Original Research

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Effect of Adipose-Derived Mesenchymal Stem Cell on the Expressions of Bax/Bcl-2, Ki67, VEGF, TNF-α, and Endometrial Implants in Metformin-Administered Endometriosis Mice (A Mouse Model in Endometriosis Study)

Bax/Bcl-2, Ki67, VEGF, TNF-α Ekspresyonları ve Metformin Uygulanan Endometriyozisli Farelerdeki Endometrial İmplantlar Üzerinde Adipoz Kaynaklı Mezenkimal Kök Hücresinin Etkisi (Endometriyozis Çalışmasında Fare Modeli)

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Abstract

Objective: Endometriosis is a gynecological syndrome that affects many women around the world. The effective management for this illness has not been determined. The aim of this study was to explore the effect of mesenchymal stem cells (MSCs) and metformin on Bax/Bcl-2, Ki67, VEGF, TNF-α, and endometrial implants in endometriosis mice.
Materials and Methods: Thirty mice with endometriosis were equally divided into 5 experimental groups (S1: 0.1 ml MSCs + 4 mg metformin; S2: 0.1 ml MSCs; S3: 4 mg metformin; S4: 0.1 ml NaCl 9%; and S5: 4 mg metformin + subsequent 0.1 ml MSCs) for 14 days. On the 15th day, peritoneal tissues of mice and endometrial implants were removed to examine the expressions of Bax/Bcl-2, Ki67, VEGF, and TNF-α using immunohistochemical staining, and Allred index and endometrial implants using image tracing method with a computer. The obtained data were analyzed using the Kruskal-Wallis and ANOVA tests, followed by the Least Significant Difference (LSD) and Mann-Whitney Post-hoc tests.
Results: There were significant differences in the expressions of Bax/Bcl-2 (p=0.002), Ki67 (p=0.004), TNF-α (p=0.017), and endometrial implants (p=0.001) in all groups, except for VEGF (p=0.079). The values of S2 didn't differ much compared to the control group (S4) in the Bax/Bcl-2 (p=0.487), TNF-α (p=0.191), and endometrial implants (p=0.2). S1 was found to have the highest Bax/Bcl-2 (1.67±0.845) and lowest TNF-α (4.67±2.15) and endometrial implant (0.86±2.11).
Conclusion: MSCs alone had not any beneficial effect on the treatment of endometriosis, whereas metformin by itself exhibited favorable results. The combination of MSCs and metformin at the same time shows superior outcomes.

Keywords: Endometriosis, mesenchymal stem cells, metformin, apoptosis, cell proliferation, endometriosis implants

Öz

Amaç: Endometriyozis, dünyada çoğu kadını etkileyen jinekolojik bir sendromdur. Bu hastalığın etkin tedavisi henüz tespit edilmemiştir. Bu çalışmanın amacı, endometriyozis farelerinde Bax/Bcl-2, Ki67, VEGF, TNF-α ve endometriyal implantlar üzerinde mezenkimal kök hücrelerinin (MSC) ve metforminin etkisini incelemektir.
Gereç ve Yöntem: Otuz endometriyozisi olan otuz fare, eşit olarak 14 günlük süre için 5 deney grubuna ayrıldı (S1: 0,1 ml MSCs + 4 mg metformin; S2: 0,1 ml MSCs; S3: 4 mg metformin; S4: 0,1 ml NaCl %9; and S5: 4 mg metformin + ardından 0,1 ml MSC). Bax/Bcl-2, Ki67, VEGF ve TNF-α ile immünohistokimyasal boyama, Allred indeksi ve endometriyal implantları bilgisayarla görüntü izleme yöntemi kullanılarak incelemek için 15. günde farelerin peritoneal dokuları ve endometriyal implantları çıkarıldı. Elde edilen veriler, Kruskal-Wallis ve ANOVA testleri ve ardından En Az Anlamlı Fark Sınaması (LSD) ve Mann-Whitney Post-hoc testleri kullanılarak analiz edildi.
Bulgular: VEGF hariç (p=0.079) tüm gruplarda, Bax/Bcl-2 (p=0.002), Ki67 (p=0.004), TNF-α (p=0.017) ekspresyonlarında ve endometriyal implantlarda (p=0.001) anlamlı farklar görüldü. S2 değerleri, Bax/Bcl-2 (p=0.487), TNF-α (p=0.191) ve endometriyal implantlar (p=0.2) açısından kontrol grubuna (S4) kıyasla çok farklılık göstermedi. S1'in en yüksek Bax/Bcl-2 (1.67±0.845) ve en düşük TNF-α (4.67±2.15) ve endometriyal implanta (0.86±2.11) sahip olduğu bulundu.
Sonuç: MSC'lerin tek başına endometriyozis tedavisinde faydalı bir etkisi yoktur ancak metforminin kendisi, olumlu sonuçlar göstermiştir. MSC ve metformin kombinasyonu, üstün sonuçlar ortaya koyar.

Anahtar kelimeler: Endometriyozis, mezenkimal kök hücreleri, metformin, apoptoz, hücre proliferasyonu, endometriyozis implantları

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Introduction

Apoptosis, a programmed cell death that doesn't induce an inflammatory response, has a significant role in the development of endometriosis where a decreased rate of apoptosis will encourage the survivability of endometrial cells.[1] This mechanism is associated with the ratio of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X-protein (Bax), in which Bcl-2 can inhibit apoptosis, whereas Bax promotes cell death against the Bcl-2 effect.[2] The ratio of Bax/Bcl-2 is assumed to be significant in determining the process of apoptosis better than the individual values of Bax and Bcl-2 and subsequently the development of endometriosis.[2] In contrast, the growth of tumor mass, recurrence, and endometriosis are linked with the increase of endometrial stem cell proliferation ability in patients with eutopic or ectopic endometriosis, identified with a higher Ki67 antigen expression.[3–5] Angiogenesis also has a major role in determining the development and survivability of endometriosis lesions, in which a decreased rate of angiogenesis will improve the condition of endometriosis lesions.[6] Approximately 80% of the blood vessel is sensitive to the most potent proangiogenic agent, vascular endothelial growth factor (VEGF).[7,8] TNF-α is a major pro-inflammatory cytokine that can be used as a sensitive and specific marker to identify and diagnose endometriosis in a patient.[7] A high concentration of TNF-α is commonly associated with an increase in TNF-α receptor concentration and endometriosis growth.[7]

At present, the management of endometriosis is based on surgical and medical therapy designed to suppress estrogen synthesis, reduce endometrial implant size, halt the menstruation cycle, resolve pain, and improve infertility conditions.[9] However, the medical therapy of endometriosis is reported to inhibit conception without any benefit to fertility.[9–11] In addition, the drugs used in the common endometriosis therapy cannot eradicate this syndrome and produce adverse side effects instead which renders long-term endometriosis therapy impossible.[9] Furthermore, the mainstream management of endometriosis still results in a high recurrence rate of 60%.[12] Therefore, it is crucial to explore innovative medical treatments to efficiently remedy endometriosis syndrome that doesn't induce unfavorable side effects.

The use of mesenchymal stem cells (MSCs) in research and clinical application has advanced considerably over the last decade. MSCs are obtained from various tissue types, such as bone marrow, adipose tissue, synovial membrane, dermis, peripheral blood, umbilical cord, and placenta.[13] Bone marrow is the most common source to collect MSC. But the harvesting procedure is very invasive and carries high risks of severe pain and infection for the donors.[14] As opposed to bone marrow, adipose tissue reveals more promising benefits since it is easier to be extracted in a large amount from numerous body parts.[13] Adipose tissue is also multipotent, can be cultured for in vitro expansion, and has the capacity to differentiate into various cell lineages and potential to be utilized in regenerative treatment.[13] It may produce angiogenic, immunosuppressive, and antioxidative cytokine profiles, as well as discourage the production of proinflammatory cytokines and trigger anti-inflammatory cytokines.[13,14] Considering the recent etiopathology theory of endometriosis where the dysfunctional endometrial stem cells are the etiologic factors of the syndrome, the treatment using MSCs is assumed to help replace damaged endometrial stem cells and improve the condition of endometriosis.[13,14]

Metformin is an antidiabetic drug that has beneficial properties for endometriosis treatment, such as lowering estradiol expression through peripheral inhibition of steroidogenic acute regulatory protein (StAR) pathway to decrease inflammation and proliferation markers; and it does not disrupt ovulation cycles of females of reproductive age.[5,15] In previous studies, the administration of this drug had been proven to decrease the Bcl-2/Bax ratio and endometrial implants in the animal models of endometriosis.[2,16] Metformin can decrease proinflammatory cytokines and growth factors, including TNF-α, IL-6, IL-8, and VEGF-23, as well as possess antitumor properties by activating AMP-activated kinase (AMPK) and inhibiting mammalian target of rapamycin (mTOR) to suppress proliferation.[17] It also has a positive effect on NFkB and PI3K/AKT/mTOR signals in the cells to lower local estradiol expression, inhibit inflammation process, reduce proliferation rates, and increase cell apoptosis, thus, providing an ideal microenvironment for stem cells to repair tissues damaged by endometriosis and encourage normal regeneration process.[8,18]

In our study, we aimed to determine the effect of adipose-derived MSCs in metformin-administered mice on the expressions of Bax/Bcl-2, Ki67, TNF-α, VEGF, and endometrial implants.

Materials and Methods

All procedures were approved by The Health Research Ethics Commission of the Faculty of Medicine, Diponegoro University (08/EC/H/FK-UNDIP/11/2019).

Stem Cell Preparation

This experiment was performed in the laboratory of the Faculty of Veterinary Medicine by a veterinarian and a

stem cell laboratory technician. The adipose tissue was extracted from the abdominal and pubic area of the mice, then washed with running water and antiseptic prior to being placed in a medium transport filled with α-MEM (R&D Systems, US) to be processed at the Stem Cell Research Laboratory. The adipose tissue sample was initially treated with 10 ml sterile phosphate-buffered saline (PBS) and 5 ml collagenase enzyme. The sample mixture was shaken using a magnetic stirrer for 20 minutes and centrifuged at 200 rpm and 38°C. After addition of 5 ml α-MEM, the mixture was centrifuged at 1600 rpm for 10 minutes and removed from the fluid. The formed clots from the mixture were treated with 10 ml α-MEM, resuspended, and placed in a Petri dish to be cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours, the culture was treated with 5 ml PBS, shaken gently, and removed from the PBS. This procedure was repeated twice. Ten ml α-MEM was added into the culture and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 5-10 days. Every 3 days, the culture was removed from the medium and 10 ml α-MEM was added to the culture media. Then 5 ml PBS and 2 ml trypsin (Thermo Fisher, US) was added to the mixture, shaken gently and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 5 minutes.

Mice

Thirty 3-month old Balb/c female mice, weighing 15-20 grams, bred in, and obtained from the Faculty of Veterinary Medicine at Airlangga University were used in this study. All mice were acclimatized for one week in standard polypropylene cages under the supervision of a veterinary surgeon. The determination of the sample size was based on the WHO principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches.[19] The mice were housed under controlled temperature (25±2°C), humidity (40-60%), and 12 hours of light and dark cycles. Throughout the experiment, each mouse received 5 g/100 g body weight/day food and water ad libitum. Interventions applied on all test subjects complied with the Declaration of Helsinki regulations and the guidelines of laboratory animals were followed throughout.

To induce endometriosis in mice, 1.8 mg of cyclosporine (Sandimmun, Novartis Indonesia) and 0.7 ml of endometriosis tissues were injected intraperitoneally into the test subjects using a 16G needle.[20] The endometriotic tissues were collected from an endometriosis patient from the Central General Hospital Dr. Kariadi Semarang and developed by being centrifuged two times at 2500 rpm with PBS, extracted from the supernatant, then treated with 200 IU/ml penicillin (Generik, Indonesia) and 200 μg/ml streptomycin (Generik, Indonesia). All mice received intramuscular injection of 0.05 ml 17β-estradiol (Ovalumon, Indonesia) on the first and fifth day. On the fifteenth day, the endometriotic mice were equally and randomly divided into five groups: S1 was given 0.1 ml of 2x105 adipose-derived MSCs and 4 mg metformin (Metformin 500 mg, Hexpharm Jaya) simultaneously for 14 days, S2 was given 0.1 ml of 2x105 adipose-derived MSCs, S3 was given 4 mg metformin for 14 days, S4 was given 0.1 ml NaCl 0.9% for 14 days, and S5 was given 4 mg metformin for 14 days and eventually 0.1 ml of 2x105 adipose-derived MSCs on the last day. The metformin was crushed, measured, and dissolved in 0.1 ml of aquadest then given to the mice orally using a food tube. MSCs were injected intravenously into the tails of the mice. After 14 days, all test subjects were anesthetized with sulfuric ether 2% (Merck, US) and sacrificed for the removal of peritoneal tissue and endometrial implants and analysis.

Immunohistochemistry

To prepare the immunohistochemistry specimen, the peritoneal tissue sample was deparaffinized using xylene solution for 5 minutes three times then rehydrated through absolute, 95%, 80%, and 70% alcohol (OneMed, Indonesia) baths each for two minutes respectively. Next, the specimen was placed in a medium filled with citrate buffer, heated in a decloaking chamber for 40 minutes at 97°C, and cooled down. H_2O_2 3% (OneMed, Indonesia) solution was added to the tissues and left for 20 minutes, washed with aquadest and PBS 1:9, treated with normal serum (BioLegend, US), left for 7.5-10 minutes, then washed with PBS 1:9. The primary antibody was dropped onto the slide, put into the cool chamber at 0-8°C overnight, and washed using PBS 1:9. The sample was treated with HRP Avidin (BioLegend, US), left for 10 minutes, washed with PBS 1:9, added with DAB solution (Sigma-Aldrich, Singapore), left for 20 minutes, and washed with aquadest for 10 minutes. Next, it was soaked in hematoxylin (Sigma-Aldrich, Singapore) for 3 minutes, lithium carbonate solution (Sigma-Aldrich, Singapore) for 1 minute, washed with running water for 5 minutes and immersed in a series concentrations of alcohol solutions (70%, 80%, 95%, and absolute) for 4 minutes each then dipped in xylene I and II for 10 times each and xylene III for 15-30 minutes. The sample was mounted in Entellan (Merck, US) and covered with cover glass. The values of Bax, Bcl-2, Ki67, VEGF, and TNF-α were acquired using the immunohistochemical staining method and measured with the Allred score index.[21] The presence of the expressions was identified with brown-colored chromogen in the immunohistochemistry staining. Two pathologists were involved in this study. The immunohistochemical staining

test using pkh-26 cell linker (Sigma-Aldrich, Singapore), fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Singapore), and rhodamine (Sigma-Aldrich, Singapore) were also performed to detect the presence of stem cells in the peritoneal tissue samples of mice (Figure 1).

Examination of Endometrial Implants

The incision of mice' endometrial implants was imaged on a millimeter block paper for the cross-sectional area of the implant to be measured in a millimeter square using the computer tracing method with the software 'Image Raster.'

Data Analysis

The normality test for all data was made using the Shapiro-Wilk test. This study is an analytic comparative research on at least two unpaired groups. The parametric bivariate analysis for normally distributed data in this study used One-way ANOVA with Least Significant Difference (LSD) Post-hoc test. The Kruskal Wallis test was used for variables with abnormally distributed data and followed with Mann Whitney Post-hoc test. Differences were considered to be statistically significant at $p<0.05$. All data analysis was performed using the SPSS software program.

Results

All experimental mice (n=30) successfully completed the study procedures until they were sacrificed.

Bax/Bcl-2

The expressions of Bax, Bcl-2, and Bax/Bcl-2 of the mice models from each study group are shown in Table 1-3 and Figure 2-3. The lowest mean Bax expression value was found in group S2, followed by groups S4, S3, S1, and S5. On the contrary, the S1 showed the lowest mean Bcl-2 expression value, followed by groups S3, S2, S5, and S4. Based on the Bax/Bcl-2 expression of the mice models, the lowest Bax/Bcl-2 ratio was found in group S4, followed by groups S2, S3, S5, and S1. The LSD test showed that the mean Bax/Bcl-2 ratio of the control group (S4) was significantly different compared to those of groups S1 (p=0.001), S3 (0.030), and S5 (p=0.025).

Ki67

The highest Ki67 expression was shown in Group S4, followed by groups S2, S3, S1, and S5 (p=0.004) (Table 4 and Figure 4). Ki67 expression in the control group (S4) was statistically significantly higher in S4 group compared to those of groups (p=0.002), S2 (p=0.028), S3 (p=0.007), and S5 (p=0.001).

VEGF

VEGF expression in control group(S4) was found to be highest, followed by S2, S3, S1, and S5 (Figure 5) (Table 5; p=0.079).

TNF-α

The highest TNF-α expression was found in the control group, followed by groups S3, S2, S5, and S1 (Figure 6); Table 6) (p=0.017).

Endometrial Implant

The highest value of endometrial implant in this study was found in S4, followed by S2, S3, S5, and S1 (p=0.001) (Table 7). Implant values were found statistically significantly higher in control group (S4) compared to S1 (p=0.003), S3 (p=0.010), and S5 (p=0.005) groups; the value of S2 group was also found to be significantly different than S1 (p=0.003) and S5 group (p=0.020), and the value of S1 was statistically significantly different than that of S3 groups (p=0.049).

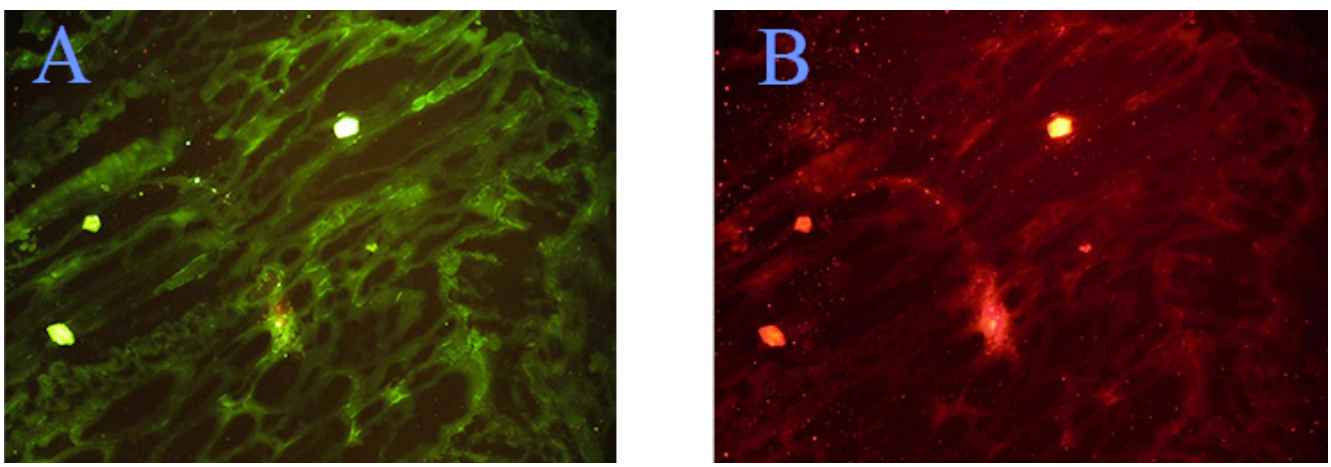

Figure 1. Immunofluorescence staining of MSCs in the mice peritoneum area using: (A) FITC and (B) rhodamine.
MSCs: Mesenchymal Stem Cells, FITC: Fluorescein Isothiocyanate

Table 1. Bax expressions in Balb/c mice of all groups analyzed using Allred score

	Bax Expression		
	Mean±SD	**Median (Minimum-maximum)**	**P***
MSCs+Mtf (S1)	4.07±1.53	4.90 (2.00-5.20)	0.036
MSCs (S2)	2.60±0.72	2.60 (1.80-3.40)	0.036
Mtf (S3)	3.97±0.48	3.80 (3.60-4.80)	0.036
NaCl (S4=control)	3.90±0.25	4.00 (3.60-4.20)	0.036
Mtf2+MSCs(S5)	4.53±1.28	3.80 (3.40-6.60)	0.036

**Kruskal-Wallis Test*

Table 2. Bcl-2 expression of Balb/c mice of all groups analyzed using Allred score.

	Bcl-2 Expression		
	Mean±SD	**Median (Minimum-maximum)**	**P***
MSCs+Mtf (S1)	2.93±1.57	2.80 (0,80-5.40)	0.003
MSCs (S2)	3.83±0.85	3.90 (2.80-4.60)	0.003
Mtf (S3)	3.53±0.47	3.40 (3.00-4.20)	0.003
NaCl (S4=control)	7.43±0.72	7.60 (6.20-8.00)	0.003
Mtf2+MSCs(S5)	3.87±0.37	3.90 (3.40-4.20)	0.003

**Kruskal-Wallis test*

Table 3. Bax/Bcl-2 expression ratio in Balb/c mice of all groups analyzed using Allred score.

	Bax/Bcl2 Expression		
	Mean±SD	**Median (Minimum-maximum)**	**P***
MSCs+Mtf (S1)	1.67±0.85	1.50 (0.77-2.75)	0.002
MSCs (S2)	0.71±0.29	0.65 (0.39-1.21)	0.002
Mtf (S3)	1.13±0.16	1.19 (0.86-1.31)	0.002
NaCl (S4=control)	0.53±0.07	0.51 (0.45-0.65)	0.002
Mtf2+MSCs (S5)	1.15±0.45	0.99 (0.81-1.94)	0.002

* *One-way ANOVA test*

* *The result of comparison between groups with Post-hoc LSD test: S1 vs S2: 0.001**; S1 vs S3: 0.050; S1 vs S4: 0.001**; S1 vs S5: 0.059; S2 vs S3: 0.122; S2 vs S4: 0.487; S2 vs S5: 0.106; S3 vs S4: 0.030**; S3 vs S5: 0.940; S4 vs S5: 0.025***

*** Significantly different*

Table 4. Ki67 expression in Balb/c mice of all groups analyzed using Allred score.

	Ki67 Expression		
	Mean±SD	**Median (Minimum-maximum)**	**P***
MSCs+Mtf (S1)	1.93±1.32	1.90 (0.60-3.80)	0.004
MSCs (S2)	2.97±1.03	3.40 (1.40-4.00)	0.004
Mtf (S3)	2.47±1.22	2.40 (0.80-4.00)	0.004
NaCl (S4=control)	4.93±2.45	3.90 (2.80-8.80)	0.004
Mtf2+MSCs (S5)	1.53±0.59	1.60 (0.60-2.20)	0.004

* *One-way ANOVA Test*

* *The result of comparison between groups with Post-hoc LSD test: S1 vs S2: 0.231; S1 vs S3: 0.532; S1 vs S4: 0.002**; S1 vs S5: 0.639; S2 vs S3: 0.558; S2 vs S4: 0.028**; S2 vs S5: 0.101; S3 vs S4: 0.007**; S3 vs S5: 0.278; S4 vs S5: 0.001***

***Significantly different*

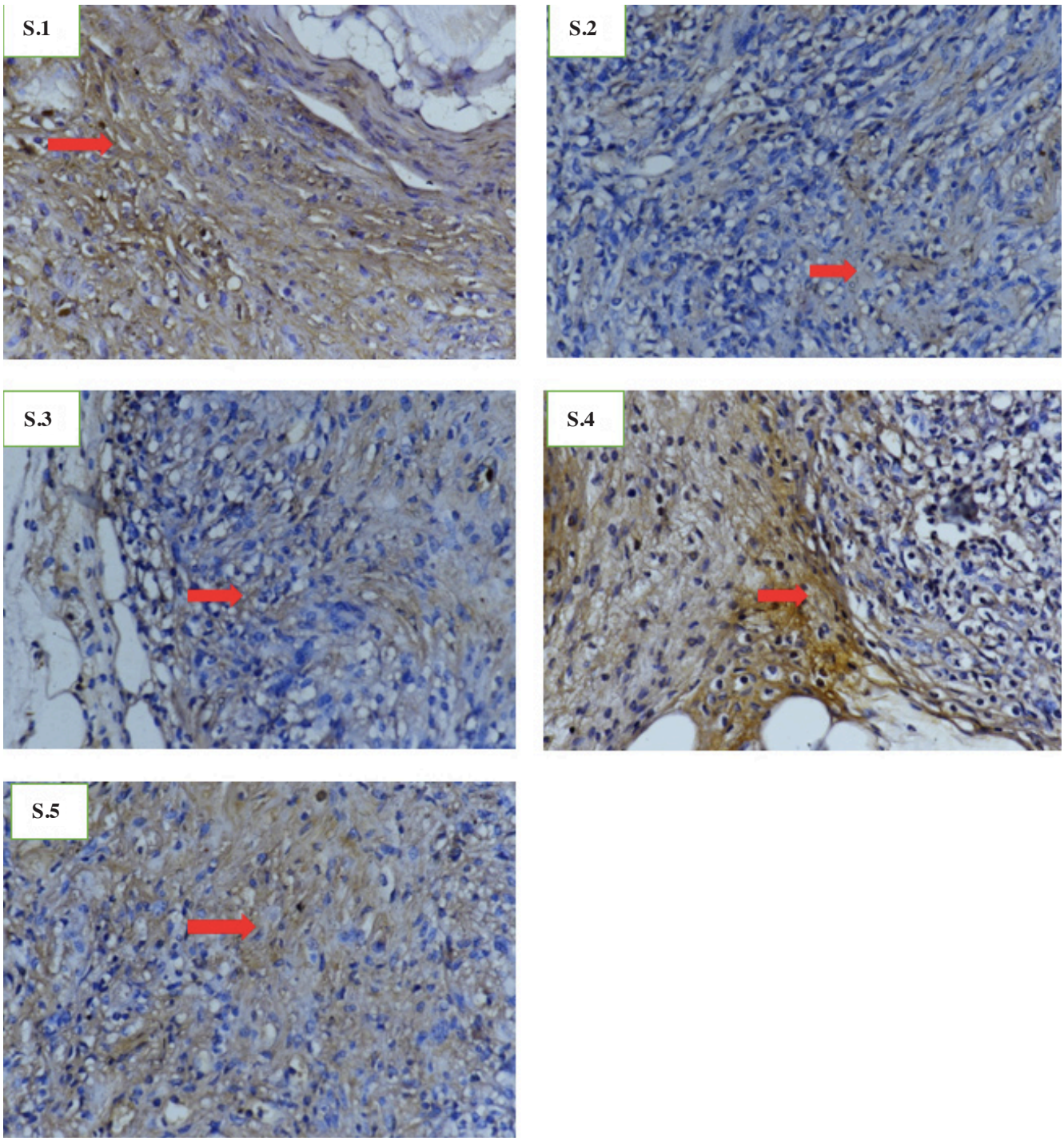

Figure 2. Comparisons of Bax expressions (red arrows) in the endometrial cells of mice among the groups. The expressions of Bax are identified with brown-coloured chromogen. (400x magnification).

Discussion

A stem cell is an undifferentiated cell that is capable to proliferate, regenerate, and differentiate into specific cells to mend damaged cells, tissues, and organs through its paracrine effect by releasing cytokines, chemokines, and immunoregulators to encourage cell regeneration.[13,22,23] Several studies propose that anomalous stem cells are the primary cause of endometriosis due to the findings of abnormal morphology, biomarker surface, gap junctional communication, and differentiation ability of stem cells in endometriosis patients.[3,15] MSCs have been used in regen-

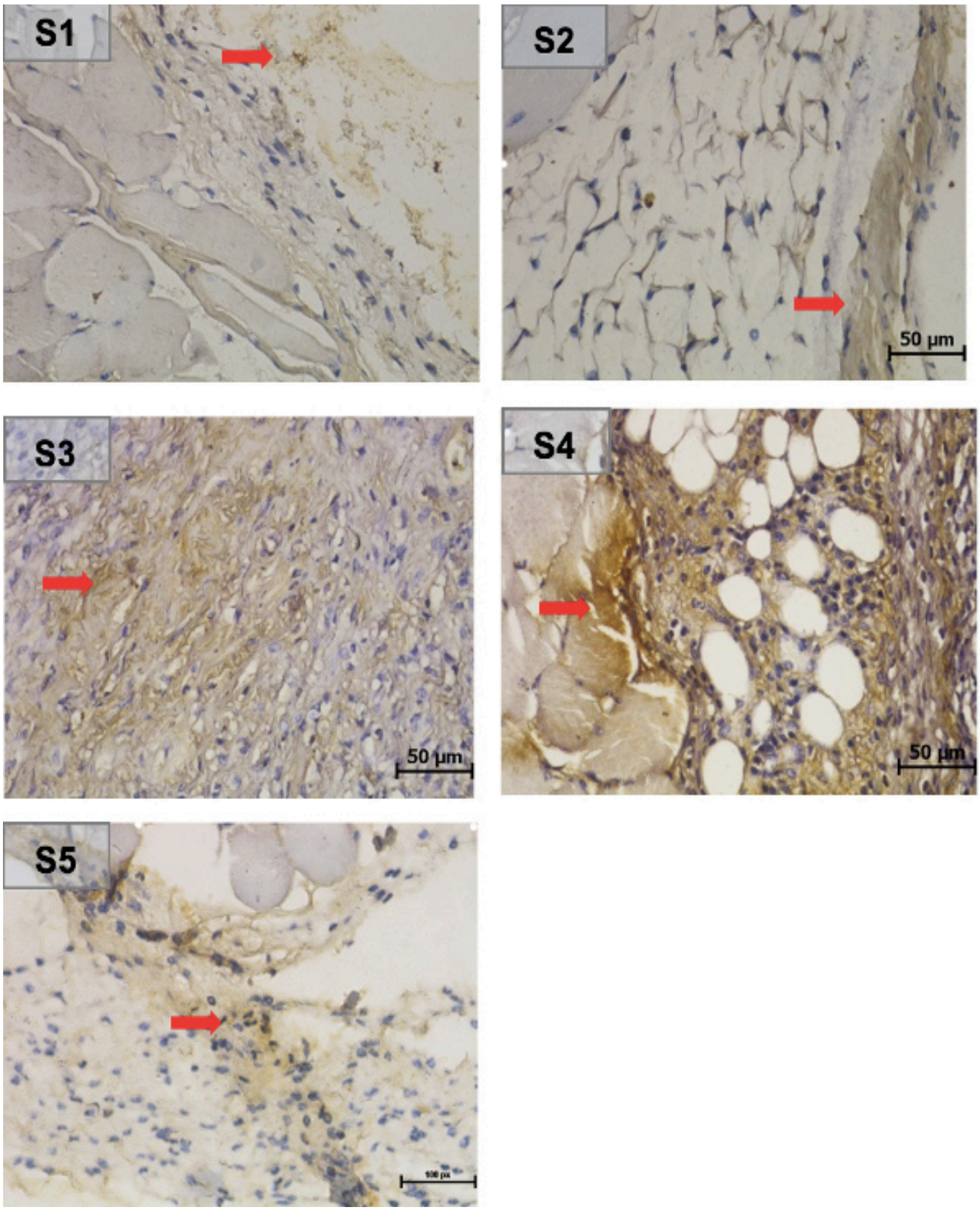

Figure 3. Intergroup comparisons of Bcl-2 expressions (red arrows) in the endometrial cells of mice The expressions of Bcl-2 are identified with brown-coloured chromogen. (400x magnification).

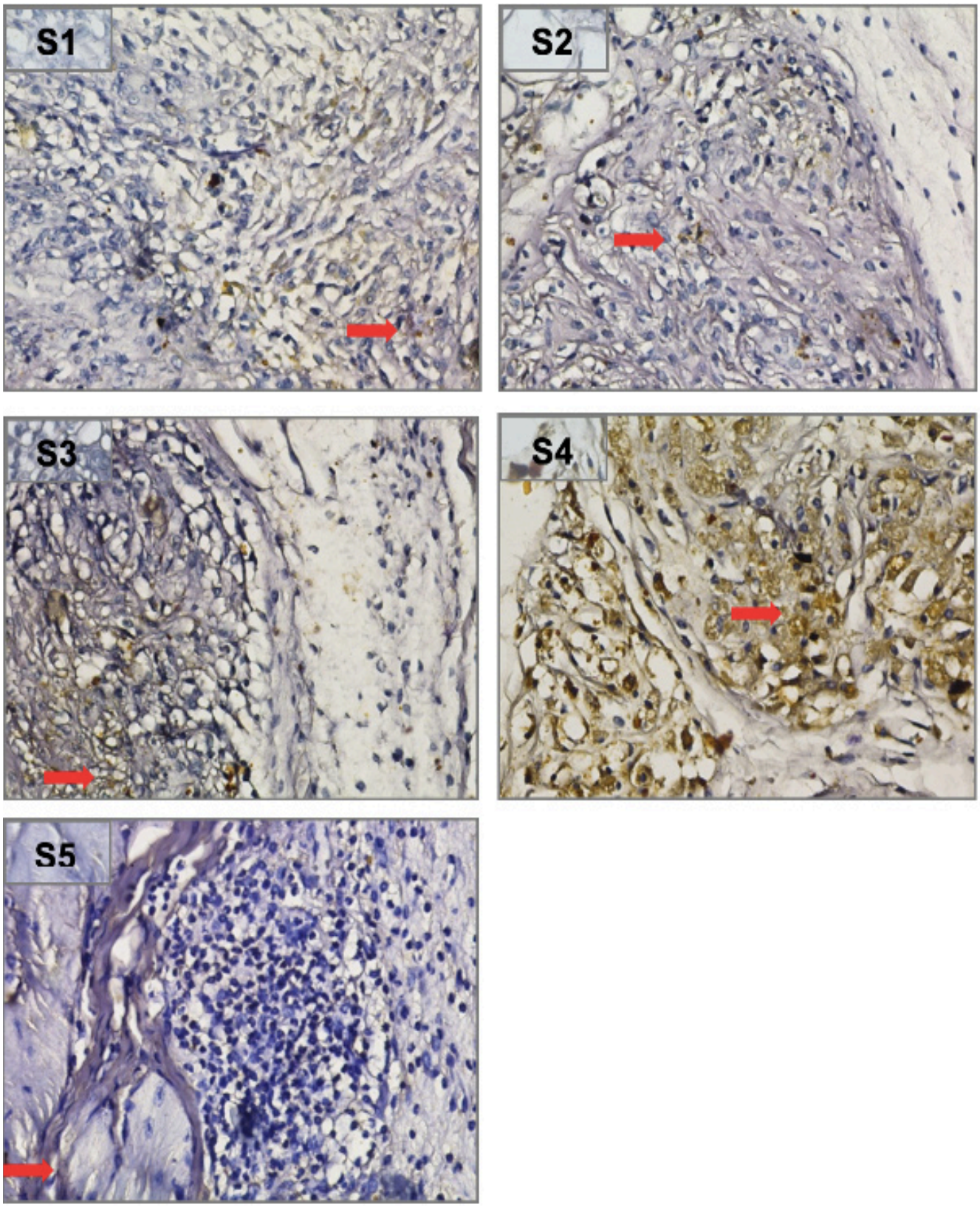

Figure 4. Intergroup comparisons of Ki-67 expressions (red arrows) in the endometrial cells of mice. The expressions of Ki67 are identified with brown-coloured chromogen. (400x magnification).

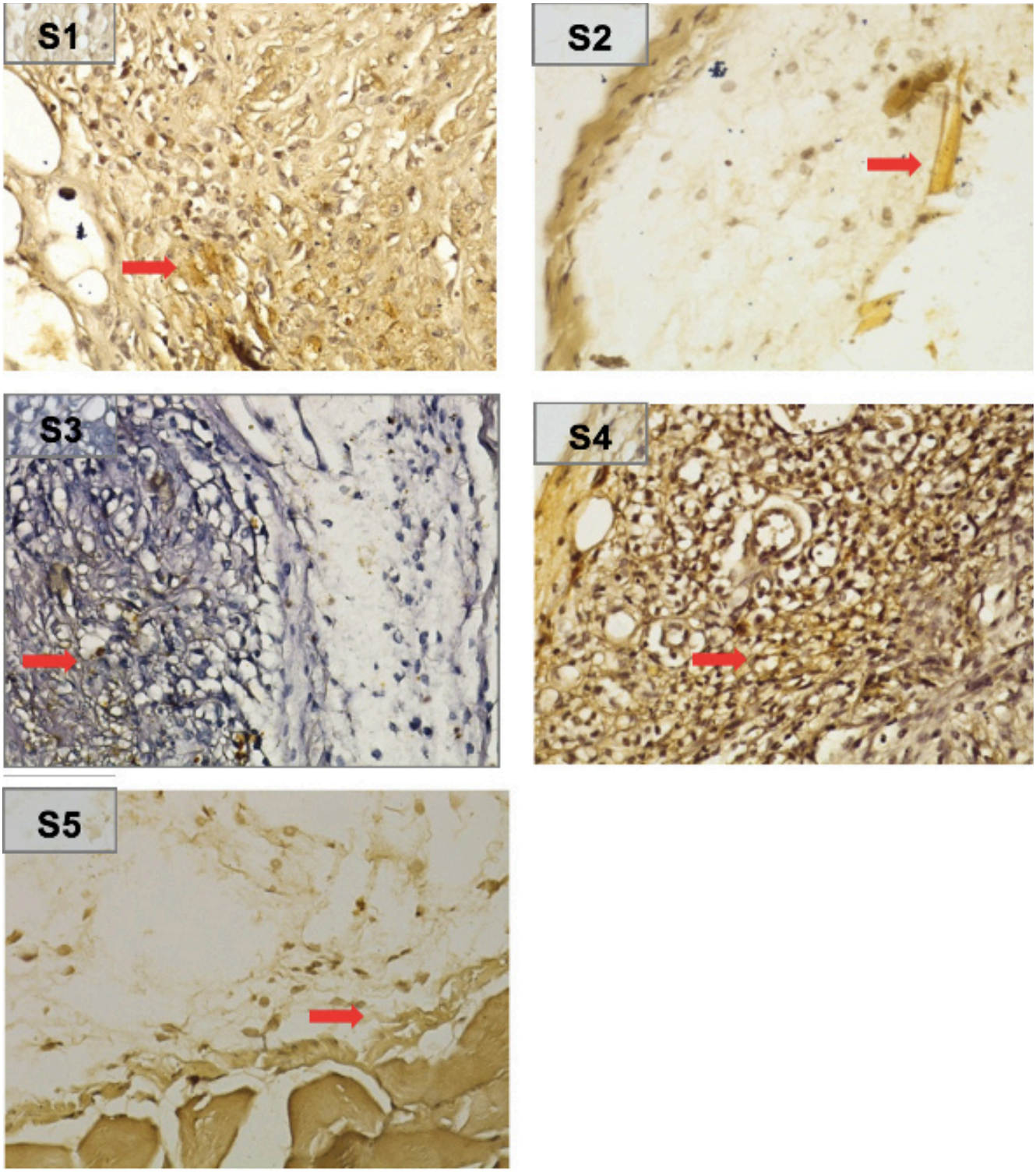

Figure 5. Intergroup comparisons of VEGF expressions (red arrows) in the endometrial cells of mice. The expressions of VEGF are identified with brown-coloured chromogen. (400x magnification) (VEGF: Vascular endothelial Growth Factor).

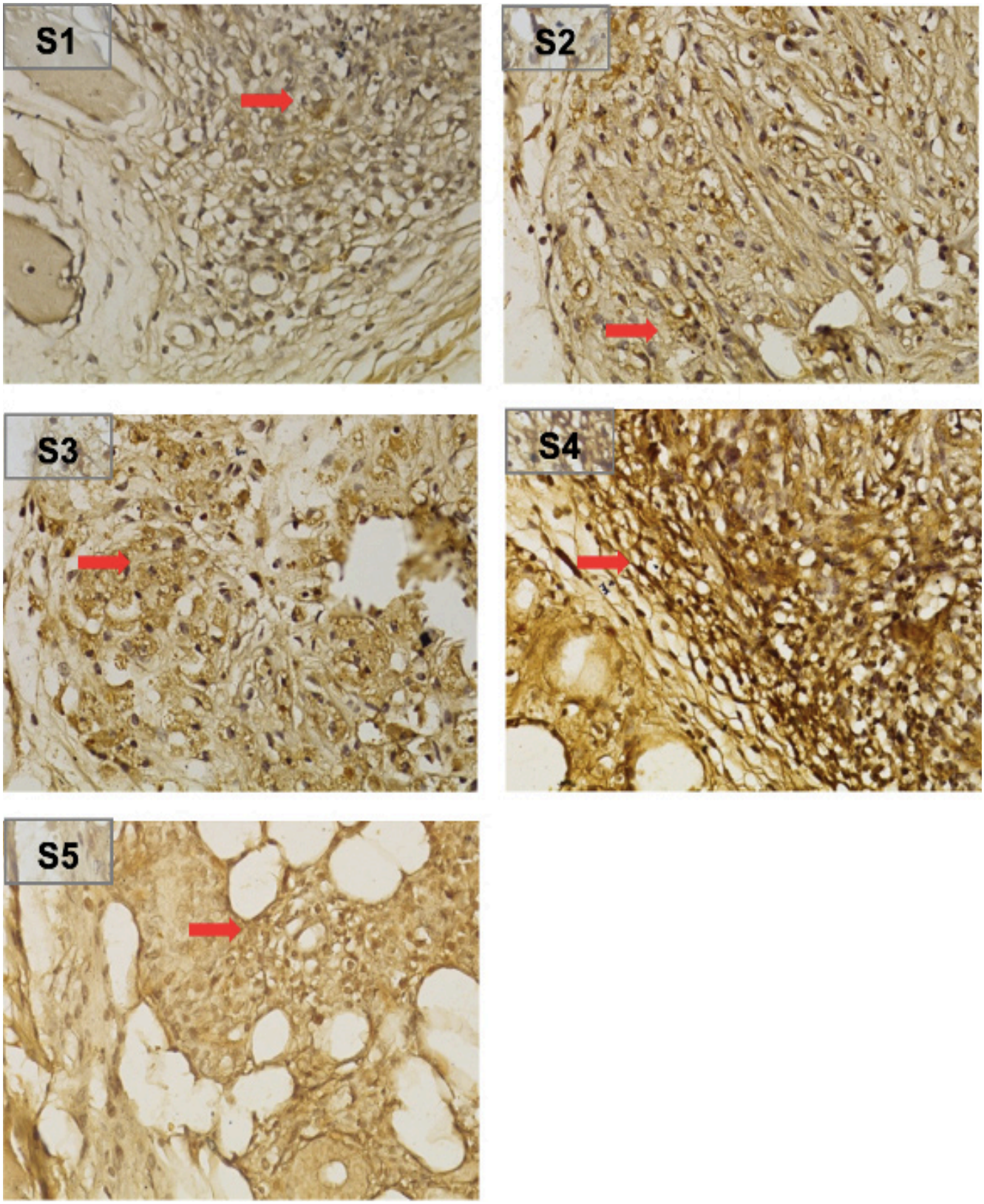

Figure 6. .Intergroup comparisons of TNF-α expressions (red arrows) in the endometrial cells of mice. The expressions of TNF-α are identified with brown-coloured chromogen. (400x magnification).

Table 5. VEGF expression in Balb/c mice of all groups analyzed using Allred score.

	VEGF Expression		
	Mean±SD (ng/mL)	**Median (Minimum-maximum) (ng/mL)**	**P***
MSCs+Mtf (S1)	6.57±2.54	6.90 (3.20-9.60)	0.079
MSCs (S2)	8.30±3.04	8.80 (4.20-11.40)	0.079
Mtf (S3)	6.57±2.80	6.60 (3.80-11.40)	0.079
NaCl (S4=control)	9.87±2.02	10.50 (7.00-12.00)	0.079
Mtf2+MSCs(S5)	6.20±1.70	6.40 (3.80-8.40)	0.079

* *One-way ANOVA Test*

Table 6. TNF-α expressions in Balb/c mice of all groups analyzed using Allred score.

	TNF-α Expression		
	Mean±SD (ng/mL)	**Median (Minimum-maximum) (ng/mL)**	**P***
MSCs+Mtf (S1)	4.67±2.15	4.00 (2.60-8.60)	0.017
MSCs (S2)	7.37±2.52	7.20 (4.60-10.80)	0.017
Mtf (S3)	7.50±2.33	6.60 (6.00-12.00)	0.017
NaCl (S4=control)	8.60±0.93	8.00 (8.00-9.80)	0.017
Mtf2+MSCs (S5)	5.60±1.89	6.20 (3.00-7.20)	0.017

* *Kruskal-Wallis Test*

* *The result of comparison between groups with Mann-Whitney test: S1 vs S2: 0.055; S1 vs S3: 0.036**; S1 vs S4: 0.022**; S1 vs S5: 0.420; S2 vs S3: 0.936; S2 vs S4: 0.191; S2 vs S5: 0.259; S3 vs S4: 0.048**; S3 vs S5: 0.218; S4 vs S5: 0.003***

** *Significantly different*

Table 7. Lengths of endometrial implants used in Balb/c mice of all groups.

	TNF-α Expression		
	Mean±SD (ml)	**Median (Minimum-maximum) (ml)**	**P***
MSCs+Mtf (S1)	0.86±2.10	0 (0-5.16)	0.001
MSCs (S2)	28.36±21.14	16.39 (7.54-59.93)	0.001
Mtf (S3)	12.19±10.24	10.57 (0-26,160)	0.001
NaCl (S4=control)	42.41±19.90	39.11 (21.18-71.15)	0.001
Mtf2+MSCs (S5)	3.61±13.63	0 (0-21.660)	0.001

* *Kruskal-Wallis Test*

* *The result of comparison between groups with Mann-Whitney test: S1 vs S2: 0.003**; S1 vs S3: 0.0495**; S1 vs S4: 0.003**; S1 vs S5: 0.902; S2 vs S3: 0.199; S2 vs S4: 0.200; S2 vs. S5: 0.020**; S3 v s S4: 0.010**; S3 vs S5: 0.108; S4 vs S5: 0.005***

** *Significantly different*

erative medicine.[13] MSCs have immunosuppressive properties to treat immune-mediated illnesses, including improving thyroiditis conditions and enhancing thin endometrium through its anti-inflammatory and immunomodulatory effects, as well as lowering VEGF receptors and TNF-α in endometrial implants.[24] MSCs can also inhibit mixed lymphocyte response and T-cell proliferation caused by mitogenic allogenic factors and regulate the immune system by enhancing regulatory T-cell response and lowering TNF-α, interferon-gamma (IFN-γ), and IL-4.[25]

Endometriosis is a disorder where ectopic endometrial cells show abnormal proliferation and apoptosis.[6] Apoptosis has a major role in maintaining tissue homeostasis and removes excessive or dysfunctional cells, which helps to eliminate endometrial cells expelled from the cavity during menstrual bleeding and prevents the development of endometriosis.[6] The inability of endometrial cells to send apoptotic signals and the ability of endometrial cells to survive from apoptosis have been linked with the increase of anti-apoptotic factors and the decrease of pro-apoptotic factors.[6] In our study the administration of metformin alone and the combination of MSCs and metformin were able to increase the Bax/Bcl-2 expression ratio, whereas MSCs alone could not improve the ratio. According to multiple preceding studies, stem cells have

the potential to induce cell apoptosis, however, the present study showed that the stem cell by itself could not increase the rate of apoptosis.[26,27] Our findings are consistent with the study done in Egypt in which the serum from subjects with endometriosis that was treated with MSCs was not able to increase apoptotic cells and instead turned the stem cells into endometriotic cells.[28] It is believed that the dominant environment of endometriosis induces inflammation, anti-apoptosis, and growth signal, and subsequently transforms healthy stem cells into endometriotic cells.[28] The administration of metformin in the mice group (S3) led to increase the Bax/Bcl-2 expression compared to that of the control group (S4). This discovery is in line with the mouse model in endometriosis research conducted by Tian et al. where mice with endometriosis that received metformin showed a decrease in Bcl-2 and an increase in p53 and Bax expressions.[29] It is also assumed that there was a synergistic effect of MSCs and metformin that contributes to the higher rates of apoptosis since the mice group that received MSCs and metformin showed the highest Bax/Bcl-2 expression.

The proliferation rate is also an important factor in measuring the success of endometriosis management.[6] Although all groups that received treatment showed a decrease in Ki67 expression compared to the control group, the mice that were given metformin-MSCs combination demonstrated the biggest reduction in the proliferation rate. Metformin suppresses angiogenesis and possesses antiproliferative activity related to the termination of cell cycle and apoptosis mediated by oxidative stress, AMP3 activation, and FOXO3a.[29–31] These findings are consistent with previous studies that showed antiproliferative properties of MSCs and metformin.[26,32,33]

Angiogenesis has a significant role in the development and growth of endometriotic lesions and is mediated with VEGF.[3] However, there was not a significant difference in terms of VEGF expression in all groups that may be related to the short duration of the experiment. According to a study conducted by Foda AA and Aal IAA, the administration of metformin for 3 months could decrease serum VEGF levels compared to control group.[34] TNF-α is a pro-inflammatory and proangiogenic cytokine that is associated with the aggravation of endometriosis.[25] This study showed that there was a decrease in TNF-α expression in all experimental groups, especially the group that received MSCs and metformin simultaneously. This discovery is in line with an experiment by Omer NA et al. where endometriosis patients who received metformin for 3 months showed a reduction in pain, dysmenorrhea, IL-8, and TNF- α.[25]

In the present study, there was a significant difference in the endometrial implant sizes in all groups, except for the MSCs group. The highest reduction was found in the group who received metformin and MSCs. In previous studies, metformin has been proven to successfully cause reduction in the size of endometrial implants.[2,16] Metformin can diminish inflammatory signals and suppress anti-inflammatory, anti-proliferative, and apoptotic signals by activating AMPK, inhibit prostaglandin and inflammation, lower aromatase enzyme activity, improve the hyperandrogenic environment by elevating sex hormone-binding globulin (SHBG), as well as trigger apoptosis in eutopic endometrium by decreasing Bcl-2 and improving p53 and Bax.[35,36] Metformin may lower PGE2-stimulated StAR expression by preventing cAMP- response binding element protein (CREB)-regulated transcription coactivator 2 (CRTC2) by phosphorylating AMPK, thus, CREB-CRTC2 and excessive estradiol would not be secreted. It is assumed that metformin activates AMPK which eventually suppresses the mTOR pathway through TSC2 and inhibits prostaglandin response and inflammation.[1] Furthermore, metformin can decrease insulin-like growth factor-1 (IGF-1) receptor signal by decreasing insulin level through AMPK-dependent insulin receptor substrate-1 (IRS-1) phosphorylation which prevents tumor cell growth via hypoxia-inducible factor 1α (HIF-1α), p53, c-Myc oncogene, DICER1, and suppression of fatty acid synthesis.[35,37–39] This antidiabetic drug can also inhibit mTORC1 independent of AMPK that will inhibit serine ataxia telangiectasia mutated (ATM) protein kinase and reduce ROS produced by mitochondria.[35,37,38] Metformin may alter negative signals to positive signals for normal cell growth and development.[35,37] Since MSCs are sensitive to the environment this signal modification enables MSCs to properly ameliorate endometriosis as an immunoregulatory, anti-inflammatory, and anti-tumor agent.[37,40]

In conclusion, our study has shown that MSC by could not alleviate endometriosis. Although metformin alone showed favorable effects in the development of endometriosis syndrome, the synergistic combination of MSCs and metformin offered more effective and promising results for the future treatment of endometriosis.

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Conflict of Interest: We do not have a conflict of interest to declare as well as an informed consent since our study was performed on mice.

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