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Alternative day fasting protocol attenuates high fructose-induced activation of the TGF-beta/Smad signaling pathway

Alternatif günlerde açlık yüksek fruktoz kaynaklı TGF-beta/Smad sinyal yolağının aktivasyonunu azaltır

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Abstract

Purpose: This study aims to explore the protective effects of alternate-day fasting (ADF) against metabolic disturbances induced by high fructose (HF) intake, with a particular focus on modulating the transforming growth factor-beta1 (TGF-β1) / mother against decapentaplegic homolog 2 (Smad2) signaling pathway.

Materials and methods: Four groups of rats (n=7 per group) were included: Control, ADF, HF (20% fructose in drinking water), and HF+ADF. The ADF protocol was applied with 24 hours of ad libitum feeding followed by 24 hours of fasting over a 5-week period. After five weeks, body weight (BW), muscle, and fat mass were measured. Serum samples were analyzed using ELISA to assess levels of TGF-β1, Smad2, connective tissue growth factor (CTGF), and total oxidant-antioxidant status (TOS-TAS).

Results: Results indicated that HF significantly increased final BW, and ADF reduced this weight gain (p=0.001). ADF also led to lower gastrocnemius-soleus muscle weights compared to controls (p=0.001), but mitigated fructose-induced retroperitoneal fat accumulation. TAS levels were higher (ADF vs control (p=0.01); HF vs HF+ADF (p=0.001)), and TOS levels were lower (ADF vs control (p=0.022); HF vs HF+ADF (p=0.001)) in the ADF groups, showing an antioxidant shift. Moreover, ADF significantly attenuated the TGF-β1/Smad2 pathway activation by decreasing serum TGF-β1 (ADF vs control (p=0.011); HF vs HF+ADF (p=0.008)), Smad2 (ADF vs control (p=0.001); HF vs HF+ADF (p=0.001)), and CTGF (ADF vs control (p=0.018); HF vs HF+ADF (p=0.001)) levels, suggesting a protective role against fructose-induced metabolic dysregulation.

Conclusions: These findings suggest that ADF could be an effective dietary intervention for mitigating the metabolic impact of excessive fructose intake, particularly by regulating oxidative stress and the TGF-β1/Smad2 pathway.

Keywords: Alternate-day fasting, high-fructose, TGF-β1/Smad2 pathway, oxidative stress, metabolic health.

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Öz

Amaç: Bu çalışma, yüksek fruktoz (HF) alımının yol açtığı metabolik bozukluklara karşı alternatif gün orucu (ADF) uygulamasının koruyucu etkilerini incelemeyi amaçlamakta olup, özellikle transformasyon büyüme faktörü-beta1 (TGF-β1) / dekapentaplejik homolog 2'ye karşı ana protein (Smad2) sinyal yolunun modülasyonuna odaklanmaktadır.

Gereç ve yöntem: Sıçanlar dört grupta (her grup için n=7) çalışmaya dahil edilmiştir: Kontrol, ADF, HF (içme suyunda %20 fruktoz), ve HF+ADF. ADF protokolü, 5 hafta boyunca 24 saat serbest yem tüketimi ve onu takiben 24 saat açlık olarak gerçekleştirilmiştir. Beş hafta sonunda vücut ağırlığı (VA), kas ve yağ kütlesi ölçülmüştür. Serum örneklerinde TGF-β1, Smad2, bağ dokusu büyüme faktörü (CTGF) ve total oksidan-antioksidan (TOS-TAS) düzeyleri ELISA yöntemi ile analiz edilmiştir.

Bulgular: Sonuçlar, HF'nin final VA'yı anlamlı derecede artırdığını ve ADF'nin bu kilo alımını azalttığını göstermiştir (p=0,001). ADF, kontrol grubuna kıyasla gastroknemius-soleus kas ağırlıklarını azaltmış (p=0,001), ancak fruktoz kaynaklı retroperitoneal yağ birikimini hafifletmiştir. ADF gruplarında TAS düzeyleri daha yüksek (ADF vs kontrol (p=0,01); HF vs HF+ADF (p=0,001)), TOS düzeyleri ise daha düşük (ADF vs kontrol (p=0,022); HF vs HF+ADF (p=0,001)) bulunmuş olup, bu da bir antioksidan kaymasına işaret etmiştir. Ayrıca, ADF, serum TGF-β1 (ADF vs kontrol (p=0,011); HF vs HF+ADF (p=0,008)), Smad2 (ADF vs kontrol (p=0,001); HF vs HF+ADF (p=0,001)) ve CTGF (ADF vs control (p=0.018); HF vs HF+ADF (p=0.001)) seviyelerini düşürerek TGF-β1/Smad2 yolunun aktivasyonunu önemli ölçüde azaltmış olup fruktoz kaynaklı metabolik düzensizliklere karşı koruyucu bir role işaret etmektedir.

Sonuç: Elde edilen veriler ADF'nin, özellikle oksidatif stres ve TGF-β1/Smad2 yolağını düzenleyerek, aşırı fruktoz alımı ile gerçekleşen metabolik etkileri hafifletmek için etkili bir diyet müdahalesi olabileceğini düşündürmektedir.

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Anahtar kelimeler: Alternatif günlerde açlık, yüksek fruktoz, TGF-β1/Smad2 yolağı, oksidatif stres, metabolik sağlık.

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Introduction

Fructose consumption has risen significantly over the past three to four decades, mainly due to its use as a food additive. It is commonly found in dietary sugars like sucrose and high fructose (HF) corn syrup, with excessive intake strongly linked to metabolic diseases and obesity [1, 2]. Studies in rats show that HF intake leads to weight gain and tissue alterations, disrupting cartilage function [3]. Unlike glucose, fructose undergoes a less restrictive metabolism, quickly converting to triglycerides and contributing to cellular ATP depletion [4]. This rapid conversion has been associated with increased visceral adipose tissue and total abdominal fat [5], as well as abnormal collagen formation [6]. However, the precise cellular and molecular mechanisms by which HF intake causes toxicity remain unclear.

Obesity and HF consumption are known to increase oxidative stress, particularly within tissue-specific contexts that regulate metabolic inflammation [7]. Excessive fructose intake can initiate a damaging cycle, where oxidative stress promotes inflammation, cellular damage, and organ dysfunction, especially in the liver, kidneys, and cardiovascular systems [8]. Increased systemic fatty acids and proinflammatory cytokines due to HF intake elevate reactive oxygen species (ROS), thereby contributing to oxidative stress and affecting peripheral tissues [9].

Transforming Growth Factor-Beta (TGF-β) is a potent fibrogenic factor with essential roles in cellular processes, including proliferation, differentiation, apoptosis, migration, and extracellular matrix (ECM) synthesis [10]. TGF-β binds to type-1 and type-2 receptors on cell surfaces, inducing phosphorylation of mother against decapentaplegic homolog 2/3 (Smad2/3) proteins and initiating intracellular signaling. Smad proteins are necessary to transmit signals from active TGF-β1 receptor complexes to the nucleus, with Smad/connective tissue growth factor (CTGF) signaling being critical for TGF-β-induced fibrogenesis [11]. This pathway plays a vital role in various fibrotic disorders with CTGF amplifying the pro-fibrogenic effects of TGF-β1 and modulating TGF-β1/Smad signaling in mesenchymal cells and fibroblasts [12]. The expressions of TGF-β, CTGF, and Smad2 have been shown to increase with HF intake, contributing to metabolic disorders in rodents [13, 14]. Moreover, high glucose intake also induces ROS production and enhances TGF-β activation, which contributes to fibrotic and inflammatory diseases [15].

Dietary strategies are promising for intervention, as reducing energy intake can create a negative energy balance and lead to weight loss [16]. Intermittent fasting (IF) protocols, including alternate-day fasting (ADF), the 5:2 diet, and time-restricted feeding (TRF), are among the most studied [17, 18]. ADF typically involves alternating feast and fast days, with food available ad libitum on feast days and restricted on fast days, typically in 24-hour intervals [19]. IF has been proposed as a strategy to improve health, potentially reducing obesity and metabolic disorders, especially those associated with aging [16]. While ADF can decrease body weight (BW) and fat levels and may affect gastrocnemius-soleus muscle weights [20], more research is needed to determine whether ADF has lasting health benefits or could pose risks over the long term [18].

IF is widely recommended due to its potential to support weight control, reduce inflammatory cytokines, and lower oxidative stress, making it an attractive option for metabolic health [17]. These benefits are largely attributed to physiological adaptations triggered by fasting periods, where short-term fasting may induce mild oxidative stress, while longer-term fasting enhances antioxidant defenses, balancing ROS levels and reducing oxidative stress [21]. Additionally, IF has been reported to increase TGF-β levels, potentially ameliorating inflammatory immune disorders caused by obesity. Although previous studies have

extensively explored the effects of HF intake on oxidative stress and fibrotic pathways, research investigating the specific interaction between HF and ADF on the TGF-β/Smad signaling pathway remains limited. To date, there has been no comprehensive study analyzing the combined effects of HF consumption and ADF on TGF-β activation, oxidative balance, and related fibrogenic markers, particularly in experimental models.

While previous studies have examined the effects of HF intake on oxidative stress and fibrotic pathways, the role of ADF in mitigating these effects remains largely unexplored. Considering this point in the literature, our study aimed to be the first to investigate the effects of ADF on oxidative stress markers and the TGF-β1/Smad signaling pathway in rats exposed to HF intake. Our study aimed to investigate the impact of ADF on serum oxidative stress and TGF-β1/Smad pathway activation.

Materials and method

Ethics Committee approval, dated 27 June 2024 and numbered PAUHADY EK-2024/60758568-020-544586 was received from the local ethics council of Animal Experiments, Pamukkale University and conducted in accordance with the guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals (USA). For this study, twenty-eight male Wistar rats, aged between 10 and 12 weeks, were obtained from the Pamukkale University Medical Experimental Research and Practice Center. The animals were housed under controlled conditions, with the temperature set at 23±2°C and humidity at 60±5%. They were exposed to a 12-hour light/dark cycle, with lights on from 7:00 A.M. to 7:00 P.M. No mortality or adverse effects were occurred during the study, and all animals remained healthy throughout the experimental period.

Experimental and study design

Twenty-eight Wistar male rats were randomly divided into four groups as shown in Table 1.

All groups were fed a standard rodent laboratory chow (Optima, Türkiye) based on the NRC-Requirement of Compounded Feed for Laboratory Mice and Rats (BIS) and the Nutrient Requirement for Maintenance, Growth, and Reproduction of Rats (NRC, 1995). The diet provided 300 kcal per 100 g of feed, consisting of 60% carbohydrates, 20% proteins, and 20% lipids.

Table 1. The experimental groups are described

Groups	Description
Control Group (n=7)	Rats fed ad libitum
ADF Group (n=7)	Rats subjected to ADF (24-hour fasting, 24-hour ad libitum feding/ 5 weeks)
HF Group (n=7)	Rats were fed ad libitum with 20% fructose added to their drinking water for 5 weeks
HF+ADF Group (n=7)	20% fructose was added to the drinking water of rats for 5 weeks + Rats subjected to ADF (24-hour fasting, 24-hour ad libitum feding / 5 weeks)

The IF protocol used was an ADF regimen, where the rats underwent a total fasting period of 24 hours, followed by 24 hours of ad libitum feeding for 5 weeks [22]. Throughout the experiment, all rats had free access to water. This ADF protocol was chosen based on rodent studies [22] and its widespread use in clinical practice, particularly for preventing metabolic diseases [23]. HF diet was created by adding 20% fructose to the drinking water of rats. The survival rate of all groups was 100%, with no observed mortality.

At the end of the experiment, the researchers sacrificed the rats under general anesthesia (using 10 mg/kg of 2% xylazine hydrochloride and 90 mg/kg of ketamine hydrochloride) after fasting them the previous night with free access to water. Blood samples were collected from the abdominal aorta into the tubes without EDTA, and then after the samples were centrifuged for 15 min at 3500 rpm for enzyme-linked immunosorbent assay (ELISA) analysis. The samples were stored at -80°C until the experimental analysis.

Measurement of Body Weight, Gastrocnemius Weight, and Visceral Fat Pad

The rats' BW was measured and recorded using a digital weighing scale at the beginning of the study and the fifth week. BW measurements were taken on non-fasting days to avoid fluctuations due to feed restriction. After sacrifice, retroperitoneal fat tissue and gastrocnemius were dissected and weighed.

Biochemical analysis

ELISA method was used to measure the serum levels TGF-β1 (Elabscience, E-EL0162, Texas/USA), Smad2 (Elabscience, E-EL-R2582, Texas/USA), CTGF (Elabscience, E-EL-R0259, Texas/USA), total antioxidant status (TAS), and total oxidant status (TOS) (BT Lab, E1512Ra, E1710Ra, Zhejiang/ China) using ready-to-use measurement kits according to the manufacturer's instructions

Statistical analysis

All calculations and power analysis were conducted using the G-power program (version 3.1.9.2. Heinrich-Heine-Universitat, Duesseldorf, Germany). The effect size reported in the reference study was substantial (d=1.22). Based on the assumption that a similar effect size (f=0.8) could be achieved in this study, which included 4 groups, a power analysis determined that a sample size of at least 28 rats (7 per group) would provide 80% power at a 95% confidence level.

Data were analyzed using IBM SPSS Statistics 23 software. Continuous variables are expressed as mean ± standard deviation. If the parametric test assumptions were met, a one-way analysis of variance (ANOVA) was performed for group comparisons, followed by the Tukey post hoc test. When the parametric test assumptions were not met, the Kruskal-Wallis variance analysis was used, with subsequent comparisons between independent groups performed using the Mann-Whitney U test with Bonferroni correction. $p<0.05$ threshold was used for statistical significance.

Results

Initial and final BW comparisons between the four groups are shown in Figure 1. At the beginning of the study, all groups had similar starting weights, with no significant differences in initial BW observed (Figure 1A). By the end of the 5-week period, the HF group showed the highest final BW, significantly exceeding the other groups. ADF reduced final BW in the HF+ADF group (240±11.59) compared to the HF group (296.25±9.91) (p=0.001), and the ADF group (237±10.73) had a significantly lower final BW than the control group (260±20.83) (p=0.031). No significant difference was observed between the ADF and HF+ADF groups (Figure 1B). These findings suggest that HF intake promotes weight gain, while ADF counteracts this effect.

Comparisons of muscle weight also showed significant differences between groups. Control group (2.51±0.56) exhibited significantly higher muscle weights than ADF group (1.67±0.23) (p=0.001). Similarly, HF group (2.54±0.46) had greater muscle weight than HF+ADF group (1.87±0.19) (p=0.005), although no significant difference was observed between control and HF groups. These findings indicate that ADF affects muscle mass, leading to reduced muscle weight (Figure 2A), potentially due to metabolic adaptations associated with fasting.

Additionally, the HF group exhibited significantly higher retroperitoneal fat weights than control group (p=0.005). The HF+ADF group (1.90±0.54) had a significantly decreased muscle weight compared to HF group (2.15±0.51) (p=0.041). Similarly, ADF group (0.86±0.15) showed a significantly lower muscle weight than control group (1.70±0.32) (p=0.001). The HF+ADF group also exhibited significantly higher retroperitoneal fat weights than ADF group (p=0.005). These results suggest that fructose consumption leads to fat accumulation, and that ADF may partially modulate this accumulation (Figure 2B).

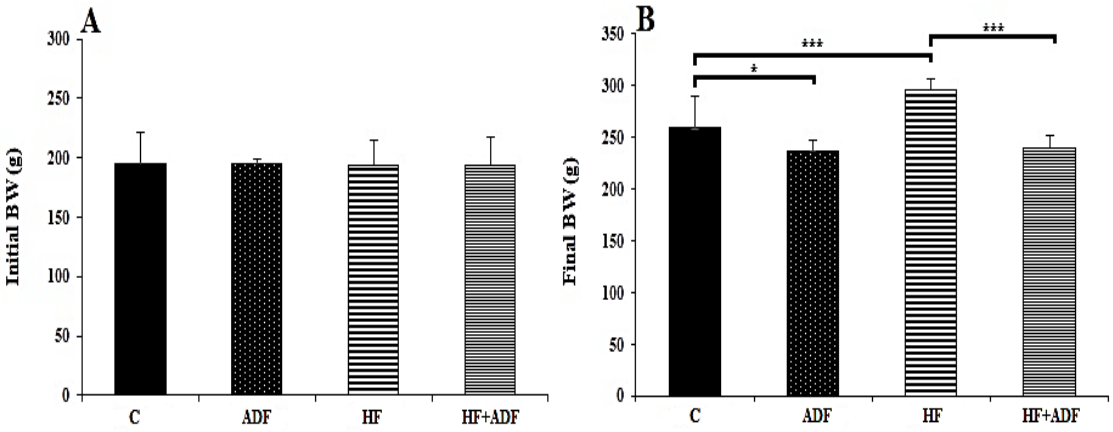

Figure 1. Initial and final BW comparisons between groups

(A) Initial BW (g) of the groups before the intervention, (B) Final BW (g) of the groups after 5 weeks of dietary and fasting interventions.Results are expressed as mean ±SD, with n=7 rats per group. Statistically significant differences are indicated as *p<0.05; ***p<0.001. The groups include C: control group, ADF: alternate-day fasting group, HF: high fructose group, and HF+ADF: high fructose and alternate-day fasting group. (BW: body weight)

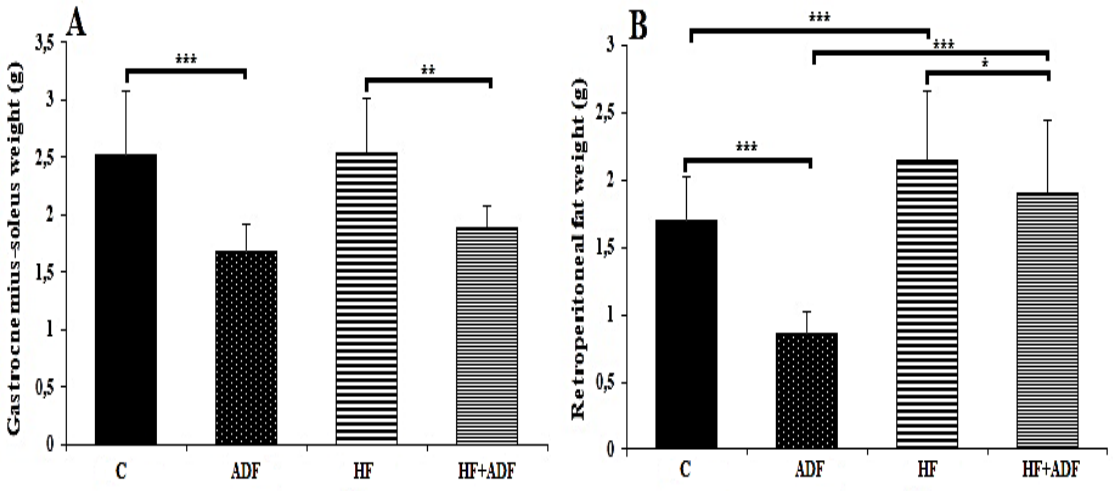

Figure 2. Comparisons of gastrocnemius-soleus weight and retroperitoneal fat weight between groups

(A) Gastrocnemius-soleus weight (g) comparisons and (B) Retroperitoneal fat weight (g) comparisons across the experimental groups. Results are expressed as mean ± SD, with n=7 rats per group. Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001. The groups include C: control group, ADF: alternate-day fasting group, HF: high fructose group, and HF+ADF: high fructose and alternate-day fasting group

ADF group (7.73±0.80) had significantly higher TAS levels than control group (6.53±0.37) (p=0.01) and in HF+ADF group (7.53±0.90) compared to HF group (5.72±0.33) (p=0.001, f=10.178) (Figure 3A). In contrast, TOS levels were significantly lower in ADF group (5.52±0.60) compared to control group (7.42±0.24) (p=0.022), and in HF+ADF group (6.55±0.56) compared to HF group (8.91±0.68) (p=0.001). HF group also had significantly higher TOS levels than control group (p=0.006, f=11.695) (Figure 3B). These findings indicate that while fructose intake shifts the oxidant balance towards pro-oxidant status, ADF counteracts this effect, shifting the balance in favor of antioxidants.

Serum TGF-β1, Smad2, and CTGF levels across the four groups are shown in Figures 4. HF group (3.17±0.42) exhibited borderline significantly higher serum TGF-β1 levels compared to control group (2.38±0.40) (p=0.066). ADF significantly reduced TGF-β1 levels in HF+ADF group (2.07±0.41) compared to HF group (p=0.008), and in ADF group (1.19±0.55) compared to control group (p=0.011, f=13.777) (Figure 4A).

Similarly, serum Smad2 levels were significantly higher in HF group (2.07±0.23) compared to control group (1.37±0.27) (p=0.003). ADF significantly reduced Smad2 levels in HF+ADF group (0.69±0.32) compared to HF group (p=0.001), and similarly, in ADF group (0.34±0.16) compared to control group (p=0.001, f=43.786) (Figure 4B).

Additionally, serum CTGF levels were significantly higher in HF group (204.71±19.67) compared to control group (121.53±34.87) (p=0.001). ADF significantly decrased CTGF levels in HF+ADF group (102.03±25.97) compared to HF group (p=0.001), and similarly, in ADF group (66.49±19.42) compared to control group (p=0.018, f=25.887) (Figure 4C).

These results indicate significant modulation of the TGF-β1/Smad pathway by both ADF and fructose consumption, suggesting a potential role in tissue remodeling and metabolic regulation.

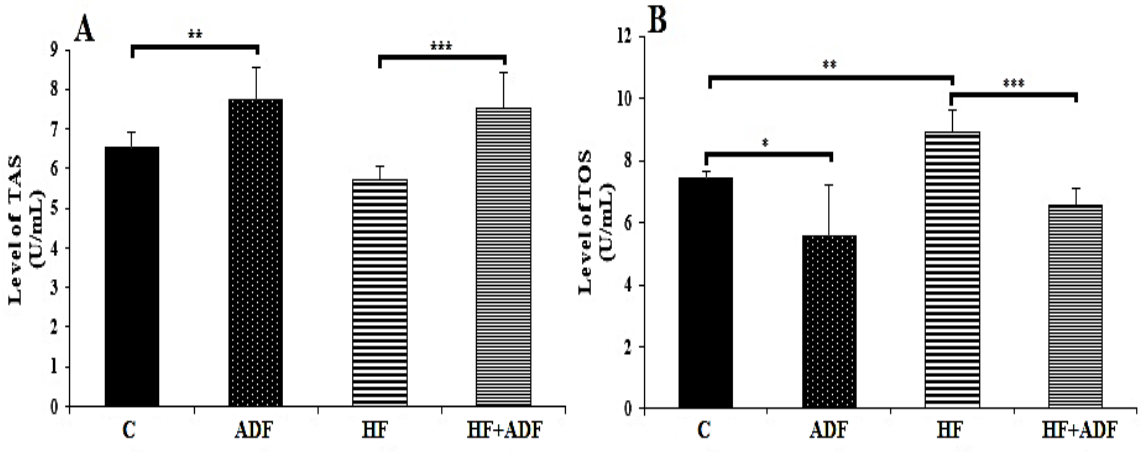

Figure 3. Serum levels of total antioxidant status (TAS) and total oxidant status (TOS) in experimental groups

(A)TAS levels, (B) TOS levels in the experimental groups. Results are expressed as mean ±SD, with n=7 rats per group. Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001. The groups include C: control group, ADF: alternate-day fasting group, HF: high fructose group, and HF+ADF: high fructose and alternate-day fasting group

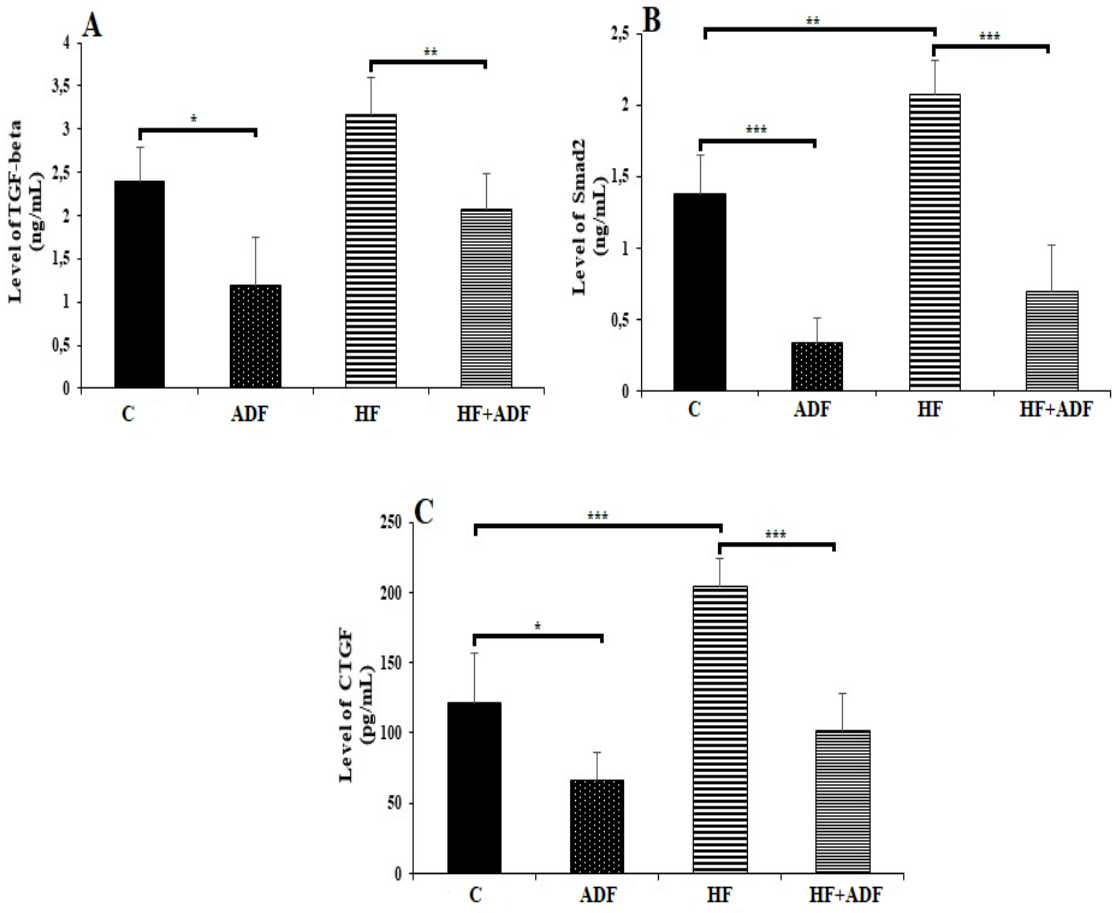

Figure 4. Serum levels of TGF-β1, Smad2, and CTGF in experimental groups.

(A) TGF-β1 levels, (B) Smad2 levels, and (C) CTGF levels in the experimental groups. Results are expressed as mean±SD, with n=7 rats per group. Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001. The groups include C: control group, ADF: alternate-day fasting group, HF: high fructose group, and HF+ADF: high fructose and alternate-day fasting group. (ERS: endoplasmic reticulum stress, TGF-β1: transforming growth factor-Beta, Smad2: mother against decapentaplegic homolog 2, CTGF: connective tissue growth factor)

Discussion

Our study revealed that HF intake and ADF had distinct effects on BW, muscle mass, adipose tissue, oxidative stress parameters, and the TGF-β1/Smad signaling pathway. Initial findings showed that HF intake significantly increased final BW compared to other groups, while ADF application lowered this weight gain. Additionally, HF intake led to increased retroperitoneal fat accumulation and decreased muscle mass, effects that were partially mitigated by ADF. The oxidative stress parameters, TAS and TOS, demonstrated a shift towards antioxidant balance with ADF, in contrast to the pro-oxidant imbalance observed with HF intake. Finally, the TGF-β1, Smad2, and CTGF levels were elevated in the HF group, while ADF reduced these fibrosis markers, particularly in the HF+ADF group.

Excessive fructose intake is linked to obesity and metabolic disturbances affecting multiple organs [1]. HF consumption through sweetened drinks and processed foods has become a public health concern, leading to increased morbidity and mortality [24]. Studies have demonstrated that prolonged intake of a 20% fructose solution significantly increases BW in animal models. For instance, Feyisa et al. [25] observed a marked weight gain after 6 weeks of fructose intake in rats, while Tanaka et al. [26] reported similar results with 8 weeks of 20% fructose consumption, showing statistically significant weight gain in both male and female rats. Additionally, Batista et al.

[27] found that rats consuming a 20% purified fructose solution over 8 weeks regulated their energy intake by reducing food consumption but compensating with the fructose solution, suggesting a direct role of fructose in promoting weight gain. Consistently, our study found that 5 weeks of 20% fructose intake led to weight gain. IF protocols, including ADF, are effective for weight loss and may offer health benefits [18, 23]. Fernandez et al. [19] reported that ADF reduced body mass gain in rats, and our findings similarly showed that ADF lowered BW in both healthy and HF rats.

Increased adipose tissue, particularly visceral fat, is closely linked to metabolic disorders. Prior studies indicate that ADF can effectively reduce visceral fat, likely by creating a moderate energy deficit that decreases overall adiposity [28, 29]. For instance, Catenacci et al. [29] observed that ADF significantly reduced visceral and truncal fat, highlighting its potential in managing adiposity-related metabolic risks. In our study, HF consumption led to an increase in retroperitoneal fat accumulation, underscoring the lipogenic effects of excessive fructose intake. However, when combined with ADF, both healthy and HF groups exhibited a notable reduction in retroperitoneal fat, suggesting that ADF may counteract fructose-induced adipose tissue expansion. These findings align with the literature, supporting ADF as an intervention to limit fat accumulation, particularly in the visceral fat.

ROS are partially reduced oxygen metabolites with strong oxidizing capabilities. At high concentrations, ROS are harmful to cells, causing oxidative damage; however, at lower concentrations, they play complex roles in cell signaling. Obesity is closely associated with elevated oxidative stress, a condition exacerbated by excess ROS [30]. Although HF intake has been shown to have detrimental metabolic effects in both humans and rodents, particularly through mechanisms such as hepatic de novo lipogenesis, lipotoxicity, oxidative stress, and hyperuricemia, it remains a significant factor in the growing incidence of metabolic disorders [31]. Chronic ROS production, in particular, plays a central role in the progression of inflammatory diseases. In response to oxidative stress, immune cells release cytokines and chemokines to recruit other immune cells, leading to further ROS generation and tissue damage at the inflammation site [32]. HF consumption caused oxidative stress, consistent with previous studies [30]. This increase in oxidative stress is consistent with literature linking excessive fructose intake to ROS accumulation and inflammation [30]. IF has shown promise in reducing oxidative stress, primarily through physiological adaptations triggered by food deprivation. Studies indicate that ROS levels in liver mitochondria increase after 36 and 72 hours of fasting in rats, resulting in lipid peroxidation and oxidative stress in the liver [33, 34]. Our previous study reported that ADF interventions over 1 to 2 months significantly reduced oxidative stress in both the liver and serum [35]. This suggests that different durations of ADF may influence oxidative stress responses: shorter fasting periods may trigger cellular resistance through low-intensity oxidative stress, while longer durations can enhance antioxidant defenses, balancing ROS production and reducing overall oxidative stress [36]. In our study, a 5-week ADF regimen increased TAS levels and decreased TOS levels in both healthy and HF rats, indicating a beneficial shift toward antioxidant balance. This finding supports the idea that ADF can mitigate oxidative stress, potentially protecting against metabolic disruptions associated with HF intake.

Fibrosis, characterized by excessive tissue growth and ECM accumulation, is largely mediated by the TGF-β/Smad signaling pathway [37, 38]. Upon activation, TGF-β phosphorylates Smad2 and Smad3, which form a complex with Smad4 to regulate fibrogenic gene expression. This process plays a central role in the development of fibrotic conditions, particularly in organs such as the liver and heart [39].

HF intake has been linked to fibrosis in both human and animal models, acting as a risk factor particularly in the liver. Studies have shown that HF consumption activates TGF-β1 and Smad3 expression, contributing to obesity, metabolic disorders, and fibrosis [40, 41]. In a previous study, HF feeding for 24 weeks induced a significant up-regulation of TGF-β1 and Smad3 mRNA levels in the renal tissue of rats [14]. Notably, Smad3 knockout models exhibit protection against HF-induced obesity and insulin resistance, highlighting Smad3's role in

fibrotic and metabolic processes [42]. Our study supports these findings, as 20% HF intake led to significant increases in TGF-β1, CTGF, and Smad3 levels, indicating the activation of the fibrotic pathway.

High concentrations of fructose have also been shown to increase TGF-β and α-SMA gene expression in hepatic stellate cells (HSCs), indicating that fructose can activate HSCs and promote liver fibrosis [13]. TGF-β1, a potent pro-fibrogenic cytokine, binds and activates its receptors, leading to the phosphorylation of Smad3, which then promotes the transcription of fibrogenic genes. Activated Smad3 is known to drive the deposition of ECM components, contributing to tissue fibrosis [43]. In line with these mechanisms, our findings revealed increased TGF-β1 and Smad3 levels in HF rats, reflecting fibrotic changes consistent with prolonged fructose exposure. Further, studies suggest that the JAK2/STAT3 pathway is also activated by HF intake and may interact with TGF-β1/Smad signaling in promoting fibrosis. Yang et al. [44] demonstrated that inhibition of JAK2 prevented the fructose-induced activation of TGF-β1/Smad signaling in liver cells, suggesting a synergistic effect between the JAK2/STAT3 and TGF-β/Smad pathways in HF-induced fibrosis. The potential interaction between these pathways warrants further investigation to better understand their roles in fructose-induced liver fibrosis. ROS production due to HF intake further influences TGF-β1 and CTGF expression, exacerbating fibrotic processes. Fructose has been shown to elevate TGF-β1 expression through ROS accumulation in cardiac cells, leading to myocardial fibrosis [45]. ROS-dependent CTGF overexpression has also been observed in myocardial fibrosis caused by hemodynamic stress, suggesting that CTGF could serve as a diagnostic marker for fructose-induced myocardial hypertrophy and fibrosis [46]. Nagayama et al. [6] found that fructose, unlike glucose, suppressed fibroblast growth and CTGF expression in vitro, which they attributed to decreased cellular viability. This finding was significant as it highlighted a unique effect of fructose on CTGF expression in fibroblasts. Other studies have observed elevated CTGF and TGF-β1 levels in myocardial tissue with chronic fructose feeding, linking these markers to increased fibrosis [47]. Additionally, Liu et al. [48] reported that high glucose levels promoted CTGF expression in vascular smooth muscle cells, implicating dietary sugars in the regulation of fibrosis-related markers at the cellular level. In our study, chronic consumption of 20% HF significantly increased serum levels of TGF-β1, CTGF, and Smad3, supporting the literature on HF's role in driving fibrotic changes through the TGF-β/Smad pathway. These findings underscore the need for further research into dietary sugars' impact on fibrosis and metabolic health, as well as potential interventions to mitigate these effects.

IF protocols, including ADF, have demonstrated favorable effects on fibrosis by reducing TGF-β1 levels and enhancing antioxidant defenses. For example, Han et al. [49] found that IF enhanced the TGF-β-producing capacity of M2 macrophages, potentially reducing inflammation related to obesity. Additionally, Raji Amirhasani et al. [50] showed that fasting regimens lowered TGF-β1 expression and improved kidney function, likely by increasing SIRT1 and reducing oxidative stress. In our study, ADF applied for 5 weeks significantly decreased TGF-β1, CTGF, and Smad3 levels in both healthy and HF rats, suggesting that ADF may effectively downregulate fibrotic signaling pathways and mitigate HF-induced fibrosis.

In conclusion, ADF effectively attenuates HF-induced activation of the TGF-β1/Smad2 signaling pathway, thereby alleviating associated metabolic disturbances. Our results demonstrate that ADF reduces weight gain, controls fat accumulation, and improves oxidative balance in rats subjected to a HF diet. The downregulation of key components of the TGF-beta/Smad pathway by ADF suggests a potential mechanism through which it promotes tissue remodeling and metabolic balance. These findings suggest the therapeutic potential of ADF in counteracting the harmful effects of HF consumption, particularly in preclinical models, supporting its role as a promising dietary approach for managing metabolic disorders. Future studies are needed to determine whether these effects can be replicated in humans and to explore the long-term implications of ADF in managing metabolic disorders.

Limitations of the study: Further research is needed to elucidate the molecular mechanisms underlying these effects and assess their long-term clinical implications.

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