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Highlights

▶ Exercise enhances free radical content. ▶ Exercise improves memory of rats. ▶ Caloric restriction is beneficial for brain.

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The effects of cocoa supplementation, caloric restriction, and regular exercise, on oxidative stress markers of brain and memory in the rat model

Zsolt Radák[a,*], Gabriella Silye[a], Csaba Bartha[a], Judit Jakus[b], Éva Stefanovits-Bányai[c], Mustafa Atalay[d], Orsolya Marton[a], Erika Koltai[a]

[a] Research Institute for Sport Sciences, Semmelweis University, Budapest, Hungary
[b] Institute of Biomolecular Chemistry, Hungarian Academy of Science, Budapest, Hungary
[c] Department of Applied Chemistry, Faculty of Food Science, Corvinus University of Budapest, Hungary
[d] Institute of Biomedicine, Physiology, University of Eastern Finland, Kuopio, Finland

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ABSTRACT

The effects of treadmill running (8 weeks, 5 times/week, 1 h/day at 27 m/min), caloric restriction, and cocoa supplementation on brain function and oxidative stress markers were tested. The Morris maze test was used to appraise rat memory. Regular exercise significantly improved spatial learning performance. The level of oxidative stress was measured by the concentration of carbonylated proteins. The free radical concentration increased in brain of the training groups but not the controls. The content of reactive carbonyl derivates did not change with exercise, suggesting that the increased production of reactive oxygen species (ROS) were well tolerated in this experimental model. Caloric restriction (CR) decreased the accumulation of free radicals in the frontal lobe. The protein content of brain-derived neutrophic factors (BDNFs) was evaluated and changes did not occur either with exercise or cocoa supplementation treatments. These data did not show significant effects of the administration of cocoa (2% w/w) on the concentration of ROS, BDNF or on spatial memory. Conversely, exercise and CR can play a role in ROS generation and brain function.

1. Introduction

It has been known for quite some time that voluntary exercise plays a significant role in decreasing the age-associated decline in cognitive function in experimental animals (Bronner et al., 1995; Johnson and McKnight, 1989; Mattson, 2000; Mayhew et al., 1998; Oliff et al., 1998). Exercise can stimulate neurogenesis (Johnson and McKnight, 1989; Oliff et al., 1998) as well as improve learning and brain plasticity (Cotman and Engesser-Cesar, 2002). Several groups have reported that reactive carbonyl derivatives (RCD) of proteins, which are the product of ROS interaction with amino acid side chains of arginyl, aspartyl, glutamyl, lysyl, prolyl and threonyl, accumulate with oxidative stress and aging (Beal, 2002; Levine, 2002; Stadtman, 1992). Accumulation of RCD in brain proteins seems to be closely related to impaired cognitive function with age, and accumulation of carbonylated proteins are not just a result of, but are probably a causative factor of, the age associated regression of physiological functions in the brain (Butterfield et al., 1997; Forster et al., 1996; Radak et al., 2001a).

Spontaneous exercise causes the production of the protein content of neutrotrophic factors, especially brain derived neutrotrophic factor (BDNF). BDNF is one of the most versatile, important neurotrophic factors in the brain. It plays a significant role in the learning process, memory, locomotion, behaviour and a wide range of stress responses (Barde, 1989). It has been suggested that BDNF regulates brain development, neuroplasticity, neurogenesis, neurite outgrowth, synaptic plasticity, and cell survival (van Praag et al., 1999). The expression and protein content of BDNF have been shown to be up-regulated by exercise, and oxidative stress (Mattson et al., 2004). Exercise does not simply up-regulate the content and expression of BDNF in different brain regions, but also impacts downstream effects of BDNF, namely the transcription factor cAMP response element binding protein (CREB). ROS are necessary for certain normal biological processes and inevitable potentially harmful products of aerobic metabolism. It has been demonstrated that ROS play an important role in signalling pathways and also regulate transactivation of transcription factors (Radak et al., 2012). Hence, ROS appear to be responsible for certain gene expression and cellular responses to internal and external challenges. In addition, exercise appears to modify the antioxidant and redox state of the brain (Somani et al., 1996, 1995). It is well known that increased reactive oxygen species are involved in the aging process and the pathogenesis of several

* Corresponding author. Address: Research Institute for Sport Sciences, Semmelweis University, Budapest, Alkotas u. 44, H-1123 Budapest, Hungary. Tel.: +36 1 4879218; fax: +36 1 3566337.
E-mail address: radak@tf.hu (Z. Radák).

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neurodegenerative diseases (Halliwell and Gutteridge, 1985). Acute exercise, depending on the intensity or duration, can readily cause increases in lipid peroxidation, carbonyl content or DNA damage (Radak et al., 2012). However, regular exercise attenuates the level of oxidative stress. Indeed, regular physical exercise is a natural means to increase longevity and to decrease the incidence of several diseases (Holloszy and Kohrt, 1995).

Diet also plays a significant role in brain function (Mattson, 2000). Many studies have shown that lifelong caloric restriction (CR) can delay aging processes. A lifelong caloric restriction reduces age-related increases in the accumulation of oxidative damage in different organs (Chen and Yu, 1994; Radak and Goto, 1998; Sohal et al., 1993).

Polyphenols are of great interest in nutrition and medicine because of their potent antioxidant capacity (Wiswedel et al., 2004) and possible protective effects on human health in reducing the risk of cardiovascular diseases and cancers (Santos-Buelga and Scalbert, 2000). Different in vitro studies have shown that cocoa flavanols prevent LDL oxidation (Pearson et al., 2001; Waterhouse et al., 1996), enhance endothelium-dependent relaxation of isolated rabbit aorta (Karim et al., 2000), modulate cytokine transcription in peripheral blood mononuclear cells (Mao et al., 2000) and suppress peroxynitrite-induced nitration of tyrosine (Arteel et al., 2000; Pannala et al., 1997). Another reported effect of cocoa procyanidins in humans is immunomodulation as interleukin 2 expression in circulating mononuclear cells has been found to be suppressed (Mao et al., 1999).

Fruit, vegetables, some beverages (tea, coffee, fruit juices, and red wine), and cocoa or chocolate are among the richest flavonoid food sources (Scalbert and Williamson, 2000). Cocoa and chocolate contain flavanols that form a unique class of polyphenols, including monomers (epicatechin and catechin), oligomers, and polymers (proanthocyanidins) (Santos-Buelga and Scalbert, 2000). The content of total polyphenols varies between 5 and 8.4 mg/g according to chocolate type and reaches 20 mg/g in cocoa powder (Waterhouse et al., 1996). The results of a very recent study suggest that epicatechin, which is a monomeric flavonoid found in cocoa, can cross the brain–blood barrier and target BDNF (Nath et al., 2012).

Therefore, the hypothesis that regular exercise, caloric restriction, cocoa supplementation and the combined effects of these three treatments affect oxidative status, BDNF content, and spatial learning performance in rat brain, were tested in the present investigation. A single bout of exercise, with a probable cause of oxidative stress, could be a good model to study the effects of cocoa supplementation on oxidative stress, but the main intent was to study the adaptive response to regular exercise and long term cocoa supplementation.

2. Materials and methods

2.1. Animals

Thirty-six four month old, male Wistar rats were used in the study and were cared for according to the guiding principles for the Care and Use of Animals based upon the Helsinki Declaration, 1964. The study was approved by the local Animal Welfare Committee. Animals were housed in standard polyethylene cages with food and water ad libitum. The rats were divided into six groups, which were subjected to running training (8 weeks) or cocoa supplementation or caloric restriction or a combination of these treatments. The supplementation of cocoa was started 6 weeks before the running program, and therefore, the cocoa supplementation program lasted 14 weeks. The enriched chow contained 2% (w/w) cocoa. Each rat ate 0.6 g cocoa daily. Cocoa was purchased from Spar Co. (Budapest, Hungary). Cocoa was mixed into the lab chow by the same company that supplied the standard food (Bioplan Co., Budapest, Hungary).

Six rats were randomly assigned to each of six groups: exercised fed cocoa enriched food (CE), non-exercised fed cocoa enriched food (CC), exercised fed normal food (NE), non-exercised fed normal food (NC), calorie restricted fed cocoa enriched food (CSC) and calorie restricted fed normal food (NSC). Non-exercised groups remained sedentary for the 14 weeks of the study. After a 1 week adaptation period consisting of 1 h of running/day with the intensity starting at 17 m/min and reaching 27 m/min the last day of the adaptation period, exercised groups were subjected to forced treadmill running for 8 weeks, 5 days/week, 1 h/day at 27 m/min intensity on a treadmill consisting of six lanes separated by glass walls. Each lane was equipped with metal bars at the rear end of the lane to provide electrical motivation to the animals, using 1–3 V electrical current. CR rats calorie intake was 60% of *ad libitium*.

2.2. Morris maze

After 14 weeks all rats were trained in a Morris water maze for five consecutive days (four trials per day). A platform 6 cm in diameter was placed in the centre of the northwest quadrant of a circular pool of 60 cm in height and 100 cm in diameter and 1 cm below the surface of the water. The water was maintained at 22–23 °C throughout training and testing. During a given training trial, each rat was introduced into the pool at one of four possible starting points (north, south, west, and east) and allowed a period of 60 s to find the platform. The order of starting points varied in a pseudorandom manner for each rat every day. The time necessary to find the platform was registered. Twenty-four hours after the final training trial, a probe test was conducted in which each rat was allowed to swim for 60 s in the pool with the platform removed. One day after the last training session, animals were sacrificed by decapitation and the brain was removed and frozen in liquid nitrogen and stored at −80 °C until analyses.

2.3. Biochemical assays

2.3.1. BDNF determination

Frontal lobes of the brain were homogenised in eight volumes of lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl (Ph 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, aprotinin (10 mg/ml), leupeptin (1 μg/ml), and 0.5 mM sodium vanadate. The protein determination was performed in duplicate as described earlier by Lowry et al. (1951).

The concentrations of BDNF were determined from the cortex, using the E-MAX ImmunoAssay System (Promega, Madison, WI). Standard 96-well flat-bottom Corning ELISA plates were incubated with carbonate coating buffer containing monoclonal anti-BDNF over-night at 4 °C. The next day, the plates were blocked with 1 × B&S buffer for 1 h at room temperature. Serial dilutions of known BDNF ranging from 500 to 0 were performed in duplicate for the standard curve for each set of tissue. For both the standards and the samples, 100 μl were added in each well in duplicate, and incubated for 2 h at room temperature. The wells were then incubated with a secondary antihuman BDNF polyclonal antibody (1 h at room temperature). Then, the wells were incubated with anti-IgY conjugated to HRP for 1 h at room temperature. A TMB was used to develop colour in the wells for 10 min at room temperature. The reaction was stopped with the addition of 1 N HCl to the wells. The absorbance was read at A450 (Molecular Devices ThermoMax microplate reader, with SOFTmax PRO v3.1 software, Sunnyvale, CA).

2.4. Protein carbonyls

The carbonyl measurements were done according to the description of Radak et al. (1999). In brief, each sample was incubated for 1 h in 500 μL of 10 mM dinitrophenylhydrazine or 2 N HCl as a blank. Later, 500 μL 20 w/w% trichloroacetic acid was added to the samples. After centrifuging for 10 min at 20,000 g, the supernatants were discarded. Samples were washed in ethanol two times and once in acetone. The remaining pellets were dissolved in 8 N urea. The pellet-urea solution was incubated for half an hour at 37 °C. The absorbance of the samples was detected by spectrophotometer at 360 nm.

2.5. Electron paramagnetic resonance

The electron paramagnetic resonance (EPR) measurements were carried out as described by Stadler et al. (2003) to detect levels of ROS in brain samples. EPR is one of the best direct methods to assess molecules with unpaired electrons and offers great specificity, since ordinary chemical solvents and matrices do not give rise to EPR spectra. In brief, the measurements were carried out with an X-Band computer-controlled spectrometer, constructed by Magnetech GmbH (Berlin, Germany). Approximately 100 mg of tissue sample from the frontal lobe was frozen into a rod-shaped form and spectra of the sample were recorded at 77 K using a quartz finger Dewar, filled with liquid nitrogen. Instrument settings were: 100 kHz modulation frequency, 0.7050 mT modulation amplitude, 18 mW microwave power, 1 min scan time, and 20.63 mT field sweep. For evaluation, the double integration method of the EPR signals, with Mn/MnO as an internal standard, was used, and the data were expressed as arbitrary units.

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2.6. Cocoa element concentration

Cocoa samples (0.2 g) were digested in a mixture of 2 ml HNO3 and 2 ml H2O2 in a Teflon bomb (PTFE) for inductively coupled plasma (ICP) analysis. The digested samples were filled with deionised water to 10 ml. The following elements were determined by ICP-OES (Thermo Jarrell Ash Co, ICAP 61): Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Na, Ni, P, Sr, Ti, V, and Zn.

2.7. Determination of total phenol content

The total amount of soluble phenols was determined using Folin–Ciocalteu's reagent according to the method of Singleton and Rossi (1965). The content of soluble phenols was calculated from a standard curve obtained using different concentrations of gallic acid.

2.8. Analysis of FRAP

The total antioxidant capacity was measured using the ferric reducing power (FRAP) assay, according to the method of Benzie and Strain (1996). The reduction of ferric to ferrous ion at low pH formed a coloured ferrous–tripyridyltriazine complex. Absorbance changes were linear over a wide concentration range with antioxidant mixtures. FRAP values were obtained using a seven-point calibration curve of known amounts of Fe2+ and expressed in mmol Fe2+/L.

2.9. Statistical analyses

Statistical significance was assayed using factorial ANOVA, followed by Tukey's posthoc test, and repeated measures ANOVA was used for the behavioural data. The significance level was set at $^{**}p < 0.05$.

3. Results

Table 1 contains the element concentrations of cocoa used in the study. The antioxidant capacity of the cocoa powder was measured by FRAP assay and the data revealed that it contained 0.343 mmol ascorbic acid/l, and 0.6 mg ascorbic acid/g cocoa. The reductive capacity of cocoa and 0.6 mg ascorbic acid were the same. When the total phenol content of the cocoa was assessed, 12.2 mg polyphenol/1 g cocoa were detected.

Exercise training and calorie restriction decreased body weight significantly ($p < 0.05$), (Fig. 1). The brain performance, spatial learning, assessed by the Morris maze test, improved significantly with exercise training. The cocoa consumption and CR did not modulate memory function (Fig. 2). Additionally, data obtained

Table 1
Minerals. demonstrates the element concentrations in cocoa (μg/g) used in this study. The elements were determined by ICP-OES.

Al	87.69	Fe	281.7	Pb	<0.5000
As	<2.500	K	32050	Se	3.589
B	19.43	Li	1.816	Si	93.97
Ba	16.28	Mg	4970	Sr	19.35
Ca	1627	Mn	febr.00	Ti	2.905
Cd	<0.5000	Mo	<0.5000	V	<0.5000
Co	1.153	Na	3280	Zn	65.34
Cr	1.567	Ni	9.542		
Cu	45.32	P	6587		

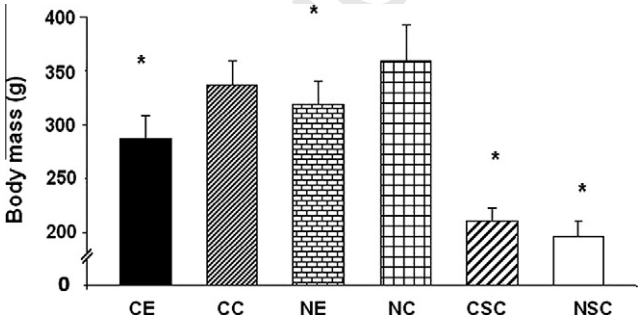

Fig. 1. *Body mass.* Exercise training and caloric restriction decreased body weight significantly ($p < 0.05$). Regular exercise and lowered caloric intake help maintain an ideal body weight.

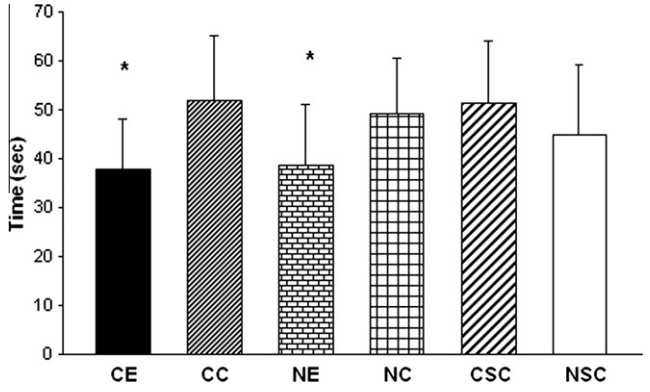

Fig. 2. *Morris maze.* The panel shows the exercise times of exercised fed cocoa enriched food (CE), non-exercised fed cocoa enriched food (CC), exercised fed normal food (NE), non-exercised fed normal food (NC), calorie restricted fed cocoa enriched food (CSC) and calorie restricted fed normal food (NSC). Exercise decreased the time to locate the platform significantly. Values are means ± S.D. for six animals per group. $^{*}p < 0.05$ vs. control.

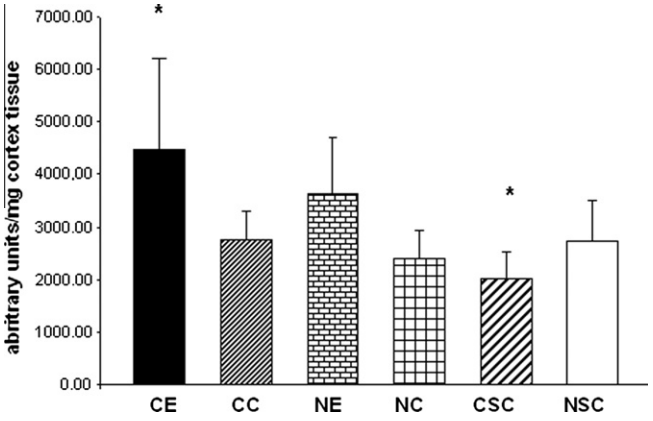

Fig. 3. *EPR data.* The EPR data revealed that the free radical concentration was increased with training, but the effect of caloric restriction and cocoa supplementation did not change significantly in the cortex of the experimental animals. Values are means ± S.D. for six animals per group. $^{*}p < 0.05$ vs. control.

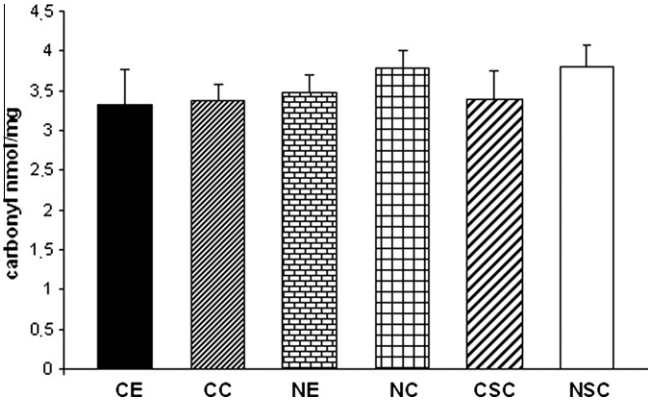

Fig. 4. *Carbonyl levels.* The carbonyl content was not significantly changed with training, caloric restriction or cocoa supplementation. Values are means ± S.D. for six animals per group.

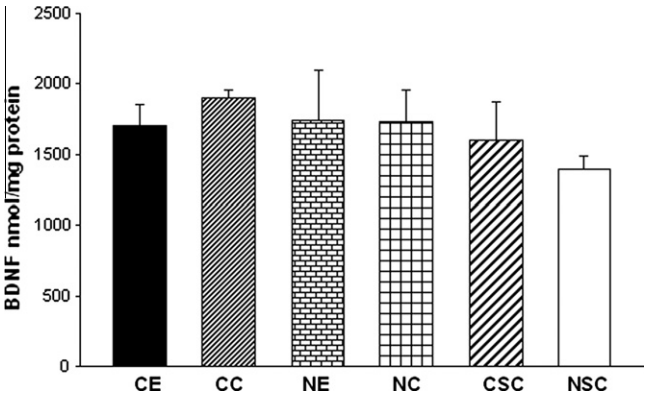

Fig. 5. *BDNF*. The concentrations of BDNF were determined from the cortex using the E-MAX ImmunoAssay System. The data revealed that the protein content of BDNF did not change significantly with training, caloric restriction or cocoa supplementation. Values are means ± S.D. for six animals per group.

by EPR measurements revealed that free radical accumulation in the frontal lobe significantly increased with exercise (Fig. 3, $p < 0.05$). The oxidative damage to brain samples was evaluated by the content of reactive carbonyl derivates. It was found that the carbonyl content did not change (Fig. 4). This result means significant oxidative modifications of proteins did not occur with training. CR decreased the accumulation of free radicals in the frontal lobe ($p < 0.05$), but cocoa supplementation did not modulate the amount of free radicals (Fig. 3). Caloric restriction, cocoa supplementation, and exercise did not alter the protein content of BDNF (Fig. 5).

4. Discussion

There are few studies concerning exercise induced oxidative stress on brain and the results are equivocal. Suzuki et al. (1983) observed that voluntary running caused advanced lipid peroxidation in rat brain. de Oliveira et al. (2003) noted increased carbonyl concentration in the brain as a result of physical exercise. However, others did not find oxidative damage in the brain after running which is in agreement with our earlier findings (Radak et al., 1995). Increased levels of ROS and oxidative damage could be initiators of a specific adaptive response, such as the stimulation of the antioxidant enzymes, thiols, and enhanced oxidative damage repair (Itoh et al., 1998; Radak et al., 2002, 2001b).

It is well demonstrated that regular exercise has beneficial effects on brain function, and improves the learning and memorising capability of animals (Radak et al., 2001a,b). Human studies also report that exercise ameliorates and/or retards the age-associated decline in cognitive function (Chodzko-Zajko and Moore, 1994; Hicks and Birren, 1970). Similarly, in the present experiment, exercise training is shown to improve the learning and memory capability of rats, after only 8 weeks of exercise training.

In the present work, it was also observed that moderate regular exercise did not change the accumulation of RCD in rat brain. Toldy et al. (2005) and Liu et al. (2000) observed a similar phenomenon. van Praag et al. (1999) showed that spontaneous exercise causes the production of neutrotrophic factors, especially BDNF. In this study the BDNF content in the frontal lobe did not change significantly which is similar to our earlier results (Toldy et al., 2005). Generally it is accepted that regular physical activity increases BDNF levels in hippocampus, which provides some explanation for the beneficial effects of exercise on brain function (Gomez-Pinilla, 2011) moreover it has also been suggested that the relationship between improved brain function and BDNF could play an evolutionary role in the development of brain (Mattson, 2012). In the present study we used the frontal lobe which contains the primary motor cortex. However, it must be noted that most of the studies showing elevated levels of BDNF with exercise are from hippocampus samples (van Praag et al., 1999). The effects of exercise on BDNF levels of the frontal lobe is not as clear as that found from hippocampus samples (Graybeal et al., 2011). We hypothesise that this is one of the reasons why no relationship between exercise of CR and related changes in BDNF levels was found. An other reason could be the young age of the animals, which could account for the lack of stimulating effects of exercise on BDNF levels in the frontal lobe.

Besides exercise, nutrition is an important means to decrease the incidence of life-style related diseases. It is well known that CR is a powerful tool to increase the maximal life span of rodents (Yu, 1994). CR can protect neurons against degeneration in animal models of Alzheimer's, Parkinson's disease and stroke (Bush, 2002; Maynard et al., 2002). Moreover, CR can stimulate the production of new neurons from stem cells (neurogenesis) and enhance sympatic plasticity, which may increase the ability of the brain to resist aging and restore function following injury. The beneficial effects of CR appear to be the result of a cellular stress response that simulates the production of proteins that enhance neuronal plasticity and resistance to oxidative and metabolic insults, including neutrotrophic factors such as BDNF (Duan et al., 2001; Mattson et al., 2003). However, in the present study we could not detect statistical differences between CR and *ad libitium* fed rats, which was probably due to the fact that the young animals were still in a developing stage.

CR has an impact on redox state, since it increases antioxidant enzyme activity, reduces oxidative damage in tissues (Seo et al., 2006), and stimulates protein turnover (Yu, 1994). CR does influence the level of free radicals, and decreases the amount of ROS in brain. Several study groups have investigated CR effects on oxidative damage, finding decreased amounts of carbonyl (Harman, 1982; Jolitha et al., 2006; Ogonovszky et al., 2005; Radak et al., 2000; Yu, 1994).

Numerous investigators have shown polyphenols to be antioxidants in cocoa which attenuate oxidative damage and thereby reduce the incidence and formation of different diseases such as cardiovascular diseases, as well as inhibiting lipid peroxidation, DNA damage, and enhancing the immune system (Keen et al., 2005; Kenny et al., 2007; Kraemer et al., 2004; Kris-Etherton and Keen, 2002; Mateos et al., 2005; Yamagishi et al., 2001). However, no groups have examined how cocoa consumption affects levels of free radicals, carbonyl, and BDNF in brain, or how these influence memory function. Cocoa supplementation did not change ROS concentrations in brain nor the levels of carbonyl, BDNF and memory function. It is possible, we were not able to measure this modification, because the cocoa ingestion dose was inadequate, albeit we used similar amounts as Orozco et al. (2003). However, it must be mentioned that in the study of Orozco et al. (2003), 8-hydroxy-2′-deoxyguanosine, F(2)-isoprostanes, thiobarbituric acid reactive substances and glutathine levels were used as markers of oxidative stress, and actually showed that cocoa supplementation reduced the levels of 8-hydroxy-2′-deoxyguanosine in testes but not in liver and heart. This observation demonstrates that the effect of cocoa supplementation could be tissue specific and that the sensitivity of oxidative stress markers to detect ROS is different.

Another possibility to explain the failure of cocoa supplementation to be pronounced could be due to the fact that cocoa polyphenols are not absorbed similarly in rat testes and brain. Proanthocyanidins are poorly absorbed through the gut barrier because of their high molecular weight (Donovan et al., 2002; Holt et al., 2002; Scalbert and Williamson, 2000). It is possible that

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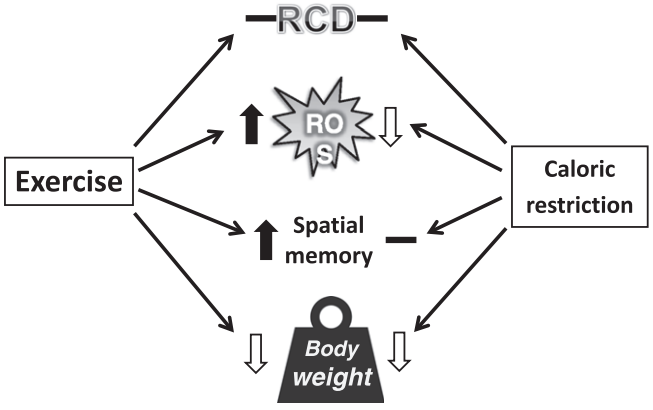

Fig. 6. *Summary*. The present figure graphically summarises the main findings of this study. Exercise and caloric restriction decreases the body mass, but exercise alone, improves spatial memory, and at moderate level the concentration of reactive oxygen species (ROS).

these biological effects, which were partially confirmed in vivo (Rein et al., 2000; Wan et al., 2001), may not be due to a direct action of proanthocyanidins themselves but to an effect of some more readily absorbed low-molecular-weight metabolites. Chocolate proanthocyanidins have been shown to be stable in the stomach of healthy human volunteers (Rios et al., 2002) but not cleaved into monomers when fed to rats (Donovan et al., 2002).

Element concentrations in cocoa were analysed, since a number of metals (e.g. Se, Mn, Fe, Zn) are involved at the active sites of antioxidant enzymes. The results revealed that indeed the cocoa used in the present study, contains elements involved in the antioxidant defence, for example selenium (Klotz and Sies, 2003; Ramoutar and Brumaghim, 2007).

The data from the present study demonstrate that exercise increases the concentration of ROS in the frontal lobe, but levels of carbonyl do not change. Therefore, it is suggested that exercise does not cause accumulation of carbonyl damage in the frontal lobe of rats, but rather enhances memory in rats. CR is able to reduce ROS in the frontal lobe, suggesting that the redox balance can be altered by CR. Thus, nutrition and exercise do play a role in brain function (see Fig. 6).

Conflict of Interest

The authors declare that there are no conflicts of interest.

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