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Protocol for Obtaining Stem Cells from Menstrual Blood

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Abstract

BACKGROUND/AIMS: Stem cells that have the ability to differentiate into other somatic cells are special cells in tissue and play a role in the repair and regeneration of tissues. Menstrual blood stem cells (MenSCs), a subtype of mesenchymal stem cells derived from the endometrium during menstruation, present an accessible and non-invasive source for stem cell research. This study aimed to create a protocol to obtain MenSCs from menstrual blood.

MATERIALS AND METHODS: Samples were collected from fertile women aged 18-45 on the second day of their menstrual cycles. Two methods: Ficoll and collagenase, were applied for cell isolation. In the Ficoll method, solution was slowly added in equal proportions and then centrifuged at 2000 rpm for twenty minutes (min). MenSCs were collected, centrifuged at 1000 rpm for 10 min, and then cultured in MenSCs culture medium at 37 °C with 5% CO_2 in air. In the collagenase technique, 0.5 mg/mL collagenase 1 was added to the samples in a 1:1 ratio and left to incubate for 1 hour at 37 °C with 5% CO_2 in air. After centrifugation, MenSCs were cultured and were then passaged three times. Distributions of CD31 and CD44 were used to characterize the cells via the indirect immunoperoxidase technique.

RESULTS: Cells isolated using both methods exhibited epithelioid morphology, cytoplasmic lipid droplets and colony formation. However, cells obtained with the collagenase method demonstrated faster proliferation and formed bigger colonies. Morphology and immunoreactivity remained consistent across passages.

CONCLUSION: MenSCs isolated with collagenase method showed better proliferation than those obtained with ficoll. The immunocytochemistry results, showing CD31 negativity and CD44 positivity, confirmed the mesenchymal stem cell characteristics of the isolated cells.

Keywords: Mesenchymal stem cells, menstrual blood stem cells, collagenase 1, ficoll

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent and, under suitable conditions, have the ability to differentiate into various cell types. MSCs can be obtained from bone marrow, dental pulp, adipose tissue, etc.[1,2] The proliferation of MSCs by adhering to plastic culture surfaces and their differentiation into at least three lines in vitro is considered to be the gold standard.[3] MSCs have become an important focus in the treatment of disease due to their biological properties, as well as their potential to repair and regenerate damaged tissues.[4] Menstrual blood stem cells (MenSCs) were adult stem cells derived from the endometrial epithelium and were first obtained in 2007. MenSCs have attracted attention due to their accessibility, potential in regenerative medicine given their non-invasive collection methods, ease of acquisition, and lack of ethical issues.[4] MenSCs have the ability to self-renew, differentiate into other types of cells, and culture long-term.[5,6] In addition, MenSCs

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have increased proliferation and differentiation potential compared to stromal bone marrow-derived MSCs.[7] For this reason, MSCs derived from menstrual blood may have the potential to be a new and easily available cell-based treatment. MenSCs are positive for markers such as CD44, CD73, CD90, SSEA-4, OCT-4, etc., but negative for CD34, STRO-1, etc.[8] Due to these advantages of MenSCs, it is very important to obtain the appropriate number of MenSCs with the desired characteristics. The aim is to analyze the culture and differentiation potential of MenSCs using different protocols.

MATERIALS AND METHODS

MenSCs Derivation and Culture

The project was supported by Manisa Celal Bayar University Health Sciences Ethics Committee (approval number: E-20478486-050.04-706680, date: 18.01.2024). All participants were given detailed information about the study and voluntary consent was obtained from the before the study started. To obtain MenSCs, samples were collected from 5 patients, and 10 mL samples were collected from each patient. The selection criteria were the collection of menstrual blood from fertile patients, on the 2nd day of their menstrual cycle. Blood samples were then divided into two tubes for two different protocols. They were first centrifuged at 1000 rpm for five minutes (min). For the first protocol, Ficoll protocol, blood samples were diluted 1:1 radio with Ficoll (Biochrom, cat no: L 6115) and centrifuged at 2000 rpm for twenty min. MenSCs in buffy coat layer were collected and washed with DMEM F12 (Sigma Life Science, cat no: RNBG7035), 5% fetal bovine serum (Gibco, 2440087), 1% pen-strep (Capricorn, PS-B), and centrifuged at 1000 rpm for five min. Culture medium was added to the pellet after discharge of the supernatant. The cells were then incubated at 37 °C in 5% CO_2. The second protocol was the collagenase protocol. 0.5 mg/mL type 1 collagenase (Sigma Aldrich, 9001-12-1) was added to the menstrual blood and incubated for sixty min in an incubator. MenSCs were collected after being centrifuged at 1000 rpm for five min and cultured with culture medium following the same Ficoll protocol. The medium was changed every two days, and cells were passaged at least three times with Trypsin-EDTA (Capricorn, TRY-4265) solution.

Characterization of MenSCs

MenSCs from two protocols were fixed in 4% paraformaldehyde (Merck, Cat. No: TP70404 415), and the distribution of MSC markers CD34 (Santa Cruz, Cat. No: sc-74499) and CD44 (Proteintech, Cat. No: 15675-1-AP) was analyzed using a biotin-streptavidin (Thermo Scientific, Cat. No: TP-125-HL) based on indirect immunoperoxidase staining protocol. Diaminobenzidine (Thermo, TA-125-HD) was used for chromogen and Mayer's hematoxylin (Atom Scientific Ltd, Cat. No: TTSP60) for background staining.

The osteogenic, chondrogenic, and adipogenic differentiation potential of MenSCs was also evaluated. For induction of differentiation for three lineages, 1×104 cells/well were cultured for one week according to their manufacturer's protocols (Bio BASIC, cat no: C14H07SNa; Chem Cruz, cat no: sc-203749A; Carlo Erba cat no:428561, respectively). After induction, phosphate buffer solution was added for washing and fixation was performed with paraformaldehyde. Differentiation potential was analyzed using Alizarin Red S (ARS) (osteogenic), Alcian Blue (chondrogenic), and Oil Red O (ORO) (adipogenic) staining.

Statistical Analyses

There was no data for statistical analyses in this article.

RESULTS

MenSCs Derivation and Culture

Both Ficoll (Figure 1A, B) and collagenase 1 (Figure 1C, D) isolated MenSCs exhibited an epithelioid morphology after 3 days of culture time. After 1 week of culture, MenSCs derived from two protocols were also able to form colonies (Figure 1B, D). Notably, MenSCs isolated with collagenase 1 reached confluence more quickly and displayed more defined colony structures (Figure 1C, D). Additionally, no significant changes in cell proliferation were observed up to the fourth passage.

Immunocytochemical Assay

Immunocytochemical results demonstrated that MenSCs obtained from both the collagenase 1 (Figure 2) and ficoll (Figure 3) protocols were positive for CD44 (Figure 2A, Figure 3A), which is a positive marker of MSCs, and negative for CD34 (Figure 2B, Figure 3B), a negative marker for MSCs.

Differentiation Assay

After 1 week of differentiation assay, ORO staining was performed on both collagenase 1 and Ficoll-derived MenSCs. Cytoplasmic adipogenic droplets were observed (orange-red staining) in MenSCs derivatives after the collagenase 1 protocol. However, MenSCs, which were collected via Ficoll protocol, were not stained with ORO. MenSCs were not stained with ARS (osteogenic) or Alcian Blue (chondrogenic) after 1 week of culture (data not shown) (Figure 4).

DISCUSSION

In recent years, there has been a growing interest in the clinical potential of MCS due to their high proliferative potential, their remarkable versatility and the fact that they can be obtained non-invasively, unlike other sources of MCS derived from adult tissues.[7] Menstrual blood is an

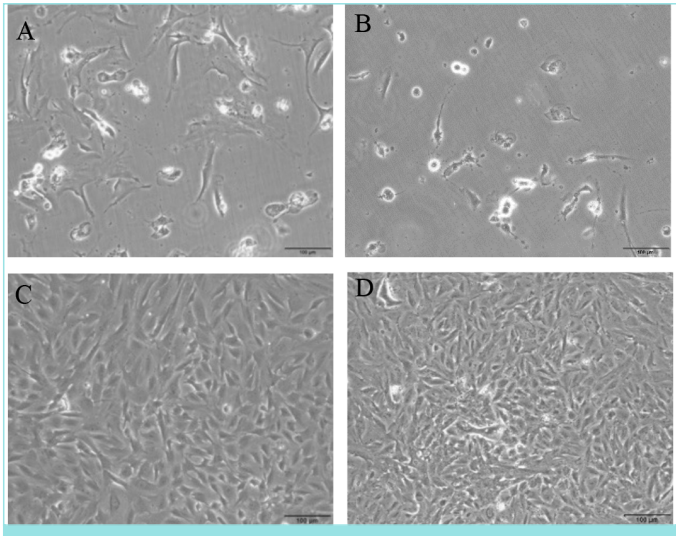

Figure 1. Menstrual blood stem cells obtained from menstrual blood by ficoll (A, B) and collagenase 1 (C, D) from day 3 (A, C) and 1 week (B, D). Scale bars:100 µm.

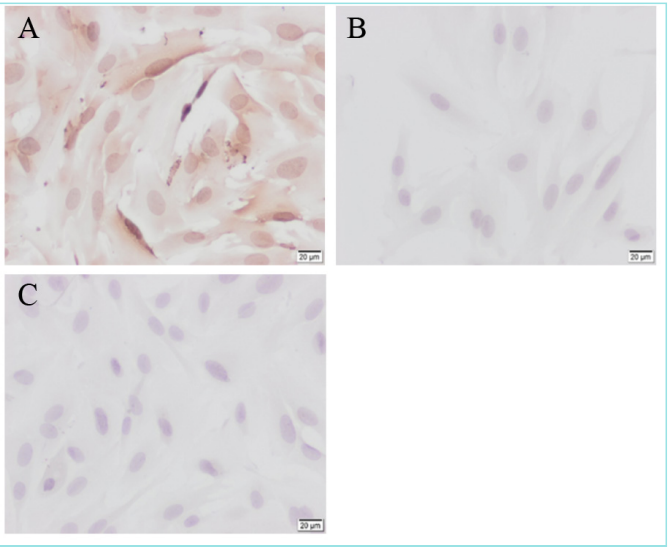

Figure 2. CD44 (A), CD34 (B), control (C) immunohistochemical staining for mesenchymal stem cell characterization obtained from menstrual blood with collagenase 1 protocol. Scale bars: 100 µm.

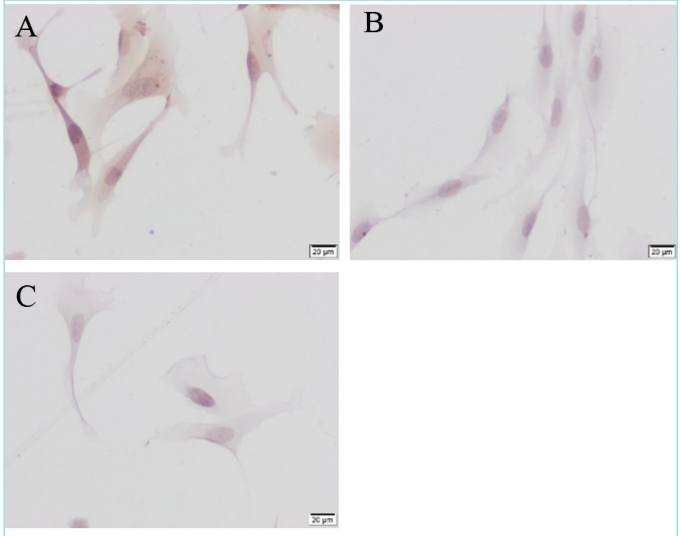

Figure 3. CD44 (A), CD34 (B), control (C) immunohistochemical staining for mesenchymal stem cell characterization obtained from menstrual blood with Ficoll protocol. Scale bars: 100 µm.

emerging and intriguing source of MenSCs for researchers, as these cells offer advantages over stem cells derived from other sources.[9] In this study, MenSCs were derived from menstrual blood using two different protocols and exhibited adherent cells with a fibroblast-like morphology, formed circular colonies and showed multiple proliferation patterns, consistent with the findings of Meng et al.[5] Dalirfardouei et al.[10] also obtained and cultured MenSCs according to two different protocols: the culture of whole blood cells and Ficoll. in this study, the first protocol was found to be more effective in obtaining MenSCs.

Our findings regarding the characterization of MenSCs revealed that CD44, one of the MSC markers, was highly expressed and exhibits a

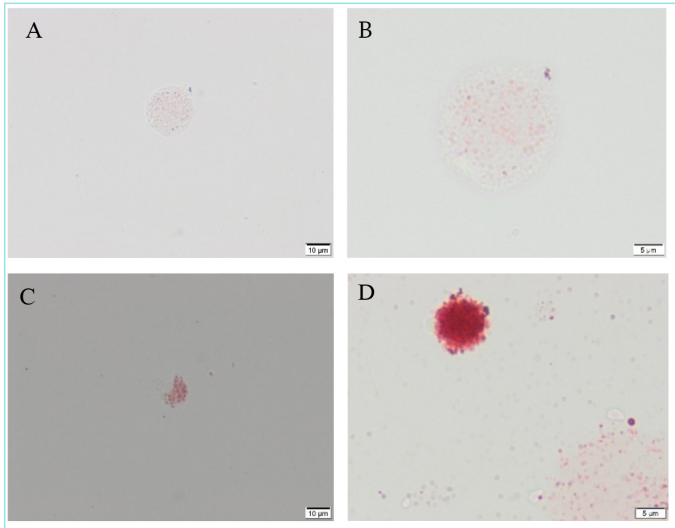

Figure 4. Oil Red staining of MenSCs obtained in the Ficoll protocol (A,B). Oil Red staining of MenSCs obtained with collagenase 1 (C,D). Scale bars: 100 µm (A,B), Scale bar: 5 µm (C).

MenSCs: Menstrual blood stem cells.

similar CD marker pattern to Sheikholeslami et al.[11] results. Also, ORO staining of both Ficoll and collagenase 1 derived cells showed that collagenase 1 derived cells have more differentiation ability compared to Ficoll-derived cells. In our study, MenSCs were derived from blood cells characterized by positive CD44 immunoreactivity, and ORO staining, leading us to identify these cells as mesenchymal-like stem cells.

Study Limitations

The limitation of this study is the insufficient number of stem cells obtained from patients. Additionally, the study was limited by a restricted number of available patients, which may have affected the generalizability of the findings. Furthermore, the study focused on a limited set of markers to characterize MenSCs, which may not fully encompass the complete spectrum of their characteristics. Future research with larger sample sizes and a broader array of markers would provide a more comprehensive understanding of MenSCs.

CONCLUSION

Menstrual blood provides a non-invasive and easily accessible source for deriving MSCs (MenSCs), offering significant potential for clinical applications due to their high proliferation rate and versatility. Therefore, obtaining a suitable protocol for having menstrual stem cells is important.

MAIN POINTS

- Collagenase 1 demonstrated a greater ability to obtain more stem cells than the Ficoll protocol.
- Menstrual blood stem cells (MenSCs) successfully expressed the stem cell marker, CD44.
- Differentiation potential of MenSCs derived from collagenase 1 was higher than Ficoll protocol.

ETHICS

Ethics Committee Approval: The project was supported by Manisa Celal Bayar University Health Sciences Ethics Committee (approval number: E-20478486-050.04-706680, date: 18.01.2024).

Informed Consent: All participants were given detailed information about the study and voluntary consent was obtained from the before the study started.

Footnotes

Authorship Contributions

Concept: H.S.V., H.K.E., A.A., Design: H.S.V., A.A., Data Collection and/or Processing: H.S.V., H.K.E., A.A., Y.U., Analysis and/or Interpretation: H.S.V., H.K.E., A.A., Literature Search: H.S.V., H.K.E., A.A., , Writing: H.S.V., H.K.E. A.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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