Modulatory effects of caffeine on oxidative stress and anxiety-like behavior in ovariectomized rats
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Abstract

Menopause is accompanied by enhanced oxidative stress and behavioral changes, effects attenuated by antioxidants. The aim of this study was to evaluate the effects of caffeine on behavior and oxidative stress in an experimental model of menopause. Female rats were divided in: sham-operated (CON), sham-operated and caffeine-treated (CAF), ovariectomized (OVX), ovariectomized and caffeine-treated (OVX+CAF). Caffeine (6 mg/kg) and vehicle were administered for 21 days (subchronic) and 42 days (chronic), using 2 experimental subsets. Behavioral tests and oxidative stress parameters in the blood, whole brain and hippocampus were assessed. The subchronic administration of caffeine decreased the lipid peroxidation and improved the antioxidant defense in the blood and brain. The GSH/GGSG ratio in the brain was improved by chronic administration, with reduced activities of antioxidant enzymes and enhanced nitric oxide and malondialdehyde levels. In particular, the lipid peroxidation in the hippocampus decreased in both experiments. The rats became hyperactive after 21 days of treatment, but no effect was observed after chronic administration. In both experimental subsets, caffeine had anxiolytic effects as tested in EPM. The administration of low doses of caffeine, for a short period of time, may be a new therapeutic approach to modulating the oxidative stress and anxiety in menopause.

Keywords: caffeine, ovariectomy, brain, blood, oxidative stress, behavior, neuroprotection, rats.

1. Introduction
Menopause, defined as the permanent cessation of menstruation, is a physiological process that occurs in women around the age of 45-55 years, being linked to the loss of ovarian activity. The estrogens have regulatory roles in many organs, therefore their decreased secretion during menopause triggers many pathological reactions: several changes in the plasmatic lipid fractions (Maynar et al. 2001) and in the circulating catecholamine levels (Knonenberg et al. 1984), the activation of the renin-angiotensin system (Yung et al. 2011), the decreased endothelial nitric oxide (NO) synthesis (Gavin et al., 2009) and the enhanced oxidative activity (Sanchez-Rodriguez et al. 2012). The estrogen depletion at menopause was also reported to be associated with cognitive decline, depression and anxiety (Walf and Frye 2006).

Generally, the reactive oxygen species (ROS) are produced during normal metabolism and have useful functions. The involvement of OS in several menopause-related conditions has gained more and more interest and the elevated level of ROS was reported to be responsible, or at least to contribute to the pathogenesis, of weight gain (Mittal and Kant 2009), osteoporosis (Rao et al. 2009), hypertension (Yanes and Reckelhoff 2011), insulin resistance (Bloch-Damti and Bashan 2005), skin aging (Bottai et al. 2013), depression (Michel et al. 2012) and cognitive decline (Droge and Schipper 2007). Moreover, the impaired oxidant/antioxidant profile and oxidative stress-related anxiety in menopause can be reversed by dietary polyphenols, suggesting the beneficial effects of natural antioxidants in anxiety (Halliwell 2006; Salim 2011).

The hormone replacement therapy was widely used to prevent or to treat the symptoms and the conditions associated with menopause. Unfortunately, the hormonal replacement therapy has important adverse effects. The increased risk of cancer, especially breast cancer, after long-term estrogens exposure, is limiting the clinical use (Stahlberg et al. 2004). In the past 10 years, the scientists
have searched for alternative therapies to reduce the OS level, to improve the quality of life in postmenopausal women.

Caffeine (1, 3, 7-trimethylxanthine), a key component of many beverages and food (coffee, tea, cocoa) is widely consumed for its stimulating effect on the central nervous system. Due to the ability to block the adenosine receptors (ARs), especially the $A_1$ and $A_{2A}$ subtypes, it has many pharmacological actions (Fredholm et al. 1999). Caffeine and its metabolites are used in clinical medicine as myorelaxants, analgesics, diuretics and bronchodilators (Kaiser and Quinn 1999). Moreover, caffeine proved to be effective as an adjuvant in the treatment of obesity (Molnar et al. 2000), Parkinson’s disease (Barbier 2001) and had preventive effects on memory impairment (Leite et al. 2011). It may also regulate the NO production in the brain by modulating the ARs and the arginase activity (Nikolic et al. 2003).

Despite all the aforementioned beneficial effects, the exact influence of caffeine on the oxidant/antioxidant status in postmenopausal conditions was never studied before. The present study was designed to investigate the effects of subchronic and chronic caffeine administration on the oxidative stress in the blood and in the brain of ovariectomized rats. In addition, the locomotion and the anxiety-like behavior of animals were evaluated.

2. Methods

2.1 Reagents

Trichloroacetic acid, o-phthalaldehyde, t-butyl hydroperoxide, glutathione reductase, reduced glutathione, Bradford reagent, cytochrome c, xanthine, xanthineoxidase, β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) and caffeine were purchased from Sigma-
Aldrich Chemicals GmbH (Munich, Germany). Guanidine hydrochloride, 2-thiobarbituric acid, and EDTA-Na$_2$ were obtained from Merck KGaA (Darmstadt, Germany), while absolute ethanol, hydrogen peroxide and n-butanol were purchased from Chimopar (Bucharest, Romania).

2.2 Animals and experimental design

The study was performed on 104 female Wistar rats (3-month-old, weighing 180 ± 15 g). The animals were supplied by the Animal Department of “Iuliu Hatieganu” University of Medicine and Pharmacy, Cluj-Napoca. They were housed in a 12 h light/dark cycle at room temperature (24 ± 2 °C). All animals were fed a standard normocaloric pellet diet and received water ad libitum. All experiments were approved by the Ethics Committee on Animal Welfare of the "Iuliu Hațieganu" University and were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Council of Europe no. 123, Strasbourg 1985.

In order to evaluate the effects of subchronic and chronic caffeine administration, the experiment was divided into two subsets (Figure 1). Each of the two experimental subsets included 4 groups of treated animals, 13 rats each. The caffeine and the drinking water were administered orally. In subset 1, two groups underwent sham surgery (opening of the abdominal cavity, exposure of the ovaries, and sewing it back), and then were given 0.2 ml of drinking water (CON) or caffeine solution (CAF). The amount of caffeine administrated daily in rats was 6 mg/kg b.w. The animals in the others groups underwent bilateral ovariectomy (surgical removal of the ovaries) and were given drinking water (OVX) or a caffeine solution, in a dose of 6 mg/kg b.w./day (OVX+CAF). After 21 days, the behavioral tests were conducted. Twenty-four hours after the last behavioral test, under anesthesia with an intraperitoneal injection of ketamine/xylazine cocktail (90 mg/kg b.w. ketamine and 10 mg/kg b.w. xylazine), all animals were euthanized. The blood and the whole brain tissues from 8 rats in each group
were harvested and frozen. From the other 5 rats in the group, only the hippocampus was harvested for the oxidative stress assays.

In subset 2, the same groups were used: two sham groups, one received drinking water (CON) and the other one was treated with caffeine in a dose of 6 mg/kg b.w./day (CAF); two groups underwent bilateral ovariectomy, one which received drinking water (OVX) and the other one was treated with the same caffeine once daily (OVX+CAF). After 42 days, the behavioral tests were conducted. Twenty-four hours after the last behavioral test, anesthesia with an intraperitoneal injection of ketamine/xyazine cocktail was induced. Then, all animals were euthanized and the same samples as in subset 1 were harvested and frozen.

2.3 Behavioral tests

The locomotor activity and the anxiety-like behavior were measured in Open Field Test (OFT) and Elevated Plus Maze (EPM). The behavior sessions, recorded by a visual tracking system (Smart Basic Software v3.0 Panlab Harvard Apparatus; Ugo Basil Animal Mazes for Video-Tracking), were carried out between 09:00 and 14:00.

The next day, after the last dose of caffeine, animals were placed in the center of the OFT apparatus (100 x 100 x 40 cm) for rats, with gray non-reflecting base and walls. The floor of the open field arena was electronically divided into 9 equal squares. Normally, rats tend to walk close to the walls, a behavior called thigmotaxis. The index of activity (total travelled distance, travelled distance in periphery, total number of entries, number of entries in periphery) and the index of emotionality (travelled distance in center, number of entries in center, time spent in center divided by the total time), during each 5-min testing session, were automatically recorded. At the end of each session, the open field arena was cleaned with 70 % ethanol and animals returned to the home cage (Carrey et al. 2000).
One day later, the rats were placed in the center of the EPM, which consists of a plus-shaped maze, 60 cm elevated from the floor with two open and two closed arms (length 50 cm, width 10 cm, closed wall height 40 cm) opposite to each other and inter-connected by a central platform. Time spent in the unprotected open arms divided by the total time spent in all arms of the maze (time ratio) was taken as an experimental measure of anxiety. Therefore, the smaller these ratios, the more "anxious" the rats (Walf and Frye 2007).

2.4 Oxidative and nitrosative stress parameters

For preparing the cytosolic fractions, the brain tissue and the hippocampus were homogenized as previously described by Filip et al (2011) and the protein content in homogenates was measured with Bradford method (Noble and Bailey 2009). In order to evaluate the oxidative/anti-oxidative status, the glutathione reduced/glutathione oxidized (GSH/GSSG) ratios were assessed in the serum and brain tissue homogenate, while the malondialdehyde (MDA) levels were assessed in the serum, hippocampus and whole brain tissue homogenate. The MDA levels were considered as markers of oxidative attack on lipids and the glutathione reduced/glutathione oxidized (GSH/GSSG) ratio was interpreted as an antioxidant biomarker. In the whole brain homogenate, we assessed the activity of antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GPx) and the NO level. The antioxidant enzymes were also assessed in the hippocampus as markers of the antioxidant defense.

The MDA levels in the blood, whole brain homogenates and hippocampus were determined by spectrofluorimetry, using the method with 2-thiobarbituric acid. The values were expressed as nanomoles/mg of protein (Conti et al. 1991). The blood and brain tissue homogenate levels of GSH and GSSG levels were fluorimetrically measured using o-phtalaldehyde. Their concentrations were determined using standard curves and the results were expressed as GSH/GSSG ratios (Hu 1994).
The CAT activity was determined in the whole brain homogenates and hippocampus by using the method described by Pippenger et al. (1998) and the values were expressed as units/mg of protein. The GPx activity was determined with a slightly modified Flohe and Gunzler method (1984) and the values were expressed as micromoles of NADP produced/min/mg of protein. The NO level in the whole brain homogenates was determined by Griess reaction for nitrite plus nitrate in a two-step procedure. The concentration of nitrite was calculated in comparison with a nitrite-standard curve and the results were expressed as nanomoles/mg of protein (Titheradge 1998).

2.5 Statistical analysis

Statistical analysis of results was conducted by using ANOVA GraphPad Prism software, version 6.0 (GraphPad, San Diego, CA, USA). Data are presented as mean ± standard error of the mean (SEM). The comparisons between the groups were made by unpaired t-test and one-way ANOVA analysis of variance, using a Turkey’s Multiple Comparison Test. A p value lower that 0.05 was considered to be statistically significant.

3. Results

3.1 Oxidative stress assessment in blood

The influence of caffeine administration for 21 days and 42 days respectively on MDA levels and GSH/GSSG ratios in blood is illustrated in Figure 2. In subset 1, MDA level in blood was significantly higher in the OVX group (2.65 ± 0.18 nmoles/mg protein) as compared to the CON group (1.74 ± 0.18 nmoles/mg protein; p = 0.01) and decreased significantly after caffeine administration (1.84 ± 0.15 nmoles/mg protein in OVX+CAF group vs. 2.65 ± 0.18 nmoles/mg protein in OVX group; p=0.01). In subset 2, the serum MDA evolution had the same pattern. Thus, the values were significantly increased
in OVX group (4.03 ± 0.32 nmoles/mg protein) as compared to CON group (3.08 ± 0.22 nmoles/mg protein; \(p=0.03\)) and were reduced significantly after caffeine exposure (2.77 ± 0.17 nmoles/mg protein in OVV+CAF group; \(p=0.004\)).

The GSH/GSSG ratio in blood diminished significantly in the OVX group (2.11 ± 0.16) as compared to the CON group (3.18 ± 0.22; \(p=0.003\)) and was increased in the OVX+CAF group (3.29 ± 0.41) as compared to the OVX group (\(p=0.01\)) in subset 1 of experiments. Also, in subset 2, the GSH/GSSG ratio in blood increased significantly in OVX+CAF group (6.93 ± 0.65) as compared to OVX group (2.09 ± 0.28; \(p<0.001\)). The evolution of GSH/GSSG ratio was more evident after 42 days of caffeine administration.

3.2 Oxidative and nitrosative stress assessments in the brain

The influence of caffeine administration for 21 days and 42 days respectively on GSH/GSSG ratios and NO levels in the whole brain homogenate is illustrated in Figure 3.

A significantly higher value of the GSH/GSSG ratio was observed in OVX+CAF group (5.09 ± 0.44) as compared to OVX group (3.25 ± 0.34; \(p=0.01\)), after 21 days of caffeine administration. The same tendency was observed after 42 days of caffeine treatment, but the increase in GSH/GSSG ratio in OVX+CAF group (9.65 ± 0.60) as compared to OVX group (6.39 ± 0.72; \(p=0.008\)) was about twice as high as in the subset 1.

Brain NO level was significantly lower in the OVX + CAF group (1.57 ± 0.53 nmoles/mg protein) as compared to OVX group (3.26 ± 0.13 nmoles/mg protein; \(p=0.03\)) in the subset 1. A significantly higher level of NO was observed in the group underwent ovariectomy and treated with caffeine (6.91 ±
0.42 nmoles/mg protein) as compared to the OVX group (5.37 ± 0.33 nmoles/mg protein; \( p = 0.02 \)) in chronic administration of caffeine.

The effect of caffeine administration for 21 days and 42 days respectively on the MDA levels, GPx and CAT activities in the brain is illustrated in Figure 4.

In the subset 1, the MDA level assessed in the whole brain homogenate, was significantly lower in OVX+CAF group (0.11 ± 0.01 nmoles/mg protein) as compared to OVX group (0.19 ± 0.02 nmoles/mg protein; \( p = 0.009 \)). Interestingly, after 42 days of caffeine administration in ovariectomized rats, the MDA increased significantly as compared to OVX group treated with vehicle (0.16 ± 0.02 nmoles/mg protein vs. 0.09 ± 0.008 nmoles/mg protein in OVX group; \( p = 0.01 \)).

After 21 days of caffeine administration, the MDA level in the hippocampus was also significantly lower in the OVX+CAF group (0.22 ± 0.02 nmoles/mg protein) as compared to the OVX group (0.45 ± 0.05 nmoles/mg protein; \( p = 0.02 \)). The same tendency was observed after 42 days of caffeine treatment: OVX+CAF group (0.19 ± 0.02 nmoles/mg protein) and OVX group (0.38 ± 0.04 nmoles/mg protein; \( p = 0.003 \)) (Figure 4A, 4B).

Regarding the GPx activity in the whole brain homogenates, in the subset 1 we did not observe significant differences between the OVX + CAF group (20.98 ± 1.22 µmoles NADP/min/mg protein) and the OVX group (22.00 ± 1.28 µmoles NADP/min/mg protein; \( p > 0.05 \)). A significantly reduced activity in OVX + CAF group (19.61 ± 0.41 µmoles NADP/min/mg protein) as compared to OVX group (24.51 ± 0.74 µmoles NADP/min/mg protein; \( p = 0.001 \)) was observed in the subset 2. In the both experimental subsets, there were no significant differences between the groups regarding the GPx activities in hippocampus (Figure 4C, 4D).
The CAT activity in the tissue homogenates increased significantly in the group which underwent bilateral ovariectomy and received caffeine 21 days ($0.27 \pm 0.03$ U/mg protein) as compared to the OVX group ($0.17 \pm 0.01$ U/mg protein; $p=0.009$) and decreased significantly after 42 days of caffeine administration ($0.26 \pm 0.06$ U/mg protein in OVX+CAF group vs. $0.50 \pm 0.02$ U/mg protein in OVX group; $p=0.01$). Regarding the enzyme activity in hippocampus, in the subset 1, we observed a lower activity in the OVX+CAF group ($0.20 \pm 0.001$ U/mg protein) as compared to the OVX group ($0.42 \pm 0.002$ U/mg protein; $p=0.001$). In the subset 2, there were not significant differences between the OVX and OVX+CAF groups (Figure 4E, 4F).

### 3.3 Anxiety-like behavior tests

The influence of subchronic and chronic caffeine administration on general locomotor activity, tested in OFT, is illustrated in Figure 5 and Figure 6.

The OFT is the most commonly used to examine locomotor activity in rodents (Carrey et al. 2000). It seems that OF is a good model to assess the effects of classical benzodiazepines and 5-HT1A receptor agonists, but not other compounds displaying anxiolytic-like effects (Prut and Belzung 2003). In the OFT, there was no significant effect of ovariectomy as compared to the control group on general locomotion (total travelled distance, total number of entries, both travelled distance and number of entries in periphery) in the 21-days experiment. Conversely, the rats became hyperactive with the subchronic caffeine administration both as compared to control (CAF vs CON; $p<0.003$) and to the OVX group (OVX+CAF vs OVX; $p<0.001$). In subset 2, the ovariectomized group travelled significantly less in the open field as compared to control (OVX vs CON), but there were no significant differences in the caffeine-treated groups as compared to control (CAF vs CON; $p>0.05$) and to the OVX group (OVX+CAF vs OVX; $p>0.05$).
The influence of 21 and 42 days of caffeine administration on emotionality, tested in OFT, is illustrated in Figure 7 and Figure 8A, 8B.

Regarding the emotionality (travelled distance in center, number of entries in center, time spent in center/total time), we observed no significant differences between the OVX and CON groups, both for subchronic and chronic caffeine administration ($p>0.05$). The ovariectomized rats that were subchronically treated with caffeine travelled a greater distance, made more entries and spent significantly more time in the center of the open field arena as compared to ovariectomized group (OVX+CAF vs OVX; $p<0.03$). In contrast, the chronic administration of caffeine in OVX rats decreased significantly both the distance and the number of entries in the center in comparison to the OVX group (OVX+CAF vs OVX; $p=0.01$), but there was no statistical difference regarding the time ratio (time spent in center divided by the total time). Thus, the subchronic, but not the chronic, caffeine administration decreased the emotionality in ovariectomized rats.

The influence of 21 and 42 days of caffeine administration on the anxiety-like behavior, tested in EPM, is illustrated in Figure 8C and 8D.

The EPM test was used to assess the anxiety-like behavior of rodents, being considered the gold standard for the evaluation of anxiety in basic research (Pires et al. 2012). Thus, more time spent in the open arms of the EPM test apparatus during a 5 minutes test session is indicative of low anxiety-like behavior. For both experimental subsets, we observed that ovariectomized rats tended to spend less time in the open arms, but the differences were not statistically significant (OVX vs CON; $p>0.05$). Both subchronic and chronic administration of caffeine in OVX groups increased significantly the time spent in the open arms (OVX+CAF vs OVX; $p<0.006$) and based on these observations it seems that subchronic and chronic caffeine administration in OVX rats decreased the anxiety level (more time spent in open arms).
4. Discussion

The results of the current work showed that both subchronic (21 days) and chronic (42 days) administration of small doses of caffeine (6 mg/kg b.w.) significantly reduced the oxidative stress in the blood of ovariectomized rats. In the whole brain homogenate, the subchronic administration decreased the oxidative stress and the NO level, while the chronic treatment diminished the activity of the antioxidant enzymes and increased the MDA and NO levels in parallel with increased GSH/GSSG ratio. In the hippocampus of ovariectomized rats, both subchronic and chronic administration of caffeine reduced the peroxidation of lipids (lower MDA levels). Regarding the behavioral effects of caffeine, the locomotor activity was increased in subchronic administration, but no change was observed with the chronic treatment. Moreover, based on the EPM test results, caffeine administration had anxiolytic effects in ovariectomized rats, in both experimental subsets.

We have chosen the experimental ovariectomy in rats to simulate the menopause in women. The menopausal age strongly correlates with important pathological changes in women’s body leading to high morbidity (Gavin et al. 2009; Kronenberg et al. 1984; Maynar et al. 2001; Sanchez-Rodriguez et al. 2012; Walf and Frye 2006; Yung et al. 2011). Thus, the postmenopausal women are at a high risk of obesity, osteoporosis, hypertension, insulin resistance, cognitive decline, depression, anxiety and other diseases, mainly due to the enhanced OS levels (Bloch-Damti and Bashan 2005; Bottai et al. 2013; Droge and Schipper 2007; Michel et al. 2012; Mittal et al. 2009; Rao et al. 2007; Salim 2011; Sanchez-Rodriguez et al. 2012; Yanes and Reckelhoff 2011).

In the present study, we provide evidence that ovariectomy in rats led to early (after 21 days) increase of the OS parameters. Therefore, we observed enhanced serum MDA levels, reduced GSH/GSSG ratios in blood and brain, reduced activities or no changes in antioxidant enzymes and
enhanced NO levels in the brain. Moreover, in ovariectomized rats (OVX group), we observed decreased locomotor activity in OFT (the most used test to examine locomotor activity in rodents) and increased anxiety-like behavior in EPM test (the gold standard test to examine the anxiolytic effect in rodents) after 42 days of vehicle administration. These observations are consistent with the literature. Several studies found a correlation between the estrogen depletion and the increased OS level in women (Bloch-Damti and Bashan 2005; Sanchez-Rodriguez et al. 2012) and also in rats (Dilek et al. 2010; Patki et al. 2013). They reported an increased level of MDA and a reduced GSH/GSSG ratio in the blood of ovariectomized rats (Dilek et al. 2010) and in postmenopausal women (Bloch-Damti and Bashan 2005). Several changes in the brain antioxidant profile were also identified in menopause: reduced GSH/GSSG ratio (Abbas and Elsamanoudy 2011; Dilek et al. 2010), increased MDA levels (Abbas and Elsamanoudy 2011) and high levels of nitrated proteins (Patki et al. 2013). It seems that hippocampus is the most susceptible region to ovariectomy-induced estrogen depletion, in terms of generation of oxidative stress (Patki et al. 2013). We have also observed enhanced MDA levels in the hippocampus of the ovariectomized rats, especially 21 days after the ovariectomy. Regarding the antioxidant enzymes, some studies showed decreased activities (Abbas et al. 2011) while others reported no changes in their levels (Dilek et al. 2010). The behavioral changes observed were also comparable with previous studies which reported anxiety-like behavior and inhibitory effect on locomotion after ovariectomy in rats (Patki et al. 2013).

Although many therapies have been tried to counteract these changes, none of them had fully proved efficiency. Caffeine was chosen to be studied in this experimental model due to the ability to block the A₁ and A₂A ARs (Fredholm et al. 1999). It has been demonstrated that adenosine receptors are involved in the regulation of ROS production, thus having protective effects in neurons and glial cells (Schubert et al. 1997). We have chosen a low dose of caffeine (6 mg/kg b.w.) taking into consideration
several adverse effects of large doses administration: insomnia, tremor, anxiety, cardiac arrhythmias, hypertension and gastrointestinal disturbances (Bhattacharya et al. 1997; Chou and Benowitz 1994). Furthermore, the dose used is equivalent to approximately 100 mg of caffeine in humans, which is the amount contained in a cup of coffee (Allometric Scaling Calculator; Fredholm et al. 1999).

We have evaluated the effect of caffeine on blood oxidative stress markers as the general indicator of oxidant/antioxidant status. The brain is highly biochemically sensitive to oxidative damage (Halliwell 2006). It is rich in polyunsaturated fatty acids which are substrates for oxidation and due to the considerable oxygen consumption; the production of ROS in brain mitochondria is higher. In addition, the brain has modest antioxidant mechanisms. As previously discussed, the hippocampus seems to be the most susceptible area to the ovariectomy-induced estrogen depletion. For these reasons we have evaluated the effect of caffeine administration on ovariectomy-induced OS in rats, by assessing the NO levels and the OS markers in the whole brain homogenate, along with the MDA levels and antioxidant enzymes activities in the hippocampus.

In this study, we noticed that caffeine improved the GSH/GSSG ratio in blood and brain, in both experimental subsets. Given that cysteine is the limiting amino acid for the glutathione synthesis (Chung et al. 1990) and caffeine may stimulate the cysteine uptake by cells (Aoyama et al. 2011), probably the GSH/GSSG ratio was improved, at least partially, by this mechanism. After subchronic caffeine administration in ovariectomized rats, the GSH/GSSG ratios in blood and brain increased, close to control levels. Interestingly, the chronic treatment led to higher ratios as compared to the control groups. These results suggest there is excessive free radicals generation early after ovariectomy or other mechanisms involved in the GSH synthesis are activated only after a long period of caffeine administration.
GPx and CAT activities had a different pattern in the brain and hippocampus, in the two experimental subsets. The subchronic treatment increased the CAT activity while the GPx activity was not changed. It is known that the stimulation of A3 receptors by adenosine is cytoprotective and enhances the activity of antioxidant enzymes (Maggirwar et al. 1994). The high free-adenosine levels due to A1 and A2A ARs blockade by caffeine (Conlay et al. 1997) and the low affinity of caffeine for the A3 receptors (Fredholm et al. 1999) are two conditions that may allow endogenous adenosine to stimulate the A3 ARs. Conversely, the chronic treatment decreased both enzyme activities and at the same time the brain MDA levels were enhanced, probably due to reduced ability of GPx to remove the lipid peroxides.

Another explanation is related to the increased production of NO and protein nitration reactions (Radi 2013). In normal concentration, NO has beneficial effects in the brain, but pathological levels inhibit nearly all enzyme-catalyzed reactions through tyrosine residues’ oxidation, resulting in nitrated proteins. In our study, the chronic administration of caffeine in ovariectomized rats enhanced the NO level in brain and at the same time the antioxidant enzymes had decreased activities. Previous studies showed caffeine had an inhibitory effect on the activity of brain arginase (Nikolic et al. 2003). Thus, it may contribute to the NO synthesis by increasing the available arginine pool. Although after chronic treatment the NO level in the OVX group was similar to control, at 21 days after ovariectomy we observed a 2-fold higher brain NO level in the OVX group as compared to control. This is consistent with the literature data which reported increased levels of nitrated proteins after the same period of time (Patki et al. 2013). Through bilateral ovariectomy, the rat’s body needs to manage not only the abrupt estrogens depletion, but also the high level of pro-inflammatory cytokines. The activation of inducible nitric oxide synthase (iNOS) in response to these cytokines could be another mechanism behind the enhanced NO secretion following ovariectomy (Garry et al. 2015).
Interestingly, the subchronic caffeine administration in ovariectomized rats decreased the NO to the control levels. The ability of caffeine to increase the GSH synthesis was previously discussed and it is also known that the pathological NO level can be scavenged by the GSH, resulting in inactive molecules called nitrosothiols (Fang et al. 2002). Furthermore, it was reported that caffeine, via adenosine A$_1$ receptors blockade, increased the cAMP production and inhibited pre-transcriptional TNF-α production in cord blood monocytes (Chavez-Valdez et al. 2009). Thus, the decreased NO level close to control, in subchronic caffeine treatment, could be also due to the inhibition of iNOS by the reduced cytokines level. In addition, Han YJ et al (2001) reported that antioxidant enzymes, especially CAT, can suppress the NO production in macrophages through the inhibition of NF-kB activation. Moreover, the fact that after 42 days of caffeine administration the NO level was enhanced and at the same time the CAT activity was decreased supports this theory. However, further studies are necessarily to clarify the mechanisms behind these changes.

The acute and chronic administration of ARs modulators has opposite effects, a phenomenon called “the effect inversion”. It was firstly described on behavior but the mechanisms involved are not yet fully understood. Some authors suggested that it may be dependent upon the duration of exposure and the selectivity of ARs ligands (Jacobson et al. 1996). In our study, the subchronic caffeine administration had antioxidant effects, while the chronic exposure exacerbated the oxidative stress in the brain of ovariectomized rats. The stimulation of the A$_{2A}$ receptors by adenosine leads to injurious effects, mainly due to the glutamate release, while the activation of the A$_1$ receptors is neuroprotective (Cunha 2005). Several A$_{2A}$ receptors antagonists (including caffeine) reduced the injury in animal models of ischemic stroke (Phillis 1995), Parkinson’s disease (Chen et al. 2001) and Huntington’s disease (Fink et al. 2004). Furthermore, the stimulation of adenosine A$_{2A}$ receptors potentiated the nitric oxide release and glutamate efflux from the glial cells (Saura et al. 2005), which in turn may stimulate the activity of
iNOS and amplify the NO production. The effect can be inhibited by A\textsubscript{2A} ARs antagonists (Garry et al. 2015; Li et al. 2001). In addition, as a response to the chronic exposure to caffeine the increased expression of A\textsubscript{1} ARs may lead to a shift in the A\textsubscript{1}/A\textsubscript{2A} ARs balance (Ferre 2008; Shi et al. 1993). Moreover, the A\textsubscript{2A} receptors have a higher affinity for caffeine compared with A\textsubscript{1} receptors (Fredholm et al. 1999) and this could explain the neuroprotective effects of caffeine after subchronic treatment. In the chronic administration of caffeine, the ARs tended to heteromerize and thereby decreased the affinity of caffeine for A\textsubscript{2A} receptors (Ciruela et al. 2006) and enhanced the oxidative damage in brain.

The increased locomotor activity in rats as a response to small or moderate doses of caffeine was previously reported (Fredholm et al. 1999), but the behavioral effects of caffeine in ovariectomized rats were never studied. Garrett et al (1994) demonstrated that caffeine inhibited the negative effects of adenosine from dopamine receptors and produced locomotor stimulant effects. Both A\textsubscript{1} and A\textsubscript{2A} receptors are involved in the motor-activating effects of caffeine (Karcz-Kubicha et al. 2003). Moreover, the caffeine had the ability to induce glutamate-dependent and glutamate-independent release of dopamine (Ferre 2008). In our study, after 21 days of caffeine administration in ovariectomized rats, they became hyperactive as seen in OFT. This effect was not observed after chronic caffeine treatment and it may be due to the tolerance of the ARs in chronic exposure.

The mechanisms involved in ARs tolerance are still under debate. Some changes in the A\textsubscript{1}-A\textsubscript{2A} heteromers (Ciruela et al. 2006), the tolerance to the effects of A\textsubscript{1} receptor blockade (Karcz-Kubicha et al. 2003), specific gene expression alterations in the striatum (Svenningsson et al. 1999), the pushed-up GABAergic activity in the brain (Mukhopadhyay and Poddar 1998) and changes in dopamine receptors (Powell et al. 2001) were proposed as underlying mechanisms for this process.
In order to assess some changes in anxiety and emotionality, we chose two behavioral tests: the OFT and the EPM. In OFT, an increase in the central locomotion (travelled distance in center, number of entries in center) or in the time spent in the central part of the device (time spent in the center/total time), can be considered as reduced emotionality behavior (Ramos 2008). Our results showed that subchronic administration of caffeine in ovariectomized rats increased significantly the center time ratio and the central locomotion (distance and entries in center). Conversely, the chronic administration of caffeine showed a significant decrease in central locomotion as compared to OVX group. Thus, the subchronic, but not the chronic, administration of caffeine decreased the emotionality index of the ovariectomized rats.

In addition, the EPM test was used in our experiments. It has been validated to assess the anxiety-like behavior in rats, based on the natural aversion of rodents for open spaces (Walf and Frye 2007) and it is recognized as the gold standard for anxiety-like behavior evaluation in rodents. The time spent in the unprotected open arms calculated as a ratio of the total time is considered a relevant parameter of anxiety (Walf and Frye 2007). Both the subchronic and chronic administration of caffeine in ovariectomized rats had anxiolytic effects in EPM, sustained by increased time spent in the open arms. As long as the hippocampus was reported to be involved in anxiety-like behavior (Shin 2006), the reduced MDA levels in the hippocampus after caffeine administration could explain the anxiolytic effect of caffeine. The decline in catalase activity in subchronic administration was probably due to the direct destruction by free radicals (Hellemans et al. 2003).

The effects of caffeine administration on anxiety are still debated in literature. Some authors have claimed caffeine induces anxiety (Bhattacharya et al. 1997) while others reported an anxiolytic effect (Tang et al. 1989), but all these studies were carried out on healthy subjects. The A$_{2A}$ ARs,
responsible for the anxiety-like effect, may become tolerant to the long-term caffeine exposure. Thus, the anxiolytic effect is becoming predominant with chronic administration. The involvements of the A2A receptors in the genesis of anxiety effect, the $A_{2A}$ antagonists capacity to ameliorate anxiety-like behaviors in rodents and the increased densities of $A_1$, but not $A_{2A}$ ARs after chronic caffeine treatment, support this idea (Shi et al. 1993; Yamada et al. 2014).

Even though, the OFT is considered the most popular test used in behavioral research, its meaning in the field of emotionality/anxiety still remains unclear. OFT has been compared with EPM and it seems they measure different aspects of emotionality in rodents, a fact sustained by Ramos (Ramos 2008). It has been mentioned that environments, such as open spaces, brightly illuminated or elevated areas, may influence the animals behavioral responses (Anchan et al. 2014; Ramos 2008). Thus, the difference, seen in regard to these tests, underscores the importance of using more than one test in the assessment of anxiety-like behavior in rodents.

In conclusion, our study demonstrates that the subchronic administration of caffeine in low dose (6 mg/kg b.w.) decreased the oxidative stress and had anxiolytic effects in ovariectomized rats. The improved GSH/GGSG ratio and increased the activity of antioxidant enzymes in parallel with decreasing the nitric oxide levels, could be the mechanisms behind the neuroprotective effects of caffeine. However, probably due to the specific changes in the adenosine receptors, the chronic caffeine administration exacerbated the oxidative stress in the whole brain homogenate, but not in the hippocampus, demonstrating also anxiolytic effects. The oxidative stress parameters in the brain of ovariectomized rats were almost the same as in the control group after 42 days of experiment and at the same time, the chronic treatment enhanced the overall oxidative stress. These observations, led us to the idea that there should be no reason for long-term caffeine administration. Taken together, these
experimental findings suggest that the administration of low doses of caffeine for a short period of time may be a new therapeutic approach to decrease the enhanced oxidative stress and the heightened anxiety in menopause. Moreover, this study suggests the necessity for additional studies to investigate the effects of caffeine and specific adenosine receptors antagonists on the brain oxidant/antioxidant status in relation to the behavioral changes in menopause.

Authors’ contributions

IC designed the experiments and wrote the paper. AB conducted the behavioral tests, performed the statistical analysis for behavioral parameters and drafted the manuscript. RM designed and implemented the research study. ND carried out and analyzed the biochemical parameters. RO contributed to the implementation of the study and critically revised the manuscript for important intellectual content. AF did the statistical analysis for oxidative stress parameters, wrote the paper and gave the final approval. All authors read and approved the manuscript.

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Competing interests

The authors declare that they have no conflict of interest.
References


**List of abbreviations used:**

ARs – adenosine receptors

CAT – catalase

EPM – elevated plus maze

GPx – glutathione peroxidase

GSH – reduced glutathione

GSSG – oxidized glutathione

iNOS – inducible nitric oxide synthase

MDA – malondialdehyde

NO – nitric oxide
OFT – open field test

OS – oxidative stress

ROS – reactive oxygen species

SEM – standard error of mean

Figures legends

**Figure 1. Experimental design.** The two subsets of experiments (subchronic-21 days; chronic-42 days) are illustrated as a diagram. OVX-ovariectomized; OFT-Open Field Test; EPM-Elevated Plus Maze.

**Figure 2. Oxidative stress parameters assessment in blood. A)** In subset 1 of experiments, MDA level increased in OVX group as compared to CON \((p=0.01)\) and decreased after CAF administration \((p=0.01)\). **B)** In subset 2, lipid peroxidation enhanced in OVX group as compared to CON \((p=0.03)\) and decreased after caffeine administration \((p=0.004)\). **C)** GSH/GSSG ratio diminished in OVX group as compared to CON \((p=0.003)\) and increased after 21 days of caffeine administration (OVX+CAF vs. OVX group \(p=0.01)\) **D)** while in subset 2 of experiments, GSH/GSSG ratio increased in OVX+CAF \((p<0.001)\) vs. OVX group. Each group consisted of 8 rats. Results are expressed as mean ± SEM; *\(p<0.05\) as compared to CON; #\(p<0.05\) as compared to OVX.

**Figure 3. The effects of caffeine administration on the GSH/GSSG ratios and NO levels in the brain A)** GSH/GSSG ratio was reduced in the OVX group as compared to CON \((p=0.008)\) and increased in OVX+CAF group as compared to OVX group \((p=0.01)\) in subset 1 of experiments. **B)** GSH/GSSG ratio increased in OVX+CAF group as compared to OVX group \((p=0.008)\) in subset 2 of experiments. **C)** NO level increased in OVX group as compared to CON group \((p<0.001)\) and diminished in OVX+CAF group as
compared to OVX group ($p=0.03$) in subset 1 while in subset 2 of experiments D) NO enhanced after caffeine administration (OVX+CAF vs. OVX group, $p=0.02$). Each group consisted of 8 rats. Results are expressed as mean ± SEM; *$p<0.05$ as compared to CON; #$p<0.05$ as compared to OVX.

Figure 4. The effects of caffeine administration on the MDA levels and the antioxidant enzymes activity in brain. A) The level of MDA decreased after 21 days of caffeine administration in OVX+CAF group as compared to OVX group in the whole brain homogenate ($p=0.009$) and also in the hippocampus ($p=0.02$). B) After 42 days of caffeine administration, the MDA level in the whole brain homogenate increased in the OVX+CAF group as compared to OVX ($p=0.01$), while in the hippocampus it was decreased ($p=0.003$). C) GPx activity was not significantly different between the OVX+CAF and OVX groups ($p>0.05$) in subset 1 of experiments while in subset 2 D) GPx activity decreased in the whole brain homogenate, but not in the hippocampus, after caffeine administration (OVX+CAF vs. OVX group, $p=0.001$). E) In the whole brain homogenate, the CAT activity increased in OVX+CAF group as compared to OVX group ($p=0.009$), while in the hippocampus it was decreased. F) The CAT activity in the brain homogenate diminished in OVX+CAF group as compared to OVX group ($p=0.01$), but these differences were not observed in the hippocampus. In each groups, 8 rats were used for the whole brain homogenate analysis and 5 rats for the hippocampus assessments. Results are expressed as mean ± SEM; *$p<0.05$ as compared to CON; #$p<0.05$ as compared to OVX.

Figure 5. The effects of caffeine on the total travelled distance and the number of entries in OFT. A) The total travelled distance was increased in CAF group as compared to CON group ($p<0.001$) and also was improved after 21 days of caffeine administration ($p<0.001$). B) The same parameter evaluated after 42 days of caffeine exposure decreased in OVX group as compared to CON group ($p=0.002$) and was not significantly different in OVX+CAF group ($p>0.05$). C) In subset 1 of experiments, the total number of
entries increased in OVX+CAF group as compared to OVX group (p<0.001) while in subset 2 D) the same parameter decreased in OVX group (p=0.01) and was not significantly different after caffeine administration (OVX+CAF vs. OVX group, p>0.05). Each group consisted of 13 rats. Results are expressed as mean ± SEM; *p<0.05 as compared to CON; #p<0.05 as compared to OVX.

**Figure 6. The effects of caffeine on the travelled distance and the number of entries in periphery in OFT.** A) In subset 1 of experiments the travelled distance in periphery increased in CAF group as compared to CON group (p<0.001) and in OVX+CAF group as compared to OVX group (p<0.001). B) In subset 2 of experiments, the same parameter diminished after ovariectomy (p=0.001) and was not significantly different after caffeine administration (OVX+CAF vs. OVX group, p>0.05). C) The number of entries in periphery increased in OVX+CAF group as compared to OVX group (p<0.001) in subset 1 of experiments while in subset 2 D) the same parameter diminished in OVX group as compared to CON group (p=0.004) and was not significantly different in OVX+CAF group (p>0.05 vs. OVX group). Each group consisted of 13 rats. Results are expressed as mean ± SEM; *p<0.05 as compared to CON; #p<0.05 as compared to OVX.

**Figure 7. The effects of caffeine on emotionality in OFT.** A) The travelled distance in center increased in OVX+CAF group as compared to OVX group (p=0.03) in subset 1 of experiments while in subset 2 B) the same parameter decreased after caffeine administration (p=0.01 vs. OVX group). C) The number of entries in center enhanced in OVX+CAF group as compared to OVX group (p=0.02) after 21 days of caffeine administration while D) after 42 days decreased (p=0.01 vs. OVX group). Each group consisted of 13 rats. Results are expressed as mean ± SEM; *p<0.05 as compared to CON; #p<0.05 as compared to OVX.
Figure 8. The effects of caffeine on emotionality in OFT and anxiety in EPM. A) The center time/total time ratio increased in OVX+CAF group as compared to OVX group ($p=0.004$) in subset 1 of experiments while in subset 2 B) the same parameter was not significantly different. C) The open arms time/total time ratio increased in OVX+CAF group as compared to OVX group ($p=0.006$) in subset 1 of experiments while in subset 2 D) the same parameter increased ($p<0.001$ vs. OVX group). Each group consisted of 13 rats. Results are expressed as mean ± SEM; *$p<0.05$ as compared to CON; #$p<0.05$ as compared to OVX.
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398x299mm (300 x 300 DPI)
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