

Effect of Exercise Training On Autophagic Process in White Adipose Tissue of High Fat Diet-Induced Obese Mice

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Abstract

Background. Some studies have established a relationship between obesity and the autophagic process in adipose tissue. This study aimed to investigate the effect of exercise training on the autophagic process in white adipose tissue (WAT) of high fat diet-induced obese mice.

Methods and Results. C57BL/6 mice were assigned into three groups included: 1) Control 2), High-Fat Diet-induced Obesity (HFD-Ob), and 3) High-Fat Diet with Exercise Training (HFD-Ex). The subjects of HFD-Ob were fed a high-fat diet for 14 weeks. The mice of HFD-Ex had eight weeks of endurance training on a treadmill in addition to having the HFD. The Real-Time-PCR and western blot methods were used to measure the mRNA and protein levels of markers of the autophagic process.

HFD caused an upregulation in the factors of the autophagosome formation, including ATG5 and ATG7, LC3, and the exercise training could augment the upregulation. Further, the training program prevented the change in LAMP2 expression (a marker of autophagolysosome), which being reduced by HFD. The lysosomal clearance factors (CTSB and CTSL) were raised in HFD-Ob and differently changed in HFD-Ex.

Conclusion. HFD-induced obesity promoted the early and last steps of autophagy whereas defected the intermediate-step of it. Interestingly, the exercise training enhanced the early phase of autophagy, which being increased by HFD. Further, the training program could modify the rising effect of HFD on the last step of autophagy. It seems that a part of the protective effect of exercise training on obesity-related complications may be mediated by modulating the autophagic process in white adipose tissue.

Introduction

Obesity is a serious risk factor for metabolic syndrome, type 2 diabetes, nonalcoholic fatty liver disease, and cardiovascular disease. A modern lifestyle characterized by high-caloric diets and lack of physical inactivity causes obesity. On the other hand, lifestyle modifications such as having a healthy diet and physical activity can prevent and treat obesity and its complications [5].

Excessive fat accumulates in an adipose depot during the onset of obesity, causing a hyper-expansion of white adipose tissue (WAT) [35]. The hyper-expansion can dysregulate the WAT function, thereby implicating obesity-associated metabolic complications [15].

Macroautophagy (generally known as autophagy) is a lysosome-dependent and self-degrading process. Some intracellular components are catabolized and recycled by autophagy when they are damaged and impaired [32]. Thereby, homeostasis is maintained by this mechanism [23]. Autophagy has been established to play an essential role in adipose tissue expansion, providing the fat-storage capacity in the adipose depots [35]. Therefore, an autophagy dysfunction could be implicated in unhealthy adipose tissue expansion, leading to developing metabolic syndrome [34, 15].

Although a close relationship between obesity and autophagy in adipose tissue has been found [13, 10, 21, 3, 4, 8, 18, 12, 30, 11], whether obesity is a reason or result of autophagy remains to be elucidated [34]. Further, it is not completely identified how obesity could affect the autophagic process in white adipose tissue.

Exercise training has a protective role in obesity and obesity-related complications. Several studies have revealed that chronic exercise training could enhance autophagy in skeletal muscle [28], heart [2], and liver [14]. Nevertheless, at the adipose-tissue levels, Tanaka et al. (2015) found a change, and Rocha-Rodrigues et al. (2017), inconsistently, did not find any change in autophagy factors following endurance training [27, 24]. It thus needs to be further investigated the effect of exercise training on the autophagic process.

Therefore, this study aimed to respond to the following questions. First, could obesity induced by a high-fat diet affect the autophagic-process factors in white adipose tissue? Second, may exercise training influence the autophagic-process agents in white adipose tissue of obese mice?

For this purpose, we designed an experiment, and the mRNA and protein levels of autophagic-process factors were measured in fat depots of trained and untrained mice that also had a high-fat diet.

Material And Methods

Animals

Twenty-four C57BL/6 male mice (age of five weeks; body weight of 12 ± 2 gram) were obtained from the animal care center of Pasteur institute of Iran and kept under controlled conditions (at $22 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$ with a humidity of $55\% \pm 10\%$ and a 12-hour light/dark cycle). The mice had normal rodent chow ad libitum with free access to water. After a one-week acclimation, the mice were randomly assigned to three groups: 1) control (C, n=7), 2) High fat diet-induced obese (HFD-obese, n=7), and 3) High-fat diet with exercise training (HFD-Ex, n=7) (Fig.1).

The subjects of the HFD-Ob group were fed a high-fat diet (HFD) for 14 weeks. The mice of the HFD-Ex group were submitted to continuous running on a treadmill for eight weeks along with feeding HFD. After the experiment, mice were sacrificed, and epididymal fat pads were collected (Fig.1).

HFD-induced obesity

The mice of the HFD-Ob group were fed a high-fat diet (HFD) for 14 weeks from 6 to 20-week old. The HFD contained 45% kcal from fat, 40% carbohydrate, 20% protein (total calorie 4.6 kcal/g) [31]. The mice in the control group were fed with normal diet (15 kcal% fat, 60 kcal% carbohydrates, and 25 kcal% protein; total calorie 3.5 kcal/g) [31]. The diets were obtained from the research and development unit of Behparvar Animal Feed Company at Karaj -Iran.

Body weights were registered weekly during the experiment using electronic digital weighing scales (*ENTRIS 3202-1S S, Artorius, Germany*). The 24-h food intake was determined weekly throughout the study.

Exercise-training protocol

Before the exercise training experiment, the mice of the HFD-Ex group were acclimatized to running on a treadmill. At 12 weeks of age, they were trained during the dark cycle by continuous running on a rodent treadmill (*Andisheh-Sanat, made in Iran*) at 0% inclination. The protocol was five sessions per week for seven weeks. Each training session consisted of continuous running for 30 minutes at 60-70% vVO_2 peak, preceded and followed by 5 min of warming up and cooling down at 50% vVO_2 peak. The control-group mice were placed on a non-moving treadmill to be exposed to the same potential environment and handle stress.

vVO_2 peak test. At the onset of the training and the end of each one-week training period, the mice were subjected to a graded progressive exercise test on the treadmill (at 0% inclination) to determine the vVO_2 peak [29]. The test consisted of two-min stages with alternating increases in speed (increments of two m/min). The vVO_2 peak was reached when the animal can not keep running at a new speed for 10 s [29]. The obtained vVO_2 peak values were utilized to define the intensity of future training programs.

Tissue and blood collection

The mice were anesthetized by intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (100 mg/kg) [1] at the end of the experiment.

The blood samples (5-10 ml) were obtained from a cardiac puncture using a heparinized syringe and put immediately into glass tubes with EDTA. Plasma was obtained from blood by centrifugation (10 min, 3000 rpm, 4°C) and frozen (-78°C) for future measurement. The epididymal fat pads were quickly dissected and immediately frozen in liquid nitrogen and stored at -78°C until assessment. The mice were sacrificed 48 h after the last training session to eliminate the acute effects of exercise.

Measurement in Plasma

Fasting plasma glucose was assessed by a glucose oxidase method using a biochemistry Auto-Analyzer (*Hitachi 902, Boehringer manneheim, Germany*). Lipid profiles including non-esterified fatty acids (NEFA), total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) concentrations were enzymatically determined by commercially available kits (*ParsAzmon, Iran*) using Auto Analyzer according to the manufacturer's instructions.

Plasma insulin concentrations were measured by ELISA technique via enzyme immunoassay (EIA) kit (*10-1247-01; Mercodia AB, Uppsala, Sweden*) and ELISA microplate reader (*Awareness, Model stat fax 2000*).

Gene expression (mRNA) evaluation

RNA Extraction. Approximately 50 mg of adipose tissue samples were homogenized in 1 ml of TRIzol reagent (*Thermo fisher Scientific, USA*). The total RNA was isolated using TRIzol under the manufacturer's instructions (*Cat. No. 15596026*). RNA concentration and purity were assessed (*OD: 260/280*) using spectrophotometers (*Ultrospec 3000, Pharmacia Biotech, Sweden*).

cDNA Synthesis. First-strand complementary DNA (cDNA) was synthesized from 1 µg of RNA using Invitrogen SuperScript II Reverse Transcriptase (*Thermo Fisher Scientific, USA*), according to the manufacturer's instructions (*Cat. No. 18-064-014*).

Primers: Primers were designed based on previous studies, then verified using primer-blast/NCBI and Oligo Analyzer (1.02), and synthesized by SinaClon (*Iran*). The characteristics of the used primers have shown in Supplemental Table 1.

Real-time PCR. The mRNA contents were determined by real-time PCR performed using the Rotor-Gene 6000 (*Corbett, Australia*) with SYBR Green RT-qPCR Kit (*QR0100-Sigma-Aldrich- Germany*) and specific synthesis primers, according to the manufacturer's instructions (*Cat. No. 1907/2006*). The thermal cycling profile was 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 55°C to 58°C for 30 s to 40 s according to annealing temperature.

Quantification of mRNA. The efficiency of PCR product and primers were determined by LinRegPCR software (*Version 2020.0*), and the melting curve confirmed PCR products' specificity. The relative gene expression was quantified by *Pfaffl* method using Genex software (7.0).

Western blot determination

Tissue preparation: Frozen adipose sample was powdered by glass mortar and pestle in liquid nitrogen. Approximately 100 mg of the powder was homogenized in RIPA lysis buffer (*Sigma-Aldrich; German*). The homogenate was centrifuged (*Rotina 380 R; HETTICH; German*) at 4°C for 15 min at 13000 × g (*3000 g, 15 min, 4°C*). The supernatant fraction was collected, and the fat cake was discarded.

Determination of total Protein. Total protein was determined via *Bradford* technique by Bradford-protein assay kit (*ZellBio; German*), in which bovine serum albumin was applied as a standard. In the method, protein concentration was assessed using spectrophotometers (in absorbance at 595 nm) (*Ultrospec 3000, Pharmacia Biotech, Sweden*).

SDS-PAGE: After preparation of SDS-polyacrylamide gel (4% and 12%), 30 µg sample was boiled for 5 minutes and loaded onto the gel. Proteins were separated using electrophoresis (*Bio-Rad, Hercules, CA*) at 110 V for 60 min.

Electroblotting: Proteins were then transferred from the gel onto nitrocellulose polyvinylidene difluoride (PVDF) membranes (*Sigma-Aldrich*) by electroblotting (*Bio-Rad, Hercules, CA*) at 80 V for 180 min.

Block of nonspecific proteins. The membranes were blocked for 60 min at room temperature with 5% non-fat milk in TBST solution (Tris buffer saline containing 0.1% Tween 20).

Incubation with antibody. The membranes were incubated with (1 µg/ml) primary antibody against ATG5, ATG7, LC3A/B, SQSTM, LAMP2, CTSB, CTSL, and B-ACTIN (as control) (diluted 1:500 or 1:1000 in blocking solution, in accordance manufacturer's instructions) by an overnight period at 4°C. After incubation by the primary antibody, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (1:1000) for two hours at room temperature. The details of used antibodies have shown in Supplemental Table 2.

Quantification of bands. Bands were visualized by Gel Doc device (*Uvidoc, England*) using Enhanced Chemiluminescent (ECL) detection method (*Sigma-Aldrich; Germany*). The bands were quantified using ImageJ software (*15.1, NIH, USA*). Each band was normalized with B-ACTIN as a control.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used for intergroup comparisons. Data were then compared between groups using the Tukey post hoc test. A P value of less than 0.05 ($p \leq 0.05$) was considered statistically significant. SPSS 21.0 (*SPSS Inc., Chicago IL, USA*) and Prism 8 (*GraphPad, USA*) software were used to analyze data and design graphs, respectively.

Results

General characteristics

Table 1 and Fig. 2 show the general characteristics of the study groups at the end of the experiment.

Table 1
General characteristics of mice in the experimental groups

	Con	HFD-ob	HFD-Ex
Body Weight (g)	25.4±0.7	31.2±1.5*	28.5±1.2*, #
Body Weight gain (g)	14.3±0.4	19.5±0.9*	16.6±0.7*, #
Epididymal pad (g)	0.41 ± 0.05	1.08 ± 0.1*	0.81 ± 0.1*, #
Food Intake (Kcal/week)	78.2±10.3	106±14.7*	101.8±12.9*
Glucose (mg/dL)	95.6 ± 5.4	130.1 ± 10.8*	111.6±11.1*, #
Insulin (ng/mL)	2.17 ±0.51	2.87 ± 0.94	2.51 ± 0.86
Triglycerides (TG)(mg/dL)	70.45 ± 9.3	90.4 ± 14.8*	80±13.6*, #
NEFA (mg/dL)	2.82±0.28	8.76±1.41	6.50±1.98
Total cholesterol (TC) (mg/dL)	180 ± 11	200 ± 20*	190 ± 18*, #
HDL cholesterol (mg/dL)	125.63±7.6	140.1±9.3*	145.63±8.5*
LDL+VLDL cholesterol (mg/dL)	85± 11	143± 18*	121±10*, #
Data are expressed as mean±SEM and were analyzed by one-way ANOVA followed by the Tukey test. * p<0.05 compared control; # p<0.05 compared HFD-Ex group. Con , Control group; HFD-Ob , High fat diet-induced obese group; HFD-Ex , High fat diet with exercise training group			

Autophagy-related genes5 (ATG5) and autophagy-related genes7 (ATG7)

At the onset of the autophagic process, the phagophore elongates, expands, and then engulfs cytosolic components, forming a double-membrane structure known as an autophagosome. ATG5 and ATG7 play a key role in constituting a conjugation system (as the early stage of the autophagic process) [32].

Data of this study showed that the mRNA and protein levels of ATG5 and ATG7 were significantly higher in HFD-induced obese mice than in control (Fig. 3). However, the HFD-Ex group did not display a significant difference in mRNA and protein levels of ATG5 compared with the HFD-Ob group (Fig. 3). Further, we observed a significantly higher ATG7 specifically at mRNA levels in the HFD-Ex group than the HFD-Ob group (Fig. 3c).

These findings reflected that the 14-week high fat diet caused the upregulation of the early stage of the autophagic process. These also reflected that the early stage of autophagy might be augmented in trained-HFD mice.

Microtubule-associated protein1- light chain 3 (LC3)

LC3 is proteolytically cleaved at post-translational modification to elicit a cytosolic form named LC3-I. A subpopulation of LC3-I is further converted to an autophagosome-membrane-associated lipidated form (LC3-II). During autophagosome formation, LC3-I (non-lipidated form) convert to LC3-II (lipidated form). Thus, LC3-II turnover (the LC3-II and LC3-II/I ratio) is implied as a progression of the autophagic process i.e.formation autophagosomes [32]. Therefore, to assess the autophagosome formation in the autophagic process, we measured the protein levels of LC3-I and LC3-II.

As can be seen in Fig. 4, the HFD-Ob mice exhibited higher LC3-II and LC3-II/LC3-I ratio levels than the control. In addition, these markers were significantly increased in HFD-Ex compared to HFD-Ob (Fig. 4b).

These data represented that the autophagosome formation was upregulated in HFD-induced obese mice and was further augmented in trained-HFD subjects.

We measured the LC3AB mRNA levels for more exploration and found higher LC3AB levels in HFD-Ob and HFD-Ex than in control (Fig. 4c). Similarly, LC3AB levels were more in HFD-Ex than in the HFD-Ob group (Fig. 4c).

Noteworthy, LC3-II accumulation implies the promoting of autophagosome formation but not necessarily the processing of autophagy. Indeed, LC3-II may be accumulated upon defection of later stages of the autophagic process such as autophagolysosome [16] or autolysosome [18] rather than accurately autophagic process. To resolve the problem, we assessed the markers of late stages of the autophagic process, i.e., LAMP2 and P62.

Lysosome-associated membrane protein 2 (LAMP2)

LAMP2 is a lysosomal protein required to fuse the autophagosome with the lysosome (autophagolysosome) [32].

Our finding showed that the protein level of LAMP2 was lower in HFD-Ob than in the control and HFD-Ex groups (Fig. 5b). We next assessed the mRNA levels of LAMP2 in the fat depot of studies groups. Consistently, we observed a lower amount of LAMP2 mRNA in HFD-Ob than in the control group (Fig. 5c). However, the LAMP2 mRNA in HFD-Ex did not significantly change compared to the control and HFD-Ob group (Fig. 5c).

Together, these findings represented that the LAMP2 expression was downregulated in obese mice induced by HFD.

Sequestosome 1 (SQSTM1/P62)

P62 is degraded along with autophagosomes as a substrate of autophagy in the lysosome. Thus, The changes at the P62 level, together with the alteration in LC3-II level, are considered the primary autophagic flux markers [20].

We observed that the p62 protein levels did not significantly change among studies groups (Fig. 5b). However, its mRNA levels were significantly higher in HFD-Ob and HFD-Ex than in control (Fig. 5c).

Cathepsin L (CTSL) and cathepsin B (CTSB)

Lysosomal cysteine proteases (also called cathepsins) play a potential role in the autophagic process's latest stage (lysosomal clearance). The main proteases that play a prominent role in adipose tissue autophagy are CTSL and CTSB [18, 11, 17].

Our finding showed that that the protein levels of CTSB and CTSL (both proform and mature form) were upregulated in HFD-Ob (Fig. 6a and 6b). In the HFD-Ex group, the protein levels of pro-CTSB (but not mature form) were downregulated (Fig. 6a), while the protein levels of CTSL (Both proform and mature form) had a tendency (not significantly) to increase as compared to HFD-Ob (Fig. 6b).

For more explore, we measured the gene expression of these cathepsins. Accordingly, our data showed that the mRNA level of CTSB and CTSL were upregulated in HFD-Ob (Fig. 6c). However, the CTSB mRNA was lessened, and CTSL mRNA was augmented in HFD-Ex compared to the HFD-Ob group (Fig. 6c).

These findings showed that the lysosome function might be increased by HFD-induced obesity and that exercise training could modify the lysosomal activity by altering proteases levels under the feeding HFD.

Discussion

The main finding of this study was that HFD-induced obesity and exercise training along with HDF could differently affect the autophagic process in white adipose tissue.

Autophagy proceeds through three main phases: (I) autophagosome formation, (II) autolysosome formation, and (III) degradation of autolysosome contents [19]. The effect of HFD-induced obesity and exercise training-HFD has been reported based on these steps in the following.

Autophagosome formation (early phase)

Exercise training could augment the HFD-induced autophagosome formation

Our findings were in line with previous evidence [13, 4, 30], showing that mRNA levels of ATG5 and ATG7 were increased in adipose tissue of obese individuals and HFD mice. Further, we found that the training protocol could augment the HFD-induced upregulation of ATG7. Consistently, Tanaka et al. (2015) reported that endurance training caused an increase in ATG7 at the fat depot of non-obese rats [27].

Together, these above results along with previous studies, suggested that a high-fat diet could develop the early phase of the autophagic process and that exercise training under HFD might augment this development.

LC3-II turnover is established as a hallmark of the autophagic process at the autophagosome-formation step [20]. In accordance with previous studies [10, 4, 18, 11], our results showed that LC3-II accumulated in the fat depot of HFD-induced obese subjects. Interestingly, we observed that exercise training could add the amount of accumulation.

This finding was in contrast to prior investigations showing an increase and non-change in LC3II protein in visceral fat depots following endurance training in chow- and HFD-fed rats, respectively [27, 24]. This discrepancy might be due to differences in methodology approach, such as diet and rodent strain differences.

The possible mechanism for promoting the early stage of the autophagic process in trained-HFD mice may be due to consecutive lipid loading and unloading in adipocytes. Indeed, long-term HFD and regular exercise training might induce synthesis and hydrolysis triglycerides in fat cells, respectively. This turnover might trigger the autophagy mechanism to maintain homeostasis. However, this needs future surveys.

LC3-II accumulation reflects the augmentation of the autophagosome [20, 16] It paradoxically represents either enhancement or deficiency in the late stage of the autophagic process [16, 18] (i.e., autolysosome formation or lysosomal degradation). Therefore, the LC3-II data were analyzed according to the late-stage indicators of the autophagic process, i.e., p62, LAMP2, and cathepsin levels.

The autophagosome-lysosome fusion step (intermediate phase)

Exercise training might prevent the alteration of LAMP2 expression being reduced by HFD

The intermediate step of the autophagic process is to fuse autophagosomes with lysosomes, forming autolysosomes (also called autophagolysosomes) [19]. LAMP2 is a ubiquitous lysosomal protein required to merge lysosomes with autophagosomes in the stage [32]. Accordingly, a defection in LAMP2 was shown to accumulate the autophagosome [6].

It was previously reported that LAMP2 was reduced in hypertrophic adipocytes at mRNA levels [33] and was increased at the protein level in adipose tissue of obese subjects [18].

We observed a low expression of LAMP2 at mRNA levels in the fat depot of HFD-induced obese mice. This finding raised a possibility of impairment in the fusing of lysosomes with autophagosomes.

Furthermore, our result showed that the exercise program could restrict the alteration of LAMP2 mRNA expression being reduced by HFD. This finding was consistent with the prior study reporting that endurance training caused an increase in LAMP2 in epididymal fat of non-obese rats [27].

Altogether, these results could suggest a protective role of exercise training in the intermediate step of the autophagic process under the feeding HFD.

The lysosome degradation step (last phase)

Exercise training could modify the lysosomal function being changed by HFD

p62 is degraded as substrate by the lysosome. Therefore its reduction can be considered as lysosome degradation. We found no considerable changes in p62 in the fat depot of HFD-obese and trained-HFD mice. However, in agreement with previous studies [26, 18], an increase in gene expression of P62 was observed in HFD-obese and trained-HFD mice.

It was speculated that the rise of P62 gene expression might compensate for the degradation-inducing reduction of P62. Therefore, it may be incorrect to analyze the degradation step based on P62 levels. For this, we explored the clearance step of the autophagic process at the lysosome-proteas phase.

We found elevated CTSB and CTSL at mRNA and protein levels in fat depots of HFD-induced obese mice. These results confirmed the previous studies showing increases in CTSL and CTSB at mRNA and protein levels [7, 9, 18, 11].

As a novel result, based on our knowledge, our finding showed that exercise training could limit the HFD-induced rise of CTSB expression while not significantly affected in CTSL.

Obese patients have been had a chronic low-grade inflammation characterized by increasing pro-inflammatory cytokines in adipose tissue [7, 22]. The cytokines such as TNF α can induce the CTSB expression in adipose tissue [11]. Thus, it was thought that the increase of CTSB levels might be due to the rising proinflammatory cytokines.

Further, exercise training has been demonstrated to alleviate inflammation by blunting proinflammatory adipokines [25]. Therefore, it was speculated that exercise training could prevent the HFD-induced CTSB expression by lessening pro-inflammatory adipokines in fat depots of obese mice.

CTSB has been established to develop insulin resistance by the following mechanism. First, by inducing inflammation in adipose tissue [7, 9, 18, 11]. Second, by degrading perilipin-1, thus impairing basal lipolysis, leading to deposit ectopic lipid [11]. Therefore, it seems that a part of the protective effect of exercise training against the development of obesity-related complications may be due to downregulating HFD-induced CTSB expression.

In summary, we found that HFD-inducing obesity promoted the early (autophagosome formation) and last (lysosome degradation) steps of the autophagic process, whereas impaired the intermediate-step (autophagosome-lysosome fusion) of it. Interestingly, exercise training could enhance the early phase of autophagy being increased by the high-fat diet, while it could restrict the reducing effect of the high-fat

diet on intermediate-step autophagy. Further, exercise training was able to modify the rising impact of the high-fat diet on the last step of autophagy (lysosomal degradation).

Consequently, It may be concluded that the protective effect of exercise training on obesity-related complications may be partly mediated by enhancing the autophagic process in white adipose tissue.

Declarations

Supplementary Information. The manuscript has a file as Supplementary Information in which there are pieces of information about materials and measurements.

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Competing Interests

The authors declare that they have no conflict of interest.

Author Contributions

Saeed Daneshyar and *Gholamreza Tavoosidana* designed the research; *Fatemeh Jalali-Moghim* and *Sadegh Amani- Shalamzari* experimented and collected data. The first draft of the manuscript was written by *Saeed Daneshar* and all authors commented on previous versions of the manuscript. All authors approved the final version of the manuscript.

Ethics approval

All experimental protocols were performed and were approved by the Research Ethics Committees of the School of Medicine- Tehran University of Medical Sciences (*IR.TUMS.MEDICINE.REC.1398.671*).

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Figures

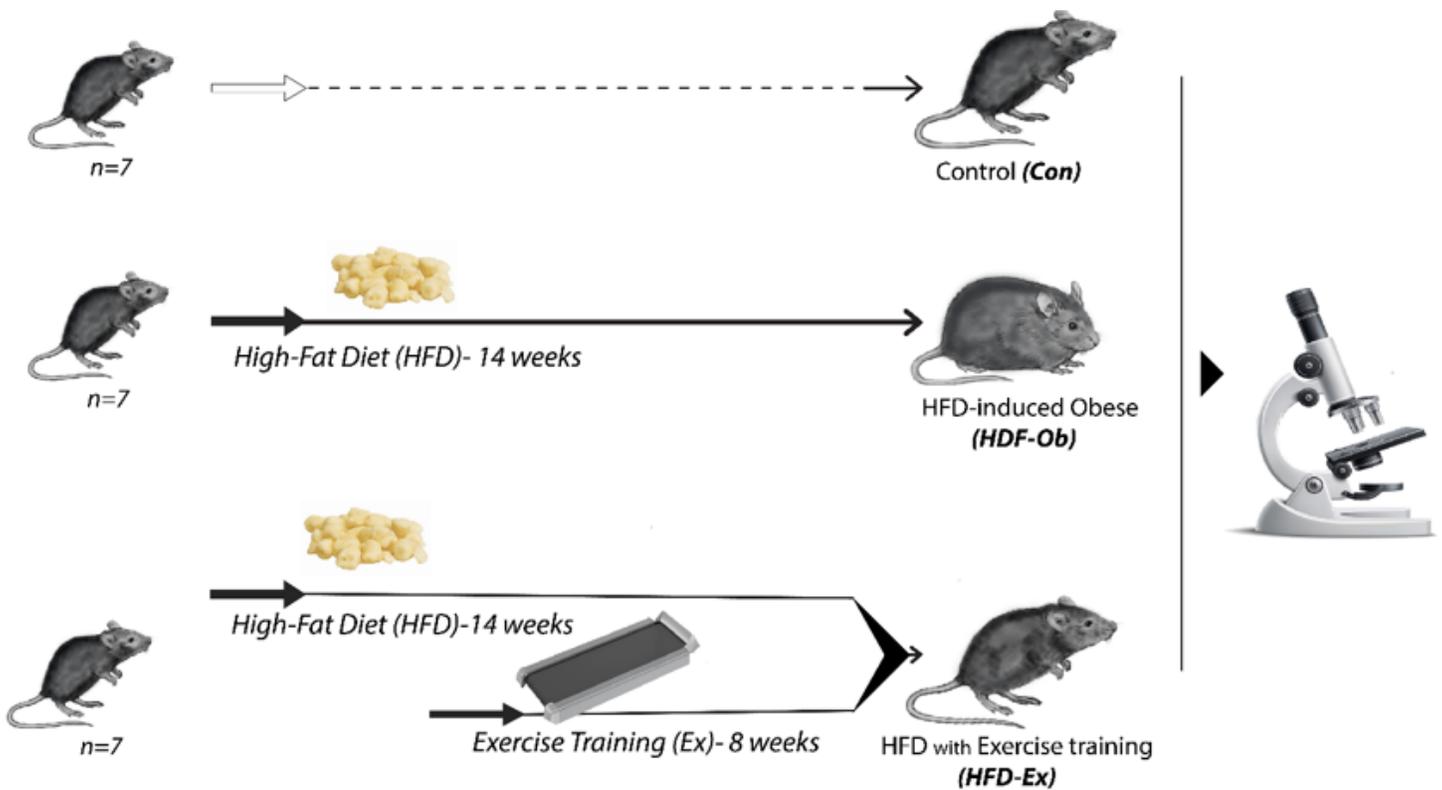


Figure 1

The protocol of the experimental research

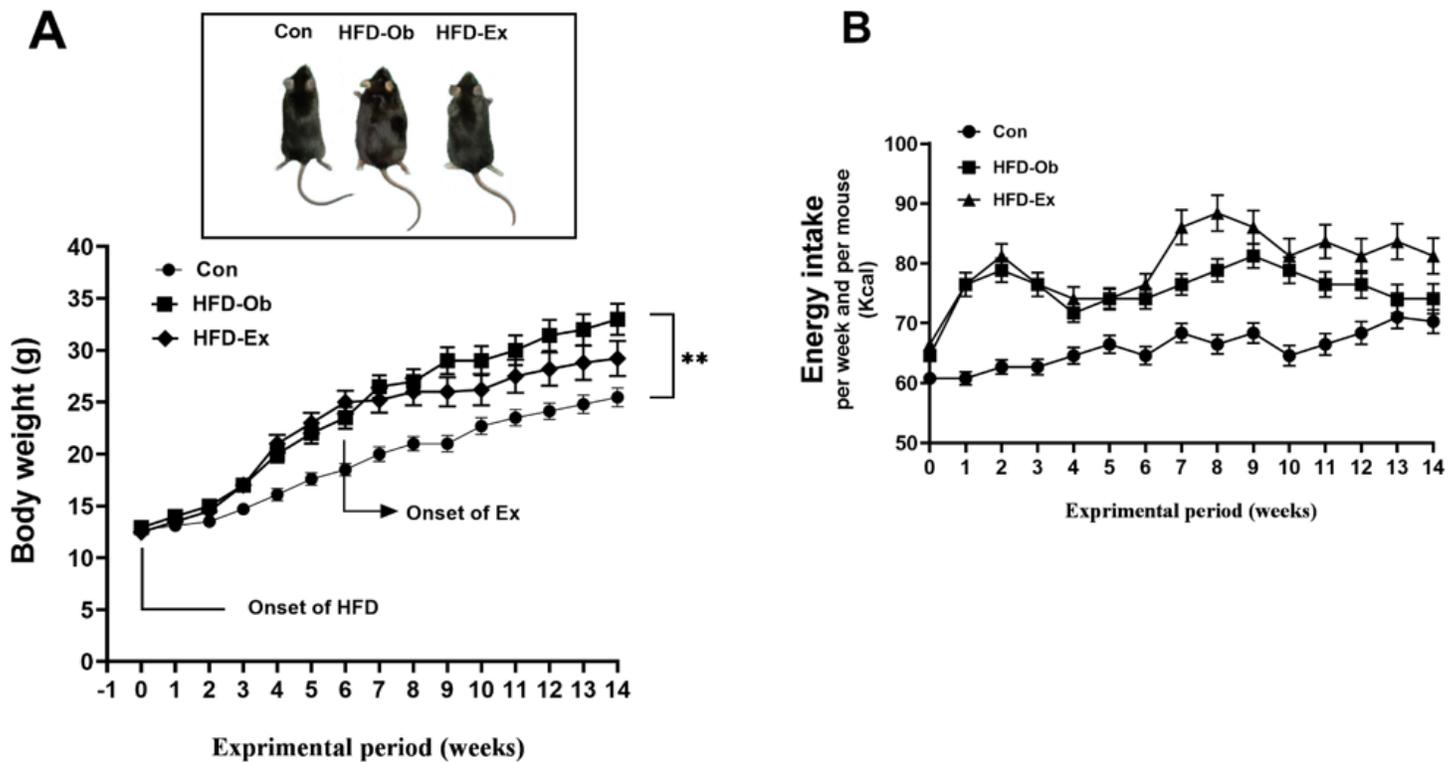


Figure 2

The changes in body weight and energy intake during the experiment

A) The changes in body weight during the experiment, along with representative images of experimental groups of mice, B) The changes in energy intake during the experiment

Data are expressed as the mean \pm SEM. ** $p < 0.01$

Con, Control group; **HFD-Ob**, High fat diet-induced obese group; **HFD-Ex**, High fat diet with exercise training group

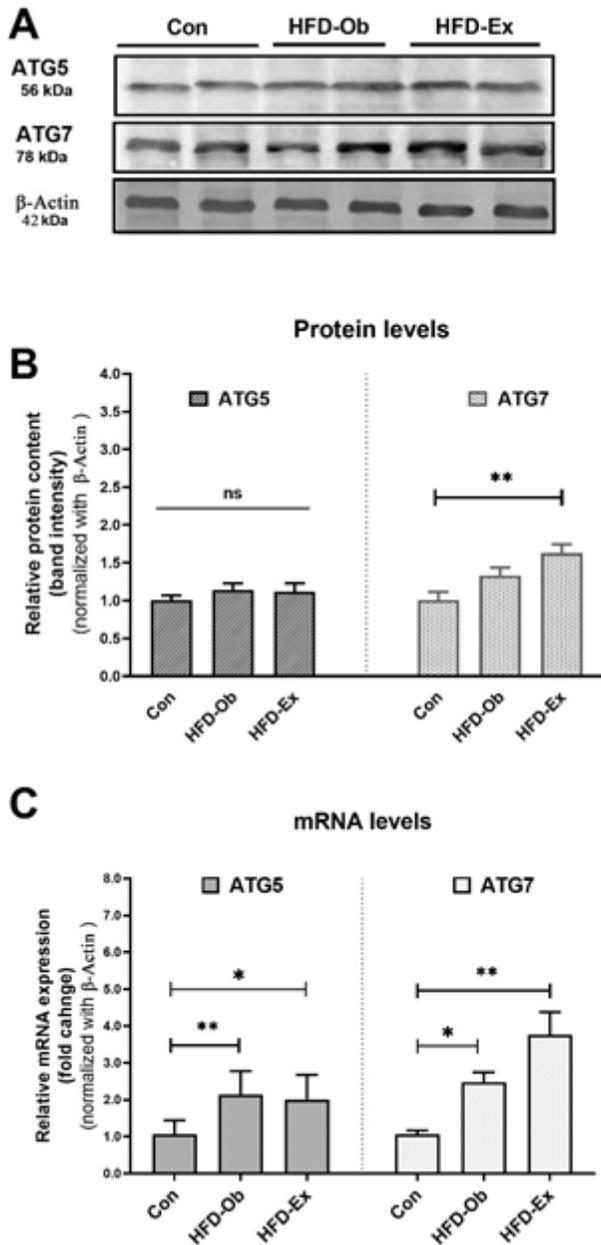


Figure 3

The protein and mRNA levels of ATG5 and ATG7 in experimental groups

A) selective western-blot images B) The comparison of protein levels C) The comparison of mRNA levels

Data are expressed as the mean±SEM. Data were analyzed by one-way ANOVA followed by the Tukey test. **ns**, not significant; * $p < 0.05$; ** $p < 0.01$

ATG5, autophagy-related gene 5; **ATG7**, autophagy-related gene 7; **Con**, Control group; **HFD-Ob**, High fat diet-induced obese group; **HFD-Ex**, High fat diet with exercise training group

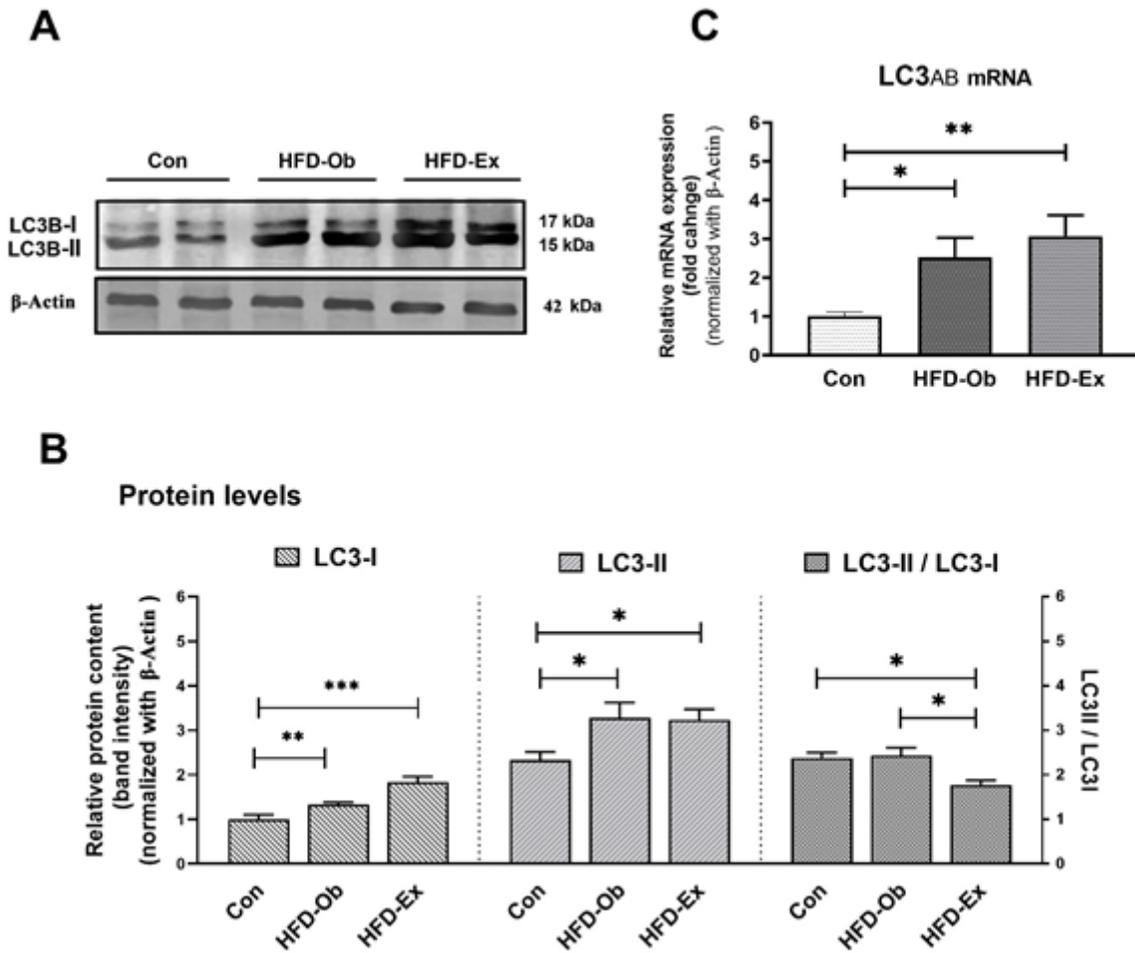


Figure 4

The levels of protein and mRNA of LC3 in experimental groups

A) selective western-blot images B) The comparison of protein levels C) The comparison of mRNA levels

Data are expressed as the mean \pm SEM. Data were analyzed by one-way ANOVA followed by the Tukey test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

LC3, microtubule-associated protein 1-light chain 3, **B-ACTIN**, Glyceraldehyde 3-phosphate dehydrogenase; **Con**, Control group; **HFD-Ob**, High fat diet-induced obese group; **HFD-Ex**, High fat diet with exercise training group

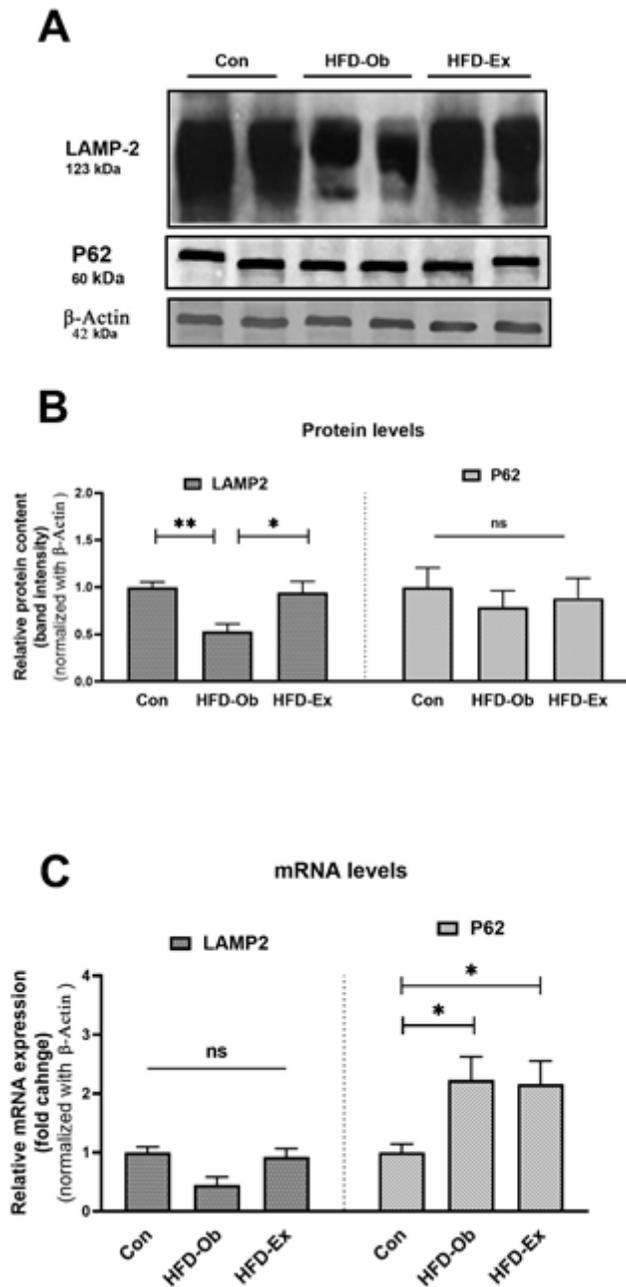


Figure 5

The levels of protein and mRNA of LAMP2 and P62 in the experimental groups

A) selective western-blot images B) The comparison of protein levels C) The comparison of mRNA levels

Data are expressed as mean±SEM. Data were analyzed by one-way ANOVA followed by the Tukey test.

ns, not significant; * p<0.05; ** p<0.01

P62, sequestosome 1; **LAMP2**, lysosome-associated membrane protein 2; **B-ACTIN**, Glyceraldehyde 3-phosphate dehydrogenase; **Con**, Control group; **HFD-Ob**, High fat diet-induced obese group; **HFD-Ex**, High fat diet with exercise training group

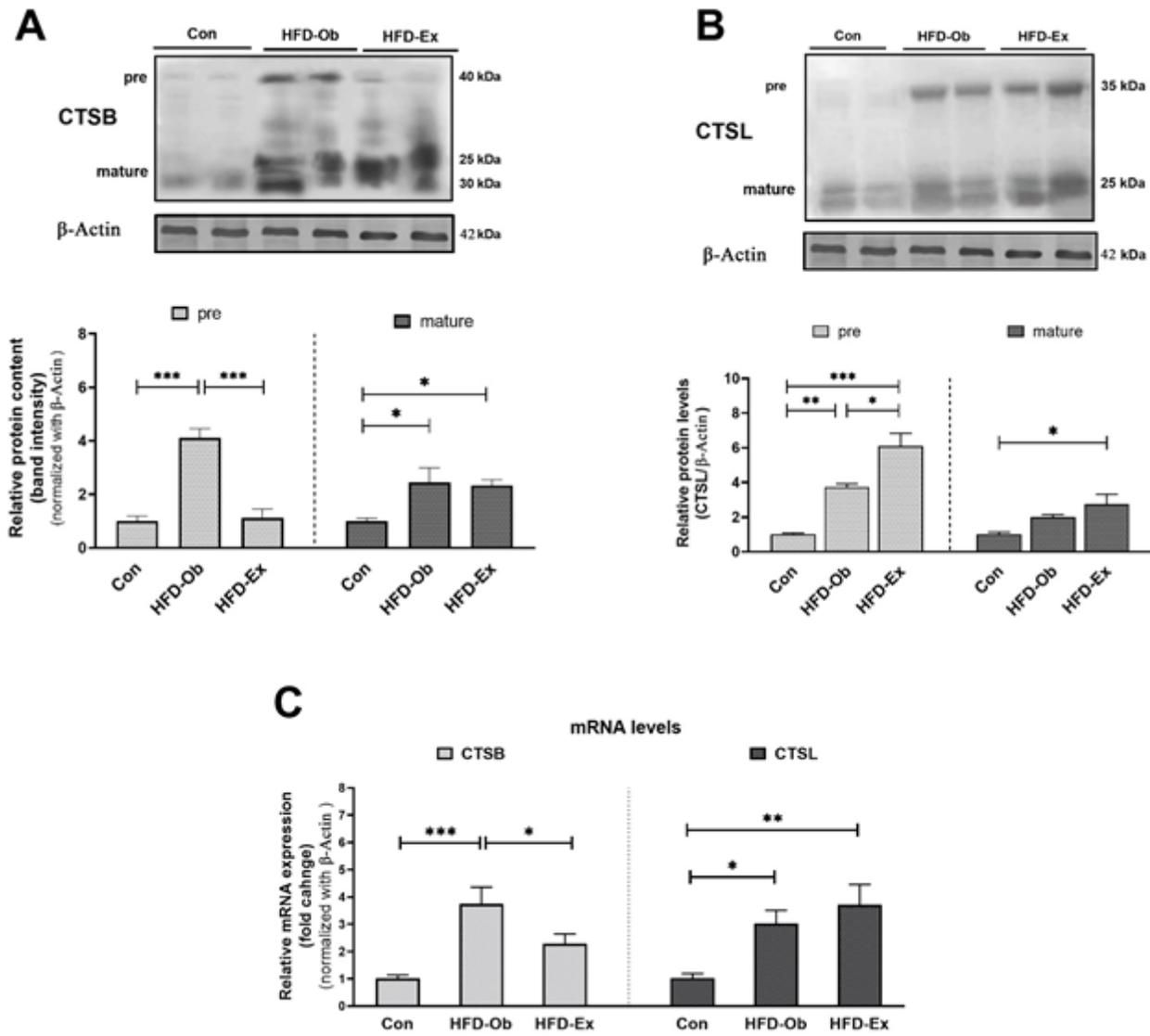


Figure 6

The levels of protein and mRNA of CTSB and CTSL in the experiment groups

A) selective western-blot images of CTSB and the comparison of protein levels among groups B) C) selective western-blot images of CTSL and the comparison of protein levels among groups C) The comparison of mRNA levels among groups

Data are expressed as mean \pm SEM. Data were analyzed by one-way ANOVA followed by the Tukey test. **ns**, not significant; * p < 0.05; ** p < 0.01

CTSB, cathepsin B; **CTSL**, cathepsin L; **B-ACTIN**, Glyceraldehyde 3-phosphate dehydrogenase. **Con**, Control group; **HFD-Ob**, High fat diet-induced obese group; **HFD-Ex**, High fat diet with exercise training group

Supplementary Files

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