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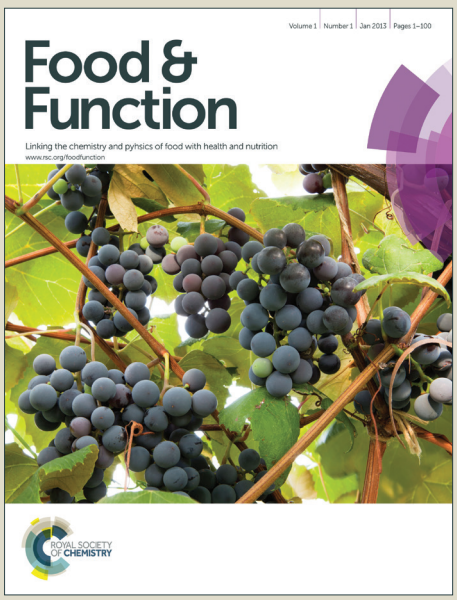

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Time course of adipose tissue dysfunction associated with antioxidant defense, inflammatory cytokines and oxidative stress in dyslipemic insulin resistant rats.

María Eugenia D´Alessandro, Dante Selenscig, Paola Illesca, Adriana Chicco, Yolanda B Lombardo*
Department of Biochemistry, School of Biochemistry, University of Litoral, Santa Fe, Argentina

* Corresponding author at: Department of Biochemistry, School of Biochemistry, University of Litoral, Ciudad Universitaria Paraje El Pozo CC 242 (3000) Santa Fe, Argentina. Tel: +54 342 4575 211; fax: +54 342 4575 221.
E-mail address: ylombard@fbcb.unl.edu.ar (Y.B. Lombardo)

Abstract

The dysfunctional adipose tissue of rats fed a sucrose-rich diet was investigated following the time course of the development of oxidative stress, changes in proinflammatory cytokines and adiponectin level and their relationship with insulin resistance. We analyzed the morphometric characteristics of epididymal adipocytes, "de novo" lipogenesis enzyme activities and cellular antioxidant defense, inflammatory mediators, adiponectin levels and insulin resistance in rats fed a sucrose-rich diet for 3, 15 or 30 weeks and compared to those fed a control diet. The results showed a depletion of antioxidant enzyme activities in the fat pads of rats fed a sucrose-rich diet, with an increase in xanthine oxidase activity and lipid peroxidation after 3, 15 and 30 weeks on the diet. Superoxide dismutase activity and the redox state of glutathione showed a significant decrease at weeks 15 and 30. This was accompanied by visceral adiposity and enhanced lipogenic enzyme activities. An increase in the plasma levels of proinflammatory markers (TNF-α and IL-6) was recorded only after 30 weeks on the diet. A reduction in plasma adiponectin levels accompanied the time course of deterioration of whole-body insulin sensitivity. The results suggest that lipid peroxidation, depletion of antioxidant defenses and changes in inflammatory cytokines induced by a sucrose-rich diet contribute to the dysregulation of adipose tissue and insulin resistance. Finally, these results show that the progressive deterioration of adipose tissue function, which begins in the absence of both visceral adiposity and overweight, is highly dependent upon the length of time on diet.

Keywords: High-sucrose diet; Antioxidant defense; Inflammatory markers; Insulin resistance; Visceral adiposity

Introduction

Adipose tissue is considered to be a major site affecting systemic insulin resistance, and visceral adiposity plays a key role in the etiology of metabolic syndrome.[1,2] Adipose tissue secretes hormones and a variety of adipocytokines.[3] There is evidence that the endocrine function of adipose tissue becomes dysregulated in enlarged fatty adipocytes, leading to changes in adipokine secretory patterns with a predominant proinflammatory profile.[3] Moreover, abdominal obesity in the presence of hyperglycemia and increased plasma free fatty acid levels results in the generation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress that affects insulin signaling and nitric oxide availability and contributing to insulin resistance.[4-7]

An experimental model resembling the characteristic feature of the human metabolic syndrome phenotype can be induced in rats by the administration of high-sucrose or -fructose diets.[8-10] Although the mechanism underlying the detrimental effects induced by high-sucrose/fructose diets in animal models is still unclear, different studies suggest that the administration of these diets leads to oxidative stress in several tissues .[11-13] In this regard, high-fructose (60%) feeding for 6 weeks was associated with increased ROS production by aortic, heart and circulatory polymorphonuclear cells.[13] In 3-week-old Wistar rats, Busserolles *et al*. [11] showed that 2 weeks of consumption of a high-sucrose diet negatively affected the balance of free radical production and antioxidant defense, increasing lipid susceptibility to peroxidation in several tissues. Alzamendi *et al*.[14] showed an increase in oxidative stress in abdominal adipose tissue in normal rats fed 10% fructose in drinking water during the same period of time.

Most of the experimental studies mentioned above have focused on the effect of the administration of fructose- or sucrose-rich diets for short periods of time (4-6 weeks), whereas dyslipidemia and insulin resistance are accompanied by hyperinsulinemia and normoglycemia. However, we previously demonstrated[15] that a different picture emerges after a long-term consumption (e.g., 15-30 weeks) of a sucrose-rich diet (SRD). In addition to altered lipid metabolism, the rats showed hyperglycemia and normoinsulinemia, insulin resistance, ectopic fat deposition in several non-adipose tissues and were moderately overweight. Adipose tissue plays a key role in these changes. We previously showed a moderate increase in basal lipolysis and a decrease in the antilipolytic action of insulin without changes in cell size distribution and epididymal tissue mass in rats fed a SRD for 3 weeks.[16] This tissue evolves with the length of time on the diet, generating enlarged adipocytes with a significant increase of basal lipolysis. At 30 weeks on the diet, impaired glucose uptake and increased “de novo” lipogenic enzyme activities and a decreased plasma leptin level were observed.[17,18]

A time-course study on the development of oxidative stress and changes in proinflammatory cytokines and adiponectin secretion in the dysfunctional adipose tissue of rats fed a SRD from a few to several weeks has not been reported so far. Therefore, the aim of the present study was to assess, in the epididymal fat pad, the time course of the effect of a high-sucrose diet on the following: i- enzymatic and non-enzymatic cellular antioxidant defense and xanthine oxidase activity as a source of reactive oxygen species and ii- inflammatory mediators, lipid peroxidation and adiponectin levels in rats fed this diet for a variable period of time (3,15 and 30 weeks) and its relationship with insulin resistance.

Methods

Animal models and diets

Male Wistar rats initially weighing 170–180 g, purchased from the National Institute of Pharmacology (Buenos Aires, Argentina), were maintained in an animal room under controlled temperature (22 ± 1 °C), humidity and airflow conditions, with a fixed 12-hour light: dark cycle (lights on 07.00 h and lights off 19.00 h). They were initially fed a standard non-purified diet (Ralston, Purina, St. Louis, MO). After 1 week of acclimation, they were randomly divided into two groups (control and experimental) and were housed individually. The control group received a semi-synthetic diet containing the following components by weight (g per 100 g): 62.5 corn starch, 17 proteins and 8 corn oil (control diet, CD). The experimental group received the same semi-synthetic diet with sucrose as the carbohydrate source (SRD). Details of the composition of the diets are given in Table 1. The preparation and handling of the diets have been reported elsewhere.[17] The rats had free access to food and water and consumed their respective diets for 3, 15 or 30 weeks. All diets provided approximately 16.3 kJ g^{-1} of food. The weight of each animal and the energy intake were recorded twice per week during the experimental period in all groups of rats. At the end of each experimental period, the food was removed at 0700 h, and unless otherwise indicated the experiments were performed between 0700 and 0900 h.

At least six rats from each dietary group were used in each procedure. They were anesthetized with sodium pentobarbital (60 mg per kg^{-1} i.p.). Blood samples were obtained from the jugular vein, collected in tubes containing sodium EDTA as an anticoagulant and rapidly centrifuged. Plasma was either immediately assayed or stored at -20 ºC until use. Epididymal, retroperitoneal and omental adipose tissues were totally removed and weighed. The epididymal fat pad was immediately processed for the preparation of adipocytes as mentioned below or frozen and stored at the temperature of liquid N_2. The visceral adiposity index (%) was calculated as previously described.[19] The experimental protocols were approved by the Human and Animal Research Committee of the School of Biochemistry, University of Litoral, Argentina.

Analytical methods

Commercially available analytical kits were employed to determine plasma glucose and triglyceride (Tg) concentration (Wiener lab., Rosario, Santa Fe, Argentina). Plasma free fatty acids (FFA) were determined using an acyl-CoA oxidase based colorimetric kit (Wako NEFA-C, Wako Chemicals, Neuss, Germany). Immunoreactive insulin was measured using the method of Herbert et al.[19] The immunoreactive insulin assays were calibrated against rat insulin standard (Novo Nordisk, Copenhagen, Denmark). Plasma adiponectin was

determined by radioimmunoassay using a kit available from Linco Research, St. Charles, MO, USA. The minimum detectable limit was 1 ng mL^{-1}. The intra- and interassay coefficients of variation (CV) were less than 4.4 % and 8.2 %, respectively. Interleukin-6 (IL-6), plasma tumor necrosis factor α (TNF-α) and the TNF-α content of the cytosolic phase of adipose tissue were measured with commercial ELISA kits (Thermo Scientific, Rockford, USA; Legend max™ Biolegend® Inc., San Diego, USA, and Thermo Scientific rat TNF-α ELISA kit, Rockford, USA) respectively. The minimum detectable limit was 16 pg mL^{-1} IL-6. The intra- and interassay CV were less than 6.8% and 14.9%, respectively. For TNF-α 4.2 pg mL^{-1} in plasma and 15 pg mL^{-1} in adipose tissue were the minimum detectable limits with the intra- and interassay CV less than 8.3% and 10.5%, respectively. All determinations were performed in triplicate.

Lipid peroxidation in plasma and adipose tissue was estimated by measuring thiobarbituric acid reactive substances (TBARS) according to the method of Lee and Csallany.[20] Briefly, TBARS were determined by a spectrophotometric method using malondialdehyde prepared by 1,1,3,3–tetraethoxypropane hydrolysis to establish the standard curve. The results were expressed as nmol mL^{-1} of plasma or nmol per epididymal fat weight.

Total glutathione (reduced (GSH) + oxidized form (GSSG)) and GSSG were assayed by the enzymatic recycling procedure in the presence of glutathione reductase as described by Griffith.[21] Total glutathione content was determined by comparison of the observed rate to a standard curve generated with known amounts of glutathione. GSSG content was determined by the same method after treating the samples with 4-vinylpyridine and triethanolamine as described by Griffith.[21] The GSSG content of the aliquot assayed was determined by comparing the observed rate to a standard curve generated with known amounts of glutathione and expressed on the basis of GSH equivalents (2 GSH →GSSG). The redox state was calculated as (oxidized form/total forms) x 100.

Euglycemic clamp studies

Whole-body peripheral insulin sensitivity was measured using the euglycemic-hyperinsulinemic clamp technique as previously described.[15,22] Briefly, the rats were anesthetized after 5 h of food deprivation. Then, a blood sample was obtained on which glucose was assessed. Afterward, an infusion of porcine neutral insulin (Actrapid; Novo, Industry, Denmark) at 0.8 U kg^{-1} h^{-1} for two hours was started. Insulin was infused through one limb of a double-lumen cannula connected to the left jugular vein. Blood samples for glucose assays were taken at 5 to 10 min intervals from the right jugular vein. The blood glucose concentration was measured using

the glucose oxidase method (Accu-Check Performa, Roche, Mannheim, Germany). Glycemia was maintained at an euglycemic level by injecting 0.2 g mL^{-1} glucose solution at a variable rate through the second limb of the double-lumen cannula. The glucose infusion began 5 minutes after the insulin infusion started. The glucose infusion rate (GIR) during the second hour of the clamp was taken as the net steady state of whole-body glucose.

Preparation of isolated adipocytes and determination of fat cell volume and number

Epididymal fat pads were removed, weighed and rinsed in isotonic saline solution at 37 ºC. Adipocytes were isolated according to the method of Rodbell.[23] Briefly, one fraction of washed cells was used for the determination of fat cell size, volume, number, and lipid content as previously described.[16] The lipid weight of the average fat cell was calculated from the mean cell volume assuming a lipid density of 0.915 (triolein density).

Lipogenic enzyme activities in epididymal fat tissue

Acetyl-CoA carboxylase (ACC) and glucose-6-phosphate dehydrogenase (G-6-PDH). Epididymal adipose tissue samples were homogenized (motor-driven Teflon glass homogenizer, Thomas Scientfic Swedesboro, NJ, USA) in an ice-cold buffer [9 mmol L^{-1} KH_2PO_4, 85 mmol L^{-1} K_2HPO_4, 1 mmol L^{-1} DTT, and 70 mmol L^{-1} $KHCO_3$, (pH 7)] and centrifuged at 100 000 g for 1 h at 4 ºC (Beckman Coulter, LE80, Palo Alto, CA, USA). The cytosolic fractions were used for the assay of enzyme activities. ACC activity was measured using an NADH-linked assay, with the slight modifications described by Zimmermann *et al.*[24] and G-6-PDH activity was investigated following the increase of NADPH absorption at 340 nm according to Cohen et al.[25] as previously described.[26]

Malic enzyme (ME). Epididymal adipose tissue samples were homogenized as described for ACC in ice-cold 0.25 mmol L^{-1}sucrose solution and then centrifuged for 10 min at 40 000g at 0–3 ºC. The resulting aqueous supernatant fractions were used for the assay of ME activity by measuring the rate of NADPH formation by spectrophotometric measurement at 340 nm and 37 ºC.[27]

Antioxidant enzyme activities in epididymal fat tissue

Powdered frozen epididymal adipose tissue was homogenized in three volumes of 30 mmol L^{-1} phosphate buffer, pH 7.4, containing 1 mmol L^{-1} EDTA and 250 mmol L^{-1} sucrose. After centrifugation for 10 min at 750 g and 4 ºC, one aliquot of the supernatant was used for the determination of both catalase (CAT) and superoxide dismutase (SOD) activities. Another aliquot was centrifuged at 100 000 g for 60 min at 4 ºC for the glutathione peroxidase (GPX) and glutathione reductase (GR) assays according to Chow .[28] The CAT activity was measured

according to the method described by Lück.[29] An enzyme unit is defined as the amount of enzyme that liberates half the peroxide oxygen from a hydrogen peroxide solution in 100 sec at 25 °C. The quantitative assessment of SOD activity was carried out with a commercial assay kit (Arbor Assays, Michigan, USA) according to the manufacturer's instructions. The sensitivity was 0.044 U mL^{-1}, and the intra- and interassay CV were less than 16.8% and 13.8%, respectively. GPX activity was determined using hydrogen peroxide as the substrate according to the method of Paglia and Valentine.[30] The enzyme activity was evaluated at 340 nm by measuring the decrease in the absorbance of NADPH. An enzyme unit is defined as the number of µmoles of NADPH oxidized per min at 20 °C. GR activity was determined by the method of Horn, [31] and one unit is defined as the amount of enzyme that reduces 1 µmol of oxidized glutathione per min at pH 6.6 and 25 °C.

Xanthine Oxidase (XO) activity in epididymal fat tissue

Adipose tissue was homogenized in 3 volumes of 50 mmol L^{-1} phosphate buffer, pH 7.8. XO activity was measured according to the method of Maia and Mira.[32] One unit of catalytic activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol per min of xanthine at 25 °C. The protein content in all enzyme assays was measured by the method of Lowry et al.[33]

Statistical Analysis

Sample sizes were calculated on the basis of measurements previously made with rats fed either a CD or SRD[7-19] considering 80% power as described by Glantz.[34] The results are expressed as the mean ± SEM. Statistical significance was determined by Student's t-test or data were subject to analysis of variance (ANOVA) when appropriate with diet and time as the main effects, followed by inspection of all differences between pairs of means by Scheffe's test.[35] Differences with $p < 0.05$ were considered to be statistically significant (SPSS 17.0 for Windows, SPSS INC. Chicago, Illinois). All reported p values are two-sided.

Results

Body weight gain, energy intake, visceral adiposity index

As previously demonstrated, [15,36] weight gain and energy intake did not differ between groups during the first 15 weeks of the study. However, significant increases in weight gain and energy intake occurred in rats fed the SRD compared to rats fed the CD between week 15 and 30. During this period, energy intake was also significantly higher ($p<0.01$) in rats fed the SRD compared to those fed the CD (Figure 1). The visceral adiposity index was similar in the control diet and SRD groups after 3 weeks. However, after 15 and 30 weeks this index significantly increased in rats fed a SRD compared with aged-matched controls fed a CD. The values (mean ± SEM) were as follows: (n = 6) 2.00 ± 0.10 in CD vs. 2.21 ± 0.06 in SRD at 3 weeks; 2.70 ± 0.09 in CD vs. 3.70 ± 0.16 in SRD at 15 weeks ($p < 0.05$); and 3.40 ± 0.20 in CD vs. 5.80 ± 0.36 in SRD at 30 weeks ($p < 0.05$).

Plasma metabolites, insulin, adiponectin levels and glucose infusion rate (GIR)

At the end of the dark period (0700 h), in agreement with previous reports from our laboratory,[15,36] plasma triglycerides and FFA levels were significantly higher in rats fed the SRD after 3 weeks compared with age-matched controls fed the CD (Table 2). A further increase of both plasma triglyceride and FFA levels was observed in the SRD group after 15 and 30 weeks on diet, whereas no changes in these metabolites occurred in rats fed the CD for the same period. Plasma glucose levels were greater after 15 and 30 weeks of consuming the SRD compared with rats fed the CD. On the other hand, plasma insulin levels were significantly higher than in controls after consumption of the SRD diet for 3 weeks, while no changes were recorded at 15 and 30 weeks. At these time values were similar to those observed in the CD group. This study also showed a significant reduction of plasma adiponectin levels in rats fed a SRD compared to rats fed a CD at each time point. Moreover, confirming previous results,[15] this was accompanied by a significant decrease of whole body peripheral insulin sensitivity estimated by GIR (clamp study) in the three experimental period (Table 2).

Epididymal adipose tissue total weight, triglyceride content, cell volume and number

Fig. 2A and 2B show that after 3 weeks of the SRD, no significant differences were observed in both, epididymal fat pad weight and triglyceride content compared to age-matched controls fed a CD. With increased length of time on the diet, the SRD groups showed a significant increase of both fat-pad weight and triglyceride content that reached the highest values after 30 weeks on the SRD. A similar behavior was observed for adipocyte cell volume (Figure 2C). No difference was observed in total number of adipocytes within or between the dietary groups throughout the experimental period (data not shown).

Lipogenic enzyme activities

The activities of enzymes related to "de novo" lipogenesis were measured in the epididymal adipose tissue in both dietary groups at 3, 15 and 30 weeks on the diet. As shown in Table 3, after 3 weeks on the SRD the enzyme activities of both G-6-PDH and malic enzyme reached values similar to those recorded in the age-matched controls fed a CD. After 15 weeks of consuming the SRD, a significant increase in both enzyme activities was observed. Moreover, confirming previous results,[18] a similar increase was recorded at 30 weeks. Although no changes in ACC activity were observed when the enzyme was expressed as mU per mg of protein, a behavior similar to that of the enzymes mentioned above was recorded when ACC activity was expressed as mU per epididymal fat weight (data not shown).

Inflammatory markers mediators

The table inserted in Figure 3 shows the plasma TNF-α level in both dietary groups at each time point of the experimental period. The plasma level of this cytokine was only significantly higher in the group of rats fed a SRD for 30 weeks compared to age-matched controls fed a CD.

Based on the above results, we examined the TNF-α content in the epididymal fat tissue and its correlation with plasma levels in both CD- and SRD-fed rats at 30 weeks on each diet (Figure 3). A high correlation between adipose tissue content and plasma TNF-α level was observed. Both parameters were significantly increased in rats fed the SRD compared to those of the CD-fed rats.

Interestingly, the plasma concentration of IL-6, another inflammatory cytokine, was also significantly higher only in rats consuming the SRD for 30 weeks. Values (mean ± SEM, pg mL^{-1}) were as follows: (n = 6) 40.2 ± 11.3 in CD vs. 80.5 ± 7.8 in SRD ($p < 0.05$). Values at 3 and 15 weeks were similar in both dietary groups.

Lipid peroxidation and redox metabolism

Figure 4 shows that lipid peroxidation estimated as TBARS concentration in plasma and adipose tissue was significantly enhanced in the SRD-fed groups compared to the CD groups at the three time points. Note, however, that the methodology used in the assay of tissue and plasma TBARS has limitations because it overestimates TBARS levels. Problems of the TBA assays using this method include: Lack of specificity for MDA ,no distinction between free and bound MDA, and the artifactual formation of the genuine MDA $(TBA)_2$ adduct from sources other than the lipid hydroperoxides. Compared with HPLC with fluorescence detection that quantifies only the genuine MDA $(TBA)_2$ adduct ,the total absorbance of several TABRS is measured by the

296 classic TBA method as shown by Breusing *et al.* [37], Lykkesfeldt *et al.*[38]and Giera *et al.*[39]. The next step was to
297 analyze changes in redox metabolism throughout the experimental period in the epididymal fat pad of both
298 dietary groups by determining the level of the enzymatic and non-enzymatic cellular antioxidant defenses. Figure
299 5 depicts a significant decrease in SOD activity in rats fed the SRD after 15 or 30 weeks on the diet compared
300 with age-matched controls fed the CD, while no change in the SOD activity was observed at 3 weeks. Compared
301 with CD-fed rats, the CAT, GPX and GR activities were significantly lower in the SRD group starting in the
302 third week on the diet and remained lower until the end of the experimental period (Figure 5). In addition, the
303 redox state of glutathione significantly decreased ($p < 0.05$) in the SRD-fed rats after 15 or 30 weeks on the diet
304 without changes in the early period (3 weeks). Values (mean ± SEM (oxidized form/total forms) x 100)) were as
305 follows: (n = 6) 40.0 ± 7.0 in CD vs. 38 ± 3.2 in SRD at 3 weeks, 43.0 ± 5.1 in CD vs. 25.2 ± 3.1 in SRD at 15
306 weeks ($p < 0.05$); and 45.3 ± 6.0 in CD vs. 25.2 ± 4.1 in SRD at 30 weeks ($p < 0.05$). A significant increase ($p <$
307 0.05) in XO activity was recorded in rats fed the SRD after 3, 15 and 30 weeks compared with age-matched
308 controls fed the CD. Values (mean ± SEM, U per total fat weight) were as follows: (n = 6) 1.05 ± 0.037 in CD
309 vs. 1.34 ± 0.10 in SRD at 3 weeks ($p<0.05$); 2.28 ± 0.05 in CD vs. 3.00 ± 0.13 in SRD at 15 weeks ($p < 0.05$) and
310 2.34 ± 0.13 in CD vs. 4.20 ± 0.52 in SRD at 30 weeks ($p < 0.05$).
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Discussion

In the adipose tissue of normal rats, we analyzed the effects of the administration of a high-sucrose diet over time (3, 15 and 30 weeks), particularly focusing on the time course of the development of oxidative stress, inflammatory mediators and adipocytokines involved in the dysregulation of epididymal fat pad and its relationship with the progressive deterioration of insulin sensitivity.

The major new findings are the following: 1- A depletion of antioxidant defense (decreased CAT, GPx and GR activities) was observed starting at week 3 and continuing through the end of the experimental period (30 weeks), while the opposite effect was recorded in the levels of the oxidant enzyme XO activity as well as TBARS both in plasma and in epididymal fat pads. SOD activity showed a significant decrease after 15 and 30 weeks. The redox state of glutathione followed the same patterns as the SOD activity. 2- Levels of the plasma inflammatory mediators IL-6 and TNF-α were significantly increased only after 30 weeks of SRD administration. In addition, a decrease of plasma adiponectin levels accompanied the deterioration of whole-body insulin sensitivity, impaired glucose homeostasis and dyslipidemia over time.

We previously demonstrated that in rats fed a SRD for 3 weeks, an oversupply of lipids deteriorated insulin sensitivity in several tissues including adipose tissue.[15,16] Expanding upon the above observation this study showed that the altered insulin action was not accompanied by an increase of "de novo" lipogenic enzymes activities or adipocytes cell size in the epididymal fat pad. Instead, short- term SRD feeding induced an imbalance between a significant increase of XO activity and TBARS as an estimation of lipid peroxidation in adipose tissue and plasma and decreased the activities of key enzymes involved in antioxidant defense. Moreover, the plasma adiponectin level also decreased. At that time, the rats fed a SRD showed normoglycemia and hyperinsulinemia that is accompanied by a moderate decrease of GIR. These results collectively suggested that an early pro-oxidative state preceding the detection of visceral adiposity (unchanged adiposity index) and could contribute to the altered production of adiponectin and whole body peripheral insulin resistance. Similarly, Fariña *et al.*[40] recently showed an increase of NADPH oxidase activity and ROS production in the abdominal adipose tissue of rats fed a fructose diet for 2 weeks, and Thirunavukkarasu *et al.*[41] observed an increase of TBARS, conjugated dienes and lipid hydroperoxide as well as a decrease of key antioxidant enzyme activities in the kidney and liver without changes in body weight in Wistar rats fed a high-fructose diet (61%). Moreover, increased TBARS and lower Cu-Zn-SOD activity were found in the hearts of rats fed a high-sucrose diet for 2 weeks without changes in body weight .[11] As previously demonstrated[15] and mentioned before, a different

metabolic milieu developed after 15 weeks and continued through 30 weeks in rats fed the SRD. At this time, dyslipidemia, ectopic fat deposition in different tissues, moderate hyperglycemia and normoinsulinemia and a more profound insulin resistance (low GIR) were present. These changes deeply affected the adipose tissue function because a further increase of the estimated lipid peroxidation, and the pro-oxidative XO and a decrease of SOD enzyme activities favored fat pad dysregulation. In addition a reduction of the redox state of glutathione in adipose tissue was observed. This could be a consequence of the higher pro-oxidant status developed in SRD fed rats. The increase of adiposity index that appeared at 15 weeks and pursue to 30 weeks on the diet was accompanied by enlarged adipocytes. The SRD induced a significant increase of several enzyme activities involved in "de novo" lipogenesis in the epididymal fat tissue. Therefore, this could be one of the possible mechanisms participating in the visceral adiposity. However, despite the increased fat pad mass, the plasma of SRD-fed rats at 15 weeks did not show an increase of inflammatory markers such as TNF-α and IL-6. Moreover, at this time, no significant changes in body weight gain or energy intake were observed compared to the control animals.

Fat accumulation in adipose tissue correlated with systemic oxidative stress in rodents and humans.[7] It is well known that hyperglycemia and increased plasma FFA result in the generation of ROS. In the absence of an appropriate compensatory response from the endogenous antioxidant network, the system becomes overwhelmed, leading to activation of stress-sensitive intracellular signaling pathways. In agreement with our results, Madani *et al*.[42]showed an increase in adiposity and TBARS concentration and a decrease in antioxidant enzyme activities in adipose tissue without changes in the body weight of rats fed a high-fructose diet for 2 months. Roberts *et al*.[43] showed up regulation of NADPH oxidase that allowed increased ROS production capacity and down regulation of several antioxidant enzyme activities in the kidney and vascular tissues in rats chronically fed a high-fat, high-refined sugar diet. Moreover, an elevated mRNA expression and enzyme activity of adipose tissue xanthine oxidoreductase (in its two forms: xanthine dehydrogenase (XDH) or xanthine oxidase (XO)) was observed in obese rats. [44]

Oxidative stress stimulates the production of a variety of pro-inflammatory cytokines, which may ultimately lead to insulin resistance. An imbalance between the production/secretion of pro- vs. anti-inflammatory cytokines induced a low level of inflammation, which is also modulated by nutrients including glucose and lipids.[5] The present study showed a significant increase of plasma levels of IL-6 and TNF-α in the SRD-fed rats when the feeding period was extended through 30 weeks. Furthermore, a positive correlation

between plasma and adipose tissue TNF-α concentration was observed. TNFα plays an important role in insulin resistance.[45] In this regard, several potential mechanisms for their metabolic effects have been described including the activation of serine kinases such as JNK and p38 mitogen activated protein kinase (MAPK) that increase serine phosphorylation of IRS-1 and IRS-2, making them poor substrates for insulin receptor –activating kinases and increasing their degradation.[46] Elevated plasma levels of TNF-α have been found to be associated with obesity.[46] A moderately overweight condition observed in the long-term SRD-fed rats is accompanied by altered insulin sensitivity in adipose tissue and skeletal muscle and a more profound deterioration of whole-body insulin resistance and hyperglycemia .[15,18,47] It is known that hyperglycemia induces oxidative stress, which is responsible for the activation of nuclear factor kappa B (NF-kB), a transcription factor that among others targets, stimulates the production of multiple inflammatory mediators including TNF-α and IL-6. Nakamura *et al*. [48] showed an increase of plasma MCP-1 level in rats fed a fructose diet, and there is a growing body of evidence showing that MCP-1 gene expression is regulated by NF-kB. Aguilera *et al*. [49] showed an increase of plasma TNF-α level in rats fed a sucrose diet for 21 weeks. In these animals, visceral adiposity and insulin resistance were also observed. Nara *et al*.[50] recorded an increase of TNF-α protein mass level in visceral fat in rats fed a high-sucrose diet for 4 weeks. The plasma level of the inflammatory adipocytokine IL-6 positively correlated with fat mass, plasma FFA levels and insulin resistance.[46] Lagathu *et al* [51] showed that chronic exposure of IL-6 in fully differentiated 3T3-LI adipocytes induces insulin resistance by inhibition of the phosphorylation of the insulin signaling components (IRβ, IRS-1, and post receptor levels ErK1/2, AKT/PKB) and a series of insulin dependent processes (eg: glucose transport). The present data show a significant increase in the plasma IL-6 concentration that was accompanied by enhanced availability of plasma FFA levels, enlarged adipose tissue mass and whole body peripheral insulin resistance. As mentioned before, basal lipolysis in isolated adipocytes of the epididymal fat pad was significantly increased; therefore, this contributed to the high levels of plasma FFA.[17] In agreement with our results, Hsieh et al.[52] demonstrated an increase of IL-6 and TNF-α concentration in the adipose tissue of rats fed a high-fructose diet. Therefore, the present results suggest that the changes observed in the pro-inflammatory cytokines and plasma adiponectin levels, the depletion of antioxidant defenses as well as the increase of plasma free fatty acids levels, adiposity and moderate overweight would favor the overall development of the dysregulation of adipose tissue function, worsening insulin resistance when the SRD diet is extended to 30 weeks.

In addition, macrophages that infiltrate the enlarged adipose tissue are an important source of inflammatory cytokines. Yang *et al*.[53] showed a significant increase of mRNA expression of genes related to the inflammation of mesenteric adipose tissue such as MAC-1, CD68 and MMP3 in male C57BL/6J mice fed a high-fat, high-sucrose diet. Moreover, Police *et al*.[54] observed a significant increase of F4/80 macrophage immunostaining in retroperitoneal adipose tissue in similar experimental conditions. Although in the present study we did not evaluate the presence of macrophages in the epididymal fat pad of SRD-fed rats, we do not discard the possibility that the infiltration of macrophages in the hypertrophied adipocytes could contribute to the enhanced release of inflammatory cytokines.

In brief the fructose moieties of the sucrose rich diet induce in the rat a sequence of events of metabolic disorders that leads to insulin resistance and abnormal glucose homeostasis and depends on both the amount of carbohydrate and the length of time the diet is administered.[55,56] Expanding our previous research, the present study provides new information regarding the evolution of the dysfunctional adipose tissue in rats fed a SRD for 3 to 30 weeks. The overall results suggest that, among other factors, the depletion of antioxidant defenses and redox state, the susceptibility to lipid peroxidation, changes in inflammatory cytokines and visceral adiposity induced by a sucrose-rich diet contribute to the dysregulation of adipose tissue function and insulin resistance in this animal model, which resembles the human metabolic syndrome phenotype. Finally and the best of our knowledge for the first time, these results show that impaired adipose tissue function, evolves as an initial event in the absence of both visceral adiposity and excess of body weight and progressively worsens with time on the diet.

Although caution is warranted before extrapolating from rodents to humans with realistic sucrose/fructose consumption, studies from humans suggest that increased intake of these nutrients may be harmful to body weight, adiposity and the metabolic indexes related to insulin resistance.[56]

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Authors' contributions: Conceived and designed the experiments: AC, YBL.

Performed the experiments: MED, DS, PI.

Analyzed the data: MED, DS, PI, AC, YBL.

Wrote the paper: MED, YBL.

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Figure legends

Fig. 1

Body weight and energy intake in rats fed a control diet (CD) or sucrose-rich diet (SRD).

Values are expressed as mean ±SEM; at least 6 animals were included in each experimental group.*p<0.05 SRD vs. CD at each time point. In the table insert in Fig 1, *p<0.05 SRD vs. CD. For more details, see animals and diets in Methods.

Fig. 2

Epididymal adipose tissue total weight (A) , triglyceride content (B) and adipocyte cell volume (C) in rats fed the control diet (CD) or the sucrose-rich diet (SRD) at 3, 15 or 30 weeks.

Values are expressed as mean ± SEM; at least six animals were included in each experimental group. Values that do not share the same superscript symbols were significantly different (p< 0.05) when one variable at a time was compared by Scheffe´s test.

Fig. 3

Plasma TNF-α level in rats fed the control diet (CD) or sucrose-rich diet (SRD) for 3, 15 or 30 weeks and correlation between TNF-α plasma and epididymal fat pad concentration at 30 weeks.

Values are expressed as mean ± SEM; at least six animals were included in each experimental group. Values that do not share the same superscript symbols were significantly different (p< 0.05) when one variable at a time was compared by Scheffe´s test. S: significant.

Fig. 4

Plasma level and epididymal fat pad concentration of TBARS in rats fed the control diet (CD) or the sucrose-rich diet (SRD) at 3, 15 or 30 weeks.

Values are expressed as mean ± SEM; at least six animals were included in each experimental group. Values that do not share the same superscript symbols were significantly different (p< 0.05) when a single variable at a time was compared by Scheffe´s test.

Fig. 5

Superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities in epididymal fat pad in rats fed the control diet (CD) or the sucrose-rich diet (SRD) at 3, 15 or 30 weeks.

Values are expressed as mean ± SEM; at least six animals were included in each experimental group. Values that do not share the same superscript symbols were significantly different ($p < 0.05$) when a single variable at a time was compared by Scheffe´s test.

Table 1- Composition of the experimental diets [a]

Diet ingredients	Control diet		Sucrose-rich diet	
	(g per 100 g)	(% Energy)	(g per 100 g)	(% Energy)
Corn starch	62.5	64.1	-	-
Sucrose	-	-	62.5	64.1
Casein free vitamin	17	17.4	17	17.4
Corn oil	8	18.5	8	18.5
Vitamin mix [b]	1		1	
Cellulose	7.5		7.5	
Salt mix [c]	3.5		3.5	
Choline bitartrate	0.2		0.2	
DL-Methionine	0.3		0.3	

[a] Diets are based on the AIN-93 diet.

[b] Vitamin mix is based on vitamin mix AIN-93M (in g per kg of mix): niacin, 3.00; calcium pantothenate, 1.60; pyridoxine HCl, 0.70; thiamin HCl, 0.60; riboflavin, 0.60; folic acid, 0.20; D-biotin, 0.02; vitamin B12, 2 500 g; vitamin E (500 IU/g), 15.00; vitamin A (500 000 IU per g) 0.80; vitamin D3 (400 000 IU per g), 0.25; vitamin K, 0.075.

[c] Salt mix is based on salt mix AIN-93M (in g per kg of mix): calcium carbonate, 357.0; potassium phosphate (monobasic) 250.0; sodium chloride, 74.0; potassium sulfate, 46.6; potassium citrate, tri-potassium (monohydrate) 28.0; magnesium oxide, 24.0; ferric citrate, 6.06; zinc carbonate, 1.65; manganese carbonate, 0.63; cupric carbonate, 0.30; potassium iodate, 0.01; sodium selenate, 0.01025; ammonium paramolybdate, 0.00795; chromium potassium sulfate, 0.275.

639 Table 2- Plasma metabolites and insulin concentration in rats fed a control (CD) or a sucrose rich (SRD) diet for up to 30 weeks[a]

Diet	Time on diet (weeks)	Triglyceride (mmol L^{-1})	Free Fatty Acids (µmol L^{-1})	Glucose (mmol L^{-1})	Insulin (µU mL^{-1})	Adiponectin (µg mL^{-1})	GIR µmol (kg x min)$^{-1}$
CD	3	0.76 ± 0.08‡	289.0 ± 19.3‡	6.18 ± 0.09†	52.1 ± 3.4†	2.93 ± 0.49*	68.1 ± 2.9*
SRD	3	1.63 ± 0.07†	542.5 ± 8.5†	6.71 ± 0.20†	81.5 ± 1.5*	1.56 ± 0.20†	50.0 ± 2.7†
CD	15	0.78 ± 0.09‡	320.7 ± 50.5‡	6.40 ± 0.13†	54.7 ± 3.5†	2.70 ± 0.21*	63.1 ± 2.1*
SRD	15	2.11 ± 0.10*	799.0 ± 16.4*	8.13 ± 0.17*	57.9 ± 2.7†	1.48 ± 0.19†	32.9 ± 1.4‡
CD	30	0.81 ± 0.07‡	334.0 ± 36.3‡	6.43 ± 0.08†	56.3 ± 2.8†	2.80 ± 0.38*	61.8 ± 1.3*
SRD	30	2.23 ± 0.10*	827.5 ± 43.9*	8.60 ± 0.17*	56.0 ± 2.6†	1.74 ± 0.15†	24.5 ± 1.0‡

2 X 3 ANOVA

Diet	S	S	S	S	S	S
Time	S	S	S	S	NS	S
Diet x Time	S	S	S	S	NS	S
Residual mean square	0.045	4340.107	0.113	51.235	0.598	20.737

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642 [a] Values are expressed as mean ± SEM; at least six animals were included in each experimental group. ANOVA: effect S: significant ($p<0.05$) or NS: not significant.

643 Values in a column that do not share the same superscript symbols were significantly different ($p< 0.05$) when one variable at a time was compared by Scheffe´s test.

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Table 3- Lipogenic enzymes activities in epididymal adipose tissue of rats fed a control (CD) or a sucrose (SRD) diet for up to 30 weeks[a]

Diet	Time on diet (weeks)	Acetyl-CoA carboxylase (mU per mg protein)	Glucose-6-phosphate dehydrogenase (mU per mg protein)	Malic enzyme (U per epididymal fat weight)
CD	3	27.62 ± 62.5	28.24 ± 1.54†	0.97 ± 0.07‡
SRD	3	29.59± 2.65	22.59 ± 3.56†	0.92 ± 0.06‡
CD	15	27.10 ± 3.67	23.27 ± 3.03†	0.88 ± 0.14‡
SRD	15	29.67 ± 2.27	44.01 ± 3.86*	1.57 ± 0.14†
CD	30	28.20 ± 2.81	30.49 ± 1.17†	1.21 ± 0.03†
SRD	30	27.86 ± 43.6	47.00 ± 5.37*	2.38 ± 0.27*

2 X 3 ANOVA

Diet	NS	S	S
Time	NS	S	S
Diet x Time	NS	S	S
Residual mean square	915.77	897.51	0.101

[a] Values are expressed as mean ± SEM; at least six animals were included in each experimental group. ANOVA: effect S: significant ($p<0.05$) or NS: not significant. Values in a column that do not share the same superscript symbols were significantly different ($p< 0.05$) when one variable at a time was compared by Scheffe´s test.

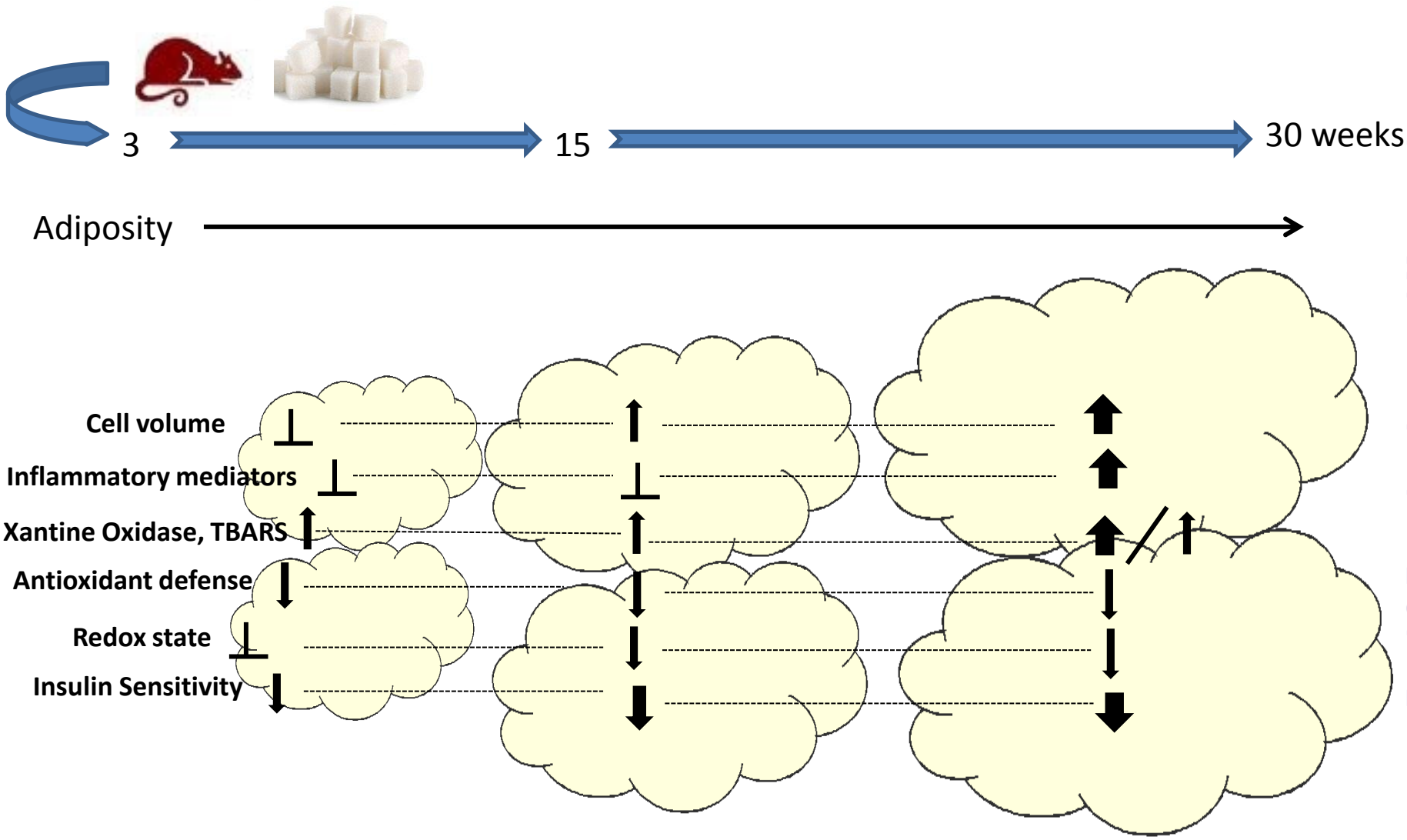
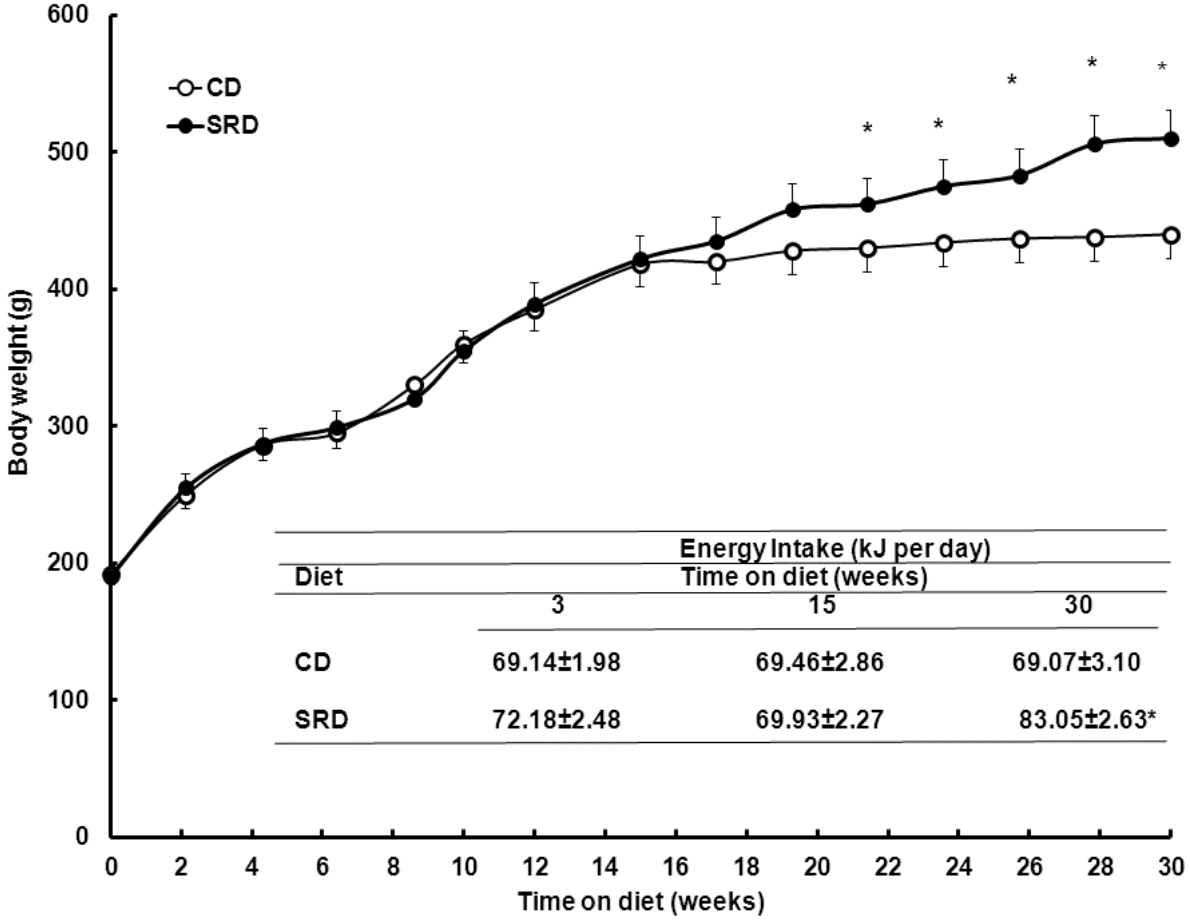


Figure 1



Diet	Energy Intake (kJ per day) Time on diet (weeks)		
	3	15	30
CD	69.14±1.98	69.46±2.86	69.07±3.10
SRD	72.18±2.48	69.93±2.27	83.05±2.63*

Figure 2

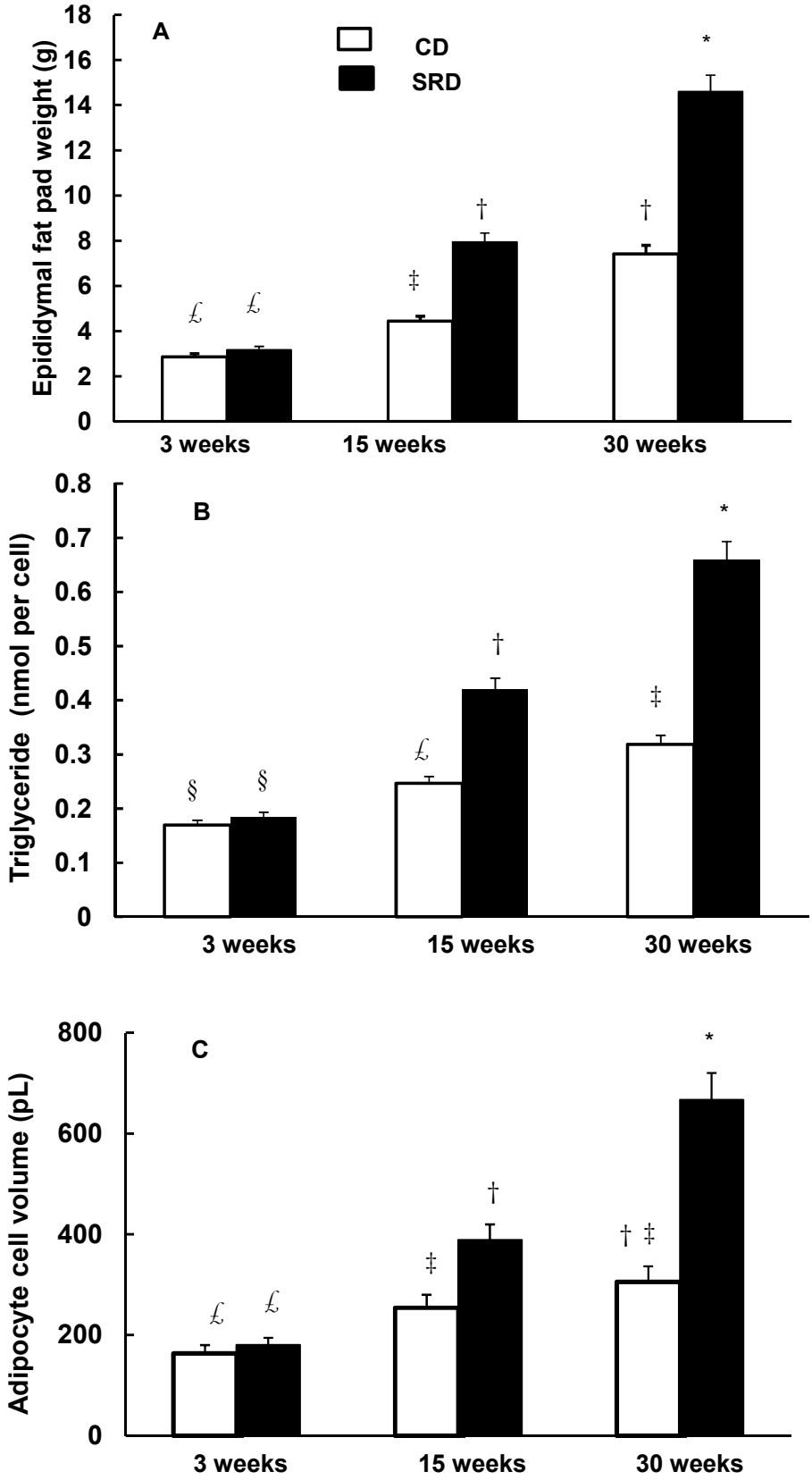


Figure 3

Time of Diet (weeks)		TNF-α (pg mL^{-1})
CD	3	5.74 ± 0.13†
SRD	3	5.80 ± 0.19†
CD	15	5.54 ± 0.18†
SRD	15	5.65 ± 0.25†
CD	30	5.81 ± 0.21†
SRD	30	14.66 ± 0.77*
2 x 3 ANOVA		
Diet		S
Time		S
Diet x Time		S
Residual mean square		12.527

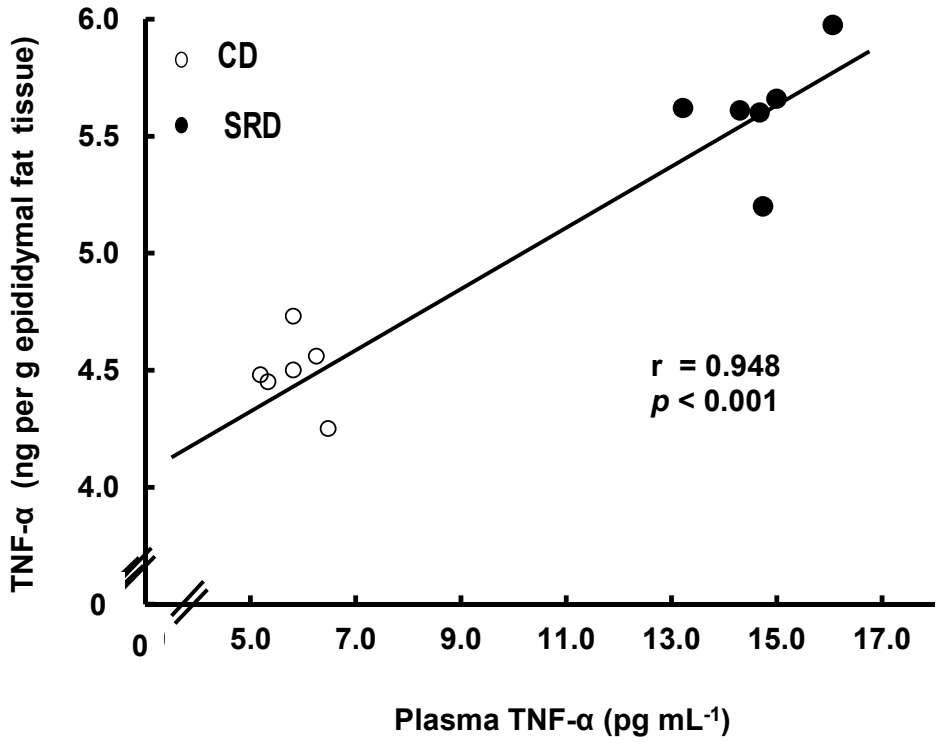


Figure 4

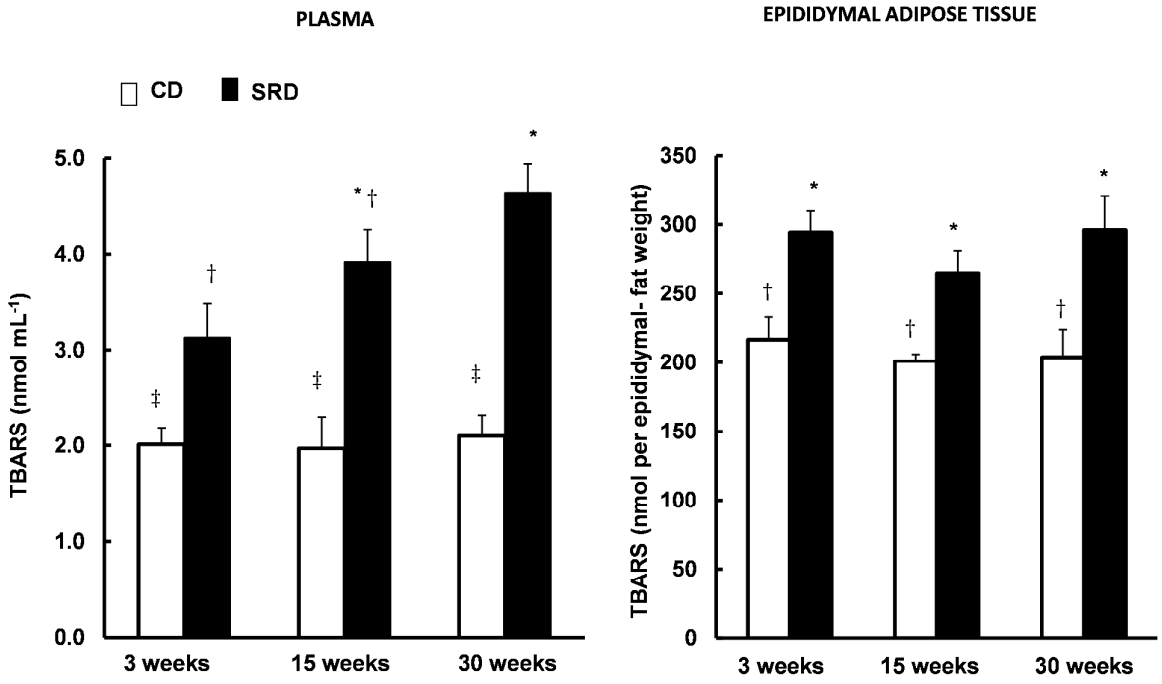


Figure 5