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Long-term effects of ageing and ovariectomy on aversive and recognition memory and DNA damage in the hippocampus of female rats

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Keywords: aged, Comet Assay, inhibitory avoidance test, object recognition test, ovariectomy

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Objective: This study investigated the influence of ageing – in particular the decrease of gonadal hormone levels during the ageing process – on the memory and the levels of DNA damage in the hippocampus of female rats.
Methods: Three groups of female Wistar rats were investigated: Group I consisted of non-ovariectomised, adult animals (6 months old); Group II consisted of non-ovariectomised, aged animals (18 months old); and Group III consisted of ovariectomised, aged animals (18 months old). The memory of the animals in these groups was examined via novel object recognition and inhibitory avoidance tests. The hippocampus tissue samples of all animals were obtained via biopsy and used to quantify the DNA damage using a Comet Assay.
Results: According to our findings, the process of ageing results in a change during the behavioural tests. To prevent genotoxic damage to the hippocampus caused by the ageing process, lowered hormone levels seem to be part of a protective biochemical mechanism in the body of rats. Animals that were previously submitted to an ovariectomy adapted better to these lower levels of hormones.
Conclusion: Our results indicate that ovariectomy can provide beneficial long-term effects on the memory. However, this could be specific to the kind of memory examined, as the aversive memory deficits caused by ageing were not affected by ovariectomy.

Significant outcomes

- The object recognition tests indicated an impaired memory in non-ovariectomised aged rats.
- The inhibitory avoidance tests suggested an affected aversive memory in the aged rats independent of the performed ovariectomy.
- Non-ovariectomised and ovariectomised aged rats showed lower concentrations of 17β-oestradiol in the blood when compared with adult rats.
- A Comet Assay showed significantly increased levels of DNA damage in the hippocampus of non-ovariectomised aged rats compared with adult rats.

Limitations

- Non-ovariectomised and ovariectomised aged rats showed similar 17β-oestradiol levels in the blood.

Introduction

Ageing is a normal and an inevitable phenomenon, which depends on the interaction of several genetic, environmental and lifestyle factors (1). It can be described as a general decline of the physiological bodily functions, which initially leads to morbidity and ultimately to mortality (2,3). This process is caused by numerous factors, all of which result in an irreversible damage to the biological macromolecules (DNA, proteins, lipids), cells and organs (4). Gonadal hormones are extremely important for proper functioning of the body, and even small hormonal imbalances can result in severe macroscopic dysfunctions of the body (5). Oestrogen, a female steroid, is synthesised inside the ovaries using blood cholesterol and acetyl coenzyme A. Only three types of oestrogen are present in the plasma of women in significant quantities: β-oestradiol, estrona and estriol. The most important oestrogen is β-oestradiol, as it is 12 times more potent than estrona and 80 times more potent than estriol (6). Oestrogens cause proliferation and growth of specific cells, which are responsible for the development of the secondary sexual characteristics. Non-pregnant women produce oestrogens in significant quantities only in the ovaries, albeit lower quantities are also produced via the adrenal cortex. During pregnancy, however, the placenta also releases significant amounts of oestrogens. Later, in the climacteric period, the levels of oestrogens start to decrease as a result of the decreased functionality of the ovaries (7). Oestrogen is also believed to be able to act as an antioxidant. The neuroprotective activity thereby arises from inhibited lipid peroxidation caused by the presence of a phenolic ring moiety in the steroid scaffold of oestrogen (5). Accordingly, the decrease in endogenous oestrogen levels can result in increased levels of free radicals and cause adverse effects in a variety of tissue types in postmenopausal women (8,9).

Oestrogen can, moreover, influence the learning performance. Learning and memory functions are fundamental to the acquisition and storage of new information. These processes are potentially affected by a change in the hormone levels. Gonadal hormones such as estriol have been shown to exhibit beneficial effects on the buildup of hippocampus-dependent memory (10). When women enter the menopausal period, the productivity of the ovarian follicles decreases, resulting in decreased levels of ovarian steroids and consequently increased levels of free radicals (11).

Ovariectomy is a common animal model used to evaluate the function of hormones in the physiological and pathological mechanisms (5). The administration of oestradiol to ovariectomised female rats over a short period of time is associated with the acquisition of memory (12) and an increased cognitive performance compared with ovariectomised animals that did not receive the same hormonal treatment (13,14).

Aims of this study

This study investigated the influence of ageing – in particular the decrease of gonadal hormone levels during the ageing process – on the memory and the levels of DNA damage in the hippocampus of female rats.

Methods and materials

Animals

Adult female Wistar rats (12 animals of 6 months and 24 animals of 18 months) were used in this study. It was ensured that none of the animals carried any offspring at any time before the study. The animals were obtained from the Fundação para Ciência e Investigação da Saúde (Porto Alegre, Brazil). They were kept in the University of Southern Santa Catarina for at least 2 weeks before the ovariectomy surgery. The animals were housed in boxes at 23 ± 1°C with access to water and food *ad libitum* and exposure to alternating bright–dark cycles of 12 h each (start of the bright period at 07:00 a.m.). Behavioural procedures were performed between 08:00 and 12:00 a.m. in a room, where exposure to excess sound and light was kept to a minimum. Animals were allowed to acclimatise to the procedure room 1 h before the tests. All experimental procedures were performed in accordance with the guidelines of the Sociedade Brasileira de Neurociências e Comportamento.

Ovariectomy

Ovariectomy procedures were carried out 15 months before the behavioural tests. Surgery was exclusively performed under anaesthesia using ketamine (60 mg/kg; Bristol Laboratories, Syracuse, NY, USA) and xylazine IP injections (12 mg/kg; Miles Laboratories, Shawnee, KS, USA) as described by Robertson et al. (15).

A small incision (1 cm) through the skin and the muscle back wall was made parallel to the bodyline. The ovaries were located and a silk thread was tightly tied around the oviduct including the ovarian blood vessels. The oviduct was sectioned and the ovaries were removed, taking good care to leave the knot intact. The skin and the muscle wall were

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subsequently sutured with a silk thread. The sham surgery control subjects received the same incisions and were sutured in the same way as the ovariectomised animals; however, their ovaries were only palpated instead of being removed. After the surgical procedure, the animals were treated with the antibiotic enrofloxacino (Flotril®, 2.5%, 0.025 mg/kg, IP injection; Schering-Plough Animal Health, Cotia, Brazil) once a day for 10 consecutive days. The animals were subsequently allowed to convalesce in their boxes (adult rats: 3 months; aged rats: 15 months) before being subjected to the behavioural tests and Comet Assay. Two weeks before the behavioural tests, vaginal smears were collected on a daily basis and analysed with a light microscope.

Vaginal cytology

Vaginal smears were collected using cotton tipped swabs, which were wetted with physiological saline solution of ambient temperature before being inserted into the vaginas of the restrained rats. The swabs were gently turned and rolled against the vaginal wall and then removed. Cells were transferred onto dry glass slides by rolling the swabs across the slides. The slides were then air-dried and stained (ca. 400 μl of Accustain/45 s; Sigma-Aldrich, St. Louis, MO, USA). The slides were then rinsed with water, overlaid with a cover slip and examined immediately at a 200-fold magnification under bright field illumination. The stage of the oestrous cycle was determined based on the presence or absence of the leukocytes, cornified epithelial and nucleated epithelial cells as described by Marcondes et al. (16).

To provide a reliable basis for the comparisons, we only considered dioeostrual adult sham rats for the statistical analysis of behavioural tests. This phase was chosen because the blood serum concentrations of oestrogens have been shown to be lower compared with other phases of the oestrous cycle (17,18). Vaginal smears were collected immediately after the behavioural tests to avoid the effects of human manipulation in the rats' responses. However, before collecting the blood samples, the vaginal cytology was not checked. Therefore, we cannot ensure that the adult sham rats were in the dioeostrus period when their blood samples were collected. In the aged sham rats, the vaginal smears were collected every day 1 week before starting the behavioural tests. To confirm the complete removal of all the ovarian tissues in the ovariectomised aged rats, vaginal smears were collected directly after the surgical procedure and subsequently every day for a period of 1 week before the behavioural tests.

Open-field test

Tests were carried out on 60×40 cm open-field grids, which were surrounded by brown plywood walls (50 cm high) with a front glass wall. The floor of the open field was divided into 12 equal squares by black lines. The animals were gently placed at the centre of the arena and allowed to explore freely for 5 min. The number of crossings and rearings of black lines were recorded to evaluate the spontaneous locomotion of the rats in this unfamiliar environment as described by de Chaves et al. (19).

Object recognition test

After a habituation period of 24 h, training sessions were conducted. Individual rats were placed for 5 min in the open field in which two identical objects (A1 and A2; A1 = A2 = cube) were positioned in two adjacent corners (distance to the walls = 10 cm). In a long-term recognition memory test taken 24 h after the initial training, the rats explored the open field for 5 min in the presence of one familiar (A; cube) and one unfamiliar object (B; pyramid with a square-shaped base). All objects had surfaces with similar smooth textures and blue colour. They were also similar in size (weight 150–200 g) but distinct in shape (cube vs. pyramid). From these test sessions, the recognition memory index was calculated for each animal, which is expressed as the ratio: TB/(TA + TB) (TA = time spent exploring the familiar object A; TB = time spent exploring the unfamiliar object B). Between the trials, objects were washed with a 10% ethanol solution. Exploration was defined as sniffing (exploring the object from a distance of 3–5 cm) or touching the object with the nose and/or the forepaws (20).

Inhibitory avoidance test

The inhibitory avoidance test is described elsewhere (21). For this test, an acrylic box ($50 \times 25 \times 25$ cm) with a floor of parallel stainless steel bars (diameter: 1 mm; spacing: 1 cm) and a platform (7×7 cm; height 2.5 cm) was used. The animals were placed on the platform and the latency to step down on the grid with all four paws was measured with an automatic device. Training sessions were conducted 10 or 30 days after the surgery. Immediately after stepping down on the grid, the animals received an electric shock (0.3 mA/2 s). In the test sessions carried out 24 h after the training, no electric shock was induced and the step-down latency period (maximum of 180 s) was used as a measure of retention. The behavioural tests were always conducted by the same person that was unrecognisable for the examined animals.

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Determination of blood serum levels of 17β-oestradiol by radioimmunoassay (RIA)

Seven days after the inhibitory avoidance test, rats were anaesthetised with thiopental (50 mg/kg) and blood samples were collected by the intracardiac puncture techniques. The blood samples were stored in Eppendorf tubes at 4°C. The blood samples were subsequently centrifuged (5000 rpm/5 min) to separate the serum. The serum was then stored at –20°C until 17β-oestradiol concentrations were determined using RIA. The RIA uses luminescent antibodies, which specifically bind to the examined hormone. These antibodies are immobilised on an ELISA plate with 96 wells. Therefore, the serum was deposited on the plates with the antibodies. After that, the plates were washed before the samples were reconstituted in butter solution, and 17β-oestradiol was added. After a short reaction time, the plates were washed again and analysed using the quantitative luminescence. The concentration of 17β-oestradiol in the blood serum of the animals was inversely proportional to the amount of luminescence recorded. The functional sensitivity of the test was 5 pg/ml.

Detection of DNA damage by Comet Assay

Comet Assay was used to assess the long-term effects of age and ovariectomy on the DNA damage in the hippocampus tissue samples of female rats. This test is a rapid, simple and sensitive technique to detect damage to the DNA molecules in individual cells. It is also considered to be a sentinel test, signalling possible damage to genetic material (22).

Comet Assay was carried out under alkaline conditions as described by Singh et al. (23) and modified by Tice et al. (24). The cell samples from different organ tissues were obtained according to Tice et al. (24). Each hippocampus tissue sample was placed in cold phosphate-buffered saline solution before being finely minced with a syringe plunger to obtain a homogenous cell suspension. To calculate the damage index (DI), the cells were visually separated into five classes according to the tail size (0 = no tails to 4 = maximum-length tails). A single DNA damage score was accordingly obtained for each sample and consequently for each group studied. The DI of any group could range from 0 (completely undamaged = 100 cells × 0) to 400 (maximum damage = 100 cells × 4). The damage frequency (DF in %) was calculated for each sample on the basis of the number of cells with tail compared with those without the tail (25).

Statistical analysis

The results obtained from the behavioural tests are expressed as median values ± interquartile interval (0.25–0.75). The differences between the training tests were evaluated by the Wilcoxon test. In the inhibitory avoidance test, the comparisons between groups were evaluated by the Kruskal–Wallis test, followed by the *post hoc* Dunn tests if necessary. Locomotor and exploratory activities and open-field crossings as well as 17β-oestradiol concentrations in the blood samples are all reported as mean values ± SEM values and assessed by the analysis of variance (ANOVA) tests, followed by the Student–Newman–Keuls tests. For the Comet Assay, the statistical analysis of the differences between DF and DI were reported as mean values ± SEM values and assessed by ANOVA, followed by the Tukey HSD tests. *P*-values of $p < 0.05$ were considered statistically significant. All analyses were performed using the SPSS statistical software package (SPSS 12.0; SPSS, Chicago, IL, USA).

Results

The results obtained from our object recognition tests (see Fig. 1) indicated that the adult rats and aged rats – ovariectomised or not – spent the same time exploring two equal objects exposed to them for the first time. When the animals were exposed to two different objects 24 h after the training, the adult rats spent more time exploring the unknown object, indicating that they recognised the previously shown object. Non-ovariectomised aged rats spent the same time exploring both objects, indicating that the ageing process impaired the recognition memory. However, aged ovariectomised rats showed statistically significant differences between the training and test sessions, indicating that some recognition memory had been preserved ($p < 0.05$, ANOVA, Tukey).

In the inhibitory avoidance tests (see Fig. 2), the adult rats had a significantly increased latency to step down from the platform during the test sessions compared with the training sessions. This is indicative of an acquisition of memory as a result of the training ($p < 0.05$, Kruskal–Wallis, Dunn). Aged rats, independent of ovariectomisation, did not exhibit any significant differences in latency between the sessions, suggesting an impairment of aversive memory in these rats. We did not observe any significant differences in the number of crossings and rearings or exploratory activity as evident from the open-field tests for all groups analysed. This suggests that the spontaneous locomotor activity was not affected by ageing or

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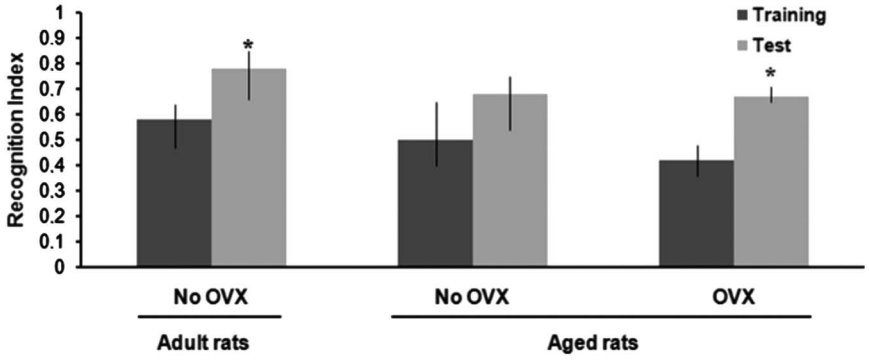

Fig. 1. Long-term effects of ovariectomy (OVX) on object recognition memory in adult rats, non-ovariectomised and ovariectomised aged rats. Bars represent median values ± interquartile range. $^{*}p < 0.05$ when compared between training and test sessions within the same group ($n = 12$).

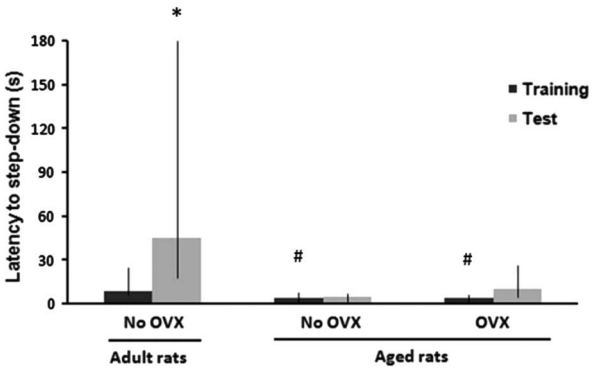

Fig. 2. Long-term effects of ovariectomy (OVX) on the latency time of adult rats, non-ovariectomised and ovariectomised aged rats submitted to inhibitory avoidance test. Bars represent median values ± interquartile range of the latency to step down from the platform. $^{*}p < 0.05$ when compared between training and test sessions within the same group ($n = 12$). $^{\#}p < 0.05$ when compared with non-ovariectomised adult rats.

ovariectomy procedures in the long term (see Fig. 3). Moreover, aged rats showed, independent of ovariectomisation, lower blood concentrations of 17β-oestradiol compared with non-ovariectomised adult rats (see Fig. 4). However, stress can increase the synthesis of ovarian hormones in female rats. We are, therefore, unable to separate the long-term effects of stress and ovariectomisation from the increased 17β-oestradiol production levels observed in the female rats ($p < 0.05$, ANOVA, Tukey) of this study. On the basis of the Comet Assay results (Fig. 5), which was made using the hippocampus cells, the non-ovariectomised aged rats had a significant increase in DNA damage ($p < 0.05$, ANOVA, Tukey) compared with the adult rats, for both index and frequency of DNA damages. Difference between the adult rats and aged rats that suffered early ovariectomy was observed.

Discussion

This study examined the changes in memory acquisition of adult (6 months) or aged (18 months) female Wistar rats in two animal memory models. We also evaluated the DNA damage in the hippocampus tissue samples of these rats. Our results indicate that non-ovariectomised aged rats display an affected memory in both behavioural tests and increased levels of DNA damage in the hippocampus compared with the adult rats.

The cognitive performance of the animals was analysed using the object recognition and the inhibitory avoidance tests. Reports in the scientific literature suggest that the cognitive perception of aged rats should be examined by using a test that eliminates the elements of motivation and favourable physical conditions (e.g. the Morris water maze test) (26). To eliminate any influence of potential deficiencies of the locomotor system in aged rats, we used the open-field test. We did not observe any alterations of the locomotor system in aged rats as a result of an ovariectomy when compared with adult animals.

The inhibitory avoidance test is a memory model task, which is used to study the acquisition and retention of memory in rodents. It has already been reported that rodents naturally suffer from an age-related cognitive decline, and the results of our inhibitory avoidance tests in this study are in agreement with these findings (27–29). We furthermore used an object recognition task to assess the recognition memory in our rodents. The results of these tests are also in accordance with the previous reports, which suggest that senescence induces memory deficiencies in rodents (28,30,31).

Considering that some clinical tests suggest deleterious effects of combined replacement with oestrogen and testosterone in some kinds of cognitive

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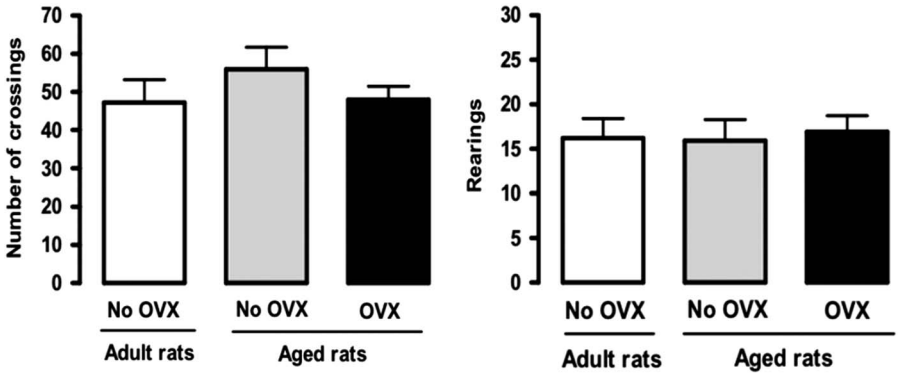

Fig. 3. Long-term effects of ovariectomy (OVX) on crossing and rearing numbers of adult rats, non-ovariectomised and ovariectomised aged rats submitted to open-field tests. Bars represent the medium values ± SEM values ($n = 12$).

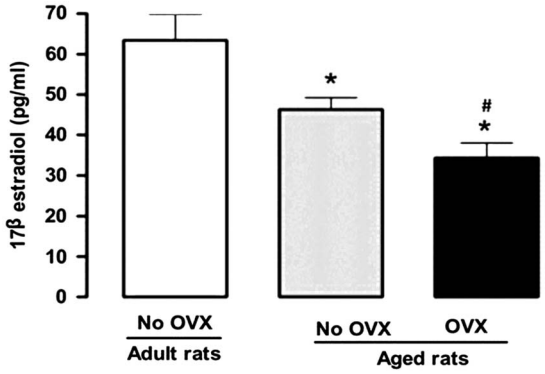

Fig. 4. Long-term effects of ovariectomy (OVX) on serum concentration of 17β-oestradiol in adult rats, non-ovariectomised and ovariectomised aged rats. Bars represent the medium values ± SEM values ($n = 12$). $^{*}p < 0.05$ when compared with non-ovariectomised adult rats. $^{\#}p < 0.05$ when compared with non-ovarectomised aged rats.

tasks in postmenopause women (32), these observations suggest that gonadal hormones can harm memory in elderly women. These results depend, however, on many factors such as patient age, type of menopause (natural or surgical), mode of hormone administration (oral, transdermal or intramuscular) and hormone dosage (33). Ovariectomised rats have been used to examine the contributions of ovarian hormones towards learning and memory in different stages of their development. Some studies have shown that the lack of gonadal hormones promotes cognitive impairment in rodents, especially in the first month after surgery (34,35). It is worth highlighting here that only very few studies have evaluated the relationship between ovarian hormones and their long-term effects on the cognitive performance in older, ovariectomised animals. Markowska and Savonenko (33) found that ovariectomies had a negative impact on the working memory of middle-age rats. However, the spontaneous alteration tests, place recognition tests and space memory tests evaluated by the Morris water maze test were unaffected. In contrast, Bimonte-Nelson et al. (36) showed that ovariectomies improved the space memory of aged rats in the long term (more than 3 months) that was evaluated by the radial maze tests. Chaves and colleagues have reported similar results. They suggested that the surgical removal of the ovarian glands in adult rats could induce short-term alterations in the structures and functions of oestradiol target neurons in the brain, which in turn could be responsible for short-term effects on emotional behaviour (19). However, compensatory mechanisms could take place in the long term, thus supporting the theory that ovarian hormones have a temporary modulating effect on behavioural despair. Pan et al. (37) demonstrated via Morris water maze tests that the phytoestrogen or oestradiol treatments can improve the acquisition and retention of spatial memory in ovariectomised rats in the long term. Interestingly, aged female monkeys that were ovariectomised when young had a significant increase in space memory compared with non-ovariectomised control monkeys (38).

In the present study, young rats (3 months of age) were ovariectomised. After 15 months, they were submitted to the object recognition and inhibitory avoidance tests. Adult rats (6 months of age) and non-ovariectomised aged rats (18 months of age) were used as control groups. Our results showed that ovariectomy improved the performance of aged rats during the object recognition tests in the long term. However, we did not observe any significant differences in the recognition memory tests between ovariectomised and non-ovariectomised aged rats. These observations suggest that ovariectomy can, in the long term, protect against recognition memory deficiencies caused by ageing. Bimonte-Nelson et al. (36) reported a short-term (3 weeks after surgery) impairment of the spatial memory after

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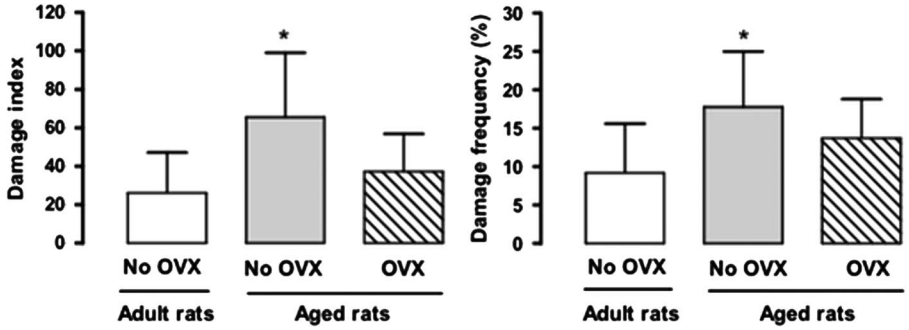

Fig. 5. Long-term effects of ovariectomy (OVX) on DNA damage in the hippocampus of adult rats, non-ovariectomised and ovariectomised aged rats. The Comet Assay produced the parameters damage index and damage frequency. Bars represent the medium values ± SEM values ($n = 12$). *$p < 0.05$ when compared with non-ovariectomised adult rats.

ovariectomy, which was restored 12 weeks after the surgical removal of the glands. These results demonstrate the need for further studies to understand the function and impact of gonadal hormones (or the lack thereof) on the memory of rats. Heikkinen et al. (39) observed that a prolonged treatment (40 days) of aged, 24-month-old female mice with oestrogen resulted in varying effects on their learning performance, depending on the memory task. For example, a decreased number of reference memory errors in the win-stay RAM task but no effect on the number of working memory errors in the T-maze test was observed. Moreover, ovariectomised mice showed improved performance in the position discrimination test in the T-maze.

The underlying biological and/or biochemical principles that explain the mechanisms through which ovariectomy could improve the memory of aged rats in the long term still remain unknown. The prolonged privation of gonadal hormones can induce many neurochemical reactions. It is, for example, known that the gradual failure of the ovaries during senescence results in a concomitant reduction of oestradiol levels, whereas increased progesterone levels can be observed in the postmenopausal phase of female rats (36). Ovariectomised animals, however, show an abrupt increase in both oestradiol and progesterone levels (36). Previous studies have shown that progesterone rules for spatial and work memory (40,41). It could be argued that the surgical removal of the ovaries causes an increase in the blood progesterone levels, and thus could mitigate the cognitive deficiencies caused by ageing and the physiological failure of ovaries in the long term. However, Hua et al. (42) have observed that the progesterone and combined progesterone/Vitamin D treatments can preserve spatial memory.

Several studies have investigated the relationship between DNA damage and ageing. The accumulation of age-dependent damage can be demonstrated by the cytogenetic and molecular assays (43–47). Heuser and colleagues have studied the basal levels of DNA damage in mice of different ages using the Comet Assay. Their results demonstrate a strong relationship between age, the inefficiency of DNA repair mechanisms and/or the accumulation of DNA damage (48).

The results presented here show that non-ovariectomised aged rats exhibit increased levels of DNA damage in the hippocampus tissue compared with adult rats. This is in agreement with the previously reported data (48). However, we also observed that rats that were ovariectomised 15 months before the experimental tests did not show any significant differences with respect to DNA damage in the hippocampus when compared with adult rats.

Previous studies have shown that extended privation of oestrogen can cause irreversible alterations to the function and structure of oestrogen-specific neurons in the brain (49–51). However, these changes could be compensated for in the long term by the removal of the ovaries: our results showed comparable results with respect to DNA damage in the hippocampus and to the recognition memory tests for ovariectomised and adult rats. In short, these results do not agree with the notion that sexual hormones are essential for a normal cognitive operation in rodents. Verma and Rana (52) reported a significantly induced breakage of strands in the DNA of the hepatic and renal cells as a result of an ovariectomy. Andersen and colleagues evaluated the genetic damage in different target organs induced by the sleep deprivation associated with an ovariectomy *in vivo*. They observed that DNA damage occurs in the brain cells independent of the phase of the oestrous cycle (53).

Our data suggest that the beneficial long-term effects of an ovariectomy on the memory could depend on the kind of memory evaluated. It seems

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that the aversive memory deficits caused by ageing do not change as a result of an ovariectomy. Our results also suggest that oestrogen is not the only hormone responsible for these behavioural and molecular alterations, as non-ovariectomised aged rats also showed decreased blood oestrogen levels compared with adult rats. The impact of the abrupt decrease in plasmatic gonadal hormone levels on ovariectomised rats and the gradual decrease of plasmatic gonadal hormone levels in non-ovariectomised aged rats could be crucial for the explanation of these observed differences with respect to the performance in the memory tests and genotoxic damage in the hippocampus.

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Authors Contributions

Daniela Dimer Leffa (graduate student) interpreted the data of the Comet Assay and assisted writing the manuscript for this paper. Adriani Paganini Damiani (graduate student) analysed the data of the Comet Assay. Daiane Dal Col Damazio (graduate student) assisted in writing the manuscript for this paper. Naiana Pereira Guerra (graduate student) performed the ovariectomy surgery. Morgana Moretti (graduate student) carried out the behavioural tests. Geovana Gomes da Silva de Brito (graduate student) conducted the vaginal cytology tests. Carina Rodrigues Boeck (Professor) was responsible for the final approval of the manuscript version to be published. Elaine Cristina Gavioli (Professor) was responsible for the experimental design. Vanessa Moraes de Andrade (Professor) was responsible for the final approval of the manuscript version to be published.

Conflicts of Interest

The authors declare no conflicts of interest.

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