

Conditioned medium from healthy women's endometrial stem cells improve inflammatory and stemness- expression genes in endometriosis women

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Research Article

Keywords: Endometriosis, menstrual blood-derived mesenchymal stem cells, inflammatory genes, stemness-related genes, conditioned medium

Posted Date: March 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-249510/v1>

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Abstract

Background. Endometriosis is a common, benign gynecological disease which is determined as an overspreading of endometrial tissue in exterior region of the uterine cavity. Evidence suggests that retrograde menstrual blood which contains mesenchymal stem cells with differential gene expression compared to healthy women may play a role in endometriosis creation. We aimed to identify whether the conditioned medium from Menstrual blood-derived stem cells (MenSCs) of healthy women can affect the expression level of inflammatory and stemness genes of MenSCs from endometriosis women.

Methods and Results. Endometriosis derived MenSCs (E-MenSCs) were treated with conditioned medium (CM) derived from healthy women's MenSCs (NE-MenSCs). Some CD markers were analyzed by flow cytometer before and after treatment compared with NE-MenSCs, and the expression level of inflammatory and stemness genes was evaluated by real-time PCR.

Results. E-MenSCs show different morphology *in vitro* culture in comparison with NE-MenSCs, which were changed in the presence of CM, into a morphology more similar to normal cells and showed significant decrease expression of CD10 after CM treatment. In our results, the IL-1, COX-2, and HIF-1 α as an inflammatory genes and OCT-4, NANOG, and SOX2 as a stemness genes showed significantly different expression level in E-MenSCs after treating with CM.

Conclusions. Our study indicates that the expression level of some inflammatory- and stemness-related genes which have differential expression in E-MenSCs compared with NE-MenSCs, could be changed to normal status by using CM derived from NE-MenSCs.

Introduction

Endometriosis is a benign gynecological disease that affects approximately 5 to 10% of women worldwide during the years of reproduction [1,2], and is described as the presence of endometrial tissues outside the uterine cavity, which leads to many complications such as chronic pelvic pain and infertility [3]. Since endometriosis is a complex disease with a multifactorial etiology, generally accepted theories reported for the causes of endometriosis include retrograde menstruation, vascular dissemination, metaplasias in pelvic cavity, and immune dysfunction. For instance, many shreds of evidence offer that immune responses are activated in endometriosis that lead to cellular changes in menstrual blood-derived mesenchymal stem cells (MenSCs) [4,3]. Regarding the differences observed in MenSCs of endometriosis women in comparison with healthy women, it seems very important to evaluate these cells in different aspects including surface markers and gene expression levels [2,4].

Some genes are differentially expressed in MenSCs from endometriosis patients which can be the leading reasons for the disease complications. These gene expression differences can help to diagnosis and treatment of endometriosis [5]. Inflammatory genes are mostly overexpressed in these patients which gradually leads to chronic inflammation and immune responses in lesion sites and the whole pelvic

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js pluripotency genes, are mostly expressed

differentially in MenSCs from endometriosis women which can result in angiogenesis, migration, and invasion [7,8]. Since there is no proper and definitive treatment for this disease, it could be important to know the basic principles that cause it, such as differentially expressed genes, to find suitable treatment methods.

One of the main inflammatory actors is interleukin 1 α (IL-1 α). It is thought that IL-1 α is a primary inflammatory factor in immune situations that stimulates prostaglandin synthesis and induces B cells to produce immunoglobulins. So, IL-1 α can play a significant role in the pathogenesis of endometriosis [9]. Also, cyclooxygenase-2 (COX-2) is an inducible enzyme that is produced by inflammatory mediators. In addition, this enzyme converts arachidonic acid to prostaglandins [10]. It has been proved that expression of COX-2 enhances in women with endometriosis [10]. Consequently, the high levels of COX-2 and Prostaglandins expression might be effective in endometriosis development [10]. Furthermore, aberrant expression of tumor necrosis factor- α (TNF- α) is identified to promote the cell proliferation and adhesion in endometriosis, because high concentrations of TNF- α induce the peritoneal macrophages to secrete inflammatory factors [11]. The inflammatory mediators such as IL-1 β and TNF- α have key function in activating nuclear factor κ B (NF- κ B) and hypoxia-inducible factor (HIF-1 α) signaling pathway. These factors regulate immune response and high expression of NF- κ B and HIF-1 α are observed in endometriosis [12].

On the other hand, stemness genes play an emerging role in endometrium regeneration in each female ovarian cycle. In addition, pluripotency genes promote cell migration in endometriosis. It was proved that the expression of stemness-related factors such as the Spalt-like protein 4 (SALL4), NANOG, octamer-binding transcription factor 4 (OCT4), and Sex determining region Y-box 2 (SOX2) are different in endometrial stem cells between endometriosis patients and healthy women [13-15]. OCT4 is an important factor for the survival of cell pluripotency and is identified to be highly expressed in endometriosis women [16]. In endometriosis, expression of the pluripotent transcription factor OCT4 encourages cell migration [7]. NANOG expression in undifferentiated stem cells and it is a critical regulator in cells pluripotency and differentiation by interaction with other factors [17,18]. Many studies revealed that SALL4 expression changes in numerous cancers and endometriosis [19,20].

Since there is no proper and definitive treatment for endometriosis, it can be very important to know its etiology to find suitable treatment methods. Extensive investigations in recent years on mesenchymal stem cells-derived conditioned medium (MSCs-CM) lead our mind to the dynamic role of endometrial stem/progenitor cells and their derivatives in the treatment of endometriosis. Growth factors, extracellular vesicles, and bioactive agents in the secretion released by stem cells into the in vitro culture medium, known as the conditioned medium, have been shown to induce many cell processes and changes in gene expression, and eventually, tissue repair and regeneration through paracrine mechanisms [21-23].

Regarding the various functions reported for MSCs-CM, it can be expected that the conditioned medium from MenSCs of healthy women can affect the surface markers and gene expression of MenSCs from endometriosis women. So, the aim of this study was to evaluate the expression of inflammatory and

stemness genes in endometriosis patients in the presence of conditioned medium from endometrial stem cells of healthy women.

2. Materials And Methods

2.1. Patient selection

This interventional experimental study was approved by ACECR biomedical research ethics committee (IR.IAU.QOM.REC.1399.016) and written informed consents were obtained from individuals before enrolling in the study. The patient group comprised endometriosis women (n = 3) (stages III–IV) undergoing laparoscopy for infertility or pain and the control group consisted of healthy women (non-endometriosis subjects) (n = 3). The inclusion criteria were as follows: (1) age ranging from 25 to 35 years; (2) history of ovulatory cycles with unregularly menstrual periods in endometriosis cases, (3) body mass index (BMI) of 18–28 kg/m², (4) no hormonal treatments for at least the last 3 months, (5) no previous surgery for endometriosis women, (6) no history of malignancies or autoimmune diseases, and (7) evidence for deep endometriosis suggested by transvaginal ultrasound and magnetic resonance imaging (Fig. 1).

2.2. MenSCs Isolation and Culture

After selecting 3 proper cases in each groups, at least 2 ml menstrual blood was collected by [pipelle](#) catheter during the second or third day of menstruation and were immediately transferred to the laboratory. 4 folds of blood sample was added EDTA 0.5 mM, an equal volume of blood sample was added to Ficoll-Paque media (Lymphodex, innotrain, Germany) carefully and centrifuged at 600 g for 30 min at room temperature. Following density gradient centrifugation, plasma and platelets in the upper layer were removed and mononuclear cell layer remained undisturbed at the interface. The mononuclear cell layer was transferred to a sterile centrifuge tube and washed twice with PBS. Cell pellets were grown in Dulbecco's modified Eagle's low glucose (DMEM-LG) medium supplemented with 10% FBS (Gibco, Grand Island, USA) and 1% penicillin/streptomycin (Gibco, Grand Island, USA) and incubated in 97% humidity, and 5% CO₂ at 37 °C. Media was changed after 24 h to remove all floating cells, followed by regular media changes every 3 days. Then Cells were trypsinized, and passaged when 70-80% confluency was achieved. The isolated menstrual blood-derived stem cells (MenSCs) from endometriosis and healthy women are defined as E-MenSCs and NE-MenSCs, respectively.

2.3. Flow Cytometry for mesenchymal stem cell markers

To confirm the isolated cells as MenSCs, the expression level of positive cell surface markers (CD29, CD90, CD105, CD44, CD73, and CD10) and negative markers (CD34, CD45, CD133, CD38) for MenSCs were determined. FITC-conjugated monoclonal antibodies against CD34, CD38, CD90, CD10, CD44, and CD133, as well as PE-conjugated monoclonal antibodies for CD105, CD45, CD29 and CD73 were purchased from BD Biosciences (San Jose, CA, USA). PE-conjugated anti-CD105 and were from R&D Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js Ig isotype-matched control antibodies were

obtained from the same companies as their test antibodies. All the antibodies were used in flow cytometry experiments at the concentrations recommended by the manufacturers. Flow cytometry was performed using a FC500 flow cytometer (Beckman Coulter, Fullerton, CA) and analyzed using Beckman Coulter CXP software.

2.4. Preparation of conditioned medium and treatment

For preparation of conditioned medium, NE-MenSCs at passages 3-4 were seeded in T-75 flasks at a density of 1×10^6 cells. Once the 70–80% confluency was obtained, the culture medium was collected after 3 days of culture. To remove cells and cell debris, culture medium was centrifuged and filter-sterilized through a 0.22- μ m filter, and then it was termed as a conditioned medium of MenSCs (MenSCs-CM). The aliquoted MenSCs-CM was stored at -80°C till use. All in vitro experiments were performed using passages 3–5 of MenSCs-CM.

Then, E-MenSCs were treated in three experimental groups with NE-MenSCs derived conditioned medium for 2, 4 and 6 days. Before and after C.M treatment the cell lines was measured by Flow Cytometry assay and Real-time PCR.

2.5. Real-time Polymerase Chain Reaction and Gene Expression Analysis

Total RNA was isolated from treated cells using “Gene All Kit (Gene All Biotechnology, Seoul, Korea) according to the manufacturer’s instructions. RNA purity and quantity were assessed using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) at 260/280 nm. The reverse transcription was used to synthesize the first-strand cDNA using transcription Kit (Yekta tajhiz, Iran)”. Quantitative real-time PCR assays were performed in triplicate to evaluate the expression of selected genes (table1). For normalizing gene expression levels, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal reference. The $2^{-\Delta\Delta\text{Ct}}$ method was used to calculate the fold change of mRNA expressions for target genes. Real-time PCR was carried out using RealQ Plus Master Mix Green (AMPLIQONIII) following the manufacturer’s instructions. Briefly, a mixture comprised of 10 μL SYBR green mix, 1 μL of cDNA (250 ng, 1 μL PCR forward primers and 1 μL PCR reverse primer in 5 pmol μL^{-1} , and millipore water to achieve a final volume of 20 μL was made. The sequences of primers are presented in Table1. The Threshold Cycle (CT) was determined manually for each run. Relative mRNA level was expressed as the relative fold change and calculated using the formula $2^{-\text{CT}} = 2^{-(\text{CT}(\text{Sample}) - \text{CT}(\text{calibrator}))}$, where each $\text{CT} = \text{CT}_{\text{Target}} - \text{CT}_{\text{GAPDH}}$. One sample without any treatment from the control group was designated as a calibrator. The quantification of mRNA was performed as a value relative to an internal reference for *GAPDH*.

2.6. Statistical analysis

Experimental data are expressed as the mean \pm standard error (SE) and compared by ANOVA. ANOVA using a multiple comparisons test was used to determine statistical significance, followed by the Tukey

test. Statistical significance was set at $p \leq 0.05$. All experiments were performed in triplicate for technical and biological repetitions.

3. Results

3.1. Morphology of MenSCs

The MenSCs are morphologically similar to bone marrow-derived mesenchymal stem cells (BM-MSCs). In our study, microscopic observations showed that MenSCs from healthy women (NE-MenSCs) had fibroblast-like spindle-shaped morphology, while, E-MenSCs were less stretched and elongated (Fig .2A, B).

The morphology of E-MenSCs was changed under treatment of CM derived from NE-MenSCs into a more elongated, spindle-shaped morphology (Fig. 2C).

3.2. Expression of specific mesenchymal stem cell markers

In order to characterize MenSCs, flow cytometry assay was performed in passage 3 for E-MenSCs (before and after treatment) and NE-MenSCs, and our results showed that both MenSC types (E-MenSC, NE-MenSCs) were positive for CD29, CD90, CD117, CD105, CD44, CD73, and CD10, and negative for CD34, CD45, CD133, CD38 (Figure 3A).

To compare E-MenSCs and NE-MenSCs concerning the expression level of CD markers, we used the mean fluorescence intensity (MFI). In our results, CD10 marker expression was significantly higher for E-MenSCs compared with that of NE-MenSCs. Also, after CM treatment, there was significantly decrease in CD10 expression for E-MenSCs ($P \leq 0.05$) (Fig. 3B).

3.3. Pro-inflammatory genes expression under MenSCs-CM treatment

In this study E-MenSCs were treated with conditioned medium derived from NE-MenSCs of 3rd passage, for 2, 4 and 6 days. According to the gene expression analysis, the best effect was related to 4 days of CM treatment and data from 2 and 6 days of treatment were omitted and not shown. Quantitative RT-PCR analysis of IL1 β , COX-2, HIF, TNF- α and NF-kB genes was performed in E-MenSCs in before and after CM treatment, as well as in NE-MenSCs as control group. As shown in Figure 4, the expression of IL-1 β in E-MenSCs was significantly higher compared to NE-MenSCs (1.00 ± 0.1 vs. 0.008 ± 0.003 , $P \leq 0.05$), that after CM treatment it was surprisingly decreased (0.013 vs. 1.00 , $P \leq 0.05$) so that was similar to NE-MenSCs (0.013 ± 0.002 vs. 0.008 ± 0.003 , $P = 0.627$). Similarly, the expression of COX2 in E-MenSCs was significantly higher compared to NE-MenSCs (1.00 ± 1.04 vs. 0.125 ± 0.09 , $p \leq 0.05$), that was significantly decreased after CM treatment (0.132 ± 1.08 , $p \leq 0.05$), which was surprisingly similar to NE-MenSCs (0.013 ± 0.002 vs. 0.008 ± 0.003 , $p \leq 0.05$). HIF-1 gene expression was significantly lower in E-MenSCs compared to NE-MenSCs (1.00 ± 1.8 vs. 8.9 ± 2.4 , $p \leq 0.05$), whereas, in treated E-MenSCs, it was significantly up-regulated (1.00 ± 1.04 vs. 4.55 ± 1.36 , $p \leq 0.05$). In the case of TNF- α gene, lower expression

was observed in E-MenSCs in comparison with NE-MenSCs (1.00 ± 1.04 vs. 6.54 ± 1.96 , $p = P \leq 0.05$). TNF- α expression was increased after CM treatment but this change was not significant. There was no significant difference in NF-kB gene expression between groups.

3.4. MenSCs-CM regulates stemness genes expression

As shown in Figure 5, the expression of some stemness genes was compared between E-MenSCs (before and after CM treatment) and NE-MenSCs. OCT4 expression showed a significant decrease in E-MenSCs compared to NE-MenSCs (1.00 ± 1.04 vs. 42.22 ± 7.4 , $p \leq 0.05$), while under CM treatment it was significantly up-regulated similar to NE-MenSCs. (1.00 ± 1.04 vs. 27.63 ± 5.32 , $p \leq 0.05$). Similar to OCT4, in the E-MenSCs, NANOG expression was significantly lower than NE-MenSCs (1.00 ± 1.04 vs. 7.1 ± 2.8 , $p \leq 0.05$). NANOG was also significantly increased in E-MenSCs after CM treatment and became almost similar to NE-MenSCs (1.00 ± 1.04 , 5.99 ± 2.3 , 7.1 ± 2.8 , $p \leq 0.05$). In E-MenSCs, SOX2 expression was significantly higher than NE-MenSCs (1.00 ± 0.88 vs. 0.05 ± 0.04 , $p \leq 0.05$). Sox2 was significantly down-regulated in E-MenSCs after CM treatment (0.14 ± 0.08 , $p \leq 0.05$), similar to NE-MenSCs with no significant difference between treated E-MenSCs and NE-MenSCs ($p = 0.235$). Furthermore, SALL4 expression show higher expression in E-MenSCs compared to NE-MenSCs (1.00 ± 0.74 vs. 0.13 ± 0.09 , $p \leq 0.05$). No significant difference was observed between E-MenSCs and treated E-MenSCs, so, SALL4 had no difference under CM treatment.

4. Discussion

Accumulating evidence showed that inflammation may be considered as a crucial mechanism in endometriosis. In addition, it was identified that stemness genes may be involved as key mediators in establish endometriosis [24-27].

It has been observed that growth factors, extracellular vesicles, and bioactive agents in secretion released by stem cell into the culture medium in vitro, which is referred to as the conditioned medium, can cause many cell processes and changes in gene expression and ultimately tissue repair and regeneration through paracrine mechanisms [28-30]. In addition, more recent evidence proposed that CM from menstrual blood-derived MSCs could support against MPP⁺-induced cytotoxicity in vitro [31].

In the present research study, we have indicated that CM derived from male factor-derived MenSCs (NE-MenSCs) could have a major impact on the phenotype and expression level of inflammatory and stemness genes of menstrual blood-derived stem cells of endometriosis patients (E-MenSCs).

According to our findings, the interleukin-1 (IL-1) and cyclooxygenase-2 (COX-2) inflammatory genes in E-MenSCs had a high level of expression, which after treatment with the CM derived from NE-MenSCs, their expression level decreased and did not show a significant difference compared to NE-MenSCs as control group.

Besides, the tumor necrosis factor- alpha (TNF- α) expression did not change significantly after treatment with CM derived from NE-MenSCs and was still significantly different from control. High expression of TNF- α was observed in E-MenSCs compared to control. In addition, the nuclear factor-kappa B (NF- κ B) expression was not significantly different before and after treatment with CM derived from NE-MenSCs. Consistent with previous results, it is possible that NF- κ B activates epithelial cells proliferation and migration most likely via Notch signaling activation resulting in enhancement of re-epithelialization [32]. Given that there is no change in epithelial cell proliferation and epithelialization in endometriosis, it seems that NF- κ B expression could not be different significantly before and after treatment with CM derived from NE-MenSCs.

Unlike TNF- α expression which was not affected, the expression of the IL-1 and COX-2 changed significantly in E-MenSCs in the presence of CM derived from NE-MenSCs compared to control.

While NE-MenSCs illustrated a more elongated, spindle-shaped morphology similar to fibroblast-like cells, E-MenSCs did not. However, the morphology of E-MenSCs changed in the presence of CM derived from NE-MenSCs into a more elongated, spindle-shaped morphology.

Whereas the E-MenSCs before treatment with CM derived from NE-MenSCs demonstrated OCT-4 and NANOG expression level lower than the control, treatment of the E-MenSCs with CM derived from NE-MenSCs resulted in the OCT-4 and NANOG expression level similar to control.

Furthermore, SOX2 and SALL4 stemness genes had a high level of expression in E-MenSCs, which after treatment with the CM derived from NE-MenSCs, their expression level decreased, however, did not show a significant difference compared to control.

In the previous studies, it was demonstrated that some pro-inflammatory genes including IL-1 and COX-2 can demonstrate a significantly dysregulated expression in some of tissues of endometriosis patients [33,12,34-36]. For instance, Chalpe et al. (2015) demonstrated that pro-inflammatory genes, including IL-1 β induce differential expressions in endometrial stromal cells [33].

Moreover, considering in vitro findings, it was identified that COX-2 expression could be increased in the eutopic and ectopic endometrium of endometriosis patients [12,34-36]. These studies were in consistent with our study results. Besides, it was cleared that COX-2 upregulation could be observed in endometriotic stromal cells due to the suppression of COUPTFII in these stem cells by pro-inflammatory cytokines via microRNA-302a [37].

In previous studies, it was observed that some stemness genes, including SOX-2 and NANOG demonstrate a significantly higher expression in some tissues of endometriosis patients [14,15,7]. For example, a study revealed that the SOX-2 stemness gene has an aberrant expression in endometriosis [38]. Furthermore, Song et al. (2014) reported that SOX-2 and NANOG genes were overexpressed in ovarian endometriosis [14].

In addition, aberrant expression of NANOG and OCT-4 stemness genes has been verified in endometriotic tissues [15,7]. The results from our study are consistent with several mentioned studies. SOX-2, OCT-4, and NANOG has been found to cooperatively activate and regulate other stem cells related genes [39,40].

Additionally, our study outcomes are in agreement with part of the results reported by Proestling et al. (2016) that some of the genes related to stemness, including OCT-4 gene show upregulation in epithelial and stromal cells of endometriotic tissue [13]. Also, a research study showed that OCT-4 and NANOG genes have an increase expression in endometriotic mesenchymal stem cells that was consistent with our study [41]. The expression of OCT-4 is influenced by the hypoxia inducible factor (HIF)-2-alpha transcription factor and HIF-2alpha regulates OCT-4 [42].

Furthermore, some studies showed that SALL4 is expressed aberrantly in some of tissues of endometriosis patients [15,43]. Our findings are in agreement with some reports which reveal that SALL4 stemness gene have upregulated expression in endometriosis disease [15,43].

Evidence demonstrated that increases in COX-2 production regulates cell survival, migration and invasion of extrauterine endometriotic tissues. It seems that the reason for this increase in COX-2 gene expression in stromal cells may be due to the regulation of its expression by pro-inflammatory cytokines such as IL-1 β or due to the high stability of COX-2 mRNA [44].

On the other hand, in a research study, the mean fluorescence intensity was used to evaluate the expression of CD10. The results obtained from this study indicated that CD10 expression was considerably higher in stromal stem cells from endometriosis compared with that of stromal stem cells from non-endometriosis [45]. Our findings are in agreement with results obtained by this study [45].

Altogether, the present study outcomes recommend that CM derived from NE-MenSCs could be exerted as an effective strategy to improve the gene expression related to endometriosis formation, such as inflammatory and stemness genes. However, further in vitro and preclinical studies for its better validation in disease treatment are needed.

Understanding and identification of the most important contents (extracellular vesicles, microRNAs, etc.) of the NE-MenSCs-derived CM which has the greatest impact on the promotion of cellular processes and changes in gene expression levels in MenSCs from endometriosis women, is an essential question that should be answered in the future research studies.

Conclusion

Our study indicates that the expression level of some inflammatory- and stemness-related genes could be changed by use of CM derived from NE-MenSCs. Furthermore, the present study identified that conditioned medium derived from NE-MenSCs could be considered as a new candidate for ameliorating the endometriosis disease.

Abbreviations

MenSCs: Menstrual blood-derived stem cells

E-MenSCs Endometriosis Menstrual blood-derived stem cells

NE-MenSCs: Non Endometriosis derived MenSCs

IL-1 α interleukin 1 α

COX2 cyclooxygenase-2

HIF-1 α hypoxia-inducible factor

TNF- α tumor necrosis factor- α

NF- κ B nuclear factor κ B

SOX2 Sex determining region Y-box 2

OCT-4 octamer-binding transcription factor 4

SALL4 Spalt-like protein 4

Declarations

Funding: NO funding

Conflicts of interest/Competing interests: The authors declare that they have no competing interests.

Ethics approval: This interventional experimental study was approved by ACECR biomedical research ethics committee (IR.IAU.QOM.REC.1399.016).

Authors' contributions ASH and SSS conceived the study. FD and LN collected the patient blood samples. SSS and FD performed the cell culture experiments. AK and SSS performed the molecular assay and analyzed the Real-time PCR data. SSS performed the Flow cytometry assay. ASH, SSS and MSh wrote the manuscript, and revised the paper. All authors read and approved the final version of the manuscript.

Data availability: All data are included in the manuscript

Availability of data and material: No available.

Code availability: No available.

Consent to participate: The participants gave their written informed consent for establishing endometrial stem cells cultures from menstrual blood samples

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Acknowledgments: The authors appreciate funding support from the Academic Center for Education, Culture and Research, Qom Branch, Qom, Iran. In addition, we are grateful to all the women who participated in this study. We also thank the staff at the midwifery section of infertility treatment clinic of ACECR.

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Table

Table 1. Primer pairs used in real-time PCR

Gene	Sequence	Accession number	Product size
IL1 β	F:TCTTCTTCGACACATGGGATA R:GTACAAAGGACATGGAGAACA	NM_000576.3	183
COX2	F:TCAGCCATACAGCAAATCCT R: TTGAAGTGGGTAAGTATGTAGTG	NM_000963.4	205
TNF-	F:GTCTGGGCAGGTCTACTTTGG R: GTTCTAAGCTTGGGTCCGAC	MH180383.1	172
NF-k β	F:GAAGTGCAGAGGAAACGTCAG R: GAAGCTATACCCTGGACCTGT	NM_001382627.1	147
HIF1A	F:GGCGAAGTAAAGAATCTGAAG R: ACCATCCAAGGCTTTCAAATA	NM_181054.3	209
OCT-4	F: GTTCTTCATTCACTAAGGAAGG R: CAAGAGCATCATTGAACTCAC	NM_001285986.2	101
NANOG	F: ACCTGAAGACGTGTGAAGATG R: ATTAGGCTCCAACCATACTCC	NM_001355281.2	187
SOX-2	F:GGGAAATGGAAGGGGTGCAAAAGAGG R:TTGCGTGAGTGTGGATGGGATTGGTG	NM_003106.4	151
SALL-4	F:GGGCAGCCACATGTCTCAGCA R:GACATGACGTTCCGGGAGCACC	NM_001318031.2	204

Figures

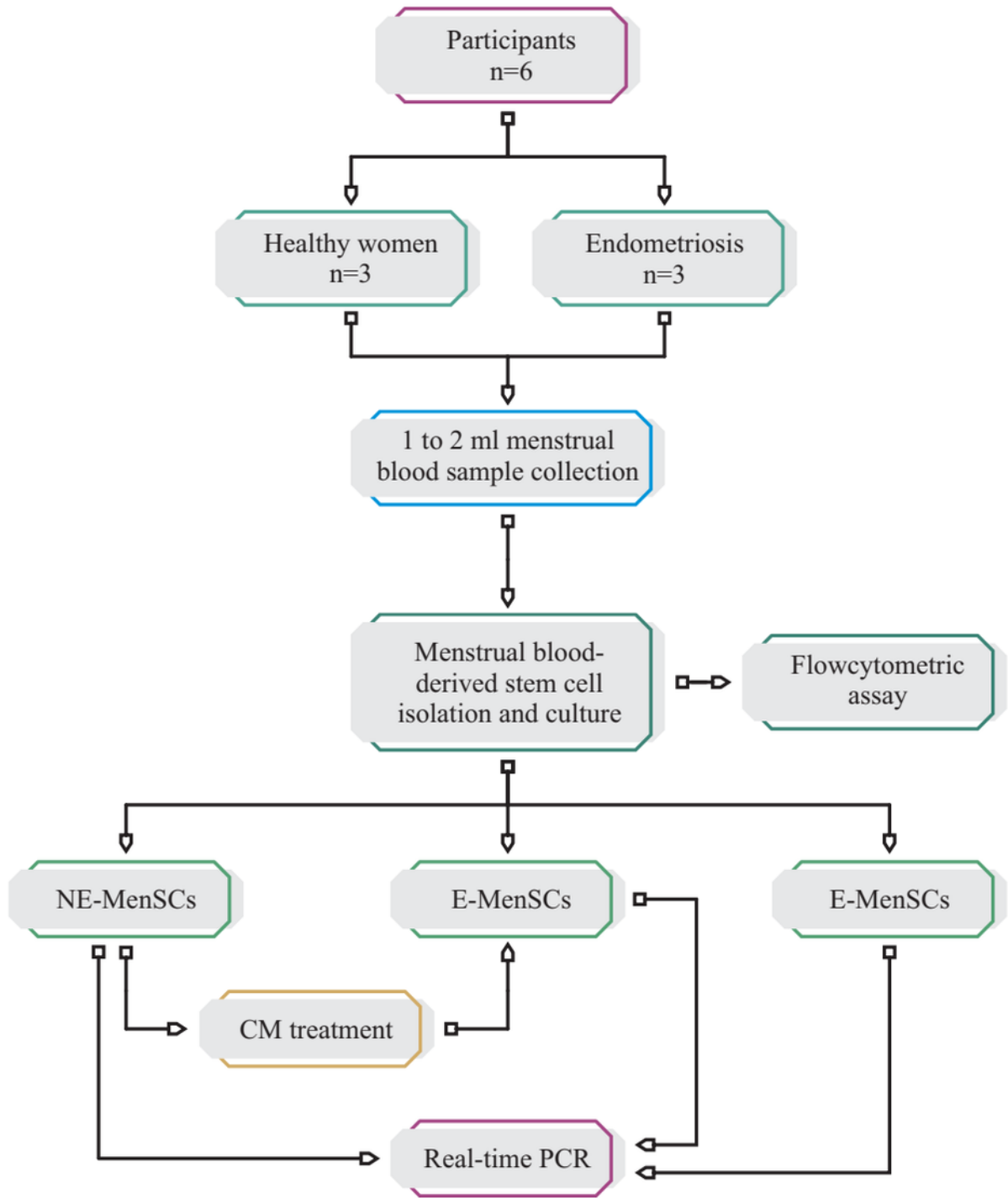


Figure 1

Study algorithm.

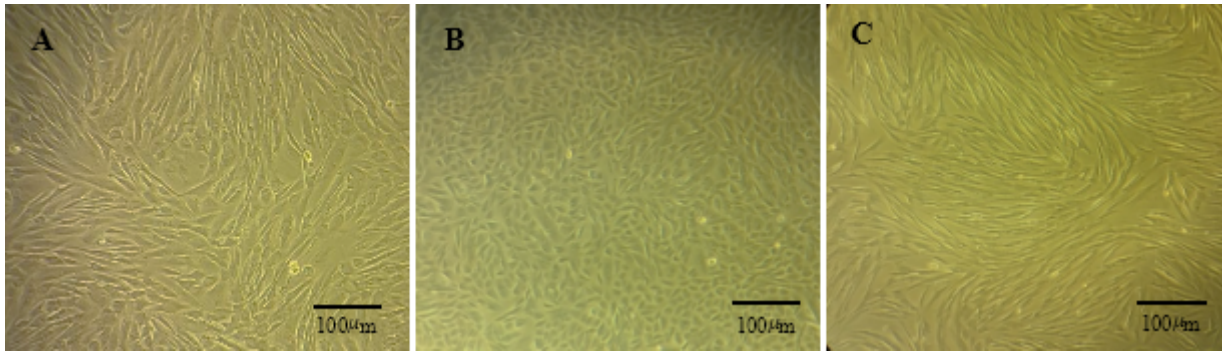


Figure 2

Morphological difference between NE-MenSCs and E-MenSCs. NE-MenSCs in 2D culture showed a fibroblast-like spindle-shaped morphology (A), while E-MenSCs were less stretched and more circular (B). After CM treatment, the cells changed to spindle-shaped morphology (C).

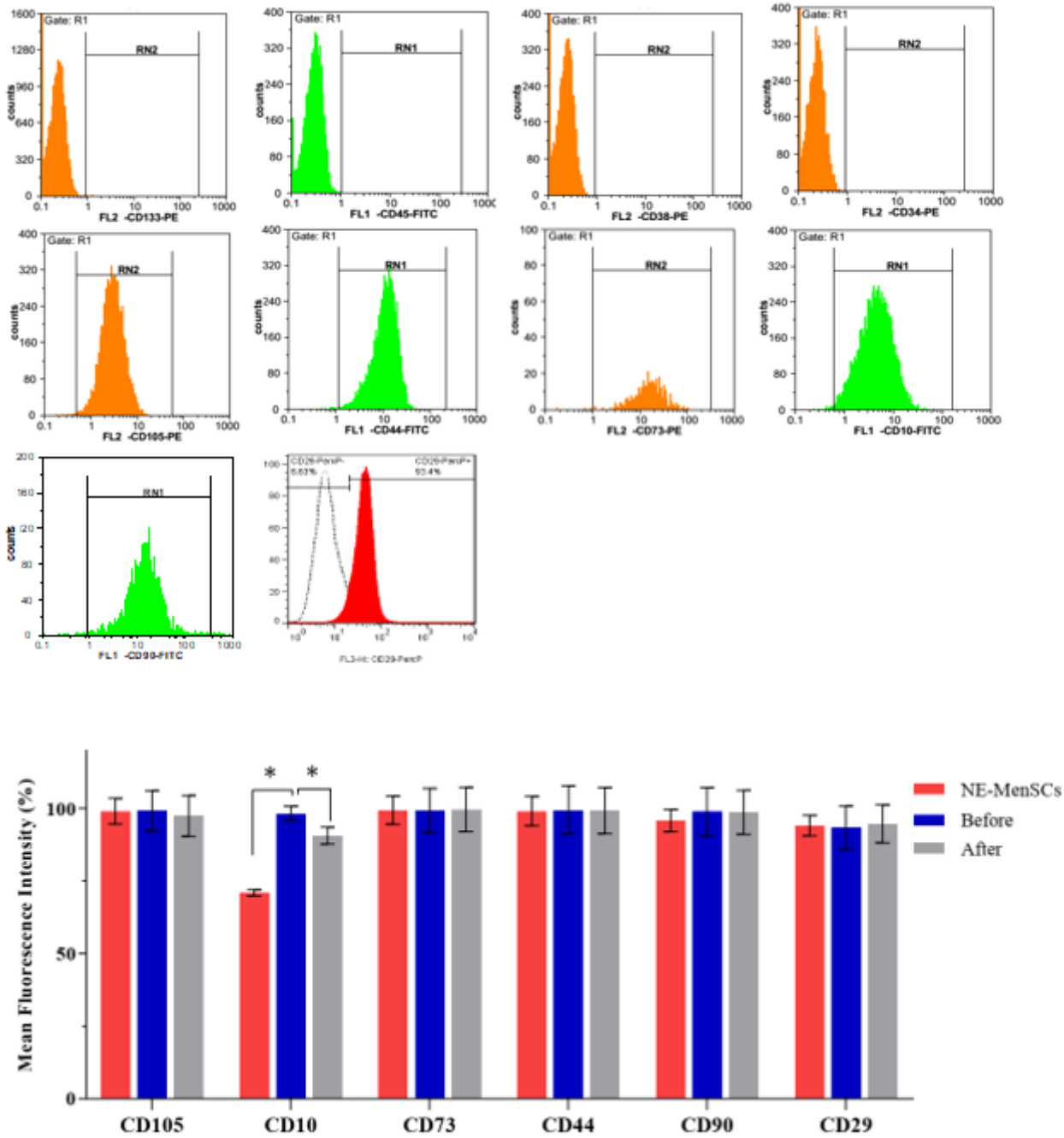


Figure 3

The expression of MenSCs surface markers was detected by flow cytometry so that they were positive for CD90, CD10, CD44, CD29, CD73, and CD105, while negative for CD34, CD133, CD45, and CD38 (Top panel-A). MFI was used to compare E- MenSCs with NE-MenSCs and E-MenSCs after CM treatment (Bottom panel-B).

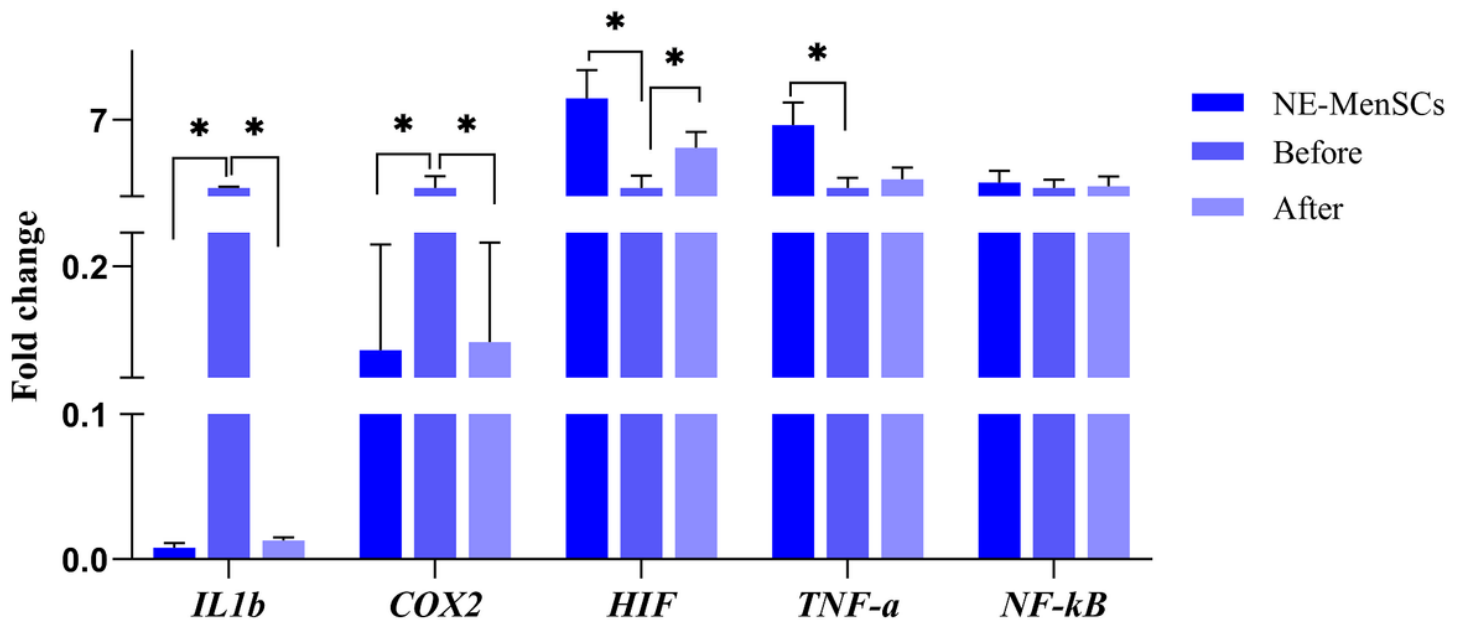


Figure 4

Real-time PCR analysis comparison of inflammatory genes expression in NE-MenSCs, EMenSCs (Before), and E-MenSCs after CM treatment (After). *p≤0.05

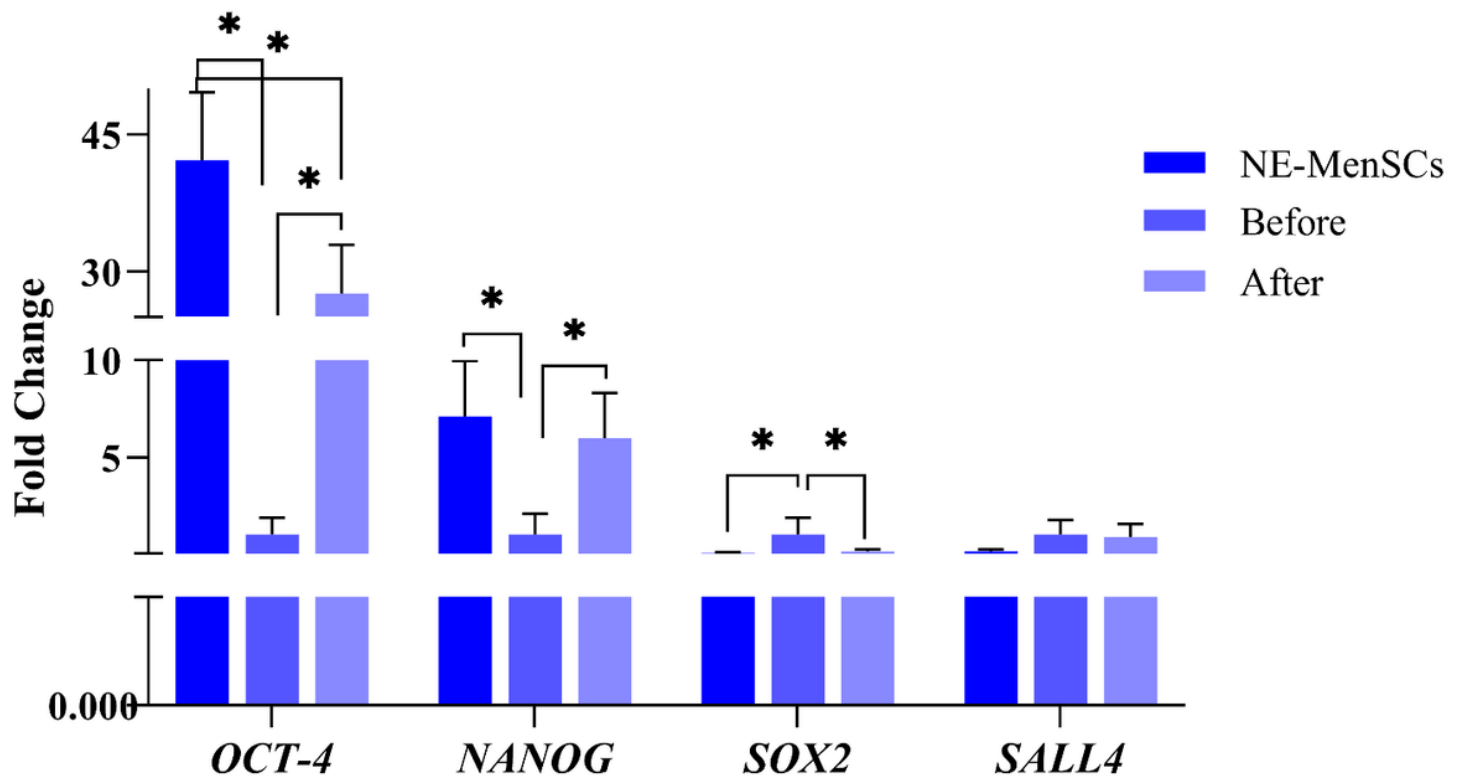


Figure 5

Real-time PCR analysis comparison of stemness genes expression in NE-MenSCs, E-MenSCs, and E-MenSCs after CM treatment. * $p \leq 0.05$