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Effects of Caloric Restriction on Cell Proliferation in Several Tissues in Mice: Role of Intermittent Feeding

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Abstract
Reduced cell proliferation may mediate anti-carcinogenic effects of caloric restriction (CR). Using heavy water (^2H_2O) labeling, the cell proliferation response to CR was investigated in detail, including the time course, effect of refeeding, and role of intermittent feeding with 5% CR. In the time course study, 8-week-old female C57BL/6J mice were placed on a 33% CR regimen (food given 3 times/week) for varying durations. Compared to *ad libitum* fed controls (AL), proliferation rates of keratinocytes, mammary epithelial cells and T-cells were markedly reduced within 2 weeks of CR. In mice fed 95% of *ad libitum* (C95, fed 3 times/week), cell proliferation was also reduced in all tissues so that the differences from 33% CR were only significant at 1 month. In the refeeding study, mice were refed a 95% *ad libitum* diet for varying durations after 1 month of 33% CR. Cell proliferation rebounded to a supra-basal rate in all tissues after 2 weeks of refeeding, then normalized after 2 months, although the C95 group again exhibited lower cell proliferation than AL. The role of intermittent feeding was studied by comparing 33% CR and C95 (both fed intermittently) to animals fed isocalorically either daily or continuously by pellet dispenser. Intermittent feeding had no additive effect on 33% CR but reduced cell proliferation in all tissues at the 95% caloric intake level. In summary, the CR effect on cell proliferation is potent, rapid and reversible in several tissues, and an intermittent feeding pattern reproduces much of the effect in the absence of substantial CR.

Key words: stable isotopes, refeeding, keratinocyte, mammary, T-cell

Introduction

Caloric restriction (CR), defined as undernutrition without malnutrition (45), was first discovered in1935 to extend maximal lifespan in rats (29). Since then, similar findings have been reported in mice, fish, flies, worms, and yeast (45). A range of 30-70% extension of maximal lifespan has been achieved using variations on CR regimens (45), including both early- and adult-onset CR (44, 46). CR also exerts a number of other beneficial health effects, including reduced carcinogenesis, enhanced insulin sensitivity, and reduced cardiovascular disease risk (15). The inhibitory effect of CR on carcinogenesis is of particular interest, as CR effectively inhibits spontaneous tumor formation as well as neoplasias in knockout/transgenic models of cancer and chemically-induced tumorigenesis (18, 19, 45). The mechanisms by which CR extends lifespan and inhibits carcinogenesis remain unknown (15, 19).

CR could affect several steps in the multi-stage carcinogenesis model (18). CR may function as an anti-initiator by decreasing carcinogen activation, enhancing carcinogen detoxification, scavenging reactive oxygen species, or enhancing DNA repair (18). CR could also function as an anti-promoter by reducing mitoses of initiated cells, altering expression of cancer-related genes, decreasing inflammation, enhancing immune competence, or stimulating apoptosis (18). Reductions in cell proliferation might be expected in view of hormonal effects of CR, such as lower GH and IGF-1 levels (18, 19) and reduced body temperature (torpor) (22, 45).

Previous work supports the hypothesis that CR reduces cell proliferation. Using ^{3}HdT labeling, Lok *et al.* (26) demonstrated a 30-60% decrease in cell proliferation after 1 month of 25% CR in mice, in the skin, esophagus, bladder, and GI tract, and a 72% decrease in the mammary gland. In mice with 30% CR and epidermal treatment with carcinogens, 25% lower cell proliferation was observed (by BrdU labeling), compared to *ad libitum* (AL) fed mice given the same carcinogens. Increased papilloma latency was also reported (10). 60% CR rats treated with 1-methyl-1-nitrosurea (MNU) had up to a 31% reduction in mammary cell proliferation compared to MNU-treated AL-fed rats, as measured by BrdU, as well as decreased mammary carcinoma size and density (47). Food reduction and fasting in rats given the hepatomitogen cyproterone acetate resulted in lower DNA replication and increased apoptosis in liver (12).

Many questions remain, however, regarding the effects of CR on cell proliferation. In particular, the details of the response of cell proliferation to CR, including the time course, dose-response relationships, effects of refeeding, and endocrine correlations, have not been established. The effect of using different control groups in the CR field (e.g. AL versus 5-15% restriction (1, 5, 13, 23, 24, 37, 41, 43, 44, 46)) also has not been systematically explored. Finally, intermittency of feeding of both controls and CR models also has varied in the field (13, 23, 37, 42, 46) and could influence the effects of CR on cell proliferation.

We recently developed a method for measuring cell proliferation *in vivo* using heavy water ($^{2}H_2O$). This technique has a number of advantages over ^{3}HdT and BrdU labeling (34, 35) and has been used to measure turnover of keratinocytes, mammary epithelial

cells, T-cells, adipocytes, colonocytes, leukemic cells, and other cells (6, 16, 17, 21, 30, 32, 39, 40). Here, we apply this quantitative method to study the details of the response of cell proliferation to CR in mice.

Materials and Methods

Mice and CR regimens. For all studies, 7-week-old female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) were acclimated for 1 week, during which time they were fed a semi-purified AIN-93M diet *ad libitum* (Bio-Serv, Frenchtown, New Jersey). Studies were then started at 8 weeks of age. 3 studies were carried out.

Study #1: Time course of CR effects (Figure 1A). A 33% CR diet was fed for varying durations of time to the 3 treatment groups (n = 8 per group): 2 weeks CR (2W), 1 month CR (1M) or 2 months CR (2M). 2W received a control diet for 6 weeks prior to onset of the CR diet, and 1M received a control diet for 1 month prior to onset of the CR diet, so that the ages of all 3 groups were matched at the end of the experiment. Accordingly, all 3 groups were sacrificed at 16 weeks of age. Two different control groups were also used (n = 8 per group): *ad libitum* fed mice (AL) and mice fed 95% of *ad libitum* intake (C95). These animals were also sacrificed at 16 weeks of age. These control groups represent the different types of control groups that have commonly been used in CR studies demonstrating lifespan extension or reduced carcinogenesis (1, 37, 41, 44, 46). One additional group of mice (n = 4) was placed on CR for a longer period of time (3 months, 3M), also starting at 8 weeks of age. This group was sacrificed at 20 weeks of age.

During non-CR periods, the treatment groups were maintained on the C95 diet regimen. During CR periods, mice were fed 67% of C95 intake, or about 64% of AL intake, as previously described (37). The CR and C95 groups were fed 3 days a week, such that 2-times the daily allotment was given on Mondays and Wednesdays, and 3-times the daily allotment was given on Fridays, as has been commonly used in previous CR studies (13, 37, 42, 44, 46). AL and C95 mice were fed a semi-purified AIN-93M diet, while CR mice were fed an enriched AIN-93M diet that contains 33% more protein, minerals, and vitamins per gram of diet (Bio-Serv). All mice were housed individually. Food intake and body weight were monitored weekly.

Study #2: Refeeding effects (Figure 1B). The time course of response to refeeding was also studied. Mice received a 33% CR diet for 1 month and were subsequently given a C95 diet (n = 8 per group) for either 2 weeks of refeeding (R2W) or 1 month of refeeding (R1M). The CR diet for the R2W group started 2 weeks into the study (10 weeks old) while CR diet for R1M started immediately (8 weeks old), so that both groups were sacrificed at 16 weeks of age. One additional group of mice received a 33% CR diet for 1 month and was refed for a longer period of time (2 months, n = 4) (R2M). These mice were sacrificed at 20 weeks of age. All mice were housed individually. Food intake and body weight were monitored weekly.

*Study #3: Intermittency of feeding study***.** The role of intermittent food intake was also investigated. 3 groups of mice were put on a 33% CR diet, administered via different feeding protocols (n = 6 per group): intermittent feeding of 3 times per week (CR-INT),

as described above (37); daily feeding (CR-DF); or continuous feeding via an electronic pellet dispenser (CR-PD). 3 other groups of mice were fed 95% of *ad libitum* diet via the same 3 feeding protocols (n = 6 per group): intermittent feeding of 3 times a week (95-INT); daily feeding (95-DF); or continuous feeding (95-PD). The 95-INT, 95-DF, and 95-PD groups were also compared with a group fed *ad libitum* (AL) concurrently. Intermittent feeding was as described above, with 2 times the daily allotment given on Mondays and Wednesdays, and 3 times the daily allotment given on Fridays. Mice fed daily were given their food allotment for each day, every morning. The amount and type of diet (33% enriched or standard AIN-93M) depended on whether the mice were in the CR groups (CR-INT, CR-DF) or the control groups (95-INT, 95-DF). Continuously fed mice were housed in cages containing an electronic pellet dispenser that delivered a 45 mg pellet of AIN-93M diet (standard for 95-PD, 33% enriched for CR-PD, Bio-Serv), into the cage every 20 to 30 minutes, depending on the caloric intake. All mice were housed individually. Food intake and body weight were monitored weekly. Mice were sacrificed at 12 weeks of age, after 4 weeks of treatment.

Heavy water (^2H_2O) labeling protocol. Cell proliferation was measured by the heavy water (^2H_2O) labeling method as described previously (34, 35). Briefly, mice were given an intraperitoneal injection of isotonic 100% ^2H_2O (0.18 mL/10 g body weight) 2 weeks prior to sacrifice to bring their ^2H_2O content in body water up to 2.5%. Mice subsequently received *ad libitum* drinking water that contained 4% ^2H_2O for 2 weeks. The mice were sacrificed by cardiac puncture under isoflurane anesthesia, followed by cervical dislocation.

Epidermal cell (keratinocyte) isolation. Dorsal hair was removed post-mortem by application of a hair removal lotion (Nair, Carter Products, New York, New York). After the lotion was cleaned off using an alcohol swab, a piece of dorsal skin was dissected (about 3 cm^2). The skin was rinsed with phosphate buffer solution (PBS) (Gibco, Grand Island, New York), divided into 3 smaller pieces, immersed in dispase II (Roche, Indianapolis, Indiana), and incubated for 3.5 hours at 37^oC on a shaker. The epidermis was then peeled from the dermis in thin white sheets, as described previously (17).

Mammary epithelial cell (MEC) isolation. MECs were isolated as described previously (32, 33). Briefly, mammary fat pads were dissected, minced, and treated with collagenase (Worthington Biochemical Corporation, Lakewood, New Jersey), prior to isolation of MECs by centrifugation in a Percoll gradient (Amersham Pharmacia Biotech, Inc., Piscataway, New Jersey).

Splenic T-cell isolation. T-cells were isolated from spleen. Briefly, the spleen was removed, minced, and filtered through a 30 μm nylon mesh. T-cells were isolated using anti-CD90 microbeads by a magnetic column method (Miltenyi Biotec, Inc., Auburn, California).

Bone marrow cell isolation. Bone marrow was collected from the femur. Marrow cells were flushed out using a needle and syringe containing PBS (Gibco), as described previously (27).

DNA enrichment. DNA was extracted from cell preparations using Qiagen kits (Qiagen Inc., Valencia, California) and hydrolyzed to deoxyribonucleosides as described elsewhere (35). In brief, DNA was heated with magnesium chloride and zinc sulfate, followed by incubation for 2 hours in a 37^{o}C water bath with DNase, nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase (Sigma, St. Louis, Missouri). The deoxyribose (dR) moiety was derivatized to pentane tetra-acetate, as described (35). Pentane tetra-acetate was analyzed by positive chemical ionization GC/MS, using a model 5973 mass spectrometer and model 6890 gas chromatograph (Agilent, Inc., Palo Alto, California). Selected ion monitoring was performed of m/z 245 and 246, representing the M_{+0} and M_{+1} ions, respectively (34, 35). The excess fractional M_{+1} enrichment (EM_1) of dR was calculated as:

$$EM_1 = \frac{(\text{Abundance m/z } 246)_{\text{sample}}}{(\text{Abundance m/z } 245 + 246)_{\text{sample}}} - \frac{(\text{Abundance m/z } 246)_{\text{STD}}}{(\text{Abundance m/z } 245+ 246)_{\text{STD}}}$$

where sample and STD represent the analyzed sample and unenriched standards, respectively. Standards of natural abundance (unlabeled) pentane tetra-acetate were analyzed concurrently with samples. Abundance matching of samples to standards and other corrections were as described in detail elsewhere (34, 35). Calculation of fractional replacement (f) of cells was by comparison to nearly fully turned over cells (bone marrow cells), as described previously (7, 34):

$$f\,(\%) = \frac{(EM_1)_{\text{sample}}}{(EM_1)_{\text{bone marrow}}} \times 100$$

Estrus cycle status. Presence or absence of estrus cycle in the 1M CR and C95 groups was determined via vaginal smear and analysis of cell morphology. Vaginal smears were taken during 4 consecutive days and samples fixed and stained on slides with hemoxylin and eosin (Histo-Tec Laboratory, Hayward, California).

Statistical analysis. Data from the time course and refeeding studies were analyzed by one-way ANOVA with Dunnett's follow-up test, comparing all groups to either AL or C95 controls ($p<0.05$ set as significance level). The intermittency of feeding study was analyzed by one-way ANOVA with Tukey's follow-up test ($p < 0.05$), comparing all pair-wise combinations in the CR and 95% of *ad libitum* intermittency experiments.

Results

Study #1: Time course

Food intake and body weight. On average, AL mice consumed 22 grams of food per week. Therefore, C95 mice were fed 21 grams of food per week and CR mice were fed

14 grams of food per week. The body weight of CR mice dropped initially by as much as 30% but stabilized over time (Figure 2A). Mice then gained weight on CR diets.

Time course. When compared to AL, proliferation of epidermal cells, MECs, and T-cells was significantly decreased in the CR groups at all time points studied (Figures 3A-3C). When compared to C95, in contrast, cell proliferation in all tissues was not significantly decreased until 1 month of CR, after which the response was again not significant. At 1 month of CR, the time of greatest effect of CR, epidermal cell proliferation was 61% of that in AL mice and 76% of that in C95 mice. MEC proliferation was only 11% of AL and 29% of C95 mice values at 1 month, while T-cell proliferation was 41% of that in AL mice and 57% of that in C95 mice.

Differences between C95 and AL control groups. C95 mice exhibited statistically significantly lower cell proliferation than AL mice in all tissues examined (Figures 3A-3C). After 2 months on respective diets, epidermal cell proliferation in C95 mice was 81% of that in AL mice, MEC proliferation was 37%, and T-cell proliferation was 71%. Thus, CR exerted significant effects on proliferation of all 3 cell types studied, but C95 also had a potent impact that appeared to account for at least part of the CR effect.

Estrus cycle. Based on cell morphology analysis of vaginal cells collected from 1M and C95 mice, CR mice were anestrus (not cycling), while C95 mice were actively cycling. The marked reduction in MEC proliferation in the CR groups might therefore in part be explained by reduction in reproductive hormone levels (31), but the substantial effect observed in the C95 groups exclude this as the primary cause of reduced MEC proliferation.

Study #2: Refeeding

Food intake and body weight. As in study #1, AL mice consumed about 22 grams of food per week. During the CR phase, mice were therefore fed 14 grams of food per week, and during the refeeding phase, mice were fed 21 grams of food per week. Refeeding resulted in a rapid gain of lost weight (Figure 2B). Body weights of CR mice had caught up to the body weights of C95 mice by the end of the study, despite the 1-month period of CR.

Time course of refeeding effects. When compared to the C95 control group, cell proliferation in all tissues rebounded to a significantly higher rate after 2 weeks of refeeding, persisting through 1 month of refeeding but normalizing after 2 months of refeeding (Figures 4A-4C). When compared to the AL group, cell proliferation in all tissues was no longer significantly different after 2 weeks of refeeding. Subsequent comparisons revealed tissue-specific differences. After 1 month of refeeding of the C95 diet, T-cell proliferation rate was statistically higher than AL levels; this was normalized after 2 months of refeeding. MEC proliferation was significantly lower than AL levels after 2 months of refeeding of C95 diet, consistent with the observation that MEC proliferation was lower in C95 mice than in AL mice (Figure 3B).

Study #3: Intermittency of feeding

Food intake and body weight. Throughout this study, all groups of CR mice were fed 14 grams of food per week, and all groups of control mice were fed 21 grams of food per week. All mice gained weight on their diets (Figure 2C). Non-significant differences in

body weight between mice with the same caloric intake but fed by different feeding patterns may be due to the presence or absence of food in the stomach during weighing.
Feeding intermittency effects among groups of CR mice. In the three tissues studied, intermittency of feeding (i.e. food given 3 times per week) had no additional effect compared to daily or continuous feeding on cell proliferation when CR was present (Figures 5A-5C).
Feeding intermittency effects among groups of control mice. There was lower cell proliferation in all tissues of the group fed intermittently at 95% of *ad libitum* diet (95-INT) compared to daily feeding (95-DF), continuous feeding (95-PD), or *ad libitum* feeding (AL), although not all comparisons were statistically significant (Figures 6A-6C). MEC proliferation was significantly lower in 95-INT than in 95PD mice, while T-cell proliferation was significantly lower in 95-INT mice compared to 95-DF and 95-PD mice. Epidermal and T-cell proliferation rates in AL were not statistically different from 95-DF or 95-PD but were significantly greater than 95-INT. An intermittent feeding regimen (i.e. food given 3 times per week) therefore caused significant reductions in cell proliferation rates compared to isocaloric diets fed by more constant patterns.

Discussion
We demonstrate here the application of a relatively simple method for measuring cell proliferation in multiple tissues in mice. By this technique, it is clear that cell proliferation rates in mice are extremely sensitive to changes in caloric intake, whether due to CR or feeding pattern.

Previous methods for measuring cell proliferation include cell-cycle indices such as Ki67 or PCNA staining (28, 38). These techniques do not accurately reveal rate of progression through the cell cycle, however (16). Dynamic measurements, including incorporation of BrdU and ^{3}HdT, also have limitations. DNA incorporation of these precursors occurs via nucleoside salvage pathways and is dependent on a number of variables, including efficiency of cellular uptake, competition with extracellular nucleosides, etc., which can differ among cell types (34, 35). Labeled deoxyribonucleosides released after cell death may also be reincorporated into other cells (16). The stable isotope labeling method used here is safe, yields quantitative kinetic information, does not depend on the deoxyribonucleoside salvage pathway, and is not susceptible to artifacts related to re-utilization (16, 34, 35).

We show here that early-onset 33% CR in C57BL/6J mice, administered by a commonly used feeding regimen in this field (i.e. food given 3 times per week) (13, 37, 42, 44, 46), reduces proliferation of epidermal cells (keratinocytes), MECs, and splenic T-cells. When mice were refed after CR, cell proliferation rates were restored within 2 weeks to values equal to *ad libitum* fed controls, and some tissues became transiently hyperproliferative in comparison to 95% *ad libitum* fed controls. These data suggest that the effects of CR on cell proliferation are rapid and reversible. Whether or not these effects on cell proliferation are sustained over extended duration of CR cannot be deduced from these data.

The mediator(s) of the CR effect on cell proliferation in multiple tissues remain uncertain. IGF-1 has been hypothesized to mediate the decrease in cell proliferation in response to CR (18, 19). Serum IGF-1 levels have been consistently reported to be reduced in CR studies (4, 9, 14, 20), and exogenous replacement of IGF-1 has been found to negate the benefits against bladder cancer conferred by CR in p53-deficient mice (9). In addition, modulations in IGF-1 signaling have been correlated to lifespan extension (3, 8, 11). We were unable to accurately compare IGF-1 levels between groups due to differences in fasting times prior to sacrifice. A priority for future studies will be to characterize the relationship between changes in cell proliferation and concentrations of potential mediators.

Our data demonstrate that an intermittent pattern of feeding, resulting in periodic fasting, contributes to the anti-proliferative effects of CR regimens, along with caloric deficit. We observed that a 5% decrease in total caloric intake, combined with an intermittent feeding pattern (food given 3 times per week), decreased cell proliferation compared to mice fed isocalorically but according to a more constant feeding pattern (daily or continuously). Intermittency of feeding did not appear to have an additive effect in CR mice. In particular, among mice receiving 95% of AL caloric intake, intermittent feeding decreased MEC and T-cell proliferation compared to continuously fed mice. Continuously and daily fed mice at 95% AL caloric intake also did not have significantly lower epidermal and T-cell proliferation compared to AL controls, whereas intermittently fed mice at 95% AL caloric intake did, ruling out an effect of the 5% reduction in caloric intake per se. Recently, intermittent feeding was found to impart greater benefits than daily feeding at a 40% level of CR (2). The intermittent feeding model employed by Anson *et al*. involved alternating *ad libitum* feeding and complete food deprivation, every other day. Although the mice compensated for food deprivation on the days during which they were fed, they were only able to attain a caloric intake of about 90% of *ad libitum* levels. Thus, their model, resulting in 10% CR with intermittent feeding, is similar to our C95 group, fed 5% CR intermittently. Anson *et al.* reported improved insulin sensitivity in this model, compared to a daily fed 40% CR model (2). Both studies therefore suggest that minimal CR in conjunction with intermittent feeding induces health effects similar to that from traditional, much more substantial CR.

Our data do not suggest, however, that the effects of substantial CR can be completely reproduced by intermittency of feeding. Although intermittent feeding with 5% CR (95-INT) resulted in lower cell proliferation than more continuous feeding at the same caloric level, it is worth noting that the degree of hypoproliferation is not as pronounced as in mice fed 33% CR, regardless of feeding intermittency. This result suggests that substantial CR still has a dominant effect over feeding intermittency. Similarly, Lee *et al*. have shown that mice fed intermittently on 41% CR have greater lifespan extension and lower tumor incidence than those fed intermittently on 15% CR as controls (25).

Nelson and Halberg also investigated the role of intermittent feeding and found that 25% CR with 6 smaller meals versus 1 big meal a day both extended lifespan to the same extent in mice but resulted in a different circadian rhythm, such that less frequent meals resulted in lower core body temperature (36). This finding may be significant, as CR-

induced torpor and cell proliferation are linked (22, 45), but cell proliferation was not measured in this study. The finding that 25% CR with increased feeding intermittency did not extend lifespan beyond daily feeding of 25% CR may suggest that substantial CR overcomes or masks any effect of intermittency on lifespan. This interpretation is also consistent with our data, as 33% CR groups had the same cell proliferation rates, despite different feeding intermittency patterns. There has yet to be a study comparing lifespan expectancy in animals with minimal CR using different feeding patterns, however. Such a study would be necessary to investigate the effect of intermittency of feeding apart from caloric deficit on lifespan extension.

The suggestion that intermittent feeding may produce benefits similar to caloric restriction is potentially of great interest to human applications. While it may be impractical to maintain humans on substantial calorically restricted diets for their lifetime, intermittent food deprivation may be feasible. If some of the health benefits of CR can be reproduced, including reduction in cancer promotion, this might be a therapeutic strategy worth pursuing. Human CR studies using the techniques described here (e.g. proliferation of skin cells and mammary epithelial cells (17, 34)) can, in principle, be performed to test this hypothesis.

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Figure Legends

Figure 1. Experimental design of (A) time course study #1 and (B) refeeding study #2.

Figure 2. Mean body weights of mice in (A) time course study #1 (n = 8 per group, n = 4 for 3M group), (B) refeeding study #2 (n = 8 per group, n = 4 for R2M group) and (C) intermittency of feeding study #3 (n = 6 per group).

Figure 3. Study #1: Effect of caloric restriction for varying durations on proliferation of (A) epidermal cells, (B) mammary epithelial cells and (C) T-cells. Data are expressed in mean ± SD (n = 8 per group, n = 4 for 3M group). * denotes significant difference from AL group ($p < 0.05$) by one-way ANOVA with Dunnett follow-up. ** denotes significant difference from C95 group ($p < 0.05$) by one-way ANOVA with Dunnett follow-up.

Figure 4. Study #2: Effect of refeeding for varying durations on proliferation of (A) epidermal cells, (B) mammary epithelial cells and (C) T-cells. Data are expressed in mean ± SD (n = 8 per group, n = 4 for R2M group). * denotes significant difference from AL group ($p < 0.05$) by ANOVA with Dunnett follow-up. ** denotes significant difference from C95 group ($p < 0.05$) by ANOVA with Dunnett follow-up.

Figure 5. Study #3: Effect of intermittency of feeding in presence of 33% caloric restriction on proliferation of (A) epidermal cells, (B) mammary epithelial cells and (C) T-cells. Data are expressed in mean ± SD (n = 6 per group). Groups with different superscript letters are significantly different ($p < 0.05$) by one-way ANOVA with Tukey follow-up.

Figure 6. Study #3: Effect of intermittency of feeding in presence of 5% caloric restriction on proliferation of (A) epidermal cells, (B) mammary epithelial cells and (C) T-cells. Data are expressed in mean ± SD (n = 6 per group). Groups with different superscript letters are significantly different ($p < 0.05$) by one-way ANOVA with Tukey follow-up.

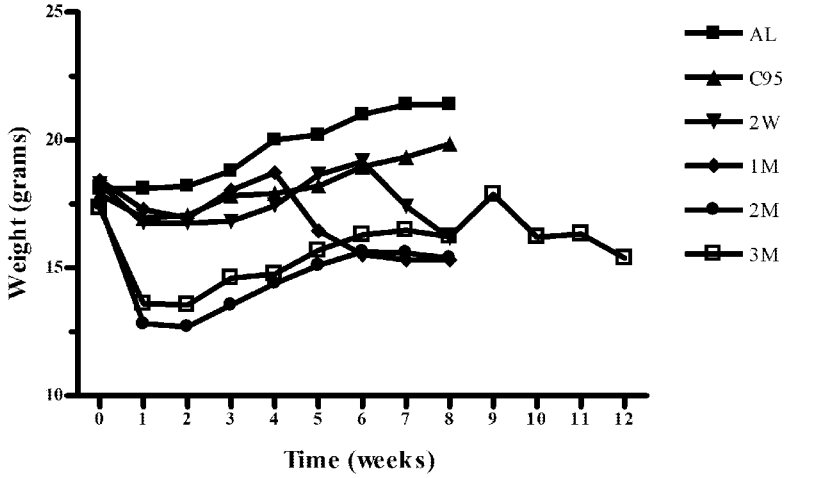
A

Age (weeks)	8	12	16	20
AL	|---------- AL--------|			
C95	|--------- C95--------|			
2W	|-----C95------|-CR-|			
1M	|---C95----|---CR---|			
2M	|--------- CR---------|			
3M	|----------CR----------|			

B

Age (weeks)	8	12	16	20
AL	|---------- AL--------|			
C95	|--------- C95--------|			
R2W	|C95|---- CR---|C95|			
R1M	|---CR----|---C95---|			
R2M	|---CR----|---------C95--------|			

A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15
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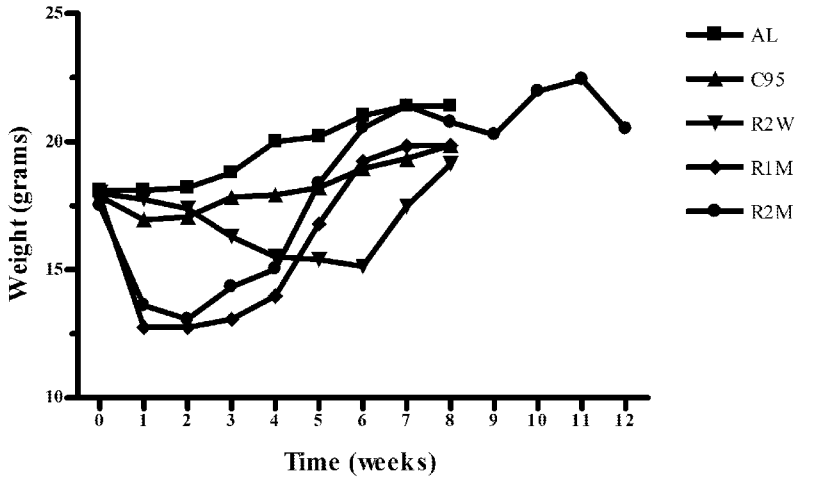
Time (weeks)
AL
C95
2W
1M
2M
3M

B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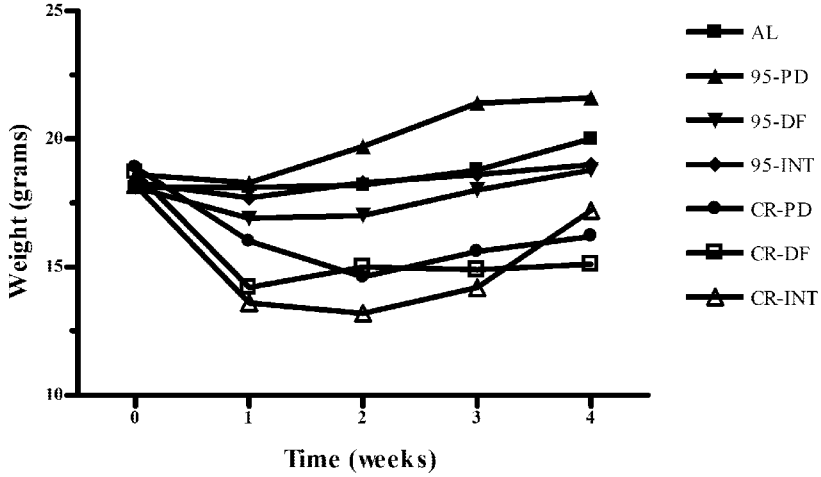
Time (weeks)
AL
C95
R2W
R1M
R2M

C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Time (weeks)
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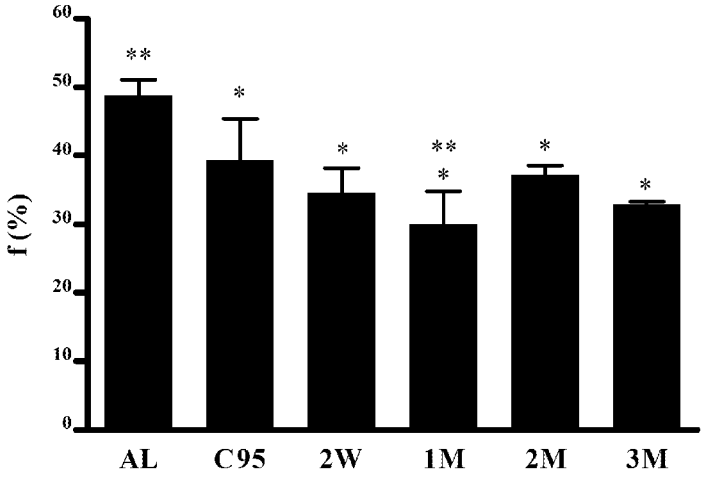
95-PD
95-DF
95-INT
CR-PD
CR-DF
CR-INT

A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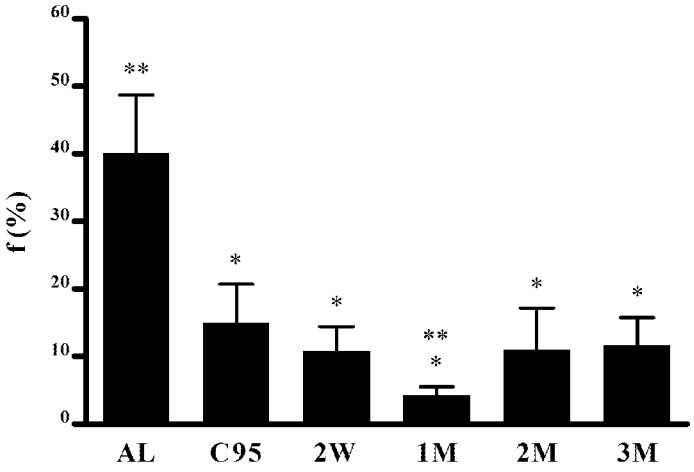
B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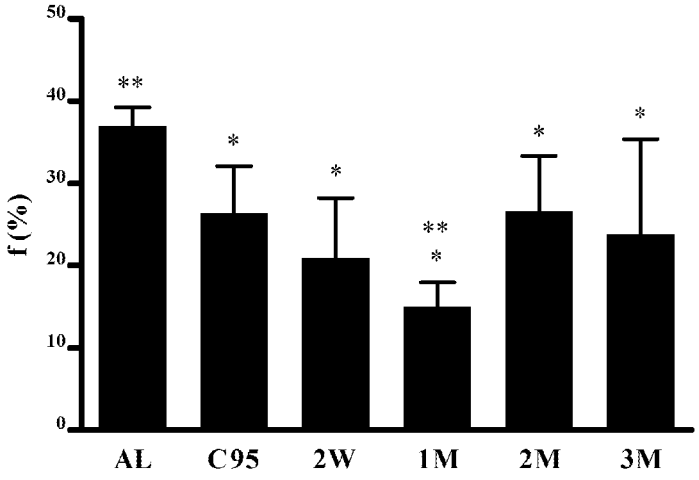
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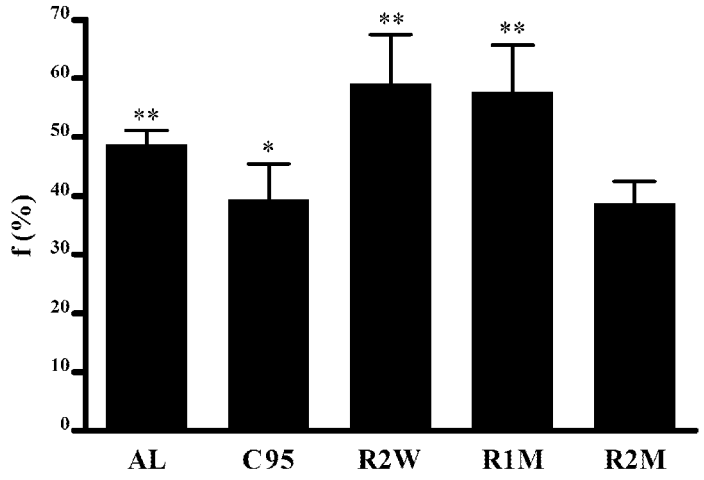
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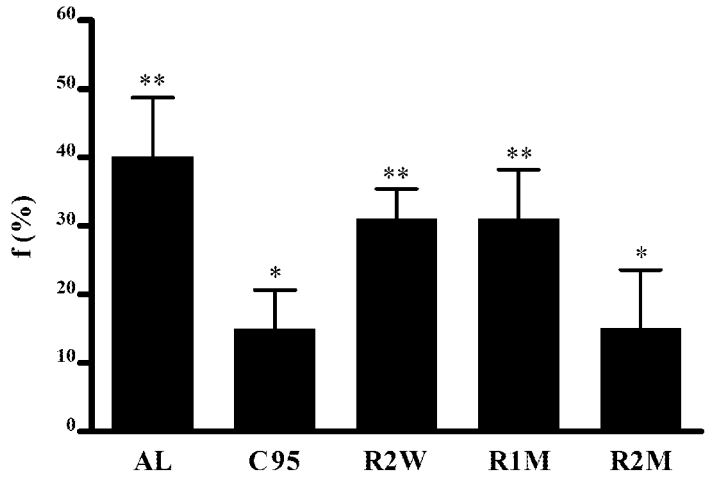
B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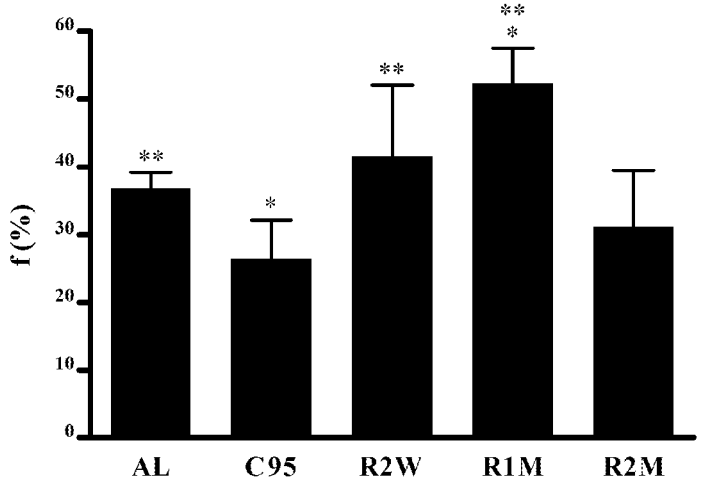
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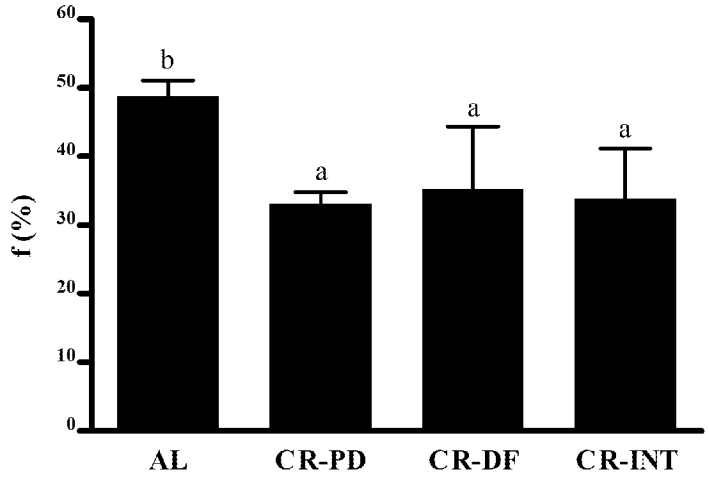
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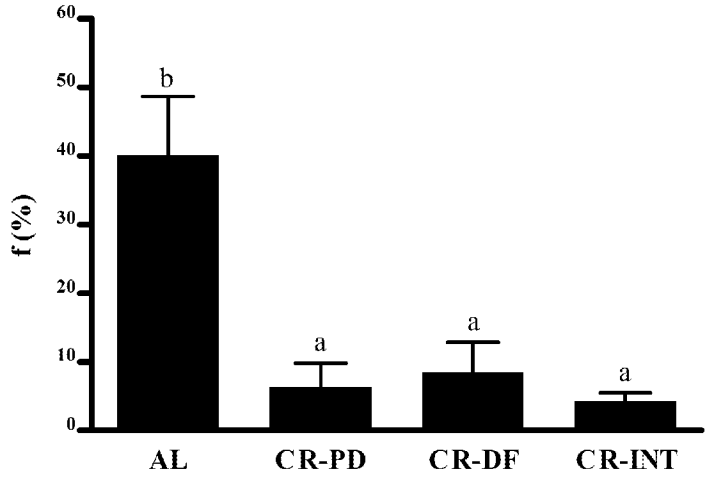
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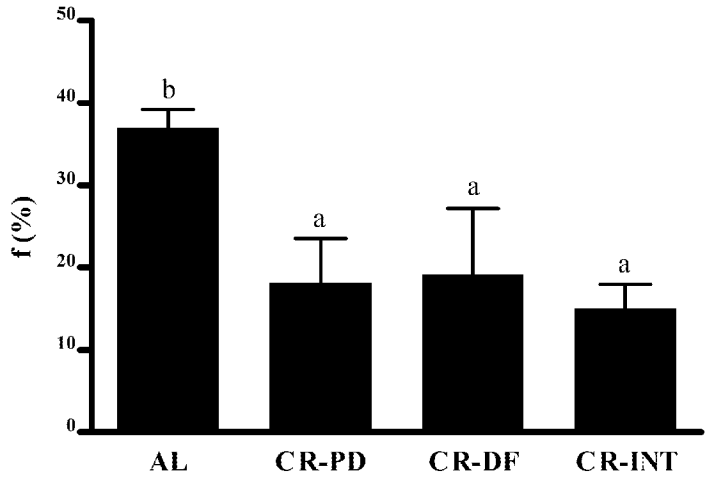
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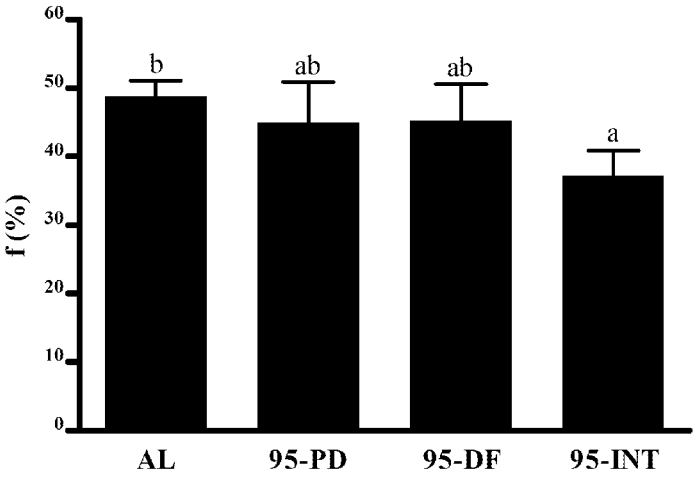
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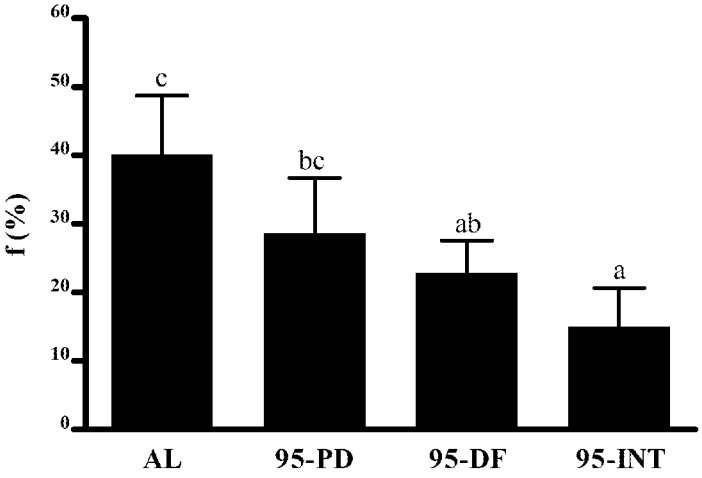
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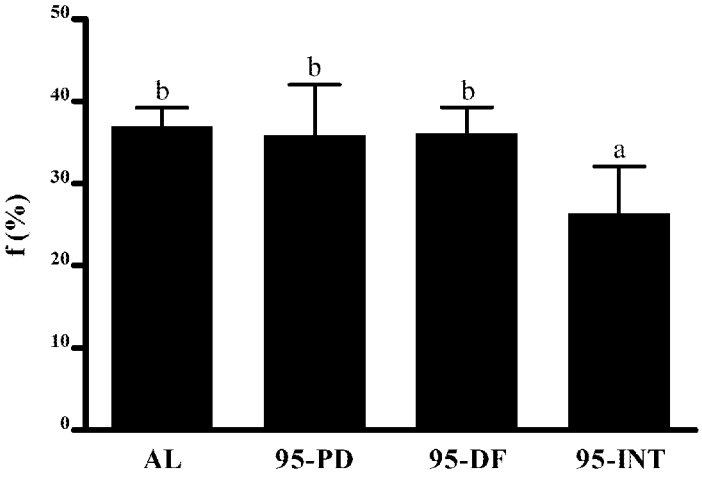
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