rats had dramatically raised. levels of IL-1, IL-6, and TNF in their kidney tissues (Figure 1). The inflammatory markers IL-1, IL-6, and TNF were significantly lower in HFFD-treated rats than in HFFD-non-treated rats when the rats fasted for 24 hours every other day from the ninth to the twelfth weeks (figure 1).

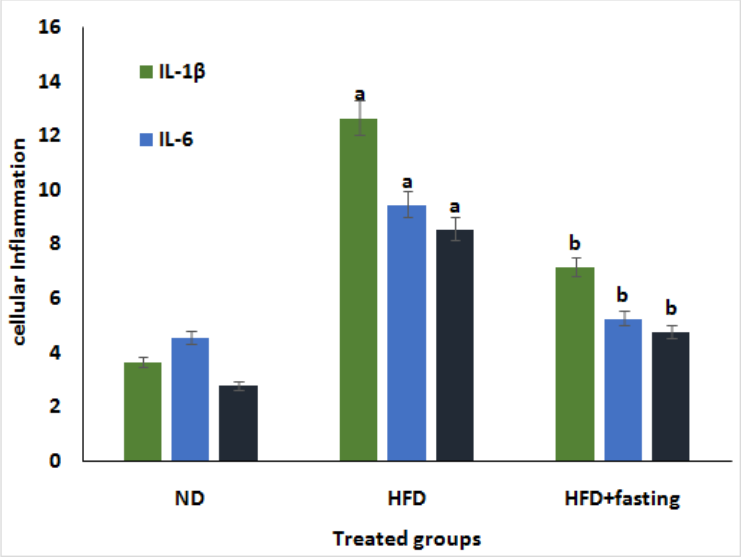


**Fig (1):** Fasting suppresses persistent inflammation induced by administration of high fat diet/fructose for 12 weeks. Representative homogenates of kidney tissues of normal and high fat diets were analyzed for renal content of interleukin 1 beta (IL-1β), IL-6, and TNFα. Data are expressed as mean ± SD. n = 6 animals per group. ND: Normal diet fed; HFD, High fat diet fed. [a] p < 0.01 HFD vs ND, [b] p < 0.001 fasting rat's vs HFD.

### 2.5. Impact of ADF , and caspase 3:□on the mRNA expression levels of IL1B, TNF

In contrast, rats given ADF from the ninth to the twelfth weeks showed significant decreases in inflammatory and apoptotic markers, as shown in figure (2), while the HFFD group showed an attenuated high levels of gene expression of the inflammatory and apoptotic markers (IL1B, TNF, and caspase 3) when compared with the control group. This demonstrated that ADF exhibited anti-inflammatory and anti-apoptotic properties that offer beneficial protection against kidney damage brought on by HFFD.

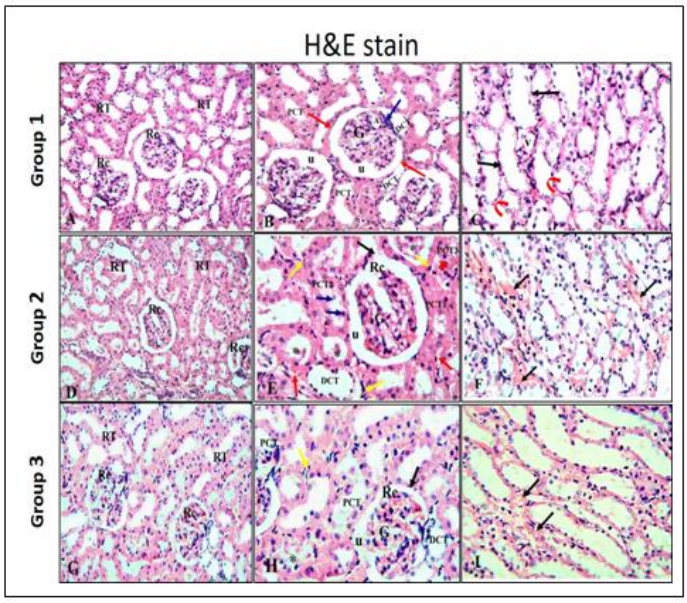


**Fig (2)**: Representative photograph of H&E stained histological sections of rat renal cortices (A&B) and renal medulla (C) of group 1 (control group) showing the typical normal structure of the kidney tissue with renal corpuscles (Rc), double wall bowman's capsule (red arrows ) enclosing glomeruli (G), normal urinary space (u) , and renal tubules including proximal convoluted tubules (PCT) and distal convoluted tubules (DCT). Renal medulla show normal medullary tubules; thick (curved arrows) and thin (black arrow) limbs of Henle with clear cavities and thin interstitial tissue . In group 2 rats (high fat diet) (E&F): the cortex shows distortion of kidney architecture, discontinuation of bowman's capsule (black arrow), glomerular swelling and congestion (G) and narrowing of urinary space(U). Tubular vacuolation (PCT1) , shedding of their lining cells (blue arrows) as in in PCT2, cast formation (stars), degeneration and separation of the cells (arrow heads) in the tubular lumen (PCT3) and dilation and thinning of their wall as in DCT and numerous peritubular fibroblasts (yellow arrows) are also seen. In (F): Marked interstitial hemorrhage are detected in between medullary tubules of group 2 rats. In group 3 (fasting rats ), (G,H&I): Normal kidney architecture is nearly preserved with normal shape and appearance of renal corpuscles (Rc) , preservation of bowman's capsules and urinary spaces(U) and less congested glomeruli (G), normal shape and lining of most of proximal (PCT) and distal (DCT) convoluted tubules but cast formation (stars) is still detected in some tubules and fewer fibroblasts (yellow arrows ) are seen peritubular. In addition to less interstitial medullary hemorrhage (black arrows) as seen in (I) of rat of this group when compared to the rats of group 2.. (H&E stain : A,D&G x200; B,C,E,F,H&I x400)

### 2.6. Effect of alternate day fasting on renal morphology and histopathological changes:

Rats in control groups had kidney cortical and medullary architecture that was visible in H&E-stained sections. (Fig. 3: A, B&C), while HFFD group showed distortion of kidney architecture, glomerular swelling and congestion narrowing of urinary spaces, discontinuity of bowman's membranes, tubular dilation, tubular vacuolations and shedding of their cells and cast formation in their lumens and interstitial medullary hemorrhage were detected (Fig.3: D,E&F). However, the fasting rat group showed relative restoration of kidney structure with minimal renal tubular,glomerular, and interstitial medullary changes (Fig.3: G, H&I). Masson trichrome stained sections showed fine collagen fibers between the tubules and glomerular capillaries of the cortex and between medullary tubuless inthe control group (Fig.4: the A&B). In the high HFFD rats' group, marked deposition of thick collagen fibers was detected (Fig.4: C&D) whereas a smaller amount of collagen fibers was noticed in fasting group rats (Fig.4: E&F) when compared to the HFFD group.

In immunohistochemicalstaining, anti-LC3 immuno-stained sections inGroup 1 rats showedmoderate

positive LC3 immunoreaction in the cytoplasm of renal tubular epithelium of the cortex and negative reaction in the cells of medullary tubules (Fig.5: A, B&C). In the HFFD group, mild LC3 immuno-positive reaction was detected in the cells of cortical and medullary tubules (Fig.5:D,E&F). Contrarily, the fasting group had a strong LC3 immunoreaction in the renal tubule cells and a minimal response in the medullary tubule cells (Fig. 5: G, H, & I). Only the walls of the renal arterioles in the kidney sections from the control group displayed SMA positive expression (Fig. 6: A, B, & C).Significantly, the cortical and medullary tubule cells in the kidneys of the HFFD group had strong expression (Fig. 6: D, E, and F). Contrarily, immunoreactivity in the fasting group was reversed and revealed a poor expression of SMA in several tubules (Fig. 6: G, H, & I).

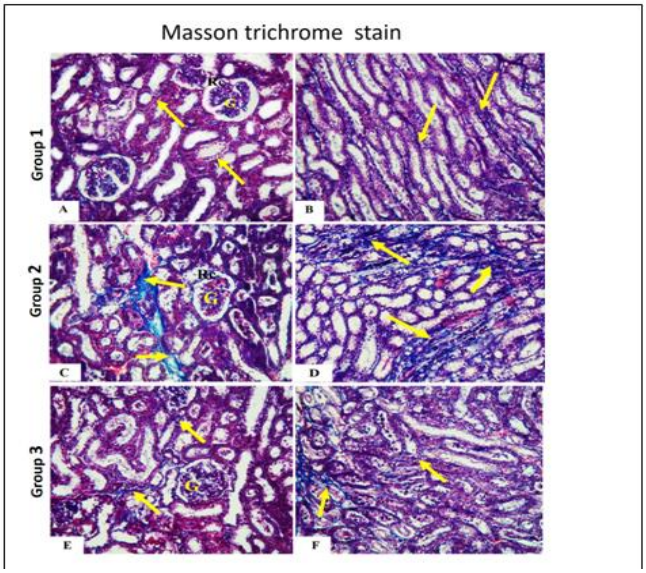


**Fig (3):** A photomicrographs of Masson trichrome stained sections in group 1 rats (A&B). A): shows renal cortex with fine blue collagen fibers between renal tubules and inside the glomeruli (G) in between the capillary tuft and also in between medullary tubules in (B). In group 2 rats (C&D), C): Shows marked increase in the amount of blue collagen fibers in the cortex which is deposited among the renal tubules, capillary tufts of glomeruli and among the medullary tubules as seen in (D). In group 3 rats (E&F), E): Shows fewer amount of collagen in between the renal tubules and glomeruli of the cortex and medullary tubules as detected in (F) when is compared with the rats of group 2. (Rc: renal corpuscle, G: glomeruli, yellow arrows: collagen)
(Masson trichrome stain; A, B, C, D, E&F x 200)

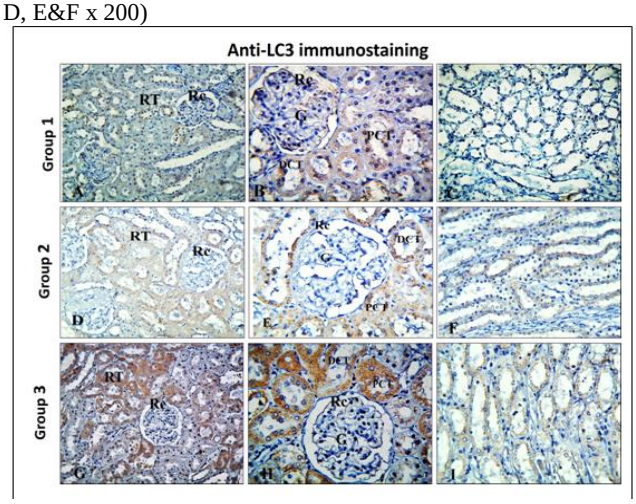


**Fig (4):** A photomicrograph of anti-LC3 immunostained sections in: Group 1 rats (A,B&C); shows moderate positive LC3 immunoreaction in the cytoplasm of renal tubular epithelium of the cortex in (A&B) and negative reaction in the cells of medullary tubules as seen in (C). Mild LC3 immunopositive reaction is detected in the cells of renal and medullary tubules of the rat of group 2 (D,E&F) .On the other hand, a strong LC3 immounoreaction is detected in the cells of renal tubules (G&H) and mild reaction in the cells of medullary tubules as seen in (I).
( (Rc: renal corpuscle, RT: renal tubules G: glomeruli, PCT: proximal convoluted tubules, DCT: distal convoluted tubules) (Anti-LC3 immunostain : A,D&G x200; B,C,E,F,H&I x400)

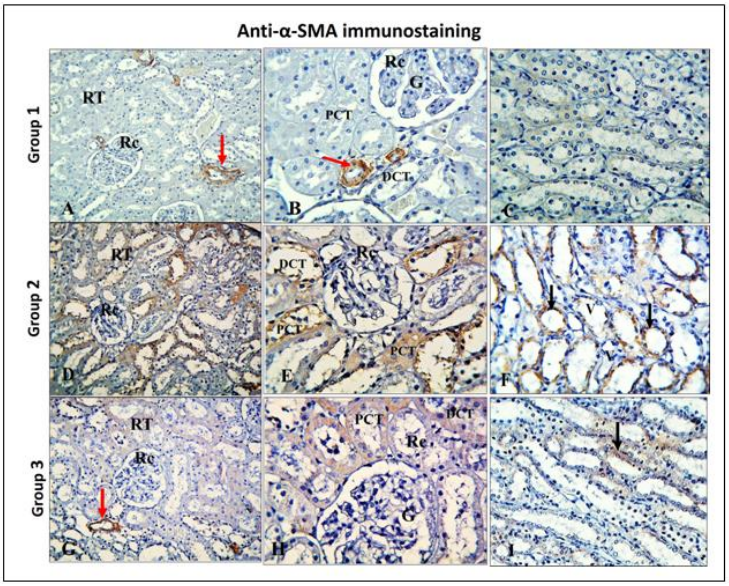


**Fig (5)**: A photomicrograph of anti-α SMA immunostained sections in: Group 1 rats (A,B&C); shows positive α SMA immunoreaction only in the cells of blood vessels wall (red arrows) whereas renal tubular and medullary tubules epithelium show negative reaction . In group 2 rats (D,E&F), a strong α SMA immunoexpression by the cells of renal tubules and medullary tubules (black arrows) is detected . On the other hand, weak α SMA immunoexpression by the cells of some renal tubules and positive reaction in the blood vessels in the renal cortex (G&H) and very weak reaction in the cells (black arrows) of medullary tubules (I) are detected when compared to group 2 rats .
(Rc: renal corpuscle, RT: renal tubules G: glomeruli, PCT: proximal convoluted tubules, DCT: distal convoluted tubules) (Anti- α SMAimmunostain : A,D&G x200; B,C,E,F,H&I x400)

## 3. Discussion

The impact of HFFD in metabolic and renal disorders has been widely recognized [29]. This is particularly relevant at the increased consumption of Processed and refined food items with high fat, red meat, and sugary beverage contents and minimum to no content of fiber, fruits, and vegetables.

In the present investigation, HFFD considerably raised body mass index, lee index, body weight, weight gain, and kidney weight. Data from both adult and pediatric autopsy have previously shown a relationship between kidney weight and body mass index (BMI). Among the organs that expand in obese patients are the liver, heart, and kidneys [30]. Hemodynamic alterations, fluid retention, and glomerular and tubular hypertrophy have all been linked to increased kidney size [31]. The majority of the evidence for the likelihood of intra-renal fat accumulation and altered lipid metabolism comes from investigations of obesity and high-fat diet models.

Our study's findings also showed that feeding rats a high-fat, high-fructose diet for 12 weeks dramatically increased their blood sugar levels and insulin resistance compared to rats on a regular diet. Additionally, rats with HFFD substantially experienced renal damage, which is characterized by diminished renal function, antioxidant enzymes, and greater levels of oxidative stress and cellular inflammation.

Our findings were consistent with those of earlier studies that linked HFFD intake to a higher risk of developing the metabolic syndrome (MS) [32], non-alcoholic fatty liver disease (NAFLD), and diabetes [33] diseases. The metabolic disorders obesity, dyslipidemia, hyperglycemia, hypertension, and cardiac illnesses are also strongly linked to HFFD[34], making them one of the major risk factors for chronic kidney disease [35]. Additionally, the increased prevalence of obesity among those who consume a high-fat diet is well acknowledged as an independent risk factor for the onset of chronic kidney disease and end-stage renal disease [36].

In this study, the HFFD group's disrupted lipid metabolism significantly contributed to kidney injury.

In mice and rats, renal damage is caused by cellular lysosomal dysfunction and chronic inflammation, as seen by the production of IL-6, TNF-α, and IL-1[37]. As a result of the buildup of extra lipids in the kidney and lipotoxicity, pro-inflammatory, pro-fibrogenic, and pro-apoptotic pathways are activated, leading to cellular damage, renal failure, and even end-stage renal disease [38]. Decrease in antioxidant defenses,the key inducible variables that result in kidney damage and the beginning of fibrosis in HFD-rats, isdue to elevated oxidative stress and chronic inflammation.[39].

The findings of this study's renal function tests showed that HFFD led to the worsening of kidney functions, which was shown by an increase in blood urea nitrogen, serum creatinine, and albuminuria as well as a decrease in urine volume and creatinine excretion through the urine. These findings corroborated those of other researchers who noted that proteinuria, albuminuria, decreased creatinine excretion, proximal tubule hypertrophy, severe tubular loss, and tubulointerstitial fibrosis are all signs of renal failure in obese people and animals [40].

Interestingly, it has been demonstrated that using fasting as an alternative medicine for 24 hours from the ninth to the twelfth week can decrease metabolic disturbances, cellular oxidative stress, and inflammation, and increase antioxidant status, improving metabolic syndrome and preventing kidney damage linked to HFFD consumption for a 12-week period.

Due primarily to its antioxidant and anti-inflammatory properties, dietary restriction has gained widespread recognition as an alternative medicine with a potent non-genetic and non-pharmacological nature that increases multisystemic protection against stress and the development of age-related chronic diseases. The reduction of tubular damage, inflammation, oxidative stress, cell death, and mitochondrial dysfunction that were shown one day after IF has been proven to have renoprotective benefits in early acute kidney injury (AKI) [41].

Under various stressful circumstances, autophagy is a critical mechanism in cell recycling and the depletion of resources for cell homeostasis. AMPK, ULK1, and mTORC1 are three upstream active components that regulate this pathway. An unstable condition of cell homeostasis results from changes in autophagy in vascular endothelium, epithelium, pericytes, podocytes, and mesangial cells. Obesity and decreased renal autophagy have been linked in several studies [42, 43].

Regarding histopathological changes, in our study the effect of induced kidney injury was demonstratedwith an assessment of LC3; a marker of autophagy, SMA; a marker of fibrosis, and caspase 3; a marker of apoptosis.The results showed distortion of kidney architecture, glomerular swelling and congestion and narrowing of urinary spaces, discontinuity of bowman’s membranes, tubular dilation, tubular vacuolations and shedding of their cells and cast formation in their lumens, interstitial medullary hemorrhage in addition to increased renal collagen deposition and fibrosis were detected in HFFD diet group. Our results are consistent with earlier studies, which demonstrate that lipid buildup affects renal tubular epithelial cells, mesangial cells, and podocytes by altering their structural and functional characteristics [44]. Lipotoxicity, a condition caused by lipid buildup in non-adipose tissue, is becoming more widely acknowledged to be a factor in organ harm. In animal models of metabolic illness (metabolic syndrome, obesity, and DM), chronic renal disease, acute kidney damage of various etiologies, as well as agingprocess, it was noted that excessive renal lipids might harm the kidneys. Numerous processes, such as the release of proinflammatory and profibrotic substances, contribute to the malfunction and damage of lipotropic cellular structures. Furthermore, the physical compression that

visceral adiposity puts on the kidneys has been linked to obesity-related renal disease.

The ADF rat group, on the other hand, displayed quantities of collagen and a relative repair of kidney structure with little renal tubular, glomerular, and interstitial medullary alterations. An earlier study that revealed intermittent fasting (IF) has been proven to provide cardioprotective benefits in the heart may complement our findings regarding the impact of fasting-induced HFFD-induced kidney injury [45, 46]. The benefits of IF on lifespan, myocardial triglyceride buildup, prevention of cardiac cell death, cardiac diastolic parameters, and a cardio-protective metabolic program have been demonstrated by clinical and experimental data. Furthermore, IF lessens the cardiac damage brought on by the high-fructose diet through altering the left ventricular renin-angiotensin system [47]. Additionally, in HFD-induced obese mice, IF improves gut microbiota, which has positive effects on lipid metabolism [48]

The present work demonstrated that autophagy was impaired in HFFD rat kidney tissue whereas it was proved to be enhanced in fasting rats. Such data was supported by decreased LC3 immuno-expression in HFFDrat's kidney and increased expression in the kidney tissue of the fasting group. Our present data could be supported by many reviews on relationship between the IF and autophagy, Dietary restriction (DR) has been proven in preclinical research to increase longevity and lessen the occurrence of age-related illnesses such cancer, diabetes, and neurological and cardiovascular disorders [49]. Most cultured cells and tissues, including the muscle and liver, are also induced to undergo autophagy as a stress response [50, 51].

Our research demonstrated that fasting reduced renal fibrosis as seen by a reduction in the amount of collagen deposited in the renal interstitium and the expression of the fibrogenic marker SMA. This corresponds to a study from 2019 by Morales et al. [52], which discovered that fasting prepares the kidney to withstand tubular damage and the onset of fibrosis through a number of mechanisms, including the preservation of redox balance, maintenance of both mitochondrial and endoplasmic reticulum homeostasis, and suppression of inflammatory processes that persist long after the initial ischemic injury.

Furthermore, we showed that fasting had an antiapoptotic effect on HFFD-induced kidney damage, as evidenced by the decrease in caspase 3 and TNF gene expression as well as lower caspase 3 immunoexpression in the kidney of the fasting rat group. This finding was corroborated by a related study [53] that looked at how fasting affected HFD-induced cardiomyopathy. Using TUNEL labeling and western blot analysis, the researchers found that myocardial lipid accumulation significantly increased cardiac apoptotic cell death and that IF intervention significantly reversed HFD-induced effects. Previous studies have revealed that cardiac apoptosis increased in obese rats as shown by an increase in TUNEL-positive cells, the Bax/Bcl-2 ratio, and the creation of the Cleaved Caspase-3 protein [54]. Food restriction was demonstrated in another study to decrease the Bax/Bcl-2 ratio and Cleaved Caspase-3 protein synthesis in TUNEL-positive cells, which may have inhibited the development of cardiac apoptosis in obese rats on a high-fat diet [55].

**Conclusion:** According to the findings of our study, alternate-day fasting from the ninth to the twelfth week might be beneficial in reducing the metabolic and renal damage brought on by diets rich in sugar and fat. Alternate-day fasting significantly improved cellular antioxidant parameters and reduced oxidative stress and inflammation. These results were supported by biochemical findings including suppression of inflammatory and apoptotic markers besides histopathological findings in which enhancement of renal autophagy, decreasing fibrosis, and apoptosis were proved.

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