Dose effects of modified alternate-day fasting regimens on in vivo cell proliferation and plasma insulin-like growth factor-1 in mice

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Varady KA, Roohk DJ, Hellerstein MK. Dose effects of modified alternate-day fasting regimens on in vivo cell proliferation and plasma insulin-like growth factor-1 in mice. J Appl Physiol 103: 547–551, 2007. First published May 10, 2007; doi:10.1152/japplphysiol.00209.2007.—Reduced cancer risk by decreasing cell proliferation. The effect of modified regimens of ADF on cell proliferation, however, has not been examined. This study measured the effects of modified ADF regimens on prostate and splenic T-cell proliferation and circulating insulin-like growth factor-1 (IGF-1) levels in mice. In a 4-wk study, 24 male C57BL/6J mice were randomized to one of four interventions: 1) ADF-25% [25% calorie restriction (CR) on fast day], 2) ADF-50% (50% CR on fast day), 3) ADF-100% (100% CR on fast day), and 4) control. Body weight of the ADF-100% group was less (P < 0.005) than that of the ADF-25% and ADF-50% groups posttreatment. On the feast day, the ADF-100% and ADF-50% groups ate 85% and 45% more food, respectively, than controls, indicating a hyperphagic response to fasting. Proliferation rates of T-cells were 6% and 30% lower (P < 0.05) in the ADF-50% and ADF-100% groups, respectively, relative to controls. Prostate cell proliferation was reduced (P < 0.05) by 49% in the ADF-100% group, relative to controls, but did not change in the other groups. IGF-1 levels were reduced (P < 0.05) by 40%, relative to controls, in the ADF-100% group. These findings confirm the beneficial effects of ADF-100% on cancer risk by decreasing cell proliferation and IGF-1 levels and suggest that modified ADF regimens comprising 25–50% CR on the fast day do not replicate these effects.

Dietary restriction has been shown to decrease the risk of certain age-associated diseases, such as cardiovascular disease, type 2 diabetes, and cancer, in a variety of animal models (16, 21, 25). Most dietary restriction studies have implemented the classic calorie restriction (CR) approach, wherein the amount of total food energy consumed is decreased by a certain percentage daily. Another dietary restriction paradigm has also been employed, although less commonly. Alternate-day fasting (ADF) consists of alternating 24-h periods of ad libitum feeding and fasting, i.e., where food is consumed ad libitum on the feast day, alternating with a day where food is withheld or reduced (fast day). Although a large body of evidence exists supporting the anti-carcinogenic effects of classic CR (16), much less data are available concerning the effects of ADF. In the few studies that exist to date, ADF has been shown to increase survival rate after tumor inoculation (31), decrease lymphoma incidence (10), and inhibit hepatic neoplastic lesion development (29) in rodents. Complementary to these findings, Hsieh et al. (14) recently demonstrated reductions in epidermal, T-cell, and mammary epithelial cell proliferation rates after short-term ADF in mice. The proliferation rate of cells (mitogenesis) represents a central element in the promotional phase of carcinogenesis (36), so a reduction in global cell proliferation rates may represent a mechanism for the anti-cancer effects of ADF. Decreases in insulin-like growth factor-1 (IGF-1) levels (33) have also been reported as a result of ADF, although inconsistently (2). Since elevated concentrations of circulating IGF-1 have been implicated in cancer development (17, 36), in part by augmenting cell proliferation rates, it is possible that reductions in IGF-1 levels by ADF may beneficially modulate cancer risk.

Several questions regarding the effect of ADF on cancer risk remain unresolved. In particular, a key question that has yet to be addressed is whether 100% CR is required on the fast day in an ADF regimen, or, more specifically, what degree of CR can be alternated with ad libitum feeding and still achieve beneficial effects? The availability of a sensitive, rapidly responsive biomarker of energy restriction, i.e., cell proliferation rates (14), enables different regimens or “doses” of ADF to be compared. Moreover, how different doses of ADF affect circulating IGF-1 levels and how these changes relate to cell proliferation rates also remain uncertain. Accordingly, the objectives of the present study were to measure the dose effects of modified ADF regimens on in vivo proliferation rates of prostate and splenic T-cells and on plasma IGF-1 concentrations in mice. The relationship between circulating levels of IGF-1 and in vivo cell proliferation rates was also examined.

METHODS

Animals. Seven-week-old C57BL/6J male mice (Charles River Breeding Laboratories, Wilmington, MA) were individually housed and maintained under temperature- and light-controlled conditions (12-h light-dark cycle: lights on at 0700 and off at 1900) for 1 wk. During this acclimation period, mice were given free access to water and a semi-purified AIN-93M diet (Bio-Serv, Frenchtown, NJ). The daily amount of food consumed by each mouse during acclimation was recorded.

ADF protocols. At 8 wk of age, the mice were randomized to one of four intervention groups (n = 6 per group). The first intervention group (ADF-25%) was fed the semi-purified AIN-93M diet ad libitum on the feast day and a 25% calorie-restricted diet on alternate days (fast day). The second group (ADF-50%) was fed the AIN-93M diet ad libitum on the fast 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 feast day and underwent a complete fast on alternate
days. The fourth group acted as the control and was fed the AIN-93M diet ad libitum each day. Food was provided or taken away at 1300 each day. The degree of CR on the fast day was calculated based on mean daily food consumption during the acclimation period for each mouse. Body weight was assessed weekly on the same day and time. Food intake was weighed daily. The intervention period in all groups lasted for 4 wk, and the mice were killed at 12 wk 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.

**Blood collection and 2H2O-labeling protocol.** Blood was collected at baseline (day 1) and on the last day of the trial (day 28). On day 28, blood was collected the morning after a feast day, following an 8-h fast. Mice were given heavy water (2H2O), as described elsewhere (14, 26), starting at the beginning of week 3 (day 14) and continuing throughout the last 2 wk of the study (days 14–28). Briefly, mice were administered an intraperitoneal injection of isotonic 100% 2H2O (0.18 mL/10 g body wt) on day 14 to bring the 2H2O content of their body water up to ~5%. For the remainder of the study, animals received drinking water containing 8% 2H2O ad libitum.

**Splenic T-cell, prostate, and bone marrow cell isolation.** Immediately after death, the spleen was removed, minced, and filtered through a 30-μm nylon mesh (14). Splenic T-cells were then isolated by using anti-CD90 microbeads via a magnetic column method (Miltenyi Biotec, Auburn, CA). The anterior prostate was dissected after death, minced, and filtered through a 30-μm nylon mesh. Bone marrow was collected from the femur. As described previously (24), marrow cells were flushed out using a needle and syringe containing PBS (GIBCO).

**Measurement of label enrichment in DNA.** DNA was isolated from T-cells, prostate, and bone marrow using Qiagen kits (Qiagen, Valencia, CA) and then was hydrolyzed to deoxyribonucleosides, as described elsewhere (27). In brief, DNA was incubated overnight at 37°C with DNase, nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase (Sigma, St. Louis, MO). The deoxyribosyl (dR) moiety of the released free deoxyribonucleosides was then derivatized to pentane tetra-acetate, as previously described (27). Positive chemical ionization GC-MS was used to analyze the pentane tetra-acetate, with a model 5973 mass spectrometer and a model 6890 gas chromatograph (Agilent, Palo Alto, CA). Selected ion monitoring was performed with a model 5973 mass spectrometer and a model 6890 gas chromatograph. Positive chemical ionization GC-MS was used to analyze the pentane tetra-acetate, as previously described (27). Positive chemical ionization GC-MS was used to analyze the pentane tetra-acetate. The deoxyribose (dR) moiety of the released free deoxyribonucleosides was then derivatized to pentane tetra-acetate, and the moity of the released free deoxyribonucleosides was then derivatized to pentane tetra-acetate. The deoxyribosyl (dR) moiety of the released free deoxyribonucleosides was then derivatized to pentane tetra-acetate, as previously described (27). Positive chemical ionization GC-MS was used to analyze the pentane tetra-acetate, with a model 5973 mass spectrometer and a model 6890 gas chromatograph. Positive chemical ionization GC-MS was used to analyze the pentane tetra-acetate.

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\[
EM_1 = \frac{[(abundance \, m/z \, 246)_{\text{sample}} - (abundance \, m/z \, 245 + 246)_{\text{standard}}]}{(abundance \, m/z \, 246)_{\text{standard}}}
\]

where sample and standard represent the analyzed sample and unenriched standards, respectively. Unlabeled standards of natural abundance pentane tetra-acetate were analyzed concurrently with samples. Matching the abundance of samples to standards and other corrections have been described in detail elsewhere (26, 27). Fractional replacement (f) of cells was calculated as described previously (9, 26), by comparison to cells from the same animal that were nearly fully turned over after 2 wk of labeling with 2H2O; i.e., bone marrow cells:

\[
f(\%) = \frac{[(EM_1)_{\text{sample}} - (EM_1)_{\text{bone marrow}}] \times 100}{[(EM_1)_{\text{sample}}]}
\]

**Plasma IGF-1 determination.** Plasma IGF-1 concentrations were determined using an enzyme-linked immunosorbent assay kit (Linco Research, St. Charles, MO).

**Statistical analysis.** Differences between groups during the acclimation period and at each week during the study were analyzed by one-way ANOVA. When significant differences were noted between groups, a Tukey’s post hoc test was performed to determine the significance of differences between group means. Changes within an intervention group throughout the study were measured using a repeated-measures ANOVA. Correlation analyses were also performed to evaluate the relationship between cell proliferation rates and IGF-1 concentrations. A test for normality of data was included in the model. A P value of <0.05 was used as the criterion for statistical significance in all analyses. Data were analyzed by SPSS software (version 11 for Mac OS X, SPSS, Chicago, IL).

**RESULTS**

**Body weight.** Mean body weights throughout the study are displayed in Fig. 1. All groups weighed approximately the same during acclimation, and all gained weight at the same rate during weeks 1 and 2. By week 3, however, the ADF-100% group did not continue to gain weight at the same rate as the other groups, and by week 4 the ADF-100% group weighed significantly less (P < 0.05) than the ADF-50% group. By the end of the study, the ADF-100% group weighed less (P < 0.005) than both the ADF-25% and ADF-50% groups. Overall, all groups gained weight (P < 0.05) from the beginning to the end of the study.

**Food intake.** During acclimation, all mice consumed, on average, ~3.5 g/day of food. The ADF-100% group ate ~11% less (P < 0.001) and ~16% less (P < 0.0001) than the other treatment groups during weeks 1 and 2, respectively, indicating an acute inability of the ADF-100% group to compensate fully on the feast day for the lack of food consumption on the fast day. Mean weekly food intake of the ADF-100% group increased during week 3, and no significant differences were observed between groups. During week 4, however, the amount of food consumed by the ADF-100% group fell to ~25% less (P < 0.001) than that of the other treatment groups.

**Hyperphagic response to food restriction.** A hyperphagic response on the feast day to food restriction on the fast day (P < 0.05) was noted in each of the ADF intervention groups at week 1 (Fig. 2). During week 1, the mean daily food intake on the feast day was 3.4, 3.9, 4.7 g/day in the ADF-25%, ADF-50%, and ADF-100% groups, respectively. The hy-

![Fig. 1. Mean body weight at the beginning of each week throughout the acclimation period and 4-wk study. Values are means ± SE. Mean body in each group was similar during the acclimation period. ADF-25%, 25% calorie restriction on fast day; ADF-50%, 50% calorie restriction on fast day; ADF-100%, 100% calorie restriction on fast day. *ADF-100% group weighed more (P < 0.05) than the control group at the beginning of week 1. **ADF-100% group weighed less (P < 0.05) than the ADF-50% group at the beginning of week 4. *ADF-100% group weighed less (P < 0.005) than the ADF-25% and ADF-50% group on the last day of the study (post). One-way ANOVA with Tukey’s post hoc test was used for all between-group analyses.](http://jap.physiology.org/content/103/4/548/F1)
perphagic response persisted throughout the study, since the ADF-50% and ADF-100% groups consumed ~45% and ~85% more (P < 0.05) food, respectively, on feast days than the control group. This hyperphagic response, however, did not persist after the first week in the ADF-25% group.

In vivo splenic T-cell and prostate cell proliferation. Splenic T-cell and prostate cell proliferation rates after 4 wk of ADF are shown in Fig. 3. At the end of the study, splenic T-cell proliferation was 6% and 29% lower (P < 0.05 for each) in the ADF-50% and ADF-100% groups, respectively, relative to controls. Prostate cell proliferation was 49% lower (P < 0.05) in the ADF-100% group compared with controls. Thus restricting food completely on the fast day resulted in significant reductions in proliferation of both cell types. Restricting food by 50% on the fast day reduced splenic T-cell proliferation modestly.

IGF-1. Circulating IGF-1 levels are shown in Fig. 4. Plasma IGF-1 concentrations were markedly reduced (P < 0.01) in the ADF-100% group compared with all other groups. Relative to controls, IGF-1 levels were 40% lower in the ADF-100% group. IGF-1 concentrations were not significantly different in the ADF-25% or ADF-50% groups compared with controls. Plasma IGF-1 levels exhibited positive, although only moderate, correlations with in vivo proliferation rates of both splenic T-cell (r = 0.48, P = 0.02) and prostate cells (r = 0.43, P = 0.04).

DISCUSSION

This study is the first to demonstrate that ADF-100% results in beneficial reductions in prostate cell proliferation rates and confirms our laboratory’s previous finding (14) that ADF-100% reduces T-cell proliferation rates. The modified ADF regimens (ADF-25% and ADF-50%), however, did not reproduce these favorable effects. Also of interest is the finding that ADF-100%, but not the modified doses of ADF, reduced IGF-1 levels. These results suggest a potential role for IGF-1 or related growth factors in the effects of ADF on cell proliferation, although reductions in IGF-1 levels were only moderately correlated with the decreases in cell proliferation rates observed here.

Cell proliferation plays an important role in carcinogenesis, particularly in the promotional stage of the multi-step carcinogenesis model (36). For this reason, numerous studies aiming to test the effect of dietary interventions on cancer risk have employed cell proliferation rates as a biomarker of carcinogenesis risk (19, 20, 22, 23, 35). In a previous study conducted in

![Fig. 2. Hyperphagic response to food restriction throughout the 4-wk study. Values are means ± SE. Means not sharing a common superscript letter are significantly different (P < 0.05) based on a one-way ANOVA with Tukey’s post hoc test.](image)

![Fig. 3. Effect of 4 wk of alternate-day fasting on splenic T-cell (A) and prostate (B) cell proliferation. Values are means ± SE. T-cell proliferation in the ADF-50% and ADF-100% groups was lower (*P < 0.05) than that of the control group. Prostate cell proliferation decreased (*P < 0.05) in the ADF-100% group compared with control. One-way ANOVA with Tukey’s post hoc test was used for all between-group analyses.](image)
concentrations (15, 28). For instance, results from classic CR nutrition has been shown to be a key mediator of plasma IGF-1 ability to promote cell proliferation while inhibiting apoptosis. A key role in cancer development (17, 36), perhaps due to its ability to promote cell proliferation while inhibiting apoptosis. Although circulating levels are regulated by multiple factors, nutrition has been shown to be a key mediator of plasma IGF-1 concentrations (15, 28). For instance, results from classic CR studies in rodents indicate that IGF-1 levels decrease in response to energy restriction and that this reduction is related to decreases in cancer incidence (1, 30, 32). In the present study, ADF-100% decreased circulating IGF-1 concentrations by 40% compared with controls. Similar reductions have also been reported by Wan et al. (33) who demonstrated an ~20% decrease in IGF-1 levels after 24 wk of ADF in rats. In contrast to these findings, however, Anson et al. (2) reported an ~15% increase in IGF-1 concentrations after 20 wk of ADF in mice. The explanation for these conflicting findings is not clear. It is recognized that circulating IGF-1 concentrations are not easily interpretable due to the presence of binding factors in the bloodstream, the possibility of paracrine actions, and other complicating factors. Despite these confounding factors, we observed a significant, although moderate, relationship between circulating IGF-1 levels and in vivo cell proliferation in animals. Previous work has demonstrated a relationship between IGF-1 and prostate/T-cell proliferation rates in cell culture (12, 13, 34). In the present study, in vivo proliferation rates were assessed using a stable isotope labeling technique that measures DNA replication (26). This method is based on the incorporation of $^2\text{H}_2\text{O}$ into the deoxyribose moiety of purine deoxyribonucleotides in dividing cells (26). This technique is safe to use in animal models and yields reproducible, quantitative kinetic information (14, 26). $^2\text{H}_2\text{O}$ has been used extensively for measuring biosynthetic rates and has no known effects, at the levels used here, on cell proliferation or other physiological processes. Results with $^2\text{H}_2\text{O}$ correlate well with other methods for measuring cell proliferation in vivo, e.g., colonocyte proliferation (18). Moreover, heavy water has no effect on cell growth rates in cultured cells (8). Future studies involving mice with genetically altered levels of IGF-1 would be useful for further clarifying the relationship between circulating IGF-1 concentrations and in vivo cell proliferation rates.

In addition to IGF-1, CR regimens have also been shown to reduce levels of other hormones and growth factors implicated in cancer risk, including insulin, PDGF, and testosterone (3, 6). In a study where non-obese subjects had been on self-imposed CR regimens for an average of 6 years, both plasma insulin and PDGF levels were markedly reduced compared with age-matched controls (11). Circulating testosterone levels have also been shown to decrease after 4 wk of CR in rats (7). These hormones were not measured in the present study but will be worth exploring in future investigations of ADF.

Also examined was the hyperphagic response to fasting on alternate days. As hypothesized, animals subjected to 100% restriction on fast days compensated by eating almost twice the amount of food on feast days compared with controls. Since this group was only able to eat roughly 85% of their total weekly baseline energy needs, these animals did not gain weight after the first 2 wk of the study and weighed less than animals in the other treatment groups by the end. A similar hyperphagic response was noted in mice in the ADF-50% group. The mice in this group consumed roughly 45% more food on feast days compared with controls. Since the amount of food on feast days compared with controls. This is apparent as the ADF-50% group gained weight at exactly the same rate as the controls throughout the study. A mild hyperphagic response was noted in mice in the ADF-25% group throughout week 1, but the response either did not persist or, more likely, was too small to be detected as significant.

In summary, these findings suggest that true ADF-100% results in reductions in cell proliferation rates and plasma...
IGF-1 levels but that modified regimens of ADF (e.g., 25–50% reductions in food intake on the fast days) do not. The ability of ADF to decrease these cancer risk parameters was shown to be both potent and rapid, since reductions were shown after only 4 wk of diet. Studies testing similar hypotheses in human subjects are still required before definitive conclusions can be reached.

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REFERENCES