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Impact of physical activity on pain perception in an animal model of endometriosis

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Abstract

Background—Symptoms of endometriosis, such as pain and infertility, are considered significant sources of stress. In many chronic conditions, exercise can act as a stress buffer and influence pain perception. We tested the impact of swimming exercise on pain perception and pain receptors in an animal model of endometriosis.

Methods—Endometriosis (Endo) was induced in female rats by suturing uterine horn tissue next to the intestinal mesentery. Sham rats received sutures only. Rats were exposed to swimming exercise for 7 consecutive days, while no-exercise rats were left in the home cage. Fecal pellets were counted after swimming as an index of anxiety, and serum corticosterone levels measured. Pain perception was assessed using the hot plate test for hyperalgesia and Von Frey test for allodynia. Mu-opioid receptor (MOR) and neurokinin-1 receptor expression in the spinal cord was measured by immunofluorescence.

Results—Fecal pellet counts were higher in those animals that swam ($p < 0.05$), but no significant difference in corticosterone was found. Although Endo-exercise rats had higher colonic damage ($p < 0.05$) with more cellular infiltration, the lesions were smaller than in Endo–no exercise rats ($p < 0.05$). Exercise did not ameliorate the hyperalgesia, whereas it improved allodynia in both groups. MOR expression was significantly higher in Endo-exercise vs. Endo–no exercise rats ($p < 0.01$), similar to Sham–no exercise levels.

Conclusions—Our results point toward beneficial effects of swimming exercise during endometriosis progression. Physical interventions might be investigated further for their ability to reduce perceived stress and improve outcomes in endometriosis.

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Keywords

Anxiety; Endometriosis; Exercise; Hyperalgesia; Opioid; Pain

Introduction

Endometriosis is a major public health problem affecting 10%–20% of women at some point during their reproductive life (1, 2). The presence of endometrium-like tissue outside the uterine cavity, mostly on the pelvic peritoneum and ovaries, manifests as pain and infertility, as well as peritoneal inflammation, fibrosis, adhesions and ovarian cysts (3).

Prior studies have demonstrated that women with endometriosis have generalized hyperalgesia in body areas beyond the site of ectopic implants, mediated by sensitization of central pain circuits (4, 5). This is consistent with animal studies demonstrating that induced endometriosis results in vaginal hyperalgesia (6). Pain and infertility are considered significant sources of stress in endometriosis patients (7), and we have previously demonstrated that stress can exacerbate the size and severity of the lesions in an animal model (8, 9). Interestingly, the pain and symptoms of endometriosis do not always correlate with the severity of the condition (such as the size of the lesions) (10).

Endometriosis displays similar features to other visceral pain disorders such as irritable bowel syndrome (IBS), with both diseases displaying central hyperalgesic dysfunction that results in central sensitization, leading to secondary allodynia or generalized hyperalgesia in zones remote to the pelvic cavity (5). Substance P (Sub P), a neuropeptide involved in pain signaling, plays a role in stress and visceral hypersensitivity in IBS (11). This molecule connects the nervous system to the immune system acting primarily on neurokinin 1 receptors (NK-1R) (12). The effects of this neuropeptide extend to surrounding undamaged tissues, where it can cause secondary hyperalgesia, presenting a potential target. In our model of endometriosis, it is not yet known whether there are changes in Sub P and NK-1R expression.

In recent years there has been increased interest in how stress can affect general well-being and its possible effects on pain threshold, but few have examined its potential to ameliorate the effects of stress on physiology (13). Physical activity can act to protect highly stressed individuals through autonomic, neuroendocrine and cognitive pathways (14). Regular exercise, such as walking, swimming or running, has beneficial effects on physical and mental health, and can improve neuropathic pain (15–17). It appears from animal research that multiple analgesia systems exist (both opioid and nonopioid), and that properties of the exercise stressor are important in determining which system is activated during exercise. Through the opioid pathway, exercise triggers the release of β -endorphins that enable analgesic effects by activating mu-opioid receptors (MOR) both peripherally and centrally (18). It is also known that extended swimming reduces inflammatory and peripheral neuropathic pain in rodents (19). The effects of physical activity on endometriosis and its progression are still unclear (20). Here we studied the effect of a short period (7 days) of physical activity (swimming) on pain perception and spinal cord receptor expression in a rat model of endometriosis.

Methods

Animal model

Female Sprague Dawley rats weighing 200 to 250 g were singly housed at 23°C in 12-hour light/dark cycle with food and water *ad libitum*. They were randomly assigned to 1 of 4 groups: Sham–no exercise, Sham-exercise, Endo–no exercise and Endo-exercise, with 5 to 6 animals per group. The Institutional Animal Care and Use Committee (IACUC) at Ponce Health Sciences University approved all procedures.

Animals were handled (5 min/d) for 7 days prior to beginning the experiments in order to reduce manipulation stress, and vaginal cytological smears carried out daily to verify reproductive cycles (9). Experiments were carried out at the same time of day (9 am to 12 pm) to minimize the influence of circadian rhythms.

Induction of endometriosis

Endometriosis was induced surgically under pentobarbital anesthesia by autotransplanting pieces of the right uterine horn to the intestinal mesentery as previously described in detail (9, 21). In sham-operated groups, the right uterine horn was massaged with no uterine implants. The endometriosis was allowed to progress for 22 days before sacrificing.

Swimming protocol and corticosterone measurement

Animals were exposed to a forced swimming exercise starting on day 14 after surgery (Fig. 1A). The animals swam in a cylinder of 30-cm diameter filled with 32°C water to a height of 20 cm with 15 cm of space above the head of the rat (22). Each animal received one 3-minute session of swimming exercise per day for 7 consecutive days. The entire process was monitored and recorded by a camera connected to a computer using the Anymaze program (Stoelting). We took into consideration 4 different behaviors: struggling, swimming, diving and immobility; to indicate whether the rats were showing hopelessness (immobility), anxiety (struggling) and/or escape behavior (diving) as a consequence of being forced to swim. The average time spent on each behavior was calculated over the 7 days. Colonic propulsive activity (an index of anxiety) was assessed by counting the number of fecal pellets (FPC) expelled during each swim trial (9). No-exercise animals were transferred to clean cages for the equivalent amount of time, and FPC noted. Blood samples from the tail vein of the rats were obtained on days 1, 4 and 7 during the swim protocol and at the time of sacrifice. Serum corticosterone levels were determined by ELISA (IBL-America).

Nociception tests

Hyperalgesia was tested on a hot plate measuring the response threshold to thermal stimuli (23). Rats were allowed to acclimatize in the testing room for 10 minutes then placed inside the cylinder of a PanLab/Harvard Hot Plate Apparatus (Model LE7406) heated to 52°C. Their response was measured in seconds, taking into consideration previously described behaviors (24).

Tactile sensitivity (allodynia) was assessed by measuring the threshold for withdrawal of the abdomen and hind paws to Von Frey filaments (Stoelting). Rats were allowed to acclimate to

the testing enclosure for ~10 minutes. Filaments were tested in ascending order of force as previously described (25). We took into consideration the same behaviors as Stagg et al (26).

Severity of endometriosis and collection of tissues

At time of sacrifice, all animals had a cytological smear taken to verify stage of the estrous cycle. After performing a laparotomy, the peritoneal cavity was systematically examined for the presence of implants and the original sutures, and their longest length and width measured with a digital caliper. Classification of lesions was carried out as previously described (9, 27) whereby lesions were assigned the following grades: <2 mm in length = grade 2; 2 mm but <4.5 mm = grade 3; 4.5 mm but <6.0 mm = grade 4; 6.0 mm = grade 5. If a lesion did not develop, a grade of 1 was assigned. Tissue segments from colon, uterus and lesions were fixed in 10% formalin, or weighed and frozen.

Colonic damage and neutrophil infiltration

At the time of sacrifice, the whole colon was examined for macroscopic damage using an established, previously well-defined scoring system (28). Colon tissue slices (4 µm) were stained with hematoxylin and eosin to determine the extent of inflammatory infiltration and the appearance of the underlying muscle layers. Histological assessment of damage was performed using previously published criteria (9, 29). Tissue myeloperoxidase (MPO) activity was determined in colonic and uterine tissues as an index of granulocyte infiltration. Approximately ~100 mg each of frozen tissues that were collected from mid-colon and uterine horn were analyzed (30).

Spinal cord

After collecting tissue samples, rat spinal cord was fixed by aortic arch perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.6) (31). The thoracic and lumbar spinal cord was removed and postfixed for 1 hour in 4% paraformaldehyde in 0.1 M PB then stored at -20°C in cryoprotectant solution (30% sucrose and 30% ethylene glycol in 0.1 M PB) before sectioning on a Leica Cryostat (30-µm thick) and mounting on charged slides.

Mu opioid (MOR) and NK-1 receptor (NK-1R) quantification

Sections of the thoracic and lumbar spinal cord were single labeled for immunofluorescence analysis of the NK-1R (rabbit polyclonal, sc-15323; Santa Cruz) or MOR (rabbit polyclonal, sc-15310; Santa Cruz). Both NK-1R and MOR antibodies have been previously characterized (32–35). Sections were boiled in sodium citrate and treated with 1% sodium borohydride in PB for 30 minutes, rinsed in 0.05 M phosphate-buffered saline (PBS; pH 7.4) and incubated in 0.3% normal goat serum (NGS) in 0.05 M PBS for 48 hours (MOR 1:100; Nk-1R 1:250). All incubations were separated by washes in PBS. The secondary antisera was goat anti-rabbit Dylight 549 (1:400; Jackson ImmunoResearch Labs, Inc.). We omitted primary antisera in controls. Sections were dehydrated and cover-slipped with DPX mounting media (Sigma). Images were acquired sequentially using an Olympus BX-60 equipped with X-cite 120Q lamp and photographed with a Nikon DS-FI1 camera. All images were taken at the same time within the same illumination parameters. The number of MOR- or NK-1R-labeled neurons in 100 µm² of the dorsal area (thoracic and lumbar part)

was counted. To prevent bias, cells were counted by a blinded observer and verified by a second blinded observer. Cell counts were summed per animal and presented as means $\pm$ SEM of MOR- or NK-1-labeled neurons per treatment group.

Sub P analysis

Sections of the lumbar spinal cord were single labeled for immunohistochemical analysis of Sub P (mouse monoclonal, MAB4375; R&D Systems). The specificity of this antibody has been previously tested in rat dorsal root ganglion by immunofluorescence (36). Sections were boiled in citrate-EDTA and blocked with normal goat serum and incubated with primary antibody (1:200) overnight at 4°C. Multi-Link was used as the secondary antibody followed by an incubation with streptavidin peroxidase (20 minutes each) and developed in 3,3'-diaminobenzidine (DAB). Control slides lacked primary antisera. Sections were dehydrated and cover-slipped using Cytoseal XYL. Pictures were taken using a CCD Camera on a Nikon 200 microscope at the same illumination level for all images. Areas of interest were scored using the ImageJ threshold tool to measure the percentage of area stained within the outlined region.

Statistical analysis

Graphs were prepared using GraphPad Prism 6.0 (Graph-Pad Software) and presented as mean difference $\pm$ SEM. A p value <0.05 was considered statistically significant. A 1-way analysis of variance (ANOVA) was used for normally distributed variables (assessed by Shapiro-Wilk normality test), followed the Tukey post hoc test. The nonparametric Kruskal-Wallis H-test was used for not normally distributed variables on the MPO and Student's *t*-test for normally distributed variables on the behaviors. In the case of Von Frey filaments and corticosterone concentration, data were transformed logarithmically and analyzed using ANOVA followed by Tukey post hoc test.

Results

Swimming and anxiety

Both exercise groups had increased FPC for the duration of the swim protocol, with the Endo-exercise group having significantly higher defecation compared with the no-exercise groups, indicating higher levels of anxiety (Fig. 1B). To verify whether forced swim was causing stress we measured serum corticosterone levels on days 1, 4 and 7 during the exercise protocol and at sacrifice. Those animals exposed to the exercise tended to have higher levels during days 1 and 4, but this did not reach significance (Fig. 1C).

Forced swimming behaviors

Both Endo-exercise and Sham-exercise animals spent approximately the same amount of time swimming and struggling, indicating that they were exercising equally with no problems doing physical activity. The Endo-exercise group showed a tendency to spend more time immobile compared with the Sham-exercise (56.10 ± 14.96 seconds vs. 34.13 ± 21.58 seconds) and significantly less time diving (2.45 ± 1.04 seconds vs. 10.90 ± 1.94 seconds; $p < 0.001$) indicating that endometriosis rats developed hopelessness behaviors, perhaps akin to emotional states found in patients (37).

Nociceptive testing

We tested nociception before surgery (baseline levels), after surgery/before swim and after swim. With the hot plate test we observed that the endo groups had significantly decreased latency after exercise compared with baseline levels (Fig. 2A) implying a hyperalgesic effect. There was no difference between days in the other groups.

Using Von Frey filaments test we found that the no-exercise groups became more sensitive to applied force after surgery. Specifically, the Endo–no exercise rats had significantly less tolerance to force in the paw after swimming ($p < 0.05$ vs. baseline) (Fig. 2B). In contrast there was a tendency in both exercise groups toward increased tolerance. No significant differences were found between time points in the abdomen (Fig. 2C).

Vesicle development

There was no difference in weight between groups at the time of sacrifice, and as predicted, none of the sham animals developed lesions. One hundred percent of the Endo–no exercise rats developed a lesion at the site of implantation, versus 87.5% of the implants in the Endo-exercise group (Fig. 3A). The largest lesions were found in Endo–no exercise rats, with 40.63% being categorized as grade 5 (larger than 6 mm) (Fig. 3B, C). Further, these animals had a significantly greater lesion length per animal (Fig. 3D).

Neutrophil infiltration and colonic damage

Endo-exercise animals had significantly more colonic macroscopic damage than Sham-exercise (Fig. 4A), however microscopically there was no difference (Fig. 4B). We measured MPO levels in the colon and uterus as an indicator of neutrophil infiltration. Levels of MPO were higher in both endo groups in the colon, reaching significance in the Endo-exercise group (Fig. 4C). There was no significant difference in the uterus (Fig. 4D).

MOR, NK-1R and Sub P spinal cord expression

We observed a similar pattern of MOR labeling in both the thoracic and lumbar spinal cord dorsal region. In the thoracic segment, Sham–no exercise and Endo-exercise had higher numbers of MOR-labeled cells (Fig. 5A). Sham–no exercise rats also had significantly more labeled cells when compared with the Endo–no exercise rats, which had the least expression. In the lumbar segment, Sham–no exercise and Endo-exercise groups had the highest numbers of labeled cells, reaching significance in the Sham–no exercise when compared with the Endo–no exercise rats (Fig. 5B). Again the Endo–no exercise rats had the lowest expression. These results suggest that the endometriosis by itself is associated with a decreased expression of MOR in the spinal cord, which is reversed upon exposure to exercise such as swimming.

To observe changes in the pain receptors, we examined NK-1R and Sub P labeling. Regardless of whether the animals were exposed to exercise or not we found no difference in NK-1R expression between groups (data not shown). We examined Sub P labeling in both sides (left and right) of the lumbar spinal cord dorsal region. Endo-exercise animals had decreased expression of Sub P compared with the other groups, which reached significance on the right side when compared with Sham–no exercise (Fig. 6A). This suggests that in this

model, nociceptive changes due to endometriosis were not associated with the amount of NK-1 receptors, but were related to the presence of Sub P in the spinal cord and down-regulated by exercise.

Discussion

In the present study we demonstrated how physical activity in a rat model can impact the development of endometriosis. In contrast to humans and nonhuman primates, rats do not menstruate, neither do they develop endometriosis spontaneously, so the disease has to be induced artificially by autotransplantation of uterine tissue (38). Further, uterine fragments which are transplanted include the myometrial layer which might affect the development of the lesions. Despite these limitations, the rat model offers significant advantages. This model shows steroid hormone dependency being estrogen dependent as found in humans, and the hallmark symptoms present in women with endometriosis are also found in the rodent model, including reduced fertility, peritoneal inflammation and vaginal hyperalgesia (38). Importantly this model provides an opportunity to investigate effects of environmental influences on the establishment of lesions.

Prior studies in our laboratory found that psychological stress can exacerbate endometriosis symptoms in this model (9). Stress is known to be involved in the etiology of a variety of disease states (39, 40), yet few studies have examined how physical activity can ameliorate the effects of stress. The protective effects of regular exercise have already been described for treatment of different diseases involving inflammatory processes including inflammatory bowel diseases and colon and breast cancer (41–43). In women with endometriosis, it has been suggested that exercise can lower the risk of developing the condition and perhaps help with feeling less pain (44). However, women with the condition tend to avoid strenuous exercise during menstruation, and because of dysmenorrhea, some women choose not to exercise during their menstruation and because of dysmenorrhea, some women also choose not to exercise. The few existing studies about exercise and endometriosis are of an observational type, with little or no statistical significance. Therefore, with the knowledge currently available, it is not yet possible to prove whether exercise can have beneficial effects in endometriosis (20).

Here we subjected animals to forced swimming, which in addition to providing physical activity (exercise) might be considered a stressor (22, 45). Although the animals were found to be more anxious than those not swimming, their corticosterone levels were not significantly higher, suggesting either that they acclimatize to the protocol or that the physical activity counteracts an exaggerated corticosterone activation expected with exposure to a stressor. Our results can be related to studies where sportsmen show reduced reactivity to a psychosocial stressor, characterized by lower cortisol levels and psychological stress responses than men that did not train (46).

The swimming exercise in this model had beneficial effects on the number and size of lesions which developed; however, colonic damage and neutrophil infiltration in endometriosis animals were still higher than in sham controls confirming our prior studies and the peritoneal inflammation found in patients (47). The antiinflammatory benefits of

exercise appear to be related to the type of exercise and the muscle mass involved in the mechanical effort (48). This antiinflammatory effect may be mediated via muscle-derived peptides such as myokines (41, 43) with positive or negative effects dependent on exercise type, volume and intensity range (43). We hypothesize that our protocol duration was not enough to disrupt the inflammation occurring in our model, yet was still sufficient to be reflected in vesicle size and number.

Women with endometriosis have a higher rate of comorbid pain syndromes, with peripheral and central sensitization being common (49). Zhao et al found that after endometriosis induction animals experienced hypersensitivity to a noxious thermal stimulus (24). Similarly, our animals experienced a decreased tolerance to thermal stimulus after endometriosis induction, yet no significant change in tactile sensitivity. Following physical activity, increased pain tolerance was observed in both paw and abdomen with no beneficial effects on thermal sensitivity. Although exercise has been suggested as an alternative for effective and healthy pain management (50), few studies have examined central opioid activity after exercise (26). We focused on MOR, NK-1R and Sub P in the spinal cord lumbar dorsal area, which connects with the pelvic organs, hindlimbs and colon (51). We found that endometriosis alone (no exercise) caused a dysregulation of the opioid pathway by decreasing the amount of cells that expressed MOR. Exposing the endo animals to physical activity increased the MOR-labeled cells back toward normal levels and decreased Sub P levels, suggesting that the exercise helps counteract endometriosis pain by restoring receptor expression and as consequence decreasing Sub P expression. Prior studies demonstrated that the MOR inhibits neurotransmitter release, particularly the release of Sub P (52). However, this means that the modality of pain is modified by the exercise but is not completely abolished. We found no difference in NK-1R between groups in the current study, implying the noninvolvement of this receptor. Future studies should focus on distribution of the endogenous opioid peptides in the spinal cord to examine whether this correlates with the behavioral changes.

To our knowledge, no prior studies have identified whether acute physical exercise prevents the occurrence or progression of the endometriosis, and to what extent physical exercise could be beneficial for women with endometriosis (20). Ours is the first study to examine how physical activity might affect pain perception in this condition and whether those effects can be correlated to what happens physiologically in the spinal cord. We used aquatic exercise since exercising in water decreases the compressive weight-bearing stress on the joint that is caused by land-based exercise (53). However, it is also known that exposure to repeated forced swim stress can lead to mechanical hyperalgesia and increased inflammatory and thermal nociceptive behavior in rats (54, 55). In light of this, follow-up studies could consider exposure to different types of voluntary physical activity in addition to evaluating longer time periods.

In summary, our results point toward possible beneficial effects of physical activity in this model and how they could help decrease not only the degree and the amount of lesions in endometriosis, but also provide beneficial effects by reducing perceived pain. Thus, physical activity might provide an effective complementary therapy to the pharmacologic treatment of endometriosis.

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Fig. 1.

Stress and anxiety levels. **(A)** Animals were subjected to a swimming exercise protocol for 7 consecutive days after surgical induction of endometriosis; HPT = Hot plate test; VFT = Von Frey filaments test. **(B)** Endo- and Sham-exercise rats had increased fecal pellet counts (* $p < 0.05$, ** $p < 0.01$, vs. Endo-no exercise; # $p < 0.05$, ## $p < 0.01$, vs. Sham-no exercise); **(C)** There was no difference in corticosterone levels between groups ($n = 5-6$, $\pm$ SEM).

Fig. 2.

Effect of exercise on nociception. (A) Both endo groups reacted significantly faster to the hot plate as indicated by a decrease in latency after surgery and swim ($^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, vs. before surgery); (B) In the paw, Sham– and Endo–no exercise tolerated less force after surgery ($^{\circ}p < 0.05$, vs. before surgery); (C) In the abdomen, there was no difference over time between groups, although the Endo-exercise group showed a trend toward increased tolerance. ($n = 5-6$, $\pm$ SEM).

Fig. 3.

Effect of exercise on implant size. None of the Sham group developed lesions. **(A)** Exposure to exercise decreased the number of lesions developed (* $p < 0.05$); **(B)** Representative photograph of lesions collected (both grade 5); **(C)** The Endo-no exercise animals developed a lesion in 100% of their sutures; **(D)** Endo-exercise animals developed lesions of a smaller size than those found in the Endo-no exercise rats (* $p < 0.05$; $n = 5-6$, $\pm$ SEM).

Fig. 4.

Effect of swimming on colonic damage and neutrophil infiltration in colon and uterus. **(A)** Endo-exercise had more colonic damage compared to Sham-exercise; **(B)** there was no difference between groups at a microscopic level; **(C)** Endo-exercise had higher myeloperoxidase levels in the colon compared with Sham-no exercise; **(D)** there was no difference in myeloperoxidase levels between groups in the uterine tissue (^{\$} $p < 0.05$, vs. Sham-exercise; [#] $p < 0.05$, vs. Sham-no exercise; $n = 4-6$, $\pm$ SEM).

Fig. 5.

Spinal cord mu-opioid receptor (MOR) expression in rats exposed to forced swimming exercise. (A) Endo-exercise animals had increased expression of MOR in the thoracic spinal cord compared with Endo-no exercise rats (** $p < 0.01$) or Sham-exercise ($p < 0.05$). Sham-no exercise had higher expression of MOR in the thoracic (* $p < 0.05$) (A) and lumbar (* $p < 0.05$) (B) spinal cord when compared with Endo-no exercise rats. Representative MOR immunofluorescent staining of thoracic sections from Endo-no exercise rats (C) and Endo-exercise animals (D) (1:100 at $\times 40$; $n = 5-6$, $\pm$ SEM).

Fig. 6.

Spinal cord substance P (Sub P) expression in rats exposed to forced swimming exercise.

(**A**) Endo-exercise animals had decreased expression of Sub P in the lumbar spinal cord compared with Sham–no exercise (right side; $*p<0.05$); Representative Sub P immunohistochemistry staining of lumbar sections from Sham–no exercise (**B**), and Endo-exercise animals (**C**) (1:200 at $\times 10$; $n = 5-6$, $\pm$ SEM).