

Assessing Pain Behavioral Responses and Neurotrophic Factors in the Dorsal Root Ganglion, Serum and Peritoneal Fluid in Rat Models of Endometriosis

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Abstract

Objective: Pain is the most frequently reported symptom involving in endometriosis. The alterations of neurotrophic factors and certain neuropeptides in the dorsal root ganglion (DRG), as well as serum and peritoneal fluid (PF), were evaluated in rat models of endometriosis.

Materials and methods: Twenty-four Sprague Dawley female rats were selected and maintained in a standard condition with 12 hours' dark-light cycles. All the rats were randomly assigned to 3 groups: Control (intact rats); Sham (the operation was conducted without endometriosis induction); and Endometriosis (endometriosis induction was performed). The formalin test was performed for all groups on the first and the 21st day of the study. The assessments of Brain-Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF), Calcitonin Gene-Related Peptide (CGRP), and Substance P levels were carried out by enzyme-linked immunosorbent assay (Elisa). The data were analyzed by One-Way ANOVA. The Tukey's test was used as post-hoc.

Results: Endometriosis induction significantly increased the mean pain scores in the endometriosis group in all three phases of the formalin test. The concentrations of DRG-CGRP ($p=0.035$), BDNF ($p<0.001$), and NGF ($p=0.006$) in the endometriosis group were significantly higher than that of the other groups while serum-BDNF ($p<0.001$), Substance P ($p=0.009$), and NGF ($p=0.015$) were significantly lower in endometriosis group compared to other groups. The concentrations of PF-BDNF ($p=0.025$) and Substance P ($p=0.009$) were significantly lower than those of other groups.

Conclusion: The present results delineate that endometriosis induction could lead to hyperalgesia. This may be related to the significant increases in the BDNF, NGF, and CGRP in DRG.

Keywords: Endometriosis; Brain-Derived Neurotrophic Factor; Nerve Growth Factor; Calcitonin Gene-Related Peptide; Substance P; Rat

Introduction

Endometriosis, with a high prevalence rate (15-25%), is

defined as the presence of endometrial gland and stroma in the extra-uterine places. Pelvic pain, dyspareunia, and dysuria, dyschezia, as well as infertility, are the primary signs and symptoms of endometriosis (1-4). Pain is the most disturbing symptom of endometriosis. The endometriosis-related pain mechanisms are still unclear;

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however, numerous studies have attempted to explain the various probable mechanisms including neuroangiogenesis, inflammation, lesional progression (5, 6). Previous studies have revealed the absence of sensory C fibers in the endometrium, however, these fibers with high density were observed in the endometriosis lesions. It has been suggested that these fibers may be involved in endometriosis hyperalgesia (7). The over-expression of the prostaglandin E2 (PGE2) signaling pathway including cyclooxygenase-2, PGE2 receptor 2, PGE2 receptor 4, and the increase in the transient receptor potential cation channel subfamily V Member 1 (TRPV1; a PGE2-regulated channel involved in nociceptive neurons) were also demonstrated in the endometriosis-related pain neuronal circuits (8). The presence of certain pain signaling neurotransmitters, such as Calcitonin Gene-Related Peptide (CGRP) and Substance P (SP) in endometriosis lesions in a rat model was also demonstrated by other studies (9, 10). Schou et al. pointed to the potential roles of SP, CGRP, and their receptors in the nociception process (11).

Recently, some limited studies have focused on the role of neurotrophins (NTs) and NTs receptors in the pain-related neurogenesis of endometriosis lesions. NTs are proteins from the Growth Factor Family that cause neurogenesis. Different types of NT proteins including Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), NT 3, and NT4 have been discovered (12, 13). The role of the over-expression of the NTs, such as NGF, BDNF, NT-3, and NT-4/5, in the development of chronic pelvic pain among endometriosis patients was confirmed by Kobayashi et al. (14). Stefani et al. showed higher serum levels of BDNF in patients with chronic pain disorders, including osteoarthritis, endometriosis, fibromyalgia, and chronic tension-type headache (15). In this regard, a study by Ding et al. showed significantly higher serum and Peritoneal Fluid (PF) BDNF levels in endometriosis women with pain compared with endometriosis women without pain (16). On the one hand, Rocha et al. and Wessels et al. demonstrated that plasma BDNF could be assessed as a clinical biomarker in endometriosis patients with pelvic pain (17, 18). On the other hand, Perricos et al. indicated that this marker did not have reliable predictive power, sensitivity or specificity in women with endometriosis (19). Morroiti et al. demonstrated an increase in the neurotrophic factors and nerve fibers expression in endometriotic lesions. However, they did not find any clear link between these findings and the pathogenesis of endometriosis-associated pain (20).

Regarding the NGF expression in the PF, Arellano et al. reported no significant differences in endometriosis patients with different pain scores. They concluded that the neurotrophic properties of the endometriotic lesion might not be pain-associated factors (21).

These discrepant findings from various studies show the necessity of further investigation. Moreover, futures studies need to explore how peripheral NTs factors and nociceptive sensors in endometriosis may affect dorsal root neurons as the first station in the transmission of pain to the central nervous system. To the best of our knowledge, there has been no study evaluating the alterations of NTs in the dorsal root ganglions in endometriosis. Therefore, the present study was performed to evaluate, first, the formalin-induced pain behavioral responses in rat models of endometriosis and, secondly, the alterations of neurotrophic factors and certain neuropeptides, including CGRP and SP in the dorsal root ganglion as well as serum and PF in these rat models of endometriosis.

Materials and methods

An animal study was conducted in Physiology Division of Basic Sciences Department, School of Veterinary Medicine, Shiraz University (Shiraz, Iran) in 2019. The animal handling was conducted in compliance with the procedures confirmed by the Ethical Committee for Animal Experiments at Shiraz University.

Animals and study design: Twenty-four Sprague-Dawley female rats with a mean weight of 205.78 ± 25.45 g were included in the present study. The rats were kept in standard condition with 12 hours dark-light cycles and 20-24°C temperature. The rats were monitored for health condition and acclimatization to the new environment for one week. Then, rats were randomly assigned to each of the following 3 groups:

- 1- Control (n=8): Intact rats
- 2- Sham-operated (n=8): Operation was performed without induction of endometriosis
- 3- Endometriosis (n=8): Endometriosis induction was performed

In all groups, the formalin test was performed on days 1 and day 21 of the study by a colleague who was blinded regarding groups. The experimental design is presented in Figure 1.

All the procedures in the present study were conducted in accordance with the Institutional Research Ethics Committee guidelines of Shiraz University for the care and use of laboratory animals in experimental studies.

Endometriosis Related Pain in Rat

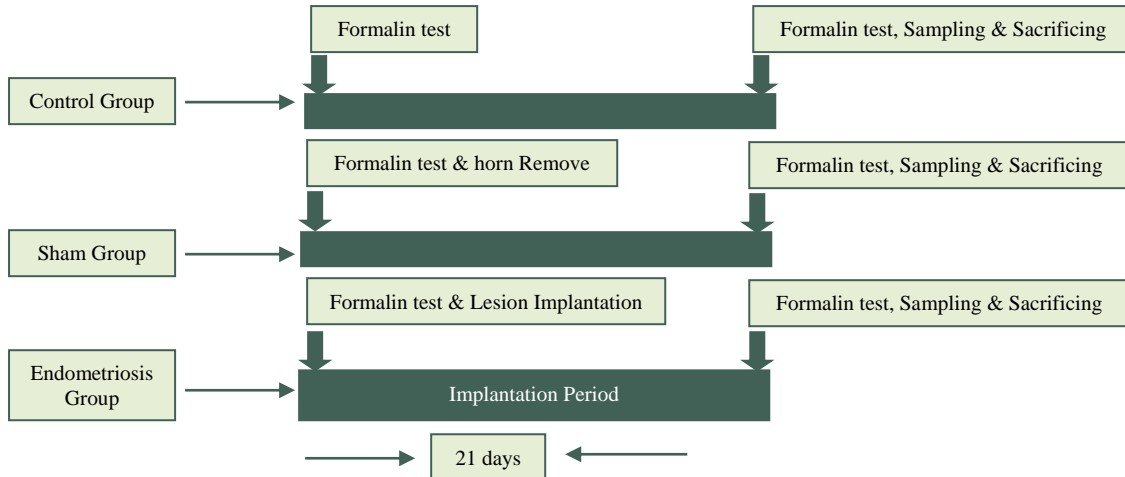


Figure 1: Experimental design paradigm

Endometriosis induction: Endometriosis was induced on the first day in the estrous phase using 0.2 mg/kg 17 β Estradiol (E8875; Sigma-Aldrich, Merck, Germany) injected subcutaneously. According to Zhixing et al. (22), the abdominal area was shaved with an electric razor under deep anesthesia with intraperitoneal (IP) injection of xylazine 2% (Alfasan Woerden, The Netherlands) and ketamine 10% (Alfasan Woerden, The Netherlands); few drops of Phosphate Buffered Saline (PBS) were placed on each rat's eye to prevent eye dryness. Subsequently, skin preparation, including prep and drape of the abdominal area, was done carefully. Through a vertical abdominal incision, the left uterine horn was ligated in both the cervical and fallopian tube regions. The left horn was cut, removed, and placed in a petri-dish containing PBS. The isolated horn was cut open longitudinally and incised into two 5 \times 5 mm measured slices. Two segments of uterine horn were sutured in different sites inside the abdominal wall (endometrium facing wall) near the main arteries using non-absorbable suture 6-0 (Figure 2A). The abdominal wall and the skin were separately sutured using non-absorbable 4-0. The injection of 17 β Estradiol continued every other day for a week and 20 μ l gentamicin was administered for 3 days intramuscularly. The second surgery was performed under deep anesthesia 21 days post endometriosis induction after performing formalin test. The abdominal cavity was opened. The dimensions of both endometriosis implanted lesions were determined by a Digital Quails (Figure 2B). The lesion size was calculated using the following formula: $S = D1 \times D2 \times \pi / 4$ (the largest (D1) and perpendicularly aligned diameter (D2) of the endometriotic lesions) (23). The lesions were removed and sent to the Department of Pathobiology for the confirmation of endometriosis histology (Figure 2C).

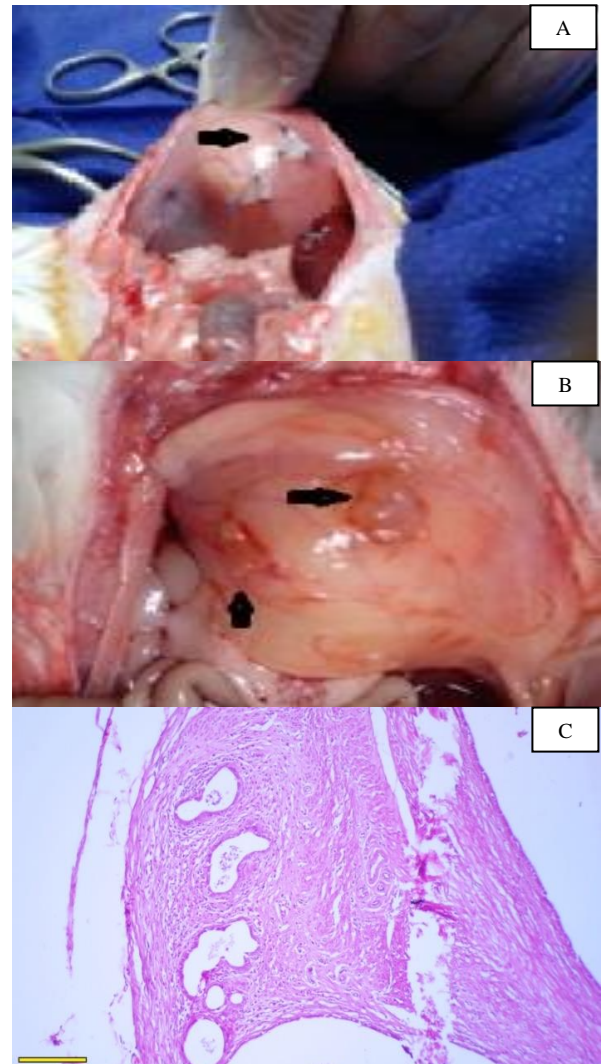


Figure 2: The representation of the experimental model of the endometriosis in rats: Endometriosis induction (A); cyst formation (B); and histological view of the endometriosis in the abdominal wall in day 22 (C). The scale bar length is 300 μ m.

Formalin test: Formalin test was performed to evaluate the pain evoked-behaviors induced by animal exposure to surgery and endometriosis. Formalin injection as an acute nociceptive model was used to demonstrate responses related to peripheral pain (driven by TRPA1 receptor and C-fiber activation) as well as responses associated with inflammatory and central sensitization of the dorsal horn (24). After the injection of formalin (50µl, 2.5%) in the hind paw, the rats' behaviors were evaluated for 60 minutes in blocks of 5 minutes every 15 seconds. The scores were assigned based on a 0-3-point scale: The injected paw was not favored, "0"; the injected paw had little or no weight, "1"; the injected paw was elevated and was not in contact with any surfaces, "2"; and the injected paw was licked, bitten, or shaken, "3". Three distinct periods of pain indices including the early (neurogenic phase), intermediate and the late (inflammatory phase) responses were identified and recorded during 0-5, 6-15 and 15-45 minutes, respectively (24). The formalin test was performed on the first and the 21st day of the study.

Sample preparation: Two ml of cardiac blood samples were collected on day 21 following a deep anesthesia achieved by the IP injection of xylazine 2% and ketamine 10%. Subsequently, for the preparation of the peritoneal fluids, 2 ml of warm PBS was slowly injected into the abdominal cavity and it was aspirated after one minute. Finally, animals were euthanized, the vertebral column was removed with a longitudinal incision, and the spinal cord was exposed. Dorsal root ganglions from T₁₃ to L₆ on each side were found, removed, and placed in a petri-dish on ice. The places of attachment of the last rib to the vertebral column and sacroiliac joints were considered as T₁₃ and L₆ spines. These ganglions receive input from both pelvic and visceral organs (25-27). The blood and peritoneal fluid samples were centrifuged and all samples were stored at -70° C.

BDNF, NGF, Substance-P, and CGRP measurements: Following the Elisa kit brochures, tubes contacting serum, peritoneal fluid, and dorsal root ganglion were placed in the refrigerator to defrost the samples. For DRG homogenization, 250 µl of PBS (8 g NaCl, 200 mg KCl, 1.44 g Na₂HPO₄, 240 mg KH₂PO₄ in 500 ml of distilled water, PH 7.4) was added to each tube and centrifuged at 2000 RPM for 20 minutes. The supernatant was collected in a new tube and labeled. BDNF, NGF, SP, and CGRP in the serum, peritoneal fluid, and

DRG samples were measured using 4 Elisa kits: 1) Bioassay Technology Laboratory; China (E0476Ra); Enzyme-linked Immunosorbent, Assay Kit (BDNF) 96 tests; Assay range 0.050-10 ng/ml and Sensitivity 0.01 ng/ml. 2) Bioassay Technology Laboratory; China (E0539Ra); Enzyme-linked Immunosorbent, Assay Kit (NGF) 96 tests; Assay range 10-3000 ng/l and Sensitivity 5.01 ng/L. 3) Bioassay Technology Laboratory; China (E0072Ra); Enzyme-linked Immunosorbent, Assay Kit (SP) 96 tests; Assay range 5-1000 ng/L and Sensitivity 2.49 ng/L. 4) Bioassay Technology Laboratory; China (E0347Ra); Enzyme-linked Immunosorbent, Assay Kit (CGRP) 96 tests; Assay range 2-600 pg./ml and Sensitivity 1.02pg/ml.

Statistical Analysis: The analyses were performed using the software package SPSS (Version 22). The data, including the weight of the animals, size of the endometriosis lesions, and the levels of NTs in different samples, were analyzed by one-way ANOVA. Tukey's test was used as post-hoc. The significant level was considered as p<0.05. The results are reported as means.

Results

Macroscopic and Histological Evaluation of Endometriotic Lesions: The present study investigates the formation of endometriosis cyst 21 days after endometriosis induction (Figure 2A) to determine the presence of endometriosis in rat. Endometriosis cysts were observed in the abdominal wall (Figure 2B). Additionally, histological examination of endometriotic lesions after day 21 revealed the presence of gland-like structures resembling human endometriotic lesions, confirming that fragments were successfully implanted in rats (Figure 2C).

Moreover, data analysis showed significant weight loss in the endometriosis group compared to the control and sham, after 21 days (p=0.0087). Although the weight gain in the control and sham groups was notable, the differences in their weight were not significant in the first and 21st days of the study (p=0.015). Also, on the first day of the study, there were no significant differences between the groups while at the 21 days of the experiment the weight of the endometriosis group was significantly lower than both the control and sham groups (Figure 3).

Pain Behaviors Evaluation in Rats with Endometriosis: The formalin test was used to evaluate the pain-related behaviors.

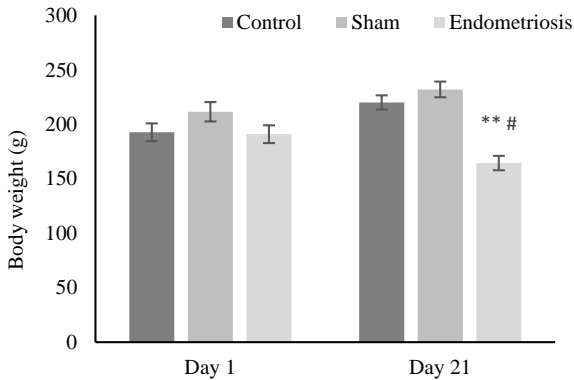


Figure 3: Comparison of weight alterations at the first and 21st days in all groups.

**p<0.01: significant relative to the control and sham
p<0.05: significant relative to the first day

Data analysis showed no significant differences between the groups in the early, intermediate, and late phases of the formalin test on the first day of the experiment (Figure 4A). On the 21st day of the experiment, the endometriosis group significantly increased pain scores in the early phase (p=0.0067), intermediate (p=0.035), and late phases (p<0.001) of the formalin test compared to the control group while there was no significant difference between the endometriosis and sham groups (Figure 4B). Comparing the first and 21st day of the experiment with regard to the mean pain scores, the results show that the pain scores for the endometriosis group increased significantly in the early (p<0.001), intermediate (p=0.0061), and late (p=0.015) phases of the formalin test compared to the day1 (Figure 4C).

BDNF, NGF, Substance-P and CGRP evaluation in rats with endometriosis: According to data analysis, the endometriosis group showed a significant decrease in PF-BDNF (p=0.025) and Serum-BDNF (p<0.001), compared to the control group, while the endometriosis group showed a significant increase in DRG-BDNF (p<0.001). There was no significant difference between the sham and both the control and endometriosis groups (Figure 5A).

The results of a comparison between the groups regarding NGF showed that there were no significant differences between them with regard to PF-NGF. Serum-NGF in the endometriosis group was significantly (p=0.015) lower than that of both the control and sham groups. However, DRG-NGF in the endometriosis group was significantly (p=0.006) higher than that of both the control and

sham groups (Figure 5B).

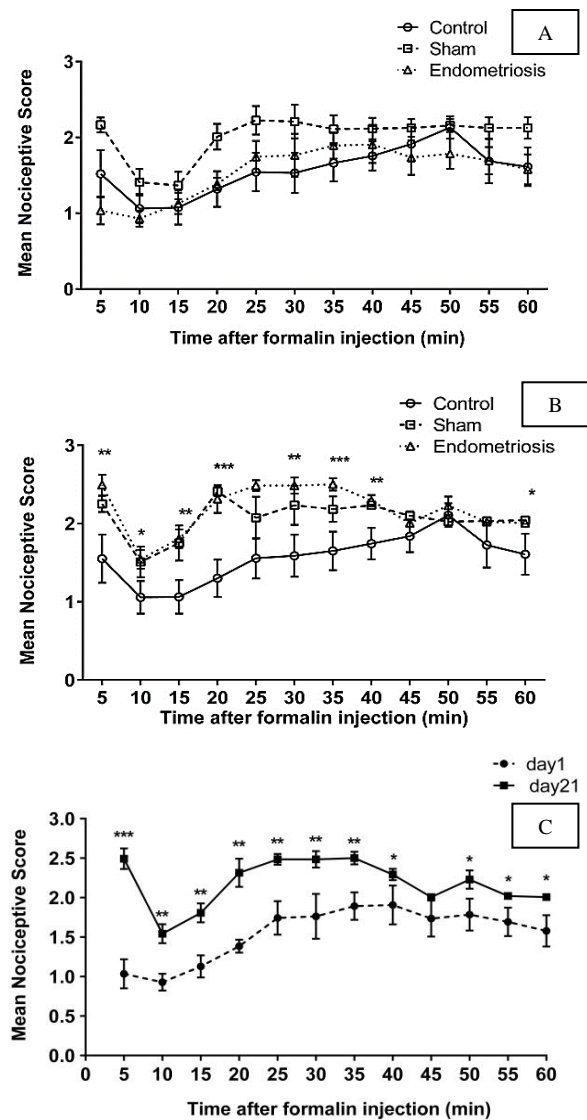


Figure 4: The effects of endometriosis induction on recorded time courses of the pain scores during the formalin test. Mean pain score comparison on day 1 (A); Mean pain score comparison on day 21 (B); and Mean pain score comparison in the endometriosis group on day1 and day 21 (C).

*p<0.05: Significant relative to the control and sham
**p<0.01: Significant relative to the control and sham
***p<0.001: Significant relative to the control and sham

Data analysis shows that Substance P in the endometriosis group was significantly (p=0.009) lower than that of both the control and sham groups in PF-Substance P, serum-Substance P, and DRG-Substance P (Figure 5C).

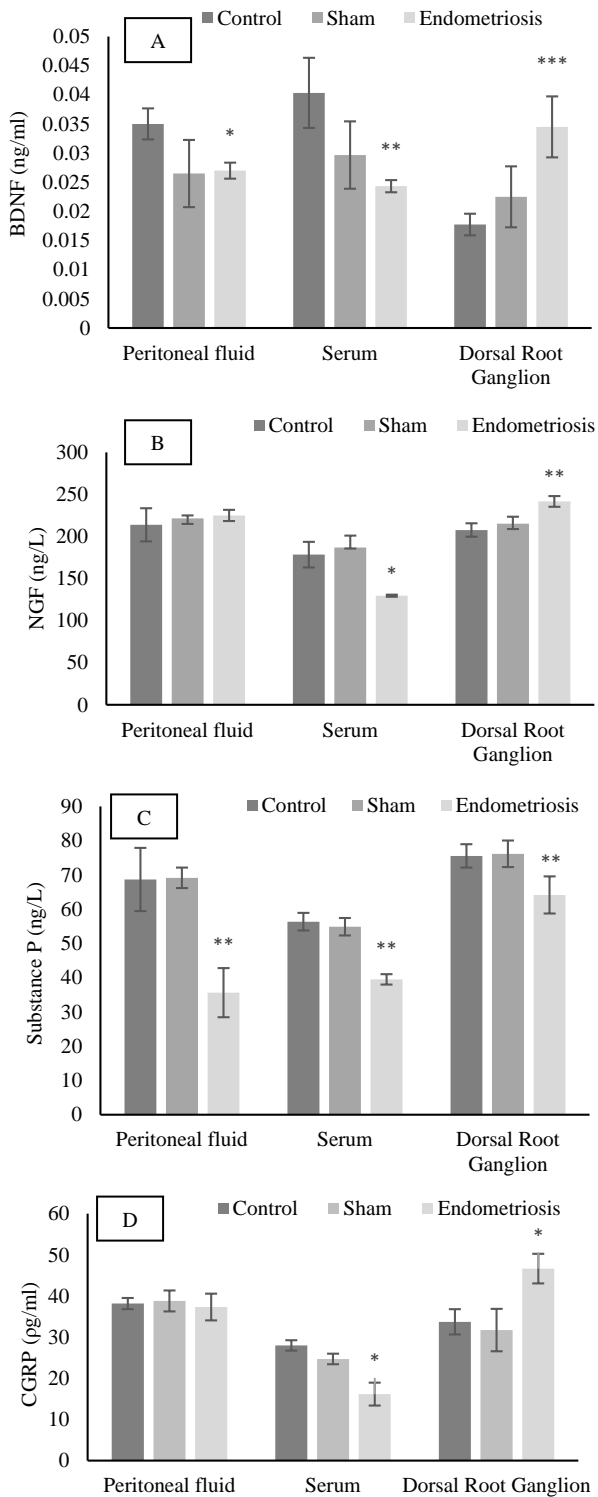


Figure 5: The relation between endometriosis and BDNF (A), NGF (B), substance P (C), and CGRP (D) concentrations in the peritoneal fluid, serum, and dorsal root ganglions.

* $p < 0.05$: Significant relative to the control and/or sham
 ** $p < 0.01$: Significant relative to the control and/or sham
 *** $p < 0.001$: Significant relative to the control and/or sham

The results of a comparison between the groups regarding CGRP show no significant differences between the endometriosis group and the two other groups in PF-CGRP. Serum-CGRP was significantly ($p=0.025$) lower in the endometriosis group compared to that of both the control and sham groups, whereas DRG-CGRP in the endometriosis group was significantly ($p=0.035$) higher than that of both the control and sham groups (Figure 5D).

Discussion

The main cause of pain-associated endometriosis is difficult to find due to the involvement of several complex processes. The presence of peritoneal lesions, adhesions, muscular spasms, ischemia, as well as other somatic and visceral pain, is among these complex processes (17).

The result of the present study shows that among the 3 groups, weight loss in the endometriosis group was significantly higher in comparison with that of the other groups 21 days after the surgery. Although very few investigations have evaluated the correlation between endometriosis and weight alteration, Goetz et al. have reported a significant weight loss related to the total body fat content in the endometriosis mice in comparison with the mice in the control group during a 6-week postsurgical period. They reported that endometriosis could affect body mass index by the over-expression of four genes, such as *Cyp2r1*, *Fabp4*, *Mrc1*, and *Rock2* in the visceral adipose tissue and liver. These genes with their anorexigenic properties, enhancing insulin sensitivity, and regulating lipid metabolism roles can protect the body against obesity and diabetes (28). In contrast to our results, Moradi et al. revealed that endometriosis could contribute to weight gain and poor body image (29). It has been suggested that factors including lack of exercise due to pain, bloating, constipation, an increase in the size of endometriosis masses, fluid retention due to estrogenic nature related to endometriosis, may be involved in weight gain. Finally, Hernandez et al. did not observe any differences between the endometriosis and sham groups regarding the animals' weights (30).

Present study delineates that 21 days after the beginning of the experiment, the endometriosis group showed significant increases in pain scores recorded during the formalin test in the early, intermediate, and late phases compared to the control group. Moreover, a comparison between the first and the 21st day of the experiment on pain scores revealed significant

increases in the score in all three phases of the formalin test in the endometriosis group through experimental time course. Previous investigations on animal models have indicated that after a successful establishment of endometriosis, multi-step processes including hyper inflammatory peritoneal environment, peritoneal attachment, the formation of fibrous bands of scar tissue, extracellular matrix degradation, neovascularization, and innervations may occur. These processes can contribute to the presentation of pain as a major symptom of endometriosis (31). Furthermore, recent studies have demonstrated that endometriosis can enhance sensitivity to subsequent painful stimuli by central sensitization. Central sensitization is a factor associated with hyperalgesia and allodynia endometriosis-associated pain (32, 33). Consistent with our findings, Hernandez et al. revealed a significant hyperalgesic effect related to endometriosis (14 days post-induction) using nociceptive testing with the hot plate test (30). Lian et al. also illustrated a significant decrease in the tail-flick latency, four weeks following the surgery in the endometriosis model group compared to pre-surgical baseline (6).

The present results show a significant increase in DRG-BDNF in the endometriosis group compared to the control group. These findings may confirm that endometriosis induction can lead to hyperinnervation resulting in pain and over-expression of BDNF in DRG as the first station of the nociceptive nerve cells (14). Although previous studies have noted the possible role of BDNF in the pathogenesis of endometriosis pain (16), there are no investigations assessing the correlation between DRG-BDNF and pain-related endometriosis. It has been shown that further investigations are strongly needed. Consistent with our findings, Huang reported a significant increase in the BDNF expression level and its receptor in DRG in some diseases with complex mechanisms and chronic pain involving peripheral nerve sensitization, increased cytokines and ischemia (34).

The present results show significant decreases in PF-BDNF and Serum-BDNF in the endometriosis group compared to the control group. These findings suggest that peritoneal adhesion due to endometriosis may prevent peritoneal cavity from enough irrigation by PBS. Different studies have indicated different findings related to the alterations of BDNF in peritoneal fluid and serum in endometriosis. Qin et al. showed an increase of BDNF concentrations in the peritoneal fluid in the rats with endometriosis (35). In contrast, other studies, for instance, that of by Ding et

al., have reported no significant differences regarding BDNF concentrations in the serum or PF of women with and without endometriosis (13, 16). Perricos et al. found that changes in the serum BDNF could be endometriosis stage- dependent and/or lesion-type-specific rather than a general endometriosis feature. They also pointed to a low prediction power of serum BDNF in discriminating women with and without endometriosis based on the results obtained from the receiver operating curve (ROC) analysis (19).

According to the present findings, DRG-NGF in the endometriosis group was significantly higher than that of other groups. Dewanto et al. observed that BDNF and NGF had a significant role in the sensory nerve development, axonal branching, and axonal elongation of sensory neurons of DRG in a mouse model of endometriosis (36). This finding maybe also compatible with that of Li et al. suggesting increased NGF levels in the DRG of the adenomyosis group compared to those of controls in a mouse model. Adenomyosis is a disease that endometrial glands and stroma are seen in the myometrium. This complication has a similar pathophysiology with endometriosis (37).

Serum-NGF in the endometriosis group was significantly lower than that of both the control and sham groups. We suggest that factors including peritoneal adhesion and ischemia in scar tissue may be involved. Moreover, Dewanto et al. pointed to TrkA and TrkB as the main receptors of BDNF and NGF in the peritoneum with a high affinity that may localize NT in the endometriosis lesions (36). This localization may be responsible for the low concentrations of NTs in the peritoneal cavity and serum. Alterations in such peritoneal factors due to angiogenesis characteristic of endometriosis or stage- and lesion-type dependency would be expectable (38).

Data analyses of the present investigation show that Substance P in PF, serum, and DRG in the endometriosis group were significantly lower those of both the control and sham groups. These findings may indicate that Substance P could not be considered as an endometriosis biomarker. Moreover, it seems that the increase in Substance P, as a pro-inflammatory agent, in newly expressed sensory nerve fibers in the peritoneum may be time-dependent (39). In line with our findings, Medina et al. indicated that Substance P was detectable in the functional and myometrium layers of the uterus in both women with and without endometriosis. They also showed that this neuropeptide with great contractility characteristics had a possible role in the generation of pain related to

other physiologic or pathologic situations, including dysmenorrhea (40). Sanfilippo et al. showed that the level of Substance P in normal controls was not significantly different from that of the endometriosis or cases with pelvic adhesions. They illustrated that Substance P was present normally in the peritoneal fluid and its levels were not affected by pelvic endometriosis or adhesions (41).

In a comparison between groups with regard to CGRP, DRG-CGRP in the endometriosis group was significantly higher than that of both the control and sham groups. Compatible to our findings, Lian et al. reported that the results of both RT-qPCR and western blot analyses showed a significant increase in CGRP protein in DRG in the endometriosis group compared with the sham group (6).

Contrary to our expectations, serum-CGRP in the endometriosis group was significantly lower than that of both the control and sham groups. Tokushige et al. observed significant increases in substance P and CGRP nerve fibers in the endometriotic lesions. However, these nerve fibers were co-localized in the endometriotic lesions, and they were frequently observed in the endometriotic cyst than elsewhere, as in the stroma (9). This co-localization in the endometriosis lesion may explain the low concentration of serum-CGRP in the endometriosis group.

The main strengths of the present study were evaluating the alterations of NTs in the dorsal root ganglions, serum, and PF simultaneously in endometriosis animal model, which were not conducted previously in other investigations. Moreover, through experimental time course, the present study tested additional factors including changes in levels of different neuropeptides and animals' weight.

Our study had several limitations. We did not evaluate the concentrations of neurotrophic factors in the endometriosis lesions. Alterations of these factors during the time may also correlate with the pain scores. Moreover, the levels of neurotrophic factors in the present study were measured using Eliza method. Implementing other laboratory methods like real-time RT-PCR (polymerase chain reaction) or western blot tests assessing gene expression or protein levels could provide further informative data. Future studies with larger sample size and using different laboratory techniques are strongly suggested.

Conclusion

The results of the present study show that

endometriosis induction could lead to hyperalgesia. This endometriosis-associated pain behavior may be related to the significant increases of BDNF, NGF, and CGRP levels in DRG, which encompasses the cell bodies of the visceral afferent neurons. Our findings may shed light on another pain mechanism associated with endometriosis.

Conflict of Interests

Authors have no conflict of interests.

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