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Effects of stem cells applications on oxidative stress and apoptosis during implantation

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ABSTRACT

Objective: To investigate the effects of bone marrow derived mesenchymal stem cell (BMSC) application into the rat endometrium on oxidative stress, cell proliferation and apoptosis. **Methods:** The female rats selected in estrous cycle were divided into three groups (saline, media and BMSC group). The intrauterine and intraperitoneal injections were performed using the saline (200 µL), culture media (200 µL) and 1×10^6 BMSCs/200 µL culture media, and then they were mated with male rats. On the 7th day of the pregnancy, uterine samples were harvested and dyed with heamatoxylin-eosin histochemically, anti-endothelial nitric oxide synthase and anti-inducible nitric oxide synthase, and anti-proliferating cell nuclear antigen immunohistochemically, with terminal deoxynucleotidyl transferase dUTP nick end labeling for apoptosis. The stainings were evaluated by H-score and the results were analyzed using one-way ANOVA test statistically. **Results:** It was found that BMSCs increased the endometrial thickness, endometrial epithelium thickness and number of endometrial glands compared to control and sham groups. The intrauterine BMSC application decreased both anti-endothelial nitric oxide synthase and anti-inducible nitric oxide synthase immunoreactivities and the number of apoptotic cells compared to the intraperitoneal applications whereas the immunoreactivity of proliferating cell nuclear antigen was increased. **Conclusions:** In current study, we define that stem cells do not cause any structural damages. Also they change the distribution of oxidative stress and cell proliferation marker. These findings support the reliability of stem cells in clinical use in the case of infertility.

1. Introduction

Implantation is important for initiation of pregnancy and takes place in three phases-apposition, adhesion and invasion, and in the

secretory phase of the endometrium. A successful implantation is due to blastocyst quality, healthy development of embryo and receptive endometrium[1–4]. It is known that implantation, which results in uterine growth and differentiation, blastocyst adhesion, and

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blastocyst invasion and placental formation, occurs in the presence of many molecular and cellular mechanisms[1,5,6]. The change in the uterine which provides the invasion of blastocysts is called uterine receptivity. The main factors determining uterine receptivity are ovarian steroids, progesterone, estrogens, cytokines, growth factors and adhesion molecules. The inadequacies of these factors may cause the failure of implantation and increase miscarriage rates, and consequently infertility occurs in women[6–8]. Furthermore, it is stated that the oxidative stress induces DNA damage, apoptosis and necrosis in blastocyst[9].

The degradation of the balance between free radicals and antioxidants in favor of free radicals is called oxidative stress. Reactive oxygen species are produced during metabolic and physiological processes and oxidative reactions may occur in the organism. Nitric oxide (NO), one of the reactive nitrogen species, is an important messenger because of its ability to carry no charge and unpaired electrons. NO is synthesized by the reaction catalyzed by the nitric oxide synthase (NOS) enzyme family in many cells: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS)[10,11]. NO can be detected by NOS activity assays, such as immunohistochemical and biochemical analysis. During implantation, NO is necessary for a healthy blood flow and feeding of the embryo in the endometrium. It was observed that there has been a relationship between oxidant/antioxidant and implantation success[12]. The oxidation-reduction disorders in the cells are toxic to all components of the cell, including protein, lipid, DNA by peroxidase and free radical production. However, excessive oxidative stress, inadequate levels of antioxidants or inhibition of antioxidant enzymes can lead to cell damage or cell death of germ cells in the reproductive system[13–15].

The apoptosis, a programmed cell death, occurs by extrinsic and intrinsic or mitochondrial pathways. Caspases are involved in both apoptotic signal pathways. The cell that receives the apoptosis induction breaks its connection with the neighboring cells and the intercellular substance by distracting from the environment and shrinking. In the nucleus, chromatin is condensed and apoptotic bodies form. The organism has some protective mechanisms to cope with the effects of oxidative stress such as enzymatic and non-enzymatic antioxidant defense systems. But in some cases, they are not insufficient[15]. Because of the ability of regeneration, differentiation, proliferation and formation of tissues and organs, stem cells can be used as an alternative to organ transplants or drugs with adverse side effects and in the treatment of many diseases. In previous study, female rats underwent intravenous infusion of bone marrow stromal stem cells at 50 000 cells/ μ L, and the uterine was removed and examined histologically in the third estrous cycle. After stem cell application, endometrial thickness increased and glandular and capillary proliferation were observed[16]. The bone marrow-derived mesenchymal stem cells (BMSCs) application was observed at the end of the first month as GFP+ cells in stromal and epithelial sections of damaged mouse uterine. These cells have been shown to be effective by inhibiting inflammation and increasing vascularity. The stem cells have also been shown in human endometrium after

transplantation in which they differentiate into epithelial, stromal and endothelial cells[17]. In our study, we aimed to compare the beneficial/pathological effects of BMSCs by intrauterine and intraperitoneal applications into the healthy rat endometrium via oxidative stress, cell proliferation and apoptosis.

2. Materials and methods

2.1. Animals

For the experiment, Wistar albino rats [male and female, (200 \pm 50) g] were obtained from Manisa Celal Bayar University Experimental Animal Research and Application Center. It was noted that the animals supplied were not mated before and were not used in any experimental work. The rats were housed under 12-hours light/12-hours dark cycle at 22 °C for adaptation to the laboratory conditions and diet and water were given *ad libitum*. The study was approved by Animal Experiments Local Ethics Committee, Faculty of Medicine, Manisa Celal Bayar University, Turkey (protocol number: 77637435-44, date of approval: 26/06/2013).

2.2. Mesenchymal stem cell isolation

BMSCs were gathered from the tibias of two male rats and following dissociation, cells were cultured in alpha-minimum essential medium (F0915, Biochrom, Berlin, Germany) containing 15% fetal calf serum (S0113, Biochrom, Berlin, Germany), 50 μ g/mL gentamycin (A2712, Biochrom, Berlin, Germany), 100 UI/mL penicillin and 100 UI/mL streptomycin (A2213, Biochrom, Berlin, Germany), 100 UI/mL amphotericin-B (A2612, Biochrom, Berlin, Germany) and 200 mM *L*-glutamine (K0282, Biochrom, Berlin, Germany) at 37 °C and 5% CO₂. Three days later, non-adherent cells were removed from the culture media. The adherent cells (mesenchymal stem cells) were maintained. Cells were passaged to passage 3 (P3)[18,19]. Under the inverted microscope (\times 71, Olympus, Japan) cell proliferation was checked.

2.3. Characterization of mesenchymal stem cell

Mesenchymal stem cell characterization was performed by immunocytochemical staining using primary antibodies of Stro-1, CD105, c-kit and CD45 at P4[20,21]. For this purpose, cells were seeded into 8-well chamber slides, and were allowed to adhere. Then cells were fixed in 4% paraformaldehyde (1.04004, Merck, Darmstadt, Germany) and washed in phosphate buffered saline (PBS). For permeabilization, cells were exposed to the 0.1% Triton- \times 100 (T8787, Sigma, St.Louis, USA) for 15 min and endogenous peroxidase activity was inhibited by 3% hydrogen peroxide (1.08600, Merck, Darmstadt, Germany). Following the blocking serum, primary antibodies anti-Stro-1 (MAB4315, Millipore), anti-CD105 (ab11414, Abcam), anti-c-kit (sc-168, Santa Cruz Biotechnology) and anti-CD45 (ab10558, Abcam) were applied to

the cells for 18 h. After washing with PBS, cells were stained with biotin-streptavidin hydrogen peroxidase secondary antibodies (85-9043, Invitrogen®-Histostain Plus Bulk Kit, CA, USA) for 30 min. Each secondary antibodies were washed 3 times with PBS for 5 min. To detect immunoreactivities, diaminobenzidine (DAB, 00-2020, Zymed, CA, USA) was used. In negative controls, the assay was performed by placing PBS in place of the primer antibodies. For counterstaining, cells were stained with Mayer's haematoxylin (72804E, Microm, Walldorf, Germany), mounted using aqueous medium (AML060, Scytek, Logan, Utah, USA) and examined under light microscope (BX43, Olympus, Japan)[22].

2.4. Experimental design

In all study groups, the menstrual cycle periods of female rats were observed by vaginal smearing method between 15.00-16.00 p.m. and rats at oestrus cycle were selected[23]. The animals were divided into three groups: saline group (200 µL, $n=10$), media group (200 µL, $n=10$), and BMSCs in media (1×10^6 cells/200 µL media, $n=10$). Female rats at oestrus period were treated with intrauterine and intraperitoneal injection of saline, media and BMSCs (P4) in media under general anesthesia (75 mg/kg ketamine and 10 mg/kg xylazine). After applications, 3 female rats and 1 male rat were taken into cages and allowed to mate. The next day, pregnant rats were separated by vaginal plaque and sperm control. Vaginal smears were obtained from the rats that had vaginal plaques on the next day to determine the pregnancy. The smear samples were fixed with methanol and stained with Giemsa solution, and samples with spermatozoa were accepted as definite pregnant[24].

2.5. Histochemistry

On the 7th day of gestation, female rats were sacrificed by cervical dislocation under general anesthesia to evaluate the implantation process. Uterine specimens were taken and fixed in 10% formalin solution for 48 h for histochemical and immunohistochemical analyzes. Uterine specimens were exposed to the routine paraffin embedding procedure and were embedded in paraffin blocks. And 5 µm thickness sections were cut using rotary microtome (RM 2135, Leica). Following the dewaxing and dehydration, sections were stained with haematoxylin and eosin. Sections were evaluated with light microscope (BX43, Olympus, Japan) and were photographed (SC50, Olympus, Germany)[25]. The samples stained with haematoxylin and eosin were evaluated in terms of endometrial thickness, endometrial epithelium thickness and number of endometrial glands. The measurements were analyzed statistically[26,27].

2.6. Immunohistochemistry

The uterine sections were dewaxed, dehydrated, and were applied with 0.5% trypsin (800.729.8350, ScyTek, Utah, USA) for 15 min. Hydrogen peroxide (3%) was used for the inhibition of endogenous peroxidase activity. After washing in PBS, sections were treated

with blocking serum (TA-125-UB, Lab Vision, Fremont, CA) for 1 h. Sections were incubated with primary antibodies anti-eNOS (eNOS, sc-654, Santa Cruz Biotechnology) ve anti-iNOS (iNOS, GTX15322, Gene Tex) and anti-proliferating cell nuclear antigen (PCNA, orb11245, Biorbyt) for 18 h. Following the washing in PBS, sections were stained with biotin-streptavidin hydrogen peroxidase secondary antibody (85-9043, Invitrogen®-Histostain Plus Bulk Kit, CA, USA) for 30 min and were washed in PBS again. Then sections were dyed with diaminobenzidine (DAB, 00-2020, Zymed, CA, USA). For negative controls, primary antibodies were not used. For counterstaining, cells were stained with Mayer's haematoxylin (72804E, Microm, Walldorf, Germany), mounted using medium and evaluated by a blinded observer under an Olympus BX40 (Tokyo, Japan) light microscope[25].

2.7. Terminal transferase dUTP nick end labeling (TUNEL) assay

TUNEL (TUNEL, S7101, Millipore, Billerica, MA, USA) staining method was used to determine apoptotic cell death. The sections were treated with 20 µg/mL proteinase K for 10 min at 37 °C in a humidity chamber and then with an equilibration buffer. Following incubation with terminal deoxynucleotidyl transferase for 1 h at 37 °C, stop wash buffer was applied to the sections for 10 min and the sections were washed in PBS. Diaminobenzidine (DAB, 00-2020, Zymed, CA, USA) staining and counterstaining with Mayer's haematoxylin was performed. Sections were mounted using entellan. TUNEL positive cells were evaluated by a blinded observer under light microscope (Olympus BX43 Tokyo, Japan) and apoptotic index was calculated[28].

2.8. Statistical analysis

Immunohistochemical stainings were evaluated by H-score. The intensity of staining was determined as follows: 0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining. In the chosen fields, the ratio of immunopositive cells were searched and the respective score was calculated using H-score formula: H-score = (% stained cells at 0) × 0 + (% stained cells at 1+) × 1 + (% stained cells at 2+) × 2 + (% stained cells at 3+) × 3. The H-score value changed in range of 0 and 300. The H-score results were analyzed using one-way ANOVA by Tukey-Kramer multiple comparisons test. For this purpose, GraphPad Software (San Diego, CA, USA) was used and P value <0.05 was considered statistically significant[25,29].

3. Results

3.1. Mesenchymal stem cell characterization

BMSCs from rat tibia and femur were maintained until confluency of 70%-80%. Cells became optimal for application after passaging

until P3. Immunocytochemical method was performed using Stro-1, CD105, CD45 and c-kit. It was observed the positivities of Stro-1, CD105 and c-kit whereas there was negativity of CD45 in BMSCs ($P<0.001$) (Figure 1).

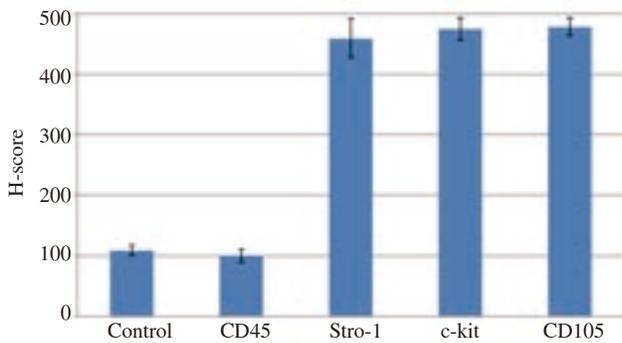


Figure 1. Characterization of BMSCs by immunocytochemistry via positivity of Stro-1, CD105, c-kit and negativity of CD45. BMSCs: bone marrow-derived mesenchymal stem cells.

3.2. Histological investigation

After morphometric examination, it was detected that intrauterine and intraperitoneal applications of BMSCs enhanced the cellular environment in the endometrium. The criteria of endometrial thickness, endometrial epithelium thickness and number of endometrial glands were significantly increased ($P<0.001$) after intrauterine application of BMSCs compared to intraperitoneal application of BMSCs. Also, it was more obvious after the both intrauterine and intraperitoneal applications of BMSCs compared to the saline and media applications (Figure 2).

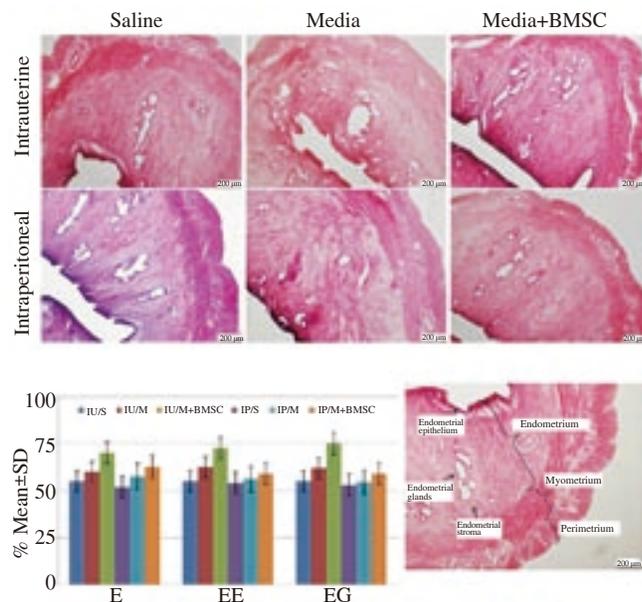


Figure 2. Effects of BMSCs applications on histological structure of endometrium via parameters of endometrial thickness (E), endometrial epithelium thickness (EE) and number of endometrial glands (EG). Scale bars: 200 µm; BMSCs: bone marrow-derived mesenchymal stem cells; IU: intrauterine; IP: intraperitoneal.

3.3. Immunohistochemical results

To detect cell proliferation, uterine samples were stained with PCNA. After intrauterine application of BMSCs, PCNA positive cells were increased in the decidua, endometrial epithelium, glands and veins of the uterine compared to the media group ($P<0.05$) (Figure 3&4). PCNA immunoreactivity was significantly increased by intraperitoneal application of BMSCs in the primary and secondary decidual areas. The increase of PCNA was more prominent in the groups of intrauterine and intraperitoneal applications of BMSCs than groups of media ($P<0.001$) (Figure 3).

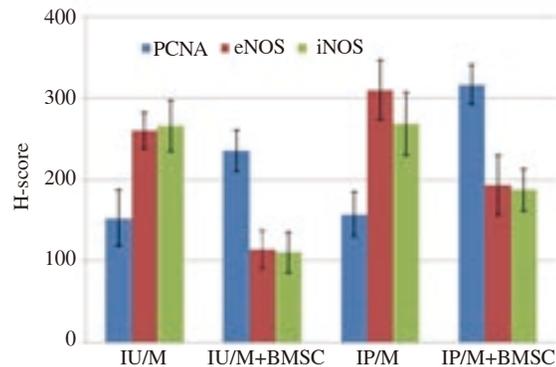


Figure 3. H-score evaluation of immunoreactivities of PCNA, eNOS and iNOS in endometrium after IU and IP applications of BMSCs and M. PCNA: proliferating cell nuclear antigen; eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; IU: intrauterine; IP: intraperitoneal; BMSCs: bone marrow-derived mesenchymal stem cells; M: media.

It was defined that eNOS immunoreactivity was significantly reduced ($P<0.001$) by the intrauterine application of BMSCs in the uterine samples. eNOS was higher in the group of intraperitoneal application of media than the group of BMSCs (Figure 3). It was seen that intrauterine application of BMSCs caused significantly the decrease of eNOS in the decidual areas ($P<0.001$) (Figure 4). There was no difference between the intrauterine and intraperitoneal administration of the media ($P>0.05$), but the application of BMSCs resulted in a significant decrease in eNOS immunoreactivity ($P<0.01$). The iNOS immunoreactivity was markedly reduced ($P<0.001$) by BMSCs in comparison with intrauterine media group (Figure 3). It was observed that especially in the decidual areas, iNOS was significantly higher in intrauterine media group than the BMSCs group ($P<0.01$) (Figure 4). While no difference was found between the intrauterine and intraperitoneal administration of media, administration of the BMSCs into the uterine was found to cause a significant decrease in eNOS immunoreactivity ($P<0.01$).

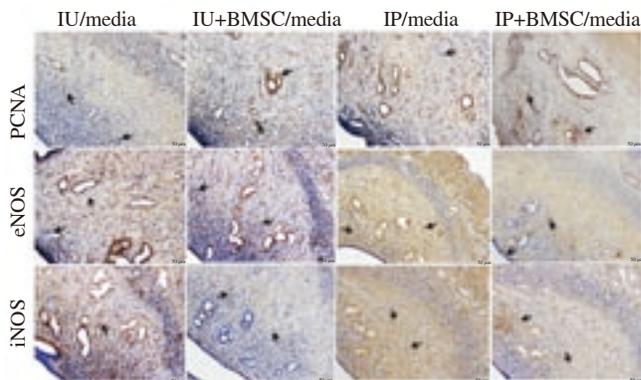


Figure 4. Immunohistochemical stainings of PCNA, eNOS and iNOS in samples of endometrium after IU and IP applications of media and BMSCs.

PCNA: proliferating cell nuclear antigen; eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; IU: intrauterine; IP: intraperitoneal; BMSCs: bone marrow-derived mesenchymal stem cells; Arrows: immunopositive cells; Scale bars: 50 µm.

3.4. Apoptosis

After intrauterine application of BMSCs, the number of apoptotic cells was reduced significantly ($P < 0.01$) in comparison with the intrauterine application of media. Also there was a decrease in the group of intraperitoneal application of BMSCs (Figure 5). The most significant decrease was seen in the group of intraperitoneal administration of BMSCs ($P < 0.05$).

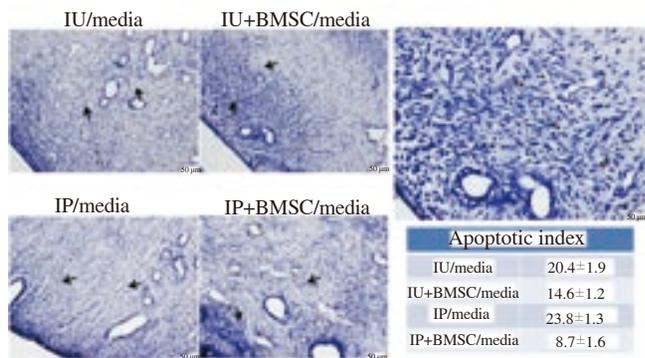


Figure 5. Distribution of apoptotic cells in endometrium samples and apoptotic index after IU and IP applications of media and BMSCs.

IU: intrauterine; IP: intraperitoneal; BMSCs: bone marrow-derived mesenchymal stem cells; Arrows: apoptotic cells; Scale bars: 50 µm.

4. Discussion

The intrauterine treatment of BMSCs is thought to be useful in uterine-related diseases. In such a cellular therapy, firstly, the implantation success will be increased and the important problem is to be resolved after the development will be recovered[30–32]. In this study, differences between intrauterine and intraperitoneal administration of BMSCs in implantation period of rat uterine were

examined by histology, morphometry and immunohistochemistry. Endometrial changes were determined in the day 7 post-administration samples to assess the effect of BMSCs, because implantation occurred on gestational day 4 and 5 in rats. On day 7 and 12, invasion of embryo into the endometrium is completed and the chorio-vitelline placenta begins to form[33,34]. After histological and morphometric examination, there was a prominent increase in endometrial thickness, endometrial epithelium thickness and number of endometrial glands. However, no negative pathological findings were found.

The studies about the effect of BMSCs on uterine tissue have shown that the mesenchymal stem cells have a initiator effect on regeneration of endometrium. In a previous study, after endometrial cell injury procedure, BMSCs application was performed into the uterine, and it was found an increase in the number of endometrial glands and endometrial thickness of rat uterine. In the group of endometrial cell injury and BMSCs, the healing effect of BMSCs was detected, and the proliferation marker and bromodeoxyuridine positive cells have seen risen[26]. The intravenous injection of BMSCs resulted in increase of epithelial thickness, the number of endometrial glands and capillaries in case of endometrial cell injury[16]. Besides, stem cells freshly isolated have been found to be more effective in the regeneration of damaged uterine. The different types of bone marrow stem cells such as unfractionated bone marrow cells, hematopoietic progenitor cells, endothelial progenitor cells, Oct4⁺ and Oct⁺ multipotent adult progenitor cells have been used in the total body irradiation animal model. After 12 weeks, the more regeneration was observed in the groups of bone marrow-derived hypoblast-like stem cells (Oct4⁺). Freshly isolated MSCs, and 5% of these cells located in the stroma and endometrial epithelia of endometrium[17]. Zhang *et al* observed that the umbilical cord-derived stem cells significantly increased the endometrial thickness and the number of endometrial glands in the injured rat endometrium. Our experimental results showed that intrauterine and intraperitoneal applications of BMSCs caused an increase in the endometrial thickness, endometrial epithelium thickness and also number of endometrial glands[35]. The increase in these parameters was prominent in the group of intrauterine application of BMSCs.

A proliferative marker Ki67 was observed to be diminished in all uterine samples treated with stem cells while the number of TUNEL⁺ cells was increased[17]. PCNA, a proliferative marker, is located at the place where intracellular stromal cells are transformed into decidual cells forming mesometrial and antimesometrial decidua by proliferation, and where they also regress by apoptosis. Concurrently, endometrial glands develop into the mesometrial area. Until the 12th day of gestation, an intense PCNA immunoreactivity was observed in the rat uterine, and then an increasing apoptosis and necrosis were stated[36]. It is thought that the differentiation ability of the stem cells may play a role in the epithelial regeneration and it has been shown that stem cells, which are quite similar to each other, have different effects within the uterine. It can be said that the functions of the stem

cells vary due to the differences in cell surface receptors[17,37]. In our study, we investigated beneficial or pathological effects of MSCs into non-damaged uterine, and it was found that BMSCs administration caused cell proliferation and increased the histological parameters of endometrium via histochemistry and PCNA staining. Moreover, there was no any pathological damages in the uterine samples. Furthermore, we ascertained the distribution of PCNA in all groups. After intraperitoneal application of BMSCs, PCNA immunoreactivity was increased markedly. These results suggest that BMSCs have a triggering effect for cell proliferation *in vivo* condition.

NO is an important mediator for multiple cell function and has been shown to be associated with plasma and urine levels during pregnancy in humans and rats. Increased nitrate levels in the circulatory system are thought to be associated with increased NO production in the maternal placenta or other tissues. The increased NO production via iNOS was shown during pregnancy in the rat uterine, and was associated with decidua and myometrium. The uterine NO level reaches maximum level on day 17 when it is low on the 13th day and then gradually decreases. The amount of NO, which is the same in decidua and myometrium in the day 13, increases to be three times the number of myometrium on the day 15 in the decidua. About the association between NOS levels and mRNA, iNOS was highly correlated and eNOS was found to be constant. The increase in NO level was found to be produced by decidua, which is thought to increase in order to prevent the contraction of the uterine muscle layer. In homozygous mice in which iNOS was knocked out, it was found the reduction of cellular density and abnormal thickening of arteries in the decidua. Thus, it was concluded that the presence of iNOS in the decidua was important for both the embryo and pregnancy process[38]. In another study, it was determined that the expression of eNOS and iNOS was seen in the gland epithelium, stroma and myometrium and they were increased in the implantation area of the mouse uterine. In particular, eNOS is more common in the endometrial vascular endothelium. One of the reasons is that increased oxidative stress enhances uterine blood flow and nutrition at the implantation window. It should be known that NO must be at optimal level in the success of implantation, and implantation failure occurs in case of increase of NO. It suggests that inhibition of oxidative stress by stem cell applications leads to optimal NO level[39]. Our study showed that the immunoreactivities of eNOS and iNOS were decreased in the group of intrauterine application of BMSCs.

In the animal experiments, the MSCs were used by intrauterine application as well as intravenously to investigate their effects *in vivo* condition. The advantageous effects of MSCs have revealed molecular pathways, such as growth factors, adhesion molecules. It has been shown that each application method of MSCs has been effective in endometrium damages[16,26,35,40,41]. However, there is no evidence comparing the application methods of MSCs. In the current study, the application methods, intrauterine and intraperitoneal

application, were compared and the data have shown that intrauterine application of BMSCs was effective on the histological parameters of endometria.

The findings of our study considers that the distribution of molecules playing an important role during implantation is critical for the success of maternal-fetal interaction. The usage of BMSCs or other type of mesenchymal stem cells may provide the treatment of infertility, but further studies are needed to show beneficial effect of stem cells *in vivo* animal or human experiments via advanced molecular techniques.

Conflict of interest statement

The authors declare that there is no conflict of interest.

References

- [1] Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, Bayless K. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Mol Hum Reprod* 2010; **16**(3): 135-152.
- [2] Cha J, Vilella F, Dey SK, Simón C. *Molecular interplay in successful implantation*. Washington, DC: Science/AAAS; 2013, p. 44-48.
- [3] Bhusane K, Bhutada S, Chaudhari U, Savardekar L, Katkam R, Sachdeva G. Secrets of endometrial receptivity: Some are hidden in uterine secretome. *Am J Reprod Immunol* 2016; **75**(3): 226-236.
- [4] Vinketova K, Mourdjeva M, Oreshkova T. Human decidual stromal cells as a component of the implantation niche and a modulator of maternal immunity. *J Pregnancy* 2016. doi: 10.1155/2016/8689436.
- [5] Tsui KH, Li HY, Cheng JT, Sung YJ, Yen MS, Hsieh SL, et al. The role of nitric oxide in the outgrowth of trophoblast cells on human umbilical vein endothelial cells. *Taiwan J Obstet Gynecol* 2015; **54**(3): 227-231.
- [6] Cha J, Sun X, Dey SK. Mechanisms of implantation: Strategies for successful pregnancy. *Nat Med* 2012; **18**(12): 1754-1767.
- [7] Salamonsen LA, Evans J, Nguyen HP, Edgell TA. The microenvironment of human implantation: Determinant of reproductive success. *Am J Reprod Immunol* 2016; **75**(3): 218-225.
- [8] Sharma S, Godbole G, Modi D. Decidual control of trophoblast invasion. *Am J Reprod Immunol* 2016; **75**(3): 341-350.
- [9] Khazaei M, Aghaz F. Reactive oxygen species generation and use of antioxidants during *in vitro* maturation of oocytes. *Int J Fertil Steril* 2017; **11**(2): 63-70.
- [10] Sugiura H, Ichinose M. Nitrate stress in inflammatory lung diseases. *Nitric Oxide* 2011; **25**(2): 138-144.
- [11] Tejero J, Stuehr D. Tetrahydrobiopterin in nitric oxide synthase. *IUBMB Life* 2013; **65**(4): 358-365.
- [12] Rahiminejad ME, Moaddab A, Ganji M, Eskandari N, Yezpe M, Rabiee S, et al. Oxidative stress biomarkers in endometrial secretions: A comparison between successful and unsuccessful *in vitro* fertilization cycles. *J Reprod Immunol* 2016; **116**: 70-75.

- [13]Kala M, Shaikh MV, Nivsarkar M. Equilibrium between anti-oxidants and reactive oxygen species: A requisite for oocyte development and maturation. *Reprod Med Biol* 2016; **16**(1): 28-35.
- [14]Peter Stein T, Scholl TO, Schluter MD, Leskiw MJ, Chen X, Spur BW, et al. Oxidative stress early in pregnancy and pregnancy outcome. *Free Radic Res* 2008; **42**(10): 841-848.
- [15]Devine PJ, Perreault SD, Luderer U. Roles of reactive oxygen species and antioxidants in ovarian toxicity. *Biol Reprod* 2012; **86**(2): 27.
- [16]Jing Z, Qiong Z, Yonggang W, Yanping L. Rat bone marrow mesenchymal stem cells improve regeneration of thin endometrium in rat. *Fertil Steril* 2014; **101**(2): 587-594.
- [17]Gil-Sanchis C, Cervelló I, Khurana S, Faus A, Verfaillie C, Simón C. Contribution of different bone marrow-derived cell types in endometrial regeneration using an irradiated murine model. *Fertil Steril* 2015; **103**(6): 1596-1605.
- [18]Tu lu MI, Özdal-Kurt F, Koca H, Sarac A, Barut T, Kazanç A. The contribution of differentiated bone marrow stromal stem cell loaded biomaterial to treatment in critical size defect model in rats. *J Faculty Vet Med Kafkas Univ* 2010; **16**(5): 783-792.
- [19]Niemeyer P, Fechner K, Milz S, Richter W, Suedkamp NP, Mehlhorn AT, et al. Comparison of mesenchymal stem cells from bone marrow and adipose tissue for bone regeneration in a critical size defect of the sheep tibia and the influence of platelet-rich plasma. *Biomaterials* 2010; **31**(13): 3572-3579.
- [20]Karaöz E, Aksoy A, Ayhan S, Sarıboyacı AE, Kaymaz F, Kasap M. Characterization of mesenchymal stem cells from rat bone marrow: Ultrastructural properties, differentiation potential and immunophenotypic markers. *Histochem Cell Biol* 2009; **132**: 533-546.
- [21]Demirayak B, Yüksel N, Çelik OS, Subası C, Duruksu G, Unal ZS, et al. Effect of bone marrow and adipose tissue-derived mesenchymal stem cells on the natural course of corneal scarring after penetrating injury. *Exp Eye Res* 2016; **151**: 227-235.
- [22]Özdal-Kurt F, Tu lu I, Vatanserver HS, Tong S, en BH, Delilo lu-Gürhan SI. The effect of different implant biomaterials on the behavior of canine bone marrow stromal cells during their differentiation into osteoblasts. *Biotech Histochem* 2016; **91**(6): 412-422.
- [23]Cora MC, Kooistra L, Travlos G. Vaginal cytology of the laboratory rat and mouse: Review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicol Pathol* 2015; **43**(6): 776-793
- [24]Kútvolgyi G, Stefler J, Kovács A. Viability and acrosome staining of stallion spermatozoa by Chicago sky blue and Giemsa [published erratum appears in *Biotech Histochem* 2007; 82(1): 45]. *Biotech Histochem* 2007; **81**(4-6): 109-117.
- [25]Gürpınar T, Ekerbiçer N, Uysal N, Barut T, Tarakçı F, Tuğlu MI. The effects of the melatonin treatment on the oxidative stress and apoptosis in diabetic eye and brain. *Sci World J* 2012. doi: 10.1100/2012/498489.
- [26]Zhao J, Zhang Q, Wang Y, Li Y. Uterine infusion with bone marrow mesenchymal stem cells improves endometrium thickness in a rat model of thin endometrium. *Reprod Sci* 2015; **22**(2): 181-188.
- [27]Kuyucu Y, Çelik LS, Kendirinan Ö, Tap Ö, Mete UÖ. Investigation of the uterine structural changes in the experimental model with polycystic ovary syndrome and effects of vitamin D treatment: An ultrastructural and immunohistochemical study. *Reprod Biol* 2018; **18**(1): 53-59.
- [28]Karabuga I, Akbay K, Turna B, Vatanserver HS, Altay B, Güzel E, et al. Effect of lisinopril on renal tissue damage in unilateral ureteral obstruction in rats. *Urol Res* 2012; **40**(1): 27-34.
- [29]Ozbilgin K, Karaca F, Turan A, Köse C, Vatanserver S, Özcakir T. The higher heparin-binding epidermal growth factor (HB-EGF) in missed abortion. *Taiwan J Obstet Gynecol* 2015; **54**(1): 13-18.
- [30]Mattar CN, Biswas A, Choolani M, Chan JK. The case for intrauterine stem cell transplantation. *Best Pract Res Clin Obstet Gynaecol* 2012; **26**(5): 683-695.
- [31]Gargett CE, Ye L. Endometrial reconstruction from stem cells. *Fertil Steril* 2012; **98**(1): 11-20.
- [32]Gargett CE, Nguyen HP, Ye L. Endometrial regeneration and endometrial stem/progenitor cells. *Rev Endocr Metab Disord* 2012; **13**(4): 235-251.
- [33]Yoshinaga K. Research on blastocyst implantation essential factors (BIEFs). *Am J Reprod Immunol* 2010; **63**(6): 413-424.
- [34]Fonseca BM, Correia-da-Silva G, Teixeira NA. The rat as an animal model for fetoplacental development: A reappraisal of the post-implantation period. *Reprod Biol* 2012; **12**(2): 97-118.
- [35]Zhang L, Li Y, Guan CY, Tian S, Lv XD, Li JH, et al. Therapeutic effect of human umbilical cord-derived mesenchymal stem cells on injured rat endometrium during its chronic phase. *Stem Cell Res Ther* 2018; **9**(1): 36.
- [36]Correia-da-Silva G, Bell SC, Pringle JH, Teixeira NA. Patterns of uterine cellular proliferation and apoptosis in the implantation site of the rat during pregnancy. *Placenta* 2004; **25**(6): 538-547.
- [37]Wang H, Jin P, Sabatino M, Ren J, Civini S, Bogin V, et al. Comparison of endometrial regenerative cells and bone marrow stromal cells. *J Transl Med* 2012; **10**: 207.
- [38]Suzuki T, Mori C, Yoshikawa H, Miyazaki Y, Kansaku N, Tanaka K, et al. Changes in nitric oxide production levels and expression of nitric oxide synthase isoforms in the rat uterus during pregnancy. *Biosci Biotechnol Biochem* 2009; **73**(10): 2163-2166.
- [39]Biyiksiz PC, Filiz S, Vural B. Is sildenafil citrate affect endometrial receptivity? An immunohistochemical study. *Gynecol Endocrinol* 2011; **27**(10): 767-774.
- [40]Kilic S, Yuksel B, Pinarli F, Albayrak A, Boztok B, Delibasi T. Effect of stem cell application on Asherman syndrome, an experimental rat model. *J Assist Reprod Genet* 2014; **31**(8): 975-982.
- [41]Wang L, Yang M, Jin M, Wu Y, Zheng T, Gu S, et al. Transplant of insulin-like growth factor-1 expressing bone marrow stem cells improves functional regeneration of injured rat uterus by NF- κ B pathway. *J Cell Mol Med* 2018; **22**(5): 2815-2825.