Research Article

Pamukkale Medical Journal
doi:https://dx.doi.org/10.31362/patd.1487708

Received: 21.05.2024

Accepted: 12.06.2024

Area of Expertise: Medical Physiology

Title: Gender-specific effects of alternate-day fasting on body weight, oxidative stress, and metabolic health in middle-aged rats.

Short title: Effects of ADF on oxidative stress in Middle-aged rats.

Abstract

Purpose: The purpose of this study was to assess the effect of alternate-day fasting (ADF) concerning sex as well as its function in systemic and tissue-level oxidative stress alterations associated with aging.

Materials and methods: Forty-two female (n=21) and male (n=21) Wistar rats (aged 16 months) were separated into six groups (n=7 each): Group-1 (control-male), Group-2 (1-month, ADF-male), Group-3 (2-month, ADF-male), Group-4 (control-female), Group-5 (1-month, ADF-female), and Group-6 (2-month, ADF-female). The ADF protocol was applied every other day for 24-h of fasting (three days/week). Serum samples were analyzed via ELISA to measure total oxidant-antioxidant status (TOS-TAS), and the oxidative stress index (OSI) was calculated.

Results: 2-months of ADF treatment reduced body weight (BW) compared compliance control groups (p<0.001). All groups' cumulative food intake and retroperitoneal fat weight decreased with ADF (p<0.05). Both 1-month and 2-month ADF interventions had positive effects on reducing TOS and OSI in both liver and serum, with a significant decrease observed in both groups compared to their respective controls (p<0.001). The liver TAS level significantly increased in female rats (p<0.05), but this increase did not reach a significant level in male rats. The difference in the serum TAS level between the groups was not significant.

Conclusions: This study evaluated the effects of ADF on BW, food consumption, and oxidative stress parameters in male and female rats. The findings highlight ADF's potential benefits in weight management and reducing oxidative stress. This study represents an important step in understanding the effects of ADF on metabolic health and in identifying potential clinical applications.

Keywords: Aging, alternate-day fasting, food intake, gender, oxidative stress.

Makale başlığı: Orta yaş sıçanlarda gün aşırı açlık protokolünün vücut ağırlığı, oksidatif stres ve metabolik sağlık üzerine cinsiyete özgü etkileri.

Kısa başlık: ADF'nin Orta yaş sıçanlarda oksidatif stres üzerine etkileri.

Öz

Amaç: Bu çalışmada, gün aşırı açlık protokolünün (ADF) cinsiyete özgü etkileri ve sistemik ve doku düzeyindeki oksidatif stres üzerine etkilerinin yaşlanmaya bağlı olarak değerlendirilmesi amaçlanmıştır.

Gereç ve yöntem: Kırk iki dişi (n=21) ve erkek (n=21) 16 aylık Wistar sıçanları altı gruba (n=7) ayrıldı: Grup-1 (kontrol-erkek), Grup-2 (1 ay, ADF-erkek), Grup-3 (2 ay, ADF-erkek), Grup-4 (kontrol-dişi), Grup-5 (1 ay, ADF-dişi) ve Grup-6 (2 ay, ADF-dişi). ADF protokolü günaşırı 24 saatlik oruç tutma şeklinde uygulandı (haftada üç gün). Serum örnekleri, ELISA yöntemiyle toplam oksidan-antioksidan durumu (TOS-TAS) ölçmek için alındı ve oksidatif stres indeksi (OSI) hesaplandı.

Bulgular: 2 aylık-ADF tedavisinin kümültatif control gruplarıyla karşılaştırıldığında vücut ağırlığında (VA) anlamlı azalma tespit edildi (p<0,001). Tüm gruplarda kümülatif gıda alımı ve retroperitoneal yağ ağırlığı ADF ile azaldığı görüldü (p<0,05). Hem 1 aylık hem de 2 aylık ADF müdahaleleri, hem karaciğer hem de serumda TOS ve OSI'yi azaltmada olumlu etkiler gösterdi ve her iki grup da kendi kontrollerine göre anlamlı bir azalma gösterdi (p<0,001). Karaciğer TAS seviyesi dişi sıçanlarda anlamlı olarak arttı (p<0,05), ancak erkek sıçanlarda bu artış anlamlı bir seviyeye ulaşmadı. Gruplar arasındaki serum TAS seviyesinde anlamlı fark saptanmadı.

Sonuç: Bu çalışma, erkek ve dişi sıçanlarda ADF'nin VA, gıda tüketimi ve oksidatif stres parametreleri üzerindeki etkilerini değerlendirdi. Bulgular, ADF'nin kilo yönetimi ve oksidatif stresi azaltmada potansiyel faydalarını vurgulamaktadır. Sonuç olarak, ADF'nin metabolik sağlık üzerindeki etkilerini anlamada ve olası klinik uygulamaları belirlemede önemli bir adımı temsil etmektedir.

Anahtar kelimeler: Yaşlanma, alternatif günlerde açlık, gıda alımı, cinsiyet, oksidatif stres.

Introduction

A progressive decline in all physiological activities is a hallmark of aging [1]. Substantial structural and functional changes occur in our organs and systems as we age. Free radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS) are highly reactive molecules with unpaired electrons [2]. The damage caused by ROS is called oxidative stress, which results from an imbalance between ROS production and antioxidant defenses. This imbalance plays a key role in aging [3].

The free radical theory of aging states that a a gradual rise in ROS and subsequent oxidative damage are pivotal in the aging process. In the absence of endogenous antioxidant defenses, free radicals damage cellular components like DNA, lipids, and proteins, contributing to aging and related disorders [4]. Overexpression of antioxidant enzymes can reduce ROS production and protect DNA, extending lifespan in mice [5]. Furthermore, long-lived mouse strains exhibit increased levels of antioxidant enzyme levels and decreased oxidative damage to proteins and lipids [6].

Rat liver mitochondria undergo oxidative stress and lose enzymatic activity during aging [7]. During aging, ROS overproduction leads to oxidative damage at both the liver and systemic levels. Luceri et al. [8] reported increased oxidative DNA damage in the livers of middle-aged rats (15 months), along with reduced DNA damage repair capacity. Many studies have shown that the aging liver exhibits signs of oxidative damage, and ROS levels in liver tissue significantly impact liver function and are linked to most age-related diseases [9].

Aging increases susceptibility to obesity due to declines in the basal metabolic rate and activity, although individual variations exist. Intermittent fasting (IF) has shown various health benefits in animal models [10, 11] and clinical trials [12, 13]. IF involves periods of eating and fasting, with different protocols such as time-restricted feeding, alternate-day fasting (ADF), or modified fasting with reduced daily food consumption [14].

Individuals aiming to lose weight often follow IF protocols, which include daily fasting periods of up to 16 hours or fasting intervals of up to 24 hours alternating with regular eating days. IF is a method to cause weight loss that has some beneficial effects. Despite the observed weight loss [13, 15] more studies are needed to evaluate whether ADF promotes health benefits or could cause undesired effects in the long term. Although ADF can reduce body weight and fat [16, 17], results vary due to differences in age, sex, and BMI among participants [18, 19]. Thus, a consensus on IF recommendations or preferred protocols is lacking, reflecting diverse study designs and divergent results [20].

The sex-dependent factors responsible for variations in oxidized macromolecule levels are largely unknown and controversial. Preliminary reports on sex-dependent variations in oxidative stress parameters in the plasma of aged subjects have been ambiguous. However, previous studies could not meaningfully correlate the relationships between sex and these oxidized macromolecules [21, 22]. We hope the present study will provide insight that clarifies this complex issue.

Considering the pivotal role of sex in feeding and aging, the question arises whether oxidative stress is influenced synchronously during prolonged food restriction followed by refeeding. Oxidative stress is known to be effective in the development of aging. However, there are no data in the literature examining the effects of age and ADF on oxidative stress parameters in different sexes. In light of this information, this study aimed to evaluate the role of ADF in aging-related oxidative stress changes at both the systemic and tissue levels and its effectiveness compared to that of sex.

Material and methods

Animal Experiments were approved by the Medical Ethics Committee of Pamukkale University (dated 15.12.2023 and numbered PAUHADYEK-2023/60758568-020-468703).

Animals and experimental design

In this study, forty-two male and female Wistar rats (16 month old) were obtained from the Pamukkale University Medical Experimental Research and Practice Center. The animals were housed in a room with controlled temperature (23±2°C) and relative humidity (60±5%). The animals were kept under adequate light conditions (7.00 a.m. to 7.00 p.m. light/dark). The rats were randomly assigned to six equivalent groups (n=7) as follows:

Group 1 (Control group, n=7): Male rats were fed ad libitum

Group 2 (1-month ADF, n=7): Male rats fasted for three days and fed ad libitum for four days per week

Group 3 (2-month ADF, n=7): Male rats fasted for three days and fed ad libitum for four days per week

Group 4 (control group, n=7): Female rats were fed ad libitum

Group 5 (1-month ADF, n=7): Female rats fasted for three days and fed ad libitum for four days per week

Group 6 (2-month ADF, n=7): Female rats fasted for three days and fed ad libitum for four days per week

Study design and ADF protocol

The ADF protocol was applied for eight weeks at a ratio of 4:3 (4 ad libitum days per week and 3 days of total fasting) [23] (Table 1). ADF rats were subjected to 24-hour fasts involving 24 hours of free access to the same chow [24].

Food consumption and body weight measurements

Food consumption was calculated by deducting the amount of food left in the box (g) from the total amount of food given. As previously described, the percentage was determined three times a week till the ADF protocols concluded [25]. Feed efficiency was determined by calculating the quotient over the 8 week of experimentation as weekly food consumption per rat. Body weight (BW) was measured at initial, 4^{th} and 8^{th} week of the experiment procedure. In the last week of treatment (4^{th} and 8^{th} weeks), both groups were subjected to a 12-hour overnight fast.

Blood and tissue sampling

The rats were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (90 mg/kg) following a fasting period of 8 h. Serum samples were collected from the abdominal aorta of the animals in plain tubes without any anticoagulant for Enzyme-Linked Immunosorbent Assay (ELISA). Liver tissues were removed and quickly frozen using liquid nitrogen. The samples were stored at 2-8°C and homogenized. After centrifugation for 20 minutes at 2000 rpm, the supernatant was extracted. After the samples were centrifuged 15 min at 3500 rpm, the blood serum was obtained from the tubes without EDTA.

Oxidative stress parameters

The total oxidant status (TOS) of the serum and tissue samples was determined via an automated colorimetric method [26]. Using this technique, the sample's ferrous ion chelator complex is changed into a ferric ion, increasing absorbance when it reacts with the chromogen in an acidic environment. The concentration of oxidant molecules in the sample is proportional to the rise in absorbance observed using spectrophotometry. The Lowry method was used to measure the tissue protein level. The solid-phase sandwich ELISA principle was applied to the assessment of the serum and tissue sample using ready-to-use measurement kits in accordance with the manufacturer's instructions. The results are reported as μmol H_2O_2Eq/L and μmol H_2O_2Eq/mg protein, respectively.

Similarly, total antioxidant status (TAS) in the serum and tissue samples was also determined using an automated colorimetric method [27]. Serum TAS was determined using the following principle: every antioxidant in the sample reduces the blue-green 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical to a colorless reduced form. The amount of antioxidants in the sample is exactly proportionate to the increase in

absorbance that is detected spectrophotometrically. The solid-phase sandwich ELISA principle was applied to the assessment of the serum and tissue sample using ready-to-use measurement kits in accordance with the manufacturer's instructions. The results are reported as mmol Trolox Eq/L and mmol Trolox Eq/mg protein, respectively.

The oxidative stability index (OSI) is defined as the ratio of TOS to TAS, calculated using the following formula:

$$\text{OSI (arbitary unit)} = \frac{\text{TOS (µmol } H_2O_2 \text{ Eq/mg protein)}}{\text{TAS (µmol Trolox Eq/mgram protein)}} \times 100$$

Statistical analysis

A power analysis was conducted using the GPower 3.1 program available online. The effect size reported in the reference study is large (d=1.22). The power analysis (f=0.9) indicated that a minimum of 42 rats (at least seven rats per group) would be required to achieve a power of 80% with a 95% confidence interval. The study used a total of 42 rats. IBM SPSS Statistics 25 program (Armonk, NY: IBM Corp.) version 23.0 was used for all statistical analyses. The standard deviation (SD) of the mean was used to define continuous variables. The normal distribution was determined using the Shapiro-Wilk tests. We employed One-way Anova analysis of variance (post hoc: Tukey method) for independent group comparisons. For statistical significance, $p<0.05$ was the threshold.

Results

Examination of body weight gain and food intake

BW (measured at the initial, 4th week and 8th week of the experiment) is shown in Figure 1A-C. The initial BW of male rats was significantly greater than that of concordant female rats (Group 1 vs Group 4, Group 2 vs Group 5, Group 3 vs Group 6, $p<0.001$), and the BW was not significantly different within the same sex groups ($p>0.05$). After 1 month of the experiment, the BW of the female rats was still significantly lower than that of the concordant male rats (Group 1 vs Group 4, Group 2 vs Group 5, Group 3 vs Group 6, $p<0.001$), and ADF did not significantly lower BW in any of the groups ($p>0.05$). At the end of the experiment, the BW of male rats was significantly greater than that of Concordant female rats (Group 1 vs Group 4, Group 3 vs Group 6, $p<0.001$), and 2-months of ADF decreased the BW in both genders (Group 1 vs Group 3, Group 4 vs Group 6, $p<0.001$).

The weekly food consumption amount per rat is shown in Figure 2. The food consumption of male rats was significantly greater than that of concordant female rats (Group 1 vs Group 4, Group 2 vs Group 5, Group 3 vs Group 6; $p<0.001$, $p<0.001$, $p<0.05$, respectively). Additionally, rats in the 1-month and 2-month ADF groups consumed significantly less chow than did those in the control group ($p<0.001$). In addition, in the 2-month ADF group compared to the 1-month group, food consumption decreased significantly in males ($p<0.05$), while this decrease did not reach a significant level in females ($p>0.05$).

Investigation of retroperitoneal fat weight in the experimental groups

The retroperitoneal adipose tissue weights (g) of the rats in the experimental groups are shown in Figure 3. Although the retroperitoneal fat weight of initial male rats was significantly lower than that of initial female rats (group 1 vs group 4, $p<0.01$), this decrease did not reach a significant level in 1-month and 2-month ADF male rats compared with concordance female rats (group 2 vs group 5, group 3 vs group 6; $p>0.05$). The 1-month ADF intervention significantly reduced the fat pad in females ($p<0.001$), whereas the reduction in males did not reach significance ($p>0.05$). At the end of the experiment, 2-month of ADF treatment significantly decreased the fat pad weight in both groups (Group 1 vs Group 3, Group 4 vs Group 6, $p<0.001$).

Evaluation of serum and liver TOS, TAS, and OSI levels

The liver TOS, TAS, and OSI levels of the experimental groups are shown in Figure 4. Although the TOS and OSI of initial male rats were significantly greater than those of female rats (group 1 vs group 4, $p<0.01$), this decrease did not reach a significant difference between 1-month and 2-month ADF male rats and concordant female rats (group 2 vs group 5, group 3 vs group 6; $p>0.05$). The liver TOS and OSI were significantly lower in Group 2 and Group 3 than in Group 1 ($p<0.001$). Similar results were observed in female rats, as the TOS and OSI were significantly lower in Group 5 and Group 6 than in Group 4 ($p<0.05$ and $p<0.001$, respectively). The 1-month and 2-month ADF interventions had a positive effect on lowering liver TOS and OSI levels, but this decrease was much more significant in males. The liver TAS level was significantly greater in Group 6 than in Group 4 and Group 5 ($p<0.001$ and $p<0.05$, respectively), but this increase did not reach a significant level in male rats.

The serum TOS, TAS, and OSI levels of the experimental groups are shown in Figure 5. The serum TOS and OSI were significantly lower in Group 2 and Group 3 than in Group 1 ($p<0.001$). Similar results were observed in female rats, as the TOS and OSI were significantly lower in Group 5 and Group 6 than in Group 4 ($p<0.001$). The 1-month and 2-month ADF interventions had a positive effect on lowering the serum TOS and

OSI. However, the difference in the serum TAS level between the groups was not significant (p>0.05).

Discussion

We conducted chronic fasting protocol experiments involving a healthy lifespan using middle-aged male and female rats to clarify the effects of ADF on age-associated changes in BW, body fat, and ROS levels. In the present study, we demonstrated that ADF positively affects BW at 2 months and that 1 month was insufficient to decrease BW in all ADF-treated rats. On the other hand, female rats had the highest retroperitoneal fat weight, which decreased with 1 month of ADF, while 2 months of ADF reduced fat weight regardless of sex. Although ADF lowered the OSI in both timelines with decreased TOS, the favorable effect of ADF was on increased TAS levels in 2-month-old ADF-treated female rats.

Previous studies have reported the beneficial effects of IF on BW and plasma glucose levels in obese individuals or obese rodent models [28, 29]. However, there is a lack of literature on the application of different IF types to increase healthspan in middle-aged rats. It has been found that IF protocols like ADF can successfully lower BW. In a research with overweight and obese participants, ADF decreased BW by 3% to 7% [18]. However, the reduction in BW induced by IF seems to vary on factors including age, the length of time spent consuming food, and the kind of diet [30]. For example, in male rats, TRF application was unable to stop the rise in BW induced by HFD in older animals as opposed to younger ones [25]. Conversely, ADF induced hyperphagia, hyperinsulinemia, and increased adiposity in juvenile female rats [24]. These consequences could result from extreme or sudden energy restriction, leading to overeating and other undesirable behaviors. These behaviors can trigger "storage signals," which promote the growth of adipose tissue [31]. It is significant to remember that in animal models, restricting access to chow or "tasty" items causes an increase in food intake [32, 33]. In this study, the effect of ADF on BW in middle-aged rats of both sexes was examined. Although 1-month of ADF treatment did not significantly affect BW in either sex, a significant decrease in weight loss was observed after 2-months of ADF treatment (Figure 1).

Adipose tissue regulates the cycling of triglycerides by controlling the uptake, esterification, and release of fatty acids, which are alternative fuel sources for the metabolically active organs [34]. During fasting periods, energy is obtained from glycogen which is followed by lipolysis of adipose tissue. This causes release of triacylglycerols and production of glucose from glycerol. Studies have also demonstrated fat loss accompanied with weight loss in ADF protocols [16, 35]. 4 weeks of ADF resulted in

decrement of visceral fat, and improvement of lipid profiles in C57BL/6 mice [36]. We still do not understand why various regions of visceral fat respond differently to the dietary protocols between genders. However, we have observed that female rats in the same age group had higher BW, and implementing ADF for 2-months reduced retroperitoneal fat tissue regardless of gender (Figure 3).

ROS can oxidatively modify many biological macromolecules, including proteins, lipids, and nucleic acids, potentially causing genetic mutations and cellular aging. The aging process has been shown to affect the redox balance of plasma proteins, lipids, DNA, and antioxidants in both rats [2, 37] and humans with age-related diseases [38, 39].

Research employing IF protocols in animal models, often utilizing adult male rats, indicates favorable outcomes in diminishing indicators of oxidative stress and inflammation [19]. These benefits are attributed to physiological adaptations triggered by periods of food deprivation. Experimental studies indicate that oxidative damage resulting from ROS plays a role in the aging process. For different animals, tissues, and cell types, the amount to which oxidative stress accelerates aging may differ [3]. Luceri et al. [8] demonstrated an increase in oxidative DNA damage in the liver with age, particularly reaching very high levels in middle-aged animals. Notably, there was a significant correlation between systemic oxidative damage and liver oxidative damage in the same animal. These findings suggest an overall disruption in the balance between pro-oxidant and antioxidant status during aging. Navarro et al. [7] reported an increase in oxidative stress markers and a decrease in antioxidant enzymes in the brain and liver during the aging process in 60-week-old and 92-week-old rats compared to 28-week-old rats. According to Cakatay et al., elevated levels of malondialdehyde (MDA) and 8-Hidroksi-2-deoksiguanozin (8-OHdG) found in elderly male rats may be a risk factor for plasma oxidation, while elevated total sulfhydryl (T-SH) levels in female rats may represent an adaptive response to oxidative damage [2].

Studies have extensively shown that levels of ROS in liver mitochondria notably rise following 36 and 72 hours of fasting in rats [40, 41], resulting in lipid peroxidation of cell membranes and oxidative stress in the liver. In contrast, ADF has been observed to mitigate oxidative stress. Interestingly, research suggests that while MDA levels in the liver significantly increase after 4 weeks of ADF treatment, prolonged ADF treatment spanning 8 to 16 weeks reduces MDA levels in visceral tissue of rats experiencing oxidative stress induced by conditions such as diabetes or spontaneous tumors [42, 43].

This phenomenon may be due to hormesis, a biological process where low doses of a harmful agent trigger a beneficial response. While higher doses can be toxic or lethal, low doses can enhance resilience and extend lifespan. Research shows that ADF, as a

mild stressor, can increase stress resistance and prolong the lifespan of various organisms [44].

The liver mitochondria of rats fed freely leak fewer electrons per unit of O2 consumed at complex III compared to rats fed normally for 72 hours. This indicates that severe food deprivation, like fasting, can increase oxidative stress in the rat liver. The increase in oxidative stress is due to factors like higher mitochondrial free radical production and greater sensitivity of liver membranes to oxidative damage. This suggests that fasting and caloric restriction may affect liver mitochondrial oxidative stress differently [41]. It is conceivable that ADF applied for different durations can modulate oxidative stress responses; short durations may activate cellular resistance with low-intensity oxidative stress, while longer durations may enhance antioxidant effects, balance the amount of ROS generated by mild stress, and thus reduce oxidative stress. In this study, in middle-aged rats, both male and female showed high levels of liver and serum TOS and OSI. Both 1-month and 2-month ADF interventions had positive effects in reducing both liver and serum TOS and OSI levels, and both groups showed a significant decrease compared to their controls. Liver TAS levels significantly increased with the 2-month ADF intervention in female rats, but this increase did not reach a significant level in male rats. Although there was an increase in serum TAS levels, it did not reach a significant level between the groups (Figure 4, 5)

There are some limitations in this study. Firstly, oxidative stress was only investigated using the TAS and TOS ELISA methods; changes in the levels of oxidative stress markers (such as MDA, glutathione, CAT, SOD, etc.) at the gene or protein levels could have been revealed. Finally, conducting analyses such as ROS analysis to examine the effects on reactive oxygen species could have further strengthened our study.

In conclusion, this study examined the effects of ADF on BW, food intake, and markers of oxidative stress in male and female middle-aged rats. Initially, male rats exhibited higher BW and food intake, but these differences decreased with ADF application. Prolonged ADF resulted in a significant decrease in retroperitoneal fat weight in both sexes. In addition, ADF significantly lowered oxidative stress markers at both tissue and systemic levels in both sexes, and particularly improved antioxidant levels in the liver of female rats. These observed differences may be due to aging processes and sex-specific homeostatic mechanisms. These findings highlight the potential benefits of ADF for weight management and mitigation of oxidative stress. Future investigations should address the molecular mechanisms and long-term effects of ADF.

Data availability: All data are presented in the manuscript.

Conflict of interest: No conflict of interest was declared by the authors

References

1. López Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell 2013;153:1194-1217. https://doi.org/10.1016%2Fj.cell.2013.05.039
2. Çakatay U, Aydin S, Yanar K, Uzun H. Gender-dependent variations in systemic biomarkers of oxidative protein, DNA, and lipid damage in aged rats. Aging Male 2010;13:51-58. https://doi.org/10.3109/13685530903236470
3. Maldonado E, Morales Pison S, Urbina F, Solari A. Aging hallmarks and the role of oxidative stress. Antioxidants 2023;12:651. https://doi.org/10.3390/antiox12030651
4. Sharifi Rad M, Anil Kumar NV, Zucca P, et al. Lifestyle, oxidative stress, and antioxidants: back and forth in the pathophysiology of chronic diseases. Front Physiology 2020;11:694. https://doi.org/10.3389/fphys.2020.00694
5. Schriner SE, Linford NJ, Martin GM, et al. Extension of murine life span by overexpression of catalase targeted to mitochondria. Science 2005;308:1909-1911. https://doi.org/10.1126/science.1106653
6. Shields HJ, Traa A, Van Raamsdonk JM. Beneficial and detrimental effects of reactive oxygen species on lifespan: a comprehensive review of comparative and experimental studies. Front Cell Dev Biology 2021;9:628157. https://doi.org/10.3389/fcell.2021.628157
7. Navarro A, Boveris A. Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging. Am J Physiol Regul Integr Comp Physiol 2004;287:1244-1249. https://doi.org/10.1152/ajpregu.00226.2004
8. Luceri C, Bigagli E, Femia AP, Caderni G, Giovannelli L, Lodovici M. Aging related changes in circulating reactive oxygen species (ROS) and protein carbonyls are indicative of liver oxidative injury. Toxicology Reports 2018;5:141-145. https://doi.org/10.1016/j.toxrep.2017.12.017
9. Lebel M, de Souza Pinto NC, Bohr VA. Metabolism, genomics, and DNA repair in the mouse aging liver. Current gerontology and geriatrics research 2011;2011:859415. https://doi.org/10.1155%2F2011%2F859415
10. Soares NL, Dorand VAM, Cavalcante HC, et al. Does intermittent fasting associated with aerobic training influence parameters related to the gut-brain axis of Wistar rats? Journal of Affective Disorders 2021;293:176-185. https://doi.org/10.1016/j.jad.2021.06.028
11. Badreh F, Joukar S, Badavi M, Rashno M, Dehesh T. The effects of age and fasting models on blood pressure, insulin/glucose profile, and expression of longevity proteins

in male rats. Rejuvenation Research 2020;23:224-236. https://doi.org/10.1089/rej.2019.2205

12. Carvajal V, Marín A, Gihardo D, Maluenda F, Carrasco F, Chamorro R. Intermittent fasting and human metabolic health. Rev Med Chile 2023;151:81-100. https://doi.org/10.4067/s0034-98872023000100081
13. Gabel K, Hoddy KK, Haggerty N, et al. Effects of 8-hour time restricted feeding on body weight and metabolic disease risk factors in obese adults: a pilot study. Nutrition and Healthy Aging 2018;4:345-353. https://doi.org/10.3233/nha-170036
14. Mattson MP, Longo VD, Harvie M. Impact of intermittent fasting on health and disease processes. Ageing Research Reviews 2017;39:46-58. https://doi.org/10.1016/j.arr.2016.10.005
15. Harris L, Hamilton S, Azevedo LB, et al. Intermittent fasting interventions for treatment of overweight and obesity in adults: a systematic review and meta-analysis. JBI Evidence Synthesis 2018;16:507-547. https://doi.org/10.11124/jbisrir-2016-003248
16. Park J, Seo YG, Paek YJ, Song HJ, Park KH, Noh HM. Effect of alternate-day fasting on obesity and cardiometabolic risk: a systematic review and meta-analysis. Metabolism 2020;111:154336. https://doi.org/10.1016/j.metabol.2020.154336
17. Moon S, Kang J, Kim SH, et al. Beneficial effects of time-restricted eating on metabolic diseases: a systemic review and meta-analysis. Nutrients 2020;12:1267. https://doi.org/10.3390/nu12051267
18. Tinsley GM, La Bounty PM. Effects of intermittent fasting on body composition and clinical health markers in humans. Nutrition Reviews 2015;73:661-674. https://doi.org/10.1093/nutrit/nuv041
19. Lee JH, Verma N, Thakkar N, Yeung C, Sung HK. Intermittent fasting: physiological implications on outcomes in mice and men. Physiology 2020;35:185-195. https://doi.org/10.1152/physiol.00030.2019
20. Catterson JH, Khericha M, Dyson MC, et al. Short-term, intermittent fasting induces long-lasting gut health and TOR-independent lifespan extension. Current Biology 2018;28:1714-1724. https://doi.org/10.1016/j.cub.2018.04.015
21. Vassalle C, Novembrino C, Maffei S, et al. Determinants of oxidative stress related to gender: relevance of age and smoking habit. Clin Chem Lab Med 2011;49:1509-1513. https://doi.org/10.1515/CCLM.2011.622
22. Takahashi M, Miyashita M, Park JH, et al. The association between physical activity and sex-specific oxidative stress in older adults. J Sports Sci Med 2013;12:571-578.

23. Bilibio BLE, Dos Reis WR, Compagnon L, et al. Effects of alternate-day fasting and time-restricted feeding in obese middle-aged female rats. Nutrition 2023;116:112198. https://doi.org/10.1016/j.nut.2023.112198
24. Munhoz AC, Vilas Boas EA, Panveloski Costa AC, et al. Intermittent fasting for twelve weeks leads to increases in fat mass and hyperinsulinemia in young female Wistar rats. Nutrients 2020;12:1029. https://doi.org/10.3390/nu12041029
25. Olsen MK, Choi MH, Kulseng B, Zhao CM, Chen D. Time-restricted feeding on weekdays restricts weight gain: a study using rat models of high-fat diet-induced obesity. Physiology Behavior 2017;173:298-304. https://doi.org/10.1016/j.physbeh.2017.02.032
26. Erel O. A new automated colorimetric method for measuring total oxidant status. Clinical Biochemistry 2005;38:1103-1011. https://doi.org/10.1016/j.clinbiochem.2005.08.008
27. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clinical Biochemistry 2004;37:277-285. https://doi.org/10.1016/j.clinbiochem.2003.11.015
28. Mattson MP, Allison DB, Fontana L, et al. Meal frequency and timing in health and disease. PNAS 2014;111:16647-16653. https://doi.org/10.1073/pnas.1413965111
29. Baumeier C, Kaiser D, Heeren J, et al. Caloric restriction and intermittent fasting alter hepatic lipid droplet proteome and diacylglycerol species and prevent diabetes in NZO mice. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 2015;1851:566-576. https://doi.org/10.1016/j.bbalip.2015.01.013
30. Sherman H, Genzer Y, Cohen R, Chapnik N, Madar Z, Froy O. Timed high-fat diet resets circadian metabolism and prevents obesity. FASEB J 2012;26:3493-3502. https://doi.org/10.1096/fj.12-208868
31. Cottone P, Sabino V, Steardo L, Zorrilla EP. Consummatory, anxiety-related and metabolic adaptations in female rats with alternating access to preferred food. Psychoneuroendocrinology 2009;34:38-49. https://doi.org/10.1016/j.psyneuen.2008.08.010
32. Wang L, Suyama S, Lee SA, et al. Fasting inhibits excitatory synaptic input on paraventricular oxytocin neurons via neuropeptide Y and Y1 receptor, inducing rebound hyperphagia, and weight gain. Front Nutr 2022;9:994827. https://doi.org/10.3389/fnut.2022.994827
33. Park S, Yoo KM, Hyun JS, Kang S. Intermittent fasting reduces body fat but exacerbates hepatic insulin resistance in young rats regardless of high protein and fat

diets. The Journal of Nutritional Biochemistry 2017;40:14-22. https://doi.org/10.1016/j.jnutbio.2016.10.003

34. Carpentier AC. 100th anniversary of the discovery of insulin perspective: insulin and adipose tissue fatty acid metabolism. Am J Physiol Endocrinol Metab 2021;320:653-670. https://doi.org/10.1152/ajpendo.00620.2020
35. Stockman MC, Thomas D, Burke J, Apovian CM. Intermittent fasting: is the wait worth the weight? Curr Obes Rep 2018;7:172-185. https://doi.org/10.1007/s13679-018-0308-9
36. Varady KA, Hudak CS, Hellerstein MK. Modified alternate-day fasting and cardioprotection: relation to adipose tissue dynamics and dietary fat intake. Metabolism 2009;58:803-811. https://doi.org/10.1016/j.metabol.2009.01.018
37. Kayali R, Çakatay U, Tekeli F. Male rats exhibit higher oxidative protein damage than females of the same chronological age. Mechanisms of Ageing and Development 2007;128:365-369. https://doi.org/10.1016/j.mad.2007.03.003
38. Höhn A, König J, Grune T. Protein oxidation in aging and the removal of oxidized proteins. Journal of Proteomics 2013;92:132-159. https://doi.org/10.1016/j.jprot.2013.01.004
39. Pandey KB, Mehdi MM, Maurya PK, Rizvi SI. Plasma protein oxidation and its correlation with antioxidant potential during human aging. Disease Markers 2010;29:31-36. https://doi.org/10.3233/dma-2010-0723
40. Marczuk Krynickaabcdef D, Hryniewieckibe T, Piątekbf J, Paluszak J. The effect of brief food withdrawal on the level of free radicals and other parameters of oxidative status in the liver. Med Sci Monit 2003;9:131-135.
41. Sorensen M, Sanz A, Gomez J, et al. Effects of fasting on oxidative stress in rat liver mitochondria. Free Radical Research 2006;40:339-347. https://doi.org/10.1080/10715760500250182
42. Bhutani S, Klempel MC, Berger RA, Varady KA. Improvements in coronary heart disease risk indicators by alternate-day fasting involve adipose tissue modulations. Obesity 2010;18:2152-2159. https://doi.org/10.1038/oby.2010.54
43. Descamps O, Riondel J, Ducros V, Roussel AM. Mitochondrial production of reactive oxygen species and incidence of age-associated lymphoma in OF1 mice: effect of alternate-day fasting. Mechanisms of Ageing and Development 2005;126:1185-1191. https://doi.org/10.1016/j.mad.2005.06.007
44. Le Bourg E. Hormesis, aging and longevity. Biochimica et Biophysica Acta (BBA)-General Subjects 2009;1790:1030-1039. https://doi.org/10.1016/j.bbagen.2009.01.004

Ethics committee approval: Animal Experiments were approved by the Medical Ethics Committee of Pamukkale University (dated 15.12.2023 and numbered PAUHADYEK-2023/60758568-020-468703).

Authors' contributions

G.G.: Methodology, data curation, investigation, resources, writing, review, and editing; O.K.E.: Conceptualization, methodology, data curation, investigation, resources, project administration, writing, review, and editing. The final manuscript has been read and approved by all of the authors.

Table 1. Arrangement of feeding and fasting windows over the entire week

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Before 7.00 AM	Food Ad libitum period	Fasting period	Food Ad libitum period	Fasting period	Food Ad libitum period	Fasting period	Food Ad libitum period
After 7.00 AM	Food Ad libitum period	Fasting period	Food Ad libitum period	Fasting period	Food Ad libitum period	Fasting period	Food Ad libitum period

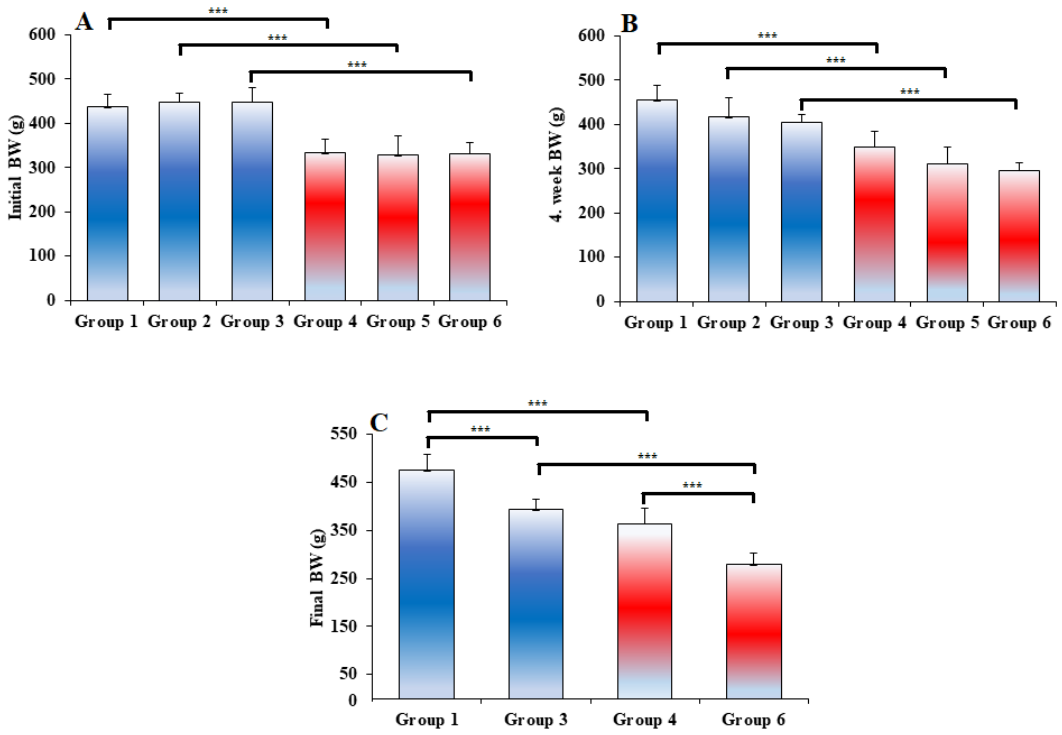

Figure 1. Changes in Body Weight Over Time Across Experimental Groups

A. Initial body weight measurements. B. Body weight measurements at the 4th week of the experiment. C. Body weight measurements at the 8th week of the experiment
Data are shown as the mean ± standard deviation; n=7, ***: p<0.001
Group 1: male control, Group 2: male 1-month ADF, Group 3: male 2-month ADF
Group 4: female control, Group 5: female 1-month ADF, Group 6: female 2-month ADF

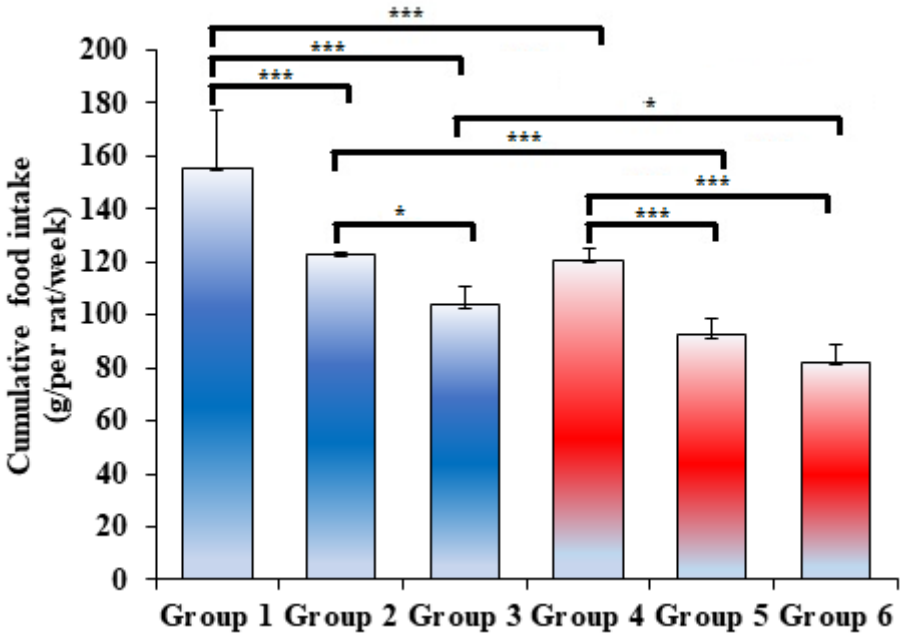

Figure 2. Cumulative food intake of the experimental groups

Data are shown as the mean ± standard deviation; n=7,
*: $p<0.05$, ***: $p<0.001$
Group 1: male control, Group 2: male 1-month ADF, Group 3: male 2-month ADF
Group 4: female control, Group 5: female 1-month ADF, Group 6: female 2-month ADF

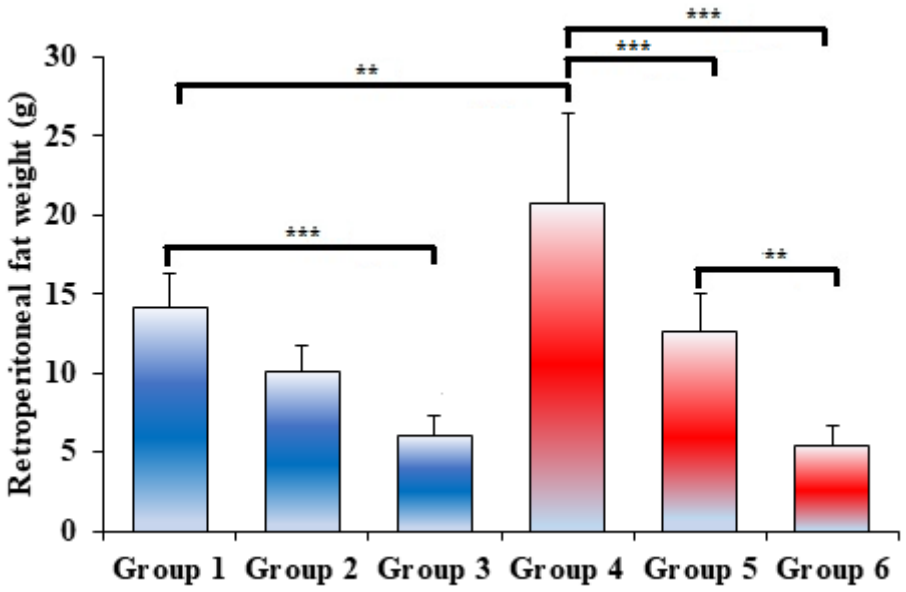

Figure 3. Retroperitoneal adipose tissue weight (g) of the experimental groups

Data are shown as the mean ± standard deviation; n=7, Statistically significant at **: $p<0.01$, ***: $p<0.001$
Group 1: male control, Group 2: male 1-month ADF, Group 3: male 2-month ADF
Group 4: female control, Group 5: female 1-month ADF, Group 6: female 2-month ADF

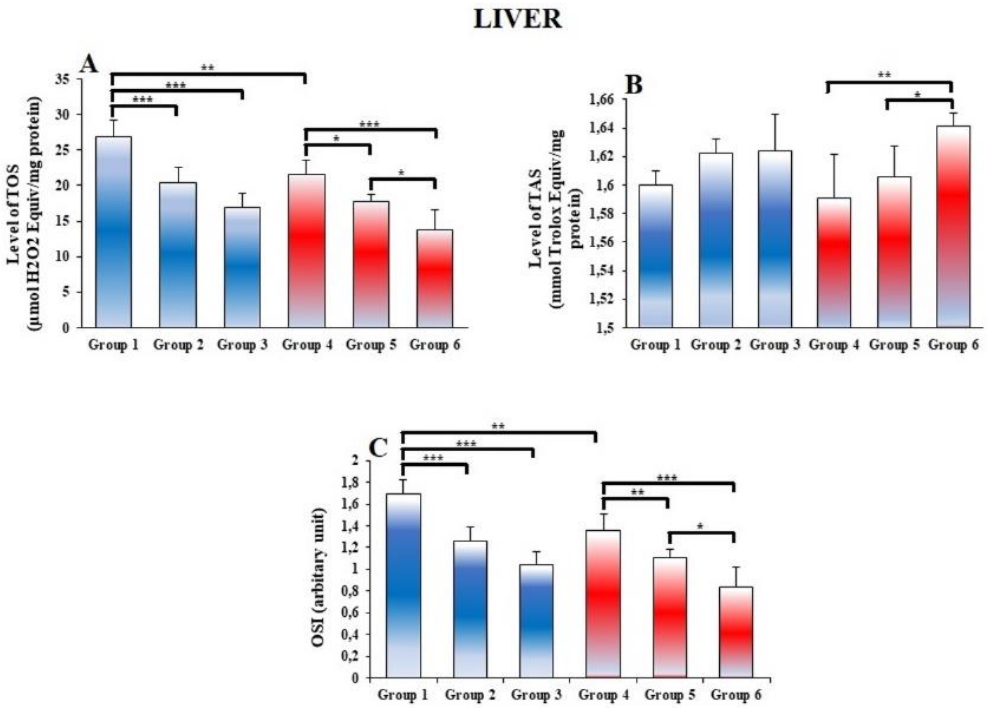

Figure 4. Levels of liver TOS, TAS, and OSI across experimental groups

A. Total Oxidant Status (TOS), B. Total Antioxidant Status (TAS), and C. Oxidative Stress Index (OSI). Data are shown as the mean ± standard deviation; n=7, Statistically significant at
*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$
Group 1: male control, Group 2: male 1-month ADF, Group 3: male 2-month ADF
Group 4: female control, Group 5: female 1-month ADF, Group 6: female 2-month ADF

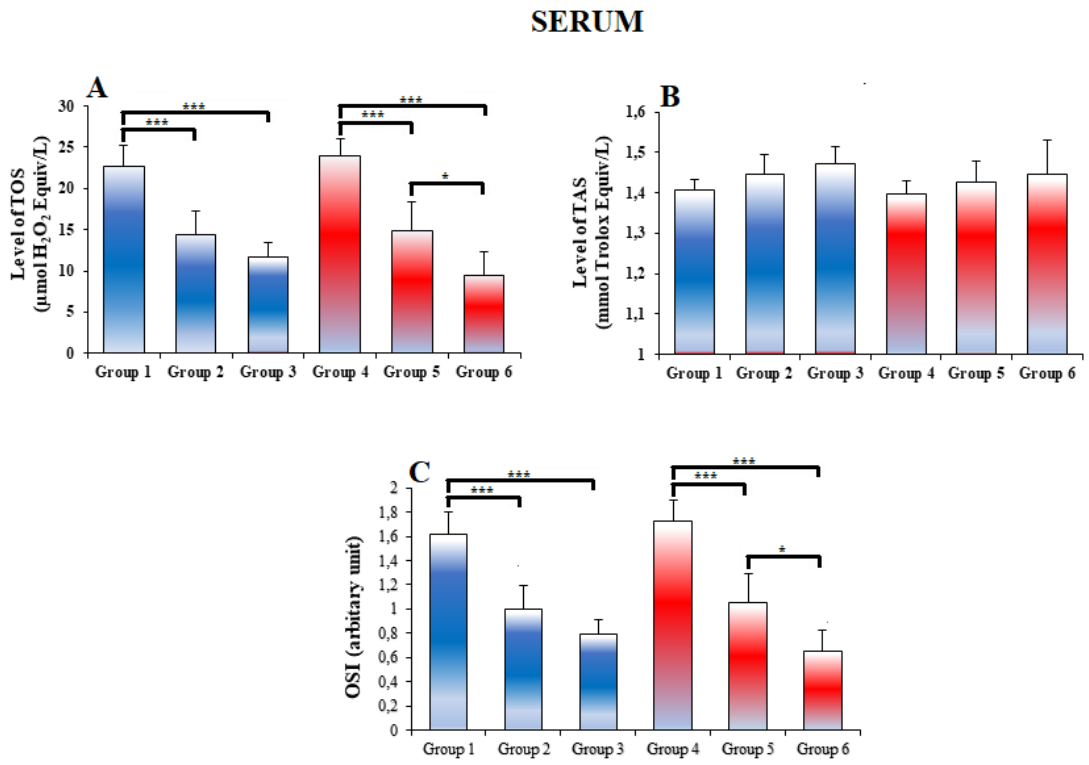

Figure 5. A. Levels of serum TOS, TAS, and OSI across experimental groups

A. Total Oxidant Status (TOS), B. Total Antioxidant Status (TAS), and C. Oxidative Stress Index (OSI). Data are shown as the mean ± standard deviation; n=7, Statistically significant at
*: $p<0.05$; ***: $p<0.001$
Group 1: male control, Group 2: male 1-month ADF, Group 3: male 2-month ADF
Group 4: female control, Group 5: female 1-month ADF, Group 6: female 2-month ADF

Kilic Erkek O, Gundogdu G. Gender-specific effects of alternate-day fasting on body weight, oxidative stress, and metabolic health in middle-aged rats. Pam Med J 2025;18:…-…

Kılıç Erkek Ö, Gündoğdu G. Orta yaş sıçanlarda gün aşırı açlık protokolünün vücut ağırlığı, oksidatif stres ve metabolik sağlık üzerine cinsiyete özgü etkileri. Pam Tıp Derg 2025;18:…-…

Özgen Kılıç Erkek, Asst. Prof. Pamukkale University, Faculty of Medicine, Department of Physiology, Denizli, Türkiye, e-mail: ozgenke@yahoo.com (https://orcid.org/0000-0001-8037-099X)

Gülşah Gündoğdu, Assoc. Prof. Pamukkale University, Faculty of Medicine, Department of Physiology, Denizli, Türkiye, e-mail: ggundogdu@pau.edu.tr (https://orcid.org/ 0000-0002-9924-5176) (Corresponding Author)