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The influence of different calorie restriction protocols on serum pro-inflammatory cytokines, adipokines and IGF-I levels in female C57BL6 mice: short term and long term diet effects

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Abstract

Calorie restriction (CR) is an effective intervention to prevent chronic diseases including cancer. Although many factors, i.e., sex hormones, IGF-I and mTOR have been studied in response to CR, the molecular mechanisms of CR remain to be identified. Our objective was to determine the short and long-term effects of different CR protocols on pro-inflammatory cytokines. Our hypothesis was that Intermittent CR (ICR) would result in greater inhibition of pro-inflammatory serum cytokines compared to Chronic CR (CCR) as we previously found ICR to be more protective in the prevention of mammary tumor development. From ten weeks of age female C57BL6 mice were maintained on either ad libitum (AL) fed, ICR or CCR protocols (overall CR of ~75% of AL) for up to 74 weeks of age. Blood samples were collected for measurements of serum interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), adiponectin, leptin, IGF-I and insulin at specified ages. For ICR mice samples were collected following 3 weeks of restriction (ICR-R) and after one week of refeeding (ICR-RF). In general, both modes of CR significantly reduced serum IL-6, TNF- α , IGF-I and leptin levels compared to AL with IL-6 levels 24 and 3.5 fold and TNF- α levels 11 and 1.5 fold lower in ICR and CCR groups, respectively at study termination. There was a trend for adiponectin and insulin to be highest in ICR-RF mice. Body weights were positively correlated with IL-6, TNF- α , insulin and leptin but negatively correlated with adiponectin-to-leptin ratio. Moreover, there was a positive correlation between IL-6 and TNF- α . Beneficial effects of ICR may function through pro-inflammatory cytokine pathways.

Keywords

Cytokines; calorie restriction; IGF-I; adipokines; inflammation; wild-type mice; female

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1. INTRODUCTION

Calorie restriction (CR) is one of the most effective experimental interventions to prevent chronic health problems such as cardiovascular disease, type-2 diabetes, dyslipidemia, asthma and cancer [1–5]. For example, researchers have reported that CR prevents both spontaneous and carcinogen induced mammary tumor (MT) incidence in rodents [1, 6–8] with up to 95% decrease when caloric intake is limited to 60–80% of their ad libitum (AL) fed consumption [1, 6, 7]. In general, two types of CR, i.e., chronic calorie restriction (CCR) and intermittent calorie restriction (ICR), a regimen for alternate periods of “on” and “off” caloric restriction, have been used [1, 8–10]. Although the effects of CCR on cancer prevention have been studied extensively, ICR investigations have been more limited. Interestingly, it has recently been reported that when compared directly in the same study, ICR was more effective than CCR in MT prevention [1, 6, 11]. For example, MMTV-TGF- α mice fed AL had MT incidence in the range of 80%, while the MT incidence rate was 27–44% in CCR mice but was only between 3–15% in ICR mice restricted to a similar overall degree but in an intermittent fashion [1, 6, 12].

Even though the protective effect of CR in many pathological conditions including cancer prevention has been well established, the mechanisms of this intervention, particularly how ICR interacts in signaling pathways have not been identified. In this context, the roles of many factors such as sex hormones, IGF-I and its binding proteins (IGFBPs), insulin, adipokines, nuclear factor kappa B (NF κ B), reactive oxygen species (ROS), prostaglandins, special microRNAs and mTOR have been studied [1, 8, 13–25], but still many questions remain unanswered. Recent findings from in-vitro and in-vivo studies demonstrated that leptin and adiponectin, which are primarily secreted from adipose tissue (adipokines), play important roles in mammary tumor development and cell proliferation through different intracellular signaling pathways [1, 16, 21, 23, 25–29] and their secretion is modulated by calorie restriction [1, 11, 30, 31].

In the current paper, we measured the effects of two different calorie restriction protocols in female C57BL6 mice on serum concentrations of pro-inflammatory cytokines as this is an understudied area of interest with respect to the preventive impact of CR especially for ICR. Wild-type mice were used so that disease conditions did not interfere with the measured factors. Systemic chronic inflammation caused by higher body mass index (BMI) is considered to be a major pathological event for many health disorders including cancer [4, 16, 22–24, 29, 30, 32–35]. Conversely, both animal and human studies have suggested that CR results in favorable changes of many biochemical factors including cytokines [2, 4, 8, 16, 19, 21, 22, 24, 25, 30, 31, 33, 36–40]. Reduction in fat mass correlates with decrease in pro-inflammatory cytokines levels in serum implying that approaches designed to promote fat loss should be useful in attenuating the pro-inflammatory setting associated with higher body weight [4, 30, 41]. To our knowledge no previous reports have been published on the long-term effects of CR specifically ICR on pro-inflammatory cytokines, adiponectin (an anti-inflammatory adipokine), IGF-I and insulin in non-transgenic mice model that mimics healthy human population with normal body mass. Therefore, the main objective of the current study was to determine the short- and long-term effects of different CR protocols on pro-inflammatory cytokines/adipokines, IGF-I and insulin levels in wild-type mice.

2. Materials and Methods

2.1. Animals and study design

For the CCR and ICR diets nutrients were altered with the goal that except for carbohydrate all other nutrients in absolute amounts were similar among the groups. Eight-week-old female C57BL6 mice obtained from a colony maintained at the Hormel Institute were fed AIN-93M diet from 8 until 10 weeks of age and then were randomly assigned to one of three experimental groups: AL-fed, ICR, and CCR. AL mice were fed AIN-93M diet through out the study. Mice assigned to the ICR group were provided with a 50% restriction diet (with 2-fold increases in protein, vitamin, mineral and fat content which was isocaloric compared to the AIN-93M diet) for three week restriction periods. Following restriction periods, ICR mice were provided the AIN-93M diet at 100% of age-matched AL consumption for three weeks. CCR mice were fed at 75% of AL mice using a diet formulated to be isocaloric with AIN-93M with 25% increases in protein, vitamin, mineral and fat content. The diets were purchased from Harlan Teklad (Madison, WI) and have previously been described in detail [1, 11]. All mice had free access to water and were individually caged. Food intakes were determined daily and body weights weekly. Animals were observed for any health problems by expert veterinary technicians on a daily basis and by veterinarian when it was necessary. At designated ages: 10 (baseline), 13, 14 (Cycle 1); 37, 38 (Cycle 5); and 73, 74 (Cycle 11) weeks of age blood samples were obtained and mice were sacrificed five hours after they were given their daily allotment of food. Tissue samples were collected and sent it to pathology department for pathological analysis by an expert on blind fashion. All the samples were used in this experiments were from healthy mice which had no tumors or any other pathophysiological conditions according to their pathological reports. Weeks 13, 37 and 73 were after three weeks of restriction for ICR mice which are designated as ICR-Restricted (ICR-R) and weeks 14, 38 and 74 following one week of refeeding for ICR mice which are designated as ICR-Refed (ICR-RF). Because there were no differences in body weights and serum measurements for AL-fed and CCR mice for the one week age differences their data were combined. Number of samples (“n” values) for each group was between 4 and 16 at each time point. This study was approved by the University of Minnesota Institutional Animal Care and Use Committee and the University of Minnesota is an AAALAC accredited facility.

2.2. Measurement of Pro-inflammatory Cytokines, Adipokines and IGF-I Levels

Serum IL-6, TNF- α , insulin and leptin levels were measured using the Mouse Adipokine LINCOplex Kit 96 Well Plate Assay (LINCO Research, St. Charles, MO) and adiponectin was measured using the Mouse Single Plex Adiponectin LINCOplex Kit 96 Well Plate Assay (LINCO Research, St. Charles, MO) and read on the Luminex100 instrument. Serum IGF-I levels were measured using mouse/rat IGF-I ELISA (DSL-10-29200, Diagnostic Systems Laboratory, Webster, TX) kit. Each serum sample was run in duplicate. Also an internal control sample was run in every plate and negative control samples were used. Inter-assay CVs in % are 7.0% for Insulin, 11.9% for Leptin, 10.0% for IL6 and 9.1% for TNF- α . Intra-assay CVs in % are 4.0% for Insulin, 6.0% for Leptin, 7.2% for IL6, 6.7% for TNF- α . The measurements were done and values were obtained by experts who have many publications in this field [42, 43]. Because some of the samples we used had below the

lowest standard value when the cytokine levels were calculated in pg/ml the fluorescence unit was used in the present study. For the correlation and ratio metric analysis, the values from the same individual mice were used.

2.3. Statistical Analysis

Results are presented as means $\pm$ SEM and analyzed using GraphPad Prism 4 (San Diego, CA). Final body weight, fat pad weight, and serum concentrations of IL-6, TNF- α , adiponectin, leptin, Adiponectin to Leptin ratio (A/L), insulin and IGF-I were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test to determine significant differences between specific groups at each age. Also within each group at each age changes were assessed. Spearman correlation test and linear regression analysis were used separately to determine whether correlation between two parameters was statistically significant. As indicated above because there were no significant difference between two consecutive time points at weeks 13 and 14, 37 and 38, and 73 and 74 for AL-fed and CCR mice data from these two time points were combined. However, for ICR mice these time points were presented separately. Statistical significance was at $p < 0.05$ or less.

3. RESULTS

3.1. The Effects of Different Calorie Restriction Protocols on Body and Fat Pad Weights (Table 1)

There were no differences in body weights among the groups when mice were assigned to the study at 10 weeks of age ($p > 0.05$). Body weights of AL mice were significantly higher than the other groups at 13/14, 37/38 and 73/74 weeks of age ($p < 0.001$) (Table 1). Although mice in the ICR-R groups had lower body weights than either ICR-RF or CCR mice at all points following three weeks of 50% calorie restriction, the difference between ICR-R and ICR-RF was significant only at weeks 13/14 and 37/38. The body weight difference between ICR-R and CCR was significant only at week 73/74 despite the greater reduction of calories for the ICR-R mice compared to CCR. There were no significant body weight differences between ICR-RF and CCR groups at any time points ($p > 0.05$) (Table 1).

Effects on total fat pad (combined parametrial, retroperitoneal and mammary fat pads) weights were in general similar to those observed for body weight (Table 1). There was highly significant positive correlation between body weights and total fat pad weights for all groups at all time points of the study ($r = 0.86$, $p < 0.0001$, data not shown). Mice in the ICR-R group had significantly lower total fat pad weights than either AL or ICR-RF groups at week 13/14 and 37/38 (Table 1). There was no significant difference between the ICR-R and CCR groups for total fat pad weights at any time point ($p > 0.05$) although the ICR-R group had lower total fat pad weights than CCR group at all three time points (Table 1). Changes in body weight from week 10 until 73/74 weeks of age were significant for all groups. This was also true for total fat pad weight except for the ICR-R mice (Table 1).

3.2. The Effects of Different Calorie Restriction Protocols on Pro-inflammatory Cytokines Level

IL-6—Mice in the ICR-R and CCR groups had significantly lower IL-6 levels compared to those in either the AL or ICR-RF groups at week 13/14 ($p<0.05$) (Table 1 and Figure 1A). Although mice in ICR-R and ICR-RF groups had four fold and eight fold lower IL-6 levels than mice in AL group, respectively; these differences were not statistically significant at week 37/38 (Table 1 and Figure 1B). Compared to the AL group, both CR protocols (ICR or CCR) significantly decreased serum IL-6 levels at week 73/74 ($p<0.01$). Compared to the AL group, IL-6 levels in ICR-R, ICR-RF and CCR groups were approximately 24, 13, and 3.5 fold lower, respectively at week 73/74 (Table 1 and Figure 1C). There was no statistically significant difference among ICR-R, ICR-RF and CCR groups at week 73/74 although both ICR groups had approximately 4 fold lower serum IL-6 levels than did the CCR group. Changes in serum IL-6 levels with aging, from week 10 through week 73/74 were significant in AL, ICR-RF and CCR groups (ANOVA, $p<0.0001$, $p<0.01$, $p<0.003$ respectively). But, change in IL-6 level was not significant in the ICR-R group (Table 1). IL-6 levels in all dietary groups (AL, ICR-R and CCR) were increased with aging, from week 10 through week 73/74 (Table 1). Taken together, these results indicate that serum IL-6 levels are substantially decreased by 50% (ICR-R) versus 25% (CCR) calorie restriction regardless of age of the mice but with increasing age all groups did have some increases.

TNF- α —Mice in the ICR-R group had significantly lower TNF- α level compared to the other groups at week 13/14 ($p<0.01$) (Table 1 and Figure 1D). Although mice in ICR-R and ICR-RF groups had three and two fold lower TNF- α levels than mice in AL and CCR groups, respectively at week 37/38, these differences were not statistically significant ($p>0.05$) (Table 1 and Figure 1E). There was a statistically significant difference among the groups for TNF- α levels at week 73/74 ($p<0.01$) (Table 1 and Figure 1F). Compared to the AL group, TNF- α levels were 11, 4, and 1.5 fold lower in ICR-R, ICR-RF and CCR groups, respectively at week 73/74 (Table 1 and Figure 1F). There was no statistically significant difference between ICR-R and ICR-RF groups at week 73/74 but the ICR-R group had significantly lower TNF- α level than the CCR group and there was no statistically significant difference between ICR-RF and CCR groups ($p>0.05$) (Table 1 and Figure 1F). Serum TNF- α levels were significantly increased from week 10 through week 73/74 of age for the AL groups ($p<0.01$). In contrast the CCR mice had little change over the course of the experiment and the ICR-R and ICR-RF groups had decreased levels with increasing age ($p<0.002$, $p<0.04$, respectively, Table 1). Taken together, these results indicate that serum TNF- α levels are decreased by calorie restriction, especially by the ICR feeding protocol and serum TNF- α levels are increased with aging AL fed mice.

IL-6 to TNF- α ratio—There was no statistically significant difference among the groups for IL-6 to TNF- α ratio at any time points. Calorie restricted groups had lower IL-6 to TNF- α ratios compared to the AL group at week 13/14 (Table 1). ICR-R, ICR-RF and CCR groups had 18, 3 and 7 fold lower IL-6 to TNF- α ratio levels than the AL group, respectively at week 13/14 (Table 1) but these differences were not statistically significant ($p>0.05$). In addition, ICR-R and CCR groups had considerably lower IL-6 to TNF- α ratio levels than the AL group at week 73/74 (Table 1). Compared to the mice in AL group, mice in ICR-R and

CCR had 4 and 2 fold lower IL-6 to TNF- α ratio levels, respectively at week 73/74 (Table 1). Although IL-6 to TNF- α ratio levels were increased with aging, from week 10 through 73/74 weeks of age in all groups, especially for AL and ICR-RF mice, this change was significant only for ICR-RF group (ANOVA, $p < 0.001$, Table 1).

3.3. The Effects of Different Calorie Restriction Protocols on Adipokine Levels

Adiponectin—Mice in the ICR-R group had significantly higher serum adiponectin level compared to mice in AL or CCR groups at week 13/14 ($p < 0.001$) (Table 2). Although mice in ICR-RF group had two fold higher adiponectin level than did mice in the AL group, this difference was not statistically significant at week 13/14 (Table 2). There was no significant difference among the dietary groups for adiponectin levels at either weeks 37/38 nor at 73/74 ($p > 0.05$). Although adiponectin levels of ICR-R and CCR mice were 1.6 fold higher than in the AL group at week 73/74, this difference was not statistically significant by ANOVA analysis (Table 2). However, the difference between either AL and ICR-R or AL and CCR mice was statistically significant when it was analyzed with Student's t-test at week 73/74 ($p < 0.05$ for AL vs ICR-R and $p < 0.02$ for AL vs CCR). There was no statistically significant difference between ICR-R and ICR-RF groups. Changes in serum adiponectin levels with aging, from week 10 through week 73/74 were significant ($p < 0.03$) for AL mice primarily due to an increased value at week 37/38. For the ICR-R mice values at all three ages were two-fold higher than the initial measurement ($p < 0.003$) (Table 2). ICR-RF and CCR mice also had a two fold increase but these values were not significantly different over time. Taken together, these results indicate that calorie restriction moderately increased serum adiponectin levels while, in mice fed ad libitum adiponectin levels either did not exhibit change or moderately increased with aging.

Leptin—There were no significant differences among the groups either at week 13/14 or week 37/38 ($p > 0.05$) (Table 2). At 73/74 weeks of age serum leptin level in the ICR-R group was significantly lower (2 fold) than in the other groups, ICR-RF, CCR, and AL which were all similar (Table 2). Serum leptin levels increased gradually with aging in all dietary groups (Table 2). These results confirm that serum leptin level was significantly increased with aging and decreased by calorie restriction at older age in wild type female mice.

Adiponectin to Leptin Ratio—There has been speculation that the adiponectin to leptin ratio may be a better reflection of how these adipokines impact tissue proliferation. Here we found that there was no statistically significant difference for this ratio among the groups at either week 13/14 or week 37/38, but it was significantly higher, 3 to 5 fold in ICR-R groups compared to the other groups at week 73/74 ($p < 0.05$) (Table 2). The adiponectin to leptin ratio was decreased dramatically with aging, from week 10 through week 73/74 in all groups but to a lesser degree in the ICR-R mice. This change was significant for all groups ($p < 0.05$ or greater for all groups) (Table 2). These results show that the adiponectin to leptin ratio was significantly decreased with aging but to a lesser degree by calorie restriction, especially by ICR.

3.4. The Effects of Different Calorie Restriction Protocols on IGF-I and Insulin Level

IGF-I—Mice in the ICR-R group had significantly lower IGF-I level compared to those in the other groups both at week 13/14 and week 37/38 ($p<0.0001$ and $p<0.05$, respectively) (Table 2). IGF-I levels of mice in the ICR groups (both ICR-R and ICR-RF) were significantly lower than those in the AL group at week 73/74 ($p<0.05$) while the value for the CCR mice was intermediate between AL and ICR groups and not significantly different from any of them. Changes in serum IGF-I levels with aging, from week 10 through week 73/74 were significant only for the ICR-R group (ANOVA, $p<0.007$) which essentially was reduced from the starting level and maintained at a lower level at older age points.

Insulin—Unexpectedly we found that mice in ICR-R group had significantly higher serum insulin level compared to the other groups (ICR-RF, CCR and AL) at week 13/14 and 37/38 ($p<0.001$ and $p<0.03$, respectively) (Table 2). There were no significant difference among AL, ICR-RF and CCR at week 13/14 or 37/38. Insulin levels of mice in all calorie restricted groups were significantly higher than those in AL group at week 73/74 ($p<0.001$). There was no significant difference between ICR-R and ICR-RF groups or ICR-R and CCR groups but, the difference between ICR-RF and CCR was statistically significant at week 73/74 ($p<0.03$) (Table 2). Changes in serum insulin levels with aging, from week 10 through week 73/74 was significant in all groups (ANOVA, $p<0.003$ or greater) (Table 2). Insulin levels gradually increased over time in all groups. These results show that serum insulin level increases with aging and is higher in ICR groups compared to AL group in wild type mice in this study.

3.5. The Effects of Different Calorie Restriction Protocols on Leptin to Insulin, IGF-I to Insulin, and Adiponectin to IGF-I Level

We also calculated different ratios of these serum measurements (leptin to insulin, IGF-I to insulin, and adiponectin to IGF-I) to determine the effects of different CR protocols (Table 3). There were no significant differences among the dietary groups for either leptin to insulin or IGF-I to insulin ratios at weeks 13/14 or 37/38. But, both ratios were significantly lower in all CR groups compared to the AL group at week 73/74 ($p<0.01$) (Table 3). Ratio of adiponectin to IGF-I in the ICR-R group was significantly higher than that of the rest of the groups (ICR-RF, CCR and AL) both at week 13/14 and 37/38 ($p<0.0001$) (Table 3). There was no significant difference between AL and CCR groups for adiponectin to IGF-I ratio levels at any time points. But, the ICR-R group had significantly higher adiponectin to IGF-I ratio compared to either AL or CCR groups at all points ($p<0.001$) (Table 3). The levels of adiponectin to IGF-I for all groups were increased with aging, from week 10 through week 73/74 (Table 3). These results indicate that ICR increases adiponectin to IGF-I ratio while it decreases IGF-I to insulin and leptin to insulin ratios at week 73/74 in wild type mice. But, the CR protocols had no significant effect on leptin to insulin and IGF-I to insulin ratio levels both at weeks 13/14 (short-term) and 37/38 (mid-term) (Table 3).

3.6. Correlation Between Body Weight and Serum Pro-inflammatory Cytokines or Adipokines

When all the data for an experimental group were pooled there was a significantly positive correlation between body weight and the following serum parameters: IL-6 ($p<0.001$, $r=0.28$), TNF- α ($p<0.0006$, $r=0.30$), leptin ($p<0.0001$, $r=0.58$), IGF-I ($p<0.0006$, $r=0.33$), insulin ($p<0.001$, $r=0.28$) (Table 4 and Figure 2). But, there was a negative correlation between body weight and adiponectin to leptin-ratio ($p<0.0001$, $r=-0.57$) while there was no correlation between body weight and adiponectin levels (Table 4). Detailed correlation analyses results for each dietary groups at each time points are reported in Table 4. When regression analysis was performed, there was a significant association between BW and the following serum parameters: IL-6 ($p<0.0001$), TNF- α ($p<0.02$), and IGF-I ($p<0.0001$). Regression analyses results are given as a supplementary data (Supplementary Table 1).

3.7. Correlation Between Serum Pro-inflammatory Cytokines or Adipokines

When all the data from different time points within the same dietary group were pooled and analyzed there was a positive correlation between IL-6 and TNF- α levels ($p<0.0001$, $r=0.40$); leptin and IL-6 ($p<0.001$, $r=0.28$); leptin and TNF- α ($p<0.002$, $r=0.29$); leptin and insulin ($p<0.0001$, $r=0.69$); as well as leptin and adiponectin ($p<0.05$, $r=0.19$) (Table 4 and Figure 2). Interestingly, there was a negative correlation between IGF-I and insulin levels ($p<0.001$, $r=-0.33$); however, no correlation was detected between IGF-I and the following parameters adiponectin, IL-6, TNF- α , leptin, or A/L ratio (Table 4). Furthermore, no correlation was detected between adiponectin and TNF- α levels while there was a weak positive correlation between serum adiponectin and insulin levels ($p<0.05$, $r=0.18$) (Table 4). Other possible correlation analyses results for each dietary subgroups at time points are reported in the Table 4. When regression analysis was performed, there was a significant association between between IL-6 and TNF- α levels ($p<0.0001$); leptin and IL-6 ($p<0.018$); leptin and TNF- α ($p<0.0008$); leptin and insulin ($p<0.0001$). Regression analyses results are given as a supplementary data (Supplementary Table 1).

4. DISCUSSION

Even though one of the main goals of cancer researchers is to prevent cancer development and/or assess its pathologic outcomes without interference of any oncogenes, most studies are done with either transgenic animals or cancer patients. Therefore, non-transgenic mice models which mimic healthy individuals with normal body mass are perhaps better to use to determine effects of interventions without the confounding presence of disease. This present study was a follow-up to a series of previously published experiments for our laboratory where as indicated in the introduction we found that ICR was superior over CCR in the prevention of mammary tumorigenesis in a transgenic mouse model of breast cancer [1, 6, 9, 12]. Overall calorie restriction level was approximately a 25% reduction for both restriction protocols and diets were based on AIN-93M diet that was formulated to be used in long term rodent experiments [44].

For the CCR and ICR diets nutrients were altered with the goal that except for carbohydrate all other nutrients in absolute amounts were similar among the groups. To our knowledge

this is the first study to report long term (64 weeks) effects of different calorie restriction protocols on pro-inflammatory cytokines in wild type female mice although a number of short term calorie restriction studies have reported on the effects of cardiovascular disease, diabetes and cancer on cytokines in obese human populations and transgenic mice models [2–4, 16, 19, 22, 30, 33, 45].

Although the effects of CR on IL-6 and TNF- α have been reported in previous studies, to our knowledge the effects of CR on IL-6 to TNF- α ratio is being reported for the first time. However, there are earlier studies which report the IL-6 to TNF- α ratio levels for innate immunity [46] and oral lichen planus [47]. It is worth noting that both IL-6 and TNF- α are pleiotropic cytokines involved in several biological processes in normal health and disease conditions such as metabolic homeostasis, inflammation and anti-tumor response. IL-6 functions through binding to a membrane receptor (IL-6R or CD126) or to its soluble form (sIL-6R). On the other hand, TNF- α binds to two distinct receptors: TNF- α receptor 1 or 2 (TNFR1 and TNFR2). Interestingly, the soluble receptors of TNF- α (sTNFR1 and sTNFR2) inhibit the biologic activity of their ligand and act as antagonists; while the sIL-6R amplifies IL-6-mediated signaling [48]. Nevertheless, IL-6 is produced by many cell lineages, including fibroblasts, muscle cells, endothelial cells, keratinocytes, macrophages, T cells and mast cells [49, 50]. IL-6 can also be produced by adipocytes and adipose-derived stromal cells or stromal vascular fraction (SVF) [51], as well as by cancer cells [49]. Whereas, TNF- α is mainly expressed by activated macrophages, NK-cells and T-lymphocytes [52]. Other cell types, e.g., Kupffer cells, fibroblasts, astrocytes, smooth-muscle cells, keratinocytes, adipocytes, and tumor cells have been shown to express TNF- α [51–53]. It has been recognized that in obesity, macrophages from SVF are the primary source of adipose-derived TNF- α and higher circulating levels of TNF- α are due to the increased infiltration of adipose tissue with M1 macrophage [53].

Here we report that both short- and long-term CR significantly reduce serum IL-6 and TNF- α levels. This change was greater in ICR-R mice after periods of 50% calorie restriction compared to CCR mice with 25% restriction. These findings are supported by earlier reports in human population. For example, levels of serum IL-6 along with other inflammatory cytokines were decreased after one year of CCR in postmenopausal obese women who had a 10% weight loss resulting from exercise combined with CCR (1,200–2,000 kcal/day based on body weight) [4]. In another study, Harvie et al., examined the effects of ICR (two days a week restricted intake) vs CCR with an overall 25% restriction per week for both groups of overweight/obese postmenopausal women on inflammatory markers after six months of intervention [30]. Both ICR and CCR groups had favorable changes with reductions in inflammatory markers. The investigators concluded that ICR is as effective as CCR and may be an alternative approach to CCR for weight loss and reducing disease risk factors [30]. Moreover, the inhibition of the expression of inflammatory genes by 41% CR for 11 months was reported in male mice [37]. Furthermore, modulation of inflammation response by dietary factors including intake of saturated fat, trans fatty acids and conjugated fatty acids have also been reported in human subjects [54]. On the other hand, serum IL-6 and TNF- α levels of 25-year-old normal-weight males who fasted for 20-hour periods on alternate days and ate their normal diet in a AL fashion for two weeks, were not significantly changed [55]. In the present study the levels of IL-6, TNF- α , and the ratio of IL-6 to TNF- α were

increased with aging in all dietary groups these increases were most apparent in the AL group and least in ICR mice. These findings indicate that one of the reasons for chronic disease including cancer could be increasing levels of IL-6, TNF- α , and their ratio with aging and/or ad libitum eating habit.

We also studied the effects of these two CR protocols (ICR and CCR) on serum adiponectin, leptin and the adiponectin to leptin ratio levels because adiponectin is considered as an anti-inflammatory protein due to its inhibitory effects on TNF- α , expression of monocyte adhesion molecules, and foam cell formation [56, 57]. In the current paper, we found that CR increased serum adiponectin levels compared to the AL group (Table 2). There was a slight increase in serum adiponectin levels with aging in the calorie restricted groups. These findings are similar to previous reports [1, 11, 58–62]. For example, 30% CR were applied to five-month-old wild type C57BL6 and transgenic male mice that overexpressed growth hormone for six weeks it resulted in significant increases in serum adiponectin levels [62]. Similar findings were also reported in human studies. For example, 25% CR in a ICR protocol over six months did not have any significant effects on adiponectin level in obese women [30], but 20 hour fasting every other day in a ICR protocol for 15 days increased serum adiponectin level by 37% in healthy men [55]. In the current paper, serum leptin level was increased with aging but was decreased by CR especially in the ICR-R mice at week 73/74 (Table 2). Interestingly, one week of refeeding erased three weeks of 50% calorie restriction on serum leptin level at week 73/74. Although, calorie restriction significantly reduced serum leptin levels in some humans and animal studies, it did not have any significant effects in others [1, 11, 30, 31, 60, 62–65].

Overall the adiponectin to leptin ratio decreased considerably with aging. The decrease was less in ICR-R mice than in the other groups resulting in the adiponectin to leptin ratio being significantly higher in ICR-R than in the groups (ICR-RF, CCR and AL) at week 73/74. This result is consistent with the preventive effects of ICR for health since a high level of adiponectin to leptin ratio is beneficially associated with human health. Previously increased serum adiponectin to leptin ratio by CR in MMTV-TGF- α transgenic mice model and obese women was reported [1, 11, 30]. In this context, a reduced adiponectin to leptin ratio has been reported in women with breast cancer and in-vitro using human breast cancer cell lines reduced proliferation resulted with higher ratios [66, 67]. Also a high ratio has been associated with the protective effect of ICR in the prevention of mammary tumors and prostate cancer in transgenic mice [11, 67–69]. These results suggest that the balance of adiponectin and leptin rather than their absolute levels may play important roles in pathophysiological events such as the development of cancer.

Here we report that although IGF-I level in the AL group does not change with aging, it decreases in both the ICR and CCR groups although it was significant only in ICR-R group (Table 2). The ICR-R mice had significantly reduced the serum IGF-I level compared to AL or CCR groups. This is in general agreement with earlier studies. For example both calorie restriction and starvation resulted in reduced serum levels of IGF-I [3, 12, 14, 31]. However, there was no significant difference for IGF-I levels between postmenopausal obese women who were on 25% calorie restriction for six months and those who were not restricted [30].

In the current study, insulin levels were increased significantly with aging in all groups. In addition, results for insulin levels in response to ICR were unexpected as the values were significantly higher compared to those of the AL group (Table 2). This is probably a reflection of the feeding protocol as we observed a similar response in an earlier study [70]. Mice in the ICR groups, who had finished all their food from the previous day, consumed their daily allotment as soon as it was available; while mice in the AL group had constant access to food and consumed most of their food overnight. Therefore, although food was provided at the same time of day for all mice when the food was consumed varied. Future studies should address this to ensure all animals are in similar post-prandial state during collection of blood samples. However, this has been a consistent finding in our laboratory when using this feeding protocol and results may also be due to the mice associating the presence of a human with feeding (MP Cleary unpublished data). To our knowledge this is the first longitudinal study to show the effects of ICR vs CCR on serum insulin levels in this mouse model. In a similar study with human subjects there was no significant difference between ICR and CCR for serum insulin levels [30].

We also calculated the ratios and correlations between the measurements made to see whether they are better indicators to assess the effects of calorie restriction (Tables 3 and 4). Changes in the ratio levels of leptin to insulin, IGF-I to insulin and adiponectin to IGF-I were similar to those of individual parameters. To our knowledge, the current paper is the first to report correlation between IL-6 and TNF- α or correlation between either IL-6 or TNF- α and adiponectin or leptin (Table 4). There is a positive correlation among these parameters especially in AL groups indicating cross relation of signaling pathways. But, this positive cross signaling is not detected in ICR group. This finding implies that either this cross signaling could be interrupted by ICR or it could be because of limited sample number. It is noteworthy that nutritional status has been shown to be linked with alterations in hypothalamus-pituitary-gonadal axis and associated hormonal secretions such as estrogen, leptin, and insulin [71–73]. In fact, there is a complex relationship between caloric restriction and estrogen. In a study on female Sprague-Dawley rats, restricted feeding (70% of ad libitum intake) resulted in anestrus and significant decreases in ovarian weight, estrogen and leptin levels [72]. Similarly, in rhesus monkeys, caloric restriction inhibited gonadotropin secretion and ovulation [74]. It has been also documented in human subjects that caloric restriction can cause considerable health consequences such as hypothalamic amenorrhea and infertility, involving hormones that participate in the hypothalamic-pituitary-ovarian axis, including estradiol, IGF-I and leptin [75, 76]. Alterations in various hormones and cytokines/adipokines, which are closely connected with the hypothalamus-pituitary-gonadal axis, have been recorded in a number of experimental studies on caloric restrictions [71, 77, 78]. Therefore, caloric restriction-linked modification of estrogen and/or hypothalamus-pituitary-gonadal axis could be a plausible explanation for the observed changes in different hormonal substances in the present study. In general, there was a positive correlation between body weight and the measurements made in the current study. In this context, our findings support previous reports of a positive correlation between body weight and serum leptin in mice [1, 11, 13]. On the other hand, we report weak positive correlation between adiponectin and leptin levels, but earlier studies reported either negative or no correlation between serum adiponectin and leptin levels [1, 11, 63, 79]. In the current

study we found a positive correlation between body weight and insulin. However, in previous studies there has been no consistent results for correlation of insulin with body fat which is highly correlated with body weight and serum adiponectin levels [80, 81]. However, it should be indicated that because of the nature of the study factors like age, diet and body weight may have contribute to changes in parameters at the same time. To determine specific effects of individual changes detailed more sophisticated statistical analysis could have been better. This is the limitation of the study.

In summary, we demonstrated that long term (64 weeks) calorie restriction, especially ICR reduced serum IL-6 and TNF- α pro-inflammatory cytokine levels in wild type female C57BL6 mice. We also demonstrated that the two different types of CR protocols significantly reduced serum leptin and IGF-I levels while it increased insulin levels in these mice. In general, body weight was positively correlated with all parameters. Also, there was a positive correlation between IL-6 and TNF- α levels. Although these findings imply that beneficial effects of ICR in chronic health problems might be through cytokine signaling pathways, further studies with transgenic mice models are needed to clarify the roles of ICR mediated pro-inflammatory cytokines in the prevention of chronic diseases including cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AIN	American Institute of Nutrition
AL	Ad libitum
A/L ratio	Adiponectin to Leptin Ratio
ICR	Intermittent Calorie Restricted
ICR-R	Intermittent Calorie Restricted- Restricted
ICR-RF	Intermittent Calorie Restricted- Refed
CCR	Chronic Calorie Restricted
IL-6	Interleukin-6
TNF-α	Tumor Necrosis Factor- α
IGF-I	Insulin-like Growth Factor-I
IGFBPs	Insulin-like Growth Factor-I Binding Proteins

mTOR	Mammalian Target of Rapamycin
NFκB	Nuclear Factor Kappa-B
ROS	Reactive Oxygen Species (ROS)
MT	Mammary Tumor
MFP	Mammary Fat Pad
RNA	Ribonucleic Acid
MMTV-TGF-α	Mouse Mammary Tumor Virus-Transforming Growth Factor- α
SVF	Stromal Vascular Fraction

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Figure 1.

The effects of different calorie restriction protocols on serum IL-6 and TNF α levels. Panels A, B, and C represent IL-6 levels at week 13/14, 37/38 and 73/74, respectively. Panels D, E, and F represent TNF- α levels at week 13/14, 37/38 and 73/74, respectively. Different letters represent significant difference among the groups ($p < 0.05$). AL = Ad libitum; CCR = Chronic Calorie Restriction; ICR-R; Intermittent Calorie Restricted- Restricted; ICR-RF; Intermittent Calorie Restricted- Refed. Values are presented as means $\pm$ SEM. “n” values are between 4 and 16.

Figure 2. Correlation figures between BW and IL-6 (A), BW and TNF- α (B), BW and IGF-I (C), IL-6 and TNF- α (D), Leptin and IL-6 (E), Leptin and TNF- α (F), Leptin and Insulin (G). Spearman correlation test was used to determine whether correlation between two parameters was statistically significant. All the data from different time points and all the dietary groups were pooled together and analyzed.

Table 1

The effects of different calorie restriction protocols on BW, total fat, IL-6, TNF- α , and IL-6 to TNF- α ratio levels.

		10	13/14	37/38	73/74	p value
Body Weight (grams)	AL	20.4 $\pm$ 0.7	22.4 $\pm$ 0.4 ^a	31.2 $\pm$ 1.2 ^a	33.5 $\pm$ 1.2 ^a	p<0.0001
	ICR-R		16.4 $\pm$ 0.5 ^b	20.9 $\pm$ 0.4 ^b	21.1 $\pm$ 0.5 ^b	p<0.0001
	ICR-RF		21.2 $\pm$ 0.5 ^c	26.3 $\pm$ 1.4 ^c	24.9 $\pm$ 0.8 ^{bc}	p<0.0001
	CCR		19.6 $\pm$ 0.4 ^{bc}	23.6 $\pm$ 0.8 ^{bc}	24.7 $\pm$ 0.4 ^c	p<0.0001
Total Fat Pad (grams)	AL	0.78 $\pm$ 0.1	1.1 $\pm$ 0.1 ^a	2.3 $\pm$ 0.3 ^a	2.9 $\pm$ 0.4 ^a	p<0.0001
	ICR-R		0.6 $\pm$ 0.04 ^b	0.8 $\pm$ 0.1 ^b	0.7 $\pm$ 0.2 ^b	NS
	ICR-RF		1.1 $\pm$ 0.1 ^a	1.9 $\pm$ 0.3 ^a	1.6 $\pm$ 0.3 ^b	p<0.001
	CCR		0.8 $\pm$ 0.1 ^{ab}	1.1 $\pm$ 0.1 ^b	1.6 $\pm$ 0.1 ^b	p<0.0001
IL-6 (Fluorescence)	AL	2.5 $\pm$ 1.1	12.13 $\pm$ 4.1 ^a	8.73 $\pm$ 3.3	70.88 $\pm$ 23.2 ^a	p<0.0001
	ICR-R		0.40 $\pm$ 0.2 ^b	2.03 $\pm$ 1.2	3.00 $\pm$ 1.1 ^b	NS
	ICR-RF		23.00 $\pm$ 12.3 ^a	1.01 $\pm$ 0.8	5.38 $\pm$ 2.0 ^b	p<0.01
	CCR		4.25 $\pm$ 0.8 ^b	5.13 $\pm$ 1.1	20.25 $\pm$ 5.6 ^b	p<0.003
TNF-α (Fluorescence)	AL	3.9 $\pm$ 1.1	4.08 $\pm$ 0.5 ^a	7.42 $\pm$ 4.1	9.07 $\pm$ 1.2 ^a	p<0.01
	ICR-R		1.40 $\pm$ 0.9 ^b	2.88 $\pm$ 1.2	0.80 $\pm$ 0.4 ^b	p<0.002
	ICR-RF		7.00 $\pm$ 1.7 ^a	3.00 $\pm$ 0.8	2.25 $\pm$ 0.8 ^{bc}	p<0.04
	CCR		4.25 $\pm$ 0.9 ^a	5.63 $\pm$ 2.0	5.60 $\pm$ 1.2 ^{ac}	NS
IL-6 / TNF-α	AL	0.70 $\pm$ 0.2	9.20 $\pm$ 5.8	2.72 $\pm$ 2.1	7.29 $\pm$ 3.3	NS
	ICR-R		0.50 $\pm$ 0.5	2.69 $\pm$ 2.1	1.88 $\pm$ 1.4	NS
	ICR-RF		2.77 $\pm$ 0.8	0.23 $\pm$ 0.2	6.04 $\pm$ 2.5	p<0.001
	CCR		1.29 $\pm$ 0.3	1.29 $\pm$ 0.4	3.23 $\pm$ 1.0	NS

Values are presented as means $\pm$ SEM. "n" values are between 4 and 16.

Values in the same column with different letters indicate a significant difference.

ICR-R is ICR-Restricted and ICR-RF is ICR-Refed. "p" value is for statistical changes through out the study (week 10 through week 74).

Table 2

The effects of different calorie restriction protocols on serum adiponectin, leptin, adiponectin to leptin ratio, IGF-I and insulin levels.

		10	13/14	37/38	73/74	p value
Adiponectin (µg/ml)	AL	11.5±0.9	11.1 ± 1.2 ^a	16.2 ± 1.6	13.2 ± 1.5	p<0.03
	ICR-R		26.5 ± 3.4 ^b	20.2 ± 1.3	22.4 ± 4.1	p<0.003
	ICR-RF		21.2 ± 5.9 ^{ab}	19.0 ± 1.9	19.2 ± 4.4	NS
	CCR		17.6 ± 2.5 ^a	19.8 ± 4.3	21.7 ± 2.3	NS
Leptin (ng/ml)	AL	0.38±0.2	0.9 ± 0.2	5.3 ± 1.0	6.4 ± 1.8 ^a	p<0.0001
	ICR-R		1.2 ± 0.4	3.1 ± 0.6	2.5 ± 0.8 ^b	p<0.0007
	ICR-RF		1.2 ± 0.3	2.8 ± 0.7	6.1 ± 0.7 ^a	p<0.0001
	CCR		1.4 ± 0.4	3.4 ± 0.4	7.0 ± 1.4 ^a	p<0.0001
Adiponectin to Leptin ratio	AL	97± 23	31.2 ± 11.0	4.5 ± 1.1	4.9 ± 1.8 ^a	p<0.0001
	ICR-R		35.8 ± 9.4	8.0 ± 2.0	16.5 ± 8.4 ^b	p<0.02
	ICR-RF		21.4 ± 8.9	8.2 ± 1.8	3.2 ± 0.8 ^a	p<0.005
	CCR		27.2 ± 9.6	6.4 ± 1.3	5.1 ± 1.4 ^a	p<0.0001
IGF-I (ng/ml)	AL	338±52	278 ± 18 ^a	343 ± 26 ^a	326 ± 34 ^a	NS
	ICR-R		139 ± 14 ^b	238 ± 31 ^b	191 ± 22 ^b	P<0.007
	ICR-RF		341 ± 20 ^a	284 ± 7 ^a	213 ± 36 ^b	NS
	CCR		315 ± 24 ^a	281 ± 17 ^a	259 ± 18 ^{ab}	NS
Insulin (ng/ml)	AL	0.44± 0.1	0.61 ± 0.1 ^a	2.2 ± 0.5 ^a	1.3 ± 0.3 ^a	p<0.003
	ICR-R		2.03 ± 0.8 ^b	4.9 ± 0.7 ^b	3.4 ± 0.8 ^{bc}	p<0.003
	ICR-RF		0.56 ± 0.1 ^a	2.1 ± 0.6 ^{ab}	4.8 ± 0.7 ^c	p<0.0001
	CCR		1.07 ± 0.3 ^a	1.6 ± 0.4 ^a	3.1 ± 0.5 ^b	p<0.0001

Values are presented as means ± SEM. “n” values are between 4 and 16.

Values in the same column with different letters indicate a significant difference.

ICR-R is ICR-Restricted and ICR-RF is ICR-Refed. “p” value is for statistical changes through out the study (week 10 through week 74).

Table 3

The effects of different calorie restriction protocols on the average of leptin to insulin, IGF-I to insulin, adiponectin to IGF-I ratio levels.

		10	13/14	37/38	73/74
Leptin to Insulin ratio	AL		1.6 ± 0.3	4.0 ± 1.0	3.6 ± 0.7 ^a
	ICR-R	0.80±0.27	1.6 ± 0.7	2.0 ± 1.2	0.9 ± 0.3 ^b
	ICR-RF		2.3 ± 0.4	2.4 ± 1.0	1.4 ± 0.3 ^b
	CCR		1.6 ± 0.3	3.2 ± 0.7	2.4 ± 0.4 ^b
IGF-I to Insulin ratio	AL		886 ± 313	301 ± 77	297 ± 82 ^a
	ICR-R	1451±717	296 ± 129	205 ± 159	65 ± 20 ^b
	ICR-RF		653 ± 53	306 ± 146	36 ± 7 ^b
	CCR		520 ± 123	227 ± 96	110 ± 23 ^b
Adiponectin to IGF-I ratio	AL		41 ± 4.4 ^a	52 ± 8.3 ^a	54 ± 10.8 ^a
	ICR-R	41.8 ± 7.1	246 ± 50.3 ^b	106 ± 6.6 ^b	178 ± 42 ^b
	ICR-RF		59 ± 16.7 ^a	63 ± 7.3 ^a	122 ± 27.3 ^{bc}
	CCR		63 ± 13.7 ^a	38 ± 9.6 ^a	88 ± 9.1 ^{ac}

Values are presented as means ± SEM. “n” values between 4 and 13.

Values in the same column with different letters indicate a significant difference.

ICR-R is ICR-Restricted and ICR-RF is ICR-Refed.

Table 4

Correlation between BW and serum parameters or between the two parameters.

	All mice	AL	ICR-R	ICR-RF	CCR
BW and IL-6	p<0.001 r=0.28	p<0.01 r=0.34	NS	p<0.01 r= - 0.69	NS
BW and TNF- α	p<0.0006 r=0.30	NS	NS	NS	NS
BW and Leptin	p<0.0001 r=0.58	p<0.0001 r=0.84	p<0.03 r=0.60	p<0.03 r=0.60	p<0.0001 r=0.55
BW and IGF-I	p<0.0006 r=0.33	NS	p<0.007 r=0.61	NS	NS
BW and Insulin	p<0.001 r=0.28	p<0.0001 r=0.84	p<0.03 r=0.60	p<0.03 r=0.60	p<0.0001 r=0.55
BW and A / L ratio	p<0.0001 r= - 0.57	p<0.0001 r= - 0.75	p<0.05 r= - 0.56	NS	p<0.001 r= - 0.44
BW and Adiponectin	NS	NS	NS	NS	NS
IL-6 and TNF- α	p<0.0001 r=0.40	p<0.003 r=0.39	NS	p<0.002 r=0.83	p<0.02 r=0.33
Leptin and IL-6	p<0.001 r=0.28	p<0.008 r=0.36	NS	NS	NS
Leptin and TNF- α	p<0.002 r=0.29	p<0.05 r=0.27	NS	NS	NS
Leptin and Insulin	p<0.0001 r=0.69	p<0.0001 r=0.59	p<0.0001 r=0.75	p<0.02 r=0.65	p<0.0001 r=0.77
Leptin and Adiponectin	p<0.05 r=0.19	p<0.05 r=0.28	NS	NS	p<0.0001 r=0.51
IGF-I and Insulin	p<0.001 r= - 0.33	NS	p<0.02 r=0.68	p<0.007 r= - 0.73	p<0.0004 r= - 0.63
IGF-I and Adiponectin	NS	NS	NS	NS	NS
IGF-I and IL-6	NS	NS	NS	NS	NS
IGF-I and TNF- α	NS	NS	NS	NS	NS
IGF-I and Leptin	NS	NS	NS	NS	NS
IGF-I and A/L ratio	NS	NS	NS	p<0.02	NS
Adiponectin and TNF- α	NS	NS	NS	NS	NS
Adiponectin and Insulin	p<0.05 r=0.18	NS	p<0.05 r=0.36	NS	p<0.02 r=0.33

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	All mice	AL	ICR-R	ICR-RF	CCR
Adiponectin and IL-6	NS	NS	NS	NS	NS
A/L ratio and TNF- α	p<0.01 r=0.22	NS	NS	NS	NS
A/L ratio and IL-6	p<0.001 r=0.28	p<0.01 r=0.33	NS	NS	p<0.04 r=0.29
A/L ratio and Insulin	p<0.0001 r= - 0.55	p<0.0001 r= - 0.58	p<0.0001 r= - 0.64		p<0.0001 r= - 0.70

ICR-R is ICR-Restricted; **ICR-RF** r= - 0.55 is ICR-Refed;r= - 0.58 **A/L** r= - is Adiponectin to Leptin 0.64 r= ratio. - 0.70