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Caloric restriction improves basal redox parameters in hippocampus and cerebral cortex of Wistar rats

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ABSTRACT

Caloric restriction (CR) has been shown to either decrease or prevent the progression of several age-related pathologies. In previous work, we demonstrated that CR modulates astrocyte functions, suggesting that CR may exert neuroglial modulation. Here, we investigated the effects of CR on hippocampal (Hc) and cortical (Cx) oxidative stress parameters of male Wistar rats. Our results showed that CR-fed rats had 17% less body weight gain after 12 weeks of treatment. CR improved locomotion performance, increased glutathione levels and decreased glutathione peroxidase activity and the production of reactive oxygen species. However, no changes were observed in lipid peroxidation, nitric oxide content and catalase activity. Single cell gel electrophoresis assay (comet assay) revealed a reduction in the extent of basal DNA damage upon CR. Our data suggest that dietary CR could induce both hippocampal and cortical modulation resulting in metabolic changes and as a consequence, significant improvement of cellular defense-associated parameters.

1. Introduction

Dietary caloric restriction (CR) is defined as a limitation of food intake below the *ad libitum* level without malnutrition and it is well known to extend the maximum lifespan in a wide range of different organisms.

Experiments in animal models have demonstrated that caloric restriction (CR) is able to either slow down or prevent the progression of several age-related pathologies (Gonzalez et al., 2011); for instance, cardiovascular disease (Mattson and Wan, 2005), multiple types of cancer (De Lorenzo et al., 2011; Klebanov, 2007) diabetes (Anson et al., 2003) and ischemic injury

Abbreviations: RC, caloric restriction; GS, glutamine synthetase; Hc, hippocampal; Cx, cortical; ROS, reactive oxygen species; GSH, glutathione; EPM, elevated plus-maze test; GPx, glutathione peroxidase; CAT, catalase; TBARS, thiobarbituric acid-reactive substances; NO, nitric oxide

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(Morris et al., 2011). CR may also reduce neuronal damage (Chouliaras et al., 2012) and consequently offer protection against neurodegenerative diseases (Bishop and Guarente, 2007; Gillette-Guyonnet and Vellas, 2008). Recent studies have shown that CR is sufficient and enough to induce neurogenesis in the hippocampus of adult mice (Lee et al., 2002), to enhances synaptic plasticity in the aging rat (Fontan-Lozano et al., 2008; Mladenovic Djordjevic et al., 2009), to modulates *a*-synuclein expression in the aging rat cortex and hippocampus (Mladenovic et al., 2007) and to attenuates age-related changes in mouse neuromuscular synapses (Valdez et al., 2010).

Moreover, our laboratory recently reported that CR also modulates astrocytic functions by increasing glutamate uptake and glutamine synthetase (GS) activity. This suggested that CR may exert certain neuroprotective effects against brain illness by a mechanism involving modulation of astrocytic functions (Ribeiro et al., 2009). Such results suggest that brain under CR could become somehow less sensitive to physiological aging process and better restore its functions after injury. With aging, brain undergoes neuronal loss in many areas, cognitive functions decline and it decreases in size as well as white matter integrity (Park and Reuter-Lorenz, 2009).

There is evidence that hippocampus seems to be particularly sensitive to aging and may be partly responsible for age-related cognitive decline (Jessberger and Gage, 2008). In addition, a large number of age-related changes within the hippocampus have already been documented, such as altered mitochondrial function, oxidative stress, changes in glutamate transmission and synaptic plasticity (Fontan-Lozano et al., 2008). Some studies indicated that the frontal cerebral cortex suffers a dramatic cell loss due to aging and its influence on synaptic loss was associated with significant cognitive decline (Asha Devi, 2009). Aging has a powerful effect on enhanced susceptibility to neurodegenerative diseases (Fratiglioni and Qiu, 2009).

Problems occur when production of reactive oxygen species (ROS) exceeds the cells ability to protect themselves against such molecules. Oxidative stress occurs as a result of imbalance between cellular production of ROS and the ability of the cells to defend themselves against them (Buonocore et al., 2010). Thus, it could trigger cellular damage as ROS is able to oxidize cellular components such as membrane lipids, proteins and DNA (Esposito et al., 2002). There is substantial evidence that the brain, which consumes large amounts of oxygen, has abundant lipid content but relative paucity of antioxidant enzymes, making it particularly vulnerable to oxidative damage.

As a matter of fact, oxidative damage has strongly been associated in the pathogenesis of several neurodegenerative diseases, including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease and stroke (brain ischemia/reperfusion injury) (Hegde et al., 2011; Kamat et al., 2008). Cells possess different physiological self-defense mechanisms against free radicals-induced damage. The major ones are for instance, antioxidant scavengers such as glutathione (GSH), vitamin C (ascorbic acid), vitamin E (α-tocopherol), carotenoids, flavonoids, polyphenols, as well as antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. These antioxidant self-defense mechanisms can be upregulated in response to increased ROS or peroxide production. Although it may confer protection against ROS, they are not completely effective in preventing aging-related oxidative damage (Esposito et al., 2002; Kamat et al., 2008). Recent studies have demonstrated that age-related increases of oxidative damage in the brain is best exemplified by lipid peroxidation-derived products, protein oxidation and oxidative modifications in nuclear and mitochondrial DNA, beyond the decrease in brain and plasma antioxidants (GSH and antioxidant enzymatic activity) (Droge and Schipper, 2007; Hegde et al., 2011).

In the present study, we investigated the effects of caloric restriction on oxidative stress parameters, basal antioxidant enzymes, lipid peroxidation and DNA damage in the hippocampus and cerebral cortex of Wistar rats. Behavioral and blood biochemical parameters were also evaluated.

2. Results

2.1. *Effect of CR diet on body weight and serum biochemistry*

Sixty-day old rats were fed with laboratory chow (Table 1) *ad libitum* (control) or underwent CR for 12 weeks, and were weighted weekly. The weight gain of the experimental protocol is shown in Fig. 1. Rats submitted to caloric restriction, had a decrease of 12% ($P < 0.05$) in body weight gain in the end of the first week of the treatment. The difference in weight gain between groups was statistically significant

Table 1 – Composition of the laboratory chow.

Composition	(g/kg)
Total fat	110
Sunflower oil	5
Proteins	220
Fibers	30
Ash	60
Vitamins	20
Carbohydrates	520

Commercial nonpurified diet, Nuvilab-CR1 (Curitiba, PR, Brazil).

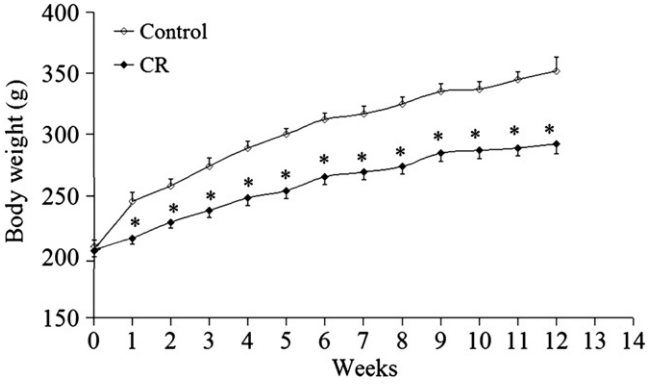

Fig. 1 – Repeated measurement of body weight. Male Wistar rats were fed with laboratory chow *ad libitum* (control) or calorie restricted (CR) diets. The figure shows the evolution in body weight of control animals (open symbols) and CR diet-fed rats (closed symbols) throughout 12 weeks. Values are means ± S.E.M. ($n = 15$). *Significantly different from controls (Student's *t*-test, $P < 0.05$).

throughout the experiment and achieved 17% ($P < 0.05$) at the end of the experiment.

The biochemistry analysis of serum (Table 2) demonstrated that there were no differences in glucose, cholesterol, triacylglycerol, corticosterone, albumin and protein, indicating a good health state in all groups.

2.2. *Behavioral parameters*

On the 12th week, behavior was also analyzed by the elevated plus-maze task (Fig. 2A) and in the open-field habitation test (Fig. 2B). Based on the Kolmogorov–Smirnov goodness-of-fit test, these data were expressed as mean and standard deviation. No differences in the total time spent the open relative to closed arms of the elevated plus maze were observed between groups. However, in the open field test, CR group produced significant increase in total locomotor activity and rearing ($P < 0.05$). In this test, the number of lines crossed and the frequency of rearing are commonly used to evaluate general locomotor activity; however, it is also possible to evaluate willingness to explore in rodents.

Table 2 – Serum biochemistry of rats fed *ad libitum* (control) and caloric restriction (CR) diets for 12 weeks.

	Control (mg/dL)	CR (mg/dL)
Glucose	220 ± 13.24	201 ± 29.51
Cholesterol	61 ± 3.10	60 ± 3.06
Triacylglicerol	100 ± 15.23	95 ± 16.45
Corticosterone	36.50 ± 5.20	30.40 ± 4.80
Albumine	2.41 ± 0.41	2.32 ± 0.58
Protein	5.81 ± 0.33	5.53 ± 0.73

Values are mean ± S.E.M. ($n = 8$).

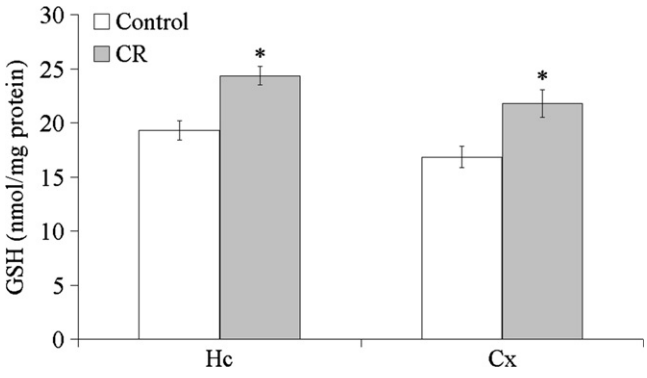

Fig. 3 – Glutathione (GSH) content in the hippocampus and cerebral cortex. Rats were fed with a control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in Section 4. Values are mean ± S.E.M. ($n = 6$). *Significantly different from controls (Student's *t*-test, $P < 0.05$).

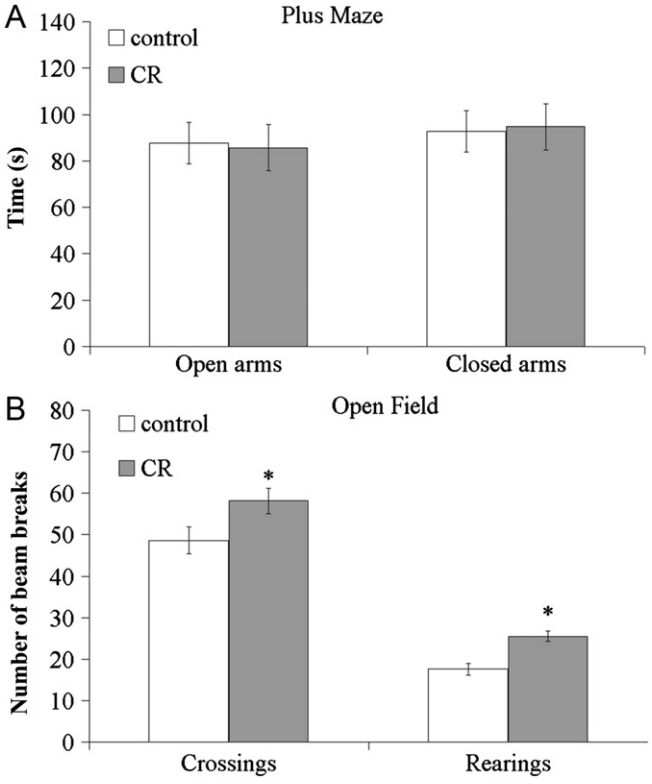

Fig. 2 – Elevated plus-maze (EPM) and behavior in open-field. Rats were fed with a control or CR diet for 12 weeks. After that, EPM and open field tasks were performed as described in Section 4. (A) EPM task: performed by placing the animal just in the center of a maze with two closed arms and two open ones. During a 2-min period, the number of entries into the closed arms and the time spent in the open ones were registered (B) open-field task. Animals were left there for 2 min during both training and test sessions, registering thereafter the number of rearings and crossings between sectors. Values are means ± S.E.M. ($n = 15$), *$P < 0.05$.

2.3. Oxidative stress parameters in hippocampus and cerebral cortex: GSH, GPx, CAT, ROS, TBARS and NO

Glutathione content was found to be higher in CR-fed animals than in the control groups (Fig. 3), increasing by 26% in Hc and to 29% in Cx and this difference was statistically significant ($P < 0.05$).

As shown in Fig. 4, the CR diet was able to significantly decrease GPx activity (about 18%) in both cerebral structures ($P < 0.05$). The CAT activity did not differ between groups and structures (Fig. 5).

CR-fed rats significantly decreased by 26% and to 14% ROS production in Hc and Cx, respectively, in comparison with control groups (Fig. 6), and this difference was statistically significant ($P < 0.05$). There were no differences in TBARS levels (Table 3) as well as NO production (Table 4) between the groups.

2.4. DNA damage in blood and hippocampal cells

Index of DNA damage did not differ between the two different groups of blood cells (Fig. 7A). On the other hand, hippocampal cells isolated from CR-fed rats showed a significant decrease in basal DNA damage index (from 12 ± 2.2 to 8 ± 1.4, $P < 0.01$) in comparison with control hippocampal cells (Fig. 7B).

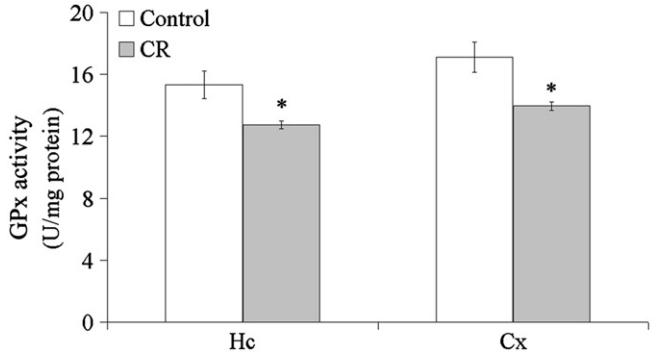

Fig. 4 – Glutathione peroxidase (GPx) activity in the hippocampus and cerebral cortex. Rats were fed with a control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in Section 4. Values are mean ± S.E.M. ($n = 6$).

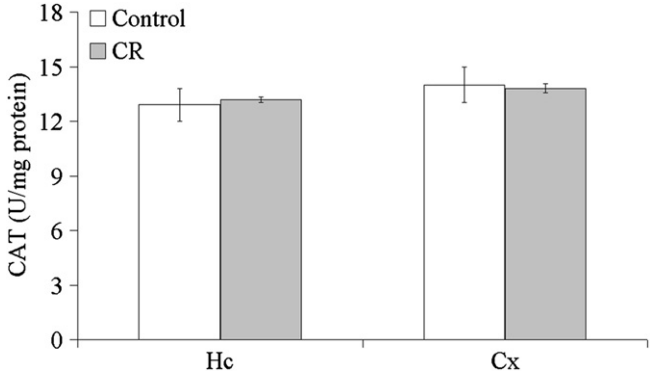

Fig. 5 – Catalase (CAT) activity in the hippocampus and cerebral cortex. Rats were fed with a control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in Section 4. Values are mean ± S.E.M. ($n = 6$).

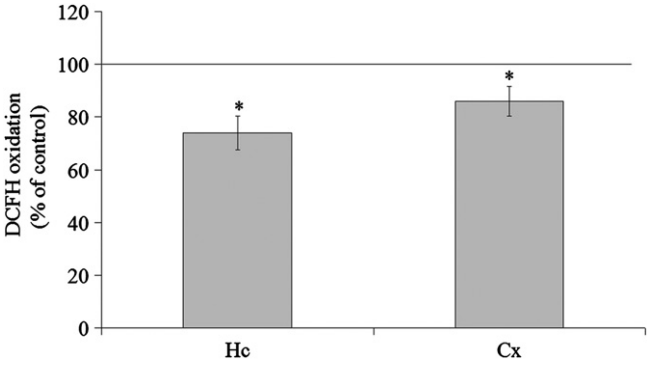

Fig. 6 – Reactive oxygen species (ROS) production in the hippocampus and cerebral cortex. Rats were fed with a control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in Section 4. Values are mean ± S.E.M. ($n = 6$). *Significantly different from controls (Student's t-test, $P < 0.05$).

Table 3 – Lipid peroxidation.

	Control (nmol/mg protein)	CR (nmol/mg protein)
Hc	36.8 ± 6.0	35.9 ± 4.9
Cx	26.9 ± 1.8	26.7 ± 1.9

Rats were fed with control or CR diet for 12 weeks. Lipid peroxidation was evaluated by the thiobarbituric acid reactive substance assay (TBARS), assayed for malondialdehyde. Homogenized tissue samples were incubated as described in Section 4. Values are mean ± S.E.M. ($n = 6$). Hc, hippocampus; Cx, cortex.

Table 4 – Nitric oxide (NO) production.

	Control (μM/mg protein)	CR (μM/mg protein)
Hc	16.6 ± 1.7	16.4 ± 1.4
Cx	15.6 ± 1.3	14.4 ± 0.9

Rats were fed with control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in the material and Methods section. Values are mean ± S.E.M. ($n = 6$). Hc, hippocampus; Cx, cortex.

3. Discussion

Benefits of dietary calorie restriction on brain aging and in particular, its putative protection against age-related neurodegenerative diseases are a target of study for several research groups within the field, nowadays. However, better comprehension about the affected biochemical parameters due to CR becomes essential for designing additional therapeutic interventions and novel pharmacological drugs aimed to treat such diseases. Since, the specific effects of CR (without malnutrition) in the brain are poorly understood, the *in vivo* treatment followed by an *ex vivo* analysis of

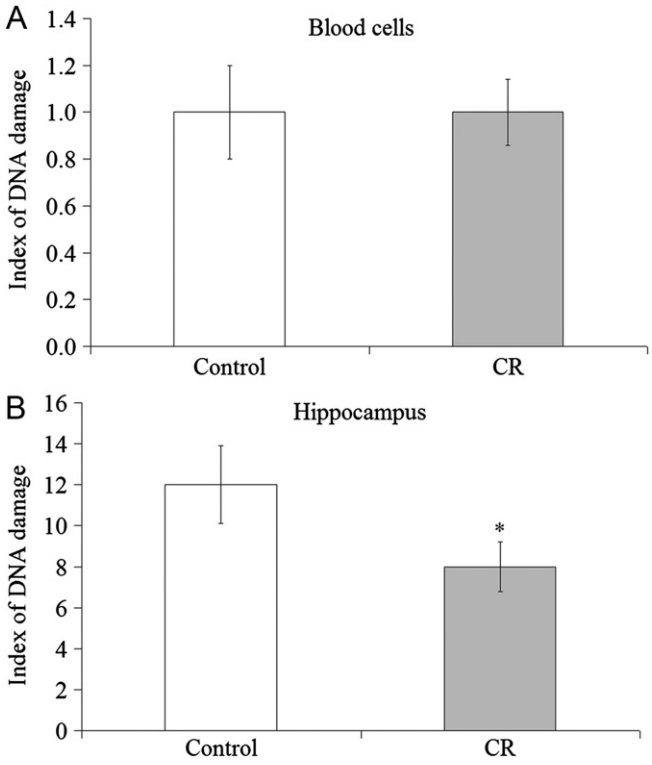

Fig. 7 – Comet assay in blood and hippocampal cells. Rats were fed with a control or CR diet for 12 weeks. Samples were prepared as described in Section 4. Values are mean ± S.E.M. ($n = 6$).

possible CR-dependent neural metabolic changes, became the primary goal of our current study.

As expected, control rats gained weight at a faster rate than animals undergoing a CR diet. In fact, such decreased body weight gain was detected in the CR group already during the first week with a 12% reduction compared to the control group and continuous decreasing reaching 17% at the end of the treatment (12 weeks). Whereas, animals under CR showed normal proteinemia, which completely discard the possibility of less efficient weight gain due to inadequate protein intake.

Interestingly, CR-fed rats significantly increased general activity levels and exploration habits in the open field tasks and as a result, higher locomotor activity than the control groups. The line crossings, rearing and center square frequencies are normally used to evaluate locomotor activity, but it can also be used to measure exploration (Brown et al., 1998). A high frequency of these behaviors may indicate increased locomotion, exploration and/or a lower level of anxiety. However, it is important to mention that CR diet did not induced anxiety, supported by: (1) The completely normal corticosterone levels; (2) The animal behavior in the plus-maze tasks, which did not vary between groups and (3) The blood parameters which indicate healthy conditions. It has been noticed with animal models, that, when rats with access to a running wheel and are restricted in their food intake, they become excessively active, and paradoxically reduce food consumption, with a possible subsequence induction of anorexic condition (Kohl et al., 2004). In the present work, as rats were not submitted to exercise protocols, they were not excessively active under CR diet and did not assume anorexic features, also observed by blood parameters, including normal proteinemia and glycemia (anorexia nervosa normally induces hypoglycemia).

Regarding glial function, our laboratory recently reported that CR was able to modulate astrocyte functions by increasing glutamate uptake and GS activity, all together suggesting a possible CR-induced neuroprotective effects *via* modulation of astrocytic functions (Ribeiro et al., 2009). Now, we wondered if GSH levels may differ upon CR, depending on the particular area of the brain. Our data showed that hippocampal and cerebral cortical GSH content was significantly higher in the CR scenario than in the control groups. Since, GSH is an extremely important non-enzymatic antioxidant for CNS; these data may provide some evidence for delineating the mechanisms by which CR may exert protective actions in the brain.

Basal values of CAT activity, TBARS levels and NO production were not different between groups except for ROS production where CR diet-fed rats gave values significantly lower than the control groups, especially in the cerebral cortex where values differed from 26% in the Hc to 14% in the Cx itself.

High levels of ROS can trigger lipid, protein and DNA damage in cells. Though hydrogen peroxide is not a free radical, it can generate hydroxyl as well as similar reactive radicals, extending oxidative damage (Halliwell, 2006). In this context, one could speculate that a significant decrease in basal ROS production could become an important strategy for the maintenance of a healthy brain. Besides, the glutathione peroxidase is capable of eliminating peroxides by reducing them to H_2O or alcohols, with GSH as reducing substrate (Dringen, 2000). In this particular case, our data showed that the CR diet was also able to significantly reduce (about 18%) GPx activity in both Hc and Cx brain structures.

Based on our past and current results we hypothesized (Fig. 8) CR-mediated modulation of neural cells may be a result of lower metabolic and mitochondrial activity (Bordone and Guarente, 2005) with a subsequent decrease of mitochondrial ROS production as we have demonstrated in this study. A decreased CR-induced production of ROS could negatively modulate GPx activity and consequently, it would justify why the detected levels of GSH were actually increased.

Finally, we have demonstrated that the CR group had 30% less of hippocampal DNA damage than the control group. Such data is in agreement with recent works showing that CR was able to reverse age-related alterations in DNA damage by enhancing its repair and reducing mutations (Heydari et al., 2007). DNA protection is believed to be a key element in cancer prevention as well as postponing aged-related phenotype. When DNA damage persists in the genome, through replicative processes and/or through transcription-associated mutagenesis, it becomes permanent in the form of mutations and/or chromosomal breakage and instability (Heydari et al., 2007). Studies by Richardson's laboratory suggested CR as an "intervention" that could alter the activation of specific "stress response genes", key enzymes in DNA repair pathways, which would result in upregulation of "DNA repair" capacity (Heydari et al., 2007; Kirkwood and Shanley, 2005). Thus, the CR diet could enhance DNA repair, decrease DNA damage and consequently, reduce mutation frequency, which would result in

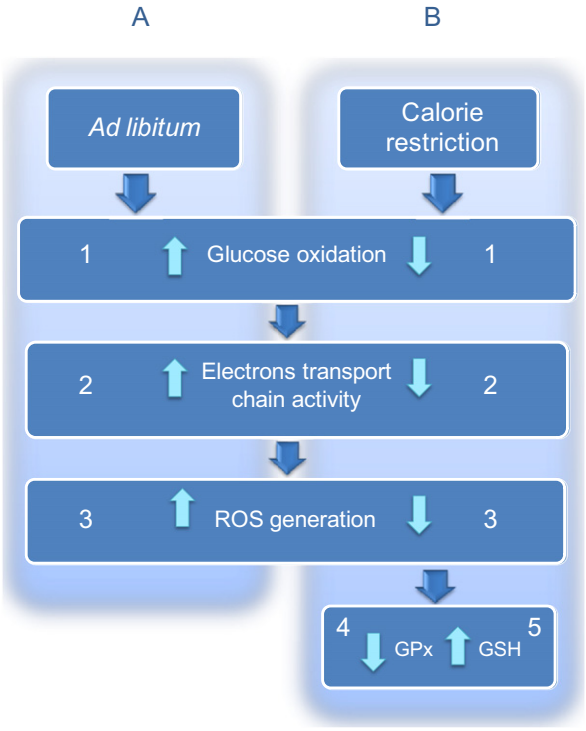

Fig. 8 – Effect of *ad libitum* and calorie restriction diets on ROS generation *via* respiratory chain. (A) Under *ad libitum* conditions, glucose is metabolized (1) and during respiration, electrons move down through the electron-transport chain. It is thought that hyper-polarization of the mitochondrial membrane can lead to electron stalling through the electron-transport chain (2) and the generation of ROS (3). (B) Proposed physiological hypothesis by which calorie restriction (CR), initiated by lower calorie intake, would decrease both glucose oxidation (1) and electron activity through the electron-transport chain (2) which would lead to decreased generation of ROS (3). Lower ROS production could negatively modulate GPx activity (4) and increased GSH levels (5).

maintenance of genomic stability. It would be interesting in future studies to investigate DNA damage in other brain structures, such as cortex, amygdala and cerebellum.

In summary, by examining calorie restriction's effects we were able to identify hippocampal and cortical modulation which gave rise to a number of metabolic changes that improved the basal status of important parameters for cellular self-defense, such as GSH upregulation and DNA damage downregulation. The maintenance of metabolic and physiological stability during aging is a prime determinant of longevity and brain function.

4. Experimental procedures

4.1. *In vivo studies and dietary caloric restriction*

Thirty male 60-day-old Wistar rats, coming from the local breeding colony (ICBS-UFRGS), were fed *ad libitum* or on CR diets for 12 weeks and maintained in a ventilated room at 21 °C with free access to water on a 12 h light/dark cycle. Experiments were performed according to *the NIH Guide for the Care and Use of Laboratory Animals* and approved by local authorities. Animals were weighted matched and divided into two different groups: Control (*ad libitum*) and calorie-restricted rats (CR). The CR group received a common/standard laboratory chow (Nuvilab-CR1, from Nuvital, Brazil) diet except for a lower caloric intake. The caloric restriction was progressive, initiated with 10% restriction during the first week and changed to 20% and 30% during the second and third weeks, respectively, until the end of treatment. Food intake was daily monitored and animals were weighted weekly (Ribeiro et al., 2009).

4.2. *Behavioral tests*

4.2.1. *Elevated plus-maze test (EPM)*

EPM task was performed by placing the animal just in the center of a maze with two closed arms and two open ones (44.5 cm × 11.5 cm for each arm). During a 2-min period, the number of entries into the closed arms and the time spent in the open ones were registered (Swarowsky et al., 2008).

4.2.2. *Open-field test for basal global activity*

In rodents, one of the most important components of exploration (a prominent activity of the animal's repertoire of spontaneous activity) is locomotion assessed in an open-field arena. The open field test is a locomotor behavior assessment paradigm that provides simultaneous measures of locomotion, exploration and anxiety. Locomotion (horizontal activity) and exploration (rearing) tasks were studied using a 50 cm high, 60 cm × 40 cm plywood box with a frontal glass-made wall and linoleum flooring divided into 12 equal rectangles. Animals were left there for 2 min during both training and test sessions, registering thereafter the number of rearings and crossings between sectors (Swarowsky et al., 2008).

4.3. *Blood sampling and analysis*

After treatment, overnight-starved animals (6th hour) were anesthetized by intramuscular injection of 75 mg/kg ketamine and 10 mg/kg of xylazine, respectively. Blood samples were obtained by intracardiac puncture and animals were killed by decapitation. Blood samples were incubated at room temperature (25 °C) for 5 min and centrifuged at 3200 rpm for 5 min. Serum was stored at -70 °C until the day of analysis. Biochemical analyses was performed by using a Multi-test Analyzer (Mega; Merck, Darmstadt, Germany) together with specific kits supplied by Merck as follows: total protein (protein-SMT, 1.19703.0001, biuret method); albumin (albumin-SMT, 1.19722.0001, bromocresol method); glucose (GLUC-DH 1.07116.0001); cholesterol (cholesterol-SMT, 1.19738.0001, CHOD-PAP method); triglycerides (SMT-triglyceride, 1.19706.0001, GPO-PAP method). For corticosterone determination, plasma was extracted with ethyl acetate and its extract evaporated and dissolved for posterior hormone evaluation by ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA). Sensitivity of the assay and intra assay coefficient of variation were 24 pg/mL and 15%, respectively.

4.4. Brain tissue dissection

Brains were removed and then placed in cold saline medium with the following composition: 120 mM NaCl; 2 mM KCl; 1 mM $CaCl_2$; 1 mM $MgSO_4$; 25 mM HEPES; 1 mM KH_2PO_4 and 10 mM glucose, adjusted to pH 7.4 and previously aerated with O_2. The hippocampi (Hc) and cerebral cortices (Cx) were dissected and cut into slices of 0.3 mm using a McIlwain Tissue Chopper for posterior analysis.

4.5. Glutathione (GSH) assay

GSH content was determined as previously described (Browne and Armstrong, 1998). Briefly, hippocampal and cortical slices were homogenized in a sodium phosphate buffer (0.1 M, pH 8.0) containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdehyde (1 mg/mL of methanol) at room temperature for 15 min. Fluorescence was measured by using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard glutathione solutions (0–500 μM). The GSH concentrations are expressed as nmol/mg protein.

4.6. Glutathione peroxidase (GPx) activity

GPx activity was measured as previously described (Wendel, 1981) by using *tert*-butyl-hydroperoxide as substrate. GPx activity was determined by monitoring NADPH (0.1 mM) disappearance at 340 nm in a medium containing 2 mM GSH, 0.15 U/mL glutathione reductase, 0.4 mM azide and 0.5 mM *tert*-butyl-hydroperoxide. One GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as U/mg protein.

4.7. Catalase (CAT) activity

CAT activity was assayed as previously described (Aebi, 1984) by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 50 μg protein. One unit (U) of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute and the specific activity is reported as U/mg protein.

4.8. Evaluation of intracellular reactive oxygen species (ROS) production

Intracellular ROS production was detected by using the non-fluorescent cell permeating compound, 2′-7′-dichlorofluorescein diacetate (DCF-DA). Samples homogenized in a sodium phosphate buffer, pH 7.4 with 140 mM KCL were treated with DCF-DA (10 μM) for 30 min at 37 °C. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm, as described previously (LeBel and Bondy, 1992), with modifications. Values are obtained as unit of fluorescence/mg protein and expressed as percentage of control.

4.9. Thiobarbituric acid-reactive substances (TBARS) measurement

Lipid peroxidation can be evaluated by the thiobarbituric acid reactive substance assay. Such method evaluates lipid peroxidation assayed for malondialdehyde, the last product of lipid breakdown caused by oxidative stress. The assay was performed as previously described (Esterbauer and Cheeseman, 1990). Briefly, 100 μL of homogenate were added to 200 μL of cold 10% trichloroacetic acid and 300 μL of 0.67% TBA in 7.1% sodium sulfate in a boiling water bath for 15 min. The mixture was placed in cold water for 1 min. Afterwards, 400 μL of butyl alcohol were added and then samples were centrifuged at 5000 × *g* for 5 min. The resulting pink stained TBARS were determined from supernatants in a spectrophotometric microtiter plate reader at 532 nm. Data were expressed as nmol TBARS/mg protein.

4.10. Nitric oxide (NO) production

NO metabolites, NO_3 (nitrate) and NO_2 (nitrite) were determined as previously described (Hevel and Marletta, 1994). Briefly, homogenates from hippocampal slices were mixed with 25% trichloroacetic and centrifuged at 1800 × *g* for 10 min. The supernatant was immediately neutralized with 2 M potassium bicarbonate. NO_3 was reduced to NO_2 by nitrate reductase. Later, the total NO_2 obtained from the incubation was measured by colorimetric assay at 540 nm, based on the Griess reaction. A standard curve was performed by using sodium nitrate (0–80 μM). Results were expressed as μM of nitrite/mg protein.

4.11. Single cell gel electrophoresis—comet assay

A standard protocol for comet assay preparation and analysis was used as previously described (Tice et al., 2000). The slides were prepared by mixing 5 μL of whole blood, or hippocampal homogenates (cold PBS), with 90 μL of low melting point agarose (0.75%). The mixture (cells/agarose) was added to a fully frosted microscope slide, previously coated with 500 μL of normal melting agarose (1%). After solidification, the coverslip was gently removed and the slides were placed in a lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5 with 1% Triton X-100 and 10% DMSO, freshly added) for 1 day. Subsequently, the slides were incubated in a freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 10 min. The DNA was electrophoresed for 20 min at 25 V (0.90 V/cm) and 300 mA. Thereafter, slides were neutralized with a Tris buffer (0.4 M; pH 7.5). Finally, DNA was stained with ethidium bromide. Neutralized and stained nuclei (from random 100-cells fields) were blindly analyzed by fluorescence microscopy (200 ×). Cells were scored from 0 (undamaged) to 4 (maximally damaged), according to the tail intensity (size and shape), resulting in a single DNA damage score for each cell, and consequently, for each group. Thus, every group could be ranged for damage index with a value from 0 (all cells no tail, 100 cells × 0) to 400 (all cells with maximally long tails, 100 cells × 4) (Collins et al., 1997). The index of DNA damage was calculated by multiplying the

number of cells by its own index score and then summed up its results.

4.12. *Protein content*

Total protein content was determined by the modified method of Lowry as previously described (Peterson, 1977), using BSA as standard.

4.13. *Statistical analysis*

Data are reported as mean ± standard error mean (S.E.M.) and were analyzed by Student's *t*-test. Values of $P < 0.05$ were considered significant. All analyses were performed using the SPSS program, Version 12.0 (SPSS, Chicago, IL).

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