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Research Article

Stem Cell Therapy in Rats with Experimental Intrauterine Adhesions Induced by Trichloroacetic Acid

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ABSTRACT

The effectiveness and role of stem cells obtained from bone marrow in removing the adhesion formed in intrauterine adhesion modeling using chemicals in rats and restoring the implantation of the blastocyst to the endometrium were investigated. The experimental model was created in a single horn using trichloroacetic acid. Three different groups and two subgroups were formed as only culture medium (CM), stem cell and 48-hour medium (Niche). A total of 30 female and 3 male rats were used in the study. Treatment was started immediately after model formation in the acute phase, and 10 days later, the subjects were placed in the same cage with male rats for conception. Then, the pregnancy status and the number of puppies born were evaluated. Histological evaluation was performed with hematoxylin-eosin staining. A statistically significant difference ($p<0.05$) was found in the stem cell applied groups compared to the other groups in both histological and morphological evaluations according to the number of newborns. It has been determined that regional stem cell application in the acute period can remove adhesions in uterine adhesions and make the endometrium more suitable for implantation. As a result, the increase in the number of newborns as a result of the increase in endometrial thickness, number of glands and vascularization, decrease in fibrous areas and regression of adhesive areas in the treatment groups is promising in terms of carrying experimental intrauterine studies to the clinic.

Sıçanlarda Trikloroasetik Asit ile İndüklenen Deneysel İntrauterin Adezyonlarda Kök Hücre Tedavisi

ÖZET

Kemik iliğinden elde edilen kök hücrelerin, sıçanlarda kimyasal kullanılarak oluşturulan intrauterin adezyon modellemesinde rahim içi yapışıklıkların giderilmesinde ve blastosistin endometriuma implantasyonunun yeniden sağlanmasında etkinliği ve rolü araştırıldı. Deney modeli, trikloroasetik asit kullanılarak tek uterin hornda meydana gelen hasara karşı, yalnızca kültür besiyeri (CM), kök hücre ve 48 saatlik Niş kullanılarak farklı grup ve iki alt grup oluşturulmuştur. Çalışmada toplam 30 dişi ve 3 erkek rat kullanıldı. Akut fazda model oluşumundan hemen sonra tedaviye başlandı ve 10 gün sonra denekler gebe kalmaları için erkek sıçanlarla aynı kafese yerleştirildi. Daha sonra gebelik durumu ve doğan yavru sayısı değerlendirildi. Histolojik değerlendirme hematoksilen-eozin boyaması ile yapıldı. Yenidoğan sayısına göre hem histolojik hem de morfolojik değerlendirmelerde kök hücre uygulanan gruplarda diğer gruplara göre istatistiksel olarak anlamlı fark ($p<0.05$) bulundu. Akut dönemde bölgesel kök hücre uygulamasının rahim içi yapışıklıkları ortadan kaldırabileceği ve endometriyumu implantasyona daha uygun hale getirebileceği belirlendi. Sonuç olarak tedavi gruplarında endometrial kalınlık, bez sayısı ve damarlanmanın artması, fibröz alanların azalması ve adeziv alanların gerilemesi sonucu yenidoğan sayısındaki artış deneysel intrauterin çalışmaların kliniğe uyarlanması açısından umut verici olmuştur.

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1. INTRODUCTION

Due to the ability of stem cells to self-renew, differentiate, multiply, and form tissues and organs, they can be used in organ transplants, in the treatment of many diseases or as an alternative to drugs with known negative side effects. Nowadays, stem cells can be obtained from many sources to be used for therapeutic purposes. The most studied sources are bone marrow derived stem cells including hematopoietic, embryonic stem cells and mesenchymal stem cells. Studies in which human embryonic stem cells are used for the treatment of many diseases such as diabetes, Parkinson's, heart failure, neurodegenerative injuries, osteogenesis imperfecta are still in the experimental stage; because embryonic stem cells must be differentiated into target tissue cells before transplantation [1-3]. Intrauterine adhesion (IUA) or Asherman's Syndrome (AS) was described by Fritsch in 1894. Defining the disease picture as "traumatic amenorrhea" for the first time, Dr. It is referred to as AS because it is Joseph G. Asherman [4]. Some authors have proposed to define the term 'Asherman's syndrome' for patients with amenorrhea, those with a completely occluded uterus, or those who have recently had a cesarean delivery (surgical operation). Others use the term 'intrauterine adhesion'; this term is clearer and more descriptive, but patients with surface deficiency of the endometrium without fibrous bridges between the uterine walls are the exception. Women with this condition similarly experience other pregnancy complications such as menstrual bleeding, infertility, recurrent pregnancy loss, uterine growth restriction, problems with implantation, and adhesions [5]. It seems indispensable for the future to develop new treatment modalities that will be more effective and alternative to surgical and hormonal treatment in patients diagnosed with IUA. Because, both in our country and in the world, the number of abortions has increased day by day. At the same time, damage to the uterine tissue may be inevitable during routine gynecological examinations. Stem cell studies in the last decade have developed so rapidly that it has increased the hopes of those seeking cures from cancer, which seems to be incurable, to metabolic diseases, even rheumatic and neurodegenerative diseases. Because these cells are known to positively affect other cells, especially cancer cells, by creating a micro-environment called a niche with the factors they secrete outside of themselves [6]. At this point, we focused on the effects of stem cell therapy as an alternative to surgical methods and medical treatments in endometrial damage.

2. MATERIAL and METHODS

2.1. Experimental Asherman model and stem cell applications

As an implantation failure model, IUA was chemically formed in the single horn of the uterus in female rats with trichloroacetic acid. Under ketamine/xylazine anesthesia, the abdomen was opened with a midline incision and the right and left uterine horns were determined, and then 0.1 ml trichloroacetic acid (IL 33, Istanbul Ilac Sanayi ve Ticaret AS, Umraniye, Istanbul, Turkey) was applied according to the experimental groups one day after the application [7].

Groups:

IUA + CM (G1, n=10); The group given freshly culturer medium (CM),

IUA + Niche (G2=10); The group given 48-hour medium,
IUA + Stem cell + Niche (G3=10); A total of three groups were formed, including the group that was given with CM medium obtained from bone marrow.

Each group was divided into two subgroups and a total of 6 groups were formed. Grouping was formed in such a way that the first subgroup pregnancy was not established, the second subgroup pregnancy was formed and the number of newborns was determined.

2.2. Bone Marrow Mesenchymal Stem Cells (BMSC)

In the study, bone marrow cells taken from the tibia and femur of male rats for bone marrow-derived stromal stem cell decomposition followed by separation in a 25cm2 culture dish with 15% fetal calf serum (FBS) (S0113, Biochrom, Berlin, Germany) added α-MEM (F0915, Biochrom, Berlin, Germany) culture medium was grown in an incubator at 370C and 5% CO2. At the end of the third day, stem cells and non-adherent cells were removed from the medium by changing the medium. Then, the medium of the cells adhering to the culture dish was changed every two days until it became confluent.

2.3. Vaginal smearing

Vaginal smear technique was used between 15.00-16.00 hours from female rats for cycle and pregnancy detection and samples were collected. To perform this procedure, 0.5 ml of saline was drawn into the micro pipette. Saline was sprayed into the vagina with a pipette tip. Without removing the tip of the pipette, the sprayed saline was drawn back into the pipette by creating negative pressure. The extracted liquid was taken on a clean slide and fixed for 5 minutes with methanol. Then, it was stained with rapid hematoxylin and eosin staining method and estrous cycle periods were evaluated cytologically under a light microscope (BX43, Olympus, Japan). In addition, after the proestrus and estrus stages were determined by this method, the subjects of the groups in which pregnancy would be created were placed in cages as three female rats and one male rat and left to mate. Vaginal smears were taken from females released for pregnancy the next day, and the first days of pregnancy were determined in rats whose sperm presence was detected by H&E staining [8].

2.4. Tissue staining

Uterine tissues were harvested by cervical dislocation under general anesthesia after treatment (10 days later). Tissues were fixed for 48 hours with 10% formalin solution for histochemical analysis. Tissues fixed in formalin were washed under running water for 1 night to remove fixative. Afterwards, it was passed through series of ethyl alcohol increasing to 60%, 70, 80 and 100% for 30 minutes each for dehydration. Then, they were kept in xylene-alcohol mixture in a one-to-one ratio for 15 minutes and in two changes of xylene for transparency for 30 minutes, and after applying xylene-paraffin in a one-to-one ratio for 15 minutes in an oven at 60˚C and immersed with two changes of paraffin for 60 minutes, the tissues were transferred to the paraffin blocks. Sections were taken with a microtome and H&E staining was performed. After tissue follow-up, sections of 4-5 µm were taken and stained with hematoxylin and eosin, endometrial thickness was determined using quantitative image processing software (ImageJ-NIH Image). Endometrial thickness measurements were obtained from the lateral edges of each slice; The measurement result was defined as the vertical distances between the luminal surfaces and the serous membranes. In addition, the number of glands was determined by taking

the average of the counts from four random areas by determining the gland abundance points in the stroma**.**

2.5. Statistical analysis

Inflammation, fibrosis, gland count and endometrial thickness were determined by H&E staining and statistical significance was determined in the results obtained from the first groups of the study. In the evaluation, 0= little inflammation and fibrosis, 1= little inflammation and moderate fibrosis, 2= moderate inflammation and fibrosis, 3= much inflammation and fibrosis. The resulting data were compared with the One Way-ANOVA Tukey statistical test, and $p<0.05$ results were considered statistically significant [9].

3. RESULTS

3.1. Vaginal spread findings

Vaginal smear procedure was performed to determine the days of pregnancies we created in our second groups. For this reason, rats in which AS was created and 10 days of medium, niche and stem cell+niche treatments were completed were taken into the same cages as male rats after proestrus and estrus cycles were determined. The next day, a vaginal smear was made again, and whether mating occurred or not was determined according to the presence of sperm detected in the smear. We considered the subjects whose sperm were observed in their vaginal smears to be pregnant. In vaginal smears, it was observed that rat sperm had a hook-shaped head and a long tail. It was observed at the end of the experiment that all of the animals in the vaginal smear preparations we observed sperm were pregnant.

The second subgroups of the groups were categorized according to the sperm findings in the vaginal smear preparations. However, it was determined that the smear was bright in color, the intermediate cells were dense, and the parabasal cells and mucus were rare in the preparations, which belonged to the proestrus group. It was determined that the group consisting of keratinized superficial cells belonged to the oestrus group smears. It was determined that the group with tightly packed multinucleated epithelial cells with cytoplasmic vacuoles and a large number of increased neutrophils belonged to rats in the metestrus cycle (Figure 1).

3.2. Histochemical findings of the groups without pregnancy

In the first groups of the experimental model, in which the IUA model was created as the implantation failure model, both the right and left uterine horns and the groups were evaluated by comparing each other. We applied different treatment agents intraperitoenally for 10 days to rats whose left uterine horns were damaged by acid treatment, and we evaluated the changes in the endometrium after 10 days under a light microscope. They were H&E stained from routine histochemical staining and the morphological and histological changes that occurred in the tissues at the end of the applications were examined (Table 1). In the study, a unilateral IUA model was created in all subject groups [7]. While damage was created by treating the left uterine horn with acetic acid, the right uterine horn was kept as a control and no damage was caused by any chemical or mechanical factor. It was observed that the endometrium degenerated intensively, the synechiae areas increased excessively in the groups to which

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medium and niche were applied (G1-1, G2-1), the number of glands decreased significantly compared to the groups in which only CM and niche were applied (G3-1).

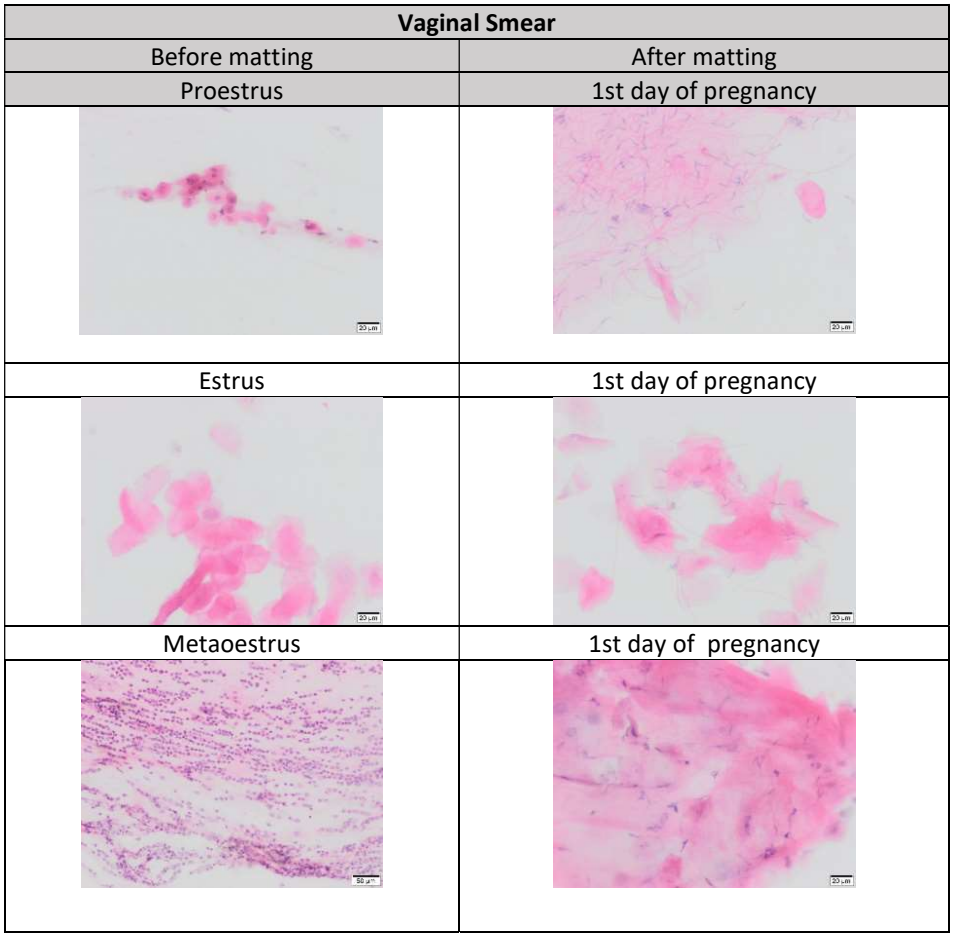

Figure 1. Evaluation results of vaginal smear preparations of IUA subgroups, H&E.

It was observed that the endometrial epithelium of the CM and niche groups was eliminated, and dense fibrous areas were formed in the lamina propria. Again in these groups, both the number of glands decreased and the tubular structure of the gland epithelium was impaired (Figure 2). In addition, the number of newborns in the lower secondary groups was determined and a statistically significant difference was observed ($p>0.05$) (Table 2).

Table 1. Fibrosis, inflammation, endometrial thickness and gland count of the sub-first groups.

	Inflammation	*Fibrosis*	*Endometrial thickness*	*Gland count*
CM	2,4 ± 0,51	2,7 ± 0,48	138, 72 ± 26 µm	5 ± 2
Niche	1,6 ± 0,51	2,3 ± 0,48	154,24 ± 26 µm	5 ± 1
BMSC-Niche	0,5 ± 0,52	0,9 ± 0,73	315,35 ± 32 µm	14 ± 3
Right horn	0,2 ± 0,30	0,7 ± 0,53	333,15 ± 18 µm	16 ± 3

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Table 2. The number of implantation and newborns of the sub-second groups.

	CM G1-2	Niche G2-2	BMSC+Niche G3-2
Newborns	4,66 ± 0,57	5 ± 0,81	8,6± 0,54

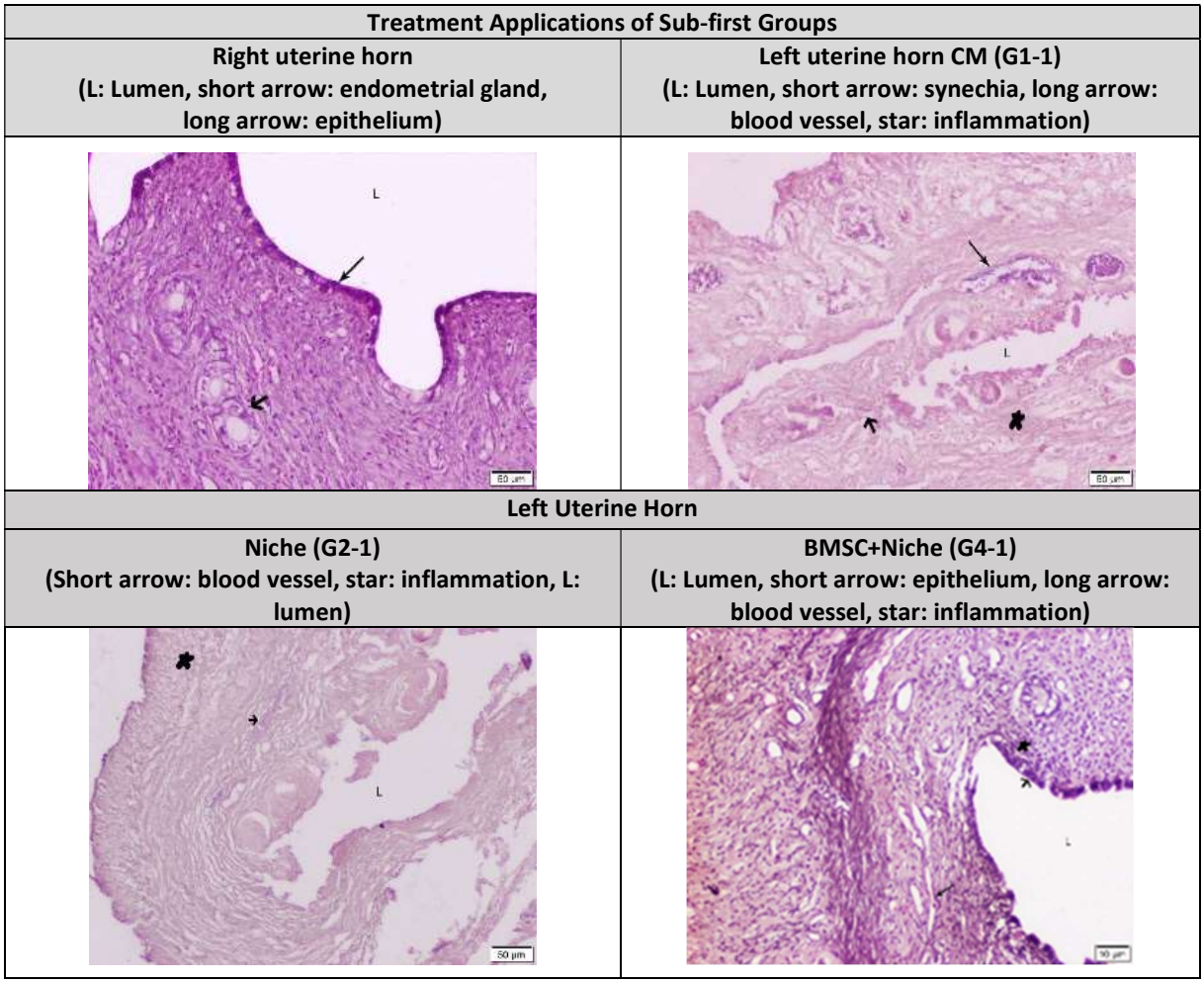

Figure 2. Images of the endometrium after treatment of the sub-first groups, H&E.

4. DISCUSSION

Macroscopic and histological stainings were used to evaluate the changes in the uterus of the CM, Niche, and BMSC + Niche groups treated in the IUA-formed rat uterus according to the number of newborn puppies before pregnancy occurred and in the subjects who became pregnant. In this study, histological images were examined in uterine samples obtained from non-pregnancy and pregnancy groups. The role of CM and its niche in adhesions caused by IUA and its effect on the implantation rate were investigated. Histological findings were obtained showing that the regeneration in the eastern part progressed in the groups treated with BMSC and its niche, new vessel formations increased and glands became more prominent, fibrosis and inflammation that inevitably occur in IUA pathology decreased, and endometrial thickness increased.

There are no randomized controlled trials for the treatment of IUAs. The standard treatment is surgical removal of adhesions by direct observation. With the removal of adhesions, it is aimed to create a cavity with normal anatomy and to provide a functional endometrium. Hysteroscopy is often preferred in current treatment methods. Although the uterine tissue is surgically restored, the recurrence of adhesions and the risk of complications from uterine surgeries have revealed the need for new treatment options. For the treatment of intrauterine formations such as synechia; There were various interventions such as serial flexible hysteroscopies [10], intrauterine adhesion barrier systems [11], fresh amniotic graft, intrauterine insertion of seprafilm [12], hysteroscopic surgery, and hyaluronic acid gels [13].

One of the main problems with intrauterine fillers is the need for concomitant use of antibiotics since a foreign body is present in the uterus. Clinical or subclinical pelvic infections in contact with the vagina may increase secondary infertility.

The point reached recently is that; Estrogen and progesterone supplementation is recommended as the primary option in order for the endometrial tissue to recover and continue its functional activity after surgical procedures. Of course, the effects of this recommendation do not show the same effect in every patient. It has been reported that vaginal administration of micro-sized estradiol further increases the endometrial estradiol concentration, since systemic side effects of oral estrogen supplementation are unavoidable, according to some researchers. However, it is still known that such applications of estrogen cause various contraindications in patients. Such problems cause researchers to work harder for new treatment approaches. Artificial hormone therapy with estrogen is frequently used to promote endometrial proliferation and angiogenesis [14]. 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 bone marrow by aspiration. Due to their extensive migratory and pluripotent potential, SCs can be easily obtained from both humans and rodents. Stem cells with endometrial regeneration and angiogenesis effects are purified from the bone marrow by immunomagnetic isolation [15]. Ultimately, they prepare the endometrium for implantation. Especially since the endometrium's own stem cells cannot provide endometrial regeneration due to the damage, it has been observed that externally applied transplant stem cells are permanent and increase the success of implantation by functioning. In this study, in which Y-positive cells can be easily demonstrated due to the use of male CM, it has been proven that transplanted cells can remain in the uterus even after three months [16]. Human endometrial tissue is one of the tissues with the strongest dynamism in completing its regeneration perfectly in every menstrual cycle. Although it is generally believed that precursor cells in the functional and basalis layers of the endometrium play a role in this dynamism, there is literature information that BMSCs are also involved in the regenerative ability of the endometrium [17]. In the study, it was tried to remove intrauterine adhesions and to remove these adhesions, which is one of the important infertility problems, by using stem cells, which will contribute to alternative methods and may be the most important treatment tool of the next century. As a matter of fact, both macroscopic findings obtained from the groups; The number of embryos, the number of newborns, the anatomical structure of the endometrium after sacrification and the histopathological findings obtained as a result of microscopic tissue follow-up proved this. Recently, populations of endometrial epithelial and stromal MSCs (Mesenchymal stem cell)

resembling adult MSCs in the basal layer of the human endometrium have been described very rarely [18]. The origin of endometrial stem cells remains unclear. However, BMSCs have been recognized to contain many sources, including menstrual blood-derived mesenchymal stem cells and adipose stem cells [19]. Experimental models have been tried to be designed on the Asherman model using many animals, especially rabbits and rodents, and alternative treatment methods for uterine adhesions have been tried. Especially in rabbits and rodents, mechanical damage, physical damage, laser damage model, curettage model, adhesion developed model with lipopolysaccharide curettage suture and experimental models created using chemicals such as 10% formalin, trichloroacetic acid, polyethylene sponge, phenyl mucilage were designed and fibrotic tissue tried to decrease and increase vascularization [20, 21]. In the light of investigations and presented findings, it was preferred to set up this experimental model using acid [7]. Because it was observed that the synechia formed by the acid creates suitable adhesion conditions for AS.

5. CONCLUSION

Surgical operations only serve to divide adhesions within the cavity, but can do little about endometrial regeneration and adhesion recurrence. So women with Asherman syndrome require more than one approach to provide optimal clinical outcomes. Although stem cells are still in the research phase, new advancing discoveries in this field are leading to new therapeutic strategies every day. We believe that the findings of this study will shed light on stem cell applications in reproductive medicine.

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The Declaration of Conflict of Interest/Common Interest

No conflict of interest or common interest has been declared by the authors.

Authors' Contribution

The authors contributed equally to the study.

The Declaration of Ethics Committee Approval

This study was conducted with the approval of the Animal Experiments Local Ethics Committee of Manisa Celal Bayar University, Faculty of Medicine, with the decision number 77637435-050.04.04 dated 05/02/2018.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of ETOXEC in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Environmental Toxicology and Ecology and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Environmental Toxicology and Ecology.

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