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Calorie restriction reduces low grade inflammation and ameliorate outcome of Non-alcoholic fatty liver disease

Ahmed Abdelsadik
Zoology Department, Faculty of Science, Aswan University
81528 Aswan. Egypt

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***Correspondence to:**
aabdelsadik@aswu.edu.eg

ABSTRACT

Cardiovascular diseases, systemic inflammation and metabolic syndromes (METS) become great global concern. Obesity mostly related with all previously described complications include non-alcoholic fatty liver (NAFLD). Different models and studies were designated to resolve the clues of obesity and related disorders. These concerns raised the alarm against fatty food especially, when allocated with physical dormancy due to the modern lifestyle.

A good alternative to avoid complication-related obesity by reducing daily calorie intake which defined globally as calorie restriction (CR) and may represent an important therapeutic approach. The current study were designed to assess the consequence of CR on body weight as an indicator for obesity and how this can reflected on the general health status in the rat model.

1. INTRODUCTION

Global economic and health burdens of obesity become the most occupant health problem for health care professionals and scientists in the current era (Tremmel et al. 2017). WHO has been reported approximately 500 million individual suffered from obesity (BMI being 30 kg/m2) in 2011, and this number are expects to enlarge at least to double or even more until 2030 *(http://www.who.int/mediacentre/factsheets/fs311/en/)* . The main problem of obesity in its participation and connection to multiple metabolic syndromes (METS) such as Type 2 diabetes, Insulin resistance (IR), inflammation, Non-alcoholic Fatty Liver (NAFLD) and asthma (Popkin and Doak 1998, Husemoen et al. 2008, Chang et al. 2016, Gorodnya et al. 2016). These side effects of obesity are interconnected with the expansion of morbidity and mortality in patients with cardiovascular and cerebrovascular diseases (Mraz and Haluzik 2014).

Obesity simplest definition as a complex and dynamic disorder that countless factors play major roles in its progression. These factors range from genetic traits that individuals can inherit from their relatives and can be explained by more distribution on particular families than others to environmental, demographical and even cultural factors. In addition, nowadays this distribution has been changed and people with non-familiar history of obesity become overweight and finally obese. This distribution of obesity in the whole world brings new questions forward, how environmental factors can predispose people more to obesity. Moreover, whether our modern lifestyle of being mostly immobile due physical inactivity is a significant key in the progression of obesity (Injae et al. 2015). Further interrogations regarding newly feeding behavior of modern man, especially consuming diet rich in fat or what's known as western high fat diet (*wHFD*) is probably one of the central factors led to obesity (Coelho et al. 2011, Waller-Evans et al. 2013). In fact, association of mean dietary fat intake is related to obesity and development of numerous METS as have been evidenced in numerous epidemiological studies (Kaur 2014).

This interplay of obesity and METS associated with quantity of fats that stored in the Adipose Tissue (AT). An individual become overweight and gradually moves ahead to obesity which is accompanied with increase in AT which is an important reservoir for excessive energy not to be used by the body, as well it is a key player in the development of METS (Choe et al. 2016). Although AT is participating in different vital processes such metabolism, hormone regulation and immunity by secreting different adipokines that includes wide spectrum of cytokines and steroids that affect locally and on remote organs (Mraz and Haluzik 2014, Trayhurn and Wood 2004) and play a crucial role in the whole-body balance (Gabrielsen et al. 2012).

Adipose tissue is mostly consisted of two subcomponents, adipocytes (mature fat cells) and the preadipocytes (immature fat cells, precursors). AT resident immune cells include most components of immune cell types, encompass most important functions in tissue homeostasis, scavenging of the remnant, and apoptosis in non-obese conditions. Therefore, redundant fat accumulation lead to functional and numerical changes in the immune cells, increasing a number of some of them while reducing others, including macrophages and various subclasses of T and B- lymphocytes. This imbalance is the central dogma of the development of obesity-related local and systemic inflammation (Cildir et al. 2013).

Despite a common feature of obesity is the increment of proinflammatory molecules with the augmented invasion of inflammatory cells into adipose tissue and adipocytes has been defined as source and target for several proinflammatory molecules (Boutens and Stienstra 2016). However, the proinflammatory cytokines show variable effects on mitochondrial metabolism of adipocytes, regulate oxidative stress, and dynamics. (Hahn et al. 2014). Moreover, overproduction of IL-6 induced hypertriglyceridemia, promotes hepatic triglyceride secretion and lipolysis (Tanaka et al. 2014).

The modern perspectives of obesity-associated AT inflammation builds on earlier studies whether using an animal models that employing different metabolic tools (e.g., high-fat diet challenges) or human studies of obese individuals (Ichimura et al. 2012, Kang et al. 2016). These studies point to important prospective, but these results did not provide information about the reduction mechanism of AT-associated inflammation when calorie restriction regime is utilized. In the current experiment variable phenotypic, biochemical and immunological factors were studied to explain the effect of CR on AT-associated inflammation. This is a vital approach in the field of obesity-related research, because therapeutics, leanness protocols, and regimes that target modest obesity and early METS become an insistent demand. Further insights into these approaches can provide innovative therapies and lease risk of METS amid obese patients and normal population.

2. MATERIALS AND METHODS

2.1. Groups

Juvenile male albino rat (40 – 50 g) obtained from Qena breeding Centre (Qena, Egypt) was housed in cages with corncob bedding in a temperature (25 ±1°C). All animals have free food access and drank water *ad libitum* for 1 week before being assigned to dietary groups. All procedures were approved by the University of Aswan, Internal Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals.

2.2. Diets.

Each 100 gm of High Fat Diet (HFD) contains 20g of fat (19 g of butter oil and 1 g of soybean oil) that provided 19.34 kJ/g of diet, including 7.74 kJ/g as fat. The calorie restriction Diet (CRD) contained 04g of fat (3 g of butter oil and 1 g of soybean oil) provided 16.12 kJ/g of diet, including 1.29 kJ as fat. Amounts of vitamins, protein and minerals were standardized for all experimental groups (Reeves et al. 1993).

2.3. Experimental groups.

Forty five male albino rat were divided into three groups HFD, CRD and control diet (CD). Because HFD and CRD groups were set to fed equal amounts of energy each day. That is, these group were fed the same proportion of dietary fat as the HFD group but had their energy intake yoked to that of the CRD group. Mean daily energy intake of CRD group was calculated every 3 days, and the yoked group were given that precise amount of energy each day, but as the HFD diet. Over the course of 90 d, the mean energy intake of the CRD and the CD groups were 83% of the energy intake of the HFD group.

2.5. Dietary manipulations.

Change in body weight were recorded daily for the first week and then once weekly for the course of the experiment.

2.6. Response to energy deprivation.

Experimental groups were assigned by equal initial body weight to HFD and CRD or CD for 90 d.

2.7. Body composition.

Carcasses were individually wrapped and frozen at -20°C for at least 24 hours. Carcass of each animal was sectioned into four equal parts and weighed accurately. These sections were dried to constant weight (3–5 d, typically) as described earlier with minor modifications (Woods et al. 2003). Briefly, each carcass treated with enough salt and dried at 60°C, then carcass was individually wrapped in Whatman paper, placed into a protective cotton sack and placed into methanol in boiling water path for 6-8 h. This duration was sufficient to remove all lipids from the carcass. Weight loss after being dried was recorded as water weight and weight loss after being treated with methanol was recorded as carcass lipid weight.

2.8. Insulin-tolerance tests.

Group withdrawn from food for a period of 5–6 hrs. at the beginning of the light period. Blood samples collected from the tail at 0, 15, 30, 45 and 60 min after an intraperitoneal injection of regular insulin (0.5 pool/kg). Blood glucose level was assessed using a handheld glucometer (Contour next; Bayer Vital Co., Basel, Switzerland).

2.9. Biochemistry tests.

Lipid profile, liver AST and ALT were performed using commercial kits from Spectrum Diagnostics using Robonik Biochemistry analyzer instrument (Robonik Pvt Ltd, India).

Liver TG were extracted from 250 mg of liver tissue as previously described (Qasem et al. 2015). Concisely, tissues were homogenized with a mixture of chloroform: methanol (2:1). Samples were washed, aqueous phase were recovered and the TG contents were determined using commercial kits from Spectrum Diagnostics (Cairo, Egypt).

2.10. ELISA assay.

TNF-α, CRP and IL-6 from serum were measured using the ELISA kits according to the manufacturers' instructions. Absorbance was read on BioTek ELx800 (BioTek Instruments Inc., USA) at 450 nm. Change in fold expression computed by cutoff values of the control group.

2.11. Serum total antioxidant status.

ABTS radical scavenging assay were assayed according to the manufacturers' instructions, using ABTS Antioxidant Assay Kit from ZenBio Inc., USA.

2.12. Liver Thiobarbituric acid reactive species (TBARS).

Homogenized liver was then mixed in a mixture of 0.25Hcl and 0.375% thiobarbituric acid. Samples and standard absorbance was read at 532 nm using Robonik Biochemistry analyzer instrument (Robonik Pvt Ltd, India), the results were expressed as μM MDA/mg tissue (Chan et al. 2012).

2.13. Histology.

Paraffin-embedded liver tissues were dewaxed and rehydrated in a chain of graded alcohols. Stained with haematoxylin-eosin (HE), then sections were mounted with DePex mounting medium (Gurr, Dorset, UK).

2.14. Statistical analyses.

Data were analyzed by parametric statistics (ANOVA and repeated measures ANOVA and t-tests, with Turkey's test used as appropriate) as described for each experiment, with set at $P<0.05$, two-tailed.

3. RESULTS

3.1. Effect of calorie restriction on body composition

Using body weight as the sole indicator of obesity, was a good choice because the difference in mean body weight were significantly different. The mean body weight of the HFD group increased about 29% more than CD group ($P\leq0.0001$). Moreover, comparing body weight of HFD to the CRD showed a dramatic increase that was about108.54% more than the CRD group ($P\leq0.0001$) after 12 weeks of being fed the respective diets. Carcass analyses were conducted on a randomly selected sample of 8 rats per group. Regarding this, the HFD group had 73.56% and 187.74% more carcass fat than the CD group and CRD group respectively. The HFD group had the uppermost proportion of an entire weight as water than all other groups ($P\leq0.01$), and it seems that carcass fat does not correlate with this value across all groups ($r=-0.883,\ P\leq0.582$) as shown in Table 1.

When body fat (as a percent of carcass weight) was normalized to that of the CD group often-used control group in such experiments (Fève and Bastard 2009), the HFD group had proportionally more fat than the CD group (158.77%, $P\leq0.05$) and the CD group was inserted for comparison purposes.

Table 1. Body weight and percentage of fat in carcass. Analysis of body weight and fat content using carcass of approximate 8 rats per group. Values are means ±SEM, n=8. Means in a row with a common superscript letter compared to control (a) and (b) to HFD group differ, P≤0.05.

Item	CD	HFD	CRD
Body weight	258.88±3.03[a]	333.84±5.2[a]	160.08±2.76[a,b]
Carcass fat,g	31.56±1.41[a]	54.77±4.32[a]	19.03±1.33[a]
Fat, g/100 gm of carcass	5.42±0.53[b]	9.082±0.602[a]	5.096±0.54[a]
water, g/100 gm of carcass	24.33±0.85[a]	40.86±0.62[a]	13.95±0.48[a]
Fat normalized to the CD group, %	-	158.77±12.7[a]	89.74±6.6[b]
Body weight normalized to the CD group, %	-	1.29±0.02[a]	0.63±0.012[a]

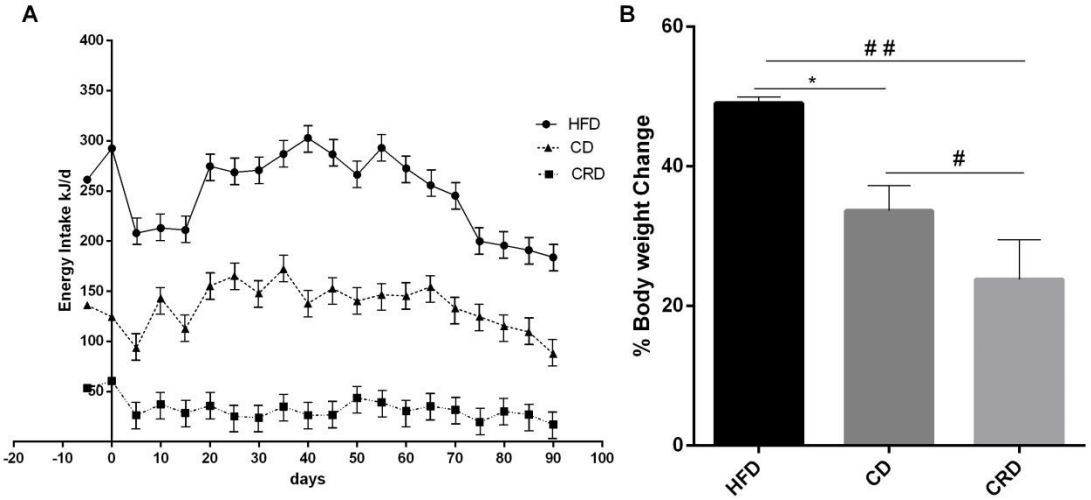

Figure 1. Energy intake and percentage of change in body weight. Energy intake showed that at the beginning of week 3, was fed more energy than CRD and CD group (A). Percentage of body weight change after 12 weeks of treatment calculated as a difference from the initial body weight of the group at the time of beginning (B). Values with differing significantly at the end of the experiment, $P \leq 0.05$. (*) represents significance difference when compared to CD group, while ($^{\#}$) when compared to the CRD group.

3.2. Effects of calorie restriction on food intake, energy intake and body weights

Although the food intake remained similar between the groups throughout the period of the experiment (data not shown), there were significant differences observed in the body weights of the different groups, the CRD group had the lowermost body weight across all the groups. It appears that calorie restriction has not the ability to yield a greater satiety, consequently reduces body weight safely without the risk of feeding relapse.

Furthermore, the CRD group initially were fed less energy per day than HFD and CD group ($P \leq 0.05$, week 1), and this trend persisted for the whole course of the experiment (Fig. 1A). This affects the body weight of the all groups lead to significant differences by the end of the twelfth week, the CRD group percentage of change in body weight was 20% while HFD and CD group were 49% and 31% respectively (Fig. 1B).

3.3. Influence of calorie restriction on lipid profile

Worsening of lipid profile is mainly associated with obesity, insulin resistance and cardiovascular diseases, while their improvement deteriorates chance for METS (Lumeng and Saltiel 2011). In this study HFD had higher levels of cholesterol and triglycerides, whereas those parameters were significantly lower in the CRD ($P \leq 0.0001$) and CD group ($P \leq 0.0004$). Moreover, CRD group show improvement in HDL and highly significant of LDL whether compared to CD or the HFD as shown in Table 2.

High fat intake increase levels of the major atherogenic lipoprotein LDL, cholesterol and levels of triglycerides while decreased level of the beneficiary anti-atherogenic HDL. In contrast calorie restriction

reverse the process and reestablish the atherogenic homeostasis.

3.4. Impact of calorie restriction on liver enzymes

Liver TG content was assessed that considered a main determinants in development of fatty liver disease. Levels of TG exceeds the acceptable value which considered a risk factor for NAFLDD. The levels of hepatic TG in HFD group was 101 mg, 74.4 and 62 mg for CD and CRD respectively. Statistically analyzed data showed that there no difference among CD and CRD group ($P>0.05$), while liver TG content was significantly higher in the HFD in comparison to CD and CRD ($P<0.001$) as shown in figure 2A. These data in accordance with previous reports on the diagnosis level of TG content in NAFLD which is ≥ 100 mg/ g liver (Vanni et al. 2010).

The higher level of TG contents in hepatocytes is associated with changes of liver enzymes ALT and AST. Level of ALT were significantly higher in the HFD group when compared to CD and CRD ($P\leq0.0025$ and $P\leq0.0018$) respectively (Fig. 2B). A slight elevation was recorded in the level of AST in the HFD rats in comparison to CRD ($P\leq 0.037$) as shown in Fig. 2C. Interestingly, TG level was not correlated with AST level ($r= 0.4211,\ P\leq 0.0818$), while it was strongly correlated with the level of ALT ($r= 0.7784, P<0.001$) as shown in figure 2D.

Table 2: Serum lipid profile

Variables	CD	HFD	CRD
Cholesterol (mmol/L)	5.95±0.46	7.29± 1.05[a,b]	1.91±0.23[a,c]
TG (mmol/L)	1.0±0.1	1.50±0.14[a,b]	0.88±0.06[a,c]
LDL (mmol/L)	4.7±0.39	5.41±0.49[b]	1.69±0.29[a,c]
HDL (mmol/L)	1.42±0.12	1.68±0.18[b]	1.08±0.04[a,c]

Table 2. Serum lipid profile. Values are means ±SEM, n=8 for cholesterol, triglycerides, HDL and LDL. Means in a row without a common superscript letter differ, $P>0.05$. The superscript letters refers to significance difference in comparison to CD group (a), CRD (b) and HFD (c)

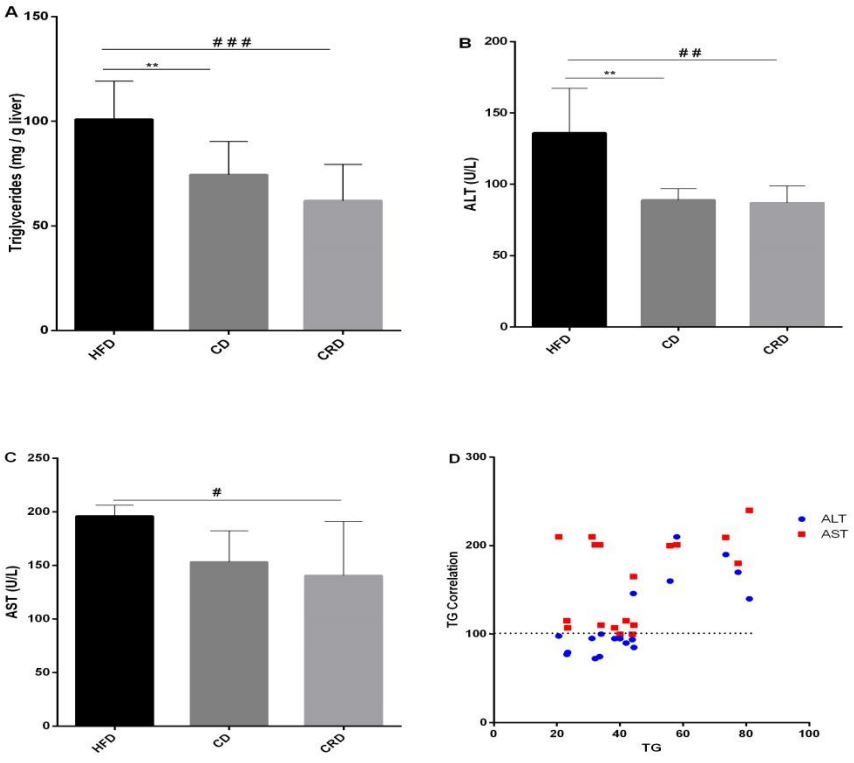

Figure 2. Liver TG content and hepatic enzymes. Hepatic TG content (A). Alanine amino transferase ALT (B); Aspartate transaminase AST (C) and TG correlation with ALT and AST (D). Error bars reflect ±SEM. *$P<0.05$; **$P<0.001$; ***$P<0.0001$ versus the CD or CRD group. (*) represents significance difference when compared to CD group, while ($^{\#}$) when compared to the CRD group.

3.5. **Calorie restriction regulate free radical activity**

Hyperlipidemia is commonly associated with augmented levels of free radicals. In the current study deteriorating of lipid profile, elevation of liver enzymes and obese phenotype in HFD group was accompanied with declining in free radical scavenging activity, this was proven by the reduction in total antioxidant status (TAS) of about 56% as shown (Fig. 3A). Furthermore, the increased lipid peroxidation and release of reactive oxygen species as indicated by MDA which increased about 64% as shown by TBARS assay in comparison with the CD group (Fig.3B). In conclusion, calorie restriction significantly improved scavenging activity by increasing TAS and reducing TBARS, consequently improve the overall health status and prevent development and progression of inflammation, atherosclerosis and aging.

Increased free radical in the liver may worsen the outcome of NAFLD and eventually can progress to cirrhosis under uncontrolled conditions (Sumida et al. 2013). Histological studies showed that dramatic changes in the hepatocytes of HFD group which characterized by increasing the lipid to cytoplasm ratio, hepatocytes ballooning and degeneration and none of these changes can be seen neither in the liver of CRD nor in the CD group (Figure 4).

3.6. Correlation of calorie restriction with plasma insulin and insulin resistance

The correlation between insulin resistance, glucose tolerance and obesity has been approved by many researchers. Based on this calorie restriction assumed to have positive effects on insulin sensitivity. Effect of calorie restriction on the levels of glucose and insulin was assessed using insulin ELISA kit and the insulin resistance index (HOMA-IR) was estimated as formerly reported (Yida et al. 2015).

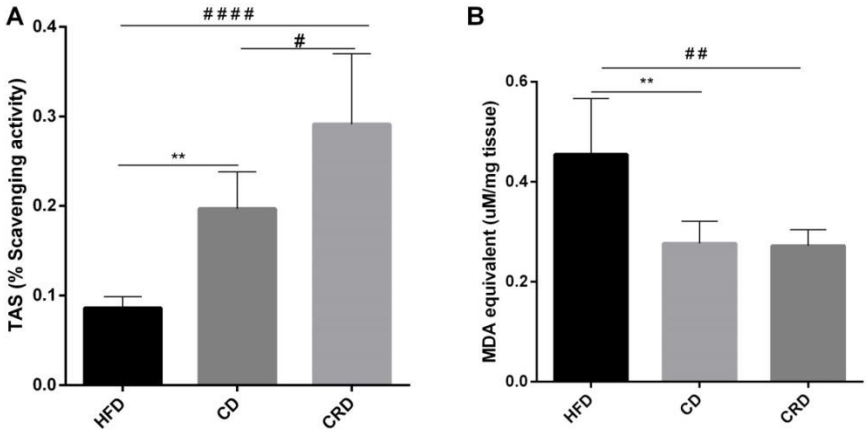

Figure 3. Free radical scavenging activity. Hepatic scavenging activity of ROS was higher in CRD group and diminished significantly in the HFD group (A). Lipid peroxidation was higher in HFD-as indicated by MDA (B). Error bars reflect ±SEM. *p < 0.05; **p < 0.001; ***p < 0.0001 versus the CD or CRD group. (*) represents significance difference when compared to CD group, while ($^{\#}$) when compared to the CRD group.

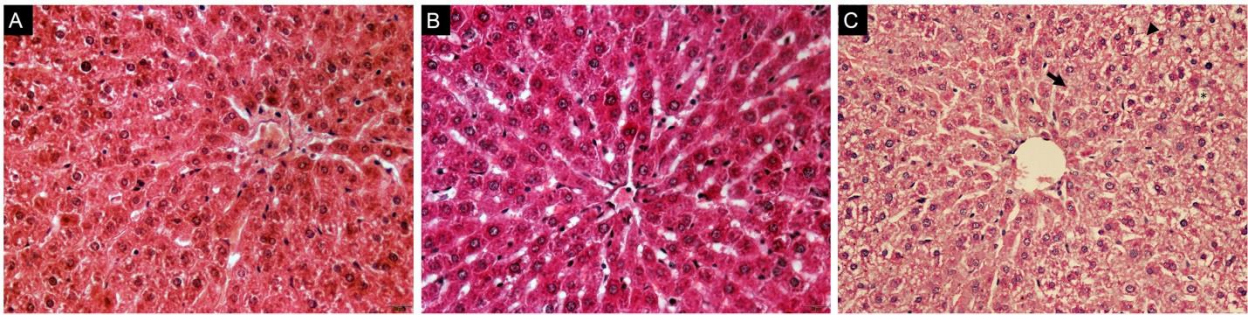

Figure 4. Histopathology of the liver stained with HE stain. Normal liver histology as shown in CD and CRD group (A) and (B). Liver of HFD group showed vacuolation (star), ballooning (arrowhead) and hepatocytes degeneration (arrow) as shown in (C).

Although serum insulin levels of the CRD and CD group were not significantly different (*P>0.05*), however the serum insulin level of the HFD group was higher than CD group (*P<0.02*) and reach its maximum differ when compared to the CRD group (*P≤0.0072*). These data, incomes that high fat raise level of insulin, while calorie restriction attenuates this elevation as shown in figure 5A. In addition, evaluation of insulin resistance using HOMA index indicated that the difference between insulin resistance values was very high among the HFD and CRD group (*P<0.003*). Moreover, CRD showed a better insulin sensitivity, even if compared to the CD group (*P<0.0036*) as shown in figure 5B.

3.7. Effects of calorie restriction on CRP and adipocytokines

C-reactive protein (CRP) is highly correlated with inflammatory disorders and metabolic syndromes, the higher CRP level the higher at risk for coronary artery diseases and type 2 diabetes (Ridker 2005, Deichgræber et al. 2016). Current study showed differentially downregulation of plasma CRP and IL-6 levels in the CRD group when compared to CD group by 27% and 23% respectively; levels of adipocytokines did not alter in normal group, but increased significantly in the HFD group. Interestingly, levels of TNF-α were highly expressed in HFD group approximately by 21% in comparison to CRD but not CD group (*P*≤0.01).The compensatory reduction of adipocytokines and CRP in CRD may due to the lower levels of lipids in the blood such as plasma free fatty acids or may as a resultant of reduction in lipid synthesis and liver triglyceride accumulation which ameliorated by CRD (Table 3).

It is obvious that reduced inflammatory cytokines and CRP reflect the beneficiary effects of calorie restriction on the general health status, contrary statement is true for consuming high fat diet and its effects on body weight gain and raise the risk for several metabolic syndromes such as T2D, coronary artery diseases and atherosclerosis.

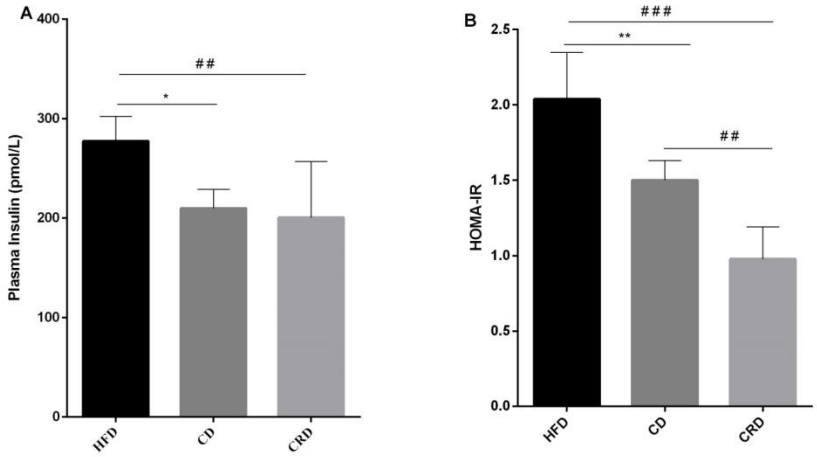

Figure 5. Plasma Insulin levels and Insulin resistance index. Plasma insulin level in the CD compared to the experimental groups (**A**). Insulin resistance index (HOMA-IR) in all groups (**B**). Data represent mean ± SD and statistical difference between any 2 groups (*P*<0.05) was calculated using Tukey's multiple comparison test**,** **P*<0.05; ***P*<0.001; ****P*<0.0001. (*) represents significance difference when compared to CD group, while ($^{\#}$) when compared to the CRD group.

Table 3. Plasma CRP, IL6 and TNF-α. Plasma Adipocytokines from different experimental groups fed control *ad libitium*, high-fat diet (HFD), calorie restriction diet (CRD) for 12 week.

Variables	CD	HFD	CRD
Plasma CRP (mg/dL)	1.75 ± 0.12	2.55 ± 0.39^{a}	1.04 ± 0.15^{a}
Plasma IL6 (mg/dL)	1.69 ± 0.14	2.06 ± 0.53 ^{a}	1.11 ± 0.17^{b}
Plasma TNF-α (mg/dL)	1.26 ± 0.16	1.55 ± 0.18^{c}	1.04 ± 0.04

Values are means ±SEM, *n=8*. Means in a row with a common superscript letter differ as follow, a) *P< 0.001*, b) *P*≤0.05 and c) *P*≤0.01.superscript represent significant values when compared to CD (^{a}) CRD (^{b}) and HFD (^{c}).

4. DISCUSSION

Consumption of food rich in fat content is a modern lifestyle aspect that negatively affect general health, with its bad consequence on the long term, alters different metabolic variables include post-processing of fats by the gut, neuronal control of meal-related signals that regulate food ingestion and metabolism that eventually led to overweight, obesity and progression of metabolic syndromes (METS). These alteration also depend on genetic and epigenetic variability and the socio-economic status of an individual (Kaur 2014). Since obesity is a complex process and a major risk factor for METS that results in a higher morbidity and mortality rate in individuals with chronic disorders such as NAFLD, study of obesity became an essential source of therapeutic approaches (Benedict and Zhang 2017). Interestingly, accumulation of fats in the body can cause a dramatic increase in production of proinflammatory adipokines in the adipose tissue (AT) regardless of their route (Rüster and Wolf 2013, Baranowska-Bik et al. 2017). Based on these data, this study proposed that CR can diminish risk of METS, particularly development and progression of NAFLD. For this, a well-established and controlled model is required to study the effects of CR on health quality and the mechanism involved to reduce inflammation and progression of NAFLD.

In the current study, despite a comparable daily based quantities of food intake throughout the whole experiment, there were notable variations in the body weight among all the groups. The lowest body weight as well as body fat content, while the HFD recorded the highest value. This points to accumulation of fat in different parts of the body rising body weight and risk of numerous obesity associated disorders such as cardiovascular diseases and type 2 diabetes. On the other hand, rats maintained on CR showed several compensatory effects have been observed in the lower level of blood glucose, improved insulin sensitivity. Furthermore, lipid profile was considerably improved as shown in the serum TG level and liver TG content, while TG in the HFD group exceeded the diagnostic level of NAFLD as previously reported in human (Vanni et al. 2010). Liver TG contents is directly allied with liver histology which showed clear signs of NAFLD such as steatosis, hepatocytes ballooning and degeneration.

Accumulation of fat in body tissue has been reported to associate with alteration in redox status, which may a major player in development of METS (Savini et al. 2013). This study showed that calorie restriction was accompanied with body weight loss and reduced ROS productivity, as deliberated by alteration in markers of free radical activity, which led to change in redox status and affected cellular integrity and damage of DNA. In addition, TNFα and IL-6 are the major cytokines produced by adipose tissue and their circulating levels are increased in obese objects (Goyal et al. 2012). It is noteworthy to point out here that our results clearly demonstrate that levels of cytokines in the HFD group were dramatically increased, whereas their level were decreased in the CRD group. These data indicated that calorie restriction protect against inflammation, which in consensus with a previous report demonstrate that fat accumulation promote inflammation in the adipose tissue (Lumeng and Saltiel 2011, Chalkiadaki and Guarente 2012). Another inflammatory marker its level were decreased by 47% in the CRD group is the CRP. Collectively these data established that high fat diet were more likely to have elevated cytokines and CRP levels than their normal-weight correlates, while CR showed significant improvements in the inflammatory cytokines and CRP.

The marvelous positive effects of CR whether locally on the liver histology, enzymes and TG content or remotely by regulating markers of inflammation, cytokines and free radical scavenging activity could be explained by lower fat content in this group. Ultimately these positive effects reflected on the liver tissue integrity alongside with improved the general health status of the individual. This support the notion that CR is an effective lifestyle in protection against development of METS and progression of the quiescent diseases such as NAFLD, as well it is a helpful approach as a therapeutic strategy.

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Compliance with ethical standards:

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflicts of interest

The author declared no conflicts of interest.

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