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The Effect of Mesenchymal Stem Cell Administration on the Expression of Nuclear Factor Kappa Beta, Neural Growth Factor Expression, and Prostaglandin E2 Concentration as Determinants of Pain in a Mouse Model of Endometriosis

Linda Margaretha Mamengko[1], Hendy Hendarto[2] and Widjiati Widjiati[3,*]

[1]*Doctoral Program in Medical Science, Faculty of Medicine, Airlangga University, Surabaya, East Java 60115, Indonesia*
[2]*Department of Obstetrics and Gynecology, Faculty of Medicine, Airlangga University, Surabaya, East Java 60115, Indonesia*
[3]*Department of Veterinary Science, Veterinary Medicine Faculty, Airlangga University, Surabaya, East Java 60115, Indonesia*

([*]Corresponding author's e-mail: widjiati@fkh.unair.ac.id)

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Abstract

Endometriosis is a significant reproductive health issue with pain being the primaru symptom. The complex pain in endometriosis involves the activation of macrophages, inflammatory activators such as NF-kB, neural growth factors (NGF), cytokines, adhesive molecules (ICAM-1), and mediators (PGE_2) which they are stimulate sensory nerve fibers and produce nociceptive signals. This study is aim for investigate the effect of Mesenchymal Stem Cell (MSC) administration on the expression of NF-kB, NGF, and PGE_2 concentration in a mouse model of endometriosis. Thirty-three mices were randomly divided into 3 groups consist non-endometriosis (P0), endometriosis without treatment (P1), and endometriosis with MSC administration (P2). The results showed MSC administration significantly reduced the expression of NF-kB by found scoring 2 groups (P1: 6.96 ± 0.13 and P2: 4.446 ± 0.228) and NGF (P1: 7.118 ± 0.629 and P2: 4.927 ± 1.187) also PGE_2 (P1:1005.862 ± 176.656 and P2: 891.218 ± 54.031. In onclusion, this study demonstrates that MSC administration can able reduce the expression of NF-kB and NGF, but not to PGE_2. The suggestion we can said is the MSC have potential therapeutic value in managing endometriosis-asssosciated pain by modulating inflammatory and neurotrophic factors.

Keywords: Endometriosis, NF-kB, PGE2, Reproductive health, Stem cell

Introduction

Endometriosis is a prevalent gynecological condition affecting a significant proportion of women in their reproductive years, with an estimated 90 million women worldwide experiencing endometriosis-related infertility and pelvic pain [1]. The condition is characterized by a markedly decreased quality of life due to the higher prevalence and severity of pain symptoms compared to women without endometriosis [2]. These symptoms encompass menstrual pain, non-menstrual pelvic pain, dyspareunia, and infertility, with the pain being reported as more intense than in women without the condition. The pathophysiology of pain in endometriosis involves 3 main pathways: The endometriotic lesion itself, the innate immune system, and the peripheral nerve system. The displaced endometrial cells undergo detachment and localization, triggering an inflammatory response through the production of cytokines and resulting in pain [3]. The activation of macrophages and inflammatory activators, such as nuclear factor kappa beta (NF-kB), leads to the activation of growth factors, cytokines, adhesive molecules, and the release of mediators like prostaglandin E2 (PGE2) [4].

These cytokines and mediators directly stimulate sensory nerve endings, producing nociceptive signals and activating sensory nerve fiber terminals, ultimately causing pain. In endometriosis, retrograde menstruation activates NF-kB, an inflammatory factor, leading to iron accumulation (in the form of heme) and initiating an inflammatory response through the NF-kB pathway. This activation results in the expression of genes related to inflammation, cellular adhesion, invasion, proliferation, and angiogenesis, with the activation of growth factors and cytokines such as NGF, interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α) [5].

NGF has been found to be present in endometriosis lesions in the peritoneum. NGF plays a crucial role in the spread of nociceptors, increases the number of sensory neurons, and contributes to persistent inflammatory pain. It induces the expression of substance P (SP) and calcitonin gene-related peptide, which are associated with neuropathic pain signaling [6]. COX-2 regulates the production of prostaglandin from arachidonic acid, and increased levels of COX-2 lead to increased prostaglandin production, which triggers the onset of pain and elevated levels of PGE2 in the peritoneal fluid of women with endometriosis contribute to the development and maintenance of endometriosis lesions. PGE2 inhibits macrophage phagocytosis by suppressing matrix metalloproteinase-9 (MMP9) and cluster of differentiation 36 (CD36), promoting the growth of endometriosis lesions. Furthermore, PGE2 is an essential lipid mediator in inflammatory pain, playing a key role in sensitizing sensory nerve fibers and causing pronociceptive effects [7,8].

The management of endometriosis involves 3 main approaches: Surgical intervention, medical therapy, or a combination of both. However, none of these approaches comprehensively resolves the challenges associated with endometriosis, such as high costs, elevated recurrence rates, impact on fertility, and limitations in administering medical therapy due to side effects, restricted administration times, and expensive medication costs. Current medical therapies for endometriosis include gonadotropin-releasing hormone agonists (GnRHa), danazol, progestogens, and combined oral contraceptives. However, nonsteroidal anti-inflammatory drugs (NSAIDs), selective COX-2 inhibitors, and painkillers often cause unintended side effects, including bleeding, ulcers, and erosion of the stomach. Moreover, extended usage of selective COX-2 inhibitors increases the risk of myocardial infarction and stroke [9].

Stem cells possess the remarkable ability to self-renew and differentiate into diverse cell types, making them valuable for regenerative and reparative therapies in disorders resulting from cell damage. While bone marrow-derived mesenchymal stem cells are commonly used, their availability is limited, and obtaining them involves complex and expensive procedures. Researchers have discovered methods to acquire, cultivate, and collect stem cells from menstrual blood, providing an alternative source of stromal stem cells that exhibit similar properties to other stem cells [10]. Numerous studies suggest that MSCs modulate inflammatory responses by targeting the NF-kB pathway. MSCs have been shown to reduce the activity of TNFR1, thereby inhibiting the NF-kB pathway in inflammatory conditions. In the presence of inflammation, stem cells secrete various factors, such as HGF, FGF, TGF, and IL-10, which activate homing and resident stem cells. These stem cells, along with exogenous stem cells, release IL-10, which suppresses NF-kB and proinflammatory cytokines in macrophages, ultimately leading to a reduction in PGE and pain [11].

Endometriosis is a debilitating condition that can cause infertility and adversely affect reproductive health. To investigate the potential of stem cells in endometriosis treatment, researchers have administered stem cells to animal models of endometriosis to evaluate their effects on the expression of NF-kB, NGF, COX2, and PGE. Since conducting immunohistochemistry on human tissue is unethical, mice are commonly employed as endometriosis animal models.

Materials and methods

This study utilized a total of 33 mice that met the inclusion and exclusion criteria. The samples were randomly divided into 3 groups: Non-endometriosis mouse group (P0), endometriosis model mouse group without treatment (P1), and endometriosis model mouse group with stem cell administration (P2). The significance level using ANOVA TEST and posthoc Dancun was set at 0.05, so if the statistical test yielded a value of $p < 0.05$, it was considered significant, whereas if the value was $p > 0.05$, it was deemed not significant.

Cyclosporin A injection

Cyclosporin A injection was administered intramuscularly in the mice's thigh. One ampule contained 50 mg/mL × 5 mL, with a human dosage of 10 mg/kgBW/day. The dosage was adjusted based on the mice's weight (20 - 30 g), resulting in a converted dose of 1.8 mg/mouse. This dose was diluted with water for injection to achieve 0.2 cc of cyclosporin A, which was then injected into the experimental mice on the first day.

Estrogen injection

Intramuscular injections were given on day 1 and day 5 after endometrial injection. Ethynil Estradiol was used, with a converted dose for mice equating to 5.4 µg, or 54 IU. Each mouse received 0.1cc of the preparation.

Isolation of bone marrow mesenchymal stem cells

An experiment named bone marrow isolation was carried out on rats (Rattus novergicus), which was then followed by the addition of MEM medium and centrifugation at 3,000 RPM for 10 min, which was done twice through. An antibiotic, an antifungal, and 15 % fetal calf serum (FCS) were added to the MEM medium that was used to culture the silt. A series of passages were carried out until a cell line was established. Positive test results were obtained for CD73, CD105, and CD90, but negative test results were obtained for CD45. This was done to guarantee that bone marrow mesenchymal stem cells were included.

Injection of bone marrow mesenchymal stem cells

An injection of rat bone marrow mesenchymal stem cells was administered to mice that were models of endometriosis. These cells were acquired from cultured bone marrow at the Tropical Disease Centre Research at Airlangga University in Surabaya. The intravenous administration of a single dosage of 10^6 cells per mouse was carried out inside the animal. Warming the tail in order to produce vasodilation in the blood vessels of the tail was done before to the injection.

Immunohistochemical examination of nuclear factor kappa beta and neural growth factor

Immunohistochemical staining was performed using NF-kB and NGF antibodies. Endogenous peroxidase activation was removed by incubating the sections with 3 % H_2O_2 in methanol, while endogenous biotin activity was eliminated using the Biotin Blocking System (Dako, Carpinteria, CA). After deparaffinization, the sections were incubated in 3 % H_2O_2 for 30 min, washed with PBS 3 times for 2 min each, then treated with 0.025 % trypsin for 6 min at 37 °C. Following the addition of primary antibodies, the sections were washed with PBS 3 times for 2 min each, and secondary antibodies were applied through a 30-minutes incubation. After secondary antibody application, the sections were washed with PBS 3 times for 2 min each, placed in chromogen substrate, and again washed with PBS 3 times for 2 min each.

Subsequently, they were rinsed with distilled water, counter-stained for 6 min, washed with running water, dehydrated, cleared, and mounted.

Prostaglandin E_2 examination using ELISA

Elisa examination for PGE_2 was conducted on mouse blood serum. The concentration was measured using the Elisa method and read with an Elisa reader.

Ethical approval

Ethical clearance was obtained from the Ethics Committee of the Faculty of Veterinary Medicine, Airlangga University, with approval number 2.KEH.097.08.2022.

Result

From **this Figure at** above, we are illustrates the depiction of stem cells used in this study. Fluorescent cells, both red and green, indicate positive Mesenchymal Stem Cells (MSCs). At the images: CD105, CD73, and CD90 are positive markers for mesenchymal stem cells, while CD45 is negative.

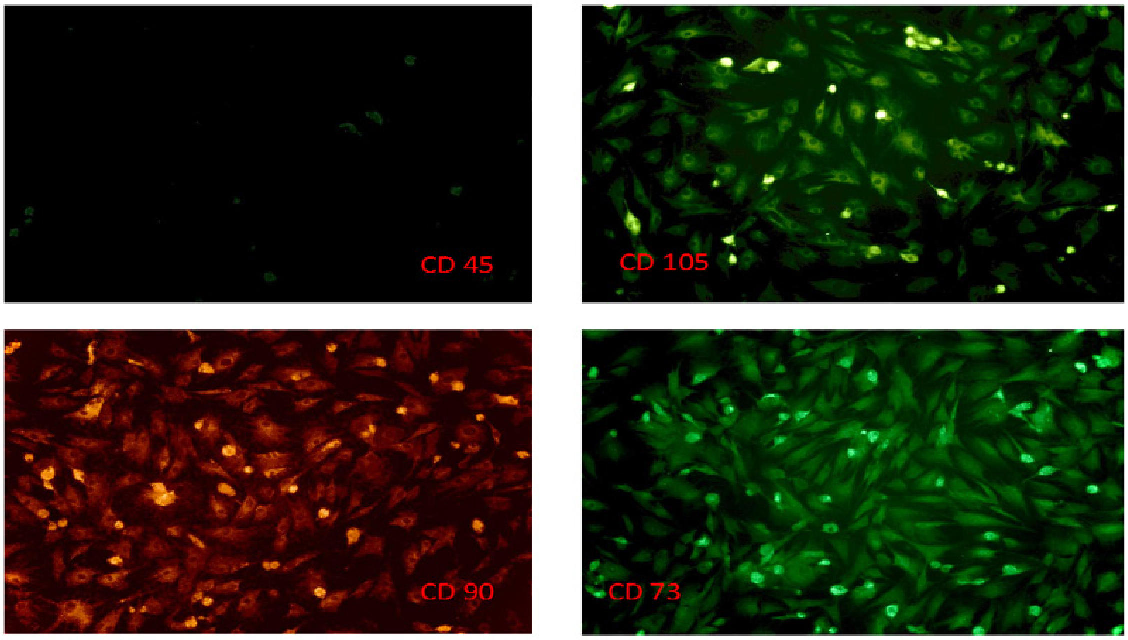

Figure 1 Immunofluorescence Examination (IF) of Stem Cells. FIT-C positive emits green fluorescence, PE emits red fluorescence. Fluorescent cells are positive for Mesenchymal Stem Cells (MSCs), while non-fluorescent cells are negative for MSCs. CD 73, 90 and 105 are positive markers for MSCs. The cell glows green if FITC. If it is labeled PE then the cells will glow red. For CD45, MSCs are negative, so they don't fluoresce or there are still cells that are green but thin.

The influence of stem cell administration on mice with endometriosis model regarding NfKb expression

There is a significant difference among the treatment groups in **Table 1**. The results of statistical analysis show a significant difference between the P0 and P1 groups, as well as between the P1 and P2 groups ($p < 0.05$). In the statistical analysis results, the P0 group does not significantly differ from the P2 group with a value of $p > 0.05$ (**Table 1**).

Table 1 Mean, Standard Deviation of NfkB Expression in the groups of untreated mice (P0), mice with endometriosis model (P1), and mice with endometriosis model receiving Stem Cell therapy (P2).

		NfKb expression	
Group	**N**	**$\bar{x}$ ± SD**	**P**
P_0	11	4.046 ± 0.229	0.001*
P_1	11	0.949 ± 0.138	
P_2	11	1.081 ± 0.279	

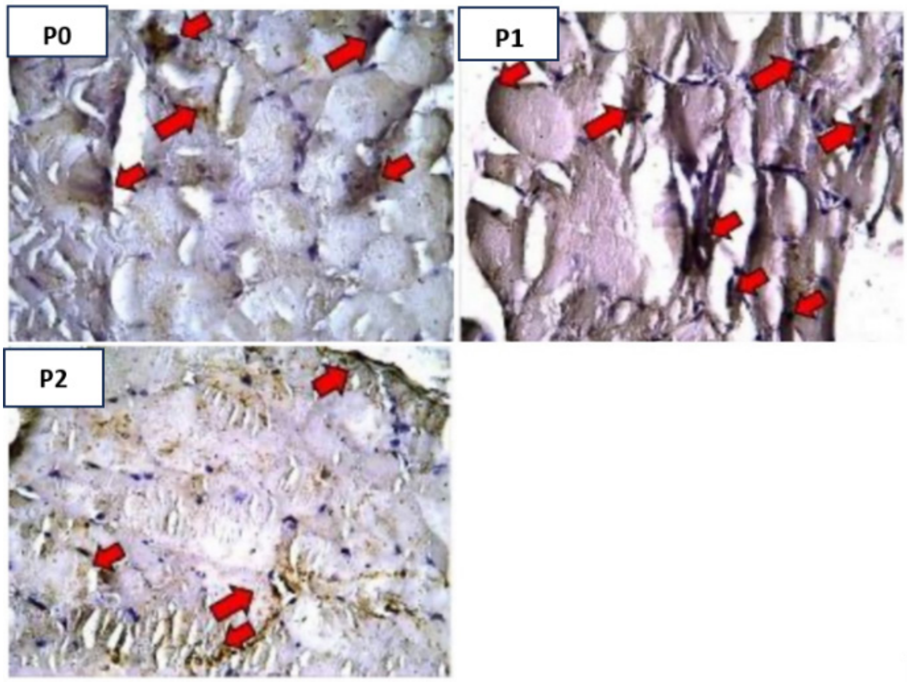

Figure 2 Expression of NFkB in the Peritoneum. Arrows indicate the expressed NFkB marked by the presence of brown chromogen color (red arrow). Observation was conducted using Nikon series microscopy with a magnification of 400x.

Table 2 Differences among treatment groups: Untreated mice (P0), mice with endometriosis model (P1), and mice with endometriosis model receiving Stem Cell therapy (P2) in NfkB expression.

Experimental groups	**P**
P_0-P_1	0.001*
P_0=P_2	0.173
P_1-P_2	0.001*

The influence of stem cell administration on mice with endometriosis model regarding NGF expression

There is a significant difference among the treatment groups in **Table 3**. The statistical analysis results show a significant difference between the P0 and P1 groups, as well as between the P1 and P2 groups ($p < 0.05$). In the statistical analysis results, the P0 group does not significantly differ from the P2 group with a value of $p > 0.05$ (**Table 3**).

Table 3 Mean, standard deviation of NGF expression in the groups of untreated mice (P0), mice with endometriosis model (P1), and mice with endometriosis model receiving stem cell therapy (P2).

Groups	N	NGF expression	
		$\bar{x} \pm$ SD	P
P_0	11	$4.656^a \pm 0.733$	0.000^*
P_1	11	$7.118^b \pm 0.629$	
P_2	11	$4.927^a \pm 1.187$	

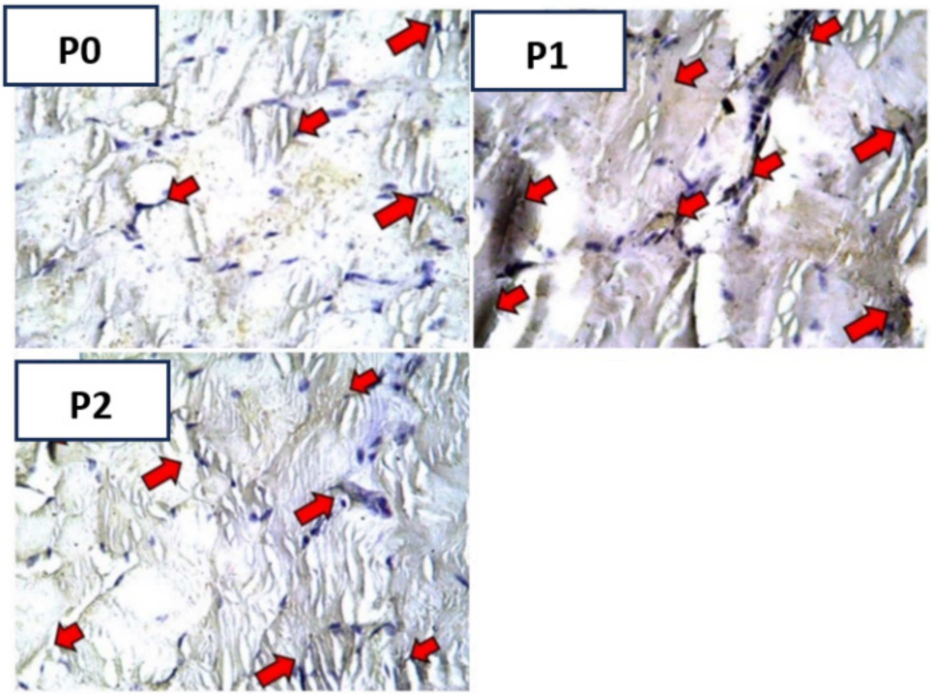

Figure 3 Histological Immunohistochemical Image of NGF Expression. Comparison of NGF expression in the peritoneum. Arrows indicate expressed NGF marked by brown chromogen color (red arrow). IHC 400×.

Table 4 Differences among treatment groups: Untreated mice (P0), mice with endometriosis model (P1), and mice with endometriosis model receiving Stem Cell therapy (P2) in NGF expression.

Groups	P
P_0-P_1	0.001^*
P_0=P_2	0.784
P_1-P_2	0.001^*

The effect of stem cell administration on mice with endometriosis model on PGE2 concentration

From the statistical analysis, it was found that the P0 group significantly differs from the P1 group ($p < 0.05$). The P0 group does not significantly differ from the P2 group, and similarly, the P1 group does not significantly differ from the P2 group with a value of $p > 0.05$ (**Table 5**).

Table 5 Mean, Standard Deviation of PGE2 Concentration in the groups of untreated mice (P0), mice with endometriosis model (P1), and mice with endometriosis model receiving Stem Cell therapy (P2).

Group	N	PGE2	
		$\bar{x} \pm SD$	P
P_0	11	$836.665^a \pm 157.657$	0.061
P_1	11	$1005.862^a \pm 176.656$	
P_2	11	$891.218^a \pm 5.031$	

Table 6 Differences among treatment groups: Untreated mice (P0), mice with endometriosis model (P1), and mice with endometriosis model receiving Stem Cell therapy (P2), in PGE2 concentration.

Experimental groups	P
P_0-P_1	0.021^*
P_0=P_2	0.439
P_1 -P_2	0.11

Discussion

NFkB expression in untreated, endometriosis model, and stem cell-treated endometriosis model mice

The control group, the endometriosis group, and the endometriosis group that had stem cell treatment all had NFkB expression levels that were significantly different from one another. Upon doing statistical analysis, it was found that there were notable distinctions existing between the P0 and P1 groups, as well as between the P1 and P2 groups ($p < 0.05$). The P0 and P2 groups, on the other hand, did not vary significantly from one another (P being more than 0.05) (**Table 1**).

Nuclear factor-kappa B (NF-kB) is a transcription factor that plays a pivotal role in various biological processes, including inflammation, immunity, cell adhesion, invasion, proliferation, apoptosis, and angiogenesis. The activation of NF-kB is triggered by numerous pro-inflammatory stimuli, such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), lipopolysaccharide, and oxidative stress. Upon activation, NF-kB promotes the expression of a wide array of genes encoding pro-inflammatory cytokines, chemokines, growth and angiogenic factors, adhesion molecules (e-selectin, ICAM-1, VCAM-1), and inducible enzymes (iNOS, COX-2). The regulation of immune and inflammatory responses is mediated by 2 distinct signaling pathways: The canonical and non-canonical (or alternative) pathways, each with its unique signaling mechanism. The canonical NF-kB pathway responds to a broad range of stimuli, including ligands from various cytokine receptors, pattern-recognition receptors (PRRs), TNF superfamily receptors (TNFR), T-cell receptors (TCR), and B-cell receptors. In contrast, the non-canonical NF-kB pathway exhibits a preferential response to specific stimulus groups, such as ligands belonging to the TNFR superfamily, including LTβR, BAFFR, CD40, and RANK. The activation of the non-canonical NF-kB pathway does not involve the degradation of IκBα; instead, it primarily focuses on the processing of the NF-kB2 precursor protein, p100 [12].

The connection NFkB along with prostaglandin include PGE_2, that we investigated here, and PGF2α can severity of baginal hyperalgesia and dysmenorrhea where this prostaglandin inflicted as mediator of acute inflammation in endometriotis and the demostration is protaglandin can interact easily with cytokines receptors then as same as explanation previous paragraph, the route created NkB the induced expression of inflammation-related genes. This signaling enchances the various NFkB-induced genes made chemokines to macrophages and neutrophil with sustained infiltration these cellk to further amplifies chronis inflammation [13].

NGF expression in untreated, endometriosis model, and stem cell-treated endometriosis model mice

The study revealed significant differences in nerve growth factor (NGF) expression among the experimental groups. Statistical analysis showed significant differences between the control group (P0) and the endometriosis model group (P1), as well as between the P1 and stem cell-treated endometriosis model group (P2) ($p < 0.05$). However, no significant difference was observed between the P0 and P2 groups ($p > 0.05$) (**Table 3**). Nerve growth factor (NGF) plays a critical role in the development and maintenance of sensory and sympathetic neurons in the peripheral nervous system. In addition to its effects on neuronal cells, NGF also influences non-neuronal cells, such as immune cells, vascular smooth muscle, and certain tumor cells. Moreover, NGF is a key player in the generation of pain and hyperalgesia in both acute and chronic pain conditions. The production of NGF is regulated by the cAMP-dependent intracellular signaling system in the brain. In the context of endometriosis, the prostaglandin E2-cAMP signaling pathway directly induces NGF mRNA expression, which is crucial for the proliferation and inflammation of endometrial tissue. Furthermore, several pro-inflammatory cytokines, including IL-β and TNF-α, have the ability to stimulate the synthesis of NGF. As a neurotrophin, NGF modulates the resilience, growth, and synaptic plasticity of neurons by altering the expression of other neurotrophin genes, cytokines, growth factors, and nociceptors [14,15].

Previous studies have utilized mice models to investigate endometriosis and its associated complications in women. Women with endometriosis are at a higher risk of developing anxiety, depression, and other physiological disorders related to their reproductive tract. Compared to normal women, the myometrium and endometrium of endometriosis patients contain a higher density of nerve fibers, with nerve fibers being more prevalent in the endometrium than in the myometrium during menstruation. Neurofilaments comprise not only nerve growth factor (NGF) but also neuropeptides, vasoactive intestinal polypeptide (VIP), protein gene product 9.5 (PGP 9.5), tyrosine hydroxylase, and a small number of acetylcholine immunoreactive nerves. Approximately 90 - 100 % of these neurofilaments are associated with the nerve growth factor receptor p75 and NGF, forming co-localized lesions in endometriosis [15].

Endometriosis-induced mice exhibit stimulus-evoked responses, leading to behavioral changes and more complex pain sensations. Chronic pain in endometriosis is associated with the presence of cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-33 (IL-33), in the vicinity of nerves in the endometrium or myocardium. Furthermore, TNF-α activates a distinct mechanism that upregulates the expression of ST2, the receptor for IL-33, which promotes neutrophil recruitment to inflammatory foci and induces proliferation and vascularization in lesions, mast cells, neutrophils, and macrophages. Additionally, the stimulation of cytokines in conjunction with estradiol triggers the production of CCL2, which leads to the formation of CGRP-, TRPA1-, and TRPV-positive nerve fibers. These fibers not only contribute to chronic pain but also enhance peripheral sensitization through the activation of NF-kB and increased calcium levels in dorsal root ganglion (DRG) neurons [16,17].

PGE2 concentration in untreated, endometriosis model, and stem cell-treated endometriosis model mice

Statistical analysis of PGE2 concentration revealed significant differences between the control (P0) and endometriosis model (P1) groups ($p < 0.05$). However, there was no significant difference between the control (P0) and stem cell-treated endometriosis model (P2) groups, nor between the endometriosis model (P1) and stem cell-treated endometriosis model (P2) groups ($p > 0.05$) (**Table 6**).

Prostaglandins are a group of bioactive lipids derived from the metabolism of arachidonic acid, playing a vital role in inflammation, fever, and pain processes as the levels keep increases then connected easily to NFkB able to created severity vaginal; hyperalgesia, and dysmenorrhea, perform acute inflammation but also chronic pain when vormulated with nerves inflammation cause endometriotis make such a complex disruption [18].

Cyclooxygenase (COX) is an enzyme that is responsible for the synthesis of prostaglandins into a number of different subtypes. PGE synthase and PGF synthase are responsible for the conversion of arachidonic acid to prostaglandin H2 (PGH2), which goes on to be transformed to PGE2 and PGE2α. This enzyme is responsible for catalyzing the conversion event. A higher quantity of PGE2 was found in women who had endometriosis, according to research conducted by Kianpour *et al*. [8]. PGE2 has its influence on peripheral nociceptors and enhances responses to stimulation in the peripheral nerves by means of transient receptor potential vanilloid 1 (TRPV1). Additionally, it induces chronic inflammatory pain by means of EP2 and EP4. The endometriosis model group showed an increase in PGE2 levels during the course of this research. When stem cells were administered, the levels of PGE2 reduced, however there was no statistically significant difference between the 2 groups [16].

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are non-hematopoietic cell precursors derived from the mesoderm and ectoderm embryonic germ layers. These cells are found in both developing embryos and adult tissues, and are valued for their multipotency, or the ability to self-renew and differentiate into various specialized cell types. MSCs give rise to bones, cartilage, tendons, ligaments, muscles, and bone marrow, and express various surface markers. Their plasticity, or ability to differentiate into new cell lineages, is one of their most fascinating characteristics. The MSC niche, or the location and environment that maintains their undifferentiated state, is believed to be found in blood vessels, as some cells from the perivascular space (PVS) of blood vessels carry specific markers and satisfy the requirements to be classified as MSCs after *in vitro* cultivation [17].

Mesenchymal stem cells (MSCs) possess both immunosuppressive and proinflammatory properties, which are dependent on their level of stimulation by various factors such as inflammatory cytokines, chemokines, metalloproteinases, indoleamine-2,3-dioxygenase (IDO1), and nitric oxide (NO). This immunosuppressive activity can be harnessed to prevent allograft rejection and abnormal autoimmune or inflammatory responses. At the molecular level, the presence of NO is crucial for MSCs to exert their immunosuppressive effects. In the absence of NO, particularly when inducible nitric oxide synthase (iNOS) is inhibited, MSCs enhance immune cell proliferation. This highlights the importance of NO production and increased NOS2 activity in MSC-mediated immunosuppression. Similarly, indoleamine 2,3-dioxygenase acts as a switch in MSC-mediated immunomodulation. While some studies suggest that MSCs may contribute to cancer development, others indicate that they have suppressive effects on tumor growth by inhibiting proliferation-related signaling pathways, inducing cell cycle arrest, and reducing cancer growth. However, it has also been shown that MSCs can differentiate into cancer-associated fibroblasts (CAFs) and actively promote cancer progression [18].

As research progresses and the understanding of endometriosis therapy using mesenchymal stem cells (MSCs) deepens, previous research by Gopalakrishnan *et al.* [9] have reported both successes and side effects of various treatments and therapies for managing endometriosis. For example, current long-term treatments still exhibit high recurrence rates, prompting researchers and clinicians to explore the potential of endometrial stem cells (EnSCs), particularly endometrial mesenchymal stem cells (eMSCs), as a gene therapy approach for endometriosis. Eutopic MSCs possess characteristics specific to the endometrium, including anti-inflammatory, immunosuppressive, and regenerative capabilities. Additionally, eutopic MSCs are flexible for gene transduction using adenoviral vectors expressing the anti-angiogenic factor sFLT-1. This is supported by research findings indicating that engineered eutopic MSCs can inhibit lesion growth and angiogenesis, as well as disrupt the expression of VEGF and MMP.

Conclusions

Based on the findings of this study, it can be concluded that the administration of Mesenchymal Stem Cells (MSCs) in the endometriosis model mice reduces the expression of NF-kB and NGF. However, the administration of Mesenchymal Stem Cells does not have a significant effect on the concentration of PGE2 in the endometriosis model mice.

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