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ORIGINAL ARTICLE

Peritoneal endometriosis induces time-related depressive- and anxiety-like alterations in female rats: involvement of hippocampal pro-oxidative and BDNF alterations

Paulo Wagner Linhares Lima Filho[1,3] · Adriano José Maia Chaves Filho[1] · Charliene Freire Xavier Vieira[1] · Tatiana de Queiroz Oliveira[1] · Michelle Verde Ramo Soares[1] · Paloma Marinho Jucá[1] · Joao Quevedo[4,5,6] · Tatiana Barichello[4,5,6] · Danielle Macedo[1,2] · Francisco das Chagas Medeiros[3]

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Abstract

Endometriosis is a gynecological condition affecting 10% of women in reproductive age. High rates of depression and anxiety are observed in these patients. The mechanisms underlying endometriosis-induced behavioral alterations are still elusive. Animal models provide a useful tool to study the temporal sequence and biological pathways involved in this disease and comorbid states. Here, we sought to characterize time-related behavioral alterations in rats submitted to endometriosis model (EM) induced by peritoneal auto-transplantation of uterine tissues weekly for three weeks. Corticosterone stress reactivity, oxidative stress markers – reduced glutathione (GSH), lipid peroxidation, activity of superoxide dismutase (SOD) and myeloperoxidase (MPO) - and brain-derived-neurotrophic factor (BDNF) levels in the hippocampus were also evaluated. We observed a progressive increase in anxiety-like behavior from 14th to 21st days post-EM. Despair-like behavior was observed from the 14th day post-EM on, while anhedonia and apathetic-like behaviors accompanied by increased corticosterone stress response were detected on 21 days post-EM. Increased pain sensitivity was observed from the 7th day post-EM and was accompanied by increased endometrioma weight. The pro-oxidative alterations, decreased GSH and increased SOD activity were observed on 21 days post-EM, except for lipid peroxidation that was altered from the 14th day. Decreased BDNF also occurred on the 21st day. Therefore, this study demonstrates that EM is related to several features of clinical depression and proposes the contribution of hippocampal oxidative state and neurotrophic support for the emergence of these changes. Our results support the use of this model as a useful tool to test new strategies for endometriosis-related neuropsychiatric symptoms.

Keywords Endometriosis · Depression · Animal models · Hippocampus · Oxidative stress · Brain-derived-neurotrophic factor

Introduction

Endometriosis is one of the most common gynecological diseases, affecting approximately 10% of women in menacme and up to 50% of infertile women. This disease is characterized by the ectopic presence of functional endometrial tissue outside the uterine cavity, and usually follows a progressive course of involvement of pelvic and abdominal structures (Janssen et al. 2013). The most common clinical signs of endometriosis are menstrual irregularities, chronic pelvic pain (CPP), dysmenorrhea, dyspareunia and infertility (Pope et al. 2015; Wickström and Edelstam 2017). Besides its impact on women health, endometriosis is associated with an enormous economic burden, in the order of $12,118 for direct costs and $15,737 for indirect ones per patient per year (Soliman et al. 2016).

Paulo Wagner Linhares Lima Filho and Adriano José Maia Chaves Filho should be considered co-first authors

Danielle Macedo and Francisco das Chagas Medeiros should be considered senior authors

Highlights
- Endometriosis model (EM) is related to several features of clinical depression;
- EM causes progressive increases in anxiety-like behavior;
- Behavioral alterations followed the cystic growth of endometriosis lesions;
- Hippocampal oxidative and neurotrophic changes underlie EM behavioral alterations

✉ Danielle Macedo
danielle.macedo@ufc.br; daniellesilmacedo@gmail.com

Extended author information available on the last page of the article

The initial explanations for the pathophysiology of endometriosis attributes this disease to retrograde menstruation. In recent years, new theories based on the involvement of immune system, apoptotic/proliferative pathways and embryogenesis disturbances in endometriosis biology were proposed (Vetvicka et al. 2016; Laganà et al. 2016). Notably, an elegant unifying theory was recently proposed by Laganà et al. 2017a, b postulating that aberrations in gene expression during embryogenesis may lead to the müllerian remnants in ectopic sites. These embryonic remnants could metaplastically originate endometriotic epithelium and stroma. In this context, immune cells and inflammatory signals are able to create the conditions for proliferation, differentiation and adhesion of these ectopic endometrial cells (Laganà et al. 2017b).

Some animal models have been proposed to study endometriosis (Tirado-González et al. 2010). In this regard, initial homologous rodent models of surgically-induced endometriosis were developed (Vernon and Wilson 1985; Cummings and Metcalf 1995). Accordingly, these animal models share several similarities to the woman's disease since the cyst-like structures present estrogen dependent-growth. The translational validity of this model is related to the occurrence of: i) endometrial glands and stroma that induce numerous adhesions in the peritoneal cavity with hemosiderin laden macrophages (Sharpe-Timms 2002; Yuan et al. 2016; Bruner-Tran et al. 2018) and ii) endometriotic lesions that mirror aberrant gene expression seen in human lesions, such as increased expression of genes associated with the extracellular matrix, cell growth and angiogenesis (Pelch et al. 2010).

Recently, the strong relationship between endometriosis and psychiatric symptoms was evidenced. Indeed, chronic pelvic pain and the other symptoms of endometriosis often affect psychological and social functioning, significantly compromising women social relationships, sexuality and mental health (Chen et al. 2016; Culley et al. 2017). In fact, a previous systematic review showed that depression and anxiety are the most common neuropsychiatric disorders associated with endometriosis (Pope et al. 2015) and, according to some literature, depressive and anxiety symptoms could be present in rates as high as 87% in this population (Sepulcri and do Amaral 2009).

To date, the possible mechanisms underlying the association between endometriosis and psychiatric symptoms are still not well understood. Some recent evidences have advocated for the participation of glutamatergic imbalance in the brain of endometriotic women with chronic pelvic pain (As-Sanie et al. 2016). Also, changes in gray matter volume and connectivity in stress-responsive brain areas, mainly the hippocampus, have been linked to the prevalence of neuropsychiatric symptoms in endometriosis (As-sanie et al. 2012; Beissner et al. 2016). Other possible mechanisms involve the participation of immune and pro-oxidative mechanisms in this disorder which, despite consitently demonstrated in endometriotic lesions and serum of endometriotic patients (Aznaurova et al. 2014; Donnez et al. 2016), were not already tested in brain areas involved in mood and anxiety regulation.

In fact, compelling evidence has shown that the progression of endometriosis is clearly related to oxidative stress (Gupta et al. 2006; Donnez et al. 2016). In endometriotic cells, the production of reactive oxygen species (ROS) is associated with increased proliferation rate and even malignancy transformation (Ngô et al. 2009). Also, increased levels of oxidative stress markers have been reported in several biological samples of these patients, such as serum, peritoneal and folicular fluid (Nasiri et al. 2017; de Lima et al. 2017). Conversely, oxidative stress plays a fundamental role in the pathogenesis of depression and anxiety disorders. Several evidences demonstrate the occurrence of decreased antioxidant status and increased pro-oxidative markers in the serum of depression and anxiety patients (Bilici et al. 2001; Herken et al. 2007; Moylan et al. 2014) and in the brain of animals submitted to depression models (Lucca et al. 2009; Mello et al. 2013; Silva et al. 2016).

Therefore, the identification of the pathways related to endometriosis-induced depression, anxiety and pain sensitization, is a fundamental step to the development of targeted treatments for these patients (Li et al. 2018). In the present study, our first outcome was to perform a broad behavioral evaluation (i.e., dispear-like, anhedonia, motivational, anxiety and pain-related symptoms) in female rats submitted to the model of peritoneal endometriosis induced by the autologous transplantation of endometrial tissue. Our second outcome was to investigate the effects of endometriosis model in corticosterone reactivity to acute stress, oxidative stress markers and brain-derived neurotrophic factor (BDNF) levels in the hippocampus of these rats. Our main hypothesis is that the induction of endometriosis model in female rats could resemble several features of neuropsychiatric symptoms seen in women with endometriosis in a time-dependent manner, and that pro-oxidative and neurotrophic support changes could take a fundamental role in the development of these alterations.

Experimental procedure

Animals

Adult female Wistar rats (10 weeks old, 220–240 g) provided by the Animal House of the Federal University of Ceara were used in the experiments. The animals were housed three per cage under standard polycarbonate cages (42 × 20.5 × 20 cm) and normal environmental conditions (22 ± 1 °C, humidity of 60 ± 5%; 12-h light/dark cycle with lights on at 7:00 AM) with free access to food (FRI-LAB Rat II, FRI-Ribe) and water. All experimental procedures were performed between 8:00 AM

and 02:00 PM. Before the induction of the model, female rats allocated to the same home cage had their reproductive cycle synchronized by transferring urine-soaked male bedding to their cages, as previously reported (Pelch et al. 2012). The research protocol was approved by the local ethics committee of the Federal University of Ceara and was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH 2011) and the Brazilian College of Animal Experimentation (COBEA).

Surgical model of endometriosis

The autologous transplantation model of endometriosis was performed as previously described (Vernon and Wilson 1985). Briefly, the animals were anesthetized with a mixture of 80 mg/kg ketamine hydrochloride and 6 mg/kg xylazine, by subcutaneous route. An adequate depth of anesthesia was determined by a negative response to toe pinch stimulus. A small (about 1 cm) midline incision ending 0.5–1.0 cm to the vaginal opening was made. Then, the area around incision was gently dissected by slowly opening and closing scissors movements such that the skin became sufficiently detached from the abdominal wall. Using small forceps, the left uterine horn was located, and a small medial incision was made with caution to do not damage the intestine. Gently, the uterine horn was pull up and with two pieces of black braided silk suture (6–8 cm) the horn was securely ligated at the utero-tubal and at the utero-cervical junctions. After few minutes of ligation, the uterine section between the ligations was cut off and placed in a sterile Petri dish with 500 μL of PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Three equal sections were obtained from the excised uterine horn using a biopsy punch (Miltex 33–31, York, PA). Then, the uterine samples were sutured to the parietal peritoneum using 4–0 vicryl sutures. The abdomen was closed with 5–0 absorbable suture and the skin was closed with 4–0 non-absorbable suture. Sham rats were treated identically to rats receiving surgery except that the suture alone was placed in the peritoneum. Two days after the transplantation surgery (early postoperatory period), estrous cycle was daily monitored through vaginal cytological smears and estrous phases were categorized as previously described (D'Souza and Sadananda 2017).

Experimental design

A total of 144 rats were randomly assigned to the endometriosis (72 rats) and sham operated groups (72 rats). The animals were kept undisturbed in their home cages until the 7th, 14th and 21st day after surgery when they were submitted to the behavioral tests by two independent observers blinded to the experimental groups. The behavioral determinations were conducted during the diestrus phase to minimize estrogen influence in animal's behavior (D'Souza and Sadananda 2017). To avoid the animal's re-exposure to the same behavioral apparatus as well as the excessive behavioral testing, each experimental group (endometriosis or sham) was equally subdivided in three different sets for temporal evaluation on 7-, 14- and 21-days post-surgery. The first subgroup ($N = 8$) was submitted to sucrose preference and forced swimming test (FST), in this order; the second subgroup (N = 8) was submitted to open field test, elevated plus maze test and splash test, in this order; and the third (N = 8) to the hot plate test and acetic acid-induced abdominal constriction test. The behavioral determinations were organized from the least to the most stressful. In the third set of experiments, more precisely the tests of pain evaluation, a time-interval of 24 h between each test was used. Endometriosis was confirmed at the time of sacrifice by identifying ectopic lesions in the abdominal cavity, and endometriosis implants were resected for weight measurement. Additionally, the rats exposed to forced swimming test (also used as acute stressor), had their trunk blood (about 1 ml/rat) collected into ice-cooled centrifugal tubes. The subjects of the subgroup 2 (the least stressful set) had their hippocampus dissected and stored at −70 °C until biochemical assays (for a timeline overview see Fig. 1).

Behavioral tests

Open field test

Locomotor activity and exploratory behavior were measured in an squared open field area, which consist of a square plastic board (40 × 40 cm, walls 40 cm high) divided into nine squares, based on model described elsewhere (Archer 1973). Under red light (28 lx in the center), each rat was placed into a corner of the area and observed for 5 min after 1 min of habituation. A solution of 20% ethanol was used for cleaning the apparatus after the removal of each animal. The parameters recorded during the test were: number of animal crossings with four legs (spontaneous locomotion), number of rearings (vertical exploratory behavior), entries in central square and time spent in the central square (in seconds).

Elevated plus maze test

The elevated plus maze apparatus, described by (Lister 1987), consisted of two open arms (50 × 10 cm, walls 40 cm high) and two closed arms (50x10cm, walls 40 cm high) connected by a central square (10 × 10 cm), and elevated to a height of 50 cm. The rat was placed in the apparatus center facing one of the open arms and allowed to explore for 5 min under conditions of red light (28 lx in the center). An entry was considered when all four limbs of the animal were inside an arm. Each rat was tested once and before the insertion of the next animal the apparatus was cleaned with 20% ethanol solution. The

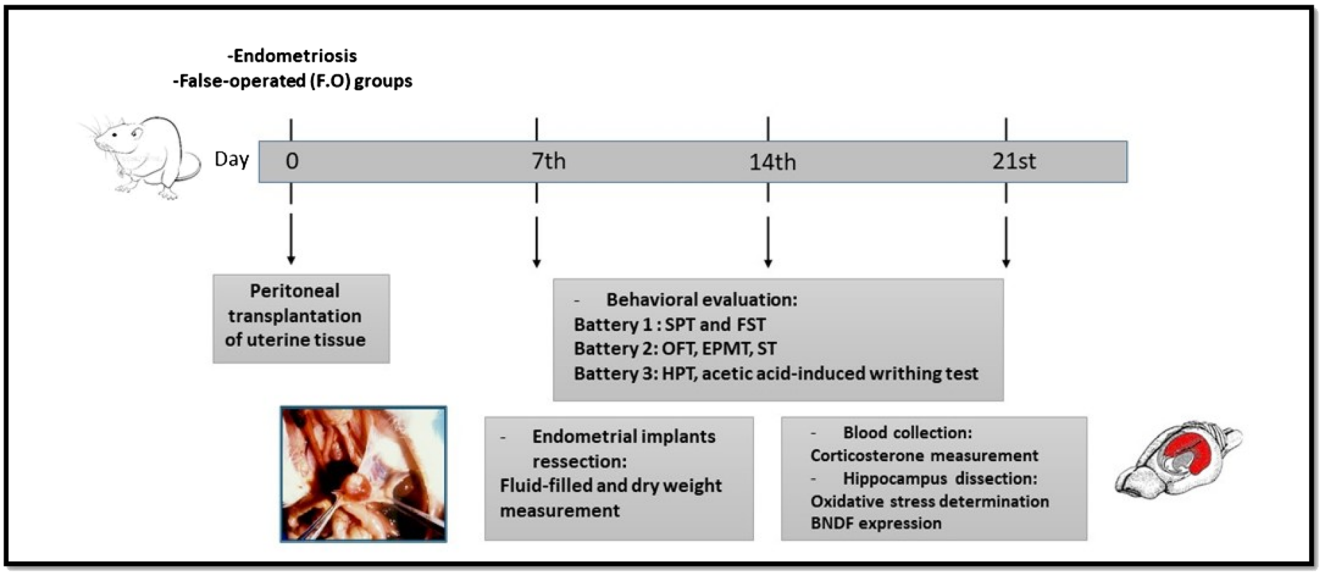

Fig. 1 Schematic overview of the experimental design. Abbreviations: SPT: sucrose preference test; FST: forced swimming test; OFT: open field test; EPMT: elevated plus maze test; ST: splash test; HPT: hot plate test; BDNF: brain-derived neurotrophic factor

behavioral measures observed during the test were the number of entries in open and closed arms and the time spent in each of these sections. These data were used to calculate the number of total entries, the % of open entries (i.e., open entries/total entries×100) and the % of time in open arms (i.e., time in open arms/total duration×100).

Forced swimming test

Forced swimming test was performed over 2 days, as previously described for rats (Porsolt et al. 1977). In the first day, the pre-swimming session consisted of an individual exposure to an acrylic cylinder (40 cm height, 22 cm diameter) containing 20 cm of water maintained at 23 ± 1 °C for 15 min. Twenty-four hours later, rats underwent the test session. The rats were again placed in cylinders filled with water, and the duration of immobility was recorded for 5 min. The immobility time was defined as the time spent by the rat in the absence of active, escape-oriented behaviors, such as swimming, jumping, rearing, sniffing, or diving, making only those movements necessary to keep its head above the water.

Sucrose preference test

This test was conducted as described elsewhere (Li et al. 2014; Qu et al. 2017). Briefly, the rats were habituated to a 1% sucrose solution for 48 h before the test day. The animals were deprived of water and food for the 4 h, and, after this, they were housed in individual cages with free access to two bottles containing 100 ml of sucrose solution (1% *w*/*v*) and 100 ml of water respectively for one hour. The volumes of consumed sucrose solution and water were recorded, and the sucrose preference was calculated as follows: % sucrose consumption = sucrose consumption/ (water + sucrose consumption) × 100.

Splash test

Splash test was conducted as previously reported (Marrocco et al. 2014). The test consists of spraying a 10% sucrose solution on the rat in a familiar cage. The sucrose solution dirtied the coat and induced a grooming behavior. After applying sucrose solution, the latency to the first grooming behavior, the number of grooming events and the time spent grooming for 5 min were registered. Previous studies in rodents have shown that in the splash test, grooming behavior represents a form of motivational behavior considered to mimic apathetic behavior as a symptom of depression (Willner 2005; Isingrini et al. 2010).

Acetic acid-induced abdominal contractions

The model of acetic acid induced-visceral pain was conducted as primarily described by Koster et al. (1959) and successively replicated (Lewter et al. 2017; Mara et al. 2018). Briefly, rats were habituated in individual house cages for 1 h. Then, the animals received an intraperitoneal injection of 0.6% acetic acid – 1 ml. The number of acid-induced writhes was observed for 20 min, starting 5 min after administration. A writhe was defined as a contraction of the abdomen following a stretch of the hind limbs. The number of writhes was cumulatively rated by an experienced observer. To reduce animal's suffering they were immediately sacrificed at the end of the test.

Hot plate test

Hot plate test is a widely used test to evaluate pain threshold to thermal stimuli in rodents (Bannon and Malmberg 2007; Mara et al. 2018). The test was performed with a commercially available hot plate meter consisting of a clear, plexiglass cylinder placed on a hotplate. Rats could walk on the hotplate (53.0 °C ± 0.1 °C) for up to 45 s (maximum allowed latency; to avoid tissue damage). Latency to jump, hind paw lick or flick was recorded, up to the maximum 45 s (if the animal did not emit such a response). Each animal was tested only once in each session.

Corticosterone determination

Corticosterone determination was performed based on a fluorescence method previously described (Mattingly 1962; Butte et al. 1978). Briefly, the fresh blood samples were centrifuged (1700×g, 10 min, 4 °C) to separate the serum. The serum free corticosteroids were extracted through dichloromethane reaction under mild rotation (33 rpm, 20 min, 4 °C). The organic phase was collected and incubated with fluorescence reagent (7 volumes of sulphuric acid to 3 volumes of purified ethyl alcohol) for 13 min, protected from the light and heating at 4 °C. The fluorescence was measured in Cytation™ 3 Plate Reader (Biotek, USA) using the wave lengths of 470 μm for excitation and 540 μm for emission. The serum corticosterone was expressed in μg /ml of serum using a standard corticosterone curve ranging from 100 to 0.5 μg of corticosterone/ml of serum.

Biochemical assays

Determination of reduced glutathione levels

The levels of reduced glutathione (GSH) were determined to estimate the endogenous antioxidant defenses. The method was based on the Ellman's reagent (DTNB) reaction with free thiol groups (Sedlak and Lindsay 1968). Briefly, the samples were mixed with 0.4 M Tris–HCl buffer, pH 8.9 and 0.01 M DTNB. GSH levels were determined using a microplate reader (Eon BioTek) set at 412 nm, and the levels were calculated based on a standard GSH curve and expressed as ng /mg wet tissue.

Measurement of lipid peroxidation

The rate of lipoperoxidation was estimated by the determination of malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) test (Huong et al. 1998; Gaweł et al. 2004). The brain areas were homogenized (10% *w*/*v*) in 50 mM potassium phosphate monobasic buffer, pH 7.4. Then, 63 μl of the homogenate was mixed with 100 μl of 35% perchloric acid. The samples were centrifuged at 5000 rpm for 10 min and 150 μl of the supernatants were removed, mixed with 50 μl of 1.2% thiobarbituric acid, and then heated in a boiling water bath for 30 min. After cooling, the levels of lipid peroxidation were determined using a microplate reader (Eon BioTek) set at 535 nm and expressed as μmol /mg wet tissue.

Measurement of superoxide dismutase (SOD) activity

Determination of SOD activity was performed according to a previously described method (Sun et al. 1988). In this assay, the photochemical reduction of riboflavin generates superoxide ($O2^{\bullet-}$) that reduces nitroblue tetrazolium (NBT) to produce a formazan salt. In the presence of SOD, the reduction of NBT is inhibited because the enzyme converts the $O2^-$ radical to peroxide. Briefly, supernatants were centrifuged for 20 min at 12,000 rpm at 4 °C, and the resulting supernatant was assayed. In a dark chamber, 1 ml of the reactant (50 mM phosphate buffer, 100 nM EDTA and 13 mML-methionine, pH 7.8) was mixed with 30 μl of the sample, 150 μl of 75 μM NBT and 300 μl of 2 μM riboflavin. The tubes containing the solution were exposed to a 15-W fluorescent light bulb for 15 min. The absorbance was determined using a microplate reader (Eon BioTek) set at 560 nm. The results are expressed as the quantity of SOD necessary to inhibit the rate of reduction of the NBT by 50% in units of enzyme per μg of protein (U/μg protein). Lowry method was used for protein determination (Lowry et al. 1951).

Analysis of myeloperoxidase (MPO) activity

Myeloperoxidase is a highly oxidative enzyme. The extracellular activity of this enzyme gives an estimate of the oxidative stress in inflammatory conditions (Pulli et al. 2013). Myeloperoxidase activity with 3,30,5,50-Tetramethylbenzidine (TMB) was measured as described elsewhere (Suzuki et al. 1983). Absorbance was measured at 450 nm in two time points, 0 and 3 min, after the beginning of reaction to estimate MPO activity (U MPO/min/μg protein).

BDNF protein expression by Western blot

Hippocampi were homogenized in RIPA lysis buffer (25 mM Tris-HCl, pH 7.6; 150 mM NaCl; 5 mM EDTA; 1% NP40; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS) with protease inhibitor (1 μL inhibitor: 100 μL RIPA). For protein extraction, hippocampi were centrifuged at 13,000 rpm for 17 min under refrigeration (4 °C) and supernatant collected. Protein concentrations were determined by the method of Lowry according to the manufacturer's protocol to extraction buffers with detergents. SDS polyacrylamide gel electrophoresis

(10%) was performed using 50 μg of protein (previously prepared with Laemmli sample buffer and heated at 95 °C for 5 min). The proteins were transferred to PVDF membrane, blocked with BSA 5% for 1 h, and incubated overnight with rabbit anti-BDNF IgG primary antibody (1:2000; ANT-010, Alomone Labs) or mouse anti-α-tubulin IgG primary antibody (1:2000; Sigma, USA). After washing, the blots were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:2000; Thermo Scientific, USA) or goat anti-mouse IgG secondary antibody (2:1000; Thermo Scientific, USA) for 90 min at room temperature. Signal was detected using the ECL system (Bio-Rad, USA) according to the manufacturer's instructions, and then the bands were captured with a CCD camera using the ChemiDoc system (Bio-Rad, USA). Densitometry quantification of bands was performed with ImageLab Biorad software. To ensure the specificity of antibody labelling, we used a recombinant mouse BDNF protein supplied by Alomone Labs (#B-240).

Statistical analysis

All data are present as mean ± standard error of the mean (SEM). Shapiro-Wilk test was performed to determine the normal distribution of data. Regular two-way analysis of variance (ANOVA) followed by Tukey's (for parametric data) or Fisher's LSD (for nonparametric data) as post hoc tests were performed. For the ANOVA analysis, the factors used were "endometriosis model" (surgically-induced endometriosis and sham groups) and "time" (7-, 14- and 21-days post-surgery). The significance level was set at $P < 0.05$. GraphPad Prism 7.0 Version for Windows, GraphPad Software (San Diego, CA, USA) was used for the analyses.

Results

Endometriosis model caused time-related anxiety-like alterations without locomotor changes in rats

In the open field test, two-way ANOVA revealed no significant interaction between factors in the analyses of the parameters crossings [F (2, 42) = 0.2109; P = 0.8104] (Fig. 2a) and rearings [F(2, 42) = 0.2227, P = 0.8011] (Fig. 2b). In the evaluation of the number of entries in the center of the open field test, two-way ANOVA showed a significant main effect "endometriosis model" [F (1, 42) = 4.12, P = 0.0476] without significant interaction between factors. Post hoc test revealed that 21 days after endometriosis surgery the rats presented decreased number of entries in the center being this decrease significant in relation to sham group and to endometriosis group when evaluated on day 7 ($P < 0.05$) (Fig. 2c). In the parameter time spent in center, two-way ANOVA also revealed a significant main effect of "endometriosis model" [F (1, 42) = 22.89, $P < 0.0001$] without significant interaction between factors. In the post hoc test, we observed a reduction in the time spent in the center of the field in endometriosis group compared to sham group, on days 14 ($P < 0.001$) and 21 ($P < 0.01$) post-endometriosis surgery (Fig. 2d).

Two-way ANOVA of the percentage of entries in the open arms (Fig. 3a) showed a significant interaction between factors [F (2, 42) = 5.159, P = 0.0124] with significant main effect of "endometriosis model" [F (1, 42) = 31.61, $P < 0.0001$] and "time" [F (2, 42) = 14.13, $P < 0.0001$]. This means that the animals submitted to endometriosis model presented a progressive decrease in the percentage of open arms entries from 14 days of surgery on in relation to sham rats (14 days, $P < 0.05$; 21 days, $P < 0.001$). Comparing only the endometriosis group along time it was observed a significant decrease in the percentage of open arms entries on day 14 when compared to day 7 ($P < 0.05$) and on day 21 when compared to days 14 ($P < 0.05$) and 7 ($P < 0.0001$).

In the evaluation of the percentage of time spent in open arms in the elevated plus maze (Fig. 3b), two-way ANOVA revealed a significant interaction between factors [F (2, 42) = 3.836, P = 0.0328] with significant main effect of "endometriosis model" [F (1, 42) = 27.09, $P < 0.0001$ and "time" [F (2, 42) = 11.09, P = 0.0002]. In the multiple comparison analysis, it was observed a progressive decrease in the time spent in the open arms in the endometriosis group at both the 14th ($P < 0.01$) and 21st days of evaluation ($P < 0.0001$) when compared to their respective sham controls. In the comparison of the endometriosis group along time it was observed a significant decrease in the time on day 14 in relation to day 7 ($P < 0.0001$) and on day 21 in relation to days 14 ($P < 0.0001$) and 7 ($P < 0.0001$).

Regarding the total entries, a parameter of locomotion inside the apparatus, no significant alterations were observed (Fig. 3c).

Endometriosis model induces time-related depression-like behaviors in rats

In the evaluation of the immobility time in the forced swimming test (Fig. 4a), two-way ANOVA revealed a significant main effect of "endometriosis model" [F (1, 42) = 25.17, $P < 0.0001$], without significant interaction between factors. In the post hoc analysis, it was observed a marked increase in immobility time in the endometriosis group compared to sham

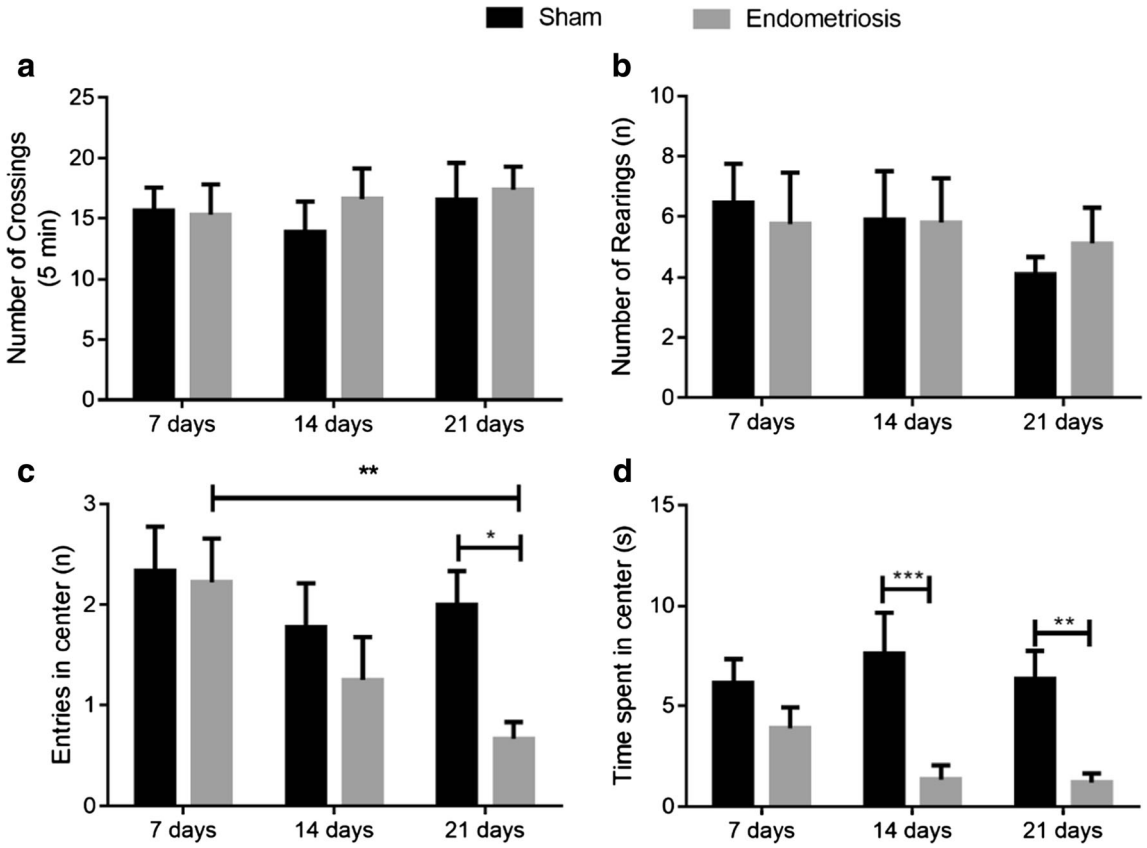

Fig. 2 Effect of endometriosis model in the number of crossings (spontaneous locomotion) (**a**), number of rearings (**b**), entries in the central square (**c**) and time spent in central square (**d**) in the open field test. Female rats were submitted to surgically-induced endometriosis or sham-operated (control surgery). The behavioral determinations were conducted on the 7th, 14th and 21st days post-induction. Bars represent means ± SEM ($n = 8$ animals/group). Data were analyzed using two-way ANOVA followed by Tukey's post hoc test. In the analysis of entries and time spent in the center, Fisher's LSD post hoc test was conducted. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

group on the 14th ($P < 0.05$) and 21st days of evaluation ($P < 0.001$).

As shown in Fig. 4b, two-way ANOVA revealed a significant main effect of "endometriosis model" [$F(1, 42) = 9.980$, $P = 0.0036$], without significant interaction between factors. Post hoc test showed a significant reduction in sucrose consumption in the endometriosis group on the 21st day of evaluation compared to sham rats ($P < 0.01$).

In the evaluation of grooming behavior in the splash test, it was observed a significant reduction in the number of grooming events in the endometriosis group on day 21st compared to sham group ($P < 0.05$) (two-way ANOVA: significant main effect of "endometriosis model" [$F (1, 42) = 16.98$, $P = 0.0002$]) (Fig. 4c). On day 21, an increase in the latency to first grooming was detected in endometriosis group when compared to sham animals. Also, this increase in latency time in endometriosis group on day 21 was significant in relation to the same group when evaluated on day 7 ($P < 0.01$) and on day 14 ($P < 0.05$) (two-way ANOVA: significant main effect of "endometriosis model" [$F (1, 42) = 12.90$, $P = 0.0010$] and "time" [$F (2, 42) = 10.77$, $P = 0.0002$] (Fig. 4d). Finally, a significant decrease in grooming duration in endometriosis group on day 21 in relation to sham group was observed ($P < 0.05$) (Fig. 4e). Again, when comparing endometriosis group along time it was observed that this reduction in grooming behavior observed on day 21 was significant in relation to day 14 ($P < 0.01$) (two-way ANOVA: significant main effect of "endometriosis model" [$F (1, 42) = 8.938$, $P = 0.0052$ and "time" [$F (2,42) = 8.818$, $P = 0.0009$].

Endometriosis caused a marked increase in corticosterone stress response at the third week post-induction

Regarding the serum levels of corticosterone evaluated after acute forced swimming stress (Fig. 4f), it was observed a significant main effect of "endometriosis model" [$F (1, 42) = 17.65$, $P = 0.0002$]. Post hoc test showed a significant increase in corticosterone levels in the endometriosis group compared to sham controls on the 21st day of evaluation ($P < 0.01$).

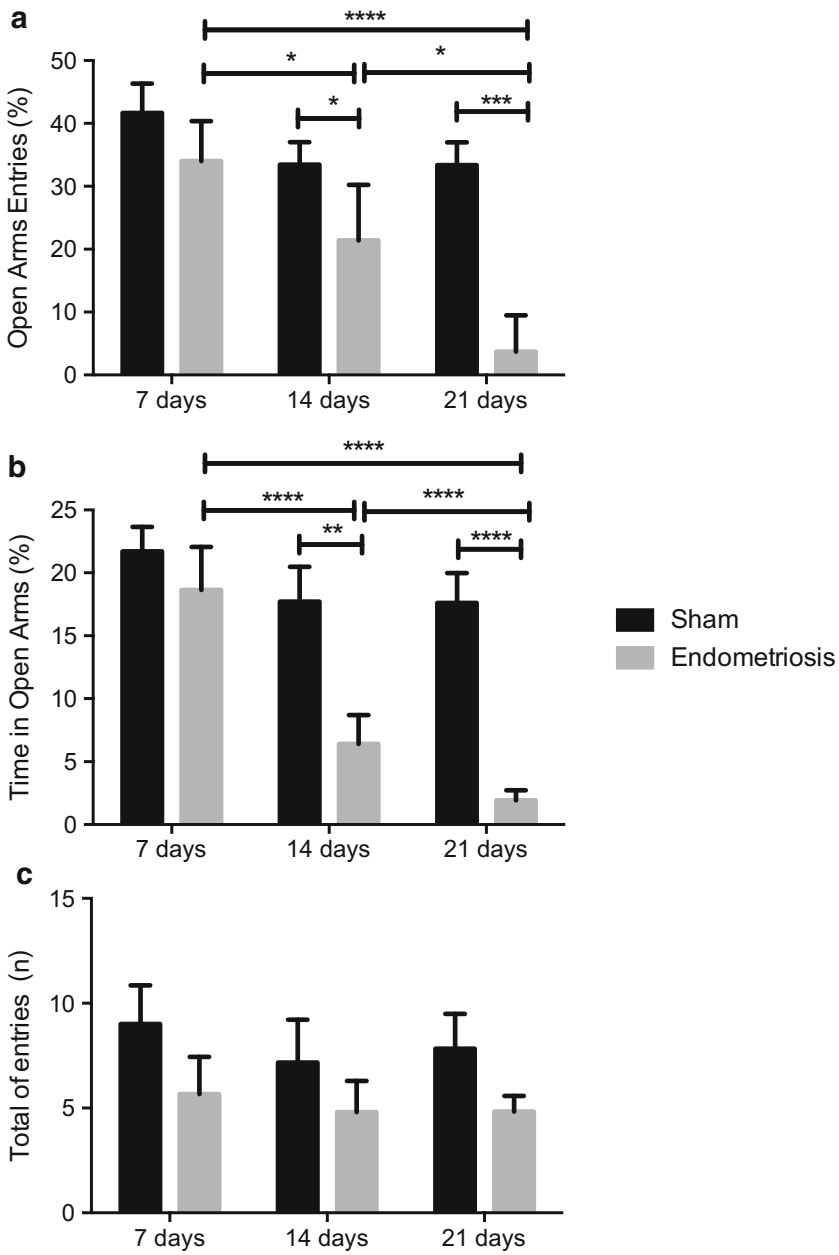

Fig. 3 Effect of endometriosis model in the percentage of open arms entries (**a**), percentage of time in open arms (**b**) and number of total entries (**c**) in the plus maze test. Female rats were submitted to surgically-induced endometriosis or sham-operated (control surgery). Female rats were submitted to surgically-induced endometriosis or sham-operated (control surgery). The behavioral determinations were conducted on the 7th, 14th and 21st days post-induction. Bars represent means ± SEM (n = 8 animals/group). Data were analyzed using two-way ANOVA followed by Fisher's LSD as post hoc test for the percentage of open arms entries and time in open arms. For total entries, Tukey's test as post hoc test was conducted. P < 0.05, **P < 0.01, ***P < 0.001, ****$P < 0.0001$

Endometriosis model cause a progressive decrease in pain sensitivity threshold in rats

Regarding the number of acetic acid-induced writhes, two-way ANOVA revealed a significant interaction between factors [F (2, 42) = 3.582, $P = 0.0403$]. In the post hoc test, it was observed a significant increase in the number of writhes in the endometriosis rats in all time-points used for evaluation: 7th ($P < 0.001$), 14th ($P < 0.05$) and 21st (P < 0.05) days. Interestingly, along the temporal course of the model, a significant reduction in the number of writhes in the endometriosis groups between the 7th and 14th days of evaluation was observed ($P < 0.05$) (Fig. 5a).

In the hot plate test, two-way ANOVA showed significant main effect of "time" [F (2, 42) = 5.499, $P = 0.0097$]. In multiple comparison test, it was observed a significant reduction in the latency to pain reaction in the endometriosis group on the 14th ($P < 0.01$) and 21st (P < 0.01) days of evaluation compared to sham rats. It was also found a significant reduction in this parameter in the comparison of endometriosis group between the 7th versus 21st days (P < 0.05) (Fig. 5b).

Time-related growth of endometriosis implants

As shown in Fig. 5c, a significant two-way interaction between factors was observed [F (3, 42) = 10.77, $P < 0.0001$]. In the post hoc analysis, a marked increase in wet weight starting on day 1 post-surgery and progressively in all evaluated time-points, i.e., 1st versus 7th (P < 0.05), 1st versus 14th

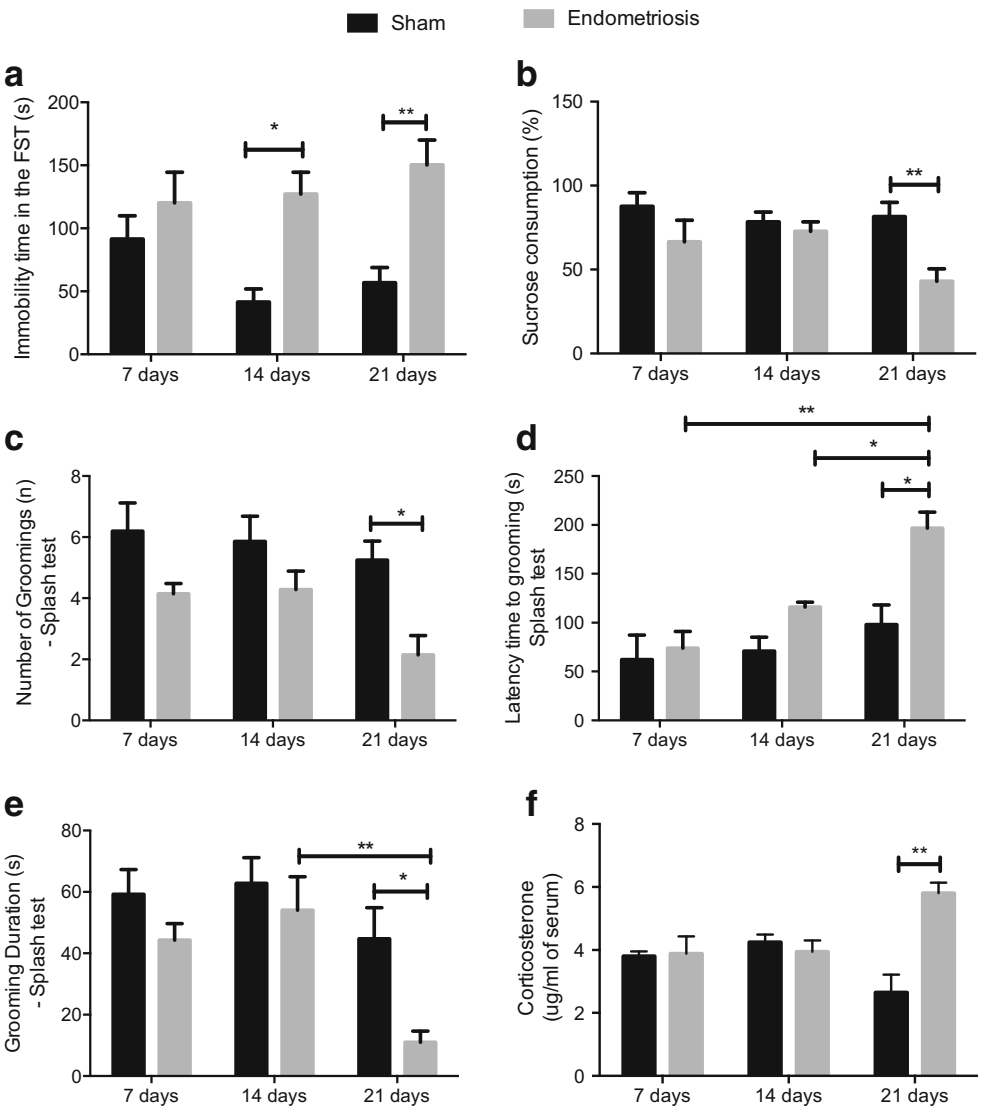

Fig. 4 Effect of endometriosis model in immobility time in the Forced Swimming Test (**a**), sucrose consumption (**b**), number of grooming events (**c**), latency to onset of grooming in splash test (**d**), total grooming duration (**e**) in the splash test as parameters of depressive-like behavior and corticosterone stress reactivity (**f**). Female rats were submitted to surgically-induced endometriosis or sham-operated (control surgery). The behavioral determinations were conducted on the 7th, 14th and 21st days post-induction. Thirty minutes, after the FST test, a sample of trunk blood was collected (about 1 ml) of each animal, and the serum concentrations of corticosterone was measured. Bars are means ± SEM (n = 8 animals/group). Data were analyzed using two-way ANOVA followed by Tukey's post hoc test. *P < 0.05, **P < 0.01

(P < 0.01), 1st versus 21st (P < 0.001), 7th versus 21st (P < 0.05) and 14th versus 21st (P < 0.01) was observed. No alterations were observed in dry weight.

Endometriosis promotes hippocampal time-related pro-oxidative alterations

As shown in Fig. 6a, a significant reduction in GSH levels in the endometriosis group compared to sham controls just on the 21st day post-surgery was observed (P < 0.05) (two-way ANOVA: significant main effect of "endometriosis model" [F (1, 30) = 6.703, $P = 0.0151$]. In the evaluation of lipid peroxidation (Fig. 6b), a significant increase in MDA levels in the endometriosis group compared to sham controls on the 14th (P < 0.05) and 21st of evaluation (P < 0.05) was detected (two-way ANOVA: significant main effect of "endometriosis model" [F (1, 30) = 18.2, P = 0.0002]). Regarding SOD activity (Fig. 6c), it was noteworthy the significant increase in SOD activity in the endometriosis group compared to sham on the 21st day of evaluation (P < 0.01). Also, when comparing the activity of this enzyme in different time points, we also observed a significant increase in SOD activity in endometriosis group at the 21st day compared to 7th day post-surgery (P < 0.01) (two-way ANOVA: significant interaction between factors [F (2, 30) = 3.516, $P = 0.0465$]). MPO activity (Fig. 6d) was significantly increased in the endometriosis group compared to sham on the 21st day post-surgery (P < 0.05). Interestingly, a significant increase was also notable between endometriosis groups when comparing day 21 with day 7 day (P < 0.05) (two-way ANOVA: ANOVA: significant interaction between factors [F (2, 36) = 3.912, $P = 0.0296$]).

Regarding BDNF protein levels (Fig. 6e), a significant decrease in the endometriosis group on the 21st day compared to its respective sham controls (P < 0.01) was detected. It was also observable a time-related decrease in BDNF levels between endometriosis group when comparing 7th day versus

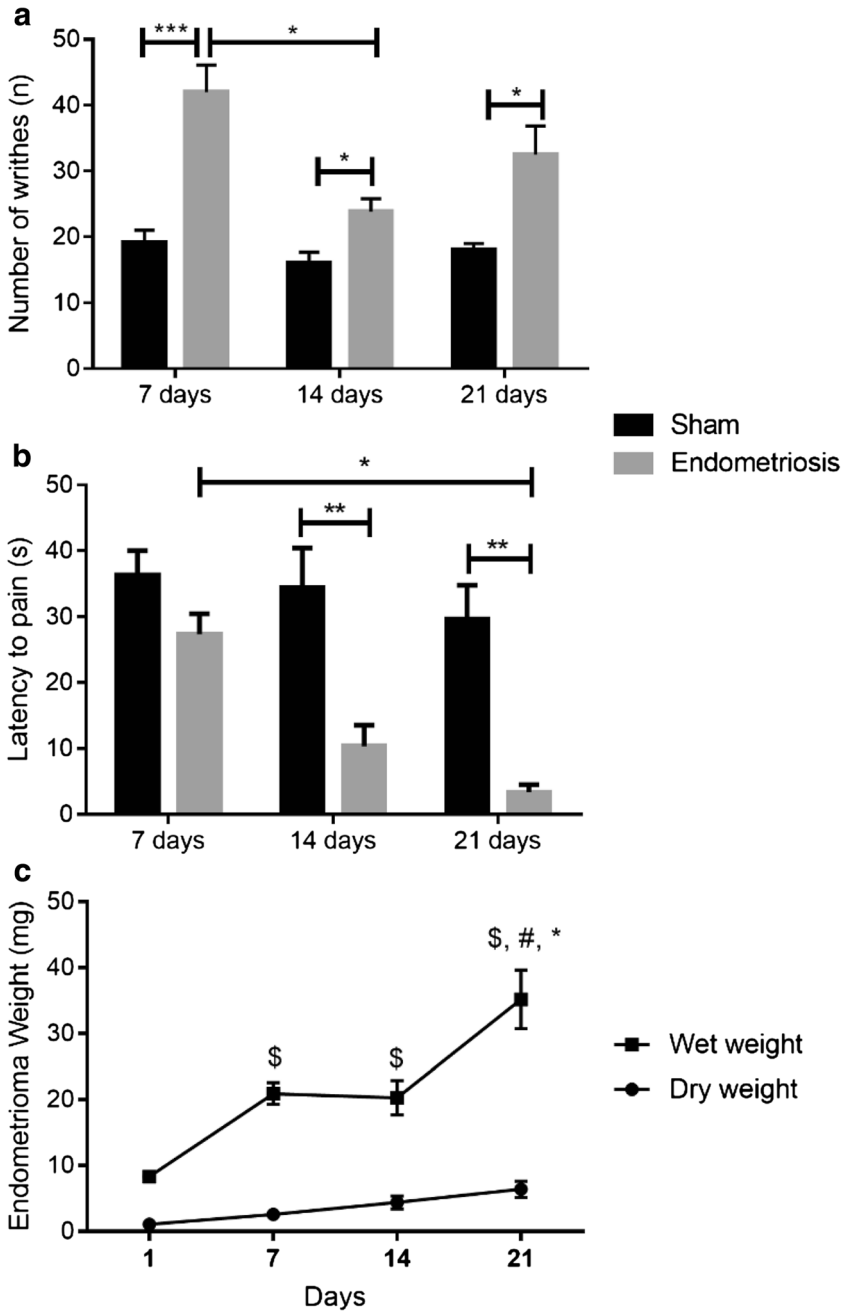

Fig. 5 Effect of endometriosis model in the number of writhes in the model of acetic acid-induced abdominal contractions (**a**) and latency to pain in the hot plate test (**b**), as measures of pain sensitivity, and endometrioma wet and dry weight (**c**). Female rats were submitted to surgically-induced endometriosis or sham-operated (control surgery). The behavioral determinations were conducted on the 7th, 14th and 21st days post-induction. In the case of endometrioma weight on the 1st, 7th, 14th and 21st days after induction the animals were sacrificed, and the endometrial implants were resected and weighted (dry and wet weight). Bars represent means ± SEM of 8 animals/group, for the measures of pain sensitivity and endometrioma weight. Data were analyzed using two-way ANOVA followed by Tukey's post hoc test. *P < 0.05, **P < 0.01, ***$P < 0.001$. In Fig. 5c, symbols represent: $^{\&}P < 0.05$ versus day 1, $^{\#}P < 0.05$ versus day 7, and *P < 0.05 versus day 14

14th ($P < 0.01$) and versus 21st day ($P < 0.01$) (two-way ANOVA: significant interaction between factors [$F(2, 18) = 4.065$, $P = 0.0349$].

Discussion

In the present study, we performed a broad and weekly behavioral evaluation of rats submitted to the endometriosis model induced by peritoneal auto-transplantation of uterine tissues for three weeks. Here, endometriosis rats displayed important time-related affective and pain sensitivity changes. Of note, endometriosis caused anxiogenic-like, despair-like behavior and pain sensitization alterations since the 14th day of evaluation, but only at the 21st day a full spectrum of depression-like changes emerged, with the emergency of anedonic- and apathetic-like behaviors. We also observed, as far as we know for the first time, that endometriosis increased corticosterone stress reactivity and induced marked hippocampal pro-oxidative changes. These findings were followed by a reduction in hippocampal BDNF expression on the 21st day post-induction. Therefore, this model represents an important tool to study the neuropsychiatric symptoms and pathophysiological basis of endometriosis.

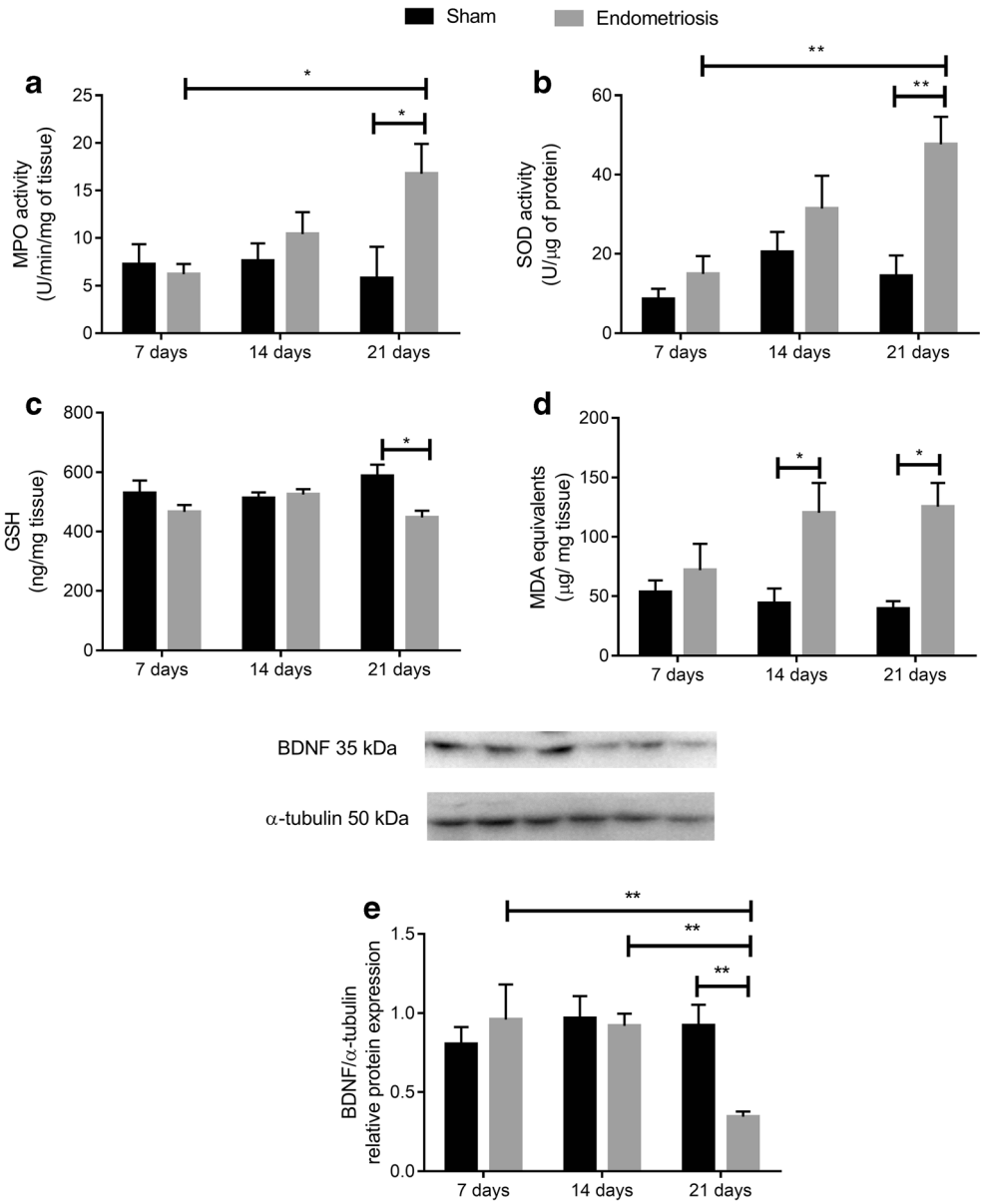

Fig. 6 Effect of endometriosis model in markers of oxidative stress (**a**-**d**) and BDNF protein levels (**e**) in the hippocampus. Female rats were submitted to surgically-induced endometriosis or sham-operated (control surgery). The neurochemical determinations were conducted in the hippocampus of animals dissected on the 7th, 14th and 21st days post-induction. Bars represent means ± SEM ($n = 6$–7 animals/group). In the case of BDNF, western blotting was performed in 4 samples/group. Data were analyzed using two-way ANOVA followed by Tukey's post hoc test for oxidative stress markers or Fisher's LSD post hoc test for the analysis of BDNF levels. *P < 0.05, **P < 0.01

Endometriosis affects social and psychological aspects of woman lives (Culley et al. 2017). Taken this into consideration, it is not surprisingly that endometriosis patients have higher rates of neuropsychiatric comorbidities and mental health problems (Pope et al. 2015).

Despite the value of animal models to the study of endometriosis pathophysiology (Tirado-González et al. 2010), few studies have consistently evaluated the presence of neuropsychiatric symptoms and their correlated biological mechanisms in these models. In this context, Li et al. 2018 recently reported that mice submitted to a similar model of endometriosis showed anxiety-like alterations in open field test two weeks post-induction accompanied by increased immobility time in the tail suspension test at four weeks. These findings were followed by interesting electrophysiological and gene expression alterations in brain areas related to mood regulation, such as insula, amygdala and hippocampus (Li et al. 2018).

We found increased anxiety-like behavior starting two weeks after the surgery and maintained at the third week post-induction. Additionally, mainly at the third week after surgery, endometriosis rats developed other behavioral alterations that resemble depressive-like symptoms, namely anhedonia (in the sucrose preference test), apathetic-like behavior (in the splash test) and despair-like behavior (in the forced swimming test).

Some apparent discrepancies between our findings and previous ones (Li et al. 2018) could be related to different animal strains used, since rats are more vulnerable to depressive-like behavior in stressful conditions (Koolhaas et al. 1997; Willner 2017). Furthermore, this previous study (Li et al. 2018) used

tail suspension, although forced swimming test seems to be more sensitive to assess depressive-like behavior.

It has been hypothesized that endometriosis-induced stress is related to a deregulation of the HPA axis response (Tariverdian et al. 2010; Cuevas et al. 2012). Indeed, both hypo- and hypercortisolism have been described for a number of chronic pain conditions including endometriosis (Gur et al. 2004; Lima et al. 2006; Hannibal and Bishop 2014). More recently, increased expression of corticotropin releasing hormone (CRH) receptors was demonstrated in endometriotic lesions in the auto-transplantation model while their blockade with a pharmacological antagonist reduced the size and number ectopic lesions (Torres-Reverón et al. 2018).

Cortisol secretion in response to a psychosocial stressor more than basal cortisol has been used to predict HPA axis reactivity (Dienes et al. 2013). Accumulated evidence demonstrated that depressed and high-risk depression individuals present higher cortisol levels during the recovery to a social stressor (Young et al. 2000; Burke et al. 2005). In rodent models, this response is achieved when submitting the animals to a typical acute stressor, such as forced swimming or restrained stress, and several studies showed that chronically stressed-animals have higher corticosterone response than controls (O'Mahony et al. 2011; Cox et al. 2011).

Here, endometriosis rats presented increased levels of corticosterone in response to an acute stressor on the 21st day of evaluation, pointing to a hyper-reactive state of the HPA axis specifically at the time point that these animals manifested the more complete depressive-like alterations (behavioral despair, anhedonic and apathetic-like alterations). This is also in agreement with a previous study showing that endometriosis caused a marked decrease in corticotropin-releasing factor expression in the hippocampus of rats, a marker of disruption in HPA axis feedback loop (Cuevas et al. 2012).

It is worth to mention that pain, such as, dysmenorrhea, dyspareunia and chronic pelvic pain, is a central feature of endometriosis and have a strong negative impact on mental health status of these patients (Carvalho et al. 2015). Some studies have reported that the experience of pain more than endometriosis itself is decisive for emotional distress and the emergence of neuropsychiatric symptoms (Souza et al. 2011). However, it is difficult to separate these aspects (pain and endometriosis itself), since both influence each other and could share common mechanisms, such as neural and immune-oxidative mechanisms, to the development of behavioral changes (Cuevas et al. 2012; Walker et al. 2014). Stress load can be a breaking factor for these manifestations, once it was recently showed that psychological stress exacerbated inflammatory manifestations and altered pain threshold in an endometriosis rat model (Hernandez et al. 2017).

In the present study, we evaluated rat pain perception based on two different tests for visceral and peripheral sensibility respectively at the development of endometriosis model. Endometriosis is related to peripheral and central pain sensitization (As-Sanie et al. 2013). In this condition, initially the peripheral nociceptive system is sensitized by tissue injury or inflammation, resulting in a decreased pain threshold and an amplified sensory input to CNS. With this continuous stimuli, central actions can become independent of any peripheral inputs due to long-term central adaptations in the process called central sensitization (Latremoliere and Woolf 2009).

In our results, we found that endometriosis rats presented increased visceral sensitivity in all time points of observation. Further, this response showed a U-shaped pattern, being more intense on the 7th day after the model induction and returning to increase at the 21st day of evaluation. Regarding pain sensitivity, the increased thermal sensitivity just became apparent after the second week, advocating for an ongoing process of central sensitization in the development of the model. Our findings are in accordance with previous studies showing decreased thermal and mechanical pain threshold in mice and rats in a similar model of endometriosis (MMed et al. 2012; Simsek et al. 2014), and with previous findings (Li et al. 2018) showing that the main changes in thermal sensibility started at 2 weeks after the implants surgery.

Here, the growth of peritoneal implants was weekly monitored by measuring their wet (fluid-filled) and dry (lanced) weight. Although we observed just a mild increase in dry weight along the course of the model, a marked increase in wet weight was noted specially on the 21st day post-surgery. This increase indicates an implant growth mainly related to the development of cystic and cystic-like lesions. These findings are in agreement with a previous work showing that two to four weeks after implants surgery the growth of lesions are mainly cyst-like, fluid filled and surrounded by peritoneal adhesions, while lesions evaluated at the first week after the model induction are markedly inflamed and hemorrhagic (Pelch et al. 2012).

Taken together, in our experimental conditions, it is possible to observe an interesting relationship between the implant's growth and the development of pain and affective-like symptoms. Notably, regarding visceral sensitivity, the U-shaped response observed here could be explained by the variations in the pro-inflammatory environment mounted around endometrial implants and involving regional pelvic and peritoneal structures. As previously mentioned, an intense local inflammatory and hemorrhagic response occurs at the beginning of the implants development in this model (Pelch et al. 2012). With the model progression, inflammation returns to increase following cystic growth and invasion of peritoneal organs (Li et al. 2016). This hypothesis needs further experimental validation, but the relationship between vaginal hyperalgesia and cystic growth and innervation was already demonstrated in rats (McAllister et al. 2009).

Considerable evidence supports the role of oxidative stress in the development of endometriosis (Ngô et al. 2009; Donnez et al. 2016; Scutiero et al. 2017; Vitale et al. 2018).

Nevertheless, no study at present evaluated the brain oxidative status of endometriosis patients (through CSF measurements or brain post-mortem samples) or the brain of animals submitted to endometriosis model.

Here we decided to evaluate oxidative alterations in the hippocampus. This brain area, besides memory, is a core anatomical area involved in the regulation of mood and anxiety (Bannerman et al. 2004), and in the transition from acute to chronic pain (Mutso et al. 2014). Recently, it was demonstrated that endometriosis patients presented abnormal connectivity in anterior hippocampus and in their afferences to somatosensory and frontoinsular cortex, areas importantly involved in processing of anxious and painful stimuli. These researchers also demonstrated a strong positive correlation between these hippocampal findings and the presence of depression/anxiety symptoms in these patients (Beissner et al. 2016).

Here, we showed that endometriosis caused a marked imbalance in oxidative state in the hippocampus of rats, as demonstrated by the decreased levels of the endogenous antioxidant, GSH combined with the increase in lipid peroxidation and augmented SOD activity. Interestingly, the main oxidative alterations occurred at the 21st day of evaluation, when the main affective and pain-related changes are also present.

In depression research, several studies have reported increased lipid peroxidation and impaired GSH levels in the serum of depressive patients (Black et al. 2015), and a recent meta-analysis found a positive correlation between depressive symptom severity and lipid peroxidation levels (Mazereeuw et al. 2015). Also, in accordance with our findings, several studies showed increased levels of lipid peroxidation and diminished GSH levels in mood-regulation areas, such as the hippocampus, in depression models namely chronic mild stress (Réus et al. 2014), lipopolyssacaryde-induced depression-like behavior (Tomaz et al. 2014) and olfactory bulbectomy (Túnez et al. 2010).

SOD is an enzyme responsible for the dismutation of O_2 – to H_2O_2, thereby reducing the hydroxyl radical (OH). Thus, SOD has been established as a useful marker of response to ROS formation and oxidative stress (Sun et al. 1988). In this context, despite oxidative stress is a consensual point in depression, there are conflicting findings in literature regarding SOD activity in clinical (Russo 2010; Tsai and Huang 2016) and preclinical studies (Moretti et al. 2013; Budni et al. 2013). Here, we found increased SOD activity in the hippocampus of endometriosis rats. We hypothesized that this increase in SOD activity occurred as an adaptive cell response to the high amount of ROS and oxidative damage, here, evidenced by the marked increase in MDA and decreased GSH levels.

Oxidative stress and inflammation are closely related pathophysiological processes (Biswas 2016). MPO is an key enzyme of innate immune system that is activated in phagocytic cells in inflammatory conditions (Arnhold and Flemmig 2010). MPO generates strong oxidant radicals, in special, hypochlorous acid (HOCl), that can covalently modify lipids and proteins (Arnhold and Flemmig 2010; Pattison et al. 2012). In line with this evidence, important studies reported an increased MPO activity and expression in serum of depressive patients (Vaccarino et al. 2008; Talarowska et al. 2015), and in the brain of rodents submitted to depression models (Maes et al. 2011; Mello et al. 2018). In the present study, we demonstrated that endometriosis promoted a time-related increase in MPO activity in rat hippocampus, reaching the higher levels at the 21st day post-surgery. Taken together, our findings advocates for the induction of an immune-oxidative milieu in the hippocampus of endometriosis rats that could represent a potential underlying mechanism for the emergence of the depression and pain sensitization changes observed here.

Neurotrophic factors are critical regulators of the brain neurogenesis and synaptic plasticity. In this context, BDNF is an important neurotrophic factor, whose expression is closely regulated by synaptic activity (Xu et al. 2000). Compelling evidence have reported that stress impaired BDNF expression in the hippocampus of rodents (Murakami et al. 2005; Lee and Kim 2010), and that this effect can be reversed by antidepressants (Baj et al. 2012). At the best of our knowledge, no study directly investigated BDNF levels in the brain of endometriosis rats, as well as its possible association with the emergence of the endometriosis-related neuropsychiatric alterations. Here, we demonstrated, for the first time, that endometriosis rats showed a progressive reduction in hippocampal BDNF levels in the temporal course of the model. This is in accordance with the other immuno-oxidative findings observed here, allowing us to propose that an oxidative/degenerative environment develops in the hippocampus of rats after the induction of endometriosis model.

In this context, Bouvier et al. 2017 demonstrated that BDNF constitutively controlled the nuclear translocation of the master redox-sensitive transcription factor Nrf2, which regulate the expression of several antioxidant defenses (Bouvier et al. 2017). Not only this, these authors showed that the persistent state of oxidative stress observable in stress vulnerable animals was due to diminished BDNF concentrations in the hippocampus (Bouvier et al. 2017). Therefore, we can infer that the endometriosis-induced impairment in hippocampal BDNF represents a central mechanism underlying the emergence of the behavioral and possibly brain pro-oxidative changes that follows this model.

Our findings also corroborates the interesting parallelism already reported between the behavioral disturbances that follows endometriosis, such as anxiety, depression, somatic symptoms, and the severity/progression of gynecological lesions and pain symptoms (Laganà et al. 2015, 2017a; Vitale et al. 2017). Despite it is not possible to delineate the specific contribution of each of these factors yet, a suggestive reciprocal relationship seems to exist between these groups of

symptoms in the course of endometriosis, and effective therapies should consider an integrated approach of them.

The present study has some important limitations since we did not perform a longer evaluation, extending for periods so long as 6 or 12 weeks and did not deeply evaluate the participation of inflammatory-immune signaling and glial cells to the development of the behavioral and brain neurochemistry changes observed here.

Conclusion

In conclusion, we noticed the presence of several behavioral features that resemble human depression, such as anxiety, anhedonia, apathy, and despair-like behavior, besides pain sensitivity changes. Further, we demonstrated that endometriosis causes a hyper-reactivity of corticosterone response, progressive increases in oxidative stress markers in the hippocampus and a marked reduction in hippocampal BDNF levels. Therefore, our data brings new evidence to better clarify the pathophysiological basis underlying the development of endometriosis-related neuropsychiatric symptoms, as well as, opens new perspectives for the use of animal models of this gynecological disease to study new drug candidates to treat both the disease and its neuropsychiatric comorbidities.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest for the present investigation.

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Affiliations

Paulo Wagner Linhares Lima Filho[1,3] · Adriano José Maia Chaves Filho[1] · Charliene Freire Xavier Vieira[1] · Tatiana de Queiroz Oliveira[1] · Michelle Verde Ramo Soares[1] · Paloma Marinho Jucá[1] · Joao Quevedo[4,5,6] · Tatiana Barichello[4,5,6] · Danielle Macedo[1,2] · Francisco das Chagas Medeiros[3]

[1] Neuropsychopharmacology Laboratory, Drug Research and Development Center, Faculty of Medicine, Department of Physiology and Pharmacology, Universidade Federal do Ceará, Rua Cel. Nunes de Melo 1000. CEP, Fortaleza, Ceará 60430-270, Brazil

[2] National Institute for Translational Medicine (INCT-TM, CNPq), Ribeirão Preto, Brazil

[3] Surgery Department, Faculty of Medicine, Universidade Federal do Ceará, Fortaleza, CE, Brazil

[4] Translational Psychiatry Program, Department of Psychiatry and Behavioral Sciences, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, USA

[5] Center of Excellence on Mood Disorders, Department of Psychiatry and Behavioral Sciences, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, USA

[6] Laboratory of Neurosciences, Graduate Program in Health Sciences, University of Southern Santa Catarina – UNESC, Criciúma, SC, Brazil