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Modulation in Reproductive Tissue Redox Profile in Sexually Receptive Female Rats after Short-Term Exposure to Male Chemical Cues

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Abstract

It is well known that antioxidants play an important role in sperm fertility, but there is no data on the literature regarding the effect of male chemical cues in the antioxidant defenses of the female reproductive tract. Here, we evaluated oxidative parameters in ovaries and uterus of virgin female rats isolated from contact to males and exposed only to male-soiled bedding (MSB). Four-month-old Wistar (regular 4-day cyclic) virgin female rats were utilized from proestrus to estrus phase of the reproductive cycle for experimental exposure. In an isolated room, female rats were exposed for 90 min to MSB. For biochemical assays, female rats were killed by decapitation at 30, 90, 180, and 240 min after the end of exposure, and the ovaries and uterus were removed for further analysis. Antioxidant enzyme activities (superoxide dismutase, catalase, and glutathione peroxidase), the nonenzymatic antioxidant potential (total radical-trapping antioxidant parameter), and the oxidative damage parameters (thiobarbituric acid–reactive species and carbonyl content) were analyzed. We observed an increase in the nonenzymatic antioxidant potential and diminished free radical oxidative damage in uterine tissue, 30 and 90 min after exposure. Furthermore, in ovaries, enzymatic defenses were modulated distinctly along the 240 min after exposure. MSB exposure modulates the antioxidant profile in ovaries and uterus of receptive female rats. It is possible that the modifications in the oxidative profile of the female genital tract may have important implications in the process of fertilization.

Key words: antioxidant defenses, female reproductive tract, male-soiled bedding, oxidative damage, pheromonal communication

Introduction

Chemical cues are widely used in intraspecific social communication in a vast majority of living organisms ranging from bacteria to mammals (Ben-Jacob et al. 2004; Brennan et al. 2004; Johansson et al. 2007). In a variety of species, sexual and reproductive behaviors are closely dependent on communication through chemical signals (Roelofs 1995; Martínez-Rícos et al. 2007). As an example, mammals release olfactory cues with urine that promote neuroendocrine modulations with changes in behavior and physiology in the receiver (Luo et al. 2003; Lin et al. 2005). In addition, some behavioral and physiological responses can occur with airborne signals, whereas other organisms require physical contact between the receiver and the chemical cue (Muroi et al. 2006). Male- and female-soiled bedding contain both volatile and nonvolatile compounds that are known to be important in intraspecific communication, for instance promoting mate selection and copulatory activity (Marchlewska-Koj et al. 2000; Moncho-Bogani et al. 2002; Briand et al. 2004). Recent developments have led to an appreciation of the diversity of chemosensory systems and their complementary roles in influencing vertebrate physiology and behavior (Brennan and Zufall 2006).

Free radicals and related reactive species play an important role in a wide range of physiological processes, including sex and reproduction (Fujii et al. 2005; Aitken and Baker 2006; Halliwell and Gutteridge 2007). Oxidative stress has long been recognized as a determinant factor in sperm fertility, as the presence of both nonenzymatic and enzymatic antioxidant defenses in sperm and epididymidal fluid have been demonstrated to be essential for sperm fertility (Drevet 2006). Besides, reduced levels of $O_2^{\bullet-}$ have been proposed to play an important role in normal sperm function during their way through the female reproductive tract (Agarwal, Makker, et al. 2008). Reactive oxygen species (ROS) may

also contribute to successful fertilization by modulation of the acrosome reaction and the passage of the sperm into the egg (Ford 2004; Rivlin et al. 2004), being also involved in oocyte maturation and regression of the corpus luteum during the normal mammalian ovarian reproductive cycle (Agarwal et al. 2005). All these evidences support the idea that free radicals exert a major role in fertilization and reproduction.

Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are pivotal for the normal function of several important biological processes in mammals, with known importance in rodent and human reproduction. SODs are highly efficient in the catalytic dismutation of $O_2^{\bullet-}$; generating H_2O_2 that can be removed by other enzymes, such as CAT and other peroxidases (e.g., GPx). SOD and GPx have an important characterized role in reproductive tract antioxidant defense, but in genital tract, the role of CAT is until now ignored (Fujii et al. 2005; Sugino 2007). Chemical cues are a primary factor to induce disturbances in behavior for a variety of species, but, to our knowledge, there is no information on the modulation of reactive species production and antioxidant defenses in the female reproductive tract by male chemical cues up to date. Here, we report oxidative stress parameters in the reproductive tract of female virgin rats induced by male-soiled bedding (MSB) exposure.

Material and methods

Animals

Four-month-old Wistar (regular 4-day cyclic) virgin female rats (250–300 g) were obtained from our breeding colony. Animals were housed in standard cages, 5 per cage, in controlled temperature room (23 ± 1 °C), with a 12-h light:12-h dark cycle, lights on at 7:00 AM. Standard laboratory chow and water were available ad libitum. Fifteen days after delivery (early before puberty) pups were sexed, males were removed (kept in male–female common room), and the females were brought to a room in complete absence of adult male chemical cues or pregnant female rats. All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1985) and were carried out according to the determinations of the Brazilian College of Animal Experimentation—COBEA.

Experimental design

To collect soiled bedding from male rats housing cage, 40 virgin male rats (120 days old, 300–350 g) were kept in cages (5 per cage) with 50 g of wood shavings for 48 h, according to a previous reported protocol (Moncho-Bogani et al. 2002). These males were housed in a room with other adult males and females. To ensure a homogeneous composition throughout the experiment, MSB from 8 cages were thoroughly mixed and utilized just before the female exposure. Control female rats were exposed to saline-soiled bedding as previous reported protocol (Tomioka et al. 2005).

We used sexually naive female rats in the passage from proestrus to estrus phase of the reproductive cycle for experimental exposure to MSB. Vaginal smear cytology analyses are used for the determination of the estrous cycle phases (Marcondes et al. 2002). Thirty-six rats were utilized for biochemical analysis ($n = 6$ for 4 treated groups and $n = 3$ for respective control groups). In an isolated room, female rats were exposed (5 or 4 per cage, in a random distribution) for 90 min to soiled bedding previously collected. All treated female rats were exposed to the same mixed MSB. After the exposure, females were housed in clean cages with naive-soiled bedding. Control- and MSB-exposed females were housed in different rooms under equivalent conditions.

For biochemical assays, female rats were killed by decapitation at 30, 90, 180, and 240 min after the end of the 90-min period of exposure to either MSB or saline-soiled bedding. Rats exposed to the MSB were considered the treated group (T30, T90, T180, and T240), whereas respective animals exposed to the saline-soiled bedding were considered control groups (C30, C90, C180, and C240). After exposure, the animals were sacrificed, and the ovaries (groups OT30, OT90, OT180, and O240) and uterus (groups UT30, UT90, UT180, and UT240), as well as the respective control groups were isolated. Sample aliquots were immediately stored at –80 °C to further analysis.

Antioxidant enzyme activities quantification

SOD (E.C. 1.15.1.1) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline autooxidation in a spectrophotometer at 480 nm, as previously described (Misra and Fridovich 1972). Results are expressed as Units SOD/mg protein. CAT (E.C. 1.11.1.6) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm (Aebi 1984). CAT activity is expressed as Units CAT/mg protein. GPx (E.C. 1.11.1.9) activity was determined by measuring the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation in a spectrophotometer at 340 nm, as previously described (Wendel 1981). The rate of glutathione oxidized by t-butyl hydroperoxide was evaluated by the decrease of NADPH in the presence of Ethylenediaminetetraacetic acid, excess reduced glutathione, and glutathione reductase. GPx1 (the main isozyme analyzed) activity was expressed as Units (nmol NADPH oxidized/min)/mg protein.

Nonenzymatic antioxidant defenses measurement

The nonenzymatic antioxidant potential of the reproductive tract structures was estimated by the total radical-trapping antioxidant parameter (TRAP) (Wayner et al. 1985). The reaction is initiated by adding luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, 4 mM)—as an external probe to monitoring radical production—and 2,2′-azobis–2-methyl-propionamidine–dihydrochloride (10 mM)—a free radical

source that produces peroxyl radical at a constant rate—in glycine buffer (0.1 M) pH 8.6 at room temperature, resulting in a steady luminescence emission (system counts). Sample addition decreases the luminescence proportionally to its antioxidant potential. Chemiluminescence was read in a liquid scintillation counter (Wallace 1409) as counts per minutes. Sample addition decreases the luminescence proportionately to its antioxidant potential. The luminescence emission was followed for 40 min after the addition of the sample (100 μg of protein) in a TRAP protocol, and the area under the curve was quantified.

Oxidative damage parameters

As an index of lipid peroxidation, we used the formation of thiobarbituric acid–reactive species (TBARS) during an acid-heating reaction, which is widely adopted as a method for measurement of lipid redox state, as previously described (Draper and Hadley 1990). Briefly, the samples were mixed with 0.6 ml of 10% trichloroacetic acid (TCA) and 0.5 ml of 0.67% TBA (4,6-dihydroxypyrimidine-2-thiol) and then heated in a boiling water bath for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm. Results were expressed as nmol malondialdehyde equivalents/mg protein.

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine (DNPH), as previously described (Levine et al. 1990). Briefly, proteins were precipitated by the addition of 20% TCA and redissolved in DNPH and the absorbance read in a spectrophotometer at 370 nm. Results were expressed as nmol carbonyl/mg protein. Antioxidant enzyme activities, TRAP, and oxidative damage parameters were normalized by the protein content using bovine albumin as standard (Lowry et al. 1951).

Statistical analysis

Biochemical results are expressed as means ± standard error of the mean. All analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc, Chicago, IL—SPSS version 15.0) software and GraphPad Prism (GraphPad Software Inc, San Diego, CA—version 4.02) software. Biochemical data were analyzed using the 2-way analysis of variance (ANOVA) with repeated-measures, with "time after the end of exposure," and "exposure to MSB or saline control" as factors. One-way ANOVA with post hoc comparisons was used to pick up detailed differences. Once significant main effects were seen, specific differences between groups were analyzed using post hoc Duncan's test. Significance level was set at $P \leq 0.05$.

Results

Antioxidant enzyme activities

Ovarian antioxidant enzyme activities presented a time-dependent modulation (Figure 1A–C), which was not observed in uterus (Figure 1D–F) after MSB exposure. At 30 min after MSB exposure, ovarian GPx activity was increased 2 fold (according to 2-way ANOVA the exposure to MSB affect the result, $F_{1,35} = 4.17$, $P = 0.0292$; and 1-way ANOVA

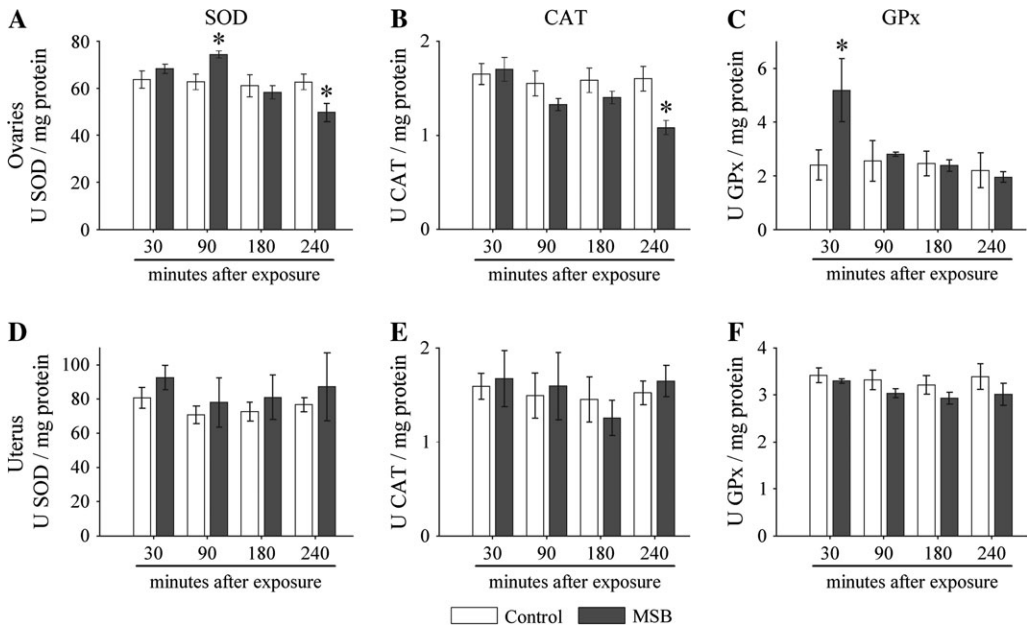

Figure 1 Antioxidant enzyme activities. Effects of MSB exposure on SOD, CAT, and GPx activity in the ovaries (**A, B,** and **C**), and uterus (**D, E,** and **F**), 30, 90, 180 and 240 min after MSB or saline-soiled bedding exposure. Data are mean ± standard error of the mean (n = 3–6 per group). *$P \leq 0.05$ when compared with respective control group.

with post hoc comparisons showed detailed differences, ovaries treated [OT]30 = 5.19 ± 1.18, OC30 = 2.41 ± 0.55, P = 0.036). Ovarian SOD activity was increased 90 min after MSB exposure ($F_{1,35}$ = 16.97, P = 0.0041; OT90 = 74.3 ± 1.5; ovaries control [OC]90 = 62.6 ± 3.24; P = 0.038) and decreased 240 min after MSB exposure (OT240 = 49.6 ± 3.85; OC240 = 61.6 ± 3.05; P = 0.029). Ovarian CAT activity was also found to be decreased 240 min after MSB exposure ($F_{1,35}$ = 8.35, P = 0.0103; OT240 = 1.08 ± 0.07; OC240 = 1.60 ± 0.13; P = 0.023).

Total radical-trapping antioxidant parameter

Uterus TRAP was found increased 30 min after MSB exposure ($F_{1,35}$ = 5.48, P = 0.0227; uterus treated [UT]30 = 11.5 ± 6.2; uterus control [UC]30 = 29.7 ± 5.4; P = 0.019) (Figure 2C and D). Ovarian TRAP did not shown differences between control and MSB exposure (Figure 2A and B).

Oxidative damage parameters

In ovarian tissue, we observed that MSB exposure induced an increase in lipid peroxidation detectable at 30 min after the end of the exposure ($F_{1,35}$ = 7.76, P = 0.0095; OT30 = 0.38 ± 0.04; OC30 = 0.25 ± 0.01; P = 0.019) (Figure 3A), with no differences in protein carbonylation among groups (Figure 3B). Nonetheless, uterus lipid peroxidation was decreased at either 30 ($F_{1,35}$ = 13.24, P = 0.0012; UT30 = 0.37 ± 0.05; UC30 = 0.62 ± 0.07; P = 0.011) and 90 (UT90 = 0.32 ± 0.05; UC90 = 0.57 ± 0.06; P = 0.008) min after MSB exposure (Figure 3C), as well as protein oxidative damage ($F_{1,35}$ = 14.68, P = 0.0019; UT30 = 2.86 ± 0.54; UC30 = 5.98 ± 0.96; P = 0.009; UT90 = 3.23 ± 0.30; UC90 = 5.72 ± 0.91; P = 0.01) (Figure 3D).

Discussion

In the present work, we suggest that chemical cues secreted by males are able to influence the capacity of fertilization in females preexposed to MSB by stimulating a favorable redox environment for the sperm, preparing/modulating in a time-dependent way the female reproductive tissues for fertilization. ROS are products of normal cellular metabolism well recognized for playing a dual role as both deleterious and essential species because they can be either harmful or beneficial to living systems (Halliwell 1999; Valko et al. 2006). Beneficial effects of ROS occur at low/moderate levels and involve physiological roles in cellular responses, especially cell signaling and signal transduction (Valko et al. 2007). One further beneficial example of ROS at low/moderate levels is the induction of mitogenic response in untransformed cells (Chiarugi and Fiaschi 2007). Our results suggest that an increase in the nonenzymatic antioxidant potential was accompanied by a diminished free radical oxidative damage in uterine tissue, 30 and 90 min after the end of exposure. Furthermore, in ovaries we observed an increase in lipid oxidative damage and in GPx activity 30 min after exposure

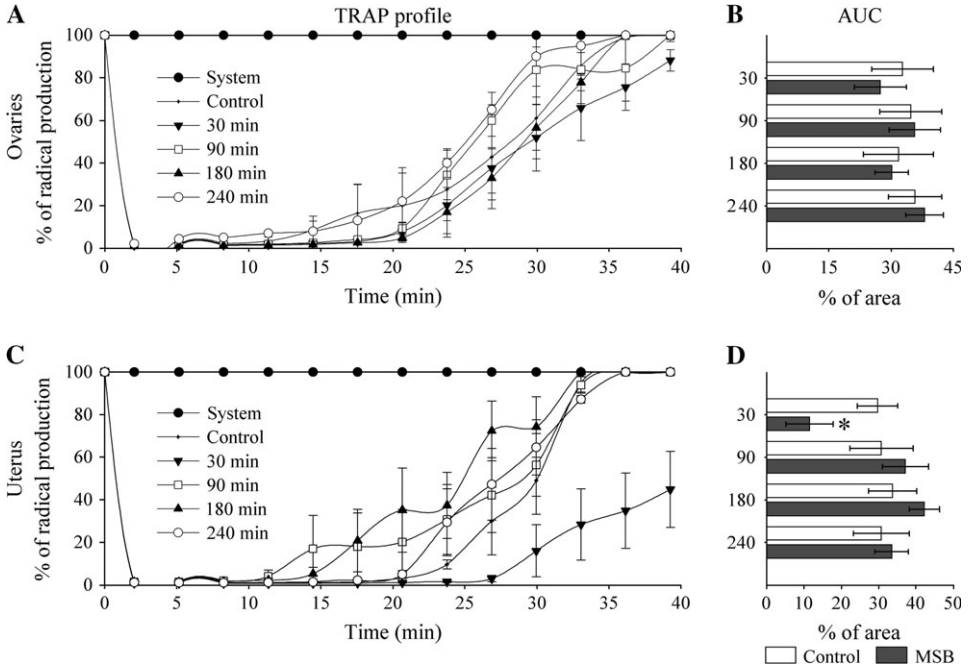

Figure 2 Total antioxidant potential (TRAP). TRAP profile and area under the curve representative of the ovaries (**A** and **B**) and uterus (**C** and **D**). Values obtained after 30, 90, 180, and 240 min after MSB or saline-soiled bedding exposure. TRAP area under curve was represented by percentage of area, and the system area was considered 100% of area. Data are mean ± standard error of the mean (n = 3–6 per group). *$P \leq 0.05$ when compared with respective control group.

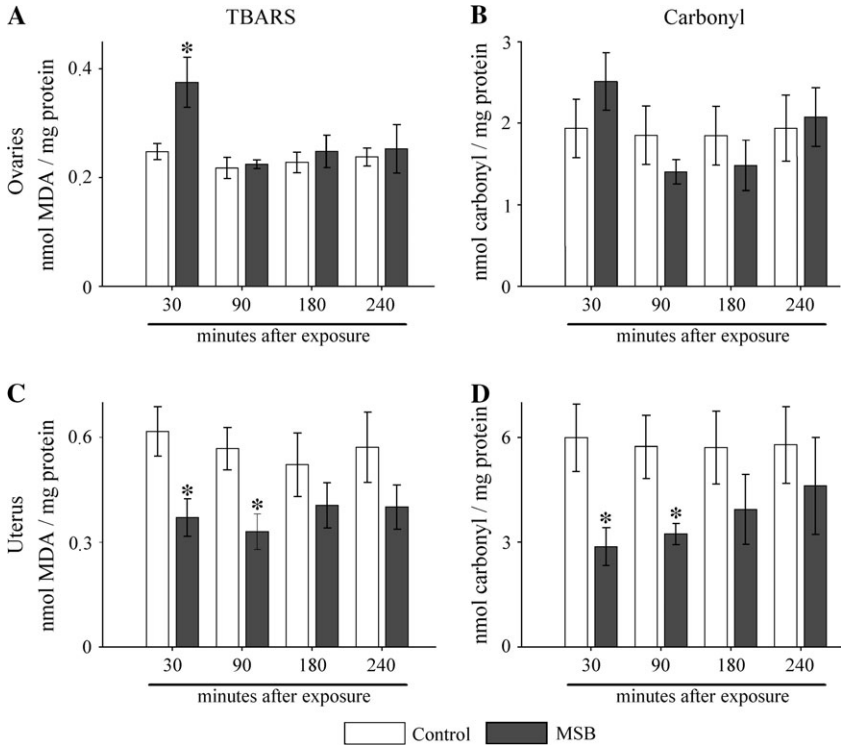

Figure 3 Oxidative damage parameters. Effects of MSB exposure on lipid peroxidation and protein carbonylation content in the ovaries (**A** and **B**) and uterus (**C** and **D**), 30, 90, 180, and 240 min after exposure to MSB or saline-soiled bedding. Data are mean ± standard error of the mean (n = 3–6 per group). *$P \leq 0.05$ when compared with respective control group.

and an increase in SOD activity 90 min after exposure. This profile was changed in ovarian tissue 240 min after exposure, showing a reduction on enzymatic defenses, with no detected oxidative damage observed. The very fast changes in GPx activity observed in ovaries suggest an increased ROS formation, principally H_2O_2. Furthermore, these findings support a predicted enhanced production in free radical species 30 min after MSB exposure, suggesting a rapid and higher pulse of H_2O_2 or other hydroperoxides in ovarian tissue. Moreover, the role of ROS as signaling agents is well understood; however, studies of ROS functions in the reproductive tissue are very recent. H_2O_2 and other hydroperoxides are not only toxic but also have signaling functions (Brigelius-Flohé 2006). Altering the redox status may impact signaling pathways, transcription factors, and epigenetic mechanisms and alter oocyte and embryo quality (Agarwal, et al. 2008). Meanwhile, oxygen radical–mediated signaling pathways play important roles in the process of reproductive physiology such as decidualization and menstruation in the human endometrium (Sugino 2007). Relatively low concentrations of H_2O_2 are beneficial for sperm capacitation but at high concentrations inhibit this process (Rivlin et al. 2004). The metabolism of oocytes and embryos is tightly regulated and importantly determined by their redox potentials (Agarwal et al. 2008). The methodology described here to analyze the GPx activity is based on the ability of the GPx to react with hydroperoxides. Indeed, all isoforms of GPx react with hydroperoxides (like t-butil hydroperoxide); however, only the GPx4 (or phospholipid hydroperoxide glutathione peroxidase) isoform react with cholesterol hydroperoxides and other phospholipids hydroperoxides. Furthermore, GPx4 was recently associated with redox-regulated transcription factors, like nuclear factor kappa B (NF-κB) and nuclear factor erythroid 2-related factor 2/Keap1 systems (Brigelius-Flohé 2006). On other hand, the methodology used here to analyze reproductive tissue GPx activity was previously described in other works that analyze ovarian and uterine samples (Sanchez et al. 2006; Al-Gubory et al. 2008). However, the main isozyme analyzed here is the cytosolic GPx1. Other possibility to the observed pattern is a non–substrate-dependent increase in antioxidant enzymes through the activity of signaling factors that promote antioxidant response elements activation (Li et al. 2007). However, this is only a speculative issue. Nonetheless, a rather interesting biological effect was observed in this work, and certainly future studies will address this issue by investigating the expression profile of the enzymes involved in this effect.

Chemical signals elicit a short-latency behavioral response in the receiver, usually involving an interaction between 2 individuals, like in aggressive attacks or mating. These signals induce delayed responses that are commonly mediated through the neuroendocrine system. In this way, it is possible

for an animal to modulate the reproductive status of another (Bigiani et al. 2005). In the present study, we observed that MSB exposure modulates antioxidant profile in receptive female rats. It is very likely that the modifications in the oxidative profile of the female genital tract induced by MSB exposure may have important implications in the process of fertilization. It is well recognized that sperm cells are very susceptible to oxidative stress, and for this reason the sperm and seminal fluid are equipped with a well-developed antioxidant apparatus, including enzymes (SOD, CAT, GPx) and nonenzymatic antioxidants (glutathione, α-tocopherol, ascorbate). Exogenous antioxidants are generally suggested to improve the fertilization capacity of frozen semen (Sikka 2004; Michael et al. 2007). Females live longer than males, and this may be due to the upregulation of longevity-associated genes by estrogens that binds to the estrogen receptors and subsequently activate the mitogen-activated protein kinase and NF-κB signaling pathways, resulting in an upregulation of antioxidant enzymes (Viña et al. 2006). In this way, the enhancement in nonenzymatic antioxidant defenses may be related to the possible increase in the estradiol-related signaling activity (Mhyre and Dorsa 2006; Pozzi et al. 2006). The crosstalk between estradiol and antioxidant pathways is well described (Viña et al. 2008). In ovaries, ROS have been implicated in several physiologic processes related to reproduction, including folliculogenesis and steroidogenesis (Shiotani et al. 1991; Behrman et al. 2001; Sugino et al. 2004).

Several works have been focused in the effects of ROS in the physiology of both male and female reproductive systems (Aitken and Baker 2004; Agarwal et al. 2005; Fujii et al. 2005; Agarwal et al. 2008), but, to our knowledge, the present paper is the first work demonstrating that the oxidative profile of female reproductive system is altered by chemical cues present in the MSB, thus suggesting that pheromonal communication is able to modulate ROS production and/or cleaning in the female reproductive system prior to fertilization. Recently, we demonstrated decreased anxiety-like behavior and locomotory/exploratory activity and modulation in hypothalamus, hippocampus, and frontal cortex redox profile in sexually receptive female rats after short-term exposure to MSB (Behr et al. in press). Here, our results point that MSB induces a decrease in the oxidative damage and increases the nonenzymatic antioxidant potential in uterus; thus, it is reasonable to suggest that chemical cues secreted by males are able to influence the capacity of fertilization in females preexposed to MSB by stimulating a favorable redox environment for the sperm. We will address this issue in future studies, by analyzing the rate of mating and fertilization in females previously exposed to MSB.

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