Mesenchymal Stem Cells Transplantation in Endometriosis Model Mice on VEGF mRNA Expression to Improve Infertility

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Abstract
The concentration of pro-inflammatory interleukin increases in the follicular fluid of patients with endometriosis, causing a decrease in the concentration of VEGF in this follicular fluid, which is thought to cause a decrease in embryo quality and a decrease in implantation rates.

This study aimed to determine the role of VEGF mRNA in endometriosis repair. The study consisted of 3 groups, the negative control group (T0), the endometriosis model group that received placebo (T1), and received bone marrow mesenchymal stem cells (T2). Examination of VEGF mRNA expression using real-time PCR method. Data analysis using ANOVA statistical test. The results showed that the expression of VEGF mRNA between the endometriosis model group was significantly different from the endometriosis model group that received bone marrow mesenchymal stem cells, but it was not different from the control group or normal mice.

This study concluded that the expression of VEGF mRNA in the group of endometriosis model mice that received bone marrow mesenchymal stem cells was lower than the group that did not. There was no relationship between VEGF mRNA expression and the number of primary follicles, secondary follicles, tertiary follicles, and de Graafian follicles.

Keywords: Reproductive Health, Endometriosis, mRNA VEGF, RT PCR, Mesenchymal Stem Cells.

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Introduction
Endometriosis is a disease that is often found in women of reproductive age and characterized by the growth of endometrial tissue outside the uterine cavity (ectopic). The incidence of endometriosis is quite high in infertile women, ranging from 10-70% and 50% in adolescents with dysmenorrhea¹. The classic symptom of endometriosis is pain, and about 90 million women of reproductive age who suffer from endometriosis develop infertility and pelvic pain¹². Until now, it is a problem that has not been completely resolved. This is because the pathophysiology and pathogenesis of endometriosis are still being debated.

Medical treatment and surgery for people with endometriosis can reduce the complaints, but cannot improve fertility. When compared with other causes of infertility such as tubal abnormalities and unexplained infertility, moderate endometriosis will provide low pregnancy success and a high incidence of abortion⁵. Endometriosis harms the function of the ovaries, tubes, and the ability of the uterus to accept conception⁶.

Endometriosis also increases the apoptosis of granulosa cells and affects the process of folliculogenesis and steroidogenesis⁷. The pro-inflammatory interleukin concentrations of IL-6, IL-1β, IL-10, and tumor necrosis factor-α (TNF-α) are increased in the follicular tract of patients with endometriosis, as well as vascular endothelial growth factor (VEGF) concentrations⁶. The concentration of VEGF in the follicular fluid is still controversial. Other studies found that decreased VEGF levels in the follicular tract. This is thought to be the main factor in the decrease in embryo quality and implantation rates⁷. Regenerative therapy can regenerate organ

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systems that have been damaged due to age, disease, or congenital defects. In this study, bone marrow mesenchymal stem cells were used because there had been a lot of research and had been widely used for therapy. Besides, bone marrow mesenchymal stem cells are a type of adult stem cells and have a small risk to develop into tumors was small when compared to embryonic stem cells. Since the use of human tissue in animal models would be advantageous for experimental research, mice with reduced immunity were established as heterologous models for endometriosis. Human endometrial tissue has been successfully transferred to SCID (severe combined immunodeficient) mice as well as NOS-SCID (non-obese diabetic-severe combined immunodeficient) mice, both of which exhibit deficiency of T and B lymphocyte function.

Materials and methods

The ethical clearance certificate number 685-KE.2017 was obtained from the Faculty of Veterinary Medicine Universitas Airlangga, Surabaya. The study was a laboratory experimental study with a post-test only control group design. This study used 42 female mice which were divided into 3 groups, which are the negative control group (T0), the endometriosis model mice that received placebo group (T1), and the endometriosis model mice that received bone marrow mesenchymal stem cells group (T2). The stages of this research are:

Endometrial Biopsy

Tissue was taken with endometrial scrapings from patients with uterine removal surgery with benign tumors who have not received hormonal treatment in the last three months. The endometrium obtained was stored in PBS (phosphate-buffered saline), then crushed and washed twice by centrifugally at a rotation of 2500 rpm. The supernatant was discarded and then added PBS, 200IU / ml penicillin, and 200ug / ml streptomycin. The wet tissue of the endometrium was taken with a syringe of 3 ml. The dose for each mouse is 0.1 ml.

Bone Isolation for Mesenchymal Stem Cells Production

The isolate of rat bone marrow (Rattus novergicus) then added with MEM medium and centrifuge at 3000 rpm for 10 minutes and repeated 2 times. The precipitate was cultured in MEM and added with antibiotics, anti-fungal, and 15% Fetal Calf Serum (FCS). The passage is repeated until a cell line is formed. To ensure that the bone marrow mesenchymal stem cells were inserted, a homing examination was performed using PKH 26 indicator on the ovaries of endometriosis model mice receiving stem cell therapy.

Mesenchymal Stem Cells Production.

Using local anesthesia, the bone marrow aspirate was collected from the rat's tibia and then put into a 15 ml heparin tube containing 3 ml α-MEM. Tubes and preparations are stored in the refrigerator prior to service. Each aspirate was transferred into a 15 ml blue cap tube and diluted with PBS. Each tube 2 times process with 5 ml PBS. Mix and coat each aspirate with ficoll. Centrifuge the tube at 1600 rpm for 15 minutes, then collect the buffy coat located on Ficoll-PBS with a Pasteur pipette and place the cells in a 15 ml tube. After that, dilute each sample with PBS, mix 3-5 times, and centrifuge again at 1,600 rpm for 10 minutes. Aspirate the supernatant and resuspend the cells with 6 ml CCM. Place the cells on a 5 or 10 cm² plate. Incubate cells at 37°C with 5% CO₂ humidity for 24 hours. After 24 hours add 2 ml of PBS to the culture, wash it 2 times. Add 10 ml CCM, incubate at 37°C humidity 5% CO₂ for 5 -10 days. Perform daily examinations with an electron microscope. Every 3 days wash with PBS 5% or 10% and add 10 ml CCM. Continue until the cells are between 60% - 80% confluent.

The production of Endometriosis Model Mice

On the first day, female mice were injected with cyclosporin A (Sandimmune produced by Novartis) intramuscularly at a dose of 10 mg/BW, 0.1 ml of endometrial tissue, and estrogen. The estrogen used with a conversion dose for each mouse 5.4 μgr (1 μgr equivalent to 10IU). On day 5, estrogen was injected again. On day 14 the model of endometriosis mice characterized by hypervascularization of the peritoneal tissue, growth of endometriosis tissue in the peritoneum.

Bone marrow mesenchymal stem cell therapy in endometriosis model mice

Endometriosis model mice were injected with single dose mesenchymal stem cells at dose 10⁵/mice⁶. Injections are carried out intravenously in the tail. Before the injection, the tail is warmed so that vasodilation occurs in the tail blood vessels.
Oocyte Collection Preparation

Endometriosis model female mice were injected with the hormone Pregnant Mare Serum Gonadotropin (PMSG) at the dose of 5 IU. 48 hours later, they were injected with the hormone Human Chorionic Gonadotropin (hCG) and ovulation induction was performed with mononating castrated male mice mixed. 17 hours after mating, the vaginal plug was examined. The female mice whose vaginal plug was positive were subsequently terminated. Then preparations are made to remove the fallopian tube. The collection of complex cumulus oocytes is done by tearing the fertilization sac under a microscope.

Cumulus Oocyte Complex sample collection for Real-Time PCR examination

A cumulus-oocyte complex (COC) sample is taken by tearing the fertilization sac of the Fallopian tube. The rip is carried out under an inverted microscope. Fertilization sac was placed in a Petri dish containing PBS media (Gibco, BRL, USA) supplemented with 3% BSA (Bovine Serum Albumin, Sigma). To release granulosa cells, COC was put in PBS added with 0.1% Hyaluronidase (Sigma, USA) for 25 seconds. The oocyte is removed and then centrifuged, the supernatant is removed, the granulose cells are deposited as a sample. The obtained granulosa cells were inserted into Trizol contained tube for real-time PCR.

VEGF-B mRNA Examination Using Real-Time PCR Method

Real-time PCR steps:

RNA Isolation.

Granulosa cells washed with 1X ice-cold NaCl 0.9% (1-2 mL). Aspirate the NaCl then add 1 mL of TRilzol to lyse the granulosa cells. Incubate at 15-30°C for 5 minutes. Add 0.2 mL of chloroform for every 0.75 mL of TRilzol. Shake the tube for 15 seconds then incubate for 2 minutes at 15-30°C. The preparation is then centrifuged for 5 minutes (10,000 rpm). Then take the aqueous phase layer (containing RNA) and add 500 microliters of isopropyl alcohol. Discard the supernatant, the RNA pellet washed once with 75% ethanol, centrifuge again at 9.500 rpm for 5 minutes. Then the ethanol is evaporated so that the RNA pellets dry up and added with a solution of water Free nuclease-free RNase.

Reverse transcriptase/ pembentukan complementary DNA (cDNA)

The isolated mRNA is then made cDNA using the reverse transcriptase enzyme. The cDNA that has been formed is then amplified using a primer that has been designed. To find out the expression of a gene, a reference gene is needed as an internal comparison (endogenous control) of the amount of DNA so that there is no misinterpretation due to different amounts of DNA. Reference genes used are genes that are not influenced by the environment. The most widely used genes are housekeeping genes such as actin and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).

The primary sequence is as follows: GAPDH (180 bp) sense, 5’-AAGGT CATCCAGAGCTGAA-3’ dan antisense, 5’-CTCAGTGTAGCCAGAATGCG-3’; p21 (169 bp) sense, 5’-GAGTGCCCTTGACGATACGC-3’ and antisense, 5’-CATGTACTGGTCCCTCATTGC-3’; survivin (180 bp) sense, 5’-GGAGCATAGGAACGTCCCTGGTG-3’ and antisense, 5’-CTTCGGGTCTTCCTCGAACTCTT-3’; Bcl-2 (173 bp) sense, 5’-AGTACCTGAACCGGATCTTG-3’ and antisense, 5’-CAGGTATGCAACCGAGTGA-3’.

Real time PCR

PCR amplification consist of denaturation for 2 minutes at 93°C followed by a temperature of 95°C for 15 seconds for 40 cycles and 60°C for 1 minute using a PCR machine (Applied Biosystems; Foster City, CA, USA). The CT value of real-time PCR was analyzed by the 2-ΔΔCT method. The PCR product results were validated by 120 V A 20 minutes electrophoresis.

Data Analysis

The data from VEGF mRNA measurements were analyzed using one-way ANOVA parametric analysis if the distribution was normal and using the Kruskal-Wallis test if the distribution was not normal.

Results

Culture and Isolation of Bone Marrow Mesenchymal Stem Cells

The isolate passages every 5 days until it reaches passage four and the stem cells reach up to 80% confluence. The stem cells from these cultures were then used as therapy in endometriosis model mice. The results of stem
cells that have reached 80% confluence are shown in the figure 1.

Figure 1. Rattus norvegicus bone marrow stem cell culture, figure shows developing and confluent stem cells after 4 passages (Inverted Microscope, 40x).

Homing of mesenchymal stem cells in mouse ovaries

The PKH26 labeling of the transplanted stem cells makes it possible to detect the position of the stem cells so the homing process can be proven. Stem cells are colored or labeled with PKH26 fluorescent membrane dye (Sigma-Aldrich) radiating at 551-567 nm waves.

Figure 2. PKH26 luminescence of endometriosis model mice ovaries labeled PKH26. Using a red-green filter (Fluorescent microscope, Magnification 4.2 x)

In Figure 2, PKH26 luminescence is visible which has colored the bone marrow mesenchymal stem cell membrane so that the cells glow green, red or orange when observed under a green, red and red-green filter fluorescent microscope. This is evidence that the bone marrow mesenchymal stem cells can be homing in the ovary.

qPCR mRNA VEGF Result

The VEGF mRNA values of the three groups were significantly different (p <0.05), so further tests were needed to determine which groups were different. The follow-up test used was LSD (because the homogeneity test was p>0.05). The follow-up test results showed that the T1 group was significantly different from the T2 group (p=0.012), but the T1 group was not significantly different from the T0 group (p=0.166). There was also no significant difference between the T0 and T2 groups (p=0.124). The results of statistical analysis can be seen in Tables 1 and 2 below.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>14</td>
<td>36.35±1.71</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>7</td>
<td>37.42±1.35</td>
<td>0.038</td>
</tr>
<tr>
<td>T2</td>
<td>11</td>
<td>35.31±1.69</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. The expression of VEGF mRNA between groups.

Comparison between group | p value |
-------------------------|---------|
T0 VS T1                 | 0.166   |
T0 VS T2                 | 0.124   |
T1 VS T2                 | 0.012   |

Table 2. Post hoc analysis of comparison of VEGF mRNA expression between groups.

Discussion

In this study, stem cells from bone marrow can grow and develop well in culture. After doing passage 4 times with a duration of 5 days, the development of stem cells reaches confluence about 80% in Figure 1 and has fulfilled the requirements for use as cell therapy following the standard in showing that proliferation and efficacy of Bone Marrow Mesenchymal Stem Cell therapy are strongly influenced by the confluent level of the stem cells. 80 percent confluent is the optimal level in culture and harvesting of Mesenchymal Stem Cell stem cells for the treatment plan compared to confluent 20, 50, 70, and 100. Many factors influence the growth of mesenchymal stem cells (MSC), including the appropriate isolation
method, culture medium, the number of initial cells in the culture, and the animal strain used\textsuperscript{11}.

In this study, stem cells were successfully homed after examination of the ovaries for PKH 26 expression in the endometriosis model mice. PKH 26 is a marker that can detect stem cell homing in target cells. This can be seen in Figure 2 after examination with immunofluorescent without the orange color. This is similar to\textsuperscript{12} which states that the PKH 26 antibody is the strongest marker and has high accuracy for detecting homing stem cells in target organs\textsuperscript{13,14}.

The assurance of homing stem cells is a process by which cells migrate and then stick firmly to the tissues where these cells have a repair function and effect. The distribution of stem cells is the first step that must be achieved so that stem cells can repair damaged tissues/organs. For that, validation is a must to prove that stem cells can reach the target tissue/organ. PKH26 fluorescent membrane dye is a chemical that color cells and glow for a certain period of time, are non-toxic, and does not inhibit cell proliferation\textsuperscript{15,16}.

The homing process can occur in several stages: chemotaxis, withdrawal and adhesion, diapedesis\textsuperscript{17}. Chemotaxis occurs after an injury, chemokines will activate local endothelial cells to increase the expression of P-selectin, E-selectin, and Vascular Cell Adhesion (VCAM 1) proteins on the cell surface. These chemokines will also be released into the systemic circulation and will activate certain leukocytes. Mobilization and adhesion occur when leukocytes migrate to the site of injury. The molecules on endothelial cells bind to leukocyte receptors to facilitate binding. This bond will slow down the flow of leukocytes and cause strong adhesions in the lumen walls of blood vessels. Mesenchymal stem cells attach to endothelial cells via P-selectin and VCAM/ VLA-4. This bond will be even stronger if there is TNF-\textgreek{a} stimulation which can increase the expression of integrin proteins. Diapedesis is the final stage of homing, known as the extravasation of mesenchymal stem cells which is trans-endothelial migration to the target tissue. The mechanism of diapedesis is not fully understood. It is suspected that this stem cell transmigration is mediated by a matrix metalloproteinase. Mesenchymal stem cells are thought to degrade the endothelial layer to enter the tissue\textsuperscript{17}. One of the chemoattractants currently known as stromal-derived factor-1 (SDF-1) binds to CXCR-4 which is expressed by mesenchymal stem cells. This bonding is thought to cause the homing process to occur\textsuperscript{18,19,20}.

mRNA VEGF Expression

The results of this study indicated that the VEGF mRNA expression was significantly different between the three groups. In this study, the VEGF mRNA expression in the T1 group was higher than the T0 group. These results are consistent with the research of Oliveira et al.\textsuperscript{21} which states that the VEGF of granulosa cells (cultured for 48 hours) is higher in infertile women without endometriosis than in women with endometriosis\textsuperscript{21}.

Through the paracrine effect, bone marrow mesenchymal stem cells can secrete several growth factors, one of which is VEGF. In this study, bone marrow mesenchymal stem cell transplantation was unable to show an increase in VEGF mRNA expression in group T2. This could be due to granulosa cell isolation techniques or granulosa culture conditions which may cause follicular compartment disorders in the endometriosis model. Although VEGF is important to form vascularization that allows the transport of nutrients to granulosa cells and the nucleus, other factors such as growth factors and oocyte secreting factors are still needed so the communication between granulosa cells and oocytes can run well and folliculogenesis can take place properly.

VEGF is a proangiogenic glycoprotein that plays a role in follicle maturation. Human Chorionic Gonadotrophin and Luteinizing Hormone directly induce VEGF production and VEGF mRNA transcription via a second messenger route involving protein kinases A and C. The level of VEGF in the follicular fluid from several existing studies have yielded controversial results. Stem cells are known to secrete IGF 1, HGF, and VEGF. IGF 1 regulates the replication of theca cells and granulosa cells, in addition to regulating aromatase enzyme activity, inhibit secretion, and formation of the corpus luteum that stimulates hormone receptors. IGF 1 also stimulates the cycle of G0 / G1 cells into S via P13K / Akt signals and inhibits the Fas receptor for apoptosis\textsuperscript{9}.

Conclusions

The expression of VEGF mRNA in the
endometriosis model group of mice that received bone marrow mesenchymal stem cells was lower than the group that did not. There was no relationship between VEGF mRNA expression and the number of primary follicles, secondary follicles, tertiary follicles, and Graafian follicles.

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Declaration of Interest

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

References