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Basic nutritional investigation

Refeeding abolishes beneficial effects of severe calorie restriction from birth on adipose tissue and glucose homeostasis of adult rats

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ABSTRACT

Objectives: Calorie restriction (CR) is an important intervention for reducing adiposity and improving glucose homeostasis. Recently we found that in rats, a severe calorie restriction (SCR) beginning at birth up to adult age promotes positive effects on cardiometabolic risk factors and heart. The aim of this study was to investigate the effects of this new model of SCR on adipose tissue and glucose homeostasis of rats and to evaluate the effects of refeeding.

Methods: From birth to 90 d of age, rats were divided into an ad libitum (AL) group, which had free access to food, and a CR50 group, which had food limited to 50% of that consumed by the AL group. From this moment, half of the CR50 animals had free access to food (the refeeding group [CR50-R]), and the other half continued 50% restricted for an additional 90-d period. Food intake was assessed daily and body weight weekly. In the final week of the SCR/refeeding protocol, oral glucose and intraperitoneal insulin tolerance tests were performed. Thereafter, rats were sacrificed and visceral fat was collected and used for histologic and Western blot analysis.

Results: Findings from this study revealed that SCR beginning at birth and up to adult life promoted a large decrease in visceral adiposity; improvement in glucose/insulin tolerance; and upregulation of adipose proliferating cell nuclear antigen, sirtuin 1, peroxisome proliferator-activated receptor-γ, and adiponectin. Refeeding abolished all of these effects. SCR from birth to adult age promoted beneficial effects on adipose tissue and glucose homeostasis; whereas refeeding abolished these effects.

Introduction

Adipose tissue is a critical regulator of systemic energy homeostasis, acting as an energetic reservoir and an endocrine organ, regulating the metabolism of glucose and lipids [1,2]. Increased adiposity, especially the accumulation of visceral adipose tissue, is considered critical for the development of disorders in glucose and insulin homeostasis [3–5].

Calorie restriction (CR) is purported to be the most effective non-pharmacologic tool for prevention and treatment of glucose metabolism disorders [6,7]. CR reduces glucose and insulin levels, improves glucose tolerance, increases peripheral insulin sensitivity, and reverses insulin resistance [8–11]. These effects are extensively associated with the reduction of fat mass [12].

The beneficial effects of long-term moderate CR (20–40% reduction) are well documented [13–15]. An important review conducted by Han and Ren [16] showed that the effects of CR are strongly associated with age and intensity, being positive when CR is started at early ages, with intensities >40%.

Recently we found that in rats, severe calorie restriction (SCR) reduces cardiometabolic risk facts and improves cardiac function at baseline conditions and in response to the insult of ischemia or reperfusion [11,17]. These results are intriguing as we found, for

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the first time, cardioprotective effects in response to a SCR initiated at birth up to the adult age. Here we focus on the effects of this model of SCR on adipose tissue and glucose homeostasis.

Of note, we also wanted to investigate the consequences of refeeding. Refeeding comprises a period after CR in which food is offered ad libitum. Despite abundant evidence of the benefits of CR, the consequences of refeeding are still poorly investigated, and its effects are not fully understood. The present evidence points to the loss of the benefits of CR and even to deleterious effects, mainly owing to the marked increase in adiposity [15,18–22]. The consequences of refeeding on adipose tissue of adult rats that initiated a SCR at birth are completely unknown.

Based on this knowledge, we hypothesized the following:

- An SCR started at birth and lasting to adulthood improves glucose and insulin tolerance and promotes a large reduction in adiposity with an upregulation of key intracellular proteins involved in these adaptations.
- These beneficial effects are abolished after an additional period of refeeding.

Thus, the purpose of this study was to investigate the effects on glucose and insulin tolerance and adipose tissue of an SCR from birth to the age of 90 d, followed by an additional period of 90 d of refeeding.

Methods

Experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 1996). The experimental protocols were approved by Ethics Committee on Animal Use/Federal University of Jequitinhonha and Mucuri Valleys, Diamantina, MG, Brazil.

Animals and CR protocol

Female pregnant Wistar rats (N = 8), ~90 d of age, were housed in individual cages and maintained in a room with controlled temperature (22°C ± 2°C) and a 12-h light/12-h dark cycle, with free access to food and water. After they gave birth, half of the mothers (n = 4) were assigned to ad libitum-fed group, and the other half (n = 4) were assigned to the CR group. A rodent-specific commercial isocaloric diet containing 22% protein, was used. Both groups had free access to water. In the mothers assigned to the CR group, the amount of food provided on a daily basis was equivalent to 50% of the amount consumed by the mothers in the ad libitum-fed group. The litters were composed of the mother and eight newborns. After weaning, part of the offspring males were randomly selected, housed in individual cages, and given the same treatment as their mothers up to the age of 90 d. The offspring were divided into rats fed ad libitum (AL rats; n = 8) and rats subjected to long-term SCR (CR50 rats; n = 16). At the end of 90 d, half of the CR50 animals began to eat freely (the refeeding group [CR50-R], n = 8). The animals were kept in their respective treatments for an additional 90 d, being sacrificed by decapitation at 180 d with ketamine (10 mg/kg) and xylazine (5 mg/kg).

Food intake, weight gain, and feed efficiency ratio

The food intake of the AL group was evaluated daily and this value was used to calculate the offer to the CR50 group. For both groups, the average daily intake was calculated weekly. Body weight (BW) was monitored weekly. Feed efficiency ratio was measured from the ratio between the total weight gain and overall food intake.

Oral glucose tolerance test

Rats were fasted for 8 h, and dextrose was administered by gavage (2 g/kg BW; 50% solution). Blood glucose levels were determined by small clipping of the rat tail immediately before (0 min) the dextrose challenge and at 30, 60, and 120 min thereafter. Blood glucose levels were determined using an ACCU-CHEK (Advantage Glucose Analyzer, Roche Diagnostics Corporation, Indianapolis, IN, USA).

Intraperitoneal insulin tolerance test

Forty-eight hours after the oral glucose tolerance test (OGTT), the animals were fasted for 8 h, and then an intraperitoneal injection of insulin (1 IU/kg BW) was administered. Blood glucose levels were determined following the same protocol as oral glucose tolerance test.

Visceral adipose tissue, adiposity, and Lee index

After 48 h of the intraperitoneal insulin tolerance test (IpITT), the animals were fasted for 8 h, anesthetized (ketamine/xilasin), and then sacrificed. Measurement of the nose to anus length of the animal was done for evaluation of the Lee index [23]. The retroperitoneal, epididymal, and mesenteric fats pads were collected for subsequent analysis and their mass measurements were used to calculate adiposity index. Total body fat was measured as the sum of the following individual fat pad weights:

Epididymal fat + retroperitoneal fat + visceral fat.

The adiposity index was calculated as described by Boustany et al. [24]:

Total body fat/final BW × 100.

Histologic examination of adipose tissue

The fragments of retroperitoneal adipose tissue were fixed in formalin (10%) for 48 h and subsequently submitted to dehydration with increasing alcohol gradient (70, 80, 90, and 100%) followed by infiltration and inclusion in paraffin. After assembly of the blocks, the samples were submitted to microtomy in sections of 5 μm with intervals of 20 cuts. The sections were stained with hematoxylin and eosin (H&E) for analysis. Cross sections were used, ~10 fields per animal, and the cell area of ≥100 adipocytes per animal was evaluated. The adipocyte size was assessed by quantification and frequency distribution as described by Parlee et al. [25].

Reverse transcription

The first-strand complementary DNA (cDNA) was synthesized from 400 ng total RNA in a final reaction volume of 20 μL using the Superscript first-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA). After denaturing the template RNA and primers (40 pmol glyceraldehyde 3-phosphate dehydrogenase [GADPH; sense: 5′ CTCCCATTCTTCCACCTTTGAT 3′ antisense: 5′ CCCTGTTGCTGTAGCCATATTC 3′], adiponectin [sense: 5′GCTCTCCTTTCCTGCCAGG 3'; antisense: 5′ GCACTCCTGGAGAGAAGGG 3′] and PPAR-γ [sense: 5′-AACCGGAACAA-ATGCCAGTA-3′; antisense: 5′-TGGCAGCAGTG-GAAGAATCG-3′]) at 70°C for 10 min, 40 U reverse transcriptase was added in the presence of real-time (RT) buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 4 μL dNTP mix (250 μM each), 40 U RNase inhibitor, and RNase-free water to complete the final volume. The reaction mixture (20 μL) was incubated at 43°C for 1 h and then stopped at 4°C and used immediately for polymerase chain reaction (PCR).

Polymerase chain reaction

PCR amplification for *GADPH*, adiponectin (*ADIPOQ*), and *PPAR-γ* genes was performed using 40 ng cDNA (2 μL RT reaction mix), using GoTaq DNA polymerase (Promega, Fitchburg, WI, USA). PCR was performed in a final volume of 25 μL, for each target, as follows: 1.25 μL (12.5 pmol) of each primer (reverse and forward), 2 μL dNTP mix (0.2 mM each dNTP), 5 μL 5 × GoTaq DNA polymerase buffer (final concentration of 1 × [1.5 mM MgCl2]) 0.25 μL (2.5 U) GoTaq DNA polymerase, and PCR grade water to complete the final volume.

Western blot

Total protein content of retroperitoneal fat was quantified with the Bradford protein assay. Protein (50 μg) was loaded onto a 10% polyacrylamide gel for electrophoresis. After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane, blocked with a 5% non-fat milk solution, and washed in phosphate-buffered saline containing 0.1% Tween 20. Membranes were incubated overnight at 4°C with the following primary antibodies: GAPDH (1:5000 dilution; Cell Signaling Technology, Danvers, MA, USA); β-actin (1:5000 dilution; Cell Signaling Technology); vascular endothelial growth factor (VEGF; 1:500 dilution; Cell Signaling Technology); proliferating cell nuclear antigen (PCNA; 1:1000 dildilution; Cell Signaling Technology); sirtuin 1 (SIRT-1; 1:500 dilution; Cell Signaling Technology); peroxisome proliferator-activated receptor-γ (PPAR-γ, 1:1000 dilution; Cell Signaling Technology); protein kinase B (p-AKT and AKT 1:1000 dilution; Cell Signaling Technology); and c-Jun N-terminal kinase (p-JNK and JNK, 1:1000 dilution; Cell Signaling Technology). Thereafter, a monoclonal antirabbit or antimouse secondary antibody conjugated with peroxidase (1:1000-5000 dilution; Cell Signaling Technology) was used. Immunodetection was carried out using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) and images were obtained by scanner (Gel Logic 200 imaging system, Kodak, Rochester, NY, USA). The protein levels were expressed as a ratio of optical densities.

Statistical analysis

All data are expressed as mean ± SD. The normality of data was evaluated using Shapiro–Wilk test. Statistical significance ($P < 0.05$) was estimated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for simple comparisons between groups and two-way ANOVA followed by identity test for data evaluated overtime. Stata software version 8 (StatCorp, College Station, TX, USA) was used.

Results

Figure 1 shows food intake, weight gain, and feed efficiency ratio during the treatment period. In the first 90 d, the AL group had free access to food, whereas the CR50 and CR50-R animals were restricted to 50% of the amount consumed by AL. Weight gain was reduced by ~40% and feed efficiency was increased by ~50 % in both the CR50 and CR50-R groups compared with the AL group. After the additional 90 d of treatment, the AL and CR50 groups continued the previous treatment, while the CR50-R rats had free access to food. CR50-R rats increased their food consumption at similar levels to AL rats in just 1 wk and had a fast increase in feed efficiency and weight gain. During this period, the weight gain of CR50-R was ~80%, whereas the weight gain in AL and CR50 rats was ~25% and ~15%, respectively. The fast weight gain in the CR50-R group was probably a reflection of the increase in food efficiency.

Next, we evaluated whether changes in body weight and food intake could be accompanied by changes in plasma glucose homeostasis. CR50-R animals showed a significantly higher fasting glucose than animals in the AL and CR50 groups (Fig. 2A). Moreover, the CR50 group had improved glucose (Fig. 2B) and (Fig. 2C) insulin tolerance than did the AL group; these effects were completely abolished in the CR50-R arm.

Confirming our assumptions, the rapid body weight gain in the CR50-R group was accompanied by a significant increase in adiposity. Table 1 shows that, despite having a lower final body weight and length, CR50-R rats had similar levels of visceral fat weight and adiposity index as AL rats. As a consequence, CR50-R had a higher Lee index (a predictor of obesity in animals models) compared with both AL and CR50 rats.

To understand how refeeding induces the remodeling of adipose tissue, we evaluated the frequency distribution of adipocytes size and expression levels of *PCNA* and *VEGF* in retroperitoneal fat. Figure 3A shows representative H&E slides of adipocytes. As expected, the CR50 rats had higher frequency of smaller adipocytes (<2000 μm^2) and lower frequency of larger adipocytes (≥2000 μm^2) compared with the AL group. Refeeding partially abolished these effects (Fig. 3B,C). The *VEGF* content, a key factor in angiogenesis and adipocyte hypertrophy, was similar between the groups (Fig. 3D). Nevertheless, *PCNA* content, a marker of proliferation, was increased in CR50-R compared with AL and CR50 rats (Fig. 3E).

Finally, we investigated the influence of SCR and refeeding on some key intracellular proteins involved with adiposity and insulin sensitivity in the retroperitoneal adipose tissue (Fig. 4). *SIRT1* (Fig. 4A) and *PPAR-γ* (Fig. 4B) contents, beyond *PPAR-γ* (Fig. 4C) and *ADIPOQ* (Fig. 4D) mRNA were increased in CR50 compared with AL rats. These effects were partially abolished in CR50-R. In addition, p-AKT (Fig. 4E) and p-JNK (Fig. 4F) contents did not differ between the groups.

Discussion

Recent data have demonstrated that beginning a SCR at birth leads to positive effects on cardiometabolic risk factors and heart function [11,17]. In this study, we evaluated for the first time the effects on rats of a 50% CR initiated at birth and maintained until the age of 90 d. followed by an additional period of 90 d of refeeding on glucose homeostasis and adipose tissue. The main finds were as follows:

1. Despite the expressive reduction in weight gain and adiposity with CR, after just 1 wk of refeeding, the CR50-R rats increased their food consumption to similar levels of AL rats and quickly increased weight gain and adiposity.
2. Although CR50 rats presented an important improvement in glucose and insulin tolerance, these effects were completely abolished in CR50-R rats.
3. These alterations were accompanied by a remodeling of adipose tissue, and changes in intracellular proteins involved with adiposity and insulin sensitivity.

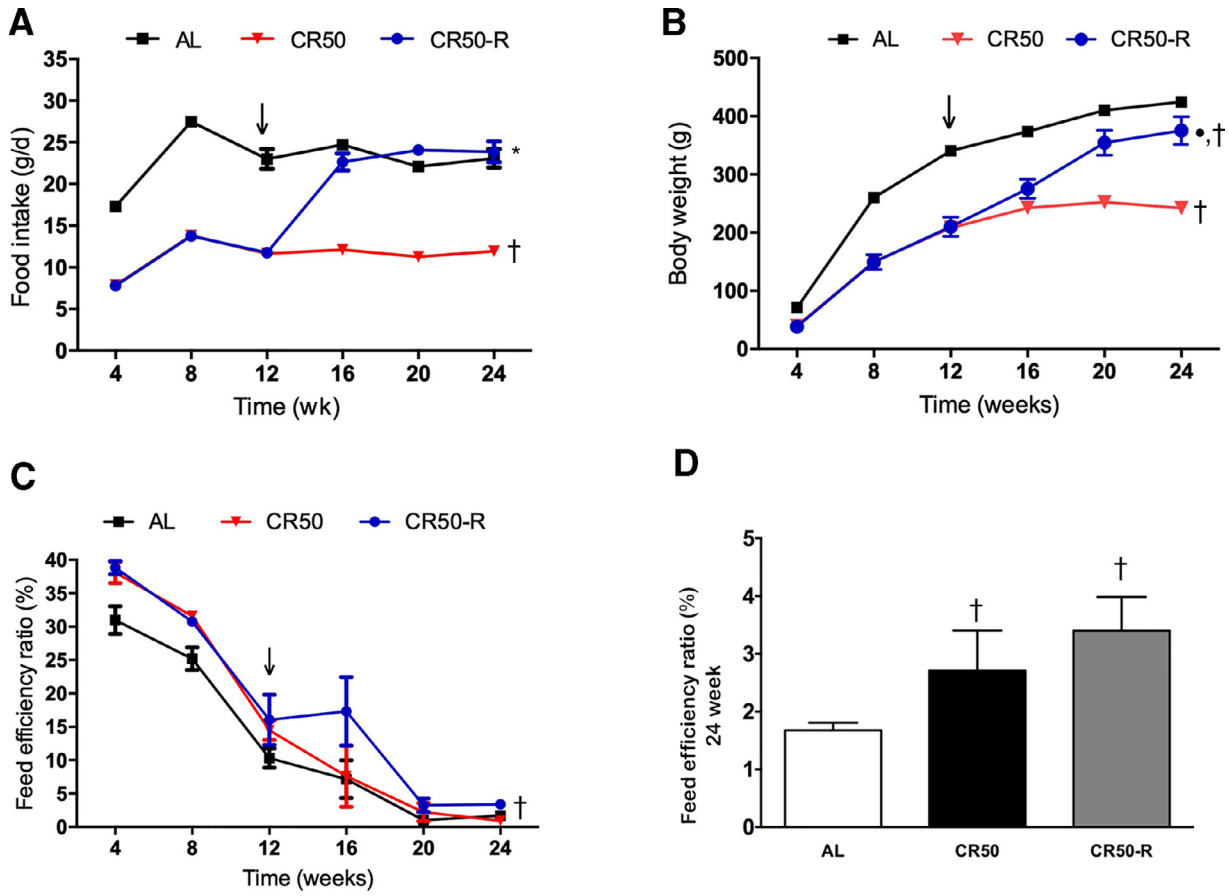

Fig. 1. Effects of 50% CR and refeeding on food intake and BW parameters. Food intake (**A**), BW (**B**), feed efficiency ratio (**C**), and final feed efficiency (**D**) of AL, CR50, and CR50-R groups during the 24 wk of treatment. Data are presented as mean ± SD. (↓) period in which CR50-R group started to be fed ad libitum. *$P < 0.05$ CR50 vs CR50-R. †$P < 0.05$ compared with AL group. One-way ANOVA followed by Tukey's post hoc test for simple comparisons between groups and two-way ANOVA followed by identity test for data evaluated overtime. AL, ad libitum; ANOVA, analysis of variance; BW, body weight; CR50, 50% calorie restriction; CR50-R, 50% calorie restriction/refeeding.

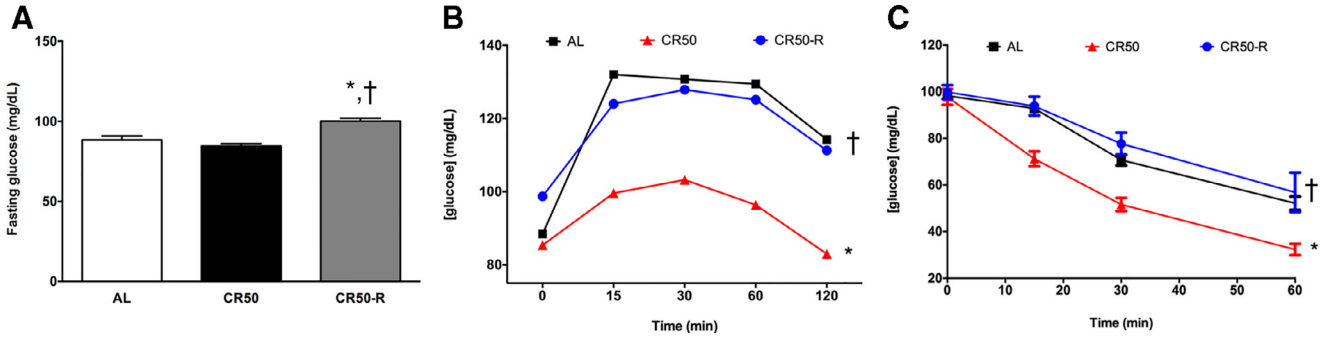

Fig. 2. Effects of 50% CR and refeeding on glucose homeostasis. Fasting glucose (**A**), time course oral tolerance glucose test (**B**) after oral glucose challenge (2 g/kg BW), time course insulin tolerance test (**C**) after intraperitoneal insulin challenge (1 U/kg BW) of AL, CR50, and CF50-R groups. Data presented as mean ± SD. * $P < 0.05$ compared with AL group. † $P < 0.05$ CR50 vs CR50-R. One-way ANOVA followed by Tukey's post hoc test for simple comparisons between groups and two-way ANOVA followed by identity test for data evaluated overtime. AL, ad libitum; ANOVA, analysis of variance; BW, body weight; CR50, 50% calorie restriction; CR50-R, 50% calorie restriction/refeeding.

Table 1
Effects of 50% calorie restriction and refeeding on final body weight and adiposity

	AL (n = 8)	CR50 (n = 8)	CR50-R (n = 8)
Final body weight (g)	421.5 ± 6.6	247.9 ± 9.2*	381.6 ± 7.4*,†
Length (cm)	22.3 ± 0.3	19 ± 0.1*	21.1 ± 0.2*,†
Lee index (g/cm)	33.3 ± 0.1	33.3 ± 0.1	34.5 ± 0.1*,†
Retroperitoneal fat (g)	6.6 ± 1.4	1.5 ± 0.5*	5.8 ± 1.3†
Epididymal fat (g)	6.6 ± 1.3	3.1 ± 1.2*	5.5 ±1.1†
Mesenteric fat (g)	5.3 ± 0.9	1.9 ± 0.5*	4.2 ± 0.9†
Total visceral fat (g)	18.5 ± 0.9	6 ± 0.8*	14.7 ± 1.3†
Total visceral fat/BW (g)	44.6 ± 2.1	26.9 ± 1.8*	38.8 ± 3.3†
Adiposity index (mg/g)	44 ± 0.5	24.2 ± 0.8*	43.3 ± 0.5†

AL, ad libitum; ANOVA, analysis of variance; BW, body weight; CR50, 50% calorie restriction; CR50-R, 50% calorie restriction/refeeding.
Data presented as mean ± SD. One-way ANOVA followed by Tukey's post hoc test.
*$P < 0.05$ compared with AL group.
†$P < 0.05$ CR50 vs CR50-R.

It is well established that CR reduces adiposity and changes the morphology of adipocytes, reducing their size and modifying the production of important adipokines, such as adiponectin [15,26]. These changes promoted by CR in adipose tissue are associated with improvement in lipid profile, glucose, and insulin tolerance in non-obese rats [27,28]. The magnitude of the effects of CR on adipose tissue appears to be associated with the age at which animals initiate the protocol. For example, Escrivá et al. [13] observed that CR was able to reverse insulin resistance associated with the accumulation of visceral adipose tissue in 8-mo-old animals but not in 24-mo-old animals.

The influence of age on the magnitude of the effects of CR appears to be associated with a metabolic reprogramming [29]. CR induces alterations in the expression of genes, especially those involved with mitochondrial energy metabolism. These adaptations lead to an improvement in mitochondrial function that favors, for example, a reduction in the production of reactive oxygen species. Therefore, it is believed that the earlier in life these adaptations are induced, the lower the cellular damage commonly observed with ageing [11,16,17,29,30]. Corroborating with this hypothesis, in the present study, beginning SCR at birth induced profound modifications on adipose tissue with changes in the morphology and signaling of adipocytes and in the improvement in glucose metabolism.

On the other hand, after a period of CR, refeeding with food that is offered ad libitum has been related to increased adiposity, adipocyte hypertrophy, or hyperplasia [15,21,31,32]. Sucajtys-Szulc et al. [21] showed that adult animals submitted to 50% CR for 30 d showed 70% reduction in visceral adipose tissue and a marked decrease in insulin levels. With only 2 d of refeeding, an important increase in adipose mass and insulin plasma levels was observed. In a longer period of CR/refeeding, Giller et al. [15] found that young mice submitted to 25% CR for 6 mo showed 85% reduction in visceral adipose tissue content and after 6 mo of refeeding these effects were completely gone. Here we found a 70% reduction in visceral adipose tissue and a marker reduction in adipocyte area after 180 d of SCR beginning at birth. These effects were no longer seen after refeeding.

CR leads to altered energy status, which is detected by molecular nutrient sensors such as SIRT1. SIRT1 has emerged as a key protein activated by CR in adipocytes [33]. CR increases SIRT1 content in adipose tissue [6,33–35] which is associated with increased lipolysis [36], decreased adipogenesis [37,38] and inflammation [39,40], as well as improved glucose and insulin tolerance [33,34]. In keeping with this, Chalkiadaki et al. [41] demonstrated that SIRT1 knockout mice presented severe glucose intolerance and insulin resistance, which was associated with a marked increase in adiposity. Yet, Xu et al. [42] found that overexpression of *SIRT1* in the adipose tissue of mice prevented aging-induced insulin sensitivity reduction.

Here, we speculated that the alterations in adipose tissue morphology, glucose, and insulin tolerance of the CR/refeeding groups would involve changes in adipose tissue SIRT1.

SIRT1 reduces adipocyte hyperplasia by reducing cell proliferation [43]. In fact, although CR50 animals had a decrease in adiposity and an increase in adipose *SIRT1* content, CR50-R rats demonstrated opposite changes: They presented an increase in adiposity and a decrease in *SIRT1* content. Moreover the decrease in *SIRT1* content in CR50-R animals was accompanied by an increase in *PCNA* expression (an important marker of cell proliferation) [44], indicating adipocyte hyperplasia in these rats.

The capacity of adipocyte *SIRT1* to improve glucose and insulin tolerance may occur by increasing *PPAR-γ* activity, which represses genes associated with insulin resistance in visceral white adipose tissue [45,46]; increasing the expression of adiponectin [47,48] and p-AKT levels [39,40], which increases the efficiency of GLUT4 translocation; and decreasing JNK phosphorylation [39], which decreases inflammatory responses. All these mechanisms contribute to improved glucose and insulin tolerance, preventing the development of insulin resistance [39,46,49].

We found that the improved glucose/insulin tolerance after SCR was accompanied by an upregulation of adipose tissue *SIRT1/PPAR-γ/ADIPOQ* expression. All these effects were abolished after refeeding. Interestingly, adipose p-AKT and p-JNK contents were not altered by either SCR or refeeding. Thus, it is plausible that the up- and downregulation of *SIRT1/PPAR-γ/ADIPQ* may contribute to the increase and decrease of glucose/insulin tolerance in CR and CR50-R groups, respectively.

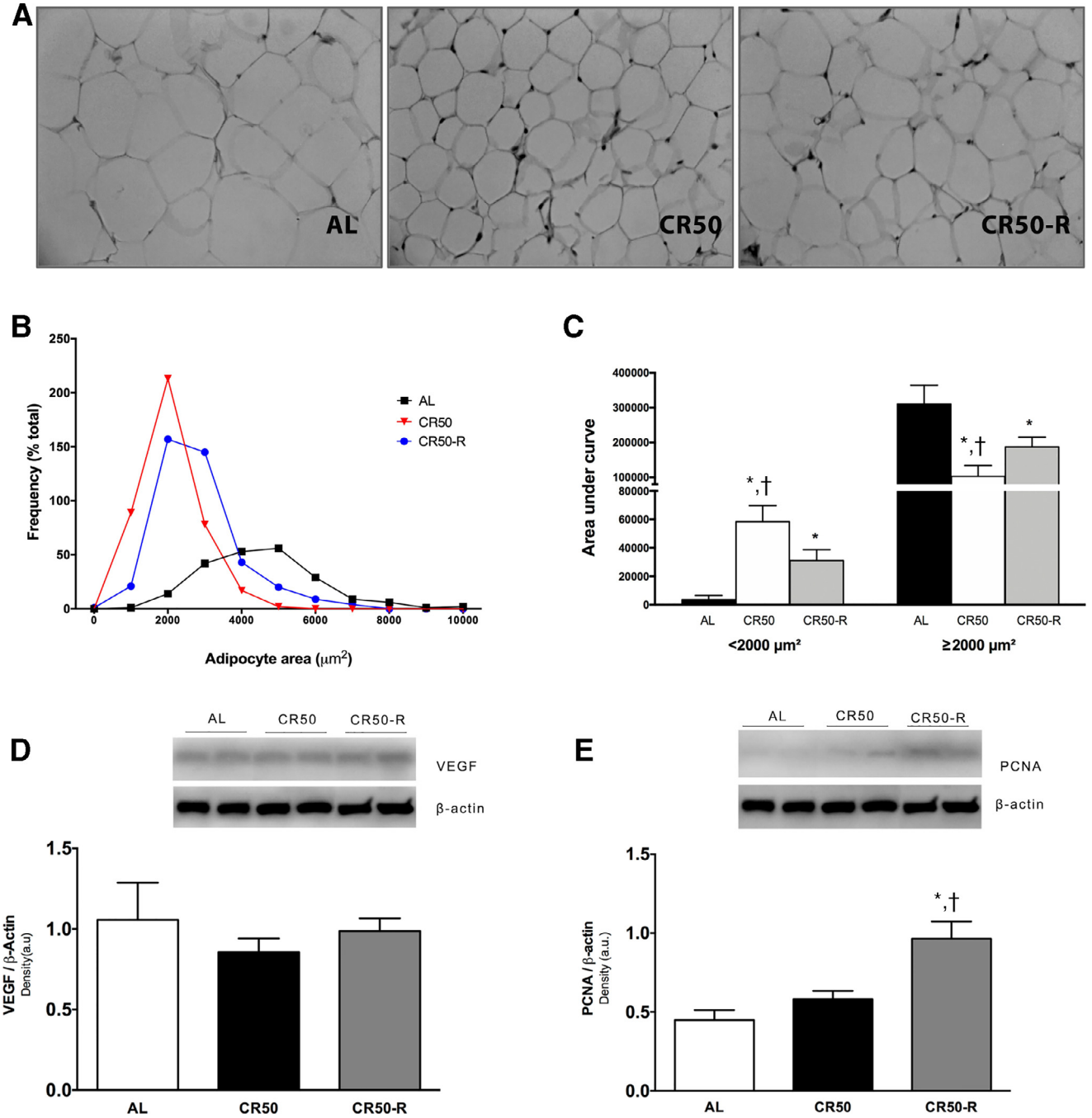

Fig. 3. Effects of 50% CR and refeeding on morphometry and protein levels of PCNA and VEGF in retroperitoneal fat. Representative H&E slides of adipocytes in the retroperitoneal fat (**A**). Analysis of frequency distribution of adipocyte size (**B,C**). Protein levels of VEGF (**D**) and PCNA (**E**) by Western blot analysis. AL, CR50, and CR50-R groups. Data presented as mean ± SD. * $P < 0.05$ compared with AL group. † $P < 0.05$ CR50 vs CR50-R. Scale bar = 20 μm. One-way ANOVA followed by Tukey's post hoc test. AL, ad libitum; ANOVA, analysis of variance; CR50, 50% calorie restriction; CR50-R, 50% calorie restriction/refeeding; H&E, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen; VEGF, vascular endothelial growth factor.

Although the mechanisms by which refeeding abolishes the beneficial effects of CR are not completely understood, recent studies have pointed to the involvement of sirtuins in this mechanism. Wronska et al. [50] showed that despite the content of SIRT-7 in retroperitoneal adipose tissue, SIRT-7 content did not change after 30 d of 40% CR in adult and older rats. Aafter only 2 d of refeeding, SIRT-7 content decreased significantly.

In previous studies, we demonstrated that the SCR model from birth to adult age reduced cardiometabolic risk factors, improved cardiac function, and protected the hearts of deleterious insults [11,17]. In the present study, we found that the beneficial effects of SCR also extended to adipose tissue. Additionally, we advanced the understanding of the effects of SCR after potentially deleterious conditions, such as refeeding, a recurrent condition in humans who practice food/CR in their daily lives.

In general, refeeding abolished the positive effects of the SCR leading to a return to baseline conditions seen in AL rats. One question that arises from the present study is whether by increasing the

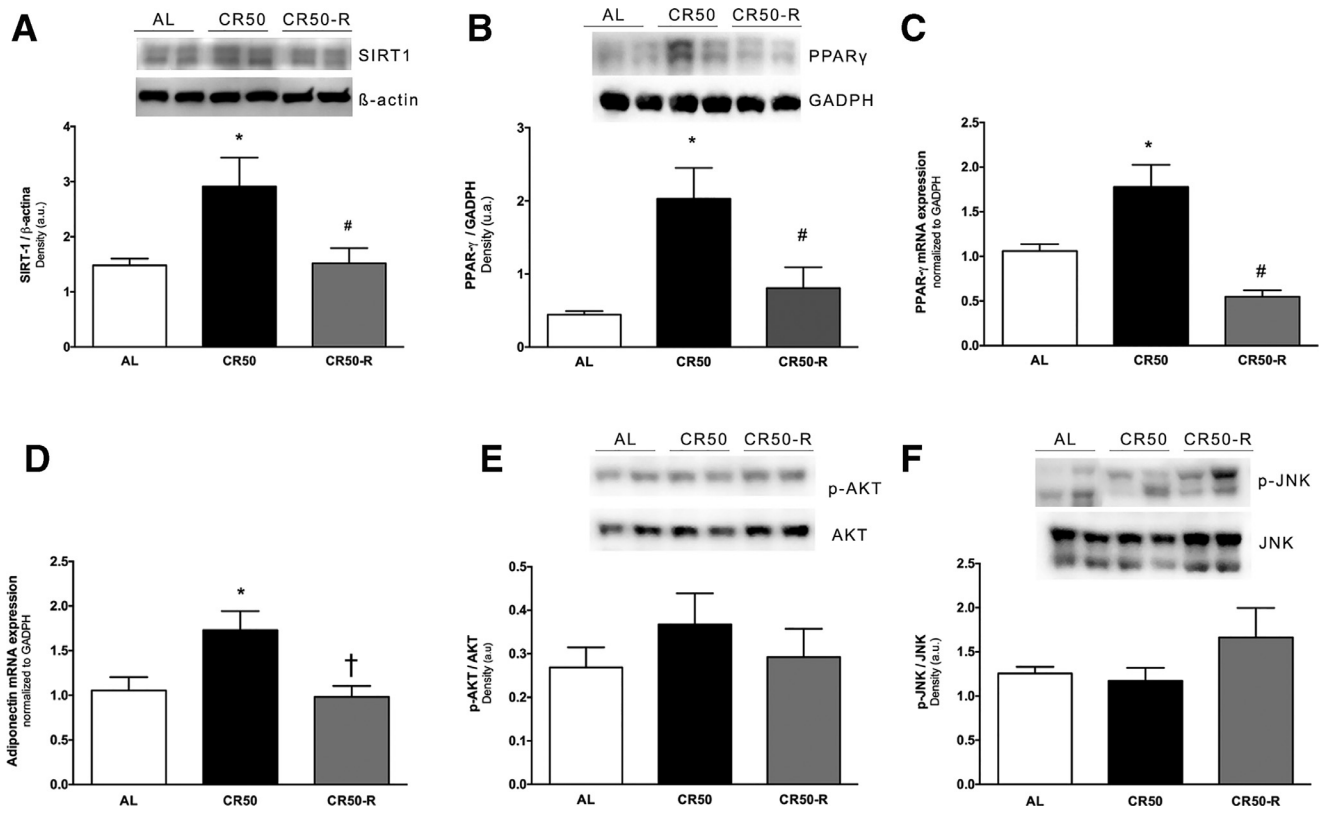

Fig. 4. Effects of 50% CR and refeeding on retroperitoneal fat SIRT1 signaling. Expression levels of Sirt-1 protein (**A**), PPAR-γ protein (**B**), PPAR-γ mRNA (**C**), adiponectin mRNA (**D**), AKT protein and JNK protein (**E**) in the retroperitoneal adipocytes of AL, CR50,and CR50-R groups. Data ae presented as mean ± SD. $^{*}P < 0.05$ compared with AL group. $^{\dagger}P < 0.05$ CR50 vs CR50-R. One-way ANOVA followed by Tukey's post hoc test. AL, ad libitum; ANOVA, analysis of variance; CR50, 50% calorie restriction; CR50-R, 50% calorie restriction/refeeding; AKT, protein kinase B; JNK, c-Jun N-terminal kinase; PPAR, peroxisome proliferator-activated receptor; Sirt, sirtuin.

period of refeeding, CR50-R animals will remain with normal glucose and insulin tolerance or will develop insulin resistance. The higher fasting glycemia of these animals may give evidence of a possible deterioration of glucose metabolism after refeeding. Recent evidence suggests that SIRT1 promoted deacetylation on PPAR-γ, leading to a browning of adipose tissue, which also improves glucose homeostasis [46,51,52].

However, it's important to note that all of the intracellular proteins investigated here participate in several signaling cascades in adipose tissue and this evaluation is beyond the scope of this study. Furthermore, in future studies we intend to evaluate the effects of SCR/refeeding in other insulin-dependent tissues. Yet, we cannot discard negative effects of this SCR protocol, such as growth retardation.

Conclusions

Our findings support the notion that in rats, beginning SCR at birth and maintaining it to the beginning of adult life promotes a large decrease in visceral adiposity, improvement in glucose or insulin tolerance, and an upregulation of adipose *SIRT1*, *PPAR-γ*, and *ADIPOQ*. All these effects are abolished after an equal period of refeeding.

References

[1] Gastaldelli A, Gaggini M, DeFronzo RA. Role of adipose tissue insulin resistance in the natural history of type 2 diabetes: results from the San Antonio Metabolism Study. Diabetes 2017;66:815–22.
[2] Coelho M, Oliveira T, Fernandes R. Biochemistry of adipose tissue: an endocrine organ. Arch Med Sci 2013;9:191–200.
[3] Gabriely I, Ma XH, Yang XM, Atzmon G, Rajala MW, Berg AH, Scherer P, Rossetti L, Barzilai N. Removal of Visceral fat prevents insulin resistance and glucose intolerance of aging. Diabetes 2002;51:2951–8.
[4] Preis SR, Massaro JM, Robins SJ, Hoffmann U, Vasan RS, Irlbeck T, et al. Abdominal subcutaneous and visceral adipose tissue and insulin resistance in the Framingham Heart Study. Obesity 2010;18:2191–8.
[5] Catalano KJ, Stefanovski D, Bergman RN. Critical role of the mesenteric depot versus other intra-abdominal adipose depots in the development of insulin resistance in young rats. Diabetes 2010;59:1416–23.
[6] Bordone L, Guarente L. Calorie restriction, SIRT1 and metabolism: understanding longevity. Nat Rev Mol Cell Biol 2005;6:298.
[7] Speakman JR, Mitchell SE. Caloric restriction. Mol Aspects Med 2011;32:159–221.
[8] Al-Regaiey KA, Masternak MM, Bonkowski MS, Panici JA, Kopchick JJ, Bartke A. Effects of caloric restriction and growth hormone resistance on insulin-related intermediates in the skeletal muscle. J Gerontol Ser A Biol Sci Med Sci 2007;62:18–26.
[9] Zheng Y, Zhang W, Pendleton E, Leng S, Wu J, Chen R, et al. Improved insulin sensitivity by calorie restriction is associated with reduction of ERK and p70(S6 K) activities in the liver of obese Zucker rats. J Endocrinol 2009;203:337–47.
[10] Cerqueira FM, da Cunha FM, Caldeira da Silva CC, Chausse B, Romano RL, Garcia CCM, et al. Long-term intermittent feeding, but not caloric restriction, leads to redox imbalance, insulin receptor nitration, and glucose intolerance. Free Radic Biol Med 2011;51:1454–60.
[11] Melo DS, Costa-Pereira LV, Santos CS, Mendes BF, Costa KB, Santos CFF, et al. Severe Calorie restriction reduces cardiometabolic risk factors and protects rat hearts from ischemia/reperfusion injury. Front Physiol 2016;7:106.
[12] Catalano KJ, Bergman RN, Ader M. Increased susceptibility to insulin resistance associated with abdominal obesity in aging rats. Obes Res 2005;13:11–20.
[13] Escrivá F, Gavete ML, Fermín Y, Pérez C, Gallardo N, Alvarez C, et al. Effect of age and moderate food restriction on insulin sensitivity in Wistar rats: role of adiposity. J Endocrinol 2007;194:131–41.
[14] Yamagishi T, Bessho M, Yanagida S, Nishizawa K, Kusuhara M, Ohsuzu F, et al. Severe, short-term food restriction improves cardiac function following ischemia/reperfusion in perfused rat hearts. Heart Vessels 2010;25:417–25.
[15] Giller K, Huebbe P, Hennig S, Dose J, Pallauf K, Doering F, et al. Beneficial effects of a 6-month dietary restriction are time-dependently abolished within 2 weeks or 6 months of refeeding—genome-wide transcriptome analysis in mouse liver. Free Radic Biol Med 2013;61:170–8.
[16] Han X, Ren J. Caloric restriction and heart function: is there a sensible link? Acta Pharmacol Sin 2010;31:1111–7.
[17] Melo DS, Riul TR, Esteves EA, Moraes PL, Ferreira FO, Gavioli M, et al. Effects of severe caloric restriction from birth on the hearts of adult rats. Appl Physiol Nutr Metab 2013;38:879–85.

[18] Evans SA, Messina MM, Knight WD, Parsons AD, Overton JM. Long-Evans and Sprague-Dawley rats exhibit divergent responses to refeeding after caloric restriction. Am J Physiol Integr Comp Physiol 2005;288:R1468–76.
[19] Mager DE, Wan R, Brown M, Cheng A, Wareski P, Abernethy DR, et al. Caloric restriction and intermittent fasting alter spectral measures of heart rate and blood pressure variability in rats. FASEB J 2006;20:631–7.
[20] Duarte FO, Sene-Fiorese M, Manzoni MSJ, de Freitas LF, Cheik NC, Garcia de Oliveira Duarte AC, et al. Caloric restriction and refeeding promoted different metabolic effects in fat depots and impaired dyslipidemic profile in rats. Nutrition 2008;24:177–86.
[21] Sucajtys-Szulc E, Goyke E, Korczynska J, Stelmanska E, Rutkowski B, Swierczynski J. Refeeding after prolonged food restriction differentially affects hypothalamic and adipose tissue leptin gene expression. Neuropeptides 2009;43:321–5.
[22] Barbosa-da-Silva S, Fraulob-Aquino JC, Lopes JR, Mandarim-de-Lacerda CA, Aguila MB. Weight cycling enhances adipose tissue inflammatory responses in male mice. PLoS One 2012;7:39837.
[23] Malafaia AB, Nassif PAN, Ribas CAPM, Ariede BL, Sue KN, Cruz MA. of obesity with sucrose in rats. ABCD Arq Bras Cir Dig 2013;6:17–21.
[24] Boustany CM, Bharadwaj K, Daugherty A, Brown DR, Randall DC, Cassis LA. Activation of the systemic and adipose renin-angiotensin system in rats with diet-induced obesity and hypertension. Am J Physiol Regul Integr Comp Physiol 2004;287:R943–9.
[25] Parlee SD, Lentz SI, Mori H, MacDougald OA. Quantifying size and number of adipocytes in adipose tissue. Methods Enzymol 2014;537:93–122.
[26] Okita N, Hayashida Y, Kojima Y, Fukushima M, Yuguchi K, Mikami K, et al. Differential responses of white adipose tissue and brown adipose tissue to caloric restriction in rats. Mech Ageing Dev 2012;133:255–66.
[27] Davidson RT, Arias EB, Cartee GD. Calorie restriction increases muscle insulin action but not IRS-1-, IRS-2-, or phosphotyrosine-PI 3-kinase. Am J Physiol Metab 2002;282:E270–6.
[28] Chen YR, Fang SR, Fu YC, Zhou XH, Xu MY, Xu WC. Calorie restriction on insulin resistance and expression of SIRT1 and SIRT4 in rats. Biochem Cell Biol 2010;88:715–22.
[29] Anderson RM, Weindruch R. Metabolic reprogramming, caloric restriction and aging. Trends Endocrinol Metab 2010;21:134–41.
[30] Mattison JA, Colman RJ, Beasley TM, Allison DB, Kemnitz JW, Roth GS, et al. Caloric restriction improves health and survival of rhesus monkeys. Nat Commun 2017;8:14063.
[31] Wronska A, Sledzinski T, Goyke E, Lawniczak A, Wierzbicki P, Kmiec Z. Short-term calorie restriction and refeeding differently affect lipogenic enzymes in major white adipose tissue depots of young and old rats. J Physiol Pharmacol 2014;65:117–26.
[32] Kliewer KL, Ke J-Y, Stout MB, Cole R, Samuel VT, Shulman GI, et al. Short-term food restriction followed by controlled refeeding promotes gorging behavior, enhances fat deposition, and diminishes insulin sensitivity in mice. J Nutr Biochem 2015;26:721–8.
[33] Cao Y, Jiang X, Ma H, Wang Y, Xue P, Liu Y. SIRT1 and insulin resistance. J Diabetes Complications 2016;30:178–83.
[34] Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, de Oliveira RM, et al. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-γ. Nature 2004;429:771.
[35] Nemoto S, Takeda K, Yu Z-X, Ferrans VJ, Finkel T. Role for mitochondrial oxidants as regulators of cellular metabolism. Mol Cell Biol 2000;20:7311–8.
[36] Chakrabarti P, English T, Karki S, Qiang L, Tao R, Kim J, et al. SIRT1 controls lipolysis in adipocytes via FOXO1-mediated expression of ATGL. J Lipid Res 2011;52:1693–701.
[37] Jin Q, Zhang F, Yan T, Liu Z, Wang C, Ge X, et al. C/EBP regulates SIRT1 expression during adipogenesis. Cell Res 2010;20:470–9.
[38] Pang W, Wang Y, Wei N, Xu R, Xiong Y, Wang P, et al. Sirt1 Inhibits Akt2-mediated porcine adipogenesis potentially by direct protein-protein interaction. PLoS One 2013;8:E71576.
[39] Yoshizaki T, Milne JC, Imamura T, Schenk S, Sonoda N, Babendure JL, et al. SIRT1 exerts anti-inflammatory effects and improves insulin sensitivity in adipocytes. Mol Cell Biol 2009;29:1363–74.
[40] Wang C-H, Wang C-C, Huang H-C, Wei Y-H. Mitochondrial dysfunction leads to impairment of insulin sensitivity and adiponectin secretion in adipocytes. FEBS J 2013;280:1039–50.
[41] Chalkiadaki A, Guarente L. High-fat diet triggers inflammation-induced cleavage of SIRT1 in adipose tissue to promote metabolic dysfunction. Cell Metab 2012;16:180–8.
[42] Xu C, Bai B, Fan P, Cai Y, Huang B, Law IKM, et al. Selective overexpression of human SIRT1 in adipose tissue enhances energy homeostasis and prevents the deterioration of insulin sensitivity with ageing in mice. Am J Transl Res 2013;5:412–26.
[43] Abdesselem H, Madani A, Hani A, Al-Noubi M, Goswami N, Ben Hamidane H, et al. SIRT1 limits adipocyte hyperplasia through c-Myc inhibition. J Biol Chem 2016;291:2119–35.
[44] Lo Y-H, Ho P-C, Chen M-S, Hugo E, Ben-Jonathan N, Wang S-C. Phosphorylation at tyrosine 114 of proliferating cell nuclear antigen (PCNA) is required for adipogenesis in response to high fat diet. Biochem Biophys Res Commun 2013;430:43–8.
[45] Janani C, Ranjitha Kumari BD. PPAR gamma gene—a review Diabetes Metab Syndr. Clin Res Rev 2015;9:46–50.
[46] Qiang L, Wang L, Kon N, Zhao W, Lee S, Zhang Y, et al. Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Pparγ. Cell 2012; 150:620–32.
[47] Qiao L, Schaack J, Shao J. Suppression of adiponectin gene expression by histone deacetylase inhibitor valproic acid. Endocrinology 2006;147:865–74.
[48] Takemori K, Kimura T, Shirasaka N, Inoue T, Masuno K, Ito H. Food restriction improves glucose and lipid metabolism through Sirt1 expression: a study using a new rat model with obesity and severe hypertension. Life Sci 2011;88:1088–94.
[49] Gillum MP, Kotas ME, Erion DM, Kursawe R, Chatterjee P, Nead KT, et al. SirT1 regulates adipose tissue inflammation. Diabetes 2011;60:3235–45.
[50] Wronska A, Lawniczak A, Wierzbicki PM, Kmiec Z. Age-related changes in sirtuin 7 expression in calorie-restricted and refed rats. Gerontology 2016;62:304–10.
[51] Boutant M, Cantó C. SIRT1 metabolic actions: integrating recent advances from mouse models. Mol Metab 2014;3:5–18.
[52] Boutant M, Joffraud M, Kulkarni SS, García-Casarrubios E, García-Roves PM, Ratajczak J, et al. SIRT1 enhances glucose tolerance by potentiating brown adipose tissue function. Mol Metab 2015;4:118–31.