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ENCÉFALO E ESTRESSE OXIDATIVO EM RATOS MACHOS REPRODUTORES

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Encéfalo e estresse oxidativo em ratos machos reprodutores

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Science is wonderfully equipped to answer the question "How?" but it gets terribly confused when you ask the question "Why?"

Erwin Chargaff (1905 - 2002)

Oxidative stress in the brain of reproductive male rats

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Abstract

Many changes occur as the result of reproduction, but little is known about how reproduction influences oxidative stress in the brains of male vertebrates. In the present study, reproductive activity was found to increases superoxide dismutase, glutathione peroxidase, and catalase antioxidant enzymes, yet increased oxidative damage in protein (carbonilation and nitration) and lipids peroxidation was still observed, compared to the naïve group. The level of testosterone also increased, suggesting that metabolic rate is increased in reproductive males, leading to more oxidative damage and induction of antioxidant enzymatic defenses.

Keywords: Oxidative stress; reproduction; male; brain; antioxidant defenses.

Introduction

Reproduction cause many changes in male vertebrates, including morphological, behavioral, and physiological alterations [1]. Many of these changes are related to brain functions, but some processes remain unclear. Reproductive success incurs some costs: previous studies have suggested that reproduction causes oxidative stress [2].

Many studies have investigated reproduction in mammals, although these have typically been limited to female (lactation, number of litters, size of litter) [3], and related to behavior for males [4]. Moreover, studies concerning reproductive activity and oxidative stress have been restricted to invertebrates such as *Drosophila melanogaster* and *Caernohabdits elegans* [2], and few are related with male vertebrates [2;5].

The brain has low antioxidant levels and consumes large amounts of oxygen, rendering it vulnerable to oxidative stress [6]. This stress can be dangerous for the organism, as many studies of neurodegeneration have suggested an influence of the free radicals on human diseases [7].

The aim of this study was to examine oxidative stress during reproductive activity in the brain of male rats. We measured oxidative stress parameters in naïve rats and experienced rats at six months of age: the activity of antioxidant enzymes; the levels of oxidative damage in lipids and proteins; and hormonal levels.

Materials and methods

Animals

Following approval from the Animal Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil, we used 20 Wistar male rats (*Rattus norvegicus*) aged six months. They were divided into two groups: naïve (n = 10) and experienced (n = 10). Naïve rats were grouped with others male rats (5 animals per box), without any contact with females. At an age of one month, each experienced rats was maintained in a box with a single female of the same age.

Condition to occur reproduction was considerate according to male sexual behaviour, which consists of normal levels of hormones (e.g. testosterone and estradiol), no brain dysfunction, and the presence of a

female [4]. Reproduction was considered to have occurred when the females gave birth to litters. After the birth, the newborns were separated from the couple at 21 days of age, before the initiation of the pubertal stage, which corresponds to an age of 30-70 days for males and 33-42 days for females [8]. The litter size ranged from 5 to 11 newborns, and each couple had 3 or 4 litters.

The animal house was kept on a 12 h light/dark cycle at a temperature of 24 ± 1°C, and animals were provided with standard lab chow and drinking water *ad libitum*.

Brain obtainment and processing

All animals were anesthetized using a mixture of ketamine and xilazine (i.p. 75 and 10 mg/kg, respectively). After saline infusion, the brain was removed from the skull and immediately frozen in liquid nitrogen for further analyses.

Organ processing began with manual maceration. The samples were sonicated in 30 mM phosphate buffer (120 mM KCl, 100 mM PMSF, pH 7.4) and centrifuged 10 min×3500 g. The supernatant was transferred to a fresh tube and a second centrifugation was performed at 10 min×15800 g. The supernatant from the second centrifugation was used for all assays.

Blood obtainment

Before saline infusion, blood was quickly collected by puncturing the left ventricle of the heart. Fresh blood was centrifuged (4 min×320 g) and the serum was separated for the subsequent radioimmunoassay.

Enzyme activities in brain

The assay to measure total superoxide dismutase (SOD) activity was based on spectrophotometric method that measures inhibition of epinephrine autoxidation using absorbance at 480nm [9]. Glutathione peroxidase (GPx) activity was evaluated by using absorbance at 340 nm to measure the oxidation of NADPH

in the presence of reduced glutathione, glutathione reductase and tert-butyl hydroperoxide [10]. Catalase (CAT) activity was evaluated by measuring the rate of hydrogen peroxide consumption using absorbance at 240nm [11]. All results were normalized against total protein content [12]. All assays were performed in three independent experiments.

Oxidative damage assays in brain

As an index of lipid peroxidation, malondialdehyde (MDA) levels were measured in a reverse-phase column (SUPELCOSIL™ LC-18-DB HPLC Column; 15cmx4.6mm, 5μm), using a flow of 1 ml/min of mobile phase, which consisted of 30mM monobasic potassium phosphate (pH 3.6) and methanol in a ratio of 82.5:17.5 (v/v). Absorbance of column effluent was measured at 250 nm. The retention time of MDA was found to be 5.6 min under these conditions (see representative chromatograms in Fig. 1) [13]. Calibration of the MDA assay was performed using standard addition. As an index of protein damage, carbonyl levels were measured using the absorbance at 370nm [14]. Indirect nitric oxide (NO) was measured using spectrophotometric method, which uses absorbance at 543 nm to determine levels of total nitrate and nitrite [15]. All results were normalized against total protein content [12]. All assays were performed in three independent experiments.

Hormonal levels measurement in blood

The levels of testosterone and 17β-estradiol were estimated by radioimmunoassay of serum using the Testosterone RIA DSL-4100® and Estradiol RIA DSL-4100® kits (Diagnostic Systems Laboratories, Inc., Oxford, UK). All assays were performed in three independent experiments.

Statistical analysis

The values are expressed as mean ± s.e.m. Statistical analysis was performed by the least-significant difference test, which consists of two steps. First, an analysis of variance was performed. The null hypothesis

was accepted for those data sets in which F was not significant at the level of $P>0.05$. Second, the data sets in which F was not significant were examined in a t-test using $P<0.05$ as the critical limit; those data sets in which F was significant were examined by a modified t-test using $P<0.05$ as the critical limit. For testosterone and estradiol, the null hypothesis was accepted ($F_{testosterone} = 0.91$, $F_{estradiol} = 0.08$); for the others assays, the null hypothesis was refused ($F_{SOD} = 1 \times 10^{-7}$, $F_{GPx} = 3 \times 10^{-3}$, $F_{CAT} = 4 \times 10^{-10}$, $F_{MDA} = 3 \times 10^{-3}$, $F_{Carbonyl} = 2 \times 10^{-3}$, $F_{NO} = 2 \times 10^{-2}$).

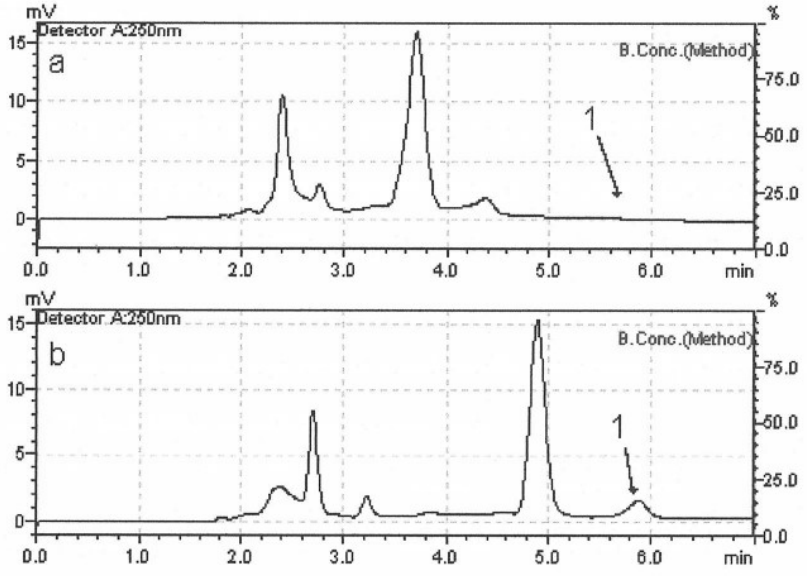

Figure 1 - Representative HPLC chromatograms from the MDA assay. Chromatograms show that MDA retention time is ~5.6 min (arrow 1). Chromatogram of a naïve rat (a) and experienced rat (b).

Results

The animals showed a body weight proportional to their age (Table 1). In addition, they showed good motor activity, and they did not show hair loss or any symptoms of disease. The brain weights did not differ significantly between the two groups (Table 1), indicating that the animals were well nourished and healthy.

Table 1 – Animals body weight, brain weight and body length (without tail). Data are expressed as mean (median, minimum value, maximum value).

	Naïve	Experienced	
Body (g)	310.5 (306, 300, 330)	366.7 (370, 330, 410)	*
Brain (g)	1.815 (1.817, 1.772, 1.855)	1.731 (1.790, 1.470, 1.883)	
Length (cm)	22 (22, 21, 23)	22 (22, 20, 24)	
n	10	10	

* $P<0.05$.

SOD, GPx, and CAT activities were found to be higher in experienced than in naïve animals (Table 2). MDA, carbonyl, NO, and testosterone levels were also higher in the experienced group (Table 2). In contrast, no statistical difference in the level of 17β-estradiol was found between groups (Table 2).

Table 2 – Enzymatic defense, oxidative damage and hormonal profile in male rats aged six months, with or without reproduction activity. Results are expressed as mean ± s.e.m.

	Naïve	Experienced	
SOD (U/mg protein)	0.36±0.03	129.88±19.34	*
GPx (U/mg protein)	20.7±2.5	12721.4±1602.1	*
CAT (U/mg protein)	25.4±3.5	1285.9±136.8	*
MDA (nmol MDA/mg protein)	2.85±0.42	139.13±11.51	*
Carbonyl (nmol carbonyl/mg protein)	0.062±0.008	0.147±0.017	*
NO (nmol $NaNO_2$/mg protein)	0.96±0.05	5.27±0.74	*
Testosterone (ng/mL)	52.0±24.39	151.5±16.44	*
Estradiol (pg/mL)	17.7±3.08	19.9±5.05	
n	10	10	

*$P<0.001$.

Discussion

The only previous study of reproduction activity and oxidative stress in vertebrate studied the pectoral muscles of male and female birds (*Taeniopygia guttata*) and their correlation with the brood size [5]. The activity of SOD was found to decrease with increasing brood size; however, GPx activity did not change [5]. In the present study, we showed that full reproductive activity altered all oxidative parameters studied, indicating an increase in both the level of oxidative damage and antioxidant enzymatic defenses.

The importance of CAT in the central nervous system during aging was suggested in the last decade [16]. CAT is thought to protect the brain from hydrogen peroxide during oxidative deamination of the neurotransmitter dopamine and to protect against lipid peroxidation of poly-unsaturated fatty acids [16]. A recent study found that an active GPx system is essential for maintaining CAT activity [17]. Thus CAT cannot compensate for a complete loss of GPx clearance of hydrogen peroxide, although GPx can fully compensate for a loss of CAT function in astroglia-rich primary cultures [17].

In females, some studies have suggested that physiological concentrations of estradiol upregulate antioxidant enzymes [18]. It is therefore possible that the longer lifespan of females is due to their higher levels of estradiol and estrogens [18]. These elevated levels may directly or indirectly increase protection against oxidative damage [18-20]. One study has suggested that mitochondria in females produce less hydrogen peroxide than those in males, resulting in less oxidative damage [18].

It is possible that testosterone increases oxidative stress, probably by increasing metabolism and thereby reducing male lifespan [21-23]. In support of this hypothesis, castrated male rats live longer than intact male rats [22]. Testosterone has also been to impair the activity of CAT, SOD, and GPx in human testes, to upregulate CAT activity in the brain, and to reduce the level of pro-oxidant enzymes in the rat prostate and the level of lipid peroxidation in the rat brain [24].

The observed difference in body weight among groups suggests two hypotheses. First, that probably is related to the number of animals in each box, because experienced males were maintained with only one female (two animals per box), and the naïve were maintained with other four animals (five animals per box), modifying the access to the food in each group. Second, the increased metabolism in experienced males demands more energy and the animal increases food intake as a consequence [2].

Endogenous gonadal hormones in mitochondria from the brain of neutered males and females rats influence oxidative defenses [25]. The rate of SOD production in neuronal-like PC-12 cells was shown to decrease following treatment with 17β-estradiol [25]. In fact, estrogen treatment of neutered rats' brain suppressed the production of reactive oxygen species and slightly reduced the level of mitochondrial SOD protein [25]. In contrast, testosterone treatment caused a small increase in the SOD level [25].

Increased GPx activity was observed in the prostate tissue of castrated male rats following testosterone treatment [26]. The various studies discussed above show that changes in testosterone levels can have different effects in different tissues.

In the present study, SOD, GPx, and CAT activities were much higher in experienced group; the respective levels were 359-, 51-, and 636-fold higher than in naïve group. Theses increases probably reflect an increase in metabolism caused by the higher level of testosterone (2.9-fold) resulted from reproductive activity. The results found are unexpected for an organ which literature mentions presenting low enzymatic activities [6], showing the capacity of adaptation of the brain to the changes promoted by reproduction.

One study reported that the amount of superoxide anion generated in the mitochondria continuously increases with aging in all brain regions of the male rat (1, 3, 6, 12, 18, and 21 months of age) [27]. Increased generation of superoxide anion may lead to generation of peroxynitrite anion (which can be formed by the reaction of superoxide anion and nitric oxide), and, in presence of high levels of hydrogen peroxide, this can mediate protein tyrosine nitration [28]. This suggests that increased metabolism resulting from reproductive activity can lead to a proportional increase in superoxide anion levels and ultimately to oxidative damage due to the action of this free radical and its downstream products. As SOD activity was found to be higher in experienced rats, high levels of hydrogen peroxide can be produced, resulting in a propitious environment to occur damage to proteins due tyrosine nitration involving peroxynitrite.

The levels of MDA, carbonyl, and NO in experienced rats coincided with the plateau of reproductive activity; these results are similar to a recent data of protein damage in brains of both males and females during aging [29]. Apart from the high activities of SOD, GPx, and CAT that we observed in experienced group, it is evident that these enzymes had not been capable to protect brain of male rats during reproduction. As Esiri has pointed out, this finding is predicted in light of the so-called 'disposable soma' view of aging, according

to which there is a trade-off between somatic maintenance and repair and other functions, e.g. reproduction, in the allocation of metabolic resources [30].

The mechanisms underlying the changes in behavior, metabolism, and other physiological processes caused by reproductive activity are unclear, and more studies should focus breeding males to elucidate the oxidative profile and costs associated with reproduction [2].

Based on our data, we speculate that full reproductive activity leads to increased oxidative damage in the brain, which can later result in disease such as Alzheimer's and Parkinson's in older men. However, more studies are required to clarify the relationship between reproduction and oxidative stress.

Acknowledgments

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Abbreviations

CAT: catalase.

GPx: Glutathione peroxidase.

MDA: Malondialdehyde.

NO: Nitric oxide.

SOD: Superoxide dismutase.

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Anexo 1

Comprovante de submissão:

Manuscript No.: FRBM-D-08-00988
Title: Oxidative stress in the brain of reproductive male rats
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Anexo 2

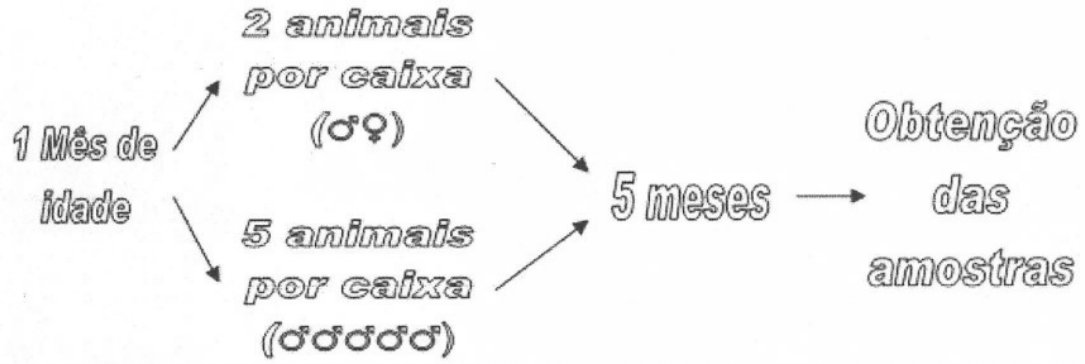

Figura Suplementar 1 - Esquema de separação entre os grupos reprodutores e não reprodutores ao longo do tempo para a obtenção das amostras de sangue e encéfalo.

Visto,
05/12/08