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Investigating Combined Treatment of Menstrual Blood-Derived and Adipose-Derived Mesenchymal Stem Cell Conditioned Media in Endometriosis Mouse Models: A Research Protocol

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Abstract
Endometriosis is a debilitating disease characterized by endometrial-like tissue abnormally growing in extrauterine regions, which causes symptoms that diminish one's quality of life and reduces fertility. Treatment currently involves invasive surgical procedures, and there is a high chance of lesion recurrence after removal. Novel research has demonstrated the potential of using conditioned media (CM) from various stem cells for regenerative properties. The proposed study aims to examine and compare the individual and coupled effects of menstrual-derived stem cell-conditioned media (menSC-CM) and adipose stem cell-conditioned media (adSC-CM) on endometrial tissue regeneration in mice models. The purity of stem cells obtained from human donors will be assessed using flow cytometric analysis with established cell markers. Nine-week-old C57BL/6 female mice will then undergo surgical engraftment of extrauterine tissue from transgenic eGFP donor mice, with groups receiving adSC-CM, menSC-CM, a combined therapy, or no treatment. A negative control group will also be included. Tissue collection will occur after the random allocation of mice to be sacrificed or mated. Previously recognized endometriotic markers, ICAM-1 and VEGF will be assessed via reverse transcription polymerase chain reaction (RT-PCR) to calculate gene expression levels and via immunohistochemistry to calculate the H-score. The H-score for GFP will also be calculated. Hematoxylin & Eosin (H&E) staining will be used for quantitative analysis of endometriosis lesion size. It is expected that all experimental groups receiving treatments will have significantly reduced endometriotic lesion size, with the group receiving the combined treatment having the largest reduction. However, as this combined therapy is yet to be studied, it could also have alternative effects. Similarly, it is predicted that mice receiving combined therapy will have the most improved pregnancy outcomes compared to the positive control and will have the lowest H-scores for ICAM-1, VEGF and GFP. This study has implications in providing insights that may be used for developing novel non-invasive treatment options, using mesenchymal stem cell source therapy, that aim to reduce endometriotic lesions and promote patients' quality of life.

Keywords: endometriosis; mesenchymal stem cells; adipose stem cells; conditioned media; RT-PCR; immunohistochemistry; H&E staining; combined therapy

Introduction

Background

Endometriosis chronically affects around 10% of women of reproductive age worldwide (190 million) and is characterized by an abnormal growth of endometrial-like tissue in extrauterine regions [1]. Generally, lesions are present in the pelvic cavity, ovaries, uterosacral ligaments, and rectouterine pouch [2]. There is no cure, and this growth leads to extreme chronic pain throughout the menstrual cycle, impairing quality of life [2]. Most endometriosis patients experience pelvic pain, dysmenorrhea, and dyspareunia. With increased endometriosis severity, there is greater scarring, which limits the ability of the oocyte to travel to the Fallopian tube, leading to irregular implantation and, ultimately, infertility [3].

Kvaskoff *et al.* reported a correlation between the presence of endometrial lesions and an increased chance of developing ovarian and breast cancer, and heart disease [4]. However, diagnosis of endometriosis takes, on average, ten years and requires invasive surgery to confirm lesions [2]. Treatment primarily involves hormonal therapy to decrease estrogen levels, block ovulation and therefore decrease endometrial tissue size, as well as surgery to extract lesions [2]. However, there is a high chance of recurrence after removal [5]. No definitive approach completely controls endometriosis development [3].

The mechanism and causes of the disease that would explain all aspects of endometriosis have yet to be identified [3]. As such, several studies have focused on finding molecular differences in the endometrium of affected and non-affected women. They have found a common factor amongst endometriosis patients: retrograde menstruation [3]. Retrograde menstruation is characterized by upward flow of menstrual blood through the Fallopian tubes and into the pelvis instead of out of the vagina [3]. Current research involves stem cell therapy, such as using various mesenchymal stem cell (MSC) sources to treat associated infertility [6]. MSCs are multipotent stromal cells with self-renewing abilities and have several tissue sources, notably from menstrual blood (menSC) and adipose tissue (adSC) [7].

Studies have shown the potential of menSC and adSCs on tissue regeneration and have tested proposed mechanisms for their effectiveness [5,8]. Interestingly, the culture medium used to grow stem cells, known as conditioned medium (CM), may be collected after the stem cells secrete bioactive molecules into it [5,8]. Huang *et al.* showed that adSCs, specifically those derived from their conditioned medium (adSC-CM), stopped endometriotic tissue growth in mice and positively affected their fertility [5]. The conditioned medium was shown to have anti-oxidative, immunomodulatory and tissue repairing properties [5,9,10]. In addition, menSCs in a cultured medium (menSC-CM) demonstrated angiogenic properties and, as a result, restored injured endometrial tissue in mice improving fertility [8].

Burns *et al.* reviewed induction methods for endometriosis mouse models [11]. Notably, surgical engraftment – suturing uterine tissue into the peritoneal cavity of the same animal or a recipient – has been repeated successfully in mice and allowed for uniform lesion development from the same starting size compared to other methods [11].

Several studies have successfully transfected mice models with the green fluorescent protein (GFP), a chromoprotein that emits green fluorescence when exposed to light in the range of blue to ultraviolet, to evaluate growth of endometrial tissue in extrauterine regions and distinguish the engrafted tissue [11–14].

Aims and Objectives

Though studies have examined the effect of either menSCs or adSCs on endometrial regeneration, there is a gap in knowledge about which source is most effective in endometrial tissue regeneration. The goal of this study is to examine and compare the effect of menSC-CMs and adSC-CMs treatment individually and in combination on endometrial tissue regeneration in mice models. It is hypothesized that the combined treatment will have the greatest reduction in endometriosis lesion size and will show the most improvement in pregnancy outcomes when compared with the negative control. It is important to pursue this question to determine effective treatment options for endometriosis patients.

Methods

AdSC and MenSC Isolation and Culture

AdSCs and menSCs will be obtained from human donors who have given informed consent. Considering the injection of human-derived cells into mice, any recordings of immune rejection, xenograft-host interactions, and differences in growth & behaviour of cells will be made. Research protocols will be approved by the local ethics board and will adhere to federal and regional regulations.

AdSCs will be isolated as described by Francis *et al.* which involves washing fat with phosphate-buffered saline (PBS), digesting with collagenase, centrifuging to separate the stromal vascular fraction (SVF), lysing red blood cells (RBCs) with ammonium chloride, purifying mononuclear cells with a Percoll gradient, and culturing the cells in Dulbecco's Modified Eagle Media (DMEM) supplemented with fetal bovine serum (FBS) and epidermal growth factor (EGF) to select for adherent cells [15]. MenSCs will be isolated from menstrual blood as described by Du *et al.* which involves depleting menstrual blood cells of RBCs, seeding, and culturing in a 1:1 mixture of DMEM and Ham's F12 medium with 10% FBS, streptomycin, and penicillin at 37ºC with 5% CO2, with medium changes every 3-4 days and passaged at 80% confluence using trypsin [16]. Studies have identified positive and negative cell markers for both adSCs and menSCS which will be used in flow cytometry assessing the purity of stem cells before transplantation into mouse models. AdSC positive cell markers include CD29, CD44, CD73, CD90, CD105 and CD166, while negative cell markers include CD11b, CD14, CD31, and HLA-DR [17]. MenSC positive cell markers include CD9, CD29, CD41a, CD44, CD59, CD73, CD90 and CD105, while negative cell markers include CD34, CD38, CD133 and CD45 [3,18–20]. ‘CD’ refers to ‘cluster of differentiation’. The conditioned medium for adSCs and menSCS will be developed using protocols created by Huang *et al.* and Zhang *et al.* [5,8]. The culture media will undergo the nucleic acid amplification test (NAAT) to ensure no mycoplasma is present and the limulus amebocyte lysate (LAL) test to ensure the CM adheres to endotoxin concentration is less than 0.5 EU/mL [5].

Endometriosis Mouse Model

The following protocol is adapted from experiments done by Huang *et al.* and Wilkosz *et al.* [5,14]. All mice (C57BL/6 and female) will be purchased from the Jackson Laboratory (California, USA). Nine-week-old mice will undergo surgical engraftment of extrauterine tissue from transgenic eGFP donor mice, except for negative controls. Temperature and light-dark cycle will remain constant for all. Mice undergoing surgical engraftment will receive the sedative anesthetic, Zoletil, via injection into their peritoneal cavity [5]. In transgenic eGFP mice, ureteral ligation at the internal cervical os will be performed, and the uterus will be removed. A longitudinal incision will be

made to the uterine horns, and a 2-mm biopsy punch will obtain four pieces of equal uterine tissue. A monofilament nylon suture will be used for engraftment [5]. The adhesion band between the peritoneal wall and endometriotic tissue will be assessed using a scale used by Huang *et al.* [5].

Study Design

Each group will consist of 20 mice. Three experimental groups consist of mice that undergo surgical engraftment and are treated with either 1 mL adSC-CM (Group A), 1 mL menSC-CM (Group B) or a combination therapy of 0.5 mL adSC-CM & 0.5 mL menSC-CM (Group C). Treatments will be administered to the peritoneal cavity, and the incision site will be closed [5]. Two types of negative controls (not receiving any treatment) will also be introduced, where Group D mice will not undergo engraftment, while Group E mice will undergo surgical engraftment. Lastly, Group F mice will undergo surgical engraftment and later surgical removal of endometriotic lesions to serve as a positive control. Half of the mice in each experimental and control group will be randomly sacrificed on day 28, and tissue collection will occur. The tissue will be fixed in 4% formaldehyde and kept at 4°C for 24 hours [5]. The other half of the mice in each group will mate with 8-week-old male C57BL/6 mice on day 28 and can deliver at term [5]. The number of offspring delivered and alive up to 1 week post-delivery will be documented.

Hematoxylin & Eosin (H&E) Staining & Fluorescence

As described by Wilkosz *et al.*, H&E staining will be conducted, followed by detecting eGFP under a fluorescence microscope [14]. Following the paraffinization of tissues, a Leica SM200R microtome will create 4 µm tissue samples that will be adhered on slides [5]. Tissue samples will then be stained using the protocol by Cardiff *et al.* [21]. Slides will be deparaffinized and rehydrated before H&E staining. After staining, slides will be cleared using xylene (ThermoFisher #X3P-1GAL) and mounted with Permount mounting medium (ThermoFisher #SP15-500). Images will be obtained using appropriate microscopes and Image-J will be used for quantitative analysis of endometriotic lesion size.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA of endometriotic lesions will be extracted and purified using the Qiagen RNeasy Plus Micro Kit (Toronto, Canada). The purified total RNA will undergo RT-PCR using thermal cyclers at the McMaster University Biointerfaces Institute (Hamilton, Canada). Primer sequences from mouse ICAM-1 and VEGF will be purchased from Qiagen, and their mRNA expression levels will be measured using the ThermoFisher Scientific PowerUp™ SYBR™ Green Master Mix (Mississauga, Canada). The $2^{-\Delta\Delta Ct}$ method will be used to compare gene expression levels [22]. The experimental process will be repeated in triplicate for each endometriotic lesion sample.

Immunohistochemistry

Following rehydration and blocking with bovine serum albumin (BSA; MilliporeSigma™ #12661525ML), the tissue sections will be incubated with the following monoclonal antibodies: goat anti-GFP, ICAM-1 and VEGF [12,23,24]. The slides will be washed, and the secondary antibody incubation will occur (anti-goat IgG antibody) [25]. The tissue sections will be washed again and 3,3'-diaminobenzidine (DAB; ThermoFisher #AC11208 0050) chromogen will be incorporated for detection. A counterstain, hematoxylin (Epredia™ #72804), will be added and dehydration using alcohol and xylene will take place [26]. The mounted tissue samples will be examined using a brightfield microscope at the Biointerfaces Institute (Hamilton, Canada), and Image-J will be used for H-score calculation to quantify expression of antigens in the tissue samples [27].

Results

Quantitative Analysis of Lesion Size

Flow cytometry is expected to reveal the absence and expression of appropriate adSCs and menSC-CMs markers mentioned previously. Compared to the Group E, experimental groups (A, B, C) are predicted to have reductions in endometriotic lesion sizes, with statistical significance. Optimistically, it is expected that the Group C mice (0.5 mL adSC-CM & 0.5 mL menSC-CM) will have the most pronounced reduction by benefiting from regenerative properties of both adSCs and menSC-CMs; however, as combined therapy has not yet been studied, this prediction should be considered with caution and could alternatively have minimal or adverse effects. We also predict that the experimental groups have reductions in lesions comparable (i.e., with no statistical difference) to Group D and the positive controls (F). Quantitative measurements obtained using Image-J analysis of the H&E-stained slides would provide an objective assessment of treatment efficacy, complementing the visual representation offered by the slides.

Gene Expression Analysis

It is expected that RT-PCR results will demonstrate lower expression levels of ICAM-1 and VEGF in the experimental groups compared to Group E, with the lowest levels in Group C. Results should also indicate similar expression levels between experimental groups and Groups D and F. ICAM-1 was found to be to be a "moderate" diagnostic marker for endometriosis. These findings will help elucidate the molecular mechanisms by which the stem cell conditioned media exerts its effects on endometrial-like tissue.

Immunohistochemistry Outcomes

H-scores for ICAM-1, VEGF, and GFP are expected to be significantly lower in the experimental groups compared to Group E, with Group C showing the lowest H-score,

indicating a significantly reduced inflammatory and angiogenic response [5,12,23,24]. H-scores are expected to be comparable between experimental groups and each of Group D and F. Such a reduction correlates with less aggressive tissue growth and potentially less pain for the subject.

Reproductive Outcomes

It is predicted that Group C mice will have the most significant pregnancy outcome improvement compared to the Group E mice measured by the survival rate of 1-week-old offspring. Improved pregnancy outcomes compared to Group E are also expected in Groups A, B, and F based on previous study results [5,8].

Discussion

Patients with endometriosis experience a significant decline in quality of life due to debilitating symptoms and chronic pain [2]. As a result of scarring, which increases with endometrial severity, there is an increased chance of infertility with the disease [3]. Treatment is primarily invasive and surgical, with a high rate of lesion return post-removal [5]. Hormonal therapy, specifically through the reduction of estrogen levels, is also used [2]. However, no single or conclusive method is used for endometriosis treatment [3]. Therefore, an effective treatment that aims to reduce lesion size and thereby mitigate symptoms is needed. The following protocol seeks to test the effectiveness of adSC-CM, menSC-CM and combined therapy in reducing endometrial lesion size.

Findings are expected to align with existing research on MSC therapy in endometriosis, offering a novel perspective on treatment strategies. For instance, adMSCs composited with a collagen scaffold have been shown to promote endometrial regeneration by enhancing tissue angiogenesis and reducing fibrosis in an endometrial rat model [28]. This research suggests that adMSCs can modulate fibrotic conditions like endometriosis. Huang *et al.* found a significant postnatal pup survival rate ($p<0.01$) in endometriotic mice with adSC-CM compared to endometriotic mice receiving no treatment [5].

Future studies should compare the effect of other MSC sources individually and in combination on endometrial tissue regeneration. It is the hope that this study will present promising results and lead to the development of an effective and non-invasive treatment method for endometriosis. This study does not administer treatments to "normal" mice with no surgical engraftment, which serves as a limitation. Future studies should ensure there are no negative impacts of treatment on "normal" mice, especially before advancing to clinical trials, and that these can be safely administered to patients with different degrees of endometriosis. Future studies should also test different volumes of menSC-CM and adSC-CM in the combined treatment to conclude which is most effective in reducing endometriotic lesion size. Lastly, to improve comparisons between mice that were treated with stem cell sources and those that undergo subsequent surgical removal of endometriotic lesions, future studies should investigate reoccurrence of lesions. Another limitation is that the male mice used for breeding could have different rates of fertility, which can affect the causal interpretations of the results. As well, given the novelty of this proposed study, adverse effects of the proposed therapies are not widely known and should be followed. It may also be difficult to accurately predict mating between mice.

Conclusion

Current treatments for endometriosis are invasive, and ~12% of patients will need a hysterectomy. Still, the recurrence rate of endometriotic lesions after a hysterectomy is 62% [29]. The significance of this study lies in its potential to develop new non-invasive treatment options for the reduction of endometriotic lesions and improve fertility. This study will contribute to endometriosis research as it will help determine which mesenchymal stem cell source therapy can lead to the most effective treatment for the disease and will act as a precursor to future studies.

List of Abbreviations Used

adSC: adipose tissue-derived stem cell
adSC-CM: adipose tissue-derived stem cell conditioned medium
BSA: bovine serum albumin
DAB: 3,3'-diaminobenzidine
GFP: green fluorescent protein
H&E: hematoxylin & eosin
LAL: limulus amebocyte lysate
menSC: menstrual-derived stem cell
menSC-CM: menstrual-derived stem cell cultured medium
MSC: mesenchymal stem cell
NAAT: nucleic acid amplification test
RT-PCR: reverse transcription-polymerase chain reaction

Conflicts of Interest

The authors disclose that DNN is a staff member at The URNCST Journal. This article was handled independently and has undergone the journal's standard peer-review process. The authors had no direct influence on the editorial decisions of this submission. This disclosure is provided to ensure full transparency and to uphold the integrity of the editorial process. The authors declare no other conflicts of interest.

Ethics Approval and/or Participant Consent

Creating this research protocol did not require any ethics approval or patient consent. Carrying out this protocol will need consent from human donors of stem cells and approval by the local ethics board. The methods carried out should adhere to regional and federal regulations.

Authors' Contributions
DNN: ideation, design of the study, collected and analyzed literature, drafted and revised the manuscript, and gave final approval of the version to be published.
ERL: ideation, design of the study, collected and analyzed literature, drafted and revised the manuscript, and gave final approval of the version to be published.

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