The effect of BMSCs on implantation mechanisms and pregnancy process in the experimental Asherman model

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Abstract. – **OBJECTIVE:** In this study, we aimed to investigate the role of bone marrow-derived mesenchymal stem cells (BMSC) on the molecular mechanisms involved in the removal of endometrial adhesions and the implantation process in the experimentally-created Asherman syndrome model in rats.
MATERIALS AND METHODS: An experimental model chemical agent was used. Culture medium (CM), only BMSC, 48-hour culture medium (Niche), and BMSC+ niche were used as therapy. Each group was divided into two subgroups, and changes in the endometrial tissue were investigated by histochemical and immunohistochemical staining.
RESULTS: The results of the study show that BMSCs exhibit therapeutic properties in endometrial damage, increase endometrial thickness and vascularization, and contribute to the implantation of embryos by reducing fibrous areas. These results are promising for the prevention of Asherman-induced infertility.
CONCLUSIONS: It has been shown that BMSC and Niche applications can be helpful in preventing adhesion molecules in the mechanisms that cause infertility.

Key Words:
 Bone marrow stem cell, Asherman's syndrome, Implantation, Infertility, Endometrium.

Introduction

Asherman syndrome (AS) is an intrauterine adhesion (IUA) condition that occurs both in cases of miscarriage and after frequent gynecological operations. Some authors have proposed to define the term "Asherman's syndrome" for patients with amenorrhea, those with a completely occluded uterus, or patients who had a recent cesarean delivery (surgical operation). Due to the increase in its prevalence in recent years, studies[1] on alternative treatments continue. It is seen as a situation where multiple deficiencies emerge with the increase in cases[2]. Most women with AS experience infertility or recurrent spontaneous abortions. In addition, AS manifests itself with menstrual disorders in many women[3]. The true incidence of AS is unknown due to the large patient group. An incidence of 0.3-21.5% was reported[4] when a wide spectrum of incidentally detected cases, postpartum abortion cases, and other cases were evaluated. Among these non-obstetric causes, curettage, uterine septum resection, hysteroscopic myomectomy, and abdominal myomectomy have a large place[2].

Stem cell studies[3] in the last decade have progressed so rapidly that it has raised the hopes of those seeking remedies from untreatable cancer to metabolic diseases, even rheumatic and neurodegenerative diseases. When tissue damage develops, stem cells separate from their microenvironments and migrate to the area where the damage develops[4-6]. BMSCs are the most common cells in living things and have the most characteristic features of human stem cells. These bone marrow-derived cells, which are adult stem cells and have the characteristics of stromal-derived support cells, are used in many areas of medicine[7,8]. Bone marrow is the primary source of stem cells in mammals, and this tissue is considered to be the main source of MSCs. MSCs are the most abundant cells in living things and have the most characteristic feature of human stem cells9. Stem cell therapy for endometrial restoration has recently moved from being an edge treatment to becoming a central treatment option. In particular, stem cells obtained from bone marrow have become the most frequently used stem cell source. These cells are isolated directly from the

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bone marrow by aspiration. BMSCs can be easily obtained from both humans and rodents due to their extensive migratory and pluripotent potential[10]. Stem cells with endometrial regeneration and angiogenesis effects are purified from the bone marrow[11]. MSC, which is easy to obtain and reproduce, has recently moved away from being an edge treatment and has become a central treatment option for endometrial restoration.

In this study, the AS model was considered with BMSCs isolated from the femoral bone of male rats, and the changes in the uterus were evaluated with histopathological parameters. Pregnancy rate, implantation morphology, and histopathology of the endometrium after AS were investigated histochemically and immunohistochemically.

Materials and Methods

Experimental Animals

A total of 43 rats (40 adult female and 3 male Wistar albino rats with a weight of 200-300 g) were involved in the study. Male rats were preferred for bone marrow-derived stem cell isolation. Standard conditions are provided for the adaptation of the experimental animals to the laboratory conditions. AS as an implantation failure model was chemically induced in a single horn of the uterus in female rats with trichloroacetic acid (left horn 0.1 ml). The next day, stem cell treatment applications were carried out according to the experimental groups.

Groups

Culture medium (CM) (n = 10): the group in which 0.1 ml of trichloroacetic acid was applied to the left uterine horn, and then freshly prepared medium (CM) was given 1 ml ip for 10 days.

Niche (n = 10): One day later, in the AS model, 1 ml of 48-hour medium (Niche) with cells removed for 10 days was applied as ip.

BMSC (n = 10): One day later, in the AS model, BMSC was centrifuged, added to the new medium, and applied as 1×10^6/ml ip for 10 days.

BMSC + Niche (n = 10): One day later, in the AS model, BMSC kept in the medium for 48 hours was applied as 1×10^6/ml ip for 10 days.

Cytological smears of female rats were examined 10 days after stem cell therapy was started, and proestrus and estrus periods were determined. Afterward, they were placed in the same cage as male rats, and the next day vaginal smears were taken, and the animals detected to have sperm were considered pregnant and placed in separate cages.

On the 12th day of pregnancy, the animals were sacrificed, and both the right and left sides of the uterine tissues with bilateral anatomical structures were removed for implantation evaluation.

Stem Cell Characterization

Stem cell characterization was performed by indirect immunohistochemistry using anti-Stro-1 antibody after BMSC was reproduced up to passage 4. Cells in passage 4 with Stro-1 and c-kit (+) were accepted as mesenchymal stem cells.

Indirect Immunohistochemical Staining

Sections of 4 μm thickness were taken from uterine tissue and, after deparaffinization, were lowered into water. The sections were diluted 1/100 in a humid chamber with primary antibodies anti-Stro-1 (MAB4315, Millipore, Cell Signaling Technology, Trask Lane Danvers, MA, USA), anti-c-kit (sc-168, Santa Cruz Biotechnology, Dallas, Texas, USA), anti-Laminin (sc-5582, Santa Cruz Biotechnology), anti-Fibronektin (sc-18825, Santa Cruz Biotechnology), anti-VEGF (ab1316, Abcam, Waltham, MA, USA) and incubated for 1 hour. Subsequently, sections washed with PBS solution for 3x5 minutes were stained with DAB for 3-5 minutes to ensure the visibility of the immunohistochemical reaction. Background staining was done with Mayer's hematoxylin, and then the sections were washed with distilled water for 10 minutes and covered with a sealing medium[11,12].

Statistical Analysis

In the evaluation, it was examined as 0 = little inflammation and less fibrosis, 1 = less inflammation and moderate fibrosis, 2 = moderate inflammation and much fibrosis, 3 = much inflammation and fibrosis, and statistical differences were determined. In addition, immunohistochemical staining results were evaluated by examining the H-score. The staining rate was graded semi-quantitatively. Descriptive statistics and statistical analysis of study variables were carried out using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). The resulting data were compared with the One Way-ANOVA Tukey statistical test, and $p < 0.05$ results were considered statistically significant[12].

Results

Definition of BMSC

Cell culture was cultured until confluent in BMSC media collected from the rat femur region.

After passaging the cells up to the third passage, it was found that there was sufficient cell density for treatment. When examined under an inverted microscope, it was seen that the cells were fibroblast-like spindle-shaped, and the cell nucleus settled in the middle (Figure 1a). Stro-1 and c-kit (Figures 1b and 1c) reactivity as stem cell markers were confirmed immunohistochemically in terms of positivity when BMSC in the P4 cell culture was confluent in the culture dish.

Vaginal Smear Results

A vaginal smear was performed to determine the gestational days of the subgroups. Subjects with sperm in their vaginal smears were considered pregnant, and on the 12th day of pregnancy, these subjects were sacrificed under general anesthesia, and their implantation numbers were determined. Sperm identified by a hook-shaped head and a long tail were observed in vaginal smears (Figures 1d and 1e).

Non-Pregnancy Groups Findings

The left uterine horn was treated with acetic acid to cause damage, while the right uterine horn was kept as the control, and no damage was caused by any chemical or mechanical element. After the AS was created, therapeutic agents were administered continuously for 10 days. 10 days after the applications, uterine samples were taken, and the results were examined in terms of endometrial thickness, number of glands in the stroma, inflammation, and fibrosis. In the media and Niche applied groups (CM, Niche), the endometrium was heavily degenerated, the synechiae areas were excessively increased, and the number of glands was significantly reduced compared to the BMSC and BMSC + Niche groups ($p < 0.001$). It was observed that the endometrial epithelium disappeared, and dense fibrous areas were observed in the lamina propria of the groups treated with CM and Niche. Again, in these groups, it was observed that both the number of glands decreased, and the tubular structure of the gland epithelium was disrupted (Figure 1j). In addition, it was determined that inflammation was observed intensively in the groups given CM and Niche. Significant histological changes in inflammation, fibrosis, number of glands, and endometrial thickness were determined in the groups in which BMSC and BMSCs + Niche compared to the groups given CM and Niche. Statistical significance was determined at $p < 0.05$ (Table I).

Pregnancy Groups

The subjects whose pregnancies were detected were sacrificed after 12 days, the embryos were fixed, and the H&E staining was performed after
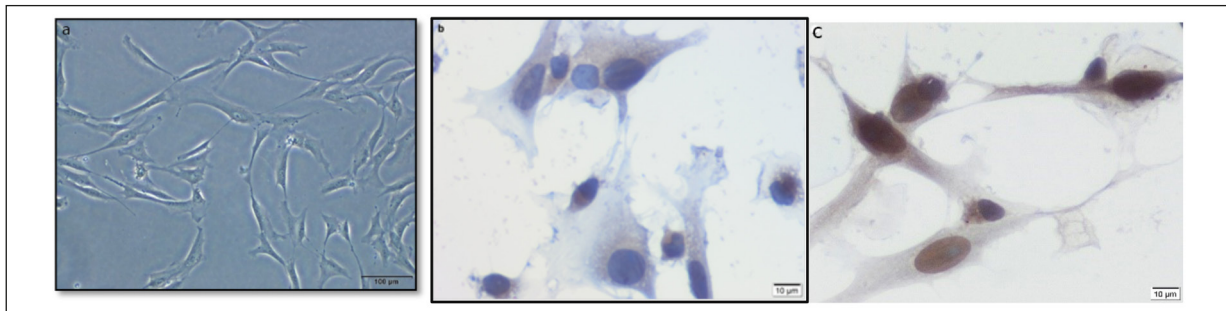

Figure 1a-c. Cell culture: (**a**) Inverted microscope images of the 4th passage cells settled and multiplied in the culture medium of BMSC (X100), (**b-c**) Positive immunohistochemistry image of BMSC stained with Stro-1 and c-kit (X400).
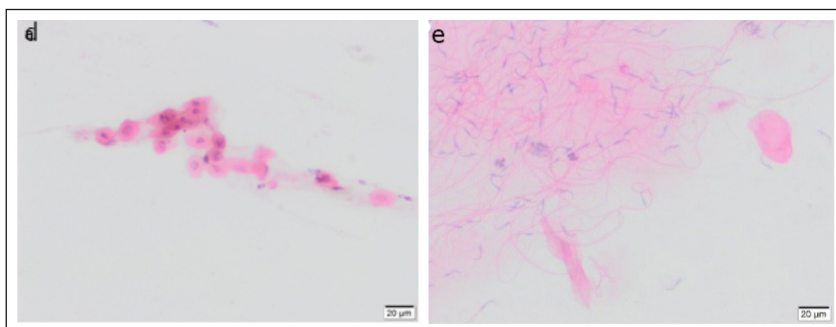

Figure 1d-e. Vaginal smear: Evaluation results of vaginal smear preparations of the groups with light microscopy, (**d**) Vaginal smear from the non-gestational cycle; proestrus (X200), (**e**) in the vaginal smear after mating, hooked sperm are noted among the epithelial cells H&E (X200).
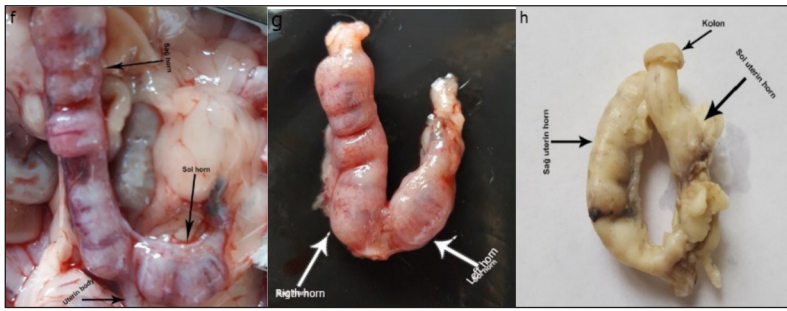
Figure 1f-h. Macroscopic presented: Embryos implanted in right and left uterine horns in post-Asherman treated groups. **f-g**: post-experimental dissection, **h**: fixed for histological follow-up.

Figure 1j. Endometrial thickness measurement of the right horn as a control (**j1**) and left horns of the AS (**j2**), left horns of niche groups (**j3**), left horns of BMSC groups (**j4**) and left horns of BMSC+Niche groups (**j5**) with the ImagJ program (X50).
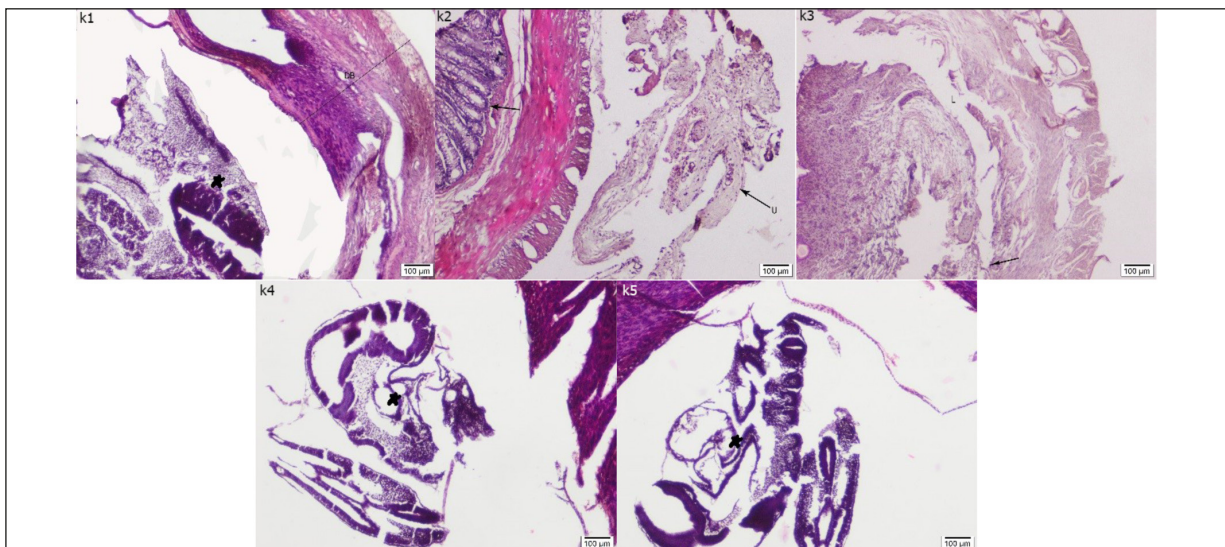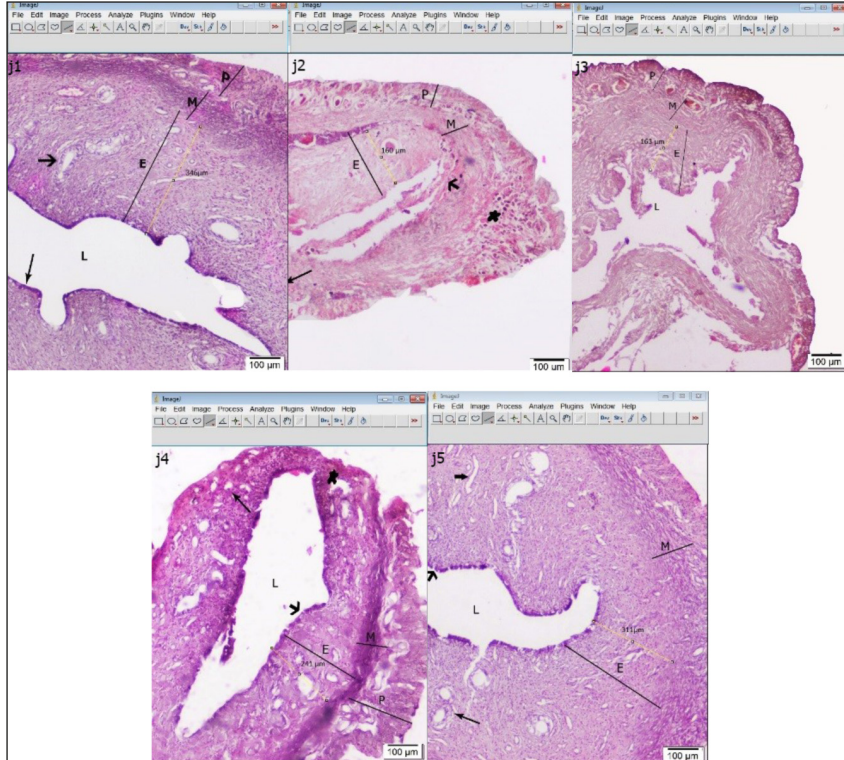

Figure 1k. Embryos implanted in right and left uterine horns in post-Asherman treated groups, H&E. **k1, k4, k5**, implanted right horn, (**k2**): non-implanted left horn, (**k3**) non-implanted left horn (star: embryo, arrow: intestinal tissue) (X50).

Table I. Fibrosis, inflammation, endometrial thickness, and gland numbers of the experimental groups were evaluated under a light microscope.

	Inflammation	Fibrosis	Endometrial thickness	Glands number
CM	2.4 ± 0.51	2.7 ± 0.48	138.72 ± 26	5 ± 2
Niche	1.6 ± 0.51	2.3 ± 0.48	154.24 ± 26	5 ± 1
BMSC	0.9 ± 0.56	1.7 ± 0.48	228.96 ± 26	10 ± 2
BMSC + Niche	0.5 ± 0.52	0.9 ± 0,.73	315.35 ± 32	14 ± 3
Right horn (Control)	0.2 ± 0.30	0.7 ± 0.53	333.15 ± 18	16 ± 3

Bone marrow-derived mesenchymal stem cells (BMSC), Culture medium (CM).

histological follow-up. No embryos with complete implantation were found in the left horns of the CM and Niche groups. Embryos implanted were found in the right uterine horns of all lower secondary groups (Figures 1f-h) and both the right and left uterine horns of the BMSC and BMSC + Niche groups.

After the number of implanted embryos of the second subgroup was determined, they were followed, and immunohistochemical staining was performed. When the implantation numbers in the groups were compared, a statistically significant difference was found ($p < 0.05$) (Table II).

Immunohistochemistry Findings

Immunohistochemical staining with c-Kit antibody revealed positive areas in the surface epithelium, endometrial glands, and myometrium in the uterine structure. The c-kit positivity was

Table II. Implantation numbers of the subgroups in the left horn.

	CM	Niche	BMSC	BMSC + Niche
Implanation of left horn	0.33 ± 0.5	0	1.5 ± 0.57	3.2 ± 0.83
Implanation of right + left horn	4.33 ± 0.57	4.75 ± 0.5	6 ± 0.81	8.2 ± 0.83

No significant difference was found between CM and Niche, and between Niche and BMSC ($p > 0.05$). Between CM and BMSC *$p < 0.05$, between CM and BMSC + Niche ****$p < 0.0001$, between BMSC + Niche and Niche only ****$p < 0.0001$ statistical significance was determined. Bone marrow-derived mesenchymal stem cells (BMSC), Culture medium (CM).
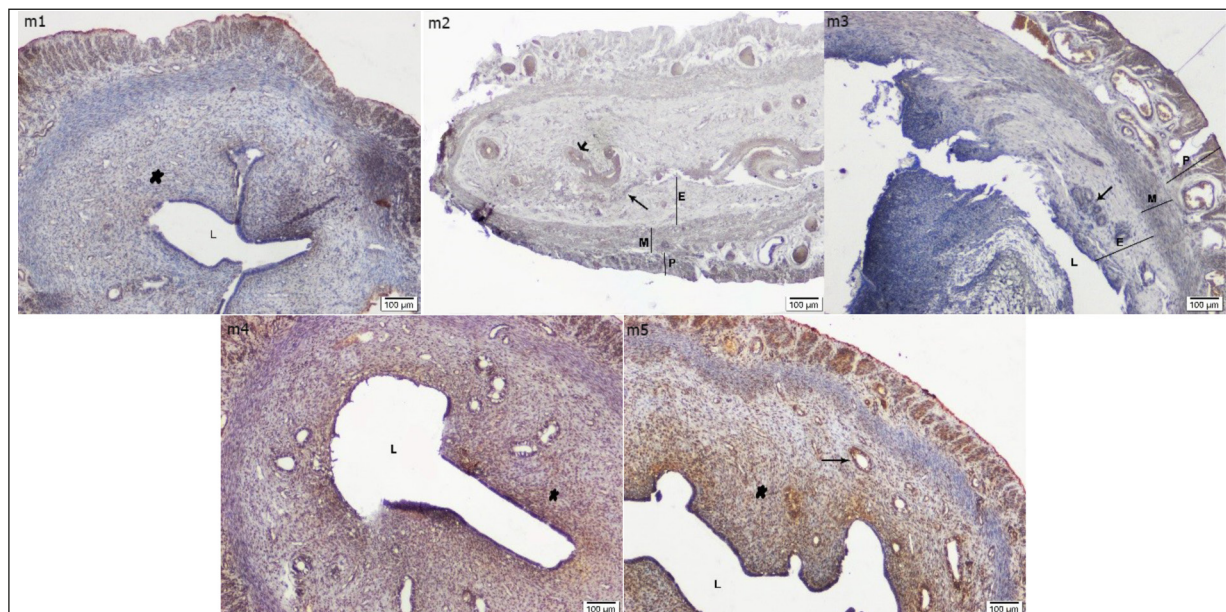

Figure 1m. c-kit staining images in the endometrium of the sub-groups. **m1**, right horn (control), (**m2**) left horn of AS, (**m3**) left horn of AS+Niche, (**m4**) left horn of AS+ BMSC, (**m5**) left horn of AS+ BMSC+ Niche (star: positive, arrow: positive cells, X50).

observed to be very pale staining around the endometrial glands of CM and Niche groups. It was observed that only stem cells (BMSC) were applied in the form of intense staining in both endometrial gland cells, myometrium, and stroma. The highest positive immunoreactivity was especially seen in the right uterine horn tissues of the same group with BMSC and Niche (Figure 1m). Nitric oxide is an important indicator of oxidative stress. In order to determine the change in nitric oxide synthase (NOS) activity in our subgroups, the distribution of eNOS synthesis in uterine tissue was determined by immunohistochemistry. The highest eNOS immunoreactivity was observed in the CM and Niche subgroups, in which there was high denervation, epithelium disappeared, and synechiae occurred. It was observed that positivity decreased in BMSC and BMSC + Niche groups, reaching values close to the immunoreactivity in the right uterine tissue kept as a control (Figure 1n). Vascular endothelial-derived growth factor (VEGF) immunoreactivity occurred intensely around the vessels, also in the lamina propria and myometrium. The immunoreactivity was quite low in the CM and Niche groups, while it was significantly increased in the BMSC and BMSC + Niche groups (Figure 1p).

L-selectin and fibronectin were detected, especially in the surface epithelium and myometrium, and it was observed that most of the tissues were stained (Figure 1q). These molecules play important roles in the adhesion of the embryo to the endometrium. Therefore, the expression of L-selectin and fibronectin, which are very important in implantation, were examined in the groups, and their statistical significance was evaluated. Although pregnancy was established in the CM and Niche groups, no embryos implanted in the left uterine horn were found (Figure 1r).

Discussion

In the study, the uterine tissue changes of rats with the AS model were evaluated macroscopically and histologically after treatment with CM, Niche, BMSC, and BMSC + Niche to eliminate synechia. The role of freshly prepared medium (CM), 48-hour BMSC and cell-free Niche in adhesions caused by AS were investigated. Histological findings were obtained indicating that regeneration of cells progressed in groups treated with BMSC and Niche, new vessel formation increased, and glands became more prominent, fibrosis and inflammation inevitably formed in AS pathology decreased, and endometrial thickness increased. On the other hand, no negative pathological findings caused by stem cells were encountered[1].

In this study, we tried to remove intrauterine adhesions, which are one of the important infertility problems, by using stem cells, which will contribute to alternative methods and will be perhaps the most important treatment tool of the next cen-
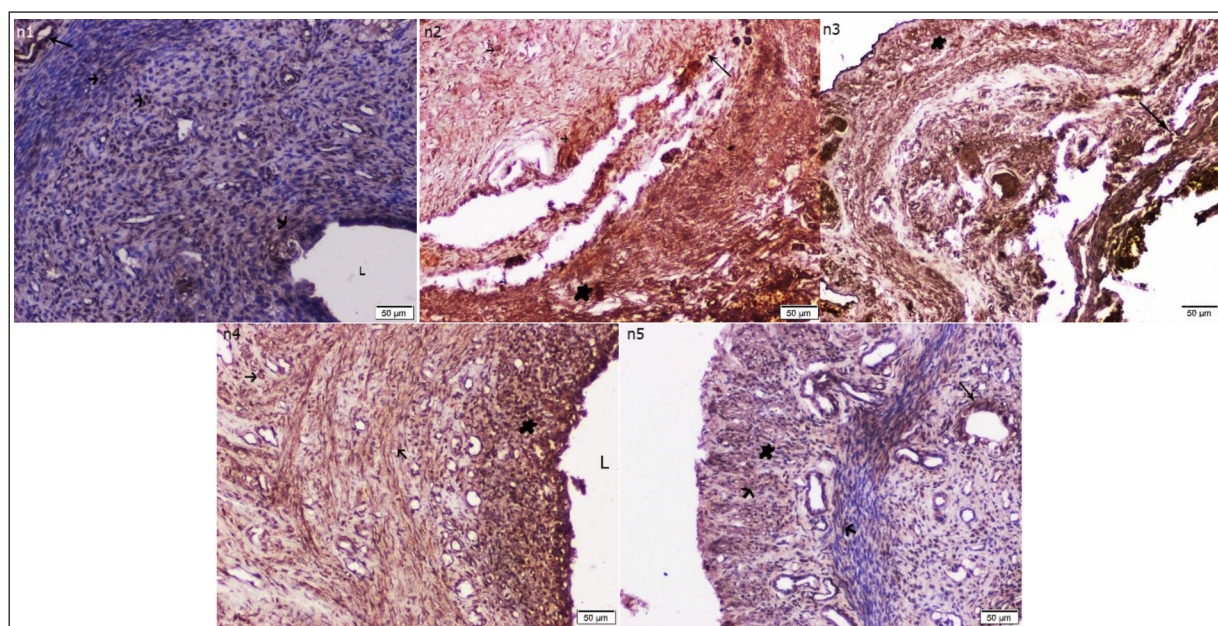

Figure 1n. eNOS staining images in the endometrium of the sub-groups. **n1**, right horn (control), **(n2)** left horn of AS, **(n3)** left horn of AS+Niche, **(n4)** left horn of AS+ BMSC, **(n5)** left horn of AS+ BMSC+ Niche (star: positive, arrow: positive cells, X100).
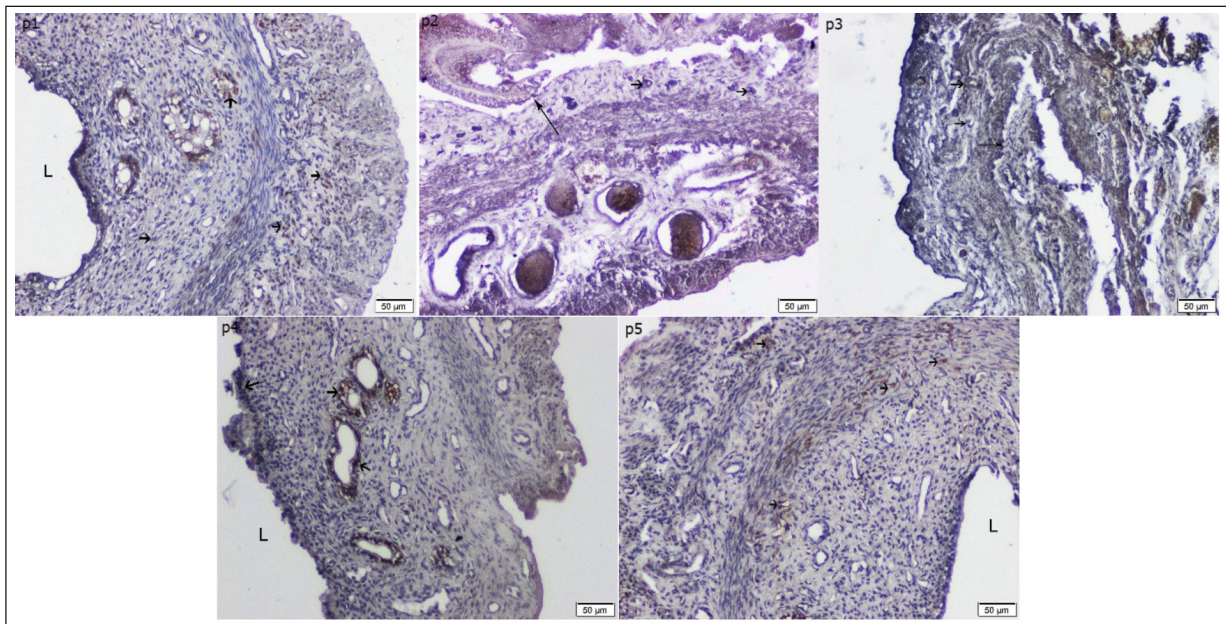
Figure 1p. VEGF staining images in the endometrium of the sub-groups. **p1**, right horn (control), (**p2**) left horn of AS, (**p3**) left horn of AS+Niche, (**p4**) left horn of AS+ BMSC, (**p5**) left horn of AS+ BMSC+ Niche (star: positive, arrow: positive cells, X100).
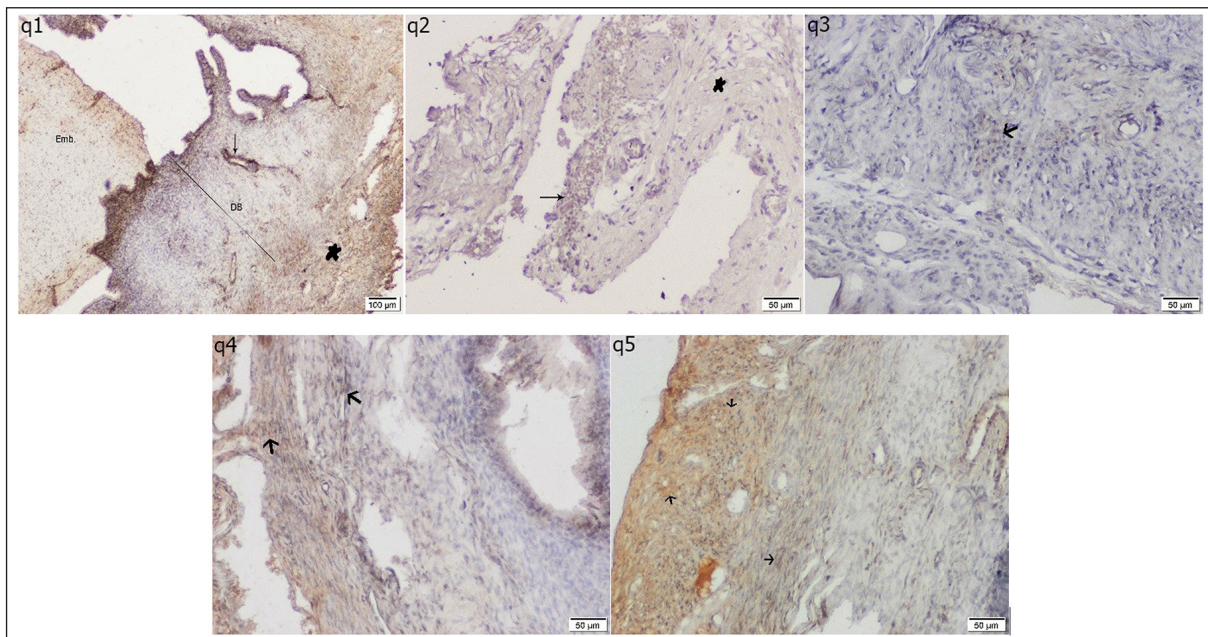

Figure 1q. L-selectin staining images in the endometrium of the sub-groups. **q1**, right horn (control), X50, (**q2**) left horn of AS, X100, (**q3**) left horn of AS+Niche, X100, (**q4**) left horn of AS+ BMSC, X100, (**q5**) left horn of AS+ BMSC+ Niche, X100 (star: positive, arrow: positive cells).

tury[1,8]. Both macroscopic findings obtained from the groups, the anatomical structure of the endometrium after sacrifice, and the histopathological findings obtained as a result of microscopic tissue follow-up proved this. In our immunohistochemical results, it was observed that c-kit and VEGF immunoreactivity increased significantly. It was observed that proliferation was triggered, and vascularity increased. When the eNOS and VEGF positivity were compared with the findings in the study, the degree of significance was similar. The characterization of mesenchymal stem cells was
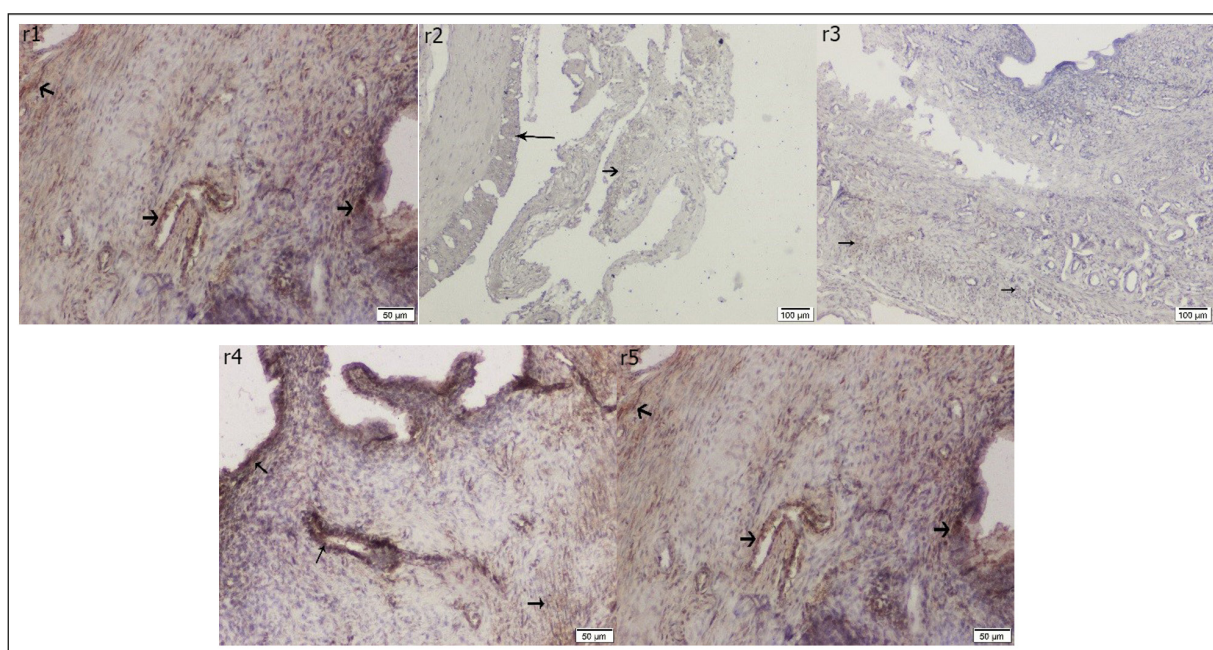
Figure 1r. Fibronectine staining images in the endometrium of the sub-groups. **r1**: right horn (control), X100, **r2**: left horn of AS, X50, **r3**: left horn of AS+Niche, X50, **r4**: left horn of AS+ BMSC, X100, **r5**: left horn of AS+ BMSC+ Niche, X100 (short arrow: positive cells, long arrow; intestinal tissue, star; positivite).

detected with anti-stro-1 and c-kit antibodies. It has been reported[13] that it is determined by CD (90+), CD (45+) and CD (49+) markers for adipose-derived mesenchymal stem cell characterization used in the experimental AS model.

Stem cell therapy for endometrial restoration has recently moved away from being an edge therapy and has become a central treatment option. Especially stem cells obtained from bone marrow have become the most used stem cell source. These cells are isolated directly from the bone marrow by aspiration. BMSCs can be easily obtained from both humans and rodents due to their extensive migration and pluripotent potential. Stem cells that can be purified from the bone marrow by immuno-magnetic isolation have effects on endometrial regeneration and angiogenesis[14]. These cells can prepare the endometrium for implantation by the repair mechanism. The entry of stem cells into the uterus creates a reparative mechanism in the tissue for injury or pregnancy, rather than replacing the endometrial cells lost during menstruation. A recent study[15] suggests that male bone marrow-derived stem cells may not contribute at all to the repair of endometrial injuries. The idea of externally supplementing stem cells arose because the number of BMSCs attached to the uterus is low, and these cells do not undergo clonal expansion to replace the entire endome-

trium. Instead, these cells secrete trophic factors that help uterine repair and regeneration[16,17]. Recently, populations of endometrial epithelial and stromal MSCs resembling adult MSCs in the basal layer of the human endometrium have very rarely been described[18]. The origin of endometrial stem cells remains uncertain. However, it has been accepted[19] to contain many sources, including BMSCs, menstrual blood-derived mesenchymal stem cells, and adipose stem cells. All of these have a strong tendency to heal tissue damage that occurs after endometrial injuries. Among these three types of stem cells, the most widely used stem cell source in human studies is BMSC.

In AS, it is not possible to correct the thinned endometrium with classical treatments, and it is thought[11] that success will be achieved with MSC application. In a study[20] conducted for this purpose, 50,000 cells/microliter bone marrow stromal stem cell was infused intravenously into female rats, and their uterus was removed and examined histologically in their third estrus. We obtained histological results in parallel with these findings (Table I). In addition, Cytokeratin, integrin β-3, and leukemia inhibitory factor (LIF) expressions have been shown[21] to be positive, especially in the cytoplasm of the endometrial epithelium, and vimentin in the cytoplasm of the endometrial stromal cells. In our study, immunohistochemical positivity in

c-kit, VEGF and eNOS staining was found to be more significant in stem cell-treated groups.

Initially, three methods were proposed for the regeneration of the endometrium. First, tissue engineering is an alternative option for endometrial repair. Although the uterus has unique physical properties in this area, reports[21,22] on uterine reconstruction are rare because of its complicated hormonal environment. The second is the use of endometrial epithelial cell infusions for epithelial repair techniques to prevent scarring. Although this is an alternative and good method, it is not preferred much because of the very limited capacity of these cells to be collected, their in vitro proliferation capacity, and highly invasive collection procedures[23]. The third is stem cell therapy. This method has been a promising treatment option for the repair and regeneration of damaged tissue[15]. In our study, the third treatment recommendation was preferred. We aimed to find new solutions for infertility by transplanting cells into experimentally induced AS. In our medium and Niche-applied groups, it was observed that the endometrium degenerated intensively, the synechiae areas increased excessively, and the number of glands decreased significantly compared to BMSC and BMSC + Niche ($p < 0.001$). It has been observed that the degenerative picture that occurs in the uterine tissue with stem cells has almost disappeared.

In experimental models of AS, it has been reported[9,21] that as a result of the adhesions in the epithelial tissue, fibroblasts in the lamina propria turn into a fibrous structure as a result of excessive collagen synthesis. In recent studies[24], it has been reported that transplantation of MSCs and their vesicles provides the inhibition of excessive fibrosis and inflammation, increases endometrial cell proliferation, and contributes to the restoration of endometrial repair and reproductive function through regulation of molecular markers related to endometrial receptivity. In a study[25] with umbilical cord-derived MSC transplantation, excessive fibrosis, and inflammation were suppressed, and endometrial cell proliferation and vascularization were remodeled, contributing to the repair of endometrial damage and restoration of fertility. In an experimental study[26], researchers transplanted autologous bone marrow-derived stem cells to rats with Asherman syndrome and endometrial atrophy to determine the genetic and paracrine mechanisms underlying endometrial regeneration. It was determined that the endometrial morphology and thickness increased in all rats with treatment. CD133+ bone marrow-derived stem cell has been reported to alter endometrial behavior and aid tissue regeneration by creating an immunomodulatory environment that targets proliferation and angiogenic processes. In another experimental AS model[27], it was reported that human umbilical cord mesenchymal stem cell-exosome gel inhibited endometrial fibrosis in rats and accelerated sub-endometrial micro angiogenesis, supporting thin endometrial receptivity and improving pregnancy rate.

In order for the endometrium to regenerate, there must be no loss of the endometrial basal. If the injury is severe in severe Asherman syndrome, limited stem cell supply may be a limiting factor in the repair process. Sufficient stem cells may need to be transplanted to supplement the limited stem cell pool resulting from basalis loss[28]. The amount of stem cells applied here may be a limiting factor in the repair of the uterus after extensive injuries. An enhanced source of stem cells likely supports endometrial regeneration and restores fertility.

Conclusions

We observed that there were differences between the treatment groups in terms of fibrosis. Bone marrow-derived stem cells may be effective in promoting endometrial proliferation. We have observed that they may be particularly effective in removing fibrosis and increasing proliferation. Restorative effects of bone marrow-derived stem cells on regeneration of endometrial tissue were seen without causing any surgical adhesions. It is now known that only adhesions in the cavity can be separated by surgical operations. However, it has little contribution to preventing endometrial regeneration and adhesion recurrence. Therefore, multiple therapeutic approaches are required to achieve optimal clinical outcomes in women diagnosed with AS. Although stem cells are still being researched for a solution in this regard, new discoveries and treatment hopes are increasing. We hope that the findings obtained in this study will contribute to reproductive medicine and shed light on the expansion of stem cell applications.

Ethics Approval

This study was conducted with the approval of the Animal Experiments Local Ethics Committee with the decision number 77738434-10 dated 01/03/2021.

Informed Consent

Not applicable.

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Authors' Contributions

Study conception and design: İÖ, US; data collection: US, İÖ, ZK; analysis and interpretation of results: US, İÖ; draft manuscript preparation: US, İÖ, ZK. All authors reviewed the results and approved the final version of the manuscript.

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