

Role of Wnt signaling on proliferation of menstrual blood derived stem cells

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Aim: Menstrual blood derived stem cells (MenSCs) are unique stem cells that have been isolated and identified recently. The special traits of MenSCs can be related to the cell signaling pathways. In this study, in order to find out the role of Wnt signaling on MenSCs proliferation, we evaluated β -catenin expression as a key participant in Wnt signaling pathway in response to Lithium chloride (LiCl).

Methods: MenSCs were isolated from healthy women by combining gradient density centrifugation with plastic adherence. After characterization of the isolated cells, cell proliferation of MenSCs in presence of 10-15 mM LiCl was evaluated by MTT assay. β -catenin expression of the treated cells was examined using immunofluorescence technique.

Results: Flow cytometric analysis revealed that both mesenchymal and embryonic stem cell markers are expressed on menstrual blood stem cells. MTT value decreased depending on the LiCl concentration. The proliferation of MenSCs cultivated in culture media containing 15mM LiCl was approximately two fold less than those grown without LiCl ($p < 0.01$). Moreover, nuclear accumulation of β -catenin protein in cells treated by LiCl was greater than cells without LiCl.

Conclusion: The MenSCs are stem cell populations with high proliferation ability and unique immunophenotyping properties. Our results demonstrated that Wnt signaling pathway regulates MenSCs proliferation via trans-localization of activated- β -catenin protein.

Key Words: Menstrual blood derived stem cells, Lithium chloride, proliferation, β -catenin

Introduction

In the recent decade, stem cell therapy has been introduced as a novel therapeutic approach for patients suffering from different diseases^[1]. The interest in adult stem cells was particularly triggered by the numerous ethical dilemmas which surround the use of embryonic stem cells (ESCs) in clinical studies^[2]. Adult stem cells have been derived from different tissues such as bone marrow, cord blood, adipose tissue and amniotic fluid^[3-6]. Although proliferation and differentiation potential of these stem cells are well established^[7-9], problems such as lesser availability, invasive methods for sample collection and lower proliferation capacity in comparison with the ESCs limit their applicability for research and clinical use. Thus, alternative resources of stem cells are of particular interest.

Recent studies have reported that menstrual blood (MB) contains a unique population of cells with properties similar to adult stem cells^[10,11]. An apparent evidence to support this assumption is the high regenerative ability of human endometrium characterized by cyclic processes of cellular proliferation, differentiation and shedding^[12]. The menstrual blood derived stem cells (MenSCs) possess *in vitro* multipotency, and appear to share some markers with mesenchymal stem cells (MSCs)^[11,13-15].

However, these cells can be distinguished from other adult stem cells by high expression of OCT-4 (an embryonic stem cell marker) and absence of STRO1 (a mesenchymal stem cell marker). Nevertheless, MenSCs proliferate more rapidly compared to bone marrow derived mesenchymal stem cells (BMSCs) or umbilical cord blood stem cells^[10, 13, 15]. The unique traits of MenSCs can be related to the cell signaling pathways that are involved in cell fate determination, regulation of cell proliferation and differentiation. Therefore, a key to successful stem cell therapy using MenSCs is finding out the molecular mechanisms governed in the distinct steps of cell proliferation and differentiation.

One major signal transduction pathway associated with stem cells development is the canonical Wnt signaling, which could regulate the downstream target genes and mediate stem cell proliferation and differentiation^[16,17].

The Wnt signaling pathway acts via the transcription factor β -catenin which binds to the promoter of Wnt-responsive genes and thus initiates their transcription. In unstimulated cells, cytoplasmic β -catenin is phosphorylated by a complex of proteins containing glycogen synthase kinase 3 (GSK3). On binding of Wnt to Frizzled, GSK3 is inactivated, resulting in the accumulation of cytoplasmic β -catenin, which will translocate to the nucleus and activate Wnt-responsive genes^[18-20].

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Till now, the role of Wnt signaling pathway in control of MenSCs proliferation is unknown. It has been shown that Lithium chloride (LiCl) affects the proliferation of MenSCs in a dose dependent manner^[21-24]. Therefore, to find out role of Wnt signaling on MenSCs proliferation, we firstly evaluated the effect of LiCl on MenSCs proliferation, and then investigated the expression of the activated- β -catenin protein in LiCl treated MenSCs.

Materials and methods

Isolation and characterization of human MenSCs

The Collection of 5 ml of MB sample was performed using a Divacup (Kitchener, ON) from healthy, female volunteers between 25–35 years on the second day of a menstrual cycle. Collection was performed by the donor, after obtaining informed consent according to the guidelines of the Medical Ethics Committee, Ministry of Health, I.R. Iran. The contents of the Diva cup were decanted into a collection tube including calcium, magnesium free phosphate buffered saline (PBS), 2.5 μ g/mL amphotericin B (Sigma-Aldrich, St Louis, MO), 100 μ g/mL streptomycin, 100 U/mL penicillin (Sigma) and 0.5mM EDTA- Na_2 (Sigma). Isolation of stem cells from menstrual blood was performed according the protocol as described in our recent publications^[15]. In brief, the mononuclear cells were collected by Ficoll-Paque (GE-Healthcare, Uppsala, Sweden) density gradient. Mononuclear cells were washed 3 times in PBS and resuspended in 5 ml complete Dulbecco's modified Eagle's medium (DMEM) -F12 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco BioCult, Paisley, Scotland, UK), 2 mM glutamine (Gibco), 100 μ g/ml streptomycin, and 100 U/ml penicillin, and plated in 75-cm² plastic cell culture flasks. The cell cultures were maintained at 37°C in a humidified 5% CO₂ incubator. Following 3 or 4 days incubation, the non-adherent cells were washed away leaving behind the adherent cell population that was growing as fibroblastic cells in clusters and the medium was replaced with a fresh medium. When cells reached 70–90% confluence, cultures were harvested with 0.25% trypsin-EDTA solution (Gibco). Cell number was determined up to three passages (P) using trypan blue stain (Sigma). Then, the expression of CD44, CD73, STRO1 and CD105 as mesenchymal stem cells markers and OCT-4 as embryonic stem cell marker was evaluated by flow cytometric analysis as described in our recent paper^[25].

MTT assay

To assess the effect of LiCl on MenSCs proliferation, cells at P 1-3 were seeded at a concentration of 1.0×10^4 cells per well in 24 well plates and cultivated in culture medium containing 0, 10, 15 mM LiCl or 10^{-7} M dexamethasone (Dex) (Sigma). After 3 days, cell proliferation was analyzed by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test based on cell growth determination protocol^[26]. Briefly, the culture medium was removed and replaced with 0.1 ml of medium without FBS; then, 25 μ l of MTT stock solution (5 mg/ml) was added to each culture being assayed. After 3 hour incubation, the medium was removed and the converted dye was solubilized with acidic anhydrous isopropanol (0.1 N HCl in absolute isopropanol). Optical densities (OD) of the converted dye were measured

at a wavelength of 570 nm using ELISA reader (Labsystem Multiskan, Finland), with background subtraction at 670 nm. Data were obtained as the mean \pm standard deviation (SD) of values from triplicate samples. Statistical analysis was performed using Student's *t*-test ($p < 0.05$).

Immunofluorescence staining

The expression of activated- β -catenin in cells cultivated in culture medium containing different concentrations of LiCl (as mentioned above) was evaluated using immunofluorescence staining technique. Briefly, the cultured cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30–45 minutes at room temperature, then permeabilized with 0.4% Triton-X-100 for 20 minutes. The cells were washed thrice with PBS and then incubated overnight at 4°C with primary monoclonal mouse anti-human antibody against activated β -catenin (1:200) (Chemicon, Billerica, MA). Subsequently, the cells were washed with PBS three times and incubated with rhodamine-labelled goat anti-mouse IgG (1:250) at 37°C for 3 hours in dark room. The cells were visualized by a fluorescence microscope (Nikon, TE-2000, Tokyo, Japan).

Results

Cell characterization

After overnight incubation, the isolated mononuclear cells from menstrual blood revealed marginal adherence with a rapid outgrowth of adherent cells toward 70-80% confluence during the 12 ± 2 days (Figure 1A). Phase contrast microscopy results of MenSCs showed that at low plating densities, MenSCs grew as a monolayer of large, flat cells and created colony forming unit fibroblasts (CFU-F). As the cells approached confluence, they assumed a more spindle-shaped, fibroblastic morphology and a homogeneous cell population with a characteristic, non-hematopoietic phenotype. This morphology was conserved at later passages (P10). The cells in the primary cultures were mainly fibroblastic in appearance and maintained this morphology during the passages (Fig.1B). Moreover, MenSCs typically expressed surface antigens associated with mesenchymal stem cells such as CD44, CD73 and CD105 while they failed to express CD34 and STRO1. Besides, the cells significantly expressed the embryonic stem cells marker OCT-4 (Fig. 1C).

The expression of β -catenin in LiCl treated MenSCs

Considering putative role of LiCl on stem cell proliferation, we expanded MenSCs in medium with 10 and 15 mM LiCl and compared their proliferative ability with cells cultured without LiCl. Morphology of MenSCs from fibroblastic-like shape changed to a flattened appearance in higher concentrations of LiCl as observed by light microscopy. As shown in figure 2A, growth curve of the MenSCs was dependent on LiCl concentration. The proliferation rate of MenSCs was highest in the absence of LiCl, and enrichment of culture media with LiCl decreased MenSCs proliferation rate in a concentration dependent manner. The proliferation of MenSCs cultivated in culture media containing 15 mM LiCl was approximately twofold less than those grown without LiCl ($p < 0.01$).

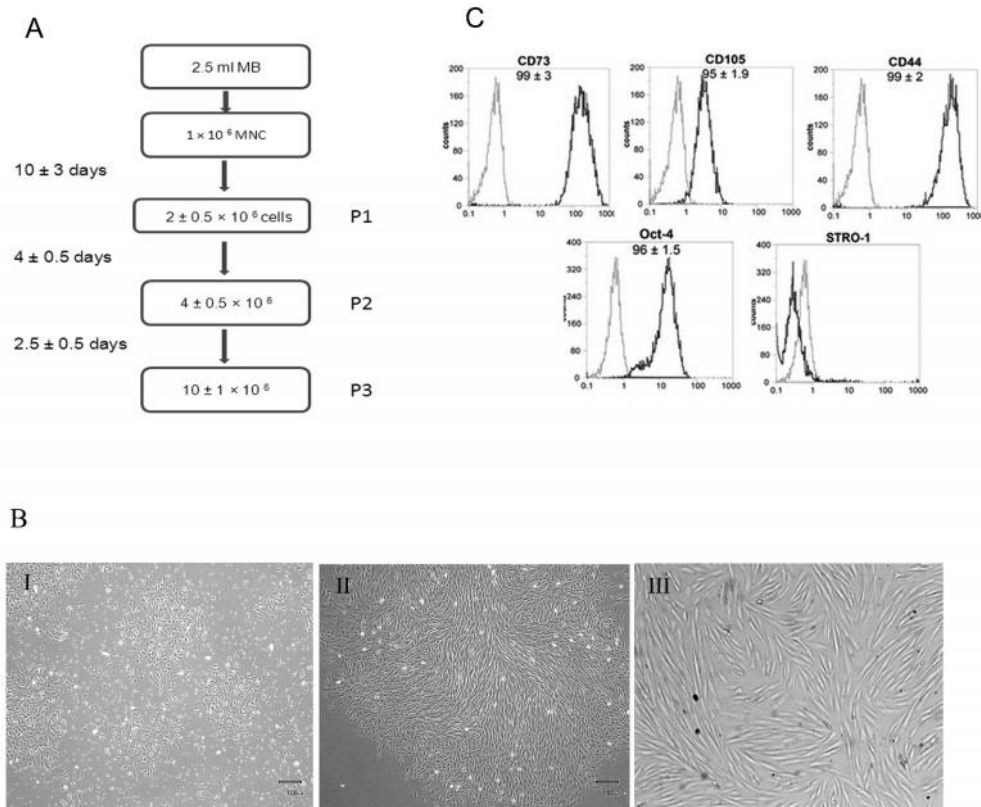


Figure 1. Characterization of isolated MenSCs. (A) Executive summary of expanded MenSCs from menstrual blood (MB) derived mononuclear cells (MNCs). The resulted cell number through three passages is indicated. (B) Morphology of MenSCs at different time intervals post retrieval: Primary cell culture showing initial colony forming unit (CFU) at day 5 (I) and day 12 (II), Part III shows cell morphology at passage 10; magnification: I & II: 10X, III: 20X. (C) Expression values of stem cell markers in the cells by flow cytometric analysis. The results are representative of 3-5 independent experiments.

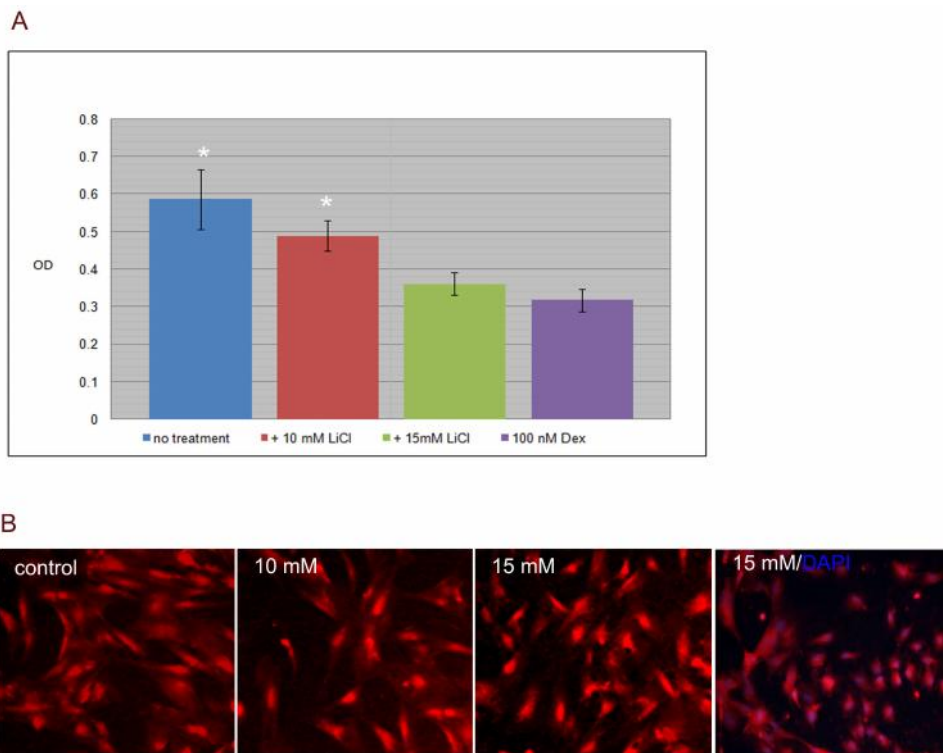


Figure 2. Effect of LiCl on proliferation and beta-catenin expression of MenSCs. (A) Cell proliferation pattern of MenSCs in the presence of 10 and 15 mM LiCl, Dex in contrast with no treatment group. Data were obtained as the mean ± SD of values from triplicate samples. * indicates significant difference (p<0.05) compared to 15 mM LiCl treated group. (B) Immunofluorescent staining of beta-catenin protein in cultured MenSCs; Magnification: 20X .

To explore the role of Wnt signaling in MenSCs proliferation, expression of β -catenin protein as a key downstream factor was evaluated in MenSCs treated by the above mentioned concentrations of LiCl using immunofluorescence staining technique. As shown in Figure 2B, the cells absolutely expressed activated- β -catenin especially with an accumulation in the nucleus. However, suppression of MenSCs proliferation by LiCl was accompanied with increase of nuclear accumulation of β -catenin protein. The ratio of nuclear to cytoplasmic accumulation of β -catenin protein in cells treated with 15 mM LiCl was more than that in cells cultured with 10 mM LiCl as well as greater than that of cells cultured without LiCl.

Discussion

In recent years, MenSCs have been regarded as a promising stem cell population for cell therapy of different diseases. However, there is no report about molecular mechanisms and signaling pathways involved in MenSCs biology. This article presents basic information about role of Wnt signaling in MenSCs proliferation. Proper and timely modification of Wnt signaling in MenSCs may help to further optimization of tissue engineering and regenerative medicine approaches using these cells.

We evaluated morphologic, proliferative and immunophenotypic characteristics of cultured MenSCs. The expanded MenSCs possess some markers of mesenchymal stem cells such as CD44, CD73 and CD105 while they lack STRO1. On the other hand, these cells typically express embryonic stem cell marker (OCT-4) that is not common in the mesenchymal stem cells. Moreover, we observed that the adherent fraction of menstrual blood cells had a mesenchymal like morphology, with high rate of proliferation (mean doubling rate of 20.5 h). Meng and his colleagues showed that menstrual blood contains a subpopulation of adherent cells which could be maintained in tissue culture for >68 doublings with higher proliferation rate than control umbilical cord derived mesenchymal stem cells [10]. Consistent to this report, our recent studies implicated that MenSCs have distinct proliferation and trans-differentiation ability compared to BMSCs [15,25]. It is clear that this cell population cannot be classified as mesenchymal stem cells since have been overgrown by BMSCs and express cell markers that are not common in mesenchymal stem cells. Therefore, investigation on cell signaling and molecular factors involving in MenSCs proliferation and differentiation pathway mediates more information about the phenotypic characteristics of MenSCs.

Previous reports showed an association of LiCl concentration with BMSCs proliferation and differentiation. While low concentration of LiCl stimulates BMSCs proliferation, at high concentration levels, LiCl causes a concentration-dependent inhibition of proliferation [24, 16]. Although there is no direct evidence about involvement of Wnt signaling pathway in proliferative response of LiCl treated BMSCs, mimicking effect of LiCl on proliferation of other cells is shown in some reports [27,28]. Therefore, in order to evaluate role of Wnt signaling in MenSCs proliferation, we exposed MenSCs to different concentrations of LiCl and followed β -catenin expression as a major protein involving in Wnt signaling pathway.

Based on our data, lithium exerted a dose-dependent inhibitory effect on MenSCs expansion. The suppression effect of LiCl on cell proliferation was accompanied by increase of intra-nuclear translocation of activated- β -catenin. Therefore, it seems that the stimulation of Wnt signaling pathway suppresses MenSCs proliferation. Previously increase of intra-nuclear translocation of activated β -catenin in response to LiCl treatment was reported in differentiated BMSCs into osteocytes [16]. The knowledge about the role of Wnt signaling in MenSCs differentiation is our future objective. However, regarding the phenomena that proliferation ability of differentiated cells may be decreased [29,30], it is suggested that hampered effect of high concentration of LiCl on MenSCs expansion, can be concomitant with differentiation induction through intra-nuclear translocation of β -catenin. Based on accumulative data, MenSCs are a unique stem cell population and Wnt signaling pathway plays a main role during its proliferation. Stimulation of Wnt signaling pathway caused inhibition of MenSCs proliferation. However, more studies are needed to clarify the involvement of Wnt signaling in the proliferation and differentiation of MenSCs into different lineages.

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Abbreviations:

MenSCs: Menstrual blood derived stem cells
LiCl: Lithium chloride
MB: Menstrual blood
ESCs: Embryonic stem cells
BMSCs: Bone marrow derived mesenchymal stem cells
GSK3: Glycogen synthase kinase 3
MSCs: Mesenchymal stem cells
PBS: Phosphate buffered saline
DMEM: Dulbecco's modified Eagle's medium
FBS: Fetal bovine serum
P: Passage
Dex: Dexamethasone
OD: Optical densities
SD: Standard deviation
CFU-F: Colony forming unit fibroblasts

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Potential Conflicts of Interests:

None

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