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Weight Regain after Alternate Day Fasting with Adipose Tissue Metabolism Changes in the Diet-Induced Obesity of Mice Model

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ABSTRACT

Background: While calorie restriction (CR) is a major weight loss method, alternate day fasting (ADF) has emerged as a promising alternative. Although ADF provides various health benefits, its impact on post-diet weight change remains widely unknown. This study aimed to investigate the effect of ADF on post-diet weight change and evaluate the potential factors associated with the weight change.

Methods: The following three types of dietary interventions were considered: *Ad libitum* (Ad lib), CR, and ADF followed by refeeding, and determining changes in weight, metabolic parameters, and gene expression levels in the adipose tissue.

Results: The ADF group gained the maximum weight during the refeeding period. However, food intake, oxygen consumption, and locomotor activity were not significantly different between the CR and ADF groups during the refeeding period. Moreover, epididymal fat weight increased in the ADF group after refeeding. In the adipose tissues, the expression of genes related to *de novo* lipogenesis and triglyceride synthesis was significantly elevated in the ADF group after the dieting period.

Conclusion: Our results suggest that ADF could not prevent weight regain after weight loss, and increased fat synthesis may contribute to weight gain and fat accumulation after ADF. Further exploration of the post-diet weight effects would help develop sustained successful weight loss strategies.

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Introduction

The prevalence of obesity has almost doubled since 1980 in more than 70 countries (1). The World Obesity Atlas 2023, published by the World Obesity Federation, predicted an increase in the global obesity rate, rising from 14% in 2020 to 24% by 2035 (2). The difficulty of losing weight and maintaining the weight hinders the treatment of obesity (3, 4). A meta-analysis, integrating 29 long-term weight loss studies reported that the individuals regained more than 75% of their initial weight loss within 5 years (5). Weight regain after weight loss was shown to be associated with various factors, such as dietary glycemic index and load (6), and also changes in adipose tissue function (3, 4, 7).

Caloric restriction (CR) is a major treatment

approach for obesity. Recently, intermittent fasting (IF) has emerged as a promising alternative to CR. IF is a dietary method involving regular repeated periods of fasting and eating. Various IF methods have been developed, including a 5:2 diet, time-restricted feeding, intermittent energy restriction, and alternate day fasting (ADF) (8, 9). ADF is a dietary method that repeats fasting and feeding cycles every alternate day (8, 9). Clinical or preclinical studies have suggested several health benefits of ADF, including a prolonged life span (10), reduced weight and fat mass (8, 9), improved insulin resistance (11), controlled blood pressure (12), and improved cognitive function (13). Some of these benefits could be attributed to a distinct alteration of lipid metabolism in ADF compared to that in CR (14, 15).

ADF can improve overall health; however, a large randomized clinical trial reported that the dropout rate is higher in the ADF group than that in the control or CR groups (16). Since the follow-up of dropout participants is unavailable, health condition after the withdrawal of ADF is elusive. Moreover, although weight management after weight loss is important for the treatment of obesity (17), body weight changes after ADF are not entirely understood. One randomized study suggested that ADF is not related to the risk of weight regain (18). However, the underlying mechanisms are still unclear. Therefore, examining weight changes and metabolic effects after ADF is essential to achieve secure and sustained weight loss. In addition, although weight regain after weight loss is correlated to changes in adipose tissue function and lipid metabolism (3, 4, 7, 19), it is still unclear whether weight changes after ADF are associated with changes in lipid metabolism induced by ADF (14, 15).

Thus, the present study aimed to determine body weight changes after ADF and explore the associated mechanisms by analyzing metabolic and biochemical parameters, adipose tissue weight, and gene expression levels. To achieve this, the study compared weight changes after the following three different dietary interventions: (1) *Ad libitum* (Ad lib), (2) CR, and (3) ADF, using a diet-induced obesity (DIO) in mouse model.

Materials and Methods

The experimental design was illustrated in Figure 1A. The 5-week-old C57/BL6J male mice (Slc Inc., Japan) were purchased and acclimated to their cages (MF, ORIENTAL YEAST Co., Ltd., Japan) for 1 week. After acclimation, a high-fat high-sucrose (HFHS) diet (D17051803, Research Diets Inc., USA) was provided *ad libitum* for 7 weeks to create DIO mice (DIO development). The daily average food intake for the last 3 days during DIO development was defined as the baseline chow intake during the following dieting period. These DIO mice were divided into three weight-matched groups, and each group was given a different dietary intervention for 5 weeks (dieting period). The first group was the Ad lib group (n=5), which continued to consume the HFHS diet *ad libitum*.

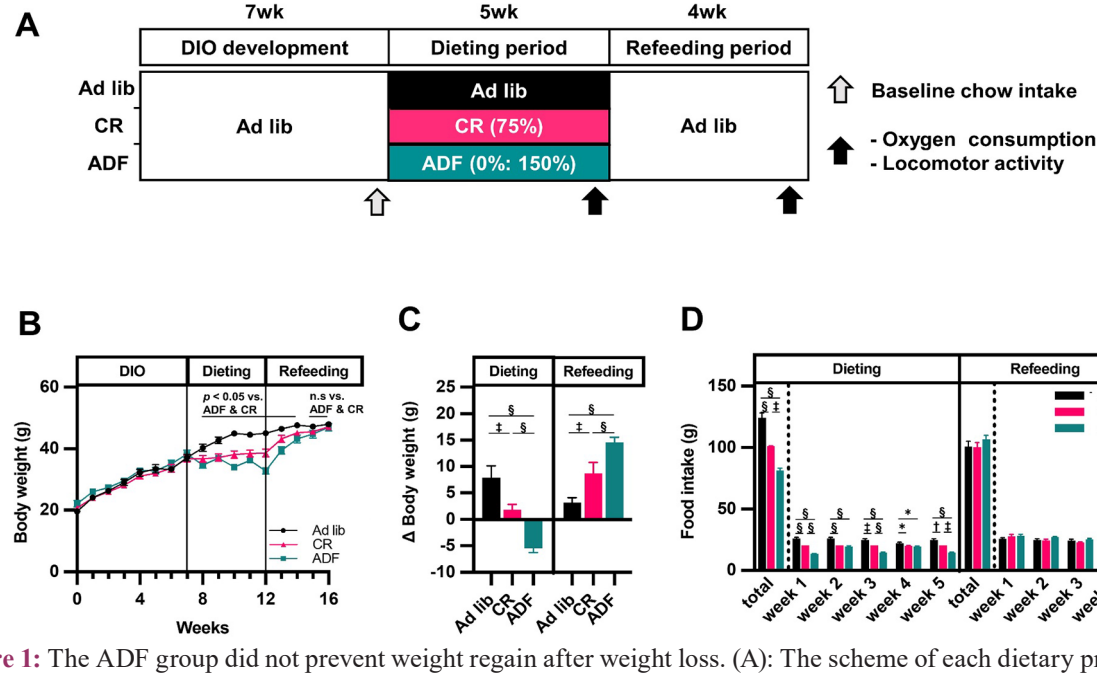

Figure 1: The ADF group did not prevent weight regain after weight loss. (A): The scheme of each dietary protocol and the time points at which baseline chow intake, oxygen consumption (VO_2), and locomotor activity were measured. (B and C): Body weight and (D): Food intake during the dieting and refeeding periods (Ad lib, n=5; CR, n=6; ADF, n=5). Circles represent the Ad lib group, triangles represent the CR group, and squares represent the ADF group values. Values represent the mean±standard error of the mean (SEM). *p<0.05, †p<0.01, ‡p<0.001, §p<0.0001, significant difference between groups. ADF: Alternate day fasting; CR: Calorie restriction; Ad lib: *Ad libitum* group.

The second group was the CR group (n=6), which received 75% of the baseline chow intake of the HFHS diet every day. The third group was the ADF group (n=5), which repeated a day without feeding (fast day) and a day with 150% of the HFHS diet (feast day) every alternate day. After the dieting period, each group was fed with the HFHS diet *ad libitum* for 7 weeks (refeeding period). After the refeeding period, cervical dislocation was performed 3 h before the onset of the dark period (at Zeitgeber time 9), and blood and tissue samples were collected. To investigate the potential mechanisms underlying the observed changes, we conducted the same experiments up to the dieting period (Ad lib, n=6; CR, n=6; ADF, n=6). After the dieting period, we performed cervical dislocation and collected blood and tissues on the last feast day, approximately 3 h before the dark period.

All the mice were housed individually in plastic cages with a 12-h light/dark cycle at 23±1°C. Body weights were measured more than once per week throughout the experiment. During the dieting period, the remaining chow was removed and the scaled chow was placed before the onset of the night cycle. From 14 weeks of age, calorie intake in all the groups was calculated from the daily weight of the remaining chow. Only male mice were used in this study, while metabolic responses to an HFHS diet were less pronounced in female mice than those in male mice (20). All experiments complied with the ARRIVE guidelines and the Declaration of Helsinki for animals, and they were approved by the Tokushima University Animal Study Committee (T2019-82).

Immediately after decapitation, trunk blood was collected in a tube and centrifuged (4°C, 9000 ×g, 15 min), for separating plasma. Plasma glucose, insulin, non-esterified fatty acids (NEFA), and corticosterone levels were measured. Plasma glucose levels were measured using the Glucose CII-Test Kit (Wako, Japan), free fatty acid levels using the NEFA C-Test Kit (Wako, Japan), insulin levels using the Mouse Insulin ELISA KIT(U-type) (AKRIN-031, Shibayagi, Gunma, Japan), and corticosterone levels using corticosterone EIA (COSMO BIO COMPANY, LIMITED, Japan). One sample from the Ad lib group after the refeeding period could not be analyzed because we could not obtain the plasma. Some samples yielded no data due to a very high sample concentration was excluded from the final statistical analysis.

Immediately after blood collection, epididymal and inguinal adipose tissues were snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from these tissues using the RNA isolation protocol (RNAisoPlus; Takara Bio, Japan). cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA, USA). Reverse transcription-polymerase chain reaction was performed using the StepOne™ Real-Time PCR System (Applied Biosystems™, USA) with FastStart Universal SYBR Green Master (Roche Applied Science, Mannheim, Germany), following the manufacturer's guidelines. In the sampled epididymal and inguinal fat following dieting and refeeding, we assessed the mRNA expression levels of genes associated with *de novo* lipogenesis (DNL: Acc1, Fas), triglyceride (TG) synthesis (Gpat3, Dgat2), and lipolysis (Atgl, Hsl, Mgl) (21). All gene expression data were normalized to β-actin expression in each tissue. Samples yielding no data values due to low mRNA concentrations were excluded from the final statistical analysis. The primer sequences for acetyl-CoA carboxylase 1 (*Acc1*), fatty acid synthase (*Fas*), glycerol-3-phosphate acyltransferase 3 (*Gpat3*), diacylglycerol acyltransferase-2 (*Dgat2*), hormone-sensitive lipase (*Hsl*), adipose triglyceride lipase (*Atgl*), monoacylglycerol lipase (*Mgl*), and β-actin were presented in Table 1.

Oxygen consumption (VO_2) and locomotor activity measurements began approximately 1 week before the dieting and refeeding periods ended. After approximately 48 h of acclimation in individual cages in LP-80LED-6ARS (NK system, Japan), VO_2 and locomotor activity were measured using a respiratory gas analyzer (ARCO-2000 mass spectrometer; ARCO system; Chiba, Japan) and animal movement analysis system (ACTIMO System; Shintechno, Fukuoka, Japan). During the measurement, the mice were allowed access to water and the HFHS diet. VO_2 and locomotor activity during the dieting period were the data from two consecutive days, including fast and feast days. The average VO_2 of the light and dark periods represented the average fast and feast day values. The total locomotor activity of the light and dark periods represented the total values of cumulative locomotor activity during the fast and feast days. During refeeding, the second day after the beginning of measurements was considered for the final statistical analysis. Data from 1 h before the dark period was excluded since food intake was measured 1 h before the dark period.

The results were expressed as mean±standard error of the mean (SEM). Data from the three groups were analyzed using one-way analysis of variance followed by the Bonferroni test. p values <0.05 were considered statistically significant. All statistical tests were performed using GraphPad Prism (Version 9 for Windows, GraphPad Software, La Jolla, California, USA).

Table 1: List of the primer sequences used for real-time PCR

Gene name	Gene sequence (5'-3')
Acetyl-CoA carboxylases 1 (*Acc1*)	Forward: AGCAGTTACACCACATACAT
	Reverse: TACCTCAATCTCAGCATAGC
Fatty acid synthase (*Fas*)	Forward: GCTGCTGTTGGAAGTCAGC
	Reverse: AGTGTTCGTTCCTCGGAGTG
Glycerol-3-phosphate acyltransferases 3 (*Gpat3*)	Forward: ACACTGGTTGGCCAGCTT
	Reverse: GCAGCAGGTCAGATGCAC
Diacylglycerol acyltransferase 2 (*Dgat2*)	Forward: CAGCAAGAAGTTTCCTGGCAT
	Reverse: CCTCCCACCACGATGATGAT
Hormone-sensitive lipase (*Hsl*)	Forward: CGAGACAGGCCTCAGTGTGA
	Reverse: GAATCGGCCACCGGTAAAG
Adipose triglyceride lipase (*Atgl*)	Forward: CCTCAGGACAGCTCCACCAA
	Reverse: TTGAACTGGATGCTGGTGTTG
Monoacylglycerol lipase (*Mgl*)	Forward: CCCAGTGGCACACCCAAG
	Reverse: TAACGGCCACAGTGTTCCC
β-actin	Forward: CTAAGGCCAACCGTGAAAAG
	Reverse: ACCAGAGGCATACAGGGACA

Results

During the dieting and refeeding periods, the three groups showed different weight changes, and the ADF group gained the most weight during the refeeding period (Figure 2B and 2C). Although differences in food intake were statistically significant among the three groups during the dieting period, no significant differences were observed during the refeeding period (Figure 2D). Food intake was the lowest in the ADF group since animals in the CR group consumed 99.6% of the food provided, calculated from the baseline chow intake, whereas those in the ADF group consumed only 78.0% (data not shown). Furthermore, after the dieting and refeeding phases, the gene expression levels of several peptides involved in regulating food consumption were relatively similar across all experimental groups (22), except for Agrp after the dieting period (Supplementary Figure 1 and 2).

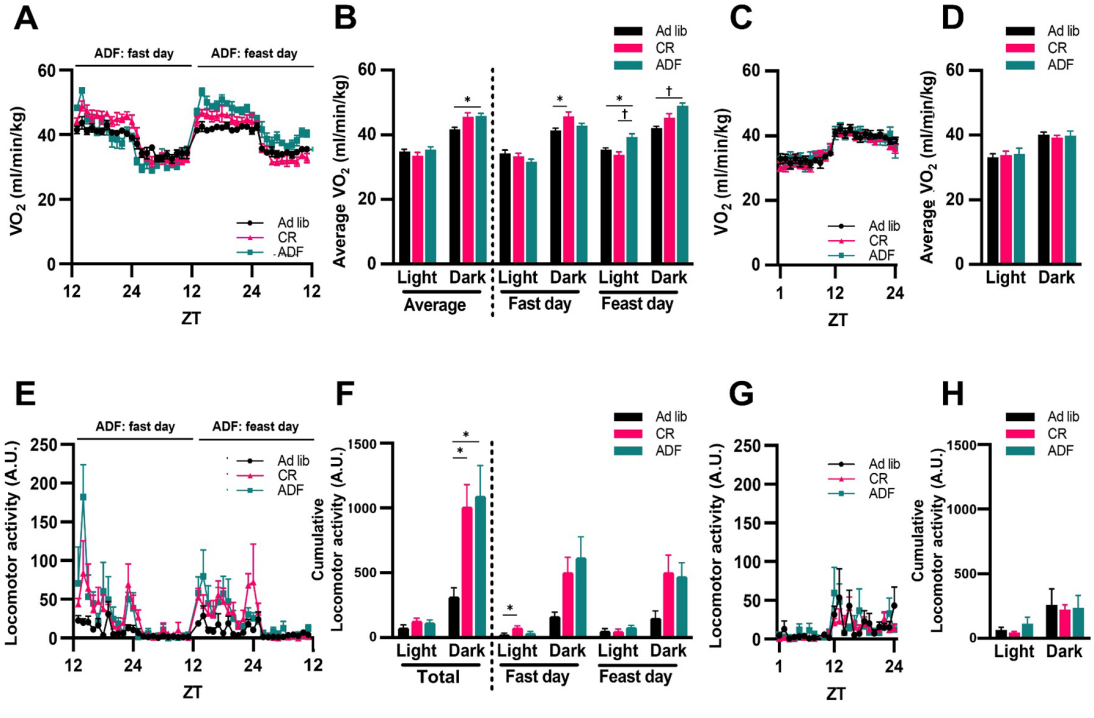

Figure 2: Oxygen consumption and locomotor activity during the dieting and refeeding periods. (A and B): Oxygen consumption (VO_2) and average VO_2 during both fasting and feeding days in the dieting period, and (C and D): During the refeeding period. (E and F): Locomotor activity and cumulative locomotor activity during both fasting and feeding days in the dieting period, and (F and G): During the refeeding period (Ad lib, n=5; CR, n=6; ADF, n=5). Circles represent the Ad lib group, triangles represent the CR group, and squares represent the ADF group values. Values represent the mean±SEM. *p<0.05, †p<0.01, ‡p<0.001, §p<0.0001, indicating significant differences between groups. ADF: Alternate day fasting; CR: Calorie restriction; Ad lib: *Ad libitum* group.

During a feast day within the dieting phase, the ADF group exhibited a notably higher average VO_2 level during the dark period than the Ad lib group (Figure 2A and 2B). Conversely, there were no significant differences in VO_2 among all experimental groups during the refeeding period (Figure 2C and 2D). Moreover, during the dieting phase, the cumulative locomotor activity was significantly greater in the CR and ADF groups than in the Ad lib group (Figure 2E and 2F). However, during the refeeding period, no significant differences were observed in locomotor activity among these three groups (Figure 2G and 2H).

Blood analyses conducted after the dieting period revealed increased blood glucose and insulin levels in the Ad lib group, while NEFA levels decreased in the ADF group (Figure 3A-3C). Notably, corticosterone levels did not differ significantly among the three groups (Figure 3D). In epididymal fat, the expression levels of Acc1, Fas, Gpat3, and Dgat2 were observed to increase in the ADF group, while the expression levels of Atgl, Hsl, and Mgl demonstrated a decrease in the Ad lib group (Figure 3E). Moreover, in the inguinal fat, elevated expression levels of Acc1 and Fas were noticed in the ADF group compared to the other groups (Figure 3E).

Blood tests after the refeeding period showed no significant differences in circulating nutrients and hormones among the three groups (Figure 4A-4D). However, epididymal fat weight was significantly higher in the ADF group than that in the Ad lib group (Figure 4E). It was demonstrated that Atgl and Hsl expression levels decreased in inguinal fat of the ADF group (Figure 4E).

Discussion

Weight gain after weight loss is a major hindrance in treating obesity (3-5). However, weight changes after ADF and their potential mechanisms are not entirely understood. The present study explored weight changes and various metabolic parameters after three dietary interventions in DIO mice. Briefly, the ADF group gained significantly more weight than the other groups during the refeeding period. However, various factors associated with weight regain, such as food intake, oxygen consumption, and locomotor activity, did not demonstrate significant differences compared to the other groups. Furthermore, the expression levels of genes related to DNL and TG synthesis were significantly elevated in the ADF group after the dieting period.

The ADF group showed maximum weight loss during the dieting period, likely due to the lower energy intake by mice in the ADF group than that by mice in the other groups. This experiment revealed that, compared to the Ad lib and CR groups, the ADF group was more likely to gain weight

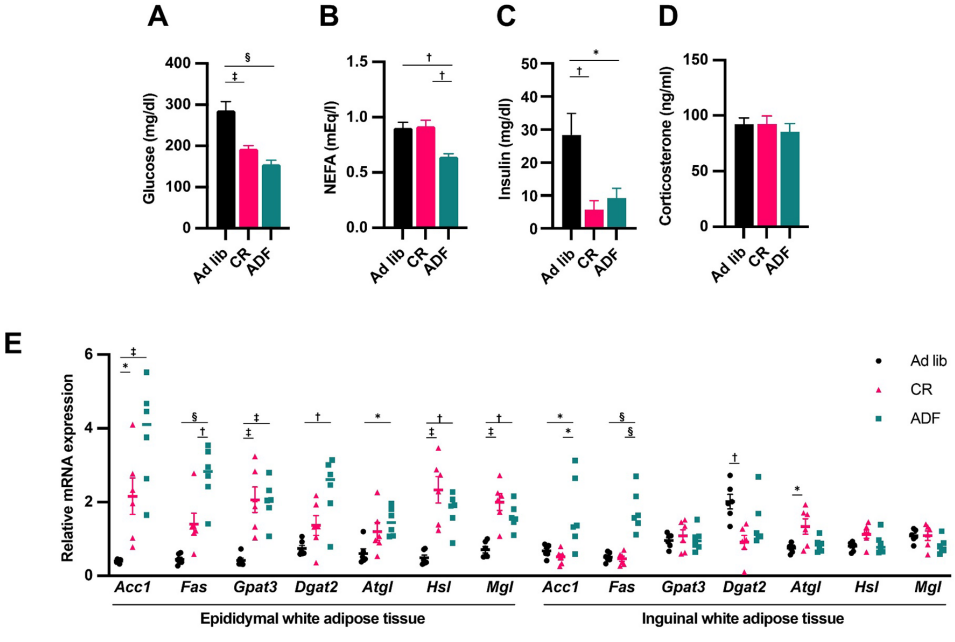

Figure 3: Lipid metabolism and gene expression in the adipose tissues changed significantly in the ADF group compared to the CR group after the dieting period. Plasma concentrations of **(A):** Glucose, **(B):** Free fatty acids, **(C):** Insulin, and **(D):** Corticosterone (Ad lib, n=5-6; CR, n=6; ADF, n=6). White, gray, and black bars represent the Ad lib, CR, and ADF groups. **(E):** mRNA expression levels in epididymal white adipose tissue and inguinal white adipose tissue collected after the refeeding period (Ad lib, n=6; CR, n=6; ADF, n=6). Circles represent the Ad lib group, triangles represent the CR group, and squares represent the ADF group values. Values represent the mean±SEM. *p<0.05, †p<0.01, ‡p<0.001, §p<0.0001, indicating significant differences between groups. ADF: Alternate day fasting; CR: Calorie restriction; Ad lib: *Ad libitum* group.

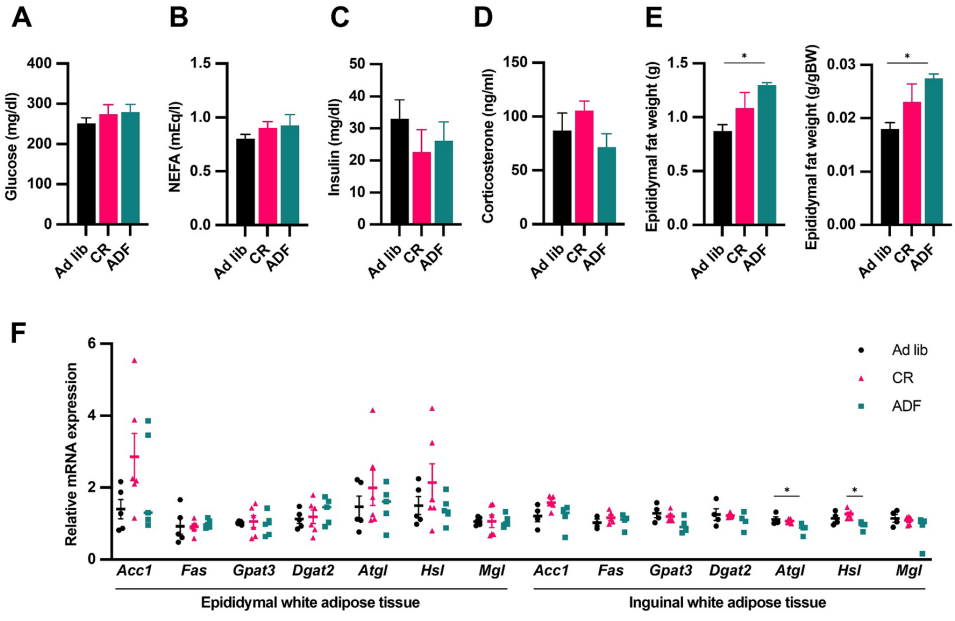

Figure 4: Epididymal fat accumulated in the ADF group after the refeeding periods. Plasma concentrations of (A): Glucose, (B): Free fatty acids, (C): Insulin, (D): Corticosterone (Ad lib, n=4; CR, n=6; ADF, n=5). (E): Epididymal white adipose tissue weight after refeeding (Ad lib, n=5; CR, n=6; ADF, n=5). White, gray, and black bars represent the values of the Ad lib, CR, and ADF groups, respectively. (F): mRNA expression levels in epididymal white adipose tissue and inguinal white adipose tissue collected after the refeeding period (Ad lib, n=3-5; CR, n=5-6; ADF, n=4-5). Circles represent the Ad lib group, triangles represent the CR group, and squares represent the ADF group values. Values represent the mean±SEM. *p<0.05, †p<0.01, ‡p<0.001, §p<0.0001, indicating significant differences between groups. ADF: Alternate day fasting; CR: Calorie restriction; Ad lib: *Ad libitum* group.

by refeeding. Several factors are assumed to be involved in regaining weight after weight loss, such as increased appetite, decreased energy expenditure, and decreased activity (4, 19). Intriguingly, between the CR and ADF groups, there were no statistically significant differences in food intake, average VO_2, and locomotor activity during the refeeding period, although the mice in the ADF group gained more weight than the ones in the CR group. Also, the gene expressions of orexigenic and anorexigenic peptides in the hypothalamus were found to be comparable in all experimental groups following both the dieting and refeeding phases, which is different from a previous study using a high-fat diet (23). Based on these findings, nutrient digestion and absorption may be altered following ADF.

Indeed, a prior study demonstrated that ADF could alter gastrointestinal motility and morphology, influencing overall digestive functions (24). Another study suggested that increased fat absorption after dieting contributed to the subsequent increase in fat mass, despite no significant changes in energy expenditure (25). However, it is important to note that this study did not use DIO mice (25). Thus, further research is warranted to explore the impact of ADF on nutrient absorption in DIO mice. Also, additional factors may be involved in the underlying mechanism of significant weight gain in the ADF group.

In adipocytes, DNL-derived fatty acids are used for TG synthesis, and the synthesized TGs are stored in fat droplets (21). The expression of *Acc1* and *Fas* in epididymal and inguinal fat after the dieting period suggests enhanced DNL in the ADF group compared with that in the other groups. This finding is in line with a previous study that suggested that DNL was augmented in the ADF group relative to the Ad lib group (26). Furthermore, the expression levels of genes related to *Gpat3* and *Dgat2* suggest that TG synthesis was enhanced in the epididymal fat of the ADF group compared to that in the other groups after the dieting period. The contribution of DNL to newly synthesized TG is known to be higher in the ADF group than in the control group (27). Therefore, in the ADF group of the present study, not only was DNL augmented, but high percentages of DNL-derived fatty acids can also be used for TG synthesis.

Moreover, the lowered NEFA concentration in the ADF group after the dieting period suggests that a distinct alteration in lipid metabolism occurred in the ADF group compared with that in the CR group. The lowered NEFA concentration in the ADF group might relate to the maximum weight regain in the ADF group based on a previous randomized controlled trial that reported a positive correlation between the decrease in plasma free fatty acid concentration during the intervention and subsequent weight gain (28). Since there were no remarkable differences in the blood test results and

the expression levels of genes after the refeeding period, the differences in adipose tissue and lipid metabolism after the diet can be transient. In the ADF group, the transient increase in DNL and TG synthesis after the dieting period could contribute to the enlargement of lipid droplets in adipose tissues and the increase in body and epididymal fat weight during the refeeding period.

Insulin may be involved in the potential mechanism underlying the observed changes in adipose tissue and lipid metabolism in the ADF group. Insulin stimulates DNL and TG synthesis, and decreases the rate of lipolysis (29). A previous study using female rats reported augmented insulin secretion in the ADF group in isolated pancreatic islets and in oral glucose tolerance test (30), supporting that increased insulin secretion in response to refeeding promotes DNL and TG synthesis in the ADF group. The reason for the lack of statistically significant differences in the blood insulin levels between the CR and ADF groups after the dieting period could be that the insulin levels were measured using blood collected 3 h before the dark period when food intake can be reduced.

To the best of our knowledge, only one study has investigated weight regain after ADF using a DIO mouse model (31). Unlike the study, we used a high-fat high-sucrose (HFHS) diet to investigate the effects after dieting. We reasoned that an HFHS diet is suitable for studying post-dieting effects based on previous research demonstrating that individuals with obesity tend to prefer foods containing both high fat and sugar (32, 33). Furthermore, HFHS diets have distinct impacts on metabolic health compared to high-fat diets (34, 35). In fact, compared to the previous study (31), the ADF group in our present study appeared to regain body weight more rapidly. This difference can be attributed to different diet types (high-fat diet vs. HFHS diet). This possibility should be further investigated in future studies.

The present study had some limitations that warrant discussion. This study did not determine whether the differences in adipose tissue and lipid metabolism observed between the CR and ADF groups after the dieting period were due to differences in the amount of energy deficit during the dieting period, dietary methods, or both. Interestingly, poor compensation for the energy deficit in ADF has been suggested in a systematic review including only human trials (36). Lower dietary energy intake during ADF compared to CR has also been observed in previous randomized studies (18), implying that low energy intake in the ADF group is not unrealistic in the clinical setting. Furthermore, even with similar weight loss rates, postprandial TG metabolism can be different between the CR and IF groups (37), suggesting that, in addition to the difference in energy deficit, the difference in dieting methods can also be related to changes in lipid metabolism after the dieting period. This study also did not examine the relationship between sex and weight change. Female mice are less likely to develop weight gain induced by an HFHS diet than male mice (20). Also, one study reported sex-specific effects of time-restricted feeding, a form of intermittent fasting, using an HFHS diet (38). Hence, the findings in female mice may vary from those observed in the current study. Finally, prior research has demonstrated distinct physiological and biochemical states between the fed and fasted states in the ADF mouse model (39, 40), suggesting that gene expressions in adipose tissues may differ between fasting and feasting periods.

Conclusion

This study showed that ADF did not prevent weight gain after weight loss, and changes in adipose tissue and lipid metabolism may be a potential mechanism for this weight gain. Thus, while ADF certainly offers various health benefits, it is imperative to investigate the post-dietary effects of ADF in future clinical and preclinical studies to achieve sustained weight loss and successful interventions for obesity.

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Authors' Contribution

S.Y. and T.S. designed the study, the main conceptual ideas, the proof outline, and collected the data. T.S., S.C., and H.S. aided in interpreting the results and worked on the manuscript. T.S. and H.S. supervised the project. S.Y. wrote the manuscript with support from T.S., S.C., and H.S. All authors discussed the results and commented on the manuscript.

Conflicts of Interest

None declared.

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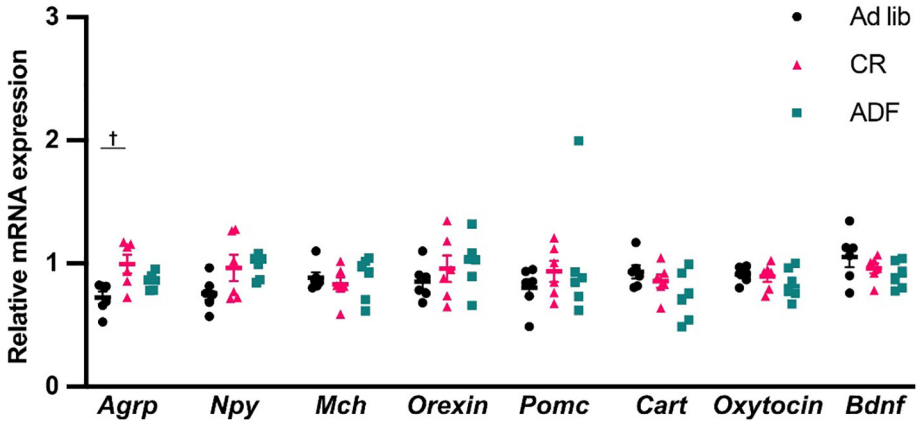
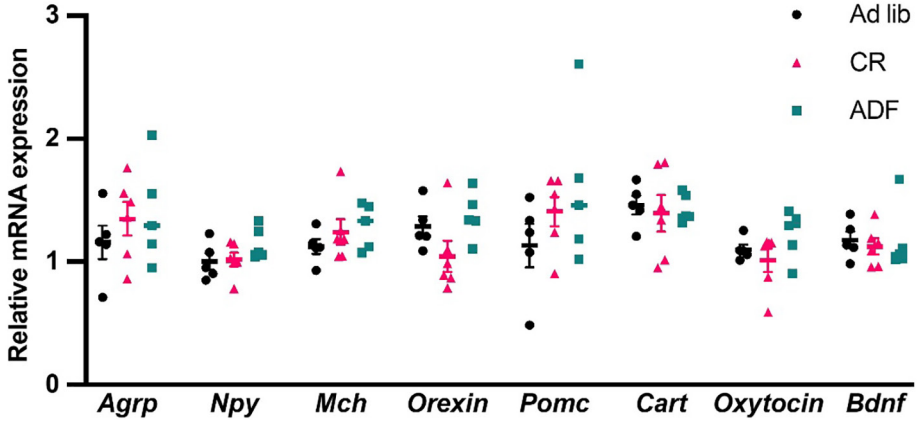


Supplementary Figure 1: Gene expressions of orexigenic and anorectic peptides in the hypothalamus after the dieting period mRNA expression levels in the hypothalamus collected after the dieting period (Ad lib, n=6; CR, n=6; ADF, n=6). Circles represent the Ad lib group, triangles represent the CR group, and squares represent the ADF group values. Values represent the mean±SEM. *p<0.05, †p<0.01, ‡p<0.001, §p<0.0001, indicating significant differences between groups. ADF: Alternate day fasting; CR: Calorie restriction; Ad lib: *Ad libitum* group.



Supplementary Figure 2: Gene expressions of orexigenic and anorectic peptides in the hypothalamus after the refeeding period mRNA expression levels in the hypothalamus collected after the refeeding period (Ad lib, n=5; CR, n=6; ADF, n=5). Circles represent the Ad lib group, triangles represent the CR group, and squares represent the ADF group values. Values represent the mean±SEM. *p<0.05, †p<0.01, ‡p<0.001, §p<0.0001, indicating significant differences between groups. ADF: Alternate day fasting; CR: Calorie restriction; Ad lib: *Ad libitum* group.