# Effects of modified alternate-day fasting regimens on adipocyte size, triglyceride metabolism, and plasma adiponectin levels in mice

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**Abstract** **Calorie restriction (CR) affects adipocyte function and reduces body weight. However, the effects of alternate-day fasting (ADF) on adipose biology remain unclear. This study examined the effects of ADF and modified ADF regimens on adipocyte size, triglyceride (TG) metabolism, and adiponectin levels in relation to changes in body weight and adipose mass. Twenty-four male C57BL/6J mice were randomized for 4 weeks among *1*) ADF-25% (25% CR on fast day, ad libitum on alternate day), *2*) ADF-50% (50% CR on fast day), *3*) ADF-100% (100% CR on fast day), and *4*) control (ad libitum). The body weight of ADF-100% mice was lower than that of the other groups ($P < 0.005$) after treatment. Adipose tissue weights did not change. Inguinal and epididymal fat cells were 35–50% smaller ($P < 0.01$) than those of controls in ADF-50% and ADF-100% animals after treatment. Net lipolysis was augmented ($P < 0.05$) in ADF-100% mice, and the contribution from glyceroneogenesis to α-glycerol phosphate increased in ADF-50% and ADF-100% mice, whereas fractional and absolute de novo lipogenesis also increased in ADF-50% and ADF-100% animals, consistent with an alternating feast-fast milieu. Plasma adiponectin levels were not affected. In summary, modified ADF (ADF-50%) and complete ADF (ADF-100%) regimens modulate adipocyte function, despite there being no change in body weight or adipose tissue weight in the former group.**—Varady, K. A., D. J. Roohk, Y. C. Loe, B. K. McEvoy-Hein, and M. K. Hellerstein. **Effects of modified alternate-day fasting regimens on adipocyte size, triglyceride metabolism, and plasma adiponectin levels in mice.** ***J. Lipid Res.*** **2007.** 48: **2212–2219.**

**Supplementary key words** calorie restriction • adipose tissue • fat cell size

Adipose tissue is an active player in whole body energy homeostasis, responding dynamically to changes in nutrient availability in several ways. For instance, the turnover of adipose tissue triglyceride (TG) is responsive to changes in macronutrient intake. Fasting increases lipolysis, whereas overfeeding or insulin reduces lipolysis (TG breakdown) (1). The pathways leading to intra-adipocyte TG synthesis also respond to nutrients and other signals. Synthesis of α-glycerol phosphate in the fat cell, the immediate biosynthetic precursor for TG synthesis, occurs through glycolysis (from blood glucose) or glyceroneogenesis (from pyruvate) (2). A high-fat, low-carbohydrate diet increases glyceroneogenesis as a source of α-glycerol phosphate for TG synthesis (2). De novo lipogenesis (DNL) also responds to dietary energy or carbohydrate intake (3). Additionally, adipose tissue cell size has been proposed to modulate the risk of obesity-related disorders (4). For instance, strong associations have been noted between large adipocytes and increased circulating levels of insulin, TG, total cholesterol, and apolipoprotein B (5). Complementary to these findings, type 2 diabetics and dyslipidemic subjects have larger subcutaneous adipocytes than controls (6). Adipocytes also produce multiple bioactive peptides, termed adipokines, which affect diverse metabolic processes throughout the body (7). One particular adipokine, adiponectin, has received considerable attention in recent years as a result of its insulin-sensitizing and antiatherogenic effects (7).

Interventions that reduce daily energy intake, also known as calorie restriction (CR), have a number of important effects on mammalian physiology, including life span extension and delay of a variety of degenerative conditions (8). CR has also been shown to beneficially modulate certain aspects of adipocyte physiology. Recently, it was reported by Larson-Meyer et al. (9) that a 25% reduction in daily energy intake (25% CR) produced significant decreases in fat cell size after 24 weeks in humans. Augmented lipolytic rates within subcutaneous fat have also been demonstrated after 4 weeks of 40% CR in obese women (10). Fatty acid synthesis is also augmented after short-term CR in rats (11). With regard to adipokines, circulating adiponectin levels are reported to increase in response to CR (12, 13).

Another form of dietary restriction that may be more feasible in practice than daily CR is alternate-day fasting (ADF). ADF regimens consist of ad libitum feeding for a

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24 h period (feed day), alternated with a 24 h fast (fast day). Although studied less frequently than traditional CR, ADF reproduces many of the beneficial effects of daily CR (14), including reductions in global cell proliferation (15). ADF is a potentially attractive strategy for implementing CR in humans, particularly if the ADF regimens can be modified (i.e., through varying degrees of energy restriction on the fast day alternated with ad libitum feeding on the feed day) while still retaining their beneficial effects.

The effects of ADF or modified ADF regimens on adipose tissue TG metabolism, adipocyte size, and circulating adiponectin levels in vivo have yet to be investigated. Accordingly, the objective of this study was to investigate the effects of ADF and modified ADF regimens on adipocyte metabolism, adipocyte size, and adiponectin levels in mice. The underlying question was whether a regimen could be identified that altered adipose tissue biology without causing loss of body weight or fat.

## MATERIALS AND METHODS

### Mice and diets

Seven week old C57BL/6J male mice (20.8 ± 0.6 g; Charles River Breeding Laboratories, Wilmington, MA) were used. Animals were housed individually and maintained under temperature- and light-controlled conditions (12 h light/dark cycle: lights on at 7:00 AM and off at 7:00 PM). Mice were acclimatized for 1 week and allowed free access to water and a semipurified AIN-93M diet (Bio-Serv, Frenchtown, NJ). During this period, the daily amount of food consumed by each mouse was recorded.

After acclimation, mice were randomized to one of four intervention groups (n = 6 per group). The first intervention group (ADF-25%) was fed the semipurified AIN-93M diet ad libitum on the feed day and a 25% calorie-restricted diet on each alternate day (fast day). The second group (ADF-50%) was fed the AIN-93M diet ad libitum on the feed day and a 50% calorie-restricted diet on the fast day. The third group (ADF-100%) was fed the AIN-93M diet ad libitum on the feed day and underwent a complete fast on each alternate day. The fourth group acted as the control and was fed the AIN-93M diet ad libitum each day. The amount of food given to the mice in the ADF-50% and ADF-25% groups on the fast day was calculated based on the mean daily food intake of each mouse during acclimation. Each intervention lasted 4 weeks, and food was given or taken away at 1:00 PM each day. Body weight was measured at the same time of day at the beginning of each week. The amount of food consumed by each mouse was weighed daily.

### Blood collection and heavy water labeling protocol

Blood was collected the morning after a feast day at baseline (day 1) and after treatment (day 28). Animals were fasted for 8 h before blood collection. The heavy water ($^2H_2O$) labeling protocol has been described previously (16). In brief, mice were administered $^2H_2O$ starting at the beginning of week 3 (day 14) and throughout the last two weeks of the study (days 14–28). An initial priming dose of 99.9% $^2H_2O$ (0.18 ml/10 g body weight) was given via intraperitoneal injection to bring the $^2H_2O$ content of body water up to ~5%. Animals then received drinking water containing 8% $^2H_2O$ ad libitum for the remainder of the study. Mice were euthanized at 12 weeks of age by cardiac puncture under isoflurane anesthesia, followed by cervical dislocation. All procedures and protocols received approval from the University of California Berkeley Animal Use Committee.

### Isolation of TG-glycerol and FA from adipose tissue

Inguinal and epididymal fat pads were dissected immediately after euthanasia and placed in glass tubes containing 1 ml of methanol-chloroform (2:1). Chloroform and water (2 ml each) were then used to extract the solution. After disposing of the aqueous fraction, the lipid phase was transesterified by incubation with 3 N methanolic HCl (Sigma-Aldrich, St. Louis, MO) for 60 min at 55°C. The Folch technique was used to separate glycerol from fatty acid methyl esters, with the modification that pure water, rather than 5% NaCl, was used for the aqueous phase. As described elsewhere (16), the aqueous phase containing glycerol was lyophilized by incubation with acetic anhydride-pyridine (2:1), thereby converting glycerol to glycerol triacetate.

### Measurement of $^2H_2O$ enrichments in body water

Plasma $^2H_2O$ enrichments in body water were measured as described previously (16). Briefly, 50–70 μl of plasma was reacted with calcium carbide to produce acetylene in an evacuated GC vial. A syringe was used to remove the acetylene gas, and the gas was then injected into a GC vial containing 10% bromine in carbon tetrachloride. The reaction was then left to incubate at room temperature for 2 h to produce tetrabromoethane. Excess bromine was neutralized with 25 μl of 10% cyclohexene, and the sample was suspended in ethyl acetate.

### GC-MS analyses of TG-glycerol, FA, and $^2H_2O$

A model 6890 gas chromatograph with a model 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA) fitted with a DB-225 fused silica column (J&W, Folsom, CA) were used for all analyses. Glycerol triacetate was analyzed under chemical ionization conditions by selected ion monitoring of *m/z* 159–161 (representing $M_0$–$M_2$). Fatty acid methyl esters were analyzed as described elsewhere (17), with selected ion monitoring of *m/z* 256–258, representing $M_0$–$M_2$ of palmitate methyl ester. Body $^2H_2O$ enrichments were analyzed as tetrabromoethane by monitoring *m/z* 265 and 266, representing $M_0$ and $M_1$ masses of the $^{79}Br^{79}Br^{81}Br$ (parent minus Br) isotopomer (16).

### Calculation of TG-glycerol synthesis and turnover (lipolysis)

The principle behind measuring all-source TG synthesis, based on the incorporation of $^2H_2O$ into the C-H bonds of TG-glycerol, has been described (16). Briefly, during glycolytic and gluconeogenic reactions, C-H bonds of α-glycerol phosphate exchange with cellular water (16). Therefore, TG molecules that are synthesized from α-glycerol phosphate during the period of $^2H_2O$ administration will be exposed to $^2H$ labeling, whereas the TG molecules that already existed will remain unlabeled in the glycerol moiety. Fractional TG-glycerol synthesis was measured as described (16):

$$f_{TG} = EM1_{TG\text{-}glycerol}/A_1\infty_{TG\text{-}glycerol}$$

where f is the fraction of newly synthesized TG molecules present, EM1 is the measured excess mass isotopomer abundance for $M_1$-glycerol at time t, and $A_1\infty$ is the asymptotic mass isotopomer abundance for $M_1$-glycerol, assuming that four of five C-H bonds of α-glycerol phosphate exchanged with $^2H_2O$ in body water (16). The absolute retained synthesis rate of adipose TG was then calculated from fractional TG synthesis multiplied by adipose TG mass (16). The rate of TG turnover (net lipolysis) was also calculated,

based on the absolute TG synthesis rate combined with the change in pool size (16):

$$\text{absolute synthesis (g/kg/day)} = (f_{TG}) \times [\text{adipose TG mass (g)/time (day)}]$$

$$\text{net lipolysis (g/kg/day)} = (f_{TG}) \times [\text{adipose TG mass (g)/time (day)}] - [\text{change in TG mass (g)/time (day)}]$$

### Calculation of glyceroneogenesis

The contribution from glyceroneogenesis to adipose tissue α-glycerol phosphate was measured as described previously (2). Briefly, the number of C-H bonds in α-glycerol phosphate in adipose tissue that derive from body water (n) is a function of the relative contributions from glycolysis (n = 3.5) versus glyceroneogenesis (n = 5). Thus, the value of n calculated in adipose TG-glycerol reveals the relative input from the two pathways (2). We have shown previously (2) that adipose glyceroneogenesis calculated in this manner responds to dietary and pharmacologic manipulations. The relative contribution of glyceroneogenesis to α-glycerol phosphate was calculated as follows (2):

$$\text{glyceroneogenesis (\%)} = [(n - 3.5)/1.5] \times 100$$

### Calculation of DNL contribution to newly synthesized TG

A combinatorial model was used to measure newly synthesized FA that is formed during $^2H_2O$ labeling, as described previously (18). Mass isotopomer distribution analysis (MIDA) is used to determine the number (n) of hydrogen atoms in C-H bonds of FA that are derived from cellular water during the de novo synthesis of FA. The value of n is calculated as described previously (18) by MIDA, using body $^2H_2O$ to represent the precursor pool enrichment (p). Fractional and absolute contributions from DNL are then calculated:

$$f_{DNL} = EM1_{FA}/A_1\infty_{FA}$$

$$\text{absolute DNL (g/day)} = [f_{DNL} \times \text{adipose TG mass (g)}]/\text{time (day)}$$

where $A_1\infty_{FA}$ is calculated from MIDA look-up tables, based on the calculated values of n and p in FA (16). The value for $f_{DNL}$ represents the fraction of total TG-palmitate in the depot that derived from DNL during the labeling period. The fraction of newly synthesized TG that came from DNL is also calculated, by correcting the measured DNL fraction present for the degree of replacement of adipose TG during the labeling period (16):

$$\text{DNL contribution to newly synthesized TG} = (f_{DNL}/f_{TG})$$

### Fat cell size

Fat cell size was estimated for inguinal and epididymal fat tissue. Briefly, 100–150 mg of adipose tissue was fixed in 4% osmium tetroxide in distilled water for 48 h. Adipose tissue was then rinsed with water for 1 h followed by an additional rinse with formalin, alcohol, and xylene. The tissue was then embedded in paraffin and sliced. Cell size was determined using an AxioImager M1 epifluorescence microscope (Zeiss) and Image J software (version 1.37 for Mac OS X; National Institutes of Health, Bethesda, MD). In each preparation, 400 cells from various parts of the tissue slice were sized for calculation of mean cell size.

### Plasma adiponectin and FFA levels

Plasma adiponectin concentrations were quantified using high-sensitivity ELISA kits (Linco Research, St. Charles, MO). The intra-assay precision of the adiponectin ELISA kit was 1.3%. Circulating FFAs were measured using a colorimetric assay kit (Roche Applied Science, Indianapolis, IN).

### Statistical analysis

Values are expressed as means ± SEM. One-way ANOVA was used to test for differences between group means. When a significant difference was noted between groups, a Tukey's post-hoc test was performed to determine the differences between group means. When baseline differences were observed for a specific parameter, analysis of covariance was performed with the baseline value as a covariate. Differences within groups from the beginning to the end of the study were measured using a repeated-measures ANOVA. Correlational analyses were performed to assess the relationship among body weight, food intake, lipid kinetic parameters, fat cell size, and adiponectin levels. A test for normality was included in the model. $P = 0.05$ was used as a criterion for statistical significance in all analyses. Data were analyzed by SPSS software (version 11 for Mac OS X; SPSS, Inc., Chicago, IL).

## RESULTS

### Body weight and food intake

Body weight and food intake throughout the 4 week study are displayed in **Table 1**. No effects of treatment on body weight were noted during the first 3 weeks of the study. By the beginning of week 4, however, the ADF-100% group weighed less ($P < 0.05$) than the ADF-50% group, and by the last day of the study, the ADF-100% group weighed less ($P < 0.05$) than both the ADF-50% and ADF-25% groups. During weeks 1 and 2 of the study, food intake of ADF-100% animals was lower ($P < 0.05$) than that of the other treatment groups. During week 3, all groups had similar food intakes, but by week 4, food consumption of the ADF-100% group was once again lower ($P < 0.05$) than that of the ADF-50% and ADF-25% groups. These results indicate that the ADF-25% and ADF-50% regimens allowed essentially full compensation of food intake on the feed day for reductions on the fast day, whereas the ADF-100% group was unable to compensate fully on the feed day for the reduced intake on the fast day.

### Inguinal and epididymal adipose tissue weight

Inguinal adipose weight was not significantly different between the ADF-25% (0.50 ± 0.07 g), ADF-50% (0.50 ± 0.06 g), ADF-100% (0.40 ± 0.06 g), and control (0.36 ± 0.04 g) groups. Similarly, epididymal adipose weight was not significantly different between the ADF-25% (0.70 ± 0.10 g), ADF-50% (0.68 ± 0.07 g), ADF-100% (0.53 ± 0.07 g), and control (0.49 ± 0.11 g) groups.

### In vivo adipose tissue TG synthesis and turnover

In vivo adipose tissue TG synthesis and breakdown rates were measured based on the incorporation of $^2H$ into the

TABLE 1. Body weight and food intake throughout the 4 week study

| Parameter | Control | ADF-25% | ADF-50% | ADF-100% |
|---|---|---|---|---|
| Body weight (g)[a] | | | | |
| Week 1 | 22.3 ± 0.2 a | 22.4 ± 0.3 a,b | 22.8 ± 0.1 a,b | 23.3 ± 0.3 b |
| Week 2 | 23.5 ± 0.4 | 23.7 ± 0.4 | 23.9 ± 0.3 | 23.8 ± 0.3 |
| Week 3 | 23.8 ± 0.5 | 24.1 ± 0.3 | 23.7 ± 0.6 | 22.9 ± 0.4 |
| Week 4 | 25.7 ± 0.5 a,b | 25.6 ± 0.5 a,b | 25.8 ± 0.5 a | 23.5 ± 0.6 b |
| After treatment | 24.8 ± 0.7 a,b | 26.2 ± 0.5 a | 26.5 ± 0.6 a | 22.6 ± 0.6 b |
| Food intake (g/mouse/day)[b] | | | | |
| Week 1 | 3.62 ± 0.15 a,b | 3.55 ± 0.07 a | 3.49 ± 0.07 a | 3.12 ± 0.05 b |
| Week 2 | 3.04 ± 0.11 a | 3.23 ± 0.05 a | 3.16 ± 0.04 a | 2.66 ± 0.07 b |
| Week 3 | 3.74 ± 0.11 | 3.53 ± 0.08 | 3.69 ± 0.10 | 3.55 ± 0.12 |
| Week 4 | 3.05 ± 0.13 a,b | 3.53 ± 0.07 a | 3.28 ± 0.11 a | 2.57 ± 0.18 b |

ADF, alternate-day fasting. Values are expressed as means ± SEM (n = 6 mice per intervention group). Means not sharing a common letter are significantly different ($P < 0.05$) based on one-way ANOVA with Tukey's post-hoc test.

[a] Body weight was taken on the first day of each week.

[b] Food intake was calculated at the end of each week.

glycerol moiety of TG (16). Fractional TG replacement ($f_{TG}$), absolute retained TG synthesis, and net lipolysis in inguinal and epididymal fat are shown in **Table 2**. If adipose TG mass is constant, $f_{TG}$ represents the cycle of lipolysis and TG resynthesis. In the inguinal fat pad, $f_{TG}$ was 47% higher ($P < 0.05$) in the ADF-100% group relative to the control group. No change in $f_{TG}$ was noted in the epididymal fat pad. When TG synthesis was expressed in absolute terms, by taking into account adipose pool size in each depot, no differences were noted between groups in either the inguinal or epididymal fat depot. Net lipolysis was calculated from the absolute synthesis rate of adipose TG combined with the loss or gain of adipose TG mass during the study. Net lipolysis in inguinal fat was 76% higher ($P < 0.05$) in the ADF-100% group and 69% higher ($P < 0.05$) in the ADF-50% group relative to the control group. Thus, changes in adipose tissue TG turnover were induced by modified ADF regimens (ADF-50%), even in the absence of net loss of body fat. No differences in net lipolysis were noted in the epididymal fat pad in any treatment group, suggesting that this effect is site-specific.

### Contribution of glyceroneogenesis to adipose tissue α-glycerol phosphate

The relative contribution from glyceroneogenesis to α-glycerol phosphate in epididymal fat was augmented ($P < 0.0001$) in the ADF-50% (78.4 ± 4.1%) and ADF-100% (94.7 ± 3.8%) groups compared with the control group (69.9 ± 5.2%) (Table 2). Similarly, in inguinal fat, the contribution of glyceroneogenesis to α-glycerol phosphate was increased ($P < 0.001$) in the ADF-50% (76.1 ± 3.0%) and ADF-100% (87.6 ± 6.8%) groups relative to the control group (58.2 ± 6.2%).

### Contribution from DNL to adipose tissue TG

In epididymal fat, fractional DNL ($f_{DNL}$), absolute DNL, and the fractional contribution from DNL to newly synthesized TG were higher ($P < 0.05$) in the ADF-100% group ($f_{DNL}$, 0.86 ± 0.11; absolute DNL, 0.03 ± 0.002 g/day) com-

TABLE 2. In vivo TG kinetic parameters in epididymal and inguinal fat depots after 4 weeks of ADF

| | Epididymal Fat Depot | | | | Inguinal Fat Depot | | | |
|---|---|---|---|---|---|---|---|---|
| Parameter | Control | ADF-25% | ADF-50% | ADF-100% | Control | ADF-25% | ADF-50% | ADF-100% |
| Fractional TG synthesis (f) | 0.64 ± 0.04 | 0.51 ± 0.03 | 0.50 ± 0.03 | 0.68 ± 0.08 | 0.59 ± 0.06a | 0.65 ± 0.07a | 0.73 ± 0.03a | 0.86 ± 0.1 b |
| Absolute TG synthesis (g/day) | 0.58 ± 0.19 | 0.67 ± 0.11 | 0.65 ± 0.08 | 0.56 ± 0.06 | 0.39 ± 0.09 | 0.58 ± 0.07 | 0.68 ± 0.07 | 0.56 ± 0.09 |
| Net lipolysis (g/day) | 0.47 ± 0.14 | 0.49 ± 0.09 | 0.47 ± 0.07 | 0.56 ± 0.04 | 0.32 ± 0.06 a | 0.43 ± 0.07 a | 0.54 ± 0.04 b | 0.56 ± 0.08 b |
| Glyceroneogenesis (%) | 69.9 ± 5.2 a | 58.2 ± 5.3 a | 78.4 ± 4.1 b | 94.7 ± 3.8 b | 58.2 ± 6.2 a | 57.4 ± 3.8 a | 76.1 ± 3.0 b | 87.6 ± 6.8 b |
| Fractional DNL (f) | 0.43 ± 0.03 a | 0.44 ± 0.04 a | 0.48 ± 0.04 a | 0.86 ± 0.11 b | 0.50 ± 0.06 a | 0.57 ± 0.05 a | 0.74 ± 0.04 b | 1.00 ± 0.07 b |
| Absolute DNL (g/day) | 0.01 ± 0.004 a | 0.02 ± 0.003 a | 0.02 ± 0.003 a | 0.03 ± 0.002 b | 0.01 ± 0.002 a | 0.02 ± 0.002 a | 0.03 ± 0.003 b | 0.03 ± 0.003 b |
| DNL contribution to new TG (f) | 0.68 ± 0.06 a | 0.88 ± 0.09 a,b | 0.97 ± 0.07 a,b | 1.29 ± 0.15 b | 0.87 ± 0.11 | 0.91 ± 0.05 | 1.01 ± 0.06 | 1.23 ± 0.15 |

DNL, de novo lipogenesis; TG, triglyceride. Values are expressed as means ± SEM (n = 6 mice per intervention group). Means not sharing a common letter are significantly different ($P < 0.05$) based on one-way ANOVA with Tukey's post-hoc test within one fat depot.

pared with the control group ($f_{DNL}$, 0.43 ± 0.03; absolute DNL, 0.01 ± 0.004 g/day) (Table 2). In the inguinal fat depot, higher values of fractional and absolute DNL were observed in both the ADF-50% (0.74 ± 0.04; 0.03 ± 0.003 g/day, respectively; $P < 0.05$ for both) and ADF-100% (1.0 ± 0.07; 0.03 ± 0.003 g/day; both $P < 0.05$) groups compared with the control group (0.50 ± 0.06; 0.01 ± 0.002 g/day). No change in the fractional contribution from DNL to newly synthesized TG was noted in the inguinal fat depot. The observation of higher DNL rates during ADF regimens, even with reduced body fat stores and higher net lipolytic rates, is consistent with a fast-feast model of alternating metabolic fluxes (i.e., new fat synthesis alternating with fat oxidation on sequential days).

### Fat cell size

Fat cell size in inguinal and epididymal fat is displayed in **Fig. 1**. After 4 weeks of treatment, inguinal fat cells were 47% smaller ($P < 0.0001$) in the ADF-50% group and 56% smaller ($P < 0.0001$) in the ADF-100% group relative to the control group (**Fig. 2**). Epididymal fat cell size was also smaller ($P < 0.01$) in the ADF-50% group (34% smaller than controls) and in the ADF-100% group (36% smaller than controls). The sizes of inguinal and epididymal cells were correlated ($r = 0.61$, $P = 0.002$). The reduction in adipocyte size in ADF-50% animals occurred in the absence of any reduction in body fat or weight. Fat cell size in either fat pad was not related to changes in any lipid kinetic parameter measured.

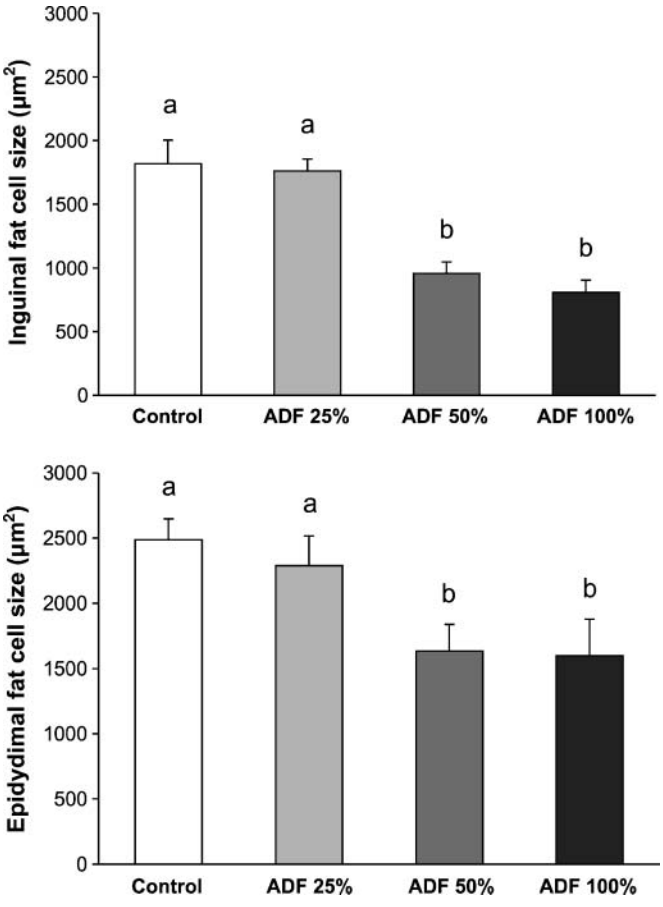


**Fig. 1.** Fat cell size in inguinal and epididymal fat after 4 weeks of alternate-day fasting (ADF). Values are expressed as means ± SEM. Inguinal fat cells were significantly smaller ($P < 0.0001$) in the ADF-50% and ADF-100% groups after treatment compared with the control and ADF-25% groups. Epididymal fat cells were also smaller ($P < 0.01$) in the ADF-50% and ADF-100% groups after 4 weeks of ADF (one-way ANOVA with Tukey's post-hoc test). Means not sharing a common letter are significantly different ($P < 0.05$) based on one-way ANOVA with Tukey's post-hoc test.

### Plasma adiponectin levels

At baseline, adiponectin levels of the ADF-50% group were lower ($P < 0.05$) than those of the other groups, but by the end of the study, no differences were noted (**Fig. 3**). No correlations between adiponectin and lipid kinetic rates or fat cell size were observed. Higher adiponectin levels were associated ($r = -0.40$, $P = 0.05$) with lower body weights.

### Plasma FFA concentrations

Circulating FFA concentrations were not different among groups at baseline (**Fig. 4**). By the end of the study, however, an increase ($P < 0.05$) in FFA levels was demonstrated in the ADF-25% group relative to the control group. Over the course of the study, FFA concentrations were increased in each of the intervention groups compared with baseline values ($P < 0.005$).

## DISCUSSION

The central purpose of this study was to determine whether adipose tissue actively responds to modified ADF regimens, particularly in the absence of changes in body weight or fat depot weight. Adipocyte size in inguinal and epididymal fat depots was markedly smaller after 4 weeks in both the ADF-100% and ADF-50% groups relative to the control group. Increases in adipose tissue TG metabolism, including higher rates of lipolysis, increased DNL rates, augmented glyceroneogenesis, and increased plasma FFA concentrations after a prior fast, were also observed in both the ADF-50% and ADF-100% regimens. Thus, ADF regimens clearly alter adipose tissue physiology and structure, even in the absence or calorie deficiency or changes in adipose mass.

Accumulating evidence suggests that fat cell enlargement may be linked to a greater risk of chronic disease (19). More specifically, large adipocytes have been shown to differ from small adipocytes for several physiologic processes, including lower capacity to store TG in settings of excess energy intake, resulting in ectopic fat storage in tissues and impairment of insulin action; less suppressible lipolysis by insulin; higher production of certain bioactive molecules, such as angiotensinogen and reactive oxygen species; and augmentation of macrophage infiltration, which may enhance proinflammatory cytokine production (19). In the few studies that have examined the effect of daily CR regimens on fat cell size, reductions

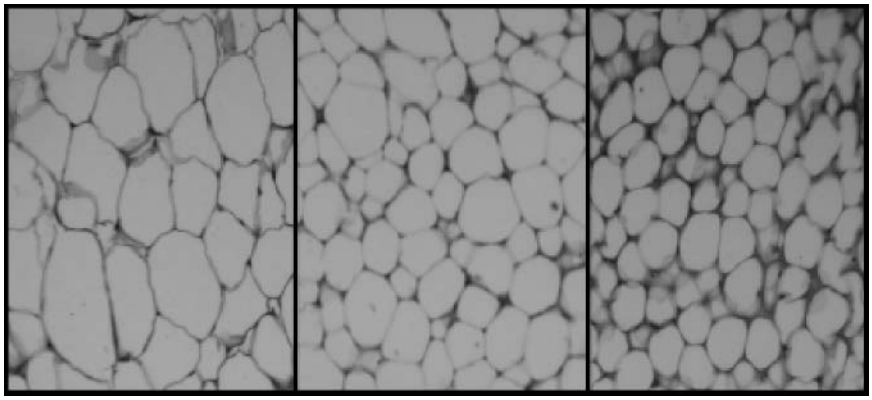

**Fig. 2.** Inguinal fat cell size in the control (left), ADF-50% (center), and ADF-100% (right) groups after 4 weeks of ADF. Inguinal fat cells were 47% smaller ($P < 0.0001$) in the ADF-50% group and 56% smaller ($P < 0.0001$) in the ADF-100% group relative to the control group.

in size have generally been reported. Recently, Larson-Meyer et al. (9) observed in humans a 27% reduction in adipocyte size after 24 weeks of 25% CR. In rodent models, two studies have demonstrated ~50% decreases in epididymal fat cell size as a result of CR (20, 21), but another study reported no effect (22). In this study, the effects of modified ADF regimens on adipocyte size in inguinal (subcutaneous) fat and epididymal (visceral) fat were substantial. After 4 weeks of treatment, adipocyte size in the ADF-100% group was reduced by ~55% in inguinal fat and by ~35% in epididymal fat compared with the control group. Similar marked reductions were demonstrated in the ADF-50% group in inguinal (~50% lower than controls) and epididymal (~35% lower than controls) depots. Accordingly, the modified ADF regimen, which allowed for the consumption of 50% of energy requirements on the fast day (ADF-50%), worked as well true ADF (ADF-100%) in decreasing fat cell size. These percentage reductions in fat cell size are comparable to those seen in the study by Tzur et al. (20), who also reported an increase in epididymal adipocyte insulin receptors and improved insulin sensitivity. Our finding of smaller fat cells suggests that ADF regimens, including modified regimens, may play a protective role against obesity-related disorders. Moreover, because visceral fat may particularly contribute to insulin resistance and the development of type 2 diabetes, compared with the effects of subcutaneous fat (4), the ability of ADF to decrease visceral fat cell size may also be beneficial.

Although fat cell size in the ADF-50% and ADF-100% groups was reduced, interestingly, no significant differences were noted for adipose depot weight between groups. This finding suggests that adipocyte number, and hence, adipogenesis, was increased in the ADF-50% and ADF-100% groups. Because augmented adipocyte differentiation has been linked to improved insulin sensitivity (23),

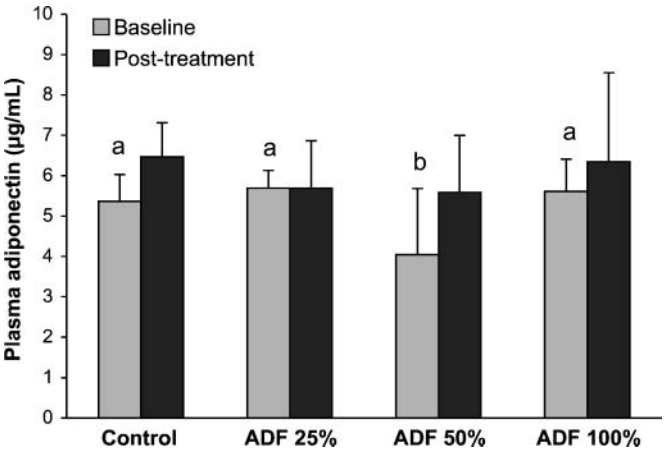


**Fig. 3.** Plasma adiponectin levels at baseline and after treatment in each intervention group. Values are expressed as means ± SEM. Adiponectin values at baseline were significantly different ($P < 0.05$) in the ADF-50% group compared with the other three groups (one-way ANOVA). No differences among groups were noted for posttreatment adiponectin levels (analysis of covariance with baseline as a covariate). Means not sharing a common letter are significantly different ($P < 0.05$) based on one-way ANOVA with Tukey's post-hoc test.

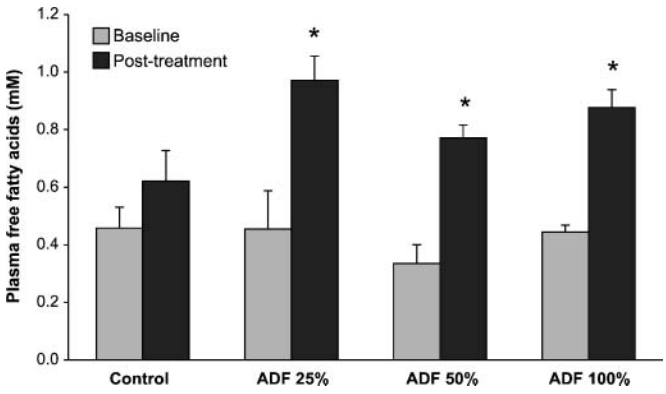


**Fig. 4.** Plasma FFA levels at baseline and after treatment in each intervention group. Values are expressed as means ± SEM. Measurements were taken after an 8 h fast after the feed day in the ADF groups. * Increase in FFA levels was significantly greater ($P < 0.005$) in the ADF-25%, ADF-50%, and ADF-100% groups from baseline to the end of the study compared with the control group (repeated-measures ANOVA).

an increase in fat cell number in these ADF groups may have beneficial effects for future diabetes risk. In the ADF-100% group, body weight was decreased significantly, yet adipose depot weights were not changed, implying that another organ must have accounted for this decrease in overall body mass in the ADF-100% group. Although not measured, it is likely that this tissue was skeletal muscle. During the complete 24 h fast every other day for a 4 week period, reduction in activity level or intermittent energy insufficiency may have impaired muscle anabolism. The effects of ADF regimens on muscle metabolism will require investigation in future studies.

Also examined was the effect of modified ADF regimens on various TG kinetic parameters. Enhanced TG turnover (net lipolysis) in inguinal fat was demonstrated in the ADF-50% and ADF-100% groups. No effect of ADF regimens on lipolysis was noted in the epididymal fat pad, suggesting that this effect may be more specific for subcutaneous adipose tissue than for visceral depots. Also observed were increases in fractional and absolute DNL in the ADF-50% (inguinal only) and ADF-100% (inguinal and epididymal) groups relative to the control group. The contribution from DNL to newly synthesized TG was also significantly higher in the ADF-100% group (epididymal only) compared with the control group. These results are similar to those of a published CR study that reported increases in DNL in both mesenteric and subcutaneous fat after 4 weeks of treatment in rats (11). The relative contribution of glyceroneogenesis to newly synthesized α-glycerol phosphate was also augmented in both the ADF-50% and ADF-100% groups. Adipose tissue glyceroneogenesis tends to be upregulated (2) in settings of reduced carbohydrate availability (e.g., low-carbohydrate diets) or active adipose TG deposition (e.g., glitazone treatment). These findings suggest that ADF regimens may enhance certain aspects of adipocyte lipid metabolism in both anabolic and catabolic directions (i.e., TG lipolysis, FA synthesis, and glyceroneogenesis), consistent with alternation between a feast and a fast metabolic milieu.

When posttreatment plasma FFA concentrations were compared with baseline concentrations, an increase was noted in each of the ADF intervention groups. This increase in fasting FFA concentrations is consistent with the observation of increased adipose TG lipolysis. Both parameters reflect enhanced lipid mobilization within the adipocyte, thus permitting lipid stores to be used as fuel during periods of fasting. The finding that ADF-50% increased both of these indices of lipolysis in the absence of net weight loss or reduction in body fat stores indicates that the cyclicity of caloric availability can alter adipose tissue lipid dynamics, even without a net catabolic state for body fat.

Also of interest was the effect of modified ADF regimens on circulating adiponectin levels. This hormone, which is synthesized only by adipose tissue, is reduced in obese, dyslipidemic, and diabetic individuals compared with healthy controls (24, 25). In view of these associations, adiponectin has been proposed to provide a link between obesity and certain obesity-related disorders, such as atherosclerosis and type 2 diabetes (7). Because previous findings indicate that ADF improves insulin sensitivity (26, 27) and vascular function (27, 28), the effects of ADF on adiponectin levels were of interest. No increases were noted after 4 weeks of treatment, however, and no relationship between adiponectin and fat cell size or TG metabolism was demonstrated in any intervention group. Our data do indicate that higher levels of adiponectin were associated with lower body weights, a result that is supported by previous findings (29). Because it appears that substantial reductions in body weight are generally required for increases in adiponectin to occur (13, 30), it is likely that adiponectin did not change because ADF had little impact on body weight.

In summary, these results indicate that true ADF (ADF-100%) and modified ADF (ADF-50%) regimens have a number of effects on adipocyte physiology, even in the absence of changes in adipose depot weight or, in the case of ADF-50%, without changes in body weight. Fat cell size was reduced in various depots, lipolysis was stimulated, DNL was increased, and glyceroneogenesis was augmented. The implications of these changes in adipose biology on disease risk, however, are not yet clear. Reduction in fat cell size has been linked directly to improved insulin sensitivity, whereas increased release of FFA and the absence of increases in adiponectin levels may represent less favorable effects. Future studies linking ADF-induced changes in adipocyte physiology to disease risk will be of interest.

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