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# Sex-specific differences on caffeine consumption and chronic stress-induced anxiety-like behavior and DNA breaks in the hippocampus

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ABSTRACT

Caffeine is widely consumed in beverages and food, and its consumption in high doses is associated with anxiety increase. Stress situations are often associated to coffee consumption, and have a strong influence on oxidative DNA damage. As there are sex-specific differences in many metabolic, neurochemical and behavioral aspects, the aim of this study is to verify the interaction between chronic consumption of caffeine and chronic stress on anxiety and DNA breaks in the hippocampus on male and female rats. Wistar rats were submitted to restraint stress for at least 50 days. The diet consisted of standard rat chow and caffeine 0.3 or 1 g/L in drinking water "ad libitum" as the only drinking source. Controls received tap water. Anxiety-like behavior and DNA breaks in the hippocampus were evaluated. Caffeine consumption and chronic stress increased anxiety-like behavior as well as DNA breaks in the hippocampus of male rats. No effect on these parameters was observed in females. These results may be related to the presence of estradiol, which may have anxiolytic and neuroprotective properties.



## 1. Introduction

Caffeine is an ingredient widely used in beverages and foods, including coffee, tea, many soft drinks, and chocolate (MacKenzie et al., 2007). Coffee is the main source of caffeine and its ingestion varies over the day (Brice and Smith, 2002). The primary action of caffeine in moderate doses is believed to be the blockage of adenosine receptors (A1 and A2A), which leads to very important secondary effects on many classes of neurotransmitters like glutamate, acetylcholine and dopamine (Sichardt and Nieber, 2007). However, in high concentrations (at mM range) it may inhibit phosphodiesterase, mobilizes intracellular calcium (Fredholm et al., 1999) and also presents antioxidant activity (Lee, 2000; Devasagayam et al., 1996). This, in turn, it will influence a large number of different physiological functions (Fredholm et al., 1999). In humans the administration of high doses of caffeine leads to an increase in the anxiety levels (Clementz and Dailey, 1988). Similarly, an acute administration of high doses of caffeine promotes anxious behaviour in different animal models, such as the social interaction test (Baldwin and File, 1989) or the elevated plus maze test (El Yacoubi et al., 2000). It has also been proposed that differences in the activity of the adenosinergic system are accompanied by differences in the anxiety level (Johansson et al., 2001; Ledent et al., 1997). Besides, caffeine and its catabolic products theobromine and xanthine exhibit both antioxidant (Gómez-Ruiz et al., 2007; Azam et al., 2003) and prooxidant properties, showing oxidative DNA breakage "in vitro" (Azam et al., 2003). However, little is known about the effect of chronic caffeine consumption on DNA damage in the central nervous system.

The stress response leads to behavioral and metabolic changes in an effort to maintain body homeostasis and increase survival chances (Chrousos and Gold, 1992; Tsigos and Chrousos, 2002). Exposure to stress induces the release of glucocorticoids (Tsigos and Chrousos, 2002), and repeated exposure to high levels of glucocorticoids produces neuronal changes in several brain regions, including the hippocampus (Sapolsky et al., 1984, 1985). On the other hand, there is an increase in adenosine extracellular concentration during stress (Scaccianoce et al., 1989) and the balance between the density of adenosine A1 and A2A receptors in the rat hippocampus is modified upon repeated immobilization stress, which leads to a down-

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regulation of A1 receptors together with an up-regulation of the A2A receptors (Cunha et al., 2006). Concerning emotional behavior, there is some evidence suggesting that repeated restraint stress produces changes in emotionality related to increased anxiety (Beck and Luine, 2002).

Several studies have examined the effect of stress on DNA integrity, as stress has been found to cause production of reactive oxygen species (ROS) resulting in oxidative stress and increased lipid peroxidation; oxidative stress-induced DNA alterations may affect replication and transcription (Perchellet and Perchellet, 1989). Besides, psychological stress has been shown to impair the repair of DNA damage induced by exposure to a carcinogen in rats (Irie et al., 2000). Additionally, it has been suggested that, in humans, work-associated stress, other than physical and environmental working conditions, has a strong influence on the oxidative DNA damage, which appears to be sex-specific (particularly in female workers) (Irie et al., 2001).

Most studies evaluating the effects of the stress were conducted in males, but the response and adaptation to stress and chronic caffeine consumption can be different on females. There are sex-specific differences in many metabolic aspects as well as in behavior and a significant body of evidence implicates interactions between stress and sex on brain adaptation, besides behavior and endocrine responses (Bujas et al., 1997; Kant et al., 1983; Troisi, 2001).

The first aim of the present study is to verify the interaction between chronic consumption of caffeine and chronic stress on anxiety-like behavior. The hypothesis is that chronic consumption of caffeine in high doses, associated with chronic stress would increase anxiety-like behavior. Since caffeine may alter a post-replication repair process in mammalian cells (Van Den Berg and Roberts, 1976), and as exposure to stress can increase oxidative stress (Perchellet and Perchellet, 1989; Fontella et al., 2005), the second aim of this study is to verify the effect of stress and caffeine on DNA breaks in the hippocampus, which is a brain structure strongly vulnerable to stress. Additionally, since it was observed that caffeine induced effects on food consumption are differently affected by stress depending on the sex of the animal (Pettenuzzo et al., 2008), the third aim of this study is to verify possible sex-differences in the effects of these chronic treatments on anxiety-like behavior and on DNA breaks.

## 2. Material and methods

### 2.1. Animals

For the present study, 58 adult males and 48 adult females Wistar rats from our breeding stock (60 days of age at the beginning of the treatment), weighing 250–300 g (males) and 150–200 g (females) were used. Animals were separated according to sex on postnatal day 21. Experiments using males and females were performed separately, at different times. Around postnatal day 60, animals from different litters were randomized, to avoid the litter effect, and maintained in groups from 3–5 animals per cage. One week later, cages were divided between six groups for males and six groups for females. Males and females were kept separated throughout all the experiments. The animals received or not caffeine (0.3 g/L and 1.0 g/L) in drinking water, and were subjected or not to repeated restraint stress during at least 50 days. The final groups were: control (non-stressed receiving water), caffeine 0.3 g/L (non-stressed receiving caffeine 0.3 g/L), caffeine 1.0 g/L (non-stressed receiving caffeine 1.0 g/L), stressed (stressed receiving water), stressed + caffeine 0.3 g/L (stressed receiving caffeine 0.3 g/L) and stressed + caffeine 1.0 g/L (stressed receiving caffeine 1.0 g/L), resulting in 12 groups, considering males and females. Cages were made of Plexiglas material (65 × 25 × 15 cm) with the floor covered with sawdust. They were kept under standard dark–light cycle (lights on between 7:00 and 19:00 h), at a room temperature of 22 ± 2 °C. The rats had free access to food (standard rat chow) and water (or caffeine solution, see below), except for the stressed group, during the periods when restraint stress was applied. Both, stress and caffeine treatment, were kept until the end of the experiments. All animal treatments were in accordance to the institutional guidelines and to the recommendations of the International Council for Laboratory Animal Science (ICLAS), and to the Federation of Brazilian Society for Experimental Biology. All efforts were done to minimize animal suffering as well as to reduce the number of animals.

### 2.2. Caffeine administration

Caffeine (Vetec, Rio de Janeiro, Brazil) at doses of 0.3 and 1.0 g/L was administered in drinking water as the only source of water during all the period of the experiment (Gasior et al., 2000). During the behavioral testing, the animals continued receiving caffeine treatment. Control animals received tap water. The volume of water and the caffeine solution consumed was measured every 48 h. Studies from the literature using similar doses of caffeine in the drinking water found caffeine levels in the blood which were directly dependent of the dose consumed, since after chronic caffeine administration in concentrations of 0.25 g/L and 1.0 g/L resulted in plasma caffeine concentrations ranging from 1.45 to 5.95 μg/mL, respectively (Gasior et al., 2000).

### 2.3. Chronic restraint stress procedure

The animals were stressed 1 h/day, five days per week, for 50 days, when behavioral tests began. Restraint stress began at the same day caffeine treatment was initiated, and was carried out by placing the animal into a 25 × 7 cm plastic tube, and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. The control group was not submitted to stress and animals were kept in their home cages. After this period, anxiety-like behavior was evaluated and restraint stress continued to be carried out. Restraint stress began at least 1 h after behavioral procedures. Previous studies using this stress model have shown that, although chronically-stressed animals exhibit a habituated corticosterone response, they still release corticosterone after a restraint section (although the levels are lower than those reached after the first exposure to restraint; Torres et al., 2001)].

### 2.4. Elevated plus maze test

The elevated plus maze test was conducted after 50 days of treatment, using a standard plus maze apparatus kept 80 cm above the floor, consisting of four arms arranged in the shape of a cross (arms measured 45 × 10 cm). The four arms were joined at the center by a 10 cm square platform. Two of the arms, opposite to each other, had no walls (open arms); the two other arms (closed arms) had 23-cm high walls. This test is considered sensitive to the anxiety state of the animal, based on the principle that exposure to an elevated and open arm maze leads to an approach conflict that is stronger than that evoked by exposure to an enclosed arm maze (Pellow and File, 1986). On the day of the experiment, the animals were acclimatized to the behavioral testing room for 5 min prior to the initiation of the test. Animals were placed individually on the center of the maze, on the junction between the open and the closed arms, facing one of the open arms, and performance was scored during 5 min. A rat was considered to have entered one arm of the maze when all four feet were within the arm. Conventional parameters of anxiety-like behavior were monitored, i.e., the number of entries into the closed arms, entries into the open arms, total entries, and the total time spent in each arm. The ratio “time spent in the open arms/time spent in all (i.e., open and closed) arms” was calculated and multiplied by 100, to yield the percentages of time spent in open arms. This parameter is considered

to reflect fear-induced inhibition from entering the open arms and can be related to the "anxiety" level experienced by the animals. Another anxiety index was obtained by dividing the number of entries into the open arms by the number of entries into the open plus closed arms and multiplying by 100.

### 2.5. Exposure to the open field

Ten days after exposure to the plus maze, the animals were exposed to an open field. A 50 cm high, 40×60 cm open field made of brown plywood with a frontal glass wall was used (Mello e Souza et al., 2000). The floor was subdivided with white lines into 12 equal 13.3 by 15.0 cm rectangles. Measurements were taken in a brightly lit room, set up so that uniform light was applied on the floor of the open field. The animals were gently placed facing the left corner and allowed to explore the arena for 3 min. The line crossings (ambulation) were counted. The open field was washed with 5% ethanol before a new animal was introduced. Twenty-four hours later, the animals were again exposed to the same apparatus. Crossings and time spent in the central part of the open field were evaluated.

### 2.6. Blood collection and adrenal dissection

Caffeine and chow were maintained until the moment of the sacrifice. Eighteen days after the behavioral procedures, animals were sacrificed by decapitation between 12:00 and 14:00 h, at least 24 h after the last restraint exposure, and the trunk blood was collected into heparinized tubes, centrifuged at 4 °C at 1000 ×*g*, and plasma separated and stored at −70 °C for corticosterone determination. All animals were killed within this interval of time in a random order considering stressed and non-stressed animals. Adrenal glands were carefully dissected and weighed using a scale with a precision of 0.0001 g.

### 2.7. Single cell gel electrophoresis — comet assay

A standard protocol for comet assay preparation and analysis was adopted (Tice et al., 2000). After the sacrifice of the animals by decapitation, the hippocampus was immediately dissected out and gently homogenized in the phosphate-buffered saline solution (PBS) pH 7.4. The slides were prepared by mixing 20 µl of hippocampus homogenate (in cold PBS), with 80 µl low melting point agarose (0.75%). The mixture (cells-agarose) was added to a microscope slide coated with a layer of 500 µl of normal melting agarose (1%). After solidification, the cover slip was gently removed and the slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.5, with freshly added 1% Triton X-100 and 10% DMSO) for one day. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 5 min. The DNA was electrophoresed during 20 min at 25 V (0.90 V/cm) and 300 mA. Afterwards, the slides were neutralized with Tris buffer (0.4 M; pH 7.5). Finally, the DNA was stained with ethidium bromide. After electrophoresis, neutralized and stained nuclei were blindly analyzed by fluorescence microscopy with visual inspection (200×). Cells were scored from zero (no breaks observed) to 4 (maximal breaks index), according to the tail intensity (size and shape), resulting in a single DNA breaks score for each cell, and, consequently, for each group. Therefore, a group index could range from zero (all cells no tail, 100 cells×0) to 400 (all cells with maximally long tails, 100 cells×4) (Collins et al., 1997) (Fig. 1). The DNA breaks index was calculated by multiplying the number of cells by its respective index score and than summing up.

### 2.8. Corticosterone determination

For corticosterone determination, plasma was extracted with ethyl acetate, the extract evaporated and the residue suspended for the hormone evaluation with an ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA).

### 2.9. Statistical analysis

Data were expressed as means ± standard error of the mean, and were analyzed using two-way ANOVA (factors were stress and caffeine) followed by the Duncan multiple range test, when indicated. Significance level was accepted as different when the $P$ value was equal or less than 0.05.

## 3. Results

### 3.1. Caffeine consumption

There were no statistical differences between the control and the stressed groups on drinking volumes consumed, therefore the mean caffeine consumption did not differ between the stressed and the control animals, neither in the males nor in the females. Rats of both sexes receiving caffeine 0.3 g/L consumed about 40 mg/kg/24 h and rats receiving 1.0 g/L consumed about 108 mg/kg/24 h. Fluid consumption was around 37 ml/day for males and 27.5 ml/day for females.

### 3.2. Adrenal weight and corticosterone measurements

A two-way ANOVA revealed no differences between the groups regarding adrenal weight (data not shown; $P>0.05$). However, on corticosterone measurements, female animals receiving caffeine 1.0 g/L showed a decrease in plasma corticosterone levels [caffeine effect, $F(2,24)=3.75$, $P<0.05$]. No differences were observed in male animals on this measurement (Fig. 2).

### 3.3. Effect of chronic consumption of caffeine and chronic stress on behavior in the open field and in the elevated plus maze test

No significant difference was observed between the groups in the number of crossings during exposure to the open field, neither in male

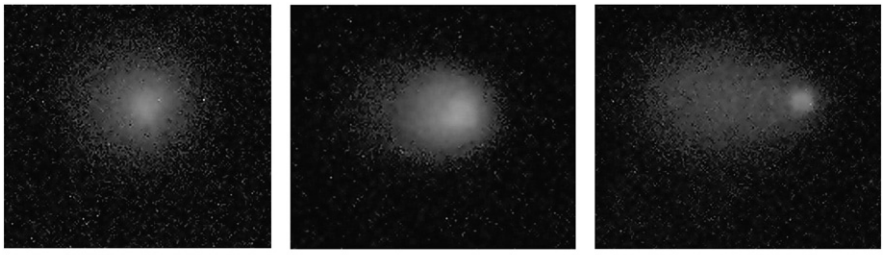


**Fig. 1.** Some typical examples of Comet assay. Evaluation of DNA breaks using ethidium bromide (200×). The cells are assessed visually and received scores from 0 (no breaks observed) to 4 (maximal breaks index), according to the size and shape of the tail. Scores were obtained using the mean score of two independent blind evaluators.

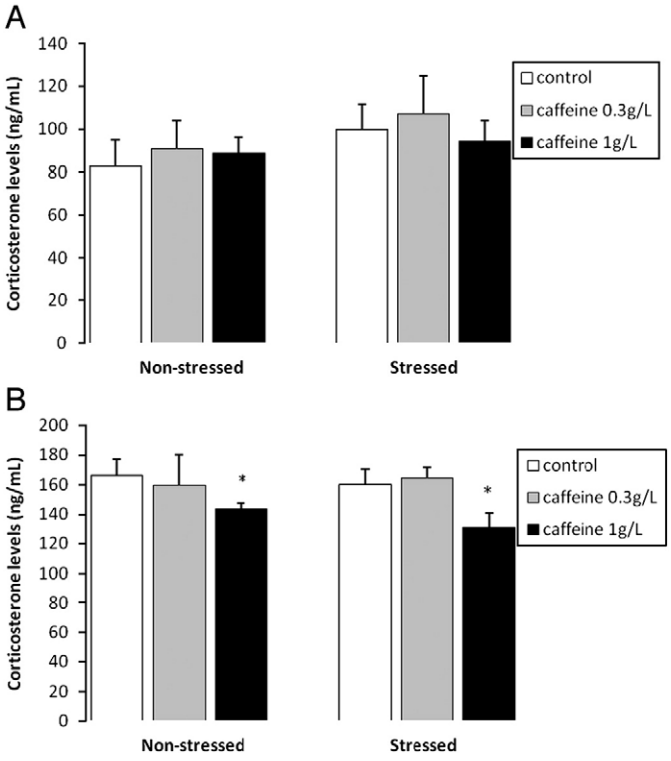


**Fig. 2.** Corticosterone measurement in stressed and non-stressed animals. Data is expressed as mean ± S.E.M. A. Males, $N = 6–7$/group. Two-way ANOVA showed no effect. B. Females, $N = 4–6$/group. Two-way ANOVA showed an effect of the caffeine group ($P<0.05$). *Significantly different compared to caffeine 0.3 g/L and control, stressed or not. (Duncan multiple range test, $P<0.05$).

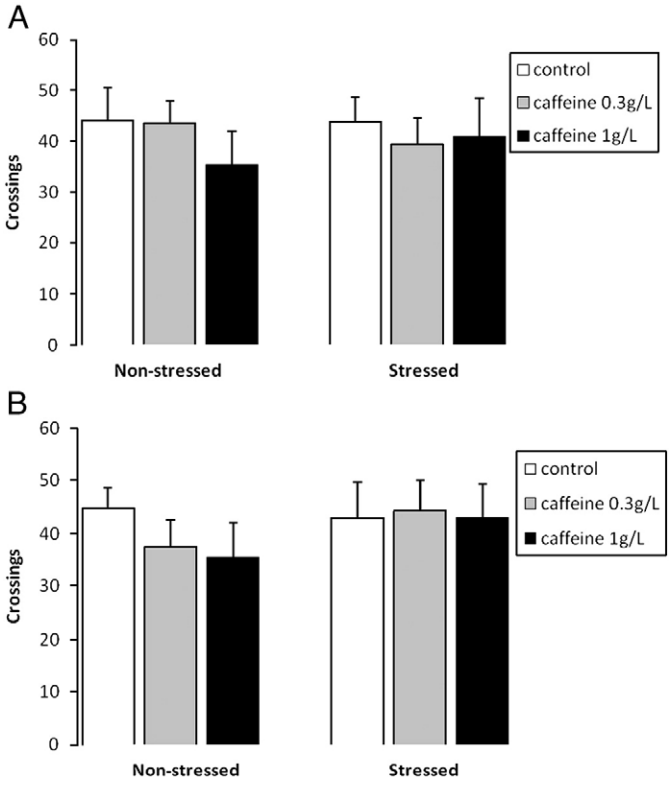


**Fig. 3.** Number of crossings in the open field in the stressed and the non-stressed animals. Data is expressed as mean ± S.E.M. A. Males, $N = 7–13$/group. Two-way ANOVA showed no effect. B. Females, $N = 8$/group. Two-way ANOVA showed no effect.

nor in female animals (Fig. 3). In male animals caffeine induced a reduction on time spent in the central part of the open field [$F(2,58) = 5.466$, $P<0.01$], while no effect of stress [$F(1,58) = 0.044$, $P>0.05$] or interaction [$F(2,58) = 0.323$, $P>0.05$] were observed. On the other hand, no differences in the time spent in the central area were observed in females (Fig. 4).

The results concerning the behavior of the rats in the elevated plus maze are shown in Table 1. For male rats, both chronic stress [$F(1,58) = 7.406$, $P<0.01$] and caffeine treatment [$F(2,58) = 3.141$, $P = 0.05$] caused a decrease in the number of entries in open arms. No difference in the number of entries in closed arms, as well as in the number of total entries, was found. Both chronic stress [$F(1,58) = 4.099$, $P<0.05$] and caffeine treatment [$F(2,58) = 4.813$, $P<0.05$] also induced a decrease in the percentage of time spent in open arms. Accordingly, there was an increase in the time spent in the closed arms [$F(1,58) = 4.086$, $P<0.05$ for the stress effect and $F(2,58) = 4.07$, $P<0.05$ for the caffeine effect]. No interactions were observed between stress and caffeine treatment in these parameters ($P>0.05$). No significant difference was observed in the performance of the female rats in any of these parameters (Table 1).

### 3.4. DNA breaks in the hippocampus

The results concerning DNA breaks index in the hippocampus are displayed in Fig. 5. Both chronic stress [$F(1,27) = 20.818$, $P<0.001$] and caffeine treatment [$F(2,27) = 12.066$, $P<0.001$] induced an

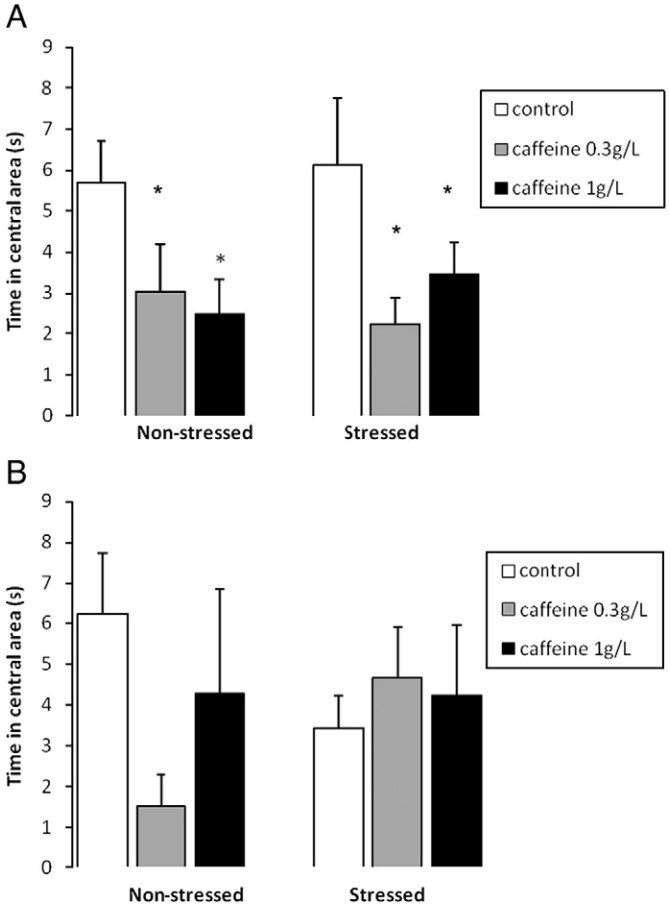


**Fig. 4.** Time spent in the central area of the open field in the stressed and the non-stressed animals. Data is expressed as mean ± S.E.M. A. Males, $N = 7–13$/group. Two-way ANOVA showed an effect of caffeine in this parameter ($P<0.01$). *Animals treated with both doses of caffeine were different from animals not receiving caffeine (Duncan multiple range test, $P<0.05$). B. Females, $N = 8$/group. Two-way ANOVA showed no effect.

**Table 1**
Effects of chronic consumption of caffeine and chronic stress on behavioral parameters measured in the elevated plus maze test.

| Group | Non-stressed | | | Stressed | | |
|---|---|---|---|---|---|---|
| | Control | Caffeine 0.3 g/L | Caffeine 1 g/L | Control | Caffeine 0.3 g/L | Caffeine 1 g/L |
| *Males* | | | | | | |
| Time in open arms[a] | 145.8 ± 16.5 | 96.1 ± 15.5 | 97.6 ± 19.9[b] | 111.1 ± 15.8 | 88.5 ± 20.1 | 52.0 ± 15.5[b] |
| Time in closed arms[a] | 104.9 ± 13.8 | 142.6 ± 10.3 | 143.9 ± 18.2[b] | 134.2 ± 15.6 | 147.9 ± 14.1 | 185.6 ± 21.6[b] |
| % time in open arms[a] | 57.5 ± 5.9 | 38.7 ± 5.4[b] | 39.6 ± 7.3[b] | 45.2 ± 6.1 | 34.7 ± 7.4[b] | 22.4 ± 6.8[b] |
| Entries in open arms[a] | 8.0 ± 0.8 | 7.8 ± 1.3 | 5.5 ± 0.7[b] | 6.4 ± 1.0 | 4.6 ± 0.7 | 3.6 ± 0.9[b] |
| Entries in closed arms | 7.6 ± 0.7 | 8.1 ± 0.8 | 8.3 ± 1.7 | 7.7 ± 0.9 | 7.5 ± 0.8 | 9.3 ± 1.6 |
| Total entries | 15.6 ± 0.9 | 15.9 ± 1.6 | 13.8 ± 1.4 | 14.1 ± 1.6 | 12.2 ± 1.1 | 12.9 ± 2.1 |
| % Open entries[a] | 51.0 ± 3.8 | 46.5 ± 4.6 | 42.9 ± 6.3 | 42.8 ± 4.9 | 37.6 ± 4.3 | 28.4 ± 6.0 |
| *Females* | | | | | | |
| Time in open arms | 150.9 ± 21.7 | 121.3 ± 18.4 | 119.1 ± 28.9 | 115.4 ± 18.1 | 122.8 ± 21.7 | 106.8 ± 17.4 |
| Time in closed arms | 108.9 ± 15.8 | 140.9 ± 13.0 | 139.0 ± 26.9 | 150.1 ± 18.6 | 135.6 ± 19.8 | 153.5 ± 12.5 |
| % Time in open arms | 56.9 ± 7.3 | 45.3 ± 6.6 | 45.3 ± 10.2 | 43.5 ± 6.9 | 47.1 ± 7.5 | 40.2 ± 6.0 |
| Entries in open arms | 10.9 ± 1.4 | 8.1 ± 1.3 | 7.8 ± 1.9 | 8.9 ± 1.2 | 9.0 ± 1.7 | 7.9 ± 1.3 |
| Entries in closed arms | 7.5 ± 0.7 | 6.8 ± 0.7 | 6.1 ± 1.3 | 8.0 ± 1.1 | 8.0 ± 1.4 | 8.6 ± 1.2 |
| Total entries | 18.4 ± 1.3 | 14.9 ± 2.0 | 13.9 ± 2.5 | 16.9 ± 1.5 | 17.0 ± 2.5 | 16.5 ± 0.7 |
| % Open entries | 57.6 ± 5.4 | 51.7 ± 5.1 | 49.0 ± 9.8 | 52.3 ± 5.0 | 52.5 ± 5.0 | 47.6 ± 7.4 |

Data are expressed as mean ± S.E.M. for each parameter.
Male, $N = 7–13$/group. Female, $N = 8$/group.
[a] Significant difference between the stressed and the control animals (two-way ANOVA, $P<0.05$).
[b] Different from control (Duncan, $P<0.05$).

increase on DNA breaks in male rats. Additionally, there was an interaction between caffeine and stress [$F$ (2,27) = 3.878, $P<0.05$], since the effect of caffeine on this parameter does not sum up with the stress effect. DNA breaks in the hippocampus of female rats were also evaluated. However, no significant effect was observed.

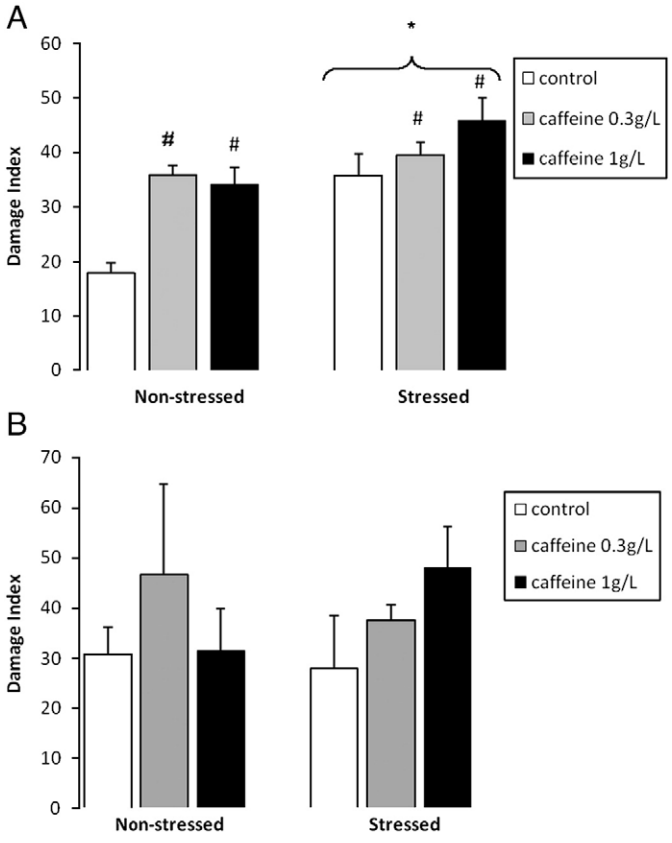


**Fig. 5.** DNA breaks in the hippocampus by Comet Assay in the stressed and the non-stressed male (A) and female (B) animals. Data is expressed as mean ± S.E.M. $N = 3–8$/group. In males, a two-way ANOVA showed effect of caffeine ($P<0.001$) and stress ($P<0.001$) groups, and there was a significant interaction between stress and caffeine treatment ($P<0.05$). No significant differences were observed in females ($P>0.05$). *Significantly different compared to the non-stressed. #Significantly different compared to the control, stressed or not (Duncan multiple range test, $P<0.05$).

## 4. Discussion

The major findings in this work are that chronic stress and caffeine treatment effects on anxiety-like behavior and on DNA breaks in the hippocampus are sex-specific. Both treatments increased anxiety-like behavior (although caffeine increased anxiety in both tasks evaluated, and chronic stress had a less notable effects on anxiety — stressed animals only showed an anxiogenic-like behavior in the plus maze task), and both treatments also lead to increased DNA breaks in the hippocampus of male rats. Furthermore, an interaction between, stress and caffeine intake, was also observed. Females, however, were more resistant to those treatments, since no effect was observed on anxiety, and only a small and non-significant increase was observed on DNA breaks in the hippocampus of females.

Basal corticosterone levels were only modified in the female animals consuming caffeine 1.0 g/L, when a decrease in the plasma corticosterone levels was found. It has been observed that high doses of caffeine produce a stress-like neuroendocrine response in rats, which is characterized by the increased serum corticosterone (Spindel, 1984); however, tolerance to the effects of caffeine on corticosterone levels is developed in seven days (Spindel et al., 1983). In the present study a chronic treatment of more than 40 days was used, therefore it is possible that the reduced corticosterone levels observed here are the results of the chronicity of the caffeine treatment. Chronic coffee consumption has been shown to decrease cortisol levels in stressed humans (Harris et al., 2007). In women, Harris et al. (2007) observed a negative correlation between coffee and cortisol levels at wake-up time and a positive correlation between coffee and cortisol levels after a working day. Lower post-stress cortisol was also observed in men after chronic black tea consumption (Steptoe et al., 2007), although caffeinated placebo was used in that study, suggesting that the effect was induced by other components of black tea. Since we measured corticosterone at least 24 h after the last restraint exposure, our result could be compared to basal levels in the Harris study (Harris et al., 2007), which suggests decreased basal cortisol levels in female coffee drinkers.

Regarding the behavioral analysis, no effect was observed on the motor activity in the open field task, showing development of tolerance against motor effects induced by caffeine intake in males

and females (Antoniou et al., 2005; Karcz-Kubicha et al., 2003). On the other hand, in the plus maze task, male rats from groups receiving caffeine (1.0 g/L and 0.3 g/L) and from stressed groups presented an increase of anxiety-like behavior. The increased anxiety induced by chronic caffeine could also be observed when evaluating time spent in the central area of the open field, another parameter of the anxiety-like behavior. In this task, however, stressed animals presented no difference.

The elevated plus maze is based on the natural aversion of rodents for open spaces (Lister, 1990; Carobrez and Bertoglio, 2005), and has been used to test drugs, as well as to study the biological basis of emotionality related to several conditions (see Carobrez and Bertoglio, 2005, for a review). We should consider, however, that these tests of anxiety (plus maze and open field) were validated pharmacologically, and may show different sensitivity. The effect of chronic stress on anxiety, as observed in the plus maze task, agrees with other reports, showing that restraint stress may induce anxiety-like behavior in the plus maze task, and that these changes are more marked in males than in females (Chakraborti et al., 2007). In that study, neurochemical alterations (lipoperoxidation) were also more evident in males than in females.

The involvement of the adenosinergic system in anxiety related behaviors has been observed (El Yacoubi et al., 2000; Kulkarni et al., 2007; Prediger et al., 2004). Other reports from the literature also agree with our results concerning caffeine and anxiety: caffeine acutely or chronically administered induced anxiety-like effects in the plus maze test (El Yacoubi et al., 2000). Since caffeine blocks both subtypes of adenosine receptors (A1/A2), and it has been shown that its anxiogenic-like effect is not shared by A2A antagonists (Cunha et al., 2008), we may suggest that the anxiogenic effect induced by chronic caffeine consumption could be due to its antagonism on the A1 receptor (Correa and Font, 2008; Prediger et al., 2004); however, adaptive alterations in the receptor levels should not be discarded (Johansson et al., 1997). Interestingly, adenosinergic antagonists have also been shown to present antidepressant activity (El Yacoubi et al., 2003; Hodgson et al., 2009).

In this study sex-specific differences were found, since female rats appear to be more resistant than males to the effects of caffeine and stress on the evaluated parameters. This observation agrees with other findings when female rats were observed to be insensitive to chronic stress in terms of facilitation and consolidation of anxiety (Mitra et al., 2005). It has been suggested that gonadal hormones, most likely estradiol (E2), may contribute to the resistance of female adult rats to chronic stress (Luine et al., 2007), since it may act as anxiolytic agents (Hill et al., 2007). Studies have shown that ovariectomy (removal of the primary source of E2, the ovaries) increases anxiety and depressive behavior, and acute, subchronic, or chronic physiological E2-replacement reduces anxiety and depressive behavior of rodents (Bernardi et al., 1989; Bowman et al., 2002), although E2's effects on anxiety of rodents may depend upon the E2 dosage and/or regimen (Walf and Frye, 2005). Different phases of the estrous cycle can influence female behavior. This cycle was not monitored in the present study, and probably females were tested in different estrous cycle phases, what would explain the higher standard deviation observed in those groups.

On male rats, caffeine consumption and chronic stress increased DNA breaks in the hippocampus. Caffeine is an alkaloid and its catabolic products, theobromine and xanthine, can reduce copper, from Cu(II) to Cu(I), leading to the generation of oxygen radicals "in vitro" (Shamsi and Hadi, 1995). The generation of oxygen radicals, increasing oxidative stress, may result in DNA breaks (Azam et al., 2003). Additionally, caffeine is, "in vitro", at concentrations between 2–50 mM, an efficient inhibitor of DNA double-strand repair (Selby and Sancar, 1990). It should be considered, however, that the study above mentioned used much higher caffeine concentrations than those in the present study. Furthermore, strand breaks in DNA arise from oxidative damage as well as from the process of DNA repair (Halliwell and Whiteman, 2004), and this increase in the index of DNA breaks, as observed here, does not mean that they are permanent. Further studies to evaluate permanent damage "in vivo" are necessary for better understanding of the chronic caffeine effect.

Concerning stress and DNA damage, it has been shown that exposure to repeated stress induces oxidative stress, especially in the hippocampus (Fontella et al., 2005). This oxidative stress indicates the production of ROS, such as peroxides, hydroxyl and superoxide anion radicals, which may induce cellular oxidative damage through DNA strand breaks and lipid peroxidation (Muqbil et al., 2006). Using restraint stress, Muqbil et al. (2006) observed no significant damage to the DNA of lymphocytes, liver and skin cells of rats, however the stressor that they used seemed to prime the cells to respond to subsequent induction of DNA damage, either by impairing the ability of cells to repair DNA, or by causing oxidative stress. In addition, it is possible that the hippocampus may be more sensitive to the DNA breaks since many endangering effects of stress are most pronounced in this structure, probably due to its high concentrations of corticosteroid receptors and sensitivity to glucocorticoids (Sapolsky, 1996). Additionally, the longer period of stress exposure in the present study, when compared to Muqbil et al. (2006), may have contributed to generate the increase in the DNA breaks.

An interaction between stress and caffeine on the DNA breaks index was detected. When these two factors were combined, the effect was not higher as it would be expected from their separate effects. This could be due to a ceiling effect, or to some inhibitory role of caffeine on the stress response; this last possibility must be considered (although the lack of effect on the plasma corticosterone measurements) since corticosterone release is just part of the complex stress response.

In females, no differences were seen between the groups on the DNA breaks index, probably due to the neuroprotective effect of estradiol. Besides, estrogens modulate the hippocampal functions by the enhancement of the different steps of neurogenesis: proliferation, migration and differentiation (De Nicola et al., 2009).

In conclusion, this study verified an increase on the anxiety-like behavior and on the DNA breaks index in male rats, induced by chronic caffeine and stress. Female rats were more resistant to the effects induced by these factors on anxiety and on the DNA breaks index; these results may be related to the presence of estradiol, which may have anxiolytic and neuroprotective properties. As far as we are concerned, this is the first study showing the effects of chronic stress and chronic consumption of caffeine (together) on the DNA breaks index in the brain of the rats. These results contribute to understand the sex-specific differences on these parameters, as well as to suggest that further studies are necessary to elucidate the mechanisms involved in these processes.

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