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Original Contribution

Long-term intermittent feeding, but not caloric restriction, leads to redox imbalance, insulin receptor nitration, and glucose intolerance

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ABSTRACT

Calorie restriction is a dietary intervention known to improve redox state, glucose tolerance, and animal life span. Other interventions have been adopted as study models for caloric restriction, including nonsupplemented food restriction and intermittent, every-other-day feedings. We compared the short- and long-term effects of these interventions to ad libitum protocols and found that, although all restricted diets decrease body weight, intermittent feeding did not decrease intra-abdominal adiposity. Short-term calorie restriction and intermittent feeding presented similar results relative to glucose tolerance. Surprisingly, long-term intermittent feeding promoted glucose intolerance, without a loss in insulin receptor phosphorylation. Intermittent feeding substantially increased insulin receptor nitration in both intra-abdominal adipose tissue and muscle, a modification associated with receptor inactivation. All restricted diets enhanced nitric oxide synthase levels in the insulin-responsive adipose tissue and skeletal muscle. However, whereas calorie restriction improved tissue redox state, food restriction and intermittent feedings did not. In fact, long-term intermittent feeding resulted in largely enhanced tissue release of oxidants. Overall, our results show that restricted diets are significantly different in their effects on glucose tolerance and redox state when adopted long-term. Furthermore, we show that intermittent feeding can lead to oxidative insulin receptor inactivation and glucose intolerance.

McCay [1] first demonstrated that rats restricted in caloric intake exhibited extended life spans. Subsequently, calorie restriction (CR) was shown to extend life span in many animal models [2–4] and attracted the attention of researchers uncovering mechanisms involved in animal aging. Unfortunately, studies involving dietary limitations and their biological effects have diverged over the years regarding the protocol adopted. Today, many diets commonly referred to as CR in the literature involve intermittent feeding and fasting cycles (IF), also known as every-other-day feedings, or food restriction (FR) without micronutrient supplementation (incurring malnutrition [5]). Although some metabolic changes similar to those observed in CR are present in FR or IF [6–9], there is little or inconsistent evidence to date that these interventions promote life-span benefits [10–14; reviewed in 5,6,15]. Furthermore, most studies adopt a single nutritional protocol, precluding side-by-side comparisons between these clearly distinct diets.

Of the many effects of CR, two are widely accepted to be connected with extended longevity: redox changes and alterations in insulin signaling pathways [16–18]. CR limits mitochondrial generation of reactive oxygen species (ROS), alters the expression and activity of antioxidant pathways, and prevents oxidative modifications of biomolecules during aging [4,18,19]. CR also prevents the loss of peripheral sensitivity to insulin, precluding many of the effects of aging associated with insulin resistance [16,17]. Changes in redox state and insulin signaling may be linked, although this cross talk is still poorly explored. Insulin receptor sensitivity has been shown to increase in response to mild oxidative imbalance [20,21], whereas excessive ROS production in diabetes and obesity can impair insulin signaling [22,23].

Here, we compare the short- and long-term effects of ad libitum feeding (AL), CR, and IF on body weight, intra-abdominal fat accumulation, glucose tolerance, and insulin signaling. We find that, although IF and CR are similar in short-term studies, they are strikingly different in long-term interventions. Long-term IF promotes redox imbalance, oxidative modification of the insulin receptor, and glucose intolerance.

Experimental procedures

Animals

All experiments were conducted in agreement with the National Institutes of Health guidelines for humane treatment of animals and were reviewed and approved by the local Animal Care and Use Committee. For short-term studies, male, 4-week-old Sprague–Dawley rats were separated into three groups: AL, fed ad libitum with an

Abbreviations: AL, ad libitum; CR, calorie restriction; FR, food restriction; IR, insulin receptor; IF, intermittent feeding/fasting; NOS, nitric oxide synthase; ROS, reactive oxygen species.

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AIN-93-M diet prepared by Rhoster (Campinas, SP, Brazil); CR, fed daily with 60% of a diet supplemented with micronutrients, to reach the vitamin and mineral levels consumed by AL animals; and IF, which had ad libitum access to the AIN-93-M diet on alternating days. Body mass and food consumption were recorded weekly. In the long-term study, the FR group was added (FR, fed daily with 60% weight of the same diet offered to AL animals), and the rats were divided into groups at 8 weeks of age. Food was offered to FR and CR rats at 6:00 PM, the same time at which IF chow was alternately placed or removed. FR and CR feedings were adjusted weekly by weight, based on AL food consumption measured 1 week prior. The animals were lodged three individuals per cage, in 12-h light/dark cycles, and given water ad libitum.

After 4 weeks or 8 months of dietary intervention, the rats were sacrificed after 12 h of overnight fasting and tissues were dissected and weighed. Total hindlimb skeletal muscle and intra-abdominal fat deposits were pooled and homogenized together. To ensure that all rats presented comparable feeding statuses, food was placed at 6:00 PM for all groups. Food was removed at 8:00 PM, and the animals were sacrificed at 8:00 AM the following day. During the long-term dietary intervention, 2 of 8 animals in the FR group and 1 of 15 AL rats died spontaneously, 2 of 16 animals from the IF group were removed because of locomotive alterations, and 1 was eliminated because of the presence of tumors. No animals were eliminated from the CR group (18 in total).

Glucose levels and glucose tolerance tests

Peripheral blood was collected from the tail of 8- or 40-week-old animals fasted for 12 h and immediately used for glucose analysis using a glucose analyzer (Accu-Check Performa, São Paulo, SP, Brazil). For glucose tolerance tests, rats were anesthetized with isoflurane (5%) using a calibrated vaporizer (35–50% O_2; 50–65% N_2O), at the end of the dietary intervention and after an overnight fasting period. A 200 g/L glucose solution was injected ip at a final dose of 2 g kg^{-1} [24], and blood samples for glycemic determinations were obtained from the paw at 0, 30, 60, and 120 min after the challenge and analyzed using a glucose analyzer.

Immunoprecipitations

Intra-abdominal adipose tissue and skeletal muscle samples were homogenized using an electric potter in lysis buffer (50 mM sodium phosphate, pH 7.4, 10% glycerol, 1% octyl phenol ethoxylate, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, supplemented with a Sigma protease inhibitor mixture). After 30 min on ice, tissue lysates were centrifuged (13,000 *g*, 20 min, 4 °C), and the resulting supernatants were collected. Solubilized proteins, at final concentration of 1 (adipose tissue) or 2 mg/ml (muscle), were incubated with anti-insulin receptor (IR) β subunit (4 μg/ml) or anti-insulin receptor substrate 1 (IRS1) (3 μg/ml) antibodies at 4 °C overnight. Protein A–agarose (Sigma) beads were added, and the incubation was continued at 4 °C for 2 h. The beads were centrifuged (13,000 *g*, 1 min, 4 °C), washed five times in lysis buffer, and suspended in Laemmli sample buffer containing 5% 2-mercaptoethanol. Immunoprecipitation specificity was verified through SDS–PAGE separation of precipitated samples followed by silver-stained polyacrylamide gels.

Western blots

Proteins from tissue lysates or protein agarose beads conjugated with IR or IRS1 were diluted in Laemmli sample buffer containing 5% 2-mercaptoethanol. After heating at 90 °C for 15 min, proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes. After membranes were blocked with 5% bovine serum albumin, detection of individual proteins was carried out by blotting with specific primary antibodies against adiponectin (Abcam; 0.5 μg ml^{-1}), IR (Upstate; 1:1000), IRS1 (Upstate; 0.54 μg ml^{-1}), phosphotyrosine (Upstate; 0.5 μg ml^{-1}), nitrotyrosine (OxisResearch; 1:2000), dinitrophenylhydrazine (Sigma; 1:5000), endothelial nitric oxide synthase (eNOS; Sigma; 1:3000), phospho-eNOS^{Ser1177} (Cell Signaling; C9C3 clone, 1:1000), inducible NOS (iNOS; Alexis Biochemicals; 1:750), catalase (Abcam; 1:1000), superoxide dismutase 2 (Abcam; 0.5 μg ml^{-1}), or γ-actin (Sigma; 1:2000), a loading control. Chemiluminescence detection using a secondary peroxidase-linked anti-rabbit (Calbiochem; 1:10,000) or anti-sheep IgG (Calbiochem; 1:13,000) and a detection system from Pierce KLP (Rockford, IL, USA) was performed. Signals were quantified by densitometry using ImageQuant (Amersham Biosciences) and corrected to actin levels. Adiponectin and dinitrophenylhydrazine were corrected using Ponceau red staining. Phospho-eNOS, phospho-Tyr-IR, phospho-Tyr-IRS1, and NO_2-Tyr-IR were corrected to the total amount of immunoprecipitate.

H_2O_2 release

Tissues were extracted and immediately segmented into fine pieces (~1 mm) in PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) supplemented with 10 mM glucose, 50 μM Amplex red, and 1 U ml^{-1} horseradish peroxidase. Amplex red reacts with peroxidase–H_2O_2 complexes producing fluorescent resorufin [25]. Fluorescence was measured at ex 563 nm and em 587 nm, using 5-nm slits, within 1 h of tissue preparation. Baseline fluorescence in the same medium was subtracted from all measurements. A calibration curve was constructed using commercial H_2O_2.

Statistical analysis

Data were analyzed using GraphPad Prism and Origin software. Figures represent averages ± SEM of 3–12 measurements and were compared using ANOVA. Two-tailed p values under 0.05 were considered significant.

Results

Fig. 1 compares the effects of short- (4 weeks) and long- (32 weeks) term dietary interventions on animal weight and intra-abdominal adiposity. FR, CR, and IF animals presented lower body weights compared to AL at both time points (Fig. 1A and B), although total food ingestion per week in IF animals was equal to that of AL (Fig. 1C and D) due to overeating during the feeding period. We found that IF animals ingested approximately 60% of their 24-h food consumption within the first 2 h after chow placement, a consumption rate of 19 g h^{-1} rat^{-1}, whereas FR and CR animals presented food intake rates of 9 g h^{-1} rat^{-1}.

Although body weights were lower in all restricted groups, IF rats presented high intra-abdominal adiposity at both time points (Fig. 1E and F). FR and CR animals, in turn, presented low levels of abdominal adiposity. Consistently, serum adiponectin levels, which are typically inversely proportional to abdominal adiposity [26], were low in AL and IF at both time points and high in CR and FR animals (Fig. 1G and H). Altogether, these results suggest that, although all feeding strategies led to weight reductions compared to AL animals, IF differs significantly from FR and CR in terms of energy intake and balance.

Short-term dietary interventions did not alter fasting glucose levels (Fig. 2A). Long-term maintenance on an AL diet led to increased fasting glucose levels, which only CR prevented significantly (Fig. 2B). Glucose levels 2 h after food was offered were in the range of 240 mg dl^{-1} in IF animals, whereas FR and CR levels were approximately 150 and 130 mg dl^{-1}, respectively, versus 150 mg dl^{-1} in AL (measured after 2 h in the dark period in 40-week-old rats).

Because of the changes observed in fasting glucose levels, we performed a glucose tolerance test. We found that short-term IF and CR improve glucose clearance (Fig. 2C and E). The same was not verified in

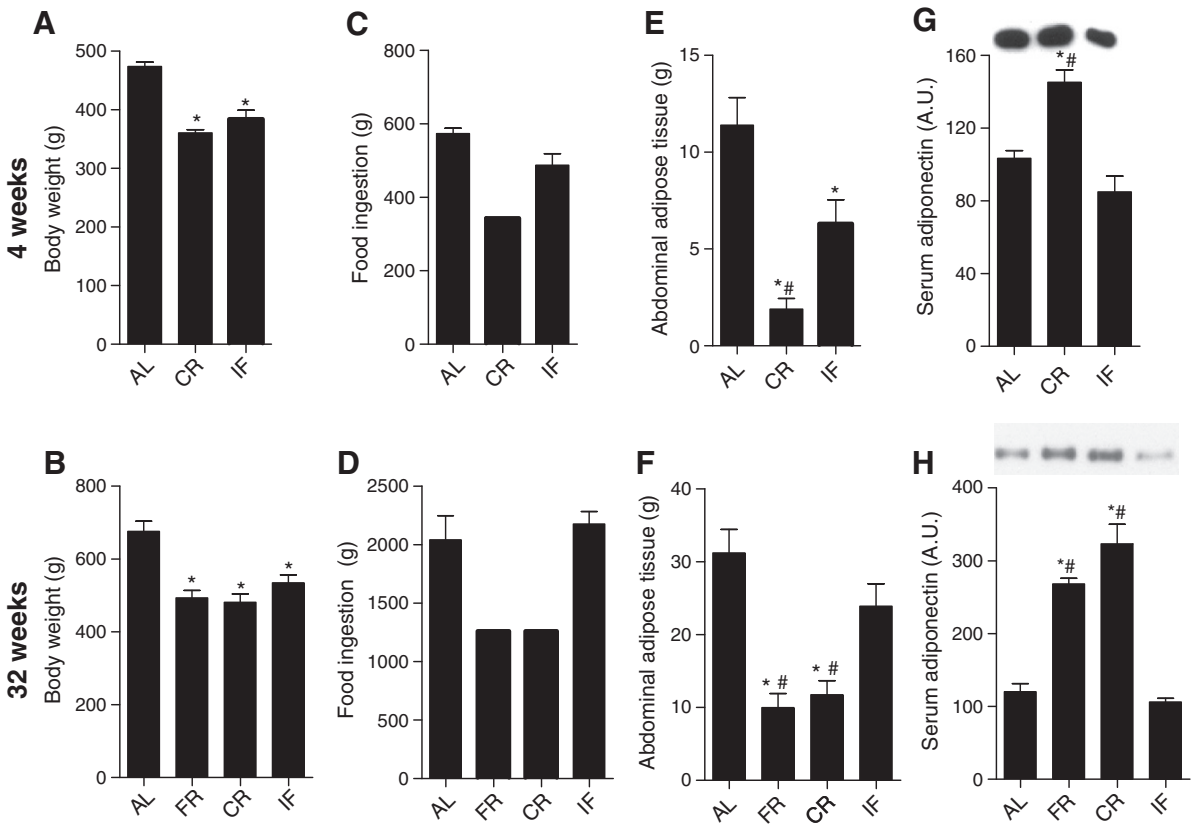

Fig. 1. IF decreases body weight, but not food ingestion or intra-abdominal adiposity. Animals were subjected to AL, FR, CR, or IF diet. Body mass after (A) 4 or (B) 32 weeks of dietary intervention, food consumption per animal over (C) 4 or (D) 32 weeks, intra-abdominal fat mass per animal after (E) 4 or (F) 32 weeks, and serum adiponectin quantifications after (G) 4 or (H) 32 weeks are shown. $^{*}p<0.05$ versus control; $^{\#}p<0.05$ versus IF, $n=15$.

the long-term experiments (Fig. 2D and F): surprisingly, whereas CR maintained good glucose tolerance long term, IF animals had significantly worse glucose clearance than AL.

To understand the changes in glucose tolerance, we measured IR expression and activation by determining Tyr phosphorylation levels in two insulin-responsive tissues: intra-abdominal adipose tissue and skeletal muscle. Short-term IF and CR strongly increased IR expression (not shown) and IR-Tyr phosphorylation in the abdominal adipose tissue and skeletal muscle (Fig. 3A and C). Long-term dietary interventions also significantly increased IR expression in the skeletal muscle (Supplementary Fig. 1) and phospho-Tyr-IR in the adipose tissue (Fig. 3B). In skeletal muscle, IR phosphorylation was increased by FR and CR, but not IF (Fig. 3D). Whereas the short-term IR phosphorylation results are consistent with glucose tolerance tests, the same was not observed in long-term results, in which IF animals presented impaired glucose clearance but not decreased IR phosphorylation relative to AL. We hypothesized that a different modification unrelated to phosphorylation could be impairing IR function.

In vitro studies have previously shown that Tyr nitration can decrease the function of this receptor [27–31]. We thus investigated whether IR nitration could explain the physiological differences between the long-term experimental groups we observed. We found (Fig. 4A and B) that CR consistently prevented IR nitration relative to AL diets. Furthermore, IF strongly increased nitration levels in the adipose tissue and muscle, and FR increased nitration in the muscle. The effect of short-term interventions on IR nitration was also measured and indicated that young rats present very low levels of this modification, virtually undetectable under our conditions (results not shown).

To verify the downstream results on IR function of the changes observed in phosphorylation and nitration, we measured the phosphorylation of the IR substrate IRS1. Expression levels of IRS1 are included in Supplemental Fig. 1C and D. Phospho-Tyr-IRS1 levels (Fig. 5A and B) were increased specifically in CR in muscle and were decreased by IF in both tissues relative to AL. The changes observed in IRS1 phosphorylation are compatible with glucose clearance, which is improved by CR and decreased in IF. We propose that the overall function of the IR is the result of both phosphorylation (activating) and nitration (inhibitory) of this receptor, as will be further discussed below.

Tyr nitration is promoted by peroxynitrite, the reaction product of superoxide radicals and nitric oxide [32]. Accordingly, we investigated whether the diets produced changes in NOS levels and the generation of ROS. We found that eNOS activation through phosphorylation was enhanced significantly relative to AL only in CR muscle samples (Fig. 6A and B), which may explain enhanced oxygen consumption levels (Supplementary Fig. 2), because mitochondrial biogenesis is stimulated by eNOS-derived nitric oxide [33–35]. iNOS expression was enhanced in all restricted diets (Figs. 6C and 4D). Overall, nitric oxide synthesis pathway activities do not explain the IR nitration patterns.

On the other hand, tissue H_2O_2 release was markedly enhanced in IF, both in muscle and in adipose tissue, and decreased in CR in muscle (Fig. 6E and F), a pattern very similar to IR nitration levels observed in Fig. 4. H_2O_2 is a relatively stable and membrane-diffusible product of superoxide radical dismutation, thus often used as a marker for ROS levels in biological samples [36] in which reliable measurements of the production of nondiffusible and unstable species such as superoxide radicals is not possible. The large increases observed in H_2O_2 diffusion from the IF tissues may represent enhanced tissue ROS generation and/or a decrease in antioxidant capacity. We measured quantities of the major antioxidants glutathione, superoxide dismutase, and catalase in these tissues (Supplementary Fig. 3). In most cases, IF increased the levels of the antioxidants measured, with the exception of catalase and glutathione in skeletal muscle. Thus, if anything, IF improved antioxidant capacity, especially in the abdominal adipose tissue. Thus, increased levels of H_2O_2 release from IF

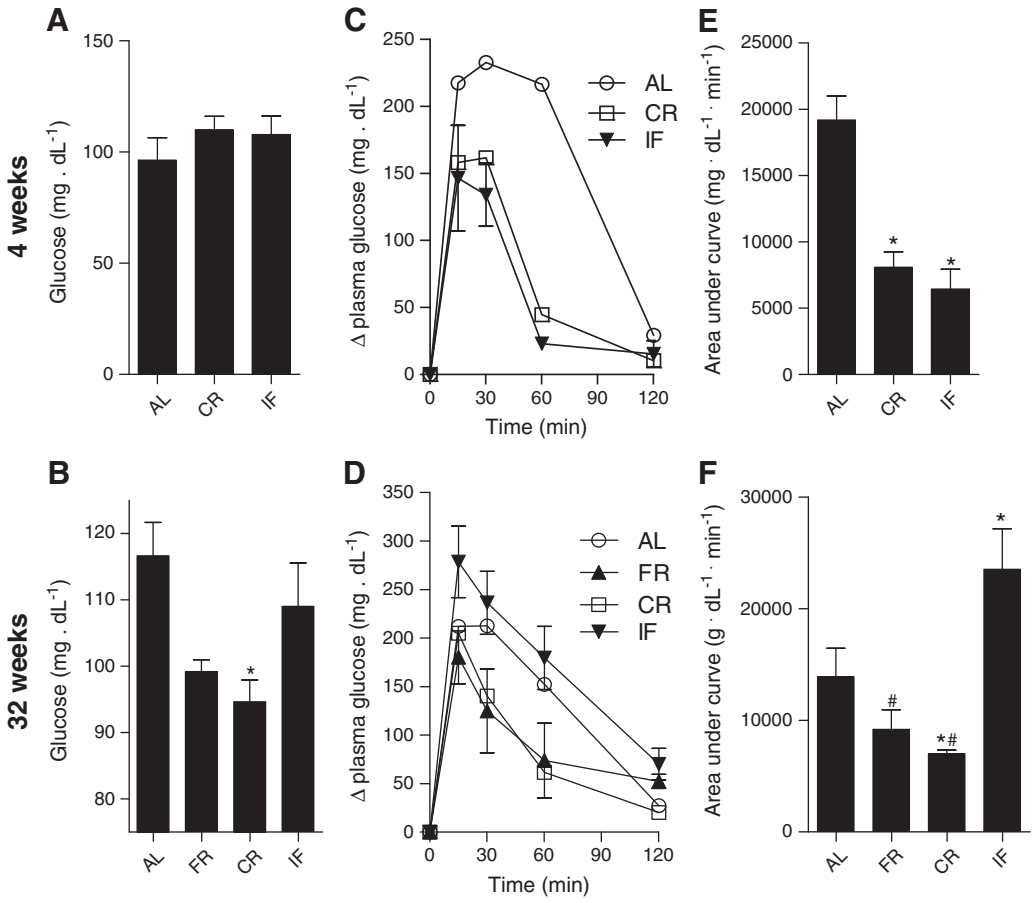

Fig. 2. Long-term, but not short-term IF leads to glucose intolerance. Rats were tested for fasting glucose after (A) 4 or (B) 32 weeks of dietary intervention. Blood glucose after a 2 g kg^{-1} intraperitoneal glucose injection after (C) 4 or (D) 32 weeks is shown. (E and F) Areas under the glucose curve in (C) and (D), respectively. $^{*}p<0.05$ vs control; $^{\#}p<0.05$ vs IF, $n=15$.

tissues probably reflects mostly enhanced ROS generation, although this point still remains to be verified directly.

Discussion

Dietary interventions such as FR and IF have been used interchangeably in the literature as equivalent to CR [5], although little is known about the long-term impact of these interventions. In this article, we compared short- and long-term effects of these diets and found that, despite some similarities, there are very significant differences that warrant attention. The most immediate difference between CR and IF is that intermittent ad libitum feeding does not limit total food intake, as reported here (Fig. 1) and in previous studies [5,6]. Some studies measured reductions in spontaneous feedings with IF [37,38], although the protocols differ in the onset of the adopted diet, which may influence feeding patterns and result in differences in food ingestion.

Despite equal food ingestion, IF animals maintained lower weights compared to animals fed ad libitum (Fig. 1), but with an interesting characteristic: abdominal fat deposits and adiponectin levels in long-term IF animals were equal to those of AL. It should be stressed that our studies involved obesity-prone Sprague–Dawley rats, previously used in IF protocols [38,39], and that further studies are necessary to determine if the results obtained here would also occur in other animal models. Despite this limitation, the use of obesity-prone animals is interesting as a model, considering human tendencies toward weight gain. In this sense, it is interesting to note that our study shows that weight loss on a restricted diet can be deceptive as a sole measurement and can occur in the presence of high abdominal fat levels and low adiponectin, risk factors in metabolic diseases associated with aging such as diabetes [40,41].

Short-term effects of CR and IF on glucose tolerance were similar and involved more favorable glucose clearance, enhanced IR expression, and phosphorylation. CR has been widely shown to promote glucose tolerance [42,43], and some short-term IF studies present similar results [44,45], even when animals were maintained on the diet for 6 months [39]. Surprisingly, however, we found that the long-term (8 months) effects of CR and IF were quite different. Only CR prevented increments in fasting glucose levels in aged rats and improved the response in glucose tolerance tests (Fig. 2). In fact, IF animals presented glucose intolerance relative to AL, as indicated by tolerance tests and hyperglycemia after feeding. Although this finding was surprising, it does have a precedent: Simon and Rosselin [45] demonstrated that glucose and insulin levels fluctuate largely in IF and can be reduced during the fasting period, but significantly increased during feeding, compared to AL animals. In this particular study, we chose to measure parameters in all animals when fasted for 12 h after a typical feeding period (see a full description under Experimental procedures), to analyze the animals under comparable conditions, which should reflect the effects of the diet on overall metabolic alterations relative to the specific feeding status.

Despite glucose intolerance in IF, phospho-Tyr-IR levels were increased in all diets (Fig. 3), demonstrating that glucose clearance did not mirror IR phosphorylation in response to insulin in FR and IF rats. Interestingly, whereas IR phosphorylation levels were not compatible with physiological measurements of glucose clearance in animals on long-term dietary interventions, IRS1 phosphorylation levels were, presenting significant increases in CR and decreases in IF relative to AL (Fig. 5). In view of this result, we speculated that in IF, and to a lesser extension in FR, IR receptors are not functionally controlled solely in response to phosphorylation levels.

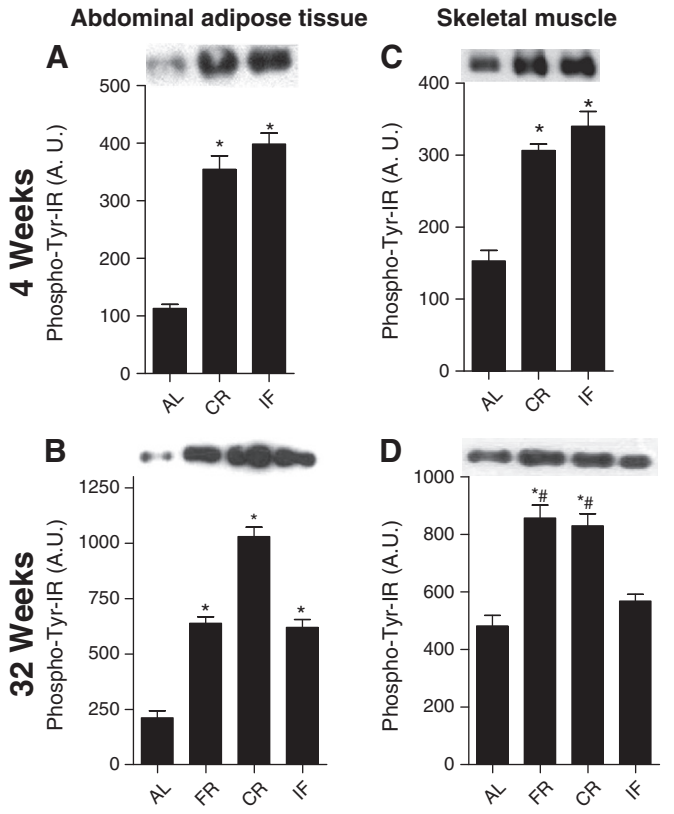

Fig. 3. FR, CR, and IF increase insulin receptor phosphorylation levels. IR from muscle and intra-abdominal adipose tissue was immunoprecipitated and blotted. Typical blots are shown above the average densitometric results. Phospho-Tyr-IR levels in intra-abdominal adipose tissue from rats in the (A) short- and (B) long-term dietary interventions and phospho-Tyr-IR in skeletal muscle from rats in the (C) short- and (D) long-term dietary interventions are shown. $^{*}p<0.05$ vs control; $^{\#}p<0.05$ vs IF, $n=4$.

A known modification that impairs IR function is tyrosine nitration [27–32], which has been previously observed in animals fed a high-fat diet [29]. We propose that phospho-Tyr sites coexist with NO_2-Tyr and that the concerted effect of both modifications determines IRS1 phosphorylation and glucose clearance. Indeed, we found that, whereas NO_2-Tyr-IR was virtually undetectable in short-term-treated animals (results not shown), older animals accumulate NO_2-Tyr-IR significantly in a manner prevented by CR (Fig. 4). IF animals exhibited large quantities of NO_2-Tyr-IR in both insulin-sensitive tissues analyzed, and FR led to an accumulation of the modified receptor in the skeletal muscle. Thus, in animals on long-term interventions, nitration of the IR

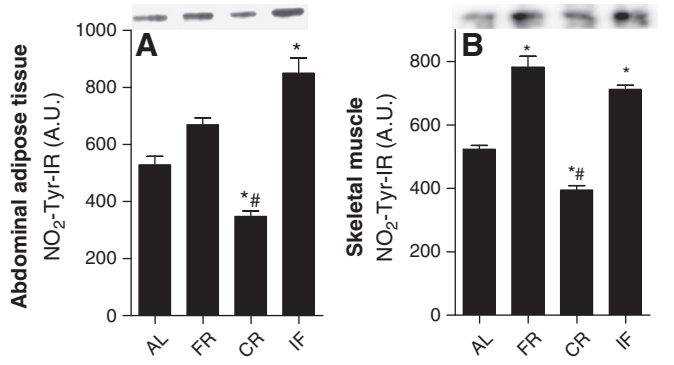

Fig. 4. IR nitration is prevented by long-term CR and enhanced by FR and IF. IR from the (A) intra-abdominal adipose tissue and (B) skeletal muscle of animals 32 weeks on the dietary interventions were immunoprecipitated and blotted. Typical blots are shown above the average densitometric results. $^{*}p<0.05$ vs control; $^{\#}p<0.05$ vs IF, $n=4$.

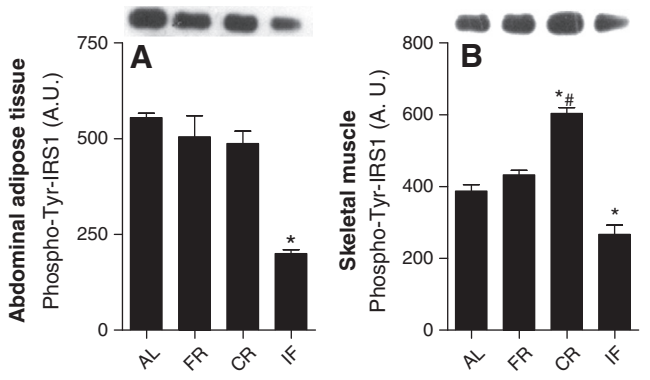

Fig. 5. IRS1 phosphorylation is enhanced by long-term CR, but decreased in IF. The IRS1 from the (A) intra-abdominal adipose tissue and (B) skeletal muscle of animals 32 weeks on the dietary interventions was immunoprecipitated and blotted for phospho-Tyr. Typical blots are shown above the average densitometric results. $^{*}p<0.05$ vs control; $^{\#}p<0.05$ vs IF, $n=4$.

is modified by the dietary protocol and, in concerted action with IR phosphorylation, can determine the activation of the downstream pathway and glucose clearance.

We conducted further experiments to determine why the dietary interventions differ in promoting IR nitration over time. Nitration is promoted by peroxynitrite, a product of nitric oxide and superoxide radicals [32]. We measured increases in eNOS phosphorylation and iNOS expression, but found that overall there was no IF- and FR-specific increase in these nitric-oxide generating systems (Fig. 6).

On the other hand, H_2O_2 release rates (Fig. 6) from both tissues closely resembled nitration patterns (Fig. 4) and were strikingly enhanced in IF. H_2O_2 release from tissues reflects overall production of this ROS versus its removal and was used as a general marker of redox state in our study because of difficulties in measuring superoxide radicals in situ in animal tissues [46]. Confirming that IF causes redox imbalance, we found that IF animals presented significantly higher oxidized glutathione levels (4939 ± 396 pmol mg protein^{-1}) than AL animals (1942 ± 648 pmol mg protein^{-1}, methods used are described in the supplementary material). Interestingly, enhanced production of ROS in IF promotes mainly oxidative modifications in proteins. IF did not increase levels of malondialdehyde, a product of lipid oxidation, relative to CR diet (results not shown, methods in supplementary materials), but resulted in strikingly higher levels of IR modification (Fig. 4) and protein carbonylation (Supplementary Fig. 3).

The opposite results of CR and IF with regard to ROS release and oxidative modifications of proteins are particularly worrisome because IF is often considered equivalent to CR in studies uncovering the impact of dietary restriction on aging [5]. On the other hand, our results show that from a redox standpoint, these diets are very distinct. Indeed, we are unaware of any other dietary intervention capable of increasing tissue ROS release under physiological conditions at this magnitude.

Overall, we find that long-term FR, CR, and IF promote different changes in energy metabolism and redox states, parameters strongly associated with life-span extension [3,4]. CR is clearly the most beneficial intervention in terms of maintaining glucose tolerance, increasing antioxidant defenses, and preventing the release of ROS from tissues. FR presents some of the benefits of CR, although the redox balance is not as favorable, possibly because of micronutrient malnutrition [5]. On the other hand, long-term IF unexpectedly leads to glucose intolerance and strongly increases ROS release rates compared to AL. This is, to our knowledge, the first report that a restrictive dietary intervention can have a negative effect on glucose tolerance associated with oxidative modifications of the IR and suggests that frequent feeding/fasting cycles may be a risk factor for age-associated obesity and insulin resistance leading to diabetes. Furthermore, the differences between CR and IF indicate that, unlike daily

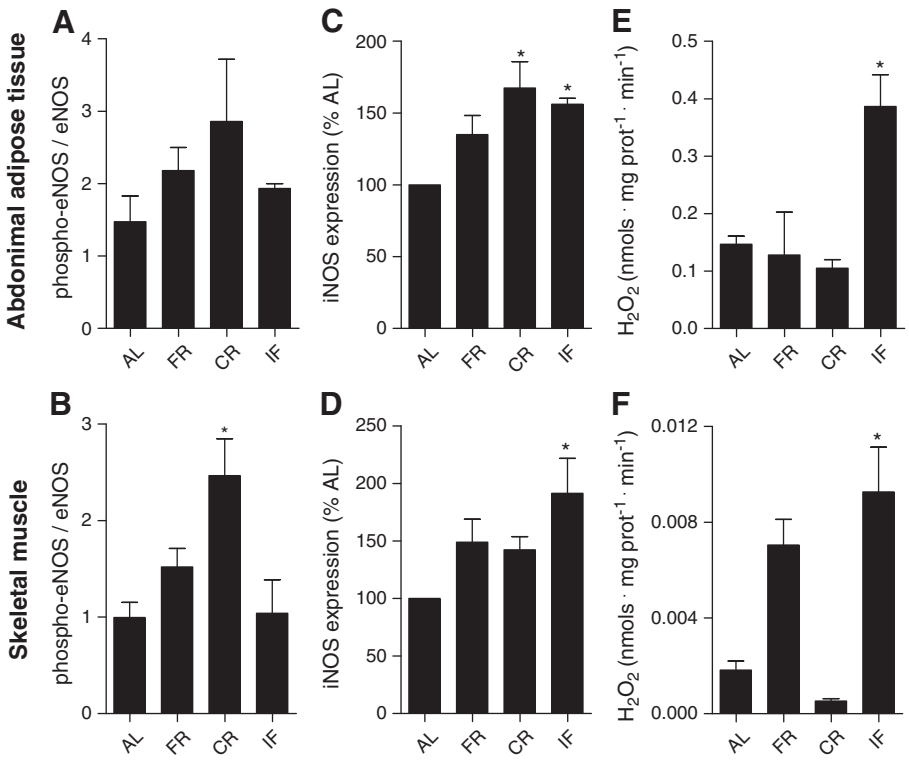

Fig. 6. Long-term IF leads to enhanced tissue reactive oxygen species release. Densitometric analysis of (A) intra-abdominal adipose tissue and (B) skeletal muscle phospho-eNOS^{Ser1177}/eNOS, densitometric analysis of (C) intra-abdominal adipose tissue and (D) skeletal muscle iNOS expression, and (E) intra-abdominal adipose tissue and (F) skeletal muscle H_2O_2 release rates are shown. $^*p<0.05$ vs control, $n=8$.

limitations of caloric intake, alternating feeding and starvation cycles in laboratory animals probably constitute an unhealthy long-term dietary intervention.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.freeradbiomed.2011.07.006.

References

[1] McCay, C. M. The effect of retarded growth upon the length of life and upon ultimate size. *J. Nutr.* **10:**63–79; 1935.
[2] Blagosklonny, M. V.; Campisi, J.; Sinclair, D. A.; Bartke, A.; Blasco, M. A.; Bonner, W. M.; Bohr, V. A.; Brosh Jr., R. M.; Brunet, A.; Depinho, R. A.; Donehower, L. A.; Finch, C. E.; Finkel, T.; Gorospe, M.; Gudkov, A. V.; Hall, M. N.; Hekimi, S.; Helfand, S. L.; Karlseder, J.; Kenyon, C.; Kroemer, G.; Longo, V.; Nussenzweig, A.; Osiewacz, H. D.; Peeper, D. S.; Rando, T. A.; Rudolph, K. L.; Sassone-Corsi, P.; Serrano, M.; Sharpless, N. E.; Skulachev, V. P.; Tilly, J. L.; Tower, J.; Verdin, E.; Vijg, J. Impact papers on aging in 2009. *Aging* **3:** 111–121; 2010.
[3] Partridge, L.; Gems, D. Mechanisms of ageing: public or private? *Nat. Rev. Genet.* **3:** 165–175; 2002.
[4] Sohal, R. S.; Weindruch, R. Oxidative stress, caloric restriction, and aging. *Science* **273:** 59–63; 1996.
[5] Cerqueira, F. M.; Kowaltowski, A. J. Commonly adopted caloric restriction protocols often involve malnutrition. *Ageing Res. Rev.* **7:**552–560; 2010.
[6] Anson, R. M.; Jones, B.; de Cabo, R. The diet restriction paradigm: a brief review of the effects of every-other-day feeding. *Age* **27:**17–25; 2005.
[7] Boily, G.; Seifert, E. L.; Bevilacqua, L.; He, X. H.; Sabourin, G.; Estey, C.; Moffat, C.; Crawford, S.; Saliba, S.; Jardine, K.; Xuan, J.; Evans, M.; Harper, M. E.; McBurney, M. W. SirT1 regulates energy metabolism and response to caloric restriction in mice. *PLoS One* **12:**e1759; 2008.
[8] Bonelli, M. A.; Desenzari, S.; Cavallini, G.; Donati, A.; Romani, A. A.; Bergamini, E.; Borghetti, A. F. Low-level caloric restriction rescues proteasome activity and Hsc70 level in liver of aged rats. *Biogerontology* **9:**1–10; 2008.
[9] Mager, D. E.; Wan, R.; Brown, M.; Cheng, A.; Wareski, P.; Abernethy, D. R.; Mattson, M. P. Caloric restriction and intermittent fasting alter spectral measures of heart rate and blood pressure variability in rats. *FASEB J.* **20:**631–637; 2006.
[10] Duffy, P. H.; Lewis, S. M.; Mayhugh, M. A.; McCracken, A.; Thorn, B. T.; Reeves, P. G.; Blakely, S. A.; Casciano, D. A.; Feuers, R. J. Effect of the AIN-93 M purified diet and dietary restriction on survival in Sprague–Dawley rats. *J. Nutr.* **132:**101–107; 2002.
[11] Goodrick, C. L.; Ingram, D. K.; Reynolds, M. A.; Freeman, J. R.; Cider, N. Effects of intermittent feeding upon body weight and life span in inbred mice: interaction of genotype and age. *Mech. Ageing Dev.* **55:**69–87; 1990.
[12] Keenan, K. P.; Laroque, P.; Ballam, G. C.; Soper, R.; Dixit, R.; Mattson, B. A.; Adams, S. P.; Coleman, J. B. The effects of diet, ad lib overfeeding, and moderate dietary restriction on the rodent bioassay. *Toxicol. Pathol.* **24:**757–768; 1996.
[13] Pearson, K. J.; Baur, J. A.; Lewis, K. N.; Peshkin, L.; Price, N. L.; Labinskyy, N.; Swindell, W. R.; Kamara, D.; Minor, R. K.; Perez, E.; Jamieson, H. A.; Zhang, Y.; Dunn, S. R.; Sharma, K.; Pleshko, N.; Woollett, L. A.; Csiszar, A.; Ikeno, Y.; Le Couteur, D.; Elliott, P. J.; Becker, K. G.; Navas, P.; Ingram, D. K.; Wolf, N. S.; Ungvari, Z.; Sinclair, D. A.; de Cabo, R. Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending lifespan. *Cell Metab.* **8:**157–168; 2008.
[14] Valle, A.; Guevara, R.; García-Palmer, F. J.; Roca, P.; Oliver, J. Caloric restriction retards the age related decline in mitochondrial function of brown adipose tissue. *Rejuvenation Res.* **11:**597–604; 2008.
[15] Pugh, T. D.; Klopp, R. G.; Weindruch, R. Controlling caloric consumption: protocols for rodents and rhesus monkeys. *Neurobiol. Aging* **20:**157–165; 1999.
[16] Hayashi, H.; Yamaza, H.; Komatsu, T.; Park, S.; Chiba, T.; Higami, Y.; Nagayasu, T.; Shimokawa, I. Calorie restriction minimizes activation of insulin signaling in response to glucose. *Exp. Gerontol.* **43:**827–832; 2008.
[17] Lambert, A. J.; Merry, B. J. Effect of caloric restriction on mitochondrial reactive oxygen species production and bioenergetics. *Am. J. Physiol.* **286:**R71–R79; 2004.
[18] Merry, B. Oxidative stress and mitochondrial function with aging—the effects of calorie restriction. *Aging Cell* **3:**7–12; 2004.
[19] Marzetti, E.; Wohlgemuth, S. E.; Anton, S. D.; Bernabei, R.; Carter, C. S.; Leewenburgh, C. Cellular mechanisms of cardioprotection by calorie restriction. *Clin. Geriatr. Med.* **25:**715–732; 2009.
[20] Mahadev, K.; Motoshima, H.; Wu, X.; Russy, M. J.; Arnold, R. S.; Cheng, G.; Lambeth, J. D.; Goldstein, B. J. The NAD(P)H oxidase homolog Nox4 modulates

insulin-stimulated generation of H_2O_2 and plays an integral role in insulin signal transduction. *Mol. Cell. Biol.* **24:**1844–1854; 2004.
[21] Ristow, M.; Zarse, K.; Oberbach, A.; Klöting, N.; Birringer, M.; Michael, K.; Stumvoll, M.; Kahn, R. C.; Blüher, M. Antioxidants prevent health-promoting effects of physical exercises in humans. *Proc. Natl. Acad. Sci. USA* **106:**8665–8670; 2009.
[22] Ceriello, A.; Motz, E. Is the oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arterioscler. Thromb. Vasc. Biol.* **24:**816–823; 2004.
[23] Evans, J. L.; Goldfine, I. D.; Maddux, B. A.; Grodsky, G. M. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endrocr. Rev.* **23:**599–622; 2002.
[24] De Campos, K. E.; Sinzato, Y. K.; Pimenta, W. de P.; Rugde, M. V. C.; Damasceno, D. C. Effect of maternal obesity on diabetes development in adult rat offspring. *Life Sci.* **81:**1473–1478; 2007.
[25] Zhou, M.; Diwu, Z.; Panchuk-Voloshina, N.; Haugland, R. P. A stable nonfluorescence derivative of resorufin for the fluorimetric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal. Biochem.* **253:**162–168; 1997.
[26] Masaki, T.; Chiba, S.; Yasuda, T.; Tsubone, T.; Kakuma, T.; Shimomura, I.; Funahashi, T.; Matsuzawa, Y.; Yoshimatsu, H. Peripheral, but not central, administration of adiponectin reduces intra-abdominal adiposity and upregulates the expression of uncoupling protein in agouti yellow (Ay/a) obese mice. *Diabetes* **52:**2266–2273; 2003.
[27] Carpenter, F. H.; Boesel, R. W.; Sakai, D. D. Tetrakis(3-nitrotyrosine)insulin. *Biochemistry* **19:**5926–5931; 1980.
[28] Morris, J. W. S.; Mercola, D. A.; Arquilla, E. R. Preparation and properties of 3-nitrotyrosine insulins. *Biochemistry* **9:**3930–3937; 1970.
[29] Duplain, H.; Sartori, C.; Dessen, P.; Jayet, P. Y.; Schwab, M.; Bloch, J.; Nicod, P.; Scherrer, U. Stimulation of peroxynitrite catalysis improves insulin sensitivity in high fat diet-fed mice. *J. Physiol.* **586:**4011–4016; 2008.
[30] Zhou, J.; Huang, K. Peroxynitrite mediates muscle insulin resistance in mice via nitration of IRβ/IRS-1 and Akt. *Toxicol. Appl. Pharmacol.* **241:**101–110; 2009.
[31] Charbonneau, A.; Marette, A. Inducible nitric oxide synthase induction underlies lipid-induced hepatic insulin resistance in mice: potential role of tyrosine nitration of insulin signaling proteins. *Diabetes* **59:**861–871; 2010.
[32] Ischiropoulos, H.; Zhu, L.; Chen, J.; Tsai, J. H. M.; Martin, J. C.; Smith, C. D.; Beckman, J. S. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch. Biochem. Biophys.* **298:**431–437; 1992.
[33] Nisoli, E.; Clementi, E.; Tonello, C.; Sciorati, C.; Briscini, L.; Carruba, M. O. Effects of nitric oxide on proliferation and differentiation of rat brown adipocytes in primary cultures. *Br. J. Pharmacol.* **125:**888–894; 1998.
[34] Nisoli, E.; Clementi, E.; Paolucci, C.; Cozzi, V.; Tonello, C.; Sciorati, C.; Bracale, R.; Valerio, A.; Francolini, M.; Moncada, S.; Carruba, M. O. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science* **299:**896–899; 2003.
[35] Cerqueira, F. M.; Laurindo, F. R.; Kowaltowski, A. J. Mild mitochondrial uncoupling and calorie restriction increase fasting eNOS, akt and mitochondrial biogenesis. *PLoS One* **6:**e18433; 2011.
[36] Kowaltowski, A. J.; de Souza-Pinto, N. C.; Castilho, R. F.; Vercesi, A. E. Mitochondria and reactive oxygen species. *Free Radic. Biol. Med.* **47:**333–343; 2009.
[37] Caro, J.; López-Torres, M.; Sánchez, I.; Naudi, A.; Portero-Otín, M.; Pamplona, R.; Barja, G. Effect of every other day feeding on mitochondrial free radical production and oxidative stress in mouse liver. *Rejuvenation Res.* **74:**621–629; 2008.
[38] Martin, B.; Pearson, M.; Brenneman, R.; Golden, E.; Keselman, A.; Iyun, T.; Carlson, O. D.; Egan, J. M.; Becker, K. G.; Wood III, W.; Prabhu, V.; de Cabo, R.; Maudsley, S.; Mattson, M. P. Conserved and differential effects of dietary energy intake on the hippocampal transcriptomes of females and males. *PLoS One* **3:**e2398; 2008.
[39] Martin, B.; Pearson, M.; Kebejian, L.; Golden, E.; Keselman, A.; Bender, M.; Carlson, O.; Egan, J.; Ladenheim, B.; Cadet, J. L.; Becker, K. G.; Wood, W.; Duffy, K.; Vinayakumar, P.; Maudsley, S.; Mattson, M. P. Sex-dependent metabolic, neuroendocrine, and cognitive responses to dietary energy restriction and excess. *Endocrinology* **148:**4318–4333; 2007.
[40] Yatagai, T.; Nagasaka, S.; Taniguchi, A.; Fukushima, M.; Nakamura, T.; Kuroe, A.; Nakai, Y.; Ishibashi, S. Hypoadiponectinemia is associated with visceral fat accumulation and insulin resistance in Japanese men with type 2 diabetes mellitus. *Metabolism* **52:**1274–1278; 2003.
[41] Matsuzawa, Y. The metabolic syndrome and adipocytokines. *FEBS Lett.* **580:** 2917–2921; 2006.
[42] Wang, Z. Q.; Floyd, Z. E.; Qin, J.; Liu, X.; Yu, Y.; Zhang, X. H.; Wagner, J. D.; Cefalu, W. T. Modulation of skeletal muscle insulin signaling with chronic caloric restriction in cynomolgus monkeys. *Diabetes* **59:**1488–1498; 2009.
[43] Zhu, M.; de Cabo, R.; Anson, R. M.; Ingram, D. K.; Lane, M. A. Caloric restriction modulates insulin receptor signaling in liver and skeletal muscle of rat. *Nutrition* **21:** 378–388; 2005.
[44] Anson, R. M.; Guo, Z.; de Cabo, R.; Lyun, T.; Rios, M.; Hagepanos, A.; Ingram, D. K.; Lane, M. A.; Mattson, M. P. Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. *Proc. Natl. Acad. Sci. USA* **100:**6216–6220; 2003.
[45] Simon, J.; Rosselin, G. Effect of intermittent feeding on glucose–insulin relationship in the chicken. *J. Nutr.* **109:**631–641; 1979.
[46] Wardman, P. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. *Free Radic. Biol. Med.* **43:**995–1022; 2007.