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Original Article

Effect of Physical Exercise on Endometriosis Experimentally Induced in Rats

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Abstract

Objective: Endometriosis is characterized by the growth of endometrial tissue outside the uterine cavity. The prevalence of endometriosis among women experiencing pain, infertility, or both is as high as 35% to 50%. The most common symptoms of endometriosis are dysmenorrhea, dyspareunia, chronic pelvic pain, and infertility. Evidence has suggested that endometriosis symptoms result from a local inflammatory peritoneal reaction caused by ectopic endometrial implants that undergo cyclic bleeding. On the other hand, regular physical exercise seems to have protective effects against diseases that involve inflammatory processes such as type 2 diabetes and colon and breast cancer. On this basis, it is possible that the practice of physical exercise may have beneficial effects on endometriosis. Therefore, the objective of this study was to evaluate the possible anti-inflammatory effect of physical exercise on endometriosis experimentally induced in rats. **Study Design:** Seventy female Wistar rats were divided into 7groups of 10 animals each. Animals performed light exercise (swimming once a week), moderate exercise (swimming 3 times a week), and intense exercise (swimming 5 times a week) before or after endometriosis induction. **Results:** At the end of the experimental protocol, a reduction in the size of endometriotic lesions was observed after physical exercise regardless of its frequency, with a greater reduction in the groups practicing moderate and intense activity; an increase in FAS levels and a decrease in matrix metalloproteinases 9 and proliferating cell nuclear antigen (PCNA)levels was also observed. The immunohistochemistry results did not lead to conclusive results. As expected, oxidative stress was reduced in all groups. These results show that the practice of physical exercise could be beneficial, at least in part, for the treatment of endometriosis.

Keywords

endometriosis, physical exercise, swimming, treatment

Introduction

Endometriosis is characterized by growth of endometrial tissue outside the uterine cavity.[1,2] The prevalence of endometriosis among women experiencing pain, infertility, or both is as high as 35% to 50%.[3] The most common symptoms of endometriosis are dysmenorrhea, dyspareunia, chronic pelvic pain, and infertility. Evidence has suggested that the symptoms of endometriosis result from a local inflammatory peritoneal reaction caused by ectopic endometrial implants that undergo cyclic bleeding.[4]

The pathogenesis of endometriosis remains controversial, and the most widely accepted theory to explain it is Sampson transplantation theory, whereby cells from the uterine cavity, moving through the fallopian tubes into the pelvic cavity during menstruation, deposit viable tissue, which implants on the peritoneal surface.[5] However, 90% of women have retrograde flow during menses, but only 10% to 15% of reproductive-age women develop endometriosis.[6] These data suggest that normal women may have protective mechanisms, whereby endometrial cells are removed from the peritoneal cavity or are prevented in some way from implanting and growing. For this reason, it is believed that immunological factors could be associated with the growth and implantation of viable endometrial cells in ectopic sites. Adhesion between endometrial tissue and

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peritoneum probably occurs by the action of integrins, transmembrane receptors that mediate attachment between cells and their surroundings.[7] This could occur by imbalance between compounds of extracellular matrix such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPS). Studies have shown an increase in some MMPs and a decrease in TIMPS in the topic and ectopic endometrium of women with endometriosis, which could explain at least in part the invasive ability of this tissue on retrograde menstrual flow.[8] After invasion, viability of the implants would be maintained by angiogenesis through vascular endothelial growth factor and cytokines. Vascular endothelial growth factor is probably produced and secreted by infiltrated peritoneal fluid macrophages, promoting endothelial growth, increased vascular permeability, and modulation of secretion of proteolytic enzymes related to angiogenesis.[9,10]

Oxidative stress also seems to be involved in the physiopathology of endometriosis, since reactive oxygen species appear to be increased in the peritoneal fluid of women with endometriosis.[11]

All of these environmental modifications somehow contribute to the development and maintenance of the inflammatory process associated with endometriosis. On the other hand, regular physical exercise seems to have protective effects against diseases involving inflammatory processes such as type 2 diabetes and colon and breast cancer.[12,13] Physical activity may also increase the levels of some hormone-binding globulin, reducing estrogen bioavailability and being beneficial in estrogen-dependent diseases such as endometriosis.[14,15] In addition, regular physical exercise is associated with a reduction of insulin resistance,[15] reduction of menstrual flow and ovarian stimulation, and increases in the systemic levels of cytokines with anti-inflammatory properties.[16,17] Despite this evidence about the possible benefits of physical exercise for the treatment of endometriosis, to date, there are no controlled and randomized studies in the literature identifying whether physical exercise prevents the occurrence or progression of endometriosis. The few studies available indicating an inverse relationship between practice of physical exercise and the risk of endometriosis are of the observational type, with little or no statistical significance.[18] Thus, the available literature does not allow us to point out the real role of physical exercise in endometriosis. Therefore, the objective of this study was to evaluate the effect of physical exercise on endometriosis experimentally induced in rats.

Methodology

Study Design

Seventy female Wistar rats were divided into 7 groups of 10 animals each: sedentary (no exercise), light exercise (once a week), moderate exercise (3 times a week), and intense exercise (5 times a week). To study a possible protective effect of physical exercise against the establishment of endometriosis, 3 groups performed physical exercise before endometriosis induction. To evaluate physical exercise as a therapy, another 3 groups performed physical exercise after endometriosis induction. Swimming was chosen as physical exercise because rats have an innate swimming ability and acclimate well to training[19,20] so that swimming is less stressful for these animals.[21,22]

Ethical Approval

The Ethics Committee on Animal Experimentation of the University of Sao Paulo approved the study (Protocol n 059/2010).

Endometriosis Induction

Endometriosis induction was performed after general anesthesia (0.4 mL thionembutal in association with 0.2 mL xylestesin). A pelvis incision of approximately 2 cm was performed. The right uterine horn was resected and a 5 × 5 mm fragment was removed and sutured in its own peritoneum with vicryl 6.0, with the endometrial side facing the abdominal cavity. No hormonal supplementation was administered before or after surgery.

Physical Exercise Protocol

Before starting the swimming protocol, the animals were allowed to adapt to water in a 0.9 × 0.9 × 1.2 m tank for 5 days.[20] On the first day, the animals were placed for 15 minutes in 5 cm deep water of 34°C ± 1°C temperature. The water level was then raised on consecutive days up to 50 cm on the fifth day. After this period, the exercise protocol was started. The sedentary group (group 0) did not exercise and was left in the home cage throughout the protocol time. The light exercise group (group 1) swam once a week; the moderate exercise group (group 3) swam 3 times a week; and the intense exercise group (group 5) swam 5 times a week. The swimming protocol lasted 5 weeks, and the intensity of exercise was increased progressively, starting with 20 minutes during the first week and ending with 60 minutes by the fifth week. The study protocol for all groups (physical exercise before or after endometriosis induction) lasted 10 weeks, that is, 4 weeks necessary for implant establishment, 1 week for adaptation to water, and 5 weeks of the swimming protocol. Throughout the swimming protocol, the investigators stood by the side of the swimming tank and constantly stimulated the animals to continue swimming.

Sample Collection and Lesion Size

At the end of the 10-week protocol, the animals were anesthetized with thiopental (50 mg/kg), and 5 mL blood was collected for the oxidative stress test. The pelvic cavity was then opened, and the endometriosis lesions were resected for histological and gene expression analysis. The contralateral uterine horn (left horn) was also resected as control.

Tissues were divided in half, partially fixed in 10% formol, processed for paraffin embedding, and stained with hematoxylin and eosin (HE) for histological analysis in order to confirm the presence of glandular and stromal endometrial tissue. The presence of endometriotic tissue was confirmed in one-half of the tissue sample in 100% of the 70 induced lesions (n = 10 per group). All animals were euthanized during the same phase of the estrous cycle (diestrus) for standardization of the results. The other half of all the collected tissue samples was used for analysis of gene expression. The widest diameters of the lesions were measured with a ruler in order to calculate the area of the lesions.

Gene Expression

The samples were washed in 1× phosphate-buffered saline (8.50 g/L sodium chloride, 1.11 g/L disodium hydrogen phosphate, 2.81 g/L disodium phosphate, 0.20 g/L monopotassium phosphate, pH 7.0) to remove the RNAlater solution. Next, total RNA (50 mg tissue) was extracted with TRIzol reagent (Invitrogen Life Technologies, Paisley, United Kingdom) and treated with DNase I (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA integrity was confirmed by the presence of the 28 S and 18 S ribosome bands when analyzed by 1% agarose gel electrophoresis with 1 × 3-(*N*-morpholino)propanesulfonic acid), (MOPS) (*N*-morpholino) propanesulfonic acid buffer. The total RNA concentration was determined by spectrophotometry (NanoDrop 2000c, Thermo Scientific, Wilmington, Delaware) at 260 nm. The extracted total RNA was stored in a freezer at −80°C for subsequent use. One microgram of total RNA from each sample was reverse transcribed using the High Capacity cDNA Transcription Kit (Applied Biosystems Life Technologies, Warrington, United Kingdom) according to the manufacturer's instructions. The tissue samples collected were analyzed by relative quantification of the *Fas, Mmp9, Timp2, and Pcna* genes using an ABI PRISM 7500 FAST instrument (Applied Biosystems Life Technologies). The reactions were conducted using the TaqMan Gene Expression Assays (TaqMan minor groove binder probes, FAM dye-labeled) from Applied Biosystems. The assay identifications of the probes used were *Fas* (Rm00685720_m1), *Mmp9* (Rm00579162_m1), *Timp2* (Rm005732332_m1), and *Pcna* (Rm00673588_m1). Quantitative real-time polymerase chain reaction (PCR) was performed in triplicate in a final volume of 20 μL using the following conditions: 10 μL TaqMan Universal PCR Master Mix (2×; Applied Biosystems Life Technologies), 1 μL TaqMan Gene Expression Assay (20×; Applied Biosystems Life Technologies), and 9 μL of complementary DNA (cDNA) diluted 1/50. The reaction conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute.

The relative quantification of the genes was calculated for each sample by the 2-delta delta Ct [$2^{-\Delta\Delta C^{T}}$] method. A pool of cDNA containing equal quantities of the samples obtained from eutopic endometrium of the control group (sedentary group) was used as calibrator sample. The Gapdh gene was used to normalize the reactions.

Immunohistochemistry

Histological sections of 4 to 5 μm were submitted to histochemistry by the antigen–antibody reaction. Deparaffinized and hydrated sections were recovered antigenically by incubation in buffered medium in a steam pot for 40 minutes. After cooling, endogenous tissue peroxidases were removed by adding hydrogen peroxide, and horse serum was added to prevent nonspecific binding of the primary antibody. The slides were then incubated with primary antibodies obtained from Novocastra Laboratories Ltd (Newcastle-upon-Tyne, United Kingdom). Cell proliferation was determined using proliferating cell nuclear antigen (PCNA) (product code NCL-PCNA, clone PC10) with nuclear labeling at 1:200 dilution. FAS (product code NCL-FAS-310, clone GM30) was used for the determination of apoptosis with membrane labeling at 1:100 dilution. Cell migration and differentiation were determined by Mmp9 (product code NCL-MMP9-493, clone 15W2) with nuclear labeling at 1:400 dilution. The tissue inhibitor of MMP9 was determined using TIMP2 (product code NCL-TIMP1-485, clone 46E5) with cytoplasmic labeling at 1:150 dilution. The material was then incubated with secondary antibody and submitted to the avidin–biotin step. The reaction was developed by treatment with 3,3′-Diaminobenzidine (Sigma-Aldrich Inc, St Louis, MO, USA) for 5 minutes, counterstained with Harris HE and mounted on slides. All slides were evaluated by 2 pathologists experienced in immunohistochemistry who were not aware of the type of tissue to be analyzed. The cells were considered to be positively or negatively labeled for each marker according to literature criteria (Immuno Reactive Score).[23] For MMP9, TIMP2, and FAS, the absence of labeling (0%) was scored as "1+." Labeling from 1% to 10% was scored as "2+," labeling from 10% to 50% was scored as "3+," cell labeling from 51% to 80% was scored as "4+," and labeling exceeding 80% was scored as "5+." For PCNA, labeling of less than 20% of the cells was scored as "1+." Labeling from 20% to 70% was scored as "2+," and labeling of more than 70% was scored as "3+."

Oxidative Stress

Oxidative stress was determined by quantification of plasma 8-isoprostane levels using the 8-Isoprostane EIA Kit (Cayman Chemical, Ann Arbor, Michigan, USA).

Statistical Analysis

Data are presented in figures in the original scale. However, the variables of gene expression were log10 transformed because one of the assumptions (linearity) in the linear model analysis was not satisfied. These analyses specify that the conditional average E (y|x = x 0) from the response variable y, given the x 0 value of the predictor vector x, is linear in x 0. The application of the linear models can be extended assuming that an

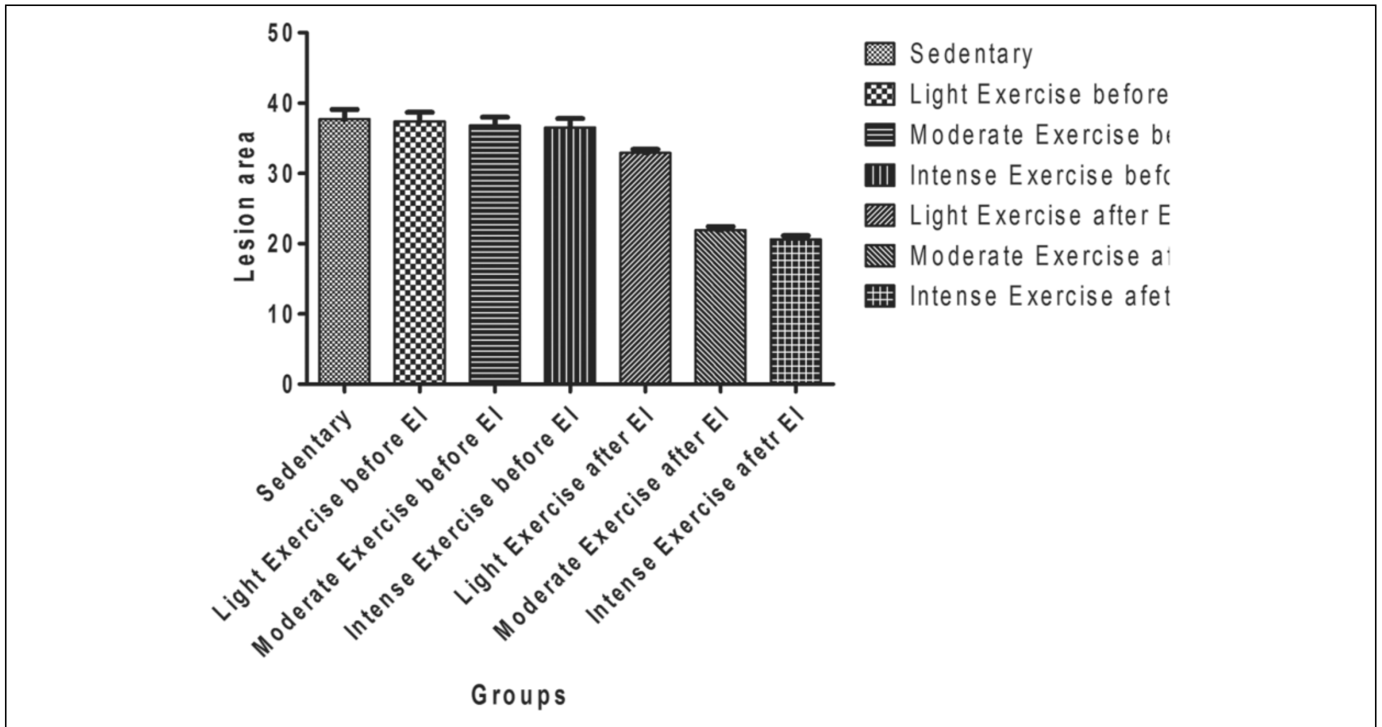

Figure 1. Comparison of the study groups regarding the variation in total lesion area. EI indicates endometriosis induction.

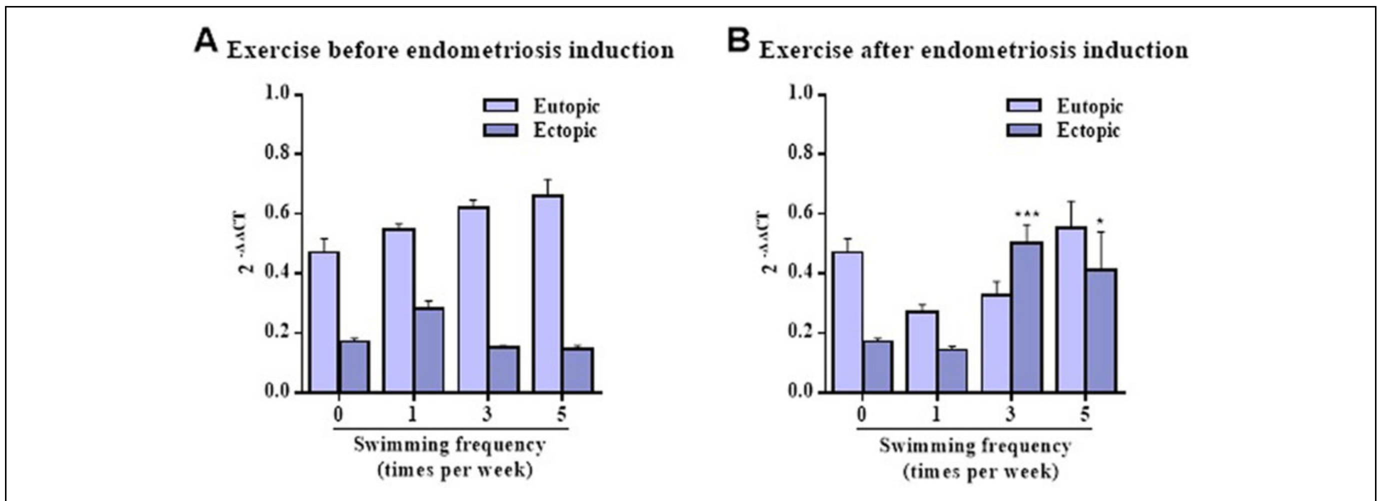

Figure 2. Real-time polymerase chain reaction (PCR) for the *Fas* gene at the end of the experimental protocol. Data are reported as means (standard deviation; n = 10 per group). **P* < .01, ****P* < .0001 compared to the eutopic endometrium of the control group (animals that did not swim [0]). There was no significant difference in the eutopic endometrium of the various study groups. Eutopic sindicates eutopic endometrium; ectopic, endometriosis lesions.

appropriate transformation of the answer variable is given by t(y), where $E\{t(y)|x\}$ is linear in x in the function $t(y) = \beta 0 + \beta T\, x + \in$, for unknown β_0 and β_T. The term $\in$ (random error) is independent of x and has an average of 0.[24] The analyses were performed using the SAS 2003 software (2002-2003 SAS Institute Inc, Cary, North Carolina). Analysis of variance was performed to compare the physical exercise groups to the control group, and the χ^2 test was used to compare the total sample to the stained sample of all study groups. The study samples were sufficient for the analyses with a power $(1-\beta)$ of at least 0.80 and a level of significance (α) of 0.05.

Results

Lesion Size

The impact of physical exercise on the induced endometriosis lesions is illustrated in Figure 1. The practice of exercise before

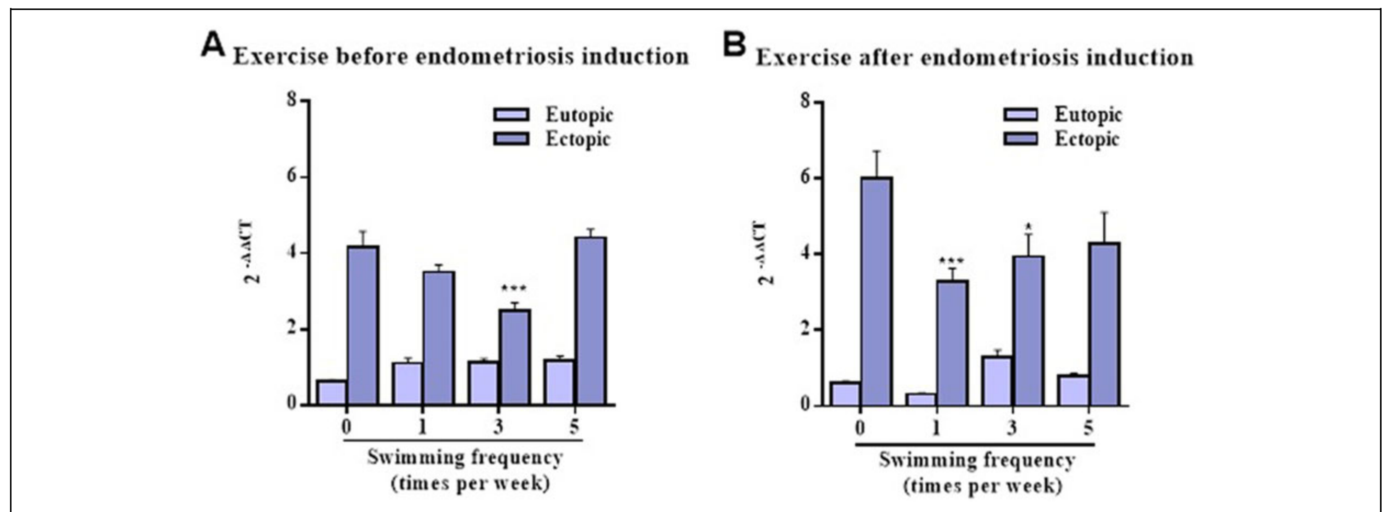

Figure 3. Real-time polymerase chain reaction (PCR) for the *Mmp9* gene at the end of the experimental protocol. Data are reported as means (standard deviation; n = 10 per group). *$P < .01$, ***$P < .0001$ compared to the eutopic endometrium of the control group (animals that did not swim [0]). Eutopic indicates eutopic endometrium; ectopic, endometriosis lesions.

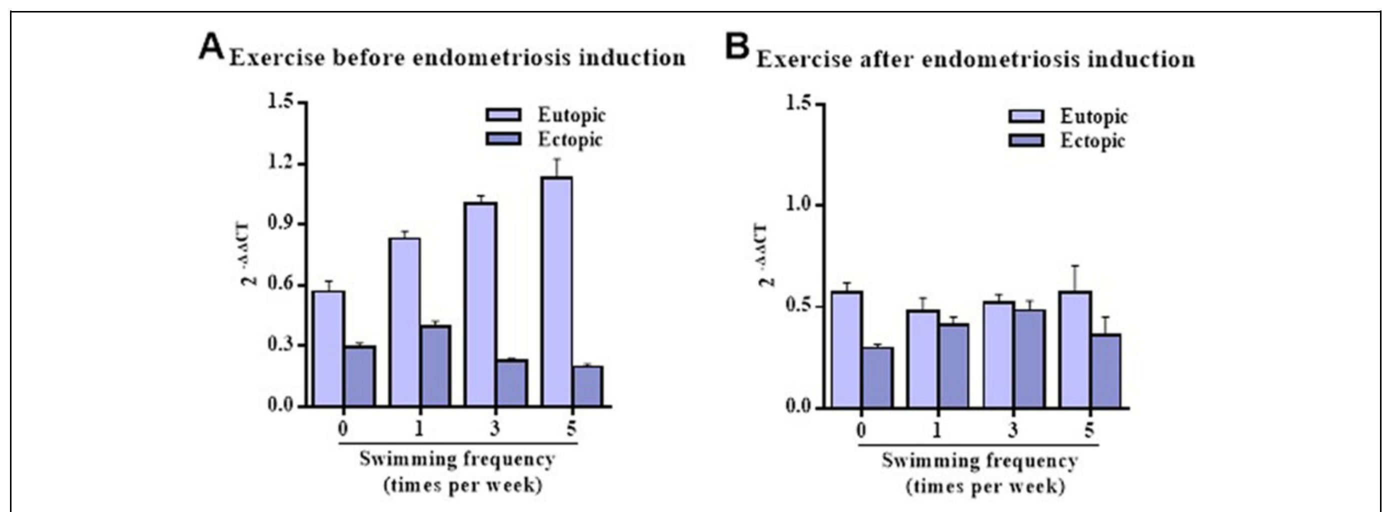

Figure 4. Real-time polymerase chain reaction (PCR) for the *Timp2* gene at the end of the experimental protocol. Data are reported as means (standard deviation; n = 10 per group). There was no statistically significant difference from the eutopic endometrium of the control group (animals that did not swim [0]). Eutopic indicates eutopic endometrium; ectopic, endometriosis lesions.

the induction of the edometriosis lesions did not prove to have a prophylactic role against endometriosis, whereas physical exercise practiced after induction of the lesions had a beneficial effect regardless of frequency (once a week or 5 times a week), reducing the size of such lesions. The animals that exercised in a moderate (3 times a week) or intense (5 times a week) manner exhibited a significant reduction of lesion size compared to the animals that exercised once a week.

Gene Expression

Our results showed increased *Fas* gene expression in the groups that practiced physical exercise after endometriosis induction ($P < .01$), suggesting greater cellular apoptosis (Figure 2). *Mmp9* expression seemed to be decreased in all exercise groups ($P < .01$), suggesting a reduction in cell migration and differentiation of endometrial tissue (Figure 3). *Timp2* expression apparently did not differ between groups (Figure 4). A decrease of *Pcna* expression ($P < .001$) can demonstrate a reduction in cell proliferation (Figure 5).

We detected no differences in gene expression between the eutopic endometria of the groups that practiced physical exercise before or after endometriosis induction, or between the different intensities of activity (once a week or 5 times a week).

Immunohistochemistry

Immunohistochemistry did not show conclusive results, with no significant differences between groups (Figures 6–9). There was no evidence of differences in protein levels when each

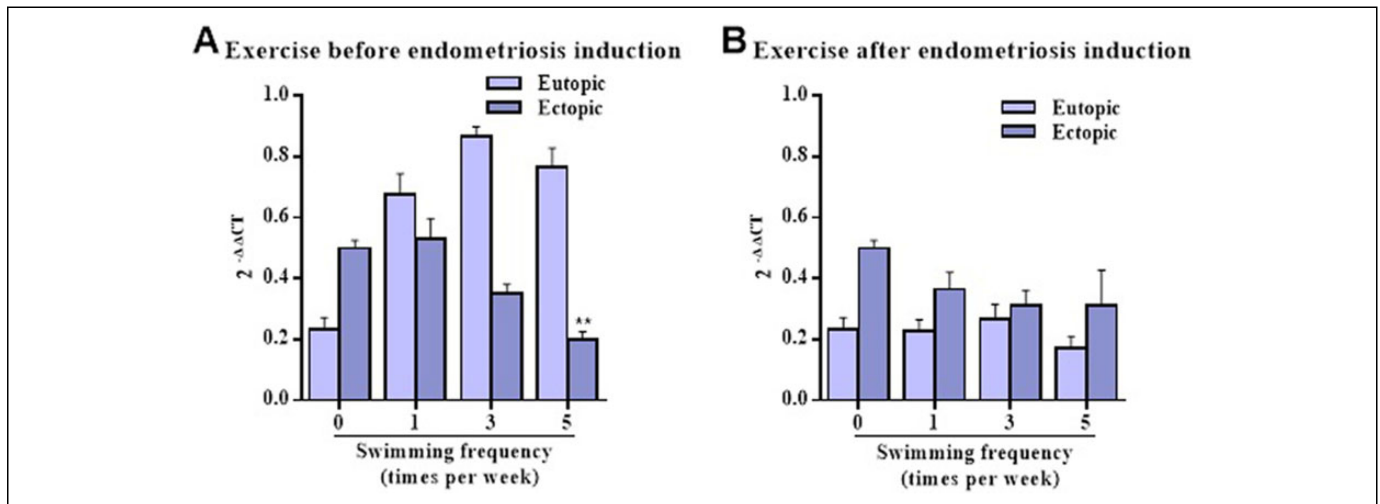

Figure 5. Real-time PCR for the *Pcna* gene at the end of the experimental protocol. Data are reported as means (standard deviation; n = 10 per group). ***P* < .001 compared to the eutopic endometrium of the control group (animals that did not swim [0]). Eutopic indicates eutopic endometrium; ectopic, endometriosis lesions.

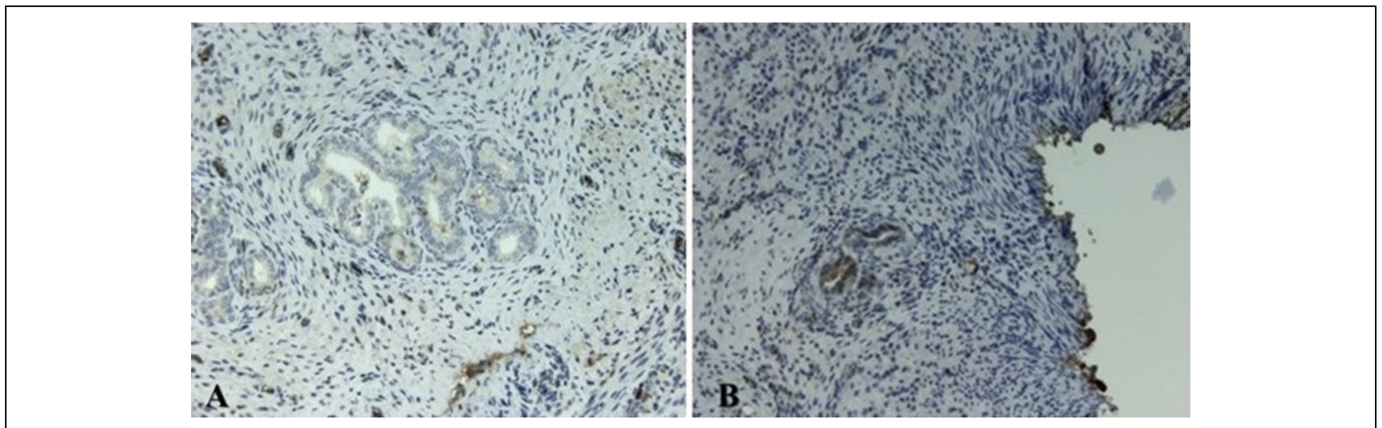

Figure 6. Immunohistochemistry for FAS protein. A, Immunohistochemistry staining of an endometriosis lesion from an animal that swam 3 times/week (moderate exercise) before endometriosis induction. B, Immunohistochemistry staining of an endometriosis lesion from an animal that swam once/week (light exercise) after endometriosis induction. The picture shows low glandular and stromal staining. All groups showed the same staining pattern. There was no statistically significant difference between groups (n = 10 per group).

group submitted to exercise (before or after lesion induction) was compared to the sedentary group, nor was there any difference when the various exercise intensities were compared.

Oxidative Stress

Oxidative stress was reduced in all exercise groups (*P* < .05; Figure 10).

Discussion

The protective effects of regular physical exercise have been extensively described in the treatment of diseases involving inflammatory processes.[12,13] Recently, striated muscle was identified as an endocrine organ, which, through contraction, stimulates the production and release of myocytokines, which may influence and change metabolism and the production of cytokines in tissues and organs.[25,26] Analyses of available literature data show that are no controlled and randomized studies identifying whether physical exercise prevents the occurrence or progression of endometriosis and how and to what extent physical exercise could be beneficial for women with endometriosis. On this basis, our results showed that the practice of physical exercise could be beneficial, at least in part, for the treatment and prevention of endometriosis since a reduction in the size of the endometriotic lesions was detected after physical exercise regardless of the frequency of execution. Additionally, as expected, oxidative stress was decreased in all groups that practiced exercise, confirming that physical exercise reduces the levels of systemic oxidative stress.

In studies of endometiosis intending to prove the efficacy of a treatment, it is often necessary to use invasive methods, a fact

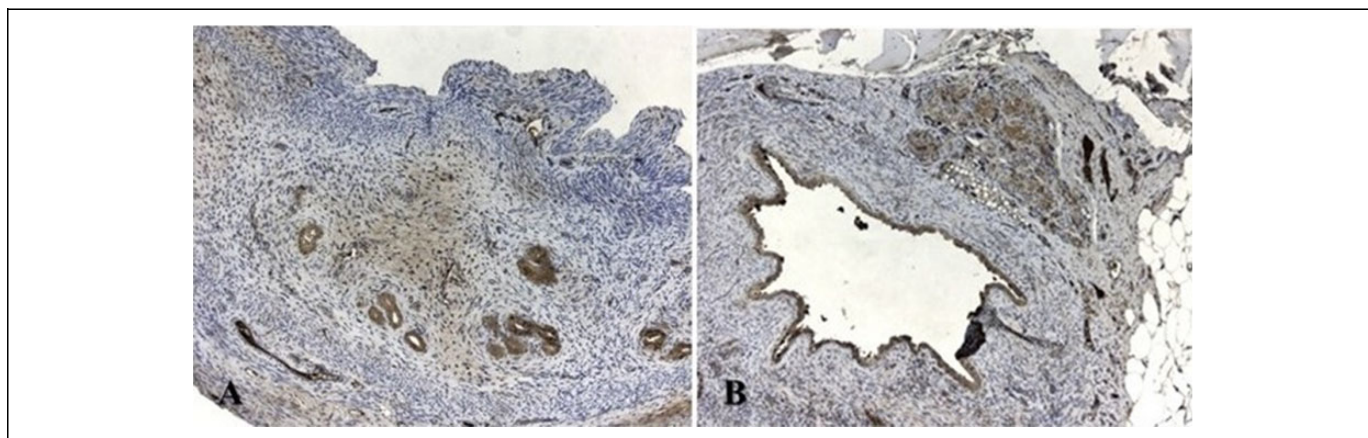

Figure 7. Immunohistochemistry for matrix metalloproteinases (MMP) 9 protein. A, Immunohistochemistry staining of an endometriosis lesion from an animal that swam once/week (light exercise) before endometriosis induction. B, Immunohistochemistry staining of an endometriosis lesion from an animal that swam 5 times/week (intense exercise) after endometriosis induction. The picture shows good glandular and superficial stromal staining. All groups showed the same staining pattern. There was no statistically significant difference between groups (n = 10 per group).

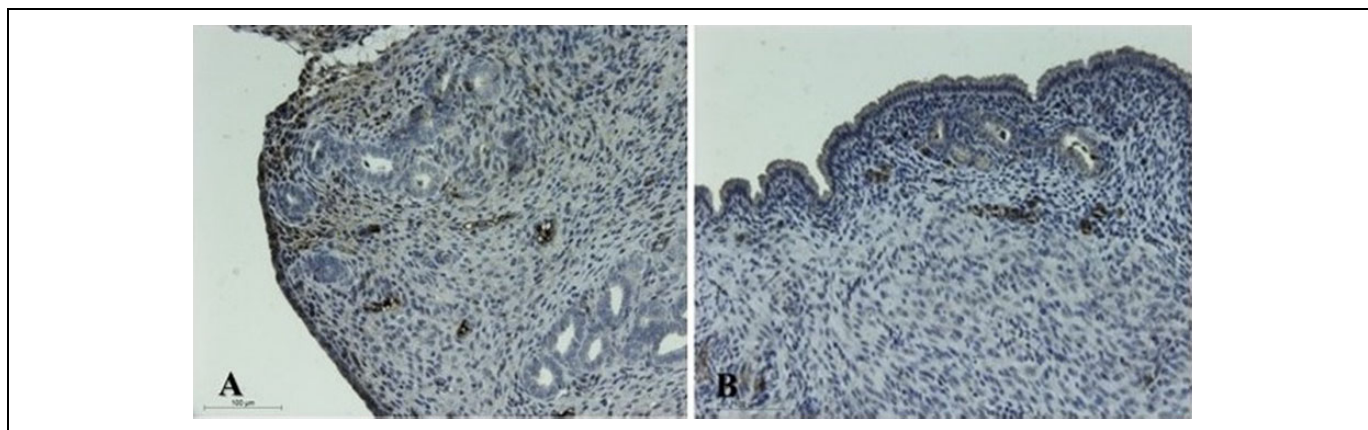

Figure 8. Immunohistochemistry for tissue inhibitors of metalloproteinase 2 protein. A, Immunohistochemistry staining of an endometriosis lesion from an animal that swam 3 times/week (moderate exercise) before endometriosis induction. B, Immunohistochemistry staining of an endometriosis lesion from an animal that swam 5 times/week (intense exercise) after endometriosis induction. The picture shows low glandular and moderate stromal staining. All groups showed the same staining pattern. There was no statistically significant difference between groups (n = 10 per group).

that limits their application to humans. Thus, an appopriate animal model is fundamental.[27] Within this context, the experimental model of endometriosis induction in rats proposed by Jones[28] is extensively used by involving a simple surgical technique, by being highly reproducible and mainly by being effective in the induction of the lesion.[29] Additionally, rats have an innate swimming ability and acclimate well to nonexhaustive training.[19,20] For these reasons, they represent a good animal model for the assessment of the impact of swimming as physical exercise on induced endometriosis.

Regarding gene expression, we can notice an increase of *Fas* levels in the groups that practiced physical exercise after endometriosis induction, suggesting greater cellular apoptosis. *Mmp9* levels were decreased in all exercise groups (before and after endometriosis induction), leading to a reduction in the proliferation, migration, and differentiation of endometriotic tissue. On the other hand, there was no change in the levels of *Timp2*, an *Mmp9* regulator. *Pcna* levels were decreased in all exercise groups, suggesting a reduction in the cell proliferation of the endometriosis lesions. However, the impact of physical exercise on these gene pathways needs to be confirmed because immunohistochemistry did not lead to conclusive results, with no significant data for protein levels. It is known that there is discordance between the expression levels of the trascriptome and of the proteins associated with the endometriosis process, which result from posttranscriptional regulation.[30] Another factor to be considered is that protein quantitation by immunohistochemistry is a less sensitive method for the detection of changes.

Considered as a whole, this evidence suggests that physical exercise can play an important role in the treatment and prevention of endometriosis. However, few data are available in

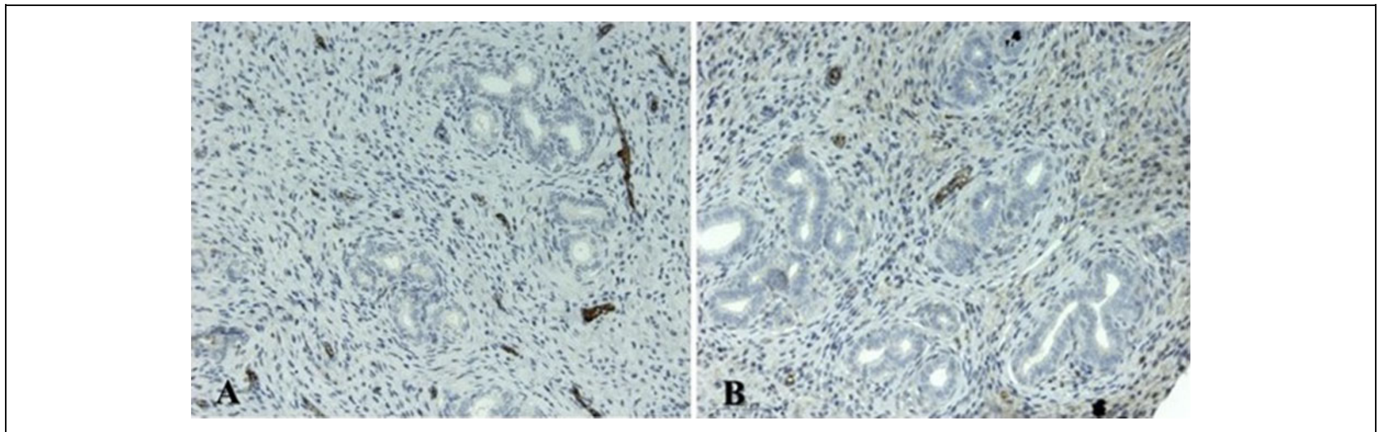

Figure 9. Immunohistochemistry for PCNA protein. A, Immunohistochemistry staining of an endometriosis lesion from an animal that swam 3 times/week (moderate exercise) before endometriosis induction. B, Immunohistochemistry staining of an endometriosis lesion from an animal that swam 5 times/week (light exercise) after endometriosis induction. The picture shows low glandular and stromal staining. All groups showed the same staining pattern. There was no statistically significant difference between groups (n = 10 per group).

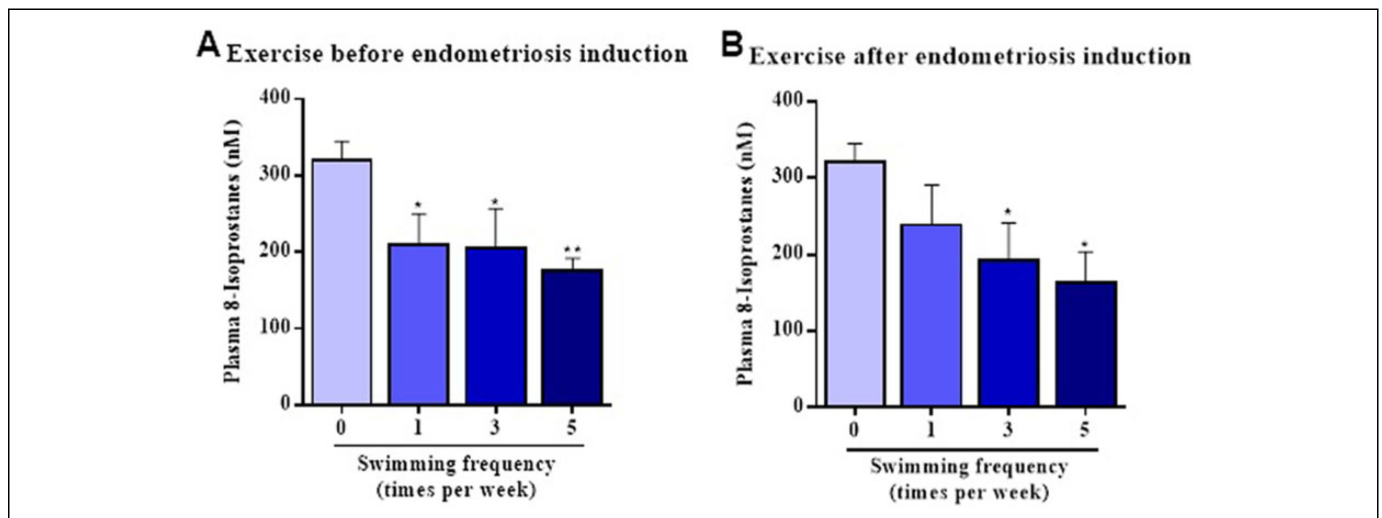

Figure 10. Plasma levels of 8-isoprostanes (nM) of the animals at the end of the experimental protocol. Data are reported as means (standard deviation; n = 10 per group). *$P < .05$, **$P < .01$ compared to plasma of the control group (animals that did not swim [0]).

the literature addressing the role of physical exercise in women with endometriosis.[18] Thus, additional controlled studies are necessary in order to clarify the real role of physical exercise in women with endometriosis.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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