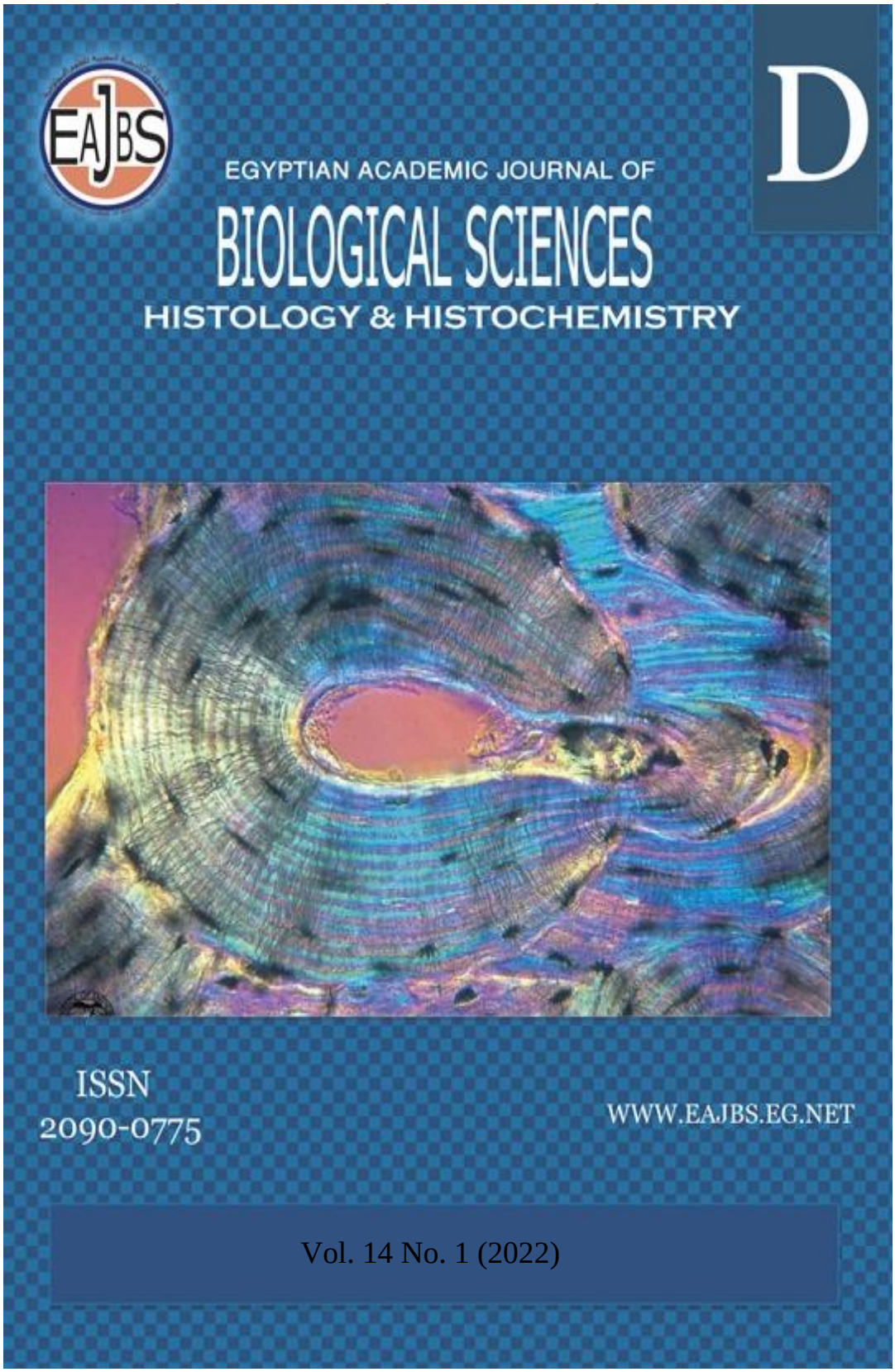


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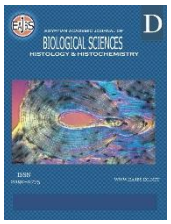

# Modulatory Effect of Intermittent Fasting on Autophagy and Apoptosis in White Adipose Tissue of A High Fat-Fed Rat Model

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**ABSTRACT**

Obesity is a worldwide public health problem, with its rate being tripled in the last four decades and continuously increasing. It is linked to varied comorbidities such as diabetes, hypertension, dyslipidemia, and cardiovascular disease. Multiple rapid weight loss protocols are used nowadays to lose weight rapidly as intermittent fasting (IF). In this study, we mimic human behavior, consuming a high-fat diet pattern (HFD), then carrying out IF. We investigated the effect of HFD and IF on the visceral and subcutaneous types of white adipose tissue (WAT) morphology, autophagy and apoptosis and to evaluate any significant differences between the 2 types of WAT, with further evaluation of blood levels of Malondialdehyde (MDA) and Superoxide dismutase (SOD).

Eighteen male rats were divided randomly into 3 groups as control group which took the basal diet, HFF fed on HFD, and IF which receive HFD accompanied by IF. Animals fasted from the 8$^{th}$ week to the end of the 12$^{th}$ week when all of them were sacrificed. Bodyweight was recorded, and blood was collected for measurement of serum glucose, insulin, cholesterol, triglyceride (TG), and oxidative stress markers. Both inguinal and epididymal WAT were dissected then processed and stained with H&E and immunohistochemical with antibodies for bax, LC3 and P62. Significant increase in body weight, glucose, cholesterol, TG and a significant decrease in SOD and insulin were detected in HFF but they were all significantly improved in IF except blood glucose which was not significantly decreased in IF rats. Besides a non-significant increase in MDA was recorded in HFF group. Also, hypertrophied WAT was observed especially the visceral one in HFF that decreased significantly after IF. Induced apoptosis and inhibited autophagy were recorded in HFF group and then reversed following IF protocol. It has been proposed that IF is effective in reversing HFF effects on WAT although IF should be investigated for a longer period and followed with more strict markers.

## INTRODUCTION

Obesity is a worldwide health problem with overweight obesity reaching an incidence of 40% of women vs. 39% of men (Blüher, 2019). High BMI is the fourth leading cause of reduced life expectancy of 5–20 years (Kinnunen *et al.*, 2021).

It is commonly linked to varied comorbidities such as diabetes, hypertension, dyslipidemia, cardiovascular disease, and cancer (Collaborators, 2017).

Adipose tissues (AT) are constituted of white adipose tissue (WAT) and brown adipose tissue (BAT) based on both color and developmental origin. WAT could be subdivided into visceral WAT (vWAT) and subcutaneous WAT (sWAT) based on localization in or outside of the abdominal cavity, respectively (Harney *et al.,* 2021). In humans and rodents, high vWAT amounts have been clearly linked to insulin resistance, cardiovascular disease, cancer, and high morbidity (Després and Lemieux, 2006; Després, 2012; Renehan *et al.,* 2008) though it is the most sensitive depot to lipolysis (Zechner *et al.,* 2017). In contrast, sWAT is the largest adipose depot in humans and mice, and its increased amount sWAT is correlated to lower metabolic disease risk (Kwon *et al.,* 2017; Vitali *et al.,* 2012). Although sWAT is more richly innervated and vascularized, it has a higher resistance to adrenergic lipolysis stimulation (Guilherme *et al.,* 2019; Farb and Gokce, 2015).

During obesity, increased lipid levels and the following hypertrophy of adipocytes lead to AT dysfunction (Klöting and Blüher, 2014). These adverse modifications in obese AT involve macrophage infiltration, apoptosis, increase in or/and secretion of inflammatory cytokines, hypoxia, in addition to insulin resistance (Grant and Dixit, 2015).

Intermittent fasting (IF) in animals and humans has been considered as one of the dietary restrictions (DR) methods that is confirmed to possess antioxidant and anti-inflammatory properties (Hu *et al.,* 2019; Morgan *et al.,* 2007). Moreover, IF has been demonstrated to lower fasted insulin levels, improve glucose tolerance, and lower blood cholesterol (Li *et al.*, 2017), although its underlying molecular mechanisms are not fully understood yet (Harney *et al.,* 2021).

Malondialdehyde (MDA) is the ultimate product of lipid oxidation and one of the commonly known markers of oxidative stress. Increased free radicals levels will cause overproduction of MDA (Elgadir et al., 2019). While superoxide dismutase (SOD) is considered one of the first-line defense antioxidants, which decreases in response to OS conditions (Ighodaro and Akinloye, 2018).

Increased adiposity has been associated with greater levels of OS compared to average-weight people (Adnan *et al.,* 2019), principally with more visceral fat volume concerns (García-Sánchez *et al.,* 2020; Akl *et al.,* 2017). Oxidative stress could aggravate and/or stimulate the development of obesity-related metabolic-related complications (Mohr *et al.,* 2021). The mechanisms involved in the OS propagation in obesity are complicated, they might involve immune cell stimulation leading to downstream production of free radicals (Sindhu *et al.,* 2018; Akhter *et al.,* 2019), pro-inflammatory cytokines synthesis (Borges *et al.,* 2018), depletion of antioxidant sources (Grenier-Larouche *et al.,* 2015), increased free fatty acid levels causing endoplasmic reticulum stress (Rennert *et al.,* 2020) and mitochondrial and peroxisomal oxidation (Barazzoni *et al.,* 2012).

Moreover, OS has been known to have a crucial role in apoptotic pathways (Redza-Dutordoir and Averill-Bates, 2016). Autophagy has a complex relation with OS; autophagy is activated in response to OS to protect the cells from apoptosis (Mizushima et al., 2008), though autophagy impairment increases the accumulation of OS (Lee *et al.,* 2012). Additionally, antioxidant molecules partially or completely inhibit autophagy (Levonen *et al.,* 2014).

Apoptosis is a complicated process, that regulates the destruction of

cells, one of its cardinal features is the involvement of mitochondria and the apoptotic proteins Bcl-2 and Bax (Tzifi *et al.,* 2012). Overexpression of Bax accelerates apoptotic death by acting on mitochondria to disrupt the electron transport chain and promote the release of cytochrome c into the cytoplasm (Ma *et al.,* 2020). Then, it activates proteolytic molecules known as caspases that are crucial for the execution of apoptosis (Elmore, 2007).

LC3-II is a standard marker indicating autophagy induction and its level is connected to the number of autophagosomes (Mizushima and Levine, 2010). After autophagosome formation, they fuse with lysosomes to form autolysosomes. On the opposite side, p62 could be used to monitor autophagic flux, so its expression level is negatively correlated to the autophagic activity (Komatsu *et al.,* 2007).

Due to the relative deficiency of previous studies on IF effects on WAT apoptosis and autophagy immunohistochemical staining and applying more concentration on other organs. This study aimed to investigate the effect of high-fat feeding and intermittent fasting on the white adipose tissue morphology, autophagy and apoptosis and to evaluate any significant differences between the 2 types of WAT, with further evaluation of blood levels of MDA and SOD.

## MATERIALS AND METHODS

### 1. Animals Used:

Eighteen male Sprague-Dawley albino rats weighing 300-350 gm, 12- 14 weeks old, were used in this study. They were housed two per cage and allowed free access to water and food under a normal day-night cycle (12-12). The experiment was carried out at Anatomy Department, Faculty of Medicine, Mansoura University following the instructions of the care and use of experimental animals of the National Institute of Health. Animal care and handling were done according to the guidelines set by the World Health Organization, Geneva, Switzerland, 1993.

### 2. Experimental Design:

Eighteen rats were enrolled in the study. After allowing animals one week to acclimatize, rats were randomly divided into 3 equal groups. The control group (C) in which the rats fed on basal diet formed of 10% fat and no fructose with free water ad libitum for 12 weeks (Hassan and Salah-Eldin, 2021). The high fat fed group (HFF), in which the rats were kept on high fat diet (HFD) + fructose throughout the experiment (Lozano *et al.,* 2016). The fasting group (IF), in which the rats had HFD for 12 weeks followed by intermittent fasting. HFD contains 40% fat + 20 g of fructose dissolved in 100 mL of tap water (Moreno-Fernández *et al.,* 2018). Fasting protocol is applied only from the $8^{th}$ week till the time of scarification, $12^{th}$ week (Varady *et al.,* 2007). The adopted fasting protocol was alternate day fasting (24 h feeding followed by 24 h fasting) (Henderson *et al.,* 2021) as well as open water access (Bhutani *et al.,* 2013). Both inguinal and epididymal white adipose tissue (represents subcutaneous fat, and visceral fat sWAT and vWAT, respectively) were dissected as described by (Zhang *et al.,* 2018).

### 3. Samples Collection:

At the assigned time, blood samples were taken from the left ventricles and collected in polyethylene tubes for biochemical tests. Then, rats were anaesthetized with isoflurane (Wong et al., 2013) followed by cervical dislocation. Body weights were measured also at beginning of the experiment and before scarification.

### 4. Biochemical Tests:

Serum levels of glucose were assessed by blood glucose assay kit and glucose oxidase method (Contournext, Parsippany, NJ, USA). Serum levels of total cholesterol and triglycerides were assessed using; the total cholesterol assay endpoint kit (MG, cat. No. MG230001), triglyceride assay endpoint kit (MG, cat. No. MG314001), respectively (Burtis et al., 2012; Young

& Friedman, 2001). All colorimetric assays were carried out according to the manufacturer's protocols utilizing Erba CHEM-7 device (ERBA Diagnostics, India). ELISA was used to measure Insulin, MDA and SOD using (Abcam, Cambridge, UK, ab273188), (Abcam, Cambridge, UK, ab238537) and (Abcam, Cambridge, UK, ab13498) respectively, following the manufacturer's instructions.

**5. Specimens Processing for Histological Examination:**

Adipose tissues were processed for paraffin sections by dehydration in graded ascending concentrations of alcohol, cleared by xylene, then embedded in soft followed by hard paraffin wax. Slides were cut using microtome at a thickness of 5-6 µm and then stained with H&E. After rehydration of paraffin sections, endogenous peroxidases were blocked by 0.3% $H_2O_2$. Antigen retrieval was done by heating in the microwave using sodium citrate buffer (pH6) for 20 min and then blocked by 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS). After that, sections were incubated overnight at 4 ℃ with a primary antibody against Bax (Abcam, Cambridge, UK, ab32503, 1:200) (Pedrana *et al.*, 2021), P62 (Abcam, Cambridge, UK, ab109012, 1:200) (Havaki *et al.*, 2017) and LC3 (Abcam, Cambridge, UK, ab192890, 1:200) (Mortezavi *et al.*, 2017). The reaction was revealed using ABC kit following the manufacturer's guidelines. The sections were then counterstained with hematoxylin, dehydrated then mounted using a synthetic resin medium.

**6. Morphometric Studies:**

Each slide was examined and photographed via Olympus SC100 digital camera installed on Olympus® CX41light microscope. To determine the fat cells diameters, we used the method adopted previously (Sjostrom *et al.*, 1971) using the Image J program. The percentage of brown colour density in Bax, LC3 and P62 immunostained sections was measured in 5 nonoverlapping randomly chosen in each slide using program NIH Image J program (National Institutes of Health, Bethesda, MD, USA), following the program instructions.

**7. Statistical Analysis:**

Data were analyzed with GraphPad PRISM 7 software (GraphPad Software Version 7, La Jolla California USA) and analytical statistics using analysis of variance (ANOVA) test to check the significance of the difference between different groups of parametric data followed by a post hoc Tukey test for multiple comparisons. P-values were considered significant if $<0.05$.

## RESULTS

**1. Bodyweight:**

Rats were weighed at the beginning of the experiment and there was no detected significant difference between the different groups. At the end of the experiment, HFF rats (385.3±18.74) showed a significant increase in weight compared to the control group (348.8±12.37), while IF group showed a significant decrease (335.5± 20.68) compared to HFF one (Fig. 1A) and a non-significant difference between C and IF groups were detected.

**2. Blood Glucose, Serum Insulin, TG, and Cholesterol:**

At the end of the $12^{th}$ week, a significant increase in the level of blood glucose (458.7 ± 169.8) and a significant decrease in serum insulin (111.7 ±11.06) was observed in HFF rats compared to control rats (125.3±5.33) and (219.7±20.01) respectively. While in IF group, blood glucose level was decreased but nonsignificant when compared to HFF group (150±10) and serum insulin was significantly increased (194 ±44.31) compared to HFF group (Fig. 1 B & C) with no recorded significant difference between C and IF groups.

Moreover, a significant increase in both levels of blood TG (192±12) and

cholesterol (151.3±10.26) was recorded in HFF rats compared to the control group (120±10) and (102.3±7.02) respectively. While in IF rats, both levels were significantly decreased (141 ±8.54) and (115.3±5.03) respectively in comparison to HFF rats (Fig. 1 D & E). In both, there was no recorded significant difference between C and IF groups.

**3. Oxidative Stress Markers:**

In HFF rats a non-significant elevation in the serum level of MDA (1.87±0.15) while SOD level was significantly decreased (36.4 ±2.42) compared to control group (1.46 ±0.50) and (61.62 ±10.38) respectively. Also, IF group showed a non-significant decrease in MDA level (1.54 ±0.51) but SOD increased significantly (53.57 ±3.5) when compared to HFF rats (Fig. 1 F & G). There was no statistical difference recorded between C and IF groups in both MDA and SOD levels.

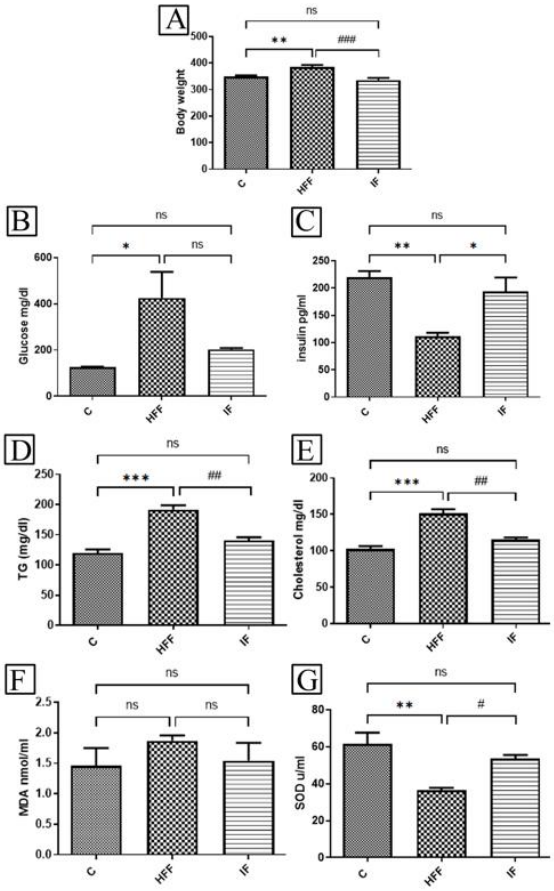


**Fig.1:** Histograms showing a comparison between 3 groups regarding: (A) bodyweights showing, a significant increase in HFF (P=0.0071) followed by a significant decrease in IF (P=0.0005). (B) level of serum glucose showing, a significant increase in HFF (P=0.037) followed by a non-significant decrease in IF group (P=.0.114) although there was also no significant decrease between the C and IF group (P=0.69). (C) Serum insulin levels showing, a significant decrease in HFF (P=0.0088) followed by a significant increase in IF rats (P=0.03). (D) Serum TG level showing, a significant increase in HFF (P=0.0003) followed by a significant decrease in IF rats (P=0.002). (E) Serum cholesterol levels showing, a significant increase in HFF (P=0.0006) followed by a significant decrease in IF (P=0.0031). (F) Serum MDA level showing, a non-significant increase in HFF (P=0.4) followed by a non-significant decrease in IF while (G) Serum SOD level showed, a significant decrease in HFF (P=0.007) followed by a significant increase in IF group (P=.04).

**4. Histopathological Examination:**

Control group sections from the white adipose subcutaneous and visceral fat groups showed normal cytoarchitecture of white adipose tissue, pale stained because lipid droplets that occupy most of cytoplasm have been dissolved during tissue preparations. The cell membrane is a thin rim, and the nucleus is compressed and displaced to one side of the cells with diameters (18.44 ±5.62) and (28.21 ±7.93) respectively (Fig. 2 A & B).

HFF group sections showed marked hypertrophy of adipocytes in white adipose subcutaneous and visceral fat groups, as cells appear enlarged but with preserved architecture and their diameters were (27 ±4.99) and (48.5±6.17) respectively (Fig. 2 C & D). However, IF group sections showed a marked reduction of diameters of adipocytes in both types of white adipose tissue with diameters (15.19 ±5.82) and (25.14±10.24) respectively (Fig. 2 E & F) with no significant statistical difference between C and IF groups and the detected differences between sWAT and vWAT was also not significant (Fig. 2 G & H).

**5. Immunohistochemical Stains:**

Bax, an apoptotic marker, immune reaction was detected minimally in the control cytoplasm of adipocytes in white adipose subcutaneous and visceral fat groups (3±1.73) (Fig. 3 A & B). The percentage of bax immune-stained area increased in HFF adipocytes sections of subcutaneous and visceral WAT (44± 2.58) and (57.86±3.34) respectively when compared to control rats (Fig. 3 C & D). Though, the percentage of bax immune-stained area decreased significantly in IF subcutaneous and visceral white adipose in comparison to HFF group (16.43± 3.51) and (22.29±5.59) correspondingly (Fig. 3 E & F). No detected significant difference between C and IF groups. Also, no significant difference was detected between sWAT and vWAT (Fig. 3 G & H).

LC3, an autophagosome marker, the immune reaction was detected in the cytoplasm of adipocytes of control rats in both subcutaneous and visceral fat groups but higher in vWAT (41.71±3.25) and (57.29±5.31) respectively (Fig. 4 A & B).

The percentage of LC3 immune-stained area decreased significantly in HFF adipocytes in subcutaneous and visceral white adipose (16.86± 3.08) and (19.29±5.25) respectively when compared to the control group (Fig. 4 C & D). While the LC3 percentage of the immune-stained area increased significantly again in adipocytes of IF group in subcutaneous and visceral WAT (33.71±3.35) and (33.71±3.35) respectively (Fig. 4 E & F) compared to HFF group. No statistically significant difference was detected between C and IF groups or sWAT and vWAT (Fig. 4 G & H).

The immune reaction of P62 was detected minimally in nuclei of adipocytes in control subcutaneous and visceral WAT (4.86±0.69) and (4.86±0.69) respectively (Fig. 5 A & B). The percentage of P62 immune-stained areas increased significantly in HFF adipocytes of subcutaneous and visceral WAT (37.86± 5.08) and (52.14±3.97) (Fig. 5 C & D) compared to control group. While the percentage of P62 immune-stained areas decreased significantly in IF adipocytes of subcutaneous and visceral WAT (14.43±4.5) and (15.86±4.15) correspondingly (Fig. 5 E & F) when compared with HFF group. Moreover, no statistically significant difference between C and IF groups was detected or between sWAT and vWAT (Fig. 5 G & H).

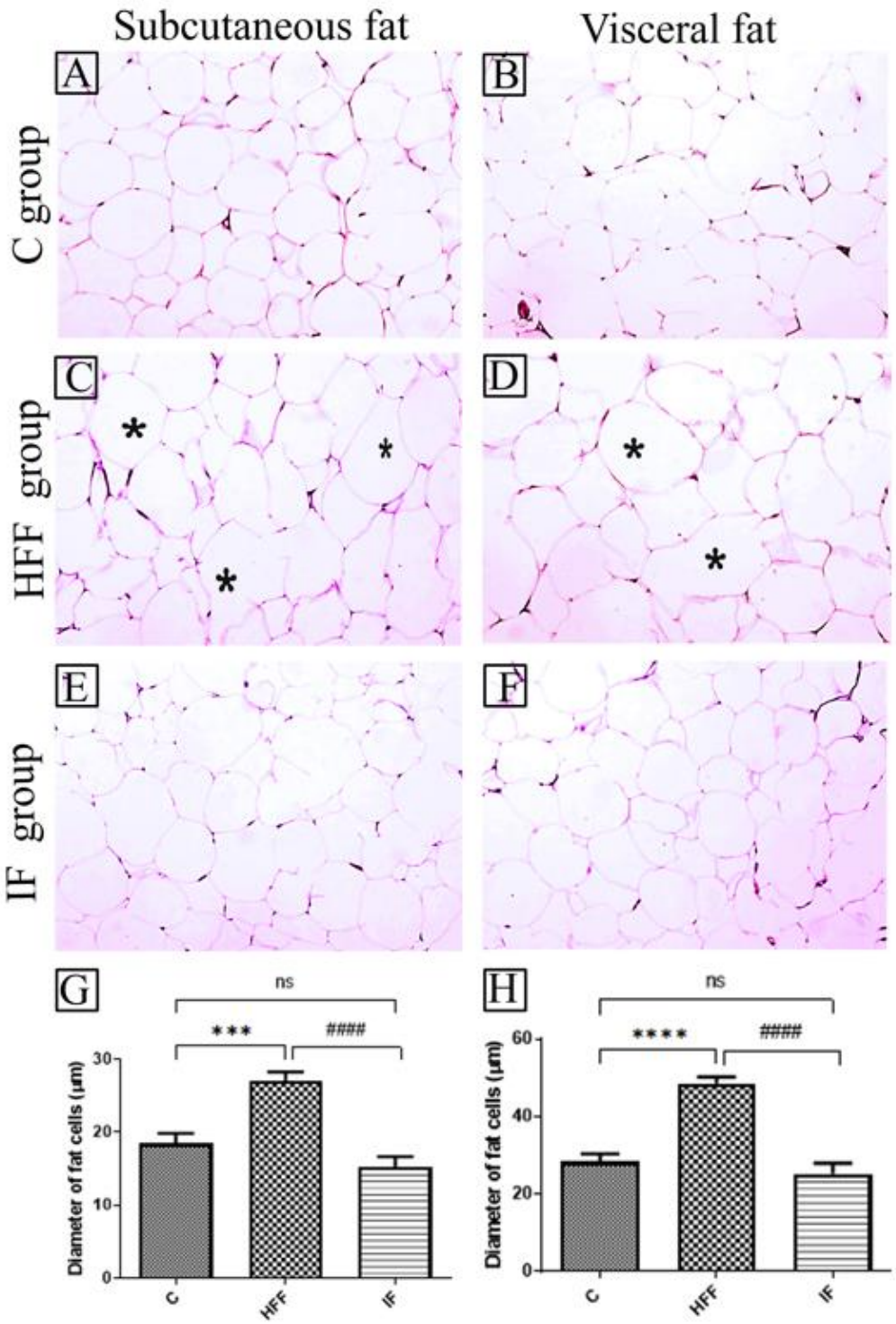


**Fig. 2:** Photomicrographs of white adipose tissue, visceral and subcutaneous, stained with H&E showing a normal appearance in the control group section in which adipocytes are pale stained and the nucleus is at one side of the cells (A, B). In HFF sections frequent multiple enlarged adipocytes (asterisks) (C, D). While in the IF sections a marked reduction in numbers of enlarged adipocytes (E, F) is seen. (Magnification X 100).

(G, H) Histograms of the Mean ±SD of diameters of subcutaneous and visceral adipocytes in µm respectively showing, a significant increase in HFF group when compared to the control group in both subcutaneous (P=0.0002) and visceral (P< 0.0001), but higher in the vWAT. Also, a significant decrease of adipocytes diameters in both subcutaneous and visceral fat in IF compared to HFF is observed (P< 0.0001).

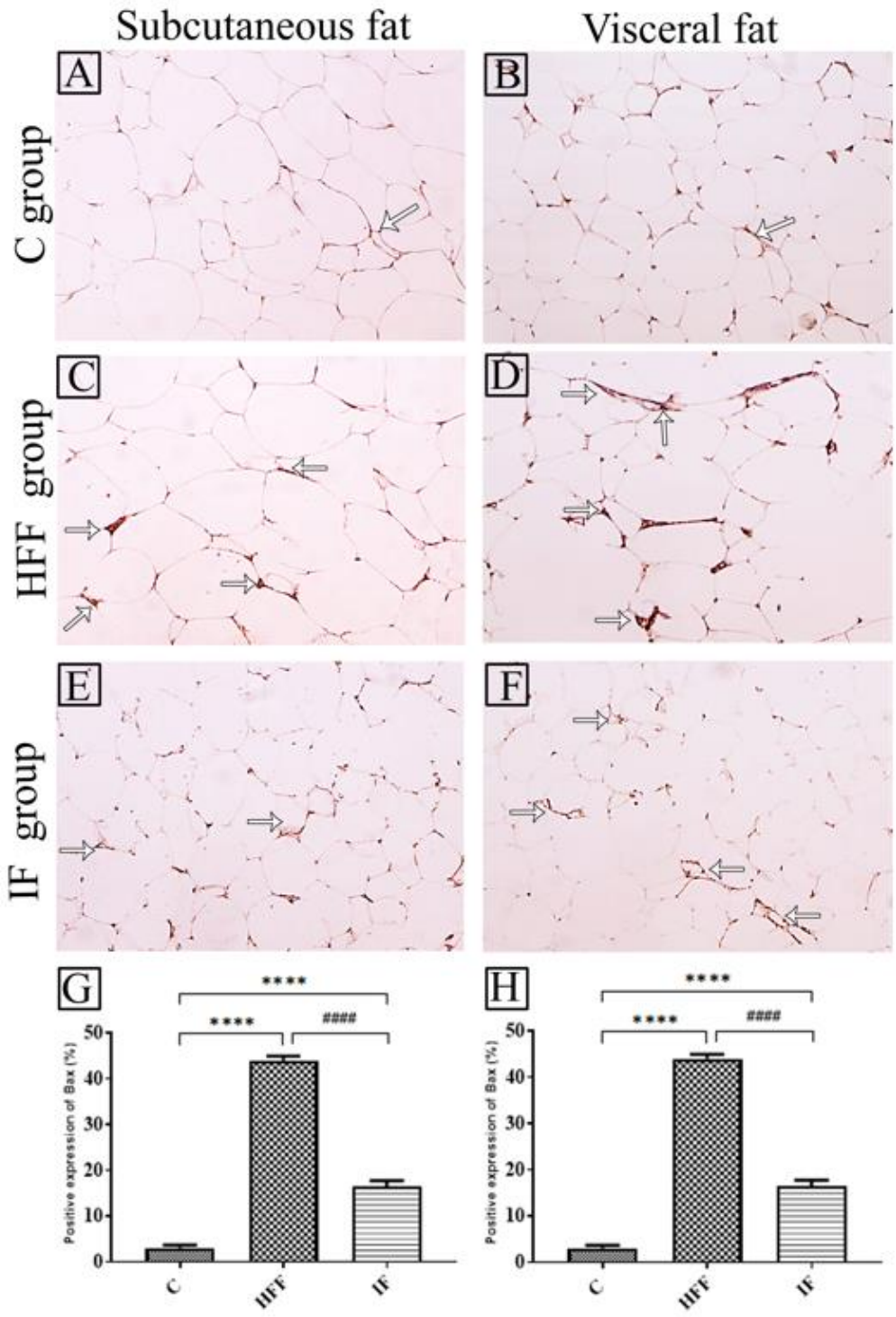


**Fig. 3:** Photomicrographs of subcutaneous and visceral WAT-stained sections with bax antibody showing, minimal bax expression in the cytoplasm of adipocytes in the control group (white arrows) but a marked increased positive brown expression (white arrows) is seen in HFF adipocytes although, bax expression was reduced markedly in IF adipocytes (white arrows). (Magnification X 100).
(G, H) Histograms comparing the Mean ±SD of the percentage of bax expression areas in subcutaneous and visceral WAT respectively among different groups showing, a significant increase in expression of bax in both subcutaneous and visceral of HFF adipocytes ($P<0.0001$) when compared to C. Then, a significant decrease of expression of bax in both subcutaneous and visceral in IF adipocytes ($P< 0.0001$) when compared to HFF was seen.

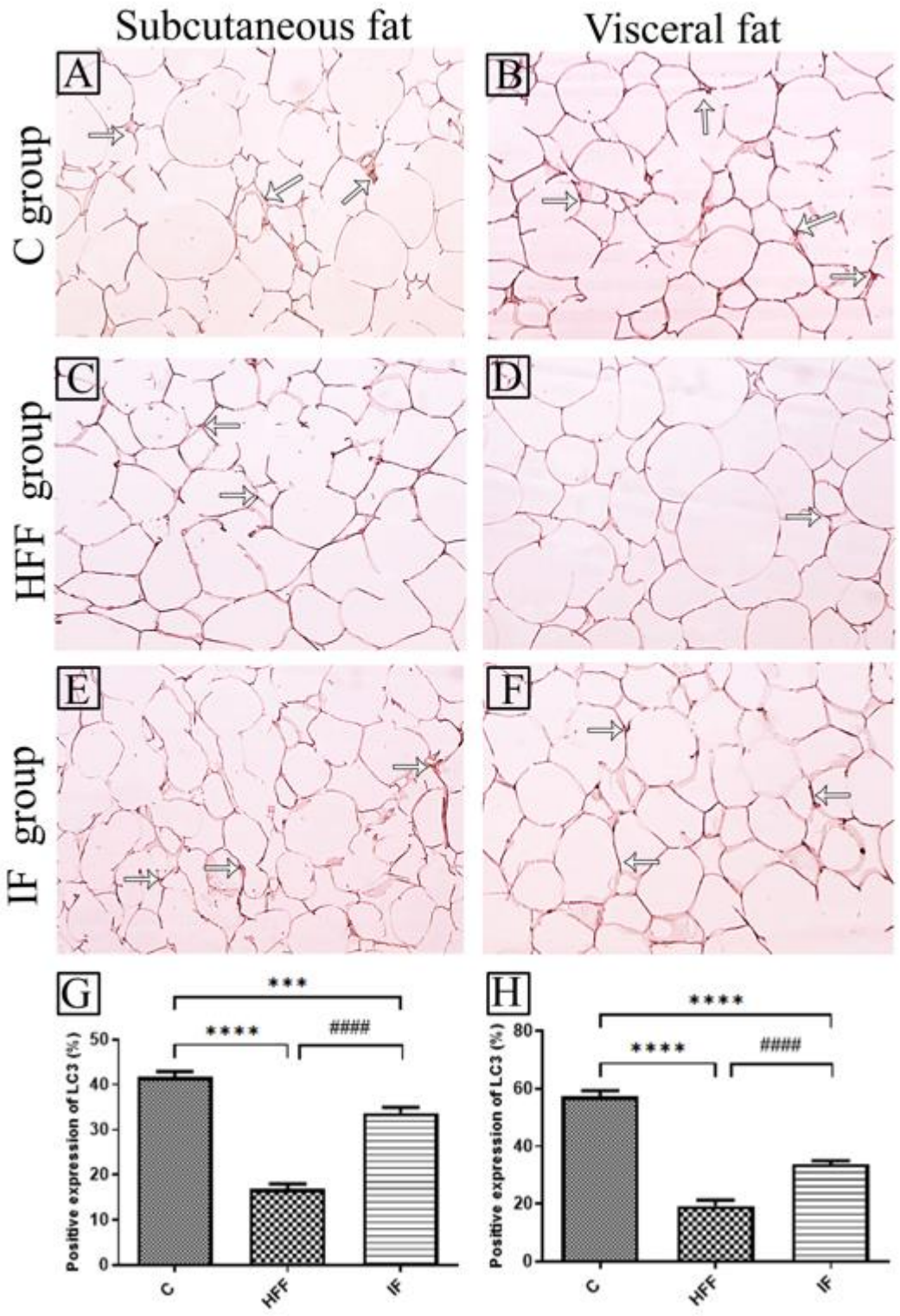


**Fig. 4:** Photomicrographs of subcutaneous and visceral WAT-stained sections with LC3 antibody showing, a brown expression of LC-3 in the cytoplasm of adipocytes of control group sections (A, B) which is markedly lowered (white arrows) in HFF section (C, D) and it increased in IF group sections (E, F). (Magnification X 100).
(G, H) Histograms comparing the Mean ±SD of the percentage of LC-3 area expression in subcutaneous and visceral WAT among different groups respectively showing, a significant decrease in LC3 expression in both types of WAT in HFF compared to control group ($P < 0.0001$ in both). Then, a significantly increased area of expression in both subcutaneous and visceral WAT in IF compared to HFF ($P < 0.0001$ in both).

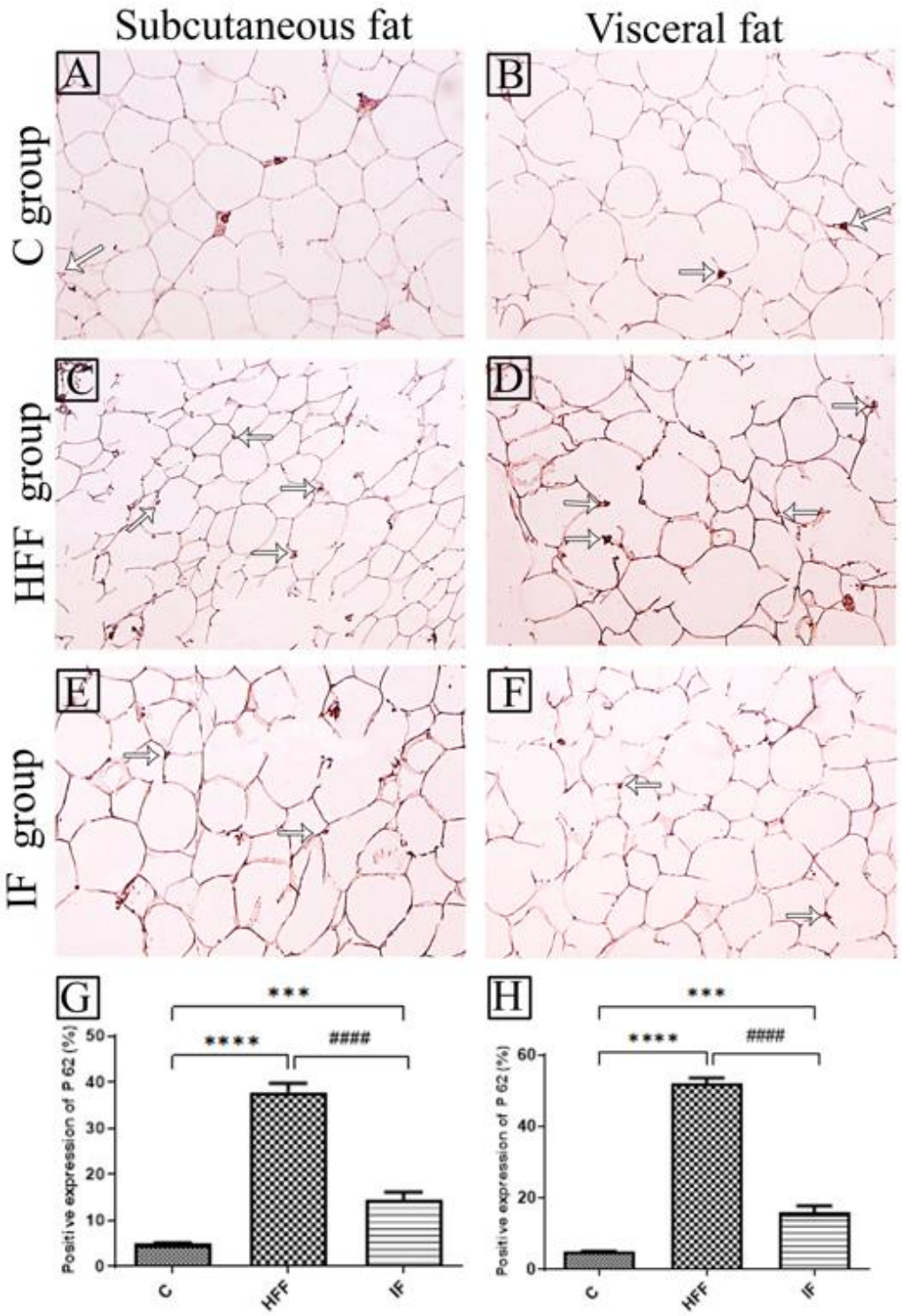


**Fig. 5:** Photomicrographs of subcutaneous and visceral WAT sections stained with P62 antibody showing, multiple positively stained nuclei of adipocytes (white arrows) in HFF (C, D) compared to the control group (A, B). Fewer positively stained nuclei of adipocytes (white arrows) are seen in IF (E, F). (Magnification X 100).
(G) Histograms showing the Mean ±SD of the percentage of P62 area expression in subcutaneous and visceral WAT among different groups respectively showing, a significant increase in HFF ($P< 0.0001$) in both when compared to control group. Also, a significant decrease of expression in both subcutaneous and visceral adipocytes in IF compared to HFF ($P< 0.0001$) was seen.

## DISCUSSION

Since the increasing global prevalence of obesity, which is the strongest risk factor for insulin resistance and type 2 diabetes, is shocking (Dedual *et al.,* 2019), multiple rapid weight loss protocols are more used especially by women due to the desire to lose weight rapidly. Therefore, we tried to mimic human behavior, in which they consume a high-fat diet pattern prolongedly, then in order to lose weight rapidly, they carry out, IF, one of the most famous programs now (Moreno-Fernández *et al.,* 2018).

The results of the current study revealed that the consumption of HFD increased rats' body weight and induced levels of total cholesterol and TG which has been reported before (Yang et al., 2019; Orabi et al., 2020). HFD could act via increasing energy intake and adiposity growth as HFD builds more fat storage rather than oxidation (Hazzaa *et al.,* 2020).

Our results are supported by former animal experiments which confirmed that chronic HFD induces obesity, hyperglycemia, and dyslipidemia in mice (Shih *et al.,* 2008; Stewart *et al.,* 2008; Wouters *et al.,* 2008). It is noted also to boost the systemic oxidative stress markers (Shertzer *et al.,* 2008) and diminish superoxide dismutase (SOD), similar results were also observed in human investigations (Furukawa *et al.,* 2017).

The levels of reactive oxygen species (ROS) depend on the balance between their rate of production and their rate of clearance by antioxidant compounds (Poljsak et al., 2013). In the present study, HFD had significantly decreased the activity of a key antioxidant SOD and increased the levels of MDA which has been reported in mice models before (Jarukamjorn *et al.,* 2016). Raised serum MDA level in the HFF rats has been also recorded (Yang *et al.,* 2019; Chung *et al.,* 2018). It can be confirmed that obesity is associated with an increased level of OS markers (Diniz *et al.,* 2006).

Lipid accumulation stimulates oxidative stress (Hauck and Bernlohr, 2016). The oxidative stress can be disseminated further through mitochondrial damages that lead to the production of ROS (Bournat and Brown, 2010). In addition, extracellular fatty acids are directly detected by toll-like receptors to stimulate stress responses (Kennedy *et al.,* 2009), and various metabolites of fatty acids indirectly provoke the activation of stress signaling pathways (Schenk *et al.,* 2008). These responses collectively lead to an aggravation of obesity-associated pathologies, such as chronic inflammation (Namkoong *et al.,* 2018).

Regarding insulin level, our results showed its reduction in HFF group and restored after IF. Previous work shows that IF increases both basal and glucose-stimulated insulin secretion in mice with diet-induced obesity (Liu *et al.,* 2017). Moreover, no significant difference in fasting serum insulin levels was recorded in both normal and HFF rats (Wu *et al.,* 2015). On the other side, IF was mentioned to increase insulin resistance, probably by decreasing hepatic insulin signaling, and reducing glycogen phosphorylase expression in spite of decreased fat mass was recorded in young male rats (Park *et al.,* 2017).

The recorded hypertrophy of adipose tissue after a high-fat diet was also recorded before and they added that there is a connection between the magnitude of weight gain and adipocyte hypertrophy (Alkhouri *et al.,* 2010). Hypertrophic AT involves an increase in the size of adipocytes as it is one of the pathways that guarantees AT adaptation of positive energy balance and consequently increased AT storage capacity (Choe *et al.,* 2016).

Consistent with our H&E finding of a reduction of fat cells diameters following intermittent fasting protocol, a reduction of the relative area of sWAT and vWAT in HFF mice as the white

adipocyte is reduced in size after acute fasting (for 36 h once) (Mao *et al.*, 2021). It has been stated that large fat cells are thought to be poorly metabolized and linked to multiple pathophysiological conditions, so fat cell size reduction is greatly associated with metabolic improvements (Ghaben & Scherer, 2019). It has been established that IF induces weight and fat loss through reduction of ROS production, increased cellular stress resistance, and diminution of inflammation (Sharsher *et al.*, 2022).

Remarkably, in our results, diameters of vWAT were increased more than the sWAT although it was not statistically significant, this could be explained in the light of previous rodent and humans' studies that attributed sex-related influence of the energy storage sites (Amengual-Cladera *et al.*, 2013; Lee and Fried, 2017). They confirmed that females had greater hypertrophy and hyperplasia of sWAT compared to visceral, which implies that fat deposition is mainly affected by hormones such as estrogen (Fernández *et al.*, 2021).

Numerous studies have proven that obesity generates a chronic low-grade inflammation in AT associated with infiltration of AT by macrophages and raised levels of cytokines, such as IL-6 and TNF-α (Lumeng and Saltiel, 2011; Olefsky and Glass, 2010). Moreover, they proposed that overnutrition generates cellular, metabolic, and inflammatory stress in adipocytes, causing the release of chemokines and other factors which attract macrophages. Then, AT macrophages release more chemokines and cytokines, that additionally aggravate AT inflammation (Osborn and Olefsky, 2012).

Additionally, apoptosis is confirmed to be dependent partially on the activation of the toll-like receptor-4(TLR4)/ (NF-κB) signaling pathway (Wang et al., 2016). Apoptotic pathways could be sorted into two: extrinsic ones initiated by receptors such as TNF- α, and intrinsic pathways instigated by mitochondrial events (Elmore, 2007). Experiments on mice and in vitro adipocytes have demonstrated that inflammatory signaling pathways including TLR4, c-Jun N-terminal kinase 1 (JNK1), or nuclear factor-κB (NF-κB) play a crucial role in obesity-associated AT inflammation (Cildir *et al.*, 2013; Sabio *et al.*, 2008; Sun *et al.*, 2011). It has been pointed out that cell stress, activates NF-*κ*B which is involved in the apoptosis pathway (Thoms *et al.*, 2007). Although JNK activation has been noted to play a significant role in inducing both pathways (Dhanasekaran and Reddy, 2008).

The elevated bax expression in our study was also recorded before in other studies on obese animals (Alkhouri *et al.*, 2010) and humans (Feng *et al.*, 2011) that showed prominent adipocyte apoptosis is in both. Under the stressful condition, relative expression of pro and anti-apoptotic Bcl-2 proteins are altered resulting in increased cellular expression of pro-apoptotic relative to anti-apoptotic Bcl-2 proteins, creating a disequilibrium that induces apoptosis (Redza-Dutordoir and Averill-Bates, 2016). Moreover, the recorded reduced bax expression in IF animals might be reinforced by recent research which confirmed that 8 weeks of IF reduced mice acetylated NF-kB and JNK1, both of which are involved in apoptosis pathways (Vemuganti and Arumugam, 2020).

Our results of elevated P62 expression in HFF group, indicated increased autophagic flux in AT with decreased expression of LC3, both signifying autophagy inhibition. A similar finding of inhibited autophagy in HFF animals was also reported before in the AT of HFF mice (Yoshizaki *et al.*, 2012). On the same hand, other human and mouse experiments showed that autophagosomes can accumulate in response to obesity and lipotoxicity in adipose tissues (Jansen *et al.*, 2012; Öst *et al.*, 2010; Nuñez *et al.*, 2013). These

findings suggested that the relationship between obesity and autophagy is not straightforward as originally thought.

On the other side, it has been thought that endoplasmic reticulum stress, provoked by obesity, might provoke autophagy (Rashid *et al.,* 2015; Senft and Ronai, 2015). In addition, inflammation and oxidative stress and other obesity-associated stresses might also stimulate autophagy (Qian *et al.,* 2017; Filomeni *et al.,* 2015). Autophagy stimulation in this situation can be considered as one of the cellular defense mechanisms exhibited to maintain cellular homeostasis under obesity-associated stresses (Namkoong *et al.,* 2018).

However, if autophagy is suppressed for a long period of time by overnutrition, as with high fat and/or fructose diet, the accumulated unnecessary proteins, and organelles in multiple sites as AT could be harmful and induce metabolic diseases such as diabetes (Rocchi and He, 2015; Zhang *et al.,* 2012).

The decreased autophagy found in our results could be interpreted as the physiological ROS induce autophagy in order to preserve the cellular homeostasis in different types of cells. Though abnormal ROS can undermine the autophagic activity (Petibone *et al.,* 2017). Autophagy is repressed by chronic hyperglycemia (Yun et al., 2020). It has been reported that NF-kB activation intermediates depression of autophagy in TNF-α-treated sarcoma cells. This repression is associated with the activation of NF-kB-dependent autophagy inhibitor mTOR (Djavaheri-Mergny *et al.,* 2006). Autophagy is thought to be a key cellular defense against oxidative stress. Moreover, autophagy inhibition leads to enhanced apoptosis, implying that autophagy has a protective effect on cellular stressful environmental conditions (Ornatowski *et al.,* 2020) which matches our reported results.

Induction of autophagy following IF in our experiment is predicted as mentioned before that autophagy is stimulated in response to various stress or physiological conditions such as food withdrawal (Badadani, 2012). A similar finding was recorded before as fasting produces rapid and intense upregulation of autophagy in the brain (Alirezaei *et al.,* 2010) and hearts (Godar *et al.,* 2015) of mice.

Finally, HFF diet in animals, mimicking obesity in humans, is proven to induce hazardous effects on multiple systems including adipose tissue, glucose, lipid level and insulin resistance. But also at the same time, the literature is full of research with conflicting findings regarding the pros and cons of IF signifying that concern should be considered when recommending IF for people with compromised glucose metabolism. Further studies on IF should be conducted applying longer time of fasting and following animals with multiple investigations.

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