Rat bone marrow mesenchymal stem cells improve regeneration of thin endometrium in rat

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Objective: To investigate whether bone marrow mesenchymal stem cell (BMSC) treatment could improve the regeneration of endometrium and improve the endometrial receptivity in an experimental model of thin endometrium.
Design: Randomized, control trial, animal research.
Setting: National key laboratory.
Animal(s): Sprague-Dawley rats.
Intervention(s): Bone marrow mesenchymal stem cell transplantation by tail vein IV injection.
Main Outcome Measure(s): Endometrial thickness, the expression of mark proteins for endometrial cell, and endometrial receptivity.
Result(s): The endometrium was significantly thicker and the expression of cytokeratin, vimentin, integrin $\alpha\gamma\beta 3$, and leukemia inhibitor factor were significantly stronger compared with the control group. Some proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) messenger RNA (mRNA) and interleukin-1β mRNA, were significantly down-regulated, and anti-inflammatory cytokines, such as fibroblast growth factor-β (bFGF) mRNA and interleukin-6 mRNA, were significantly up-regulated in the experimental group compared with the control group.
Conclusion(s): The BMSCs have beneficial effect on thin endometrium, and may play a role through migration and immunomodulatory of BMSCs. (Fertil Steril® 2014;101:587–94. ©2014 by American Society for Reproductive Medicine.)
Key Words: Bone marrow mesenchymal stem cells (BMSCs), thin endometrium, endometrial receptivity, immunodulatory

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In reproductive medicine, the failure to construct a functional endometrium with the correct morphology in patients with Asherman syndrome, or in those with inadequate, thin endometrium was believed to be one of the three main unresolved clinical issues. Several treatments, such as estrogen (E), aspirin, pentoxifylline, and endometrial injury, have been tried to improve the regeneration of the endometrium (1–4). However, these efforts have yield disappointing results with controversial conclusions. It was necessary to explore the innovative intervention for the cure of thin endometrium.

Since the identification of stem cells from human sources, such as the embryo, fetus, adult organs and tissues, their possible therapeutic applications have been explored. Clinicians and researchers have built up a new discipline, termed regenerative medicine, which aims to use these cells to treat incurable diseases and conditions that, until the present have remained untreatable with the use of drugs.

Bone marrow mesenchymal stem cells (BMSCs) are a major type of multipotent mesenchymal stem cells (MSCs) that are capable of differentiating into lineages of cells (5–8), and they could be expanded extensively in vitro and have immune-privileged properties (9–13). Therefore, BMSCs have been considered to be a strong candidate tool to treat many diseases. Preclinical studies have shown beneficial effects of BMSCs on neurological disorders. The BMSCs facilitate nerve regeneration (14), improve diabetic neuropathy (15), multiple sclerosis (16), and help functional recovery after stroke (17).

However, there was no report that evaluates the therapeutic effect of

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BMSCs on thin endometrium. This has led us to explore further into the capacity of BMSCs as a therapeutic target for thin endometrium. In light of the recent development on the therapeutic potential of MSCs, the purpose of this study was [1] to investigate whether transplantation of MSCs can improve the thin endometrium, and [2] to explore the underlying mechanisms responsible for such effects.

MATERIALS AND METHODS

Animals

Eight-week-old Sprague-Dawley rats (12 male rats, 72 female rats) weighing 200–250 g were used in all experiments. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 80-23) and approved by the Institutional Animal Care and Use Committee of the Central South University.

BMSCs from Male Rats

The BMSCs were isolated from adult, male/female, Sprague-Dawley rats, as previously described (18). In brief, the femurs and tibiae were collected from male rats killed by cervical dislocation. Bone marrow cells were harvested by flushing the marrow cavity with Dulbecco's modified Eagle's medium (DMEM) (GIBCO). The suspended cells were then collected by centrifugation at 1,500 rpm for 5 minutes. The cells were resuspended and cultured in L-DMEM media with 10% fetal calf serum (FCS) and 1% penicillin-gentamicin at 37°C in a humidified incubator with 5% CO_2. The nonadherent hematopoietic cells were removed after 48–72 hours. The culture medium was changed every 3 days. Adherent BMSCs were harvested by 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (GIBCO) when reaching 90% confluence. The BMSCs cultured to the third passage were used to transplantation. Before transplantation, at 72 hours, BMSCs were supplemented with 2 μM bromodeoxyuridine (Sigma) to label dividing cells. Cells were harvested with trypsin/EDTA, washed twice with L-DMEM, and resuspended at a concentration of 50,000 cells/μL in L-DMEM.

Before in vivo experiments, cells were characterized for their capability to differentiate toward adipocytes and osteoblasts, as previously reported. Their mesenchymal phenotype was also assessed by fluorescence-activated cell sorting (FACS).

Group and Treatment

The rats were kept under standardized laboratory conditions in an air conditioned room with free access to food and water. The thin endometrium rat model was set up by injecting anhydrous ethanol into the uterus of rats according to our preliminary study (19). A preliminary experiment was done to characterize the extent of endometrial cell injury when BMSCs were infused ($n = 3$). Seventy-two rats were randomly assigned to three groups, including the experimental group (IV-injected BMSC into tail vein 6–8 hours after modeling, $n = 24$), control group (IV-injected saline into tail vein 6–8 hours after modeling, $n = 24$), and blank group (normal rats with no treatment, $n = 24$).

All rats were anesthetized by intraperitoneal (IP) injection of overdose 10% chloral hydrate (1.0 g/kg body weight) at the third estrus phase after injection of BMSCs. The phases of the estrous cycle were determined by observing the vaginal smear. The uteri were excised after the rats were sacrificed. Portions of the uteri were sectioned and preserved in formalin and/or liquid nitrogen for further research.

Hematoxylin-eosin Staining

Hematoxylin-eosin (H & E) staining was performed as described previously (20). The sections (10–30 μm) on slides were immersed in xylene (10 minutes, twice), and rehydrated in a decreasing ethanol series diluted in distilled water (100%, 100%, 95%, 95%, 75%, 0, 1 minute each). The sections were rinsed in deionized water, stained in hematoxylin for 45 seconds, rinsed in deionized water, and finally stained in eosin for 1 second. After the color reaction, sections were dehydrated through an ethanol series in xylene and mounted using Permount mounting medium (Fisher Scientific). The thickness and the morphology of endometrium were evaluated and compared among groups.

Immunohistochemistry

After fixation in 4% paraformaldehyde, the uterine horns were embedded in paraffin and about 6-μm serial sections were prepared and placed on SuperfrostPlus microscope slides (VWR International Ltd.). Sections were deparaffinized in xylene, rehydrated through a series of ethanol washes, and rinsed in water. Endogenous peroxidase activity was blocked by incubating sections in 0.3% H_2O_2 in methanol for 40 minutes at room temperature. Slides were blocked for 1 hour in phosphate-buffered saline (PBS) supplemented with 10% normal goat serum. Expressions of cytokeratin, vimentin, integrin-β3, and leukemia inhibitor factor (LIF) proteins were performed by incubating sections of rat uteri with either rabbit polyclonal antibodies against cytokeratin, vimentin, integrin-β3, or LIF overnight at 4°C. Sections were incubated with 1:3,000 horseradish peroxidase-conjugated goat anti-rabbit IgG in 10% goat serum for 1 hour at room temperature. Sections were then briefly counterstained (10 seconds) with hematoxylin solution (Gill no. 3; Sigma), and examined using a Nikon microscope. For each protein studied, the immunohistochemical staining was repeated twice at each time point with sections obtained from different rats. The regeneration of endometrial cells and the endometrial receptivity were analyzed by immunohistochemistry with cytokeratin, vimentin, integrin-β3, and LIF.

Western Blotting

The tissues were homogenized in solubilization buffer. The homogenate was centrifuged at 10,000 $\times$ g for 10 minutes at 4°C. The supernatant was removed. The protein concentration was determined using a detergent-compatible protein assay with a bovine serum albumin standard. For detection of cytokeratin, vimentin, integrin $\alpha\gamma\beta$3, and LIF, 20 μg of protein from each sample was loaded onto an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride membrane. The blots were blocked with 5% milk in *tris*-buffer saline