



Research article

Wharton's jelly derived mesenchymal stem cells differentiate into oocyte like cells in vitro by follicular fluid and cumulus cells conditioned medium

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ABSTRACT

Wharton's jelly derived-mesenchymal stem cells (WJ-MSCs) have a same developmental origin with primordial germ cells. WJ-MSCs perhaps differentiate into oocyte and germ like-cells (OLCs/GLCs) in the presence of appropriate inducers. Human follicular fluid (FF) and cumulus cells conditioned medium (CCM) are naturally rich sources for oocyte development. The aim of this study was to evaluate WJ-MSCs potential for differentiating into OLCs and GLCs exposed to FF and CCM. WJ-MSCs were cultured in two different induction media (10% FF, 10% CCM) for 21 days. Morphological changes and expression of developmental genes were evaluated on days 0, 7, 14 and 21 of culture. Also, on 21st day of culture, the expression of oocyte and germ cell proteins investigated using immunofluorescence staining. Appearance of round shaped cells from 7th day onwards indicated that WJ-MSCs can differentiate into OLCs when exposed to FF and CCM. The size of produced OLCs and expression of oocyte specific genes and proteins were increased more positively in FF group rather than CCM group. Although, WJ-MSCs could differentiate into OLCs by FF and CCM, however, the induction potential of FF for producing OLCs was better than CCM.

1. Introduction

World infertility rate is about 15% and half of infertility problems are related to insufficiency or lack of the germ cells [1]. In the recent years, use of stem cells for producing germ cells has been considered as a solution for infertility problems. Researchers could differentiate various types of stem cells, such as embryonic stem cells [2], bone marrow and adipose tissue-derived mesenchymal stem cells [3] and induced pluripotent stem cells (iPSCs[4]) into germ cell-like cells. Mesenchymal stem cells (MSCs) can be derived from multiple tissues and organs [5, 6]. However, isolation of MSCs from some tissues like bone marrow and adipose tissue are invasive, complicated and painful. The isolation of MSCs from tissues such as umbilical cord and placenta is not complicated or invasive, and it is not faced with any risk of contamination or ethical concerns [7]. Wharton's jelly can be easily obtained from the umbilical

cords, which are collected without any harm to the donor and discarded after birth as biological waste [8]. Wharton's jelly derived-mesenchymal stem cells (WJ-MSCs) has the high differentiation potential [9]. Moreover, WJ-MSCs can express some of the embryonic stem cell markers such as Oct-4, Nanog, Sox-2 and KLF-4. So, they appear both embryonic and adult stem cell features [10, 11]. Thus, WJ-MSCs could be an attractive cell source for cell-based therapies. In the recent years, researchers could differentiate WJ-MSCs to three germ layer cells [12] and these cells have shown suitable features for differentiating toward female and male germ cells using inducers such as retinoic acid, BMP4 (Bone morphogenetic protein 4) [13,14] and co-culturing with Sertoli [15] or placenta cells [16]. In this study, we used follicular fluid (FF) and cumulus cells conditioned medium (CCM) as inducers to differentiate WJ-MSCs into oocyte-like cells (OLCs). FF is a liquid which fills the follicular antrum and surrounds the ovum in an ovarian follicle and it is known as a source

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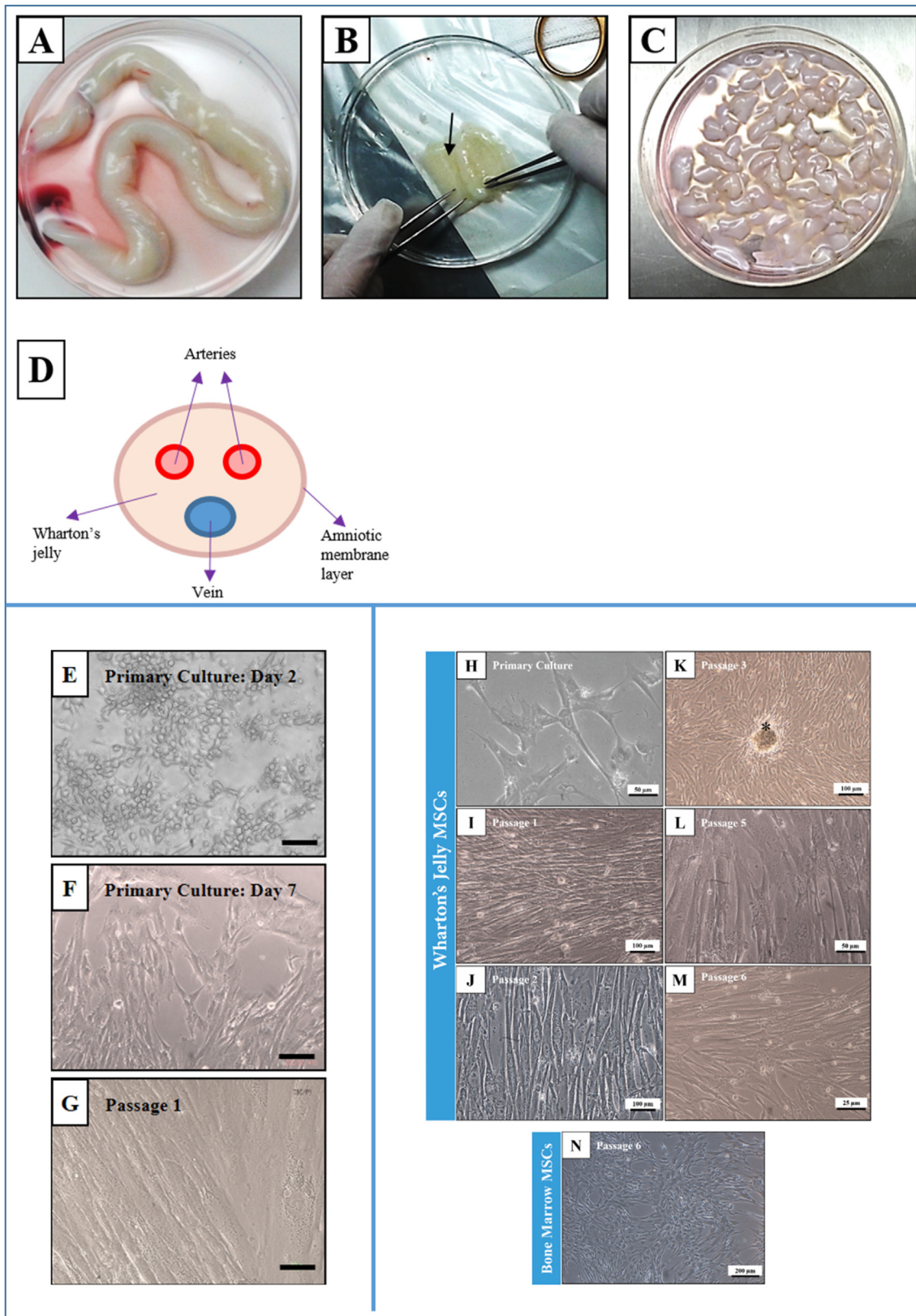


Figure 1. Wharton's jelly isolation from the umbilical cord, morphology of cultured cumulus cells and morphology of cultured Wharton's jelly cells. A-D) Wharton's jelly isolation from the umbilical cord. A) Complete discharge of umbilical cord's blood, B) Discarding of umbilical cord's arteries and vein, C) Wharton's jelly fragments preparation for culturing, D) The cross-section of umbilical cord. Arrow: the umbilical cord vessels. E-G) Morphology of cultured cumulus cells. Second and seventh day of primary culturing (E, F) and at the first passage of cumulus cells (G). Scale bar: 50 μ m. H-M) Morphology of cultured Wharton's jelly cells. H) Primary culture of WJ-MSCs. I, J, K, L and M) Morphology of WJ-MSCs in passage 1, 2, 3, 5 and 6. Asterisk: Colony formation of WJ-MSCs in passage 3. N) Morphology of bone marrow MSCs in passage 6.

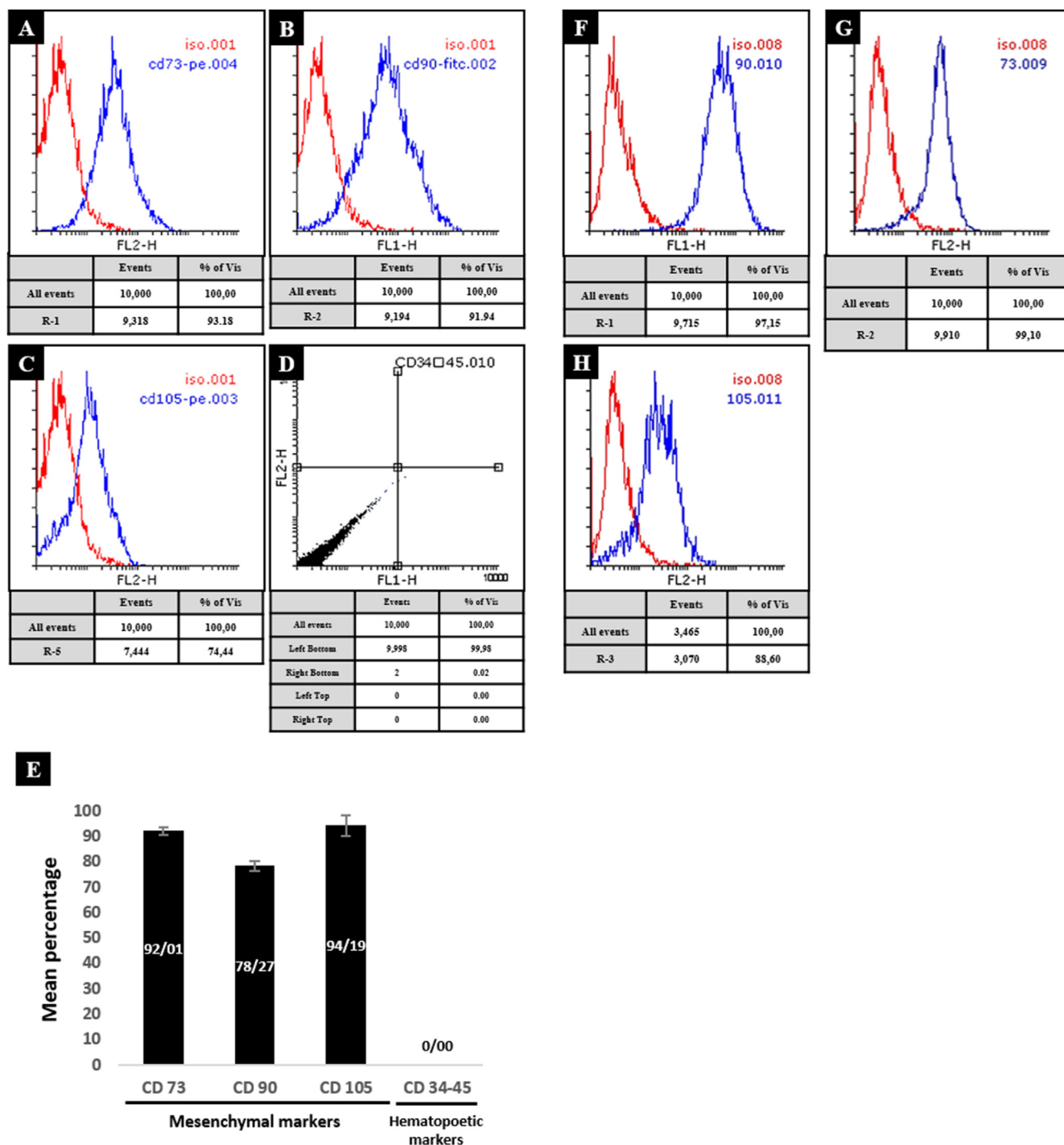


Figure 2. Flow cytometry histograms of WJ-MSCs and BM-MSCs. WJ-cells were positive for mesenchymal markers CD73 (A), CD90 (B), CD 105 (C) and negative for hematopoietic markers CD34/CD45 (D). This data compared with Positive expression of mesenchymal markers CD90 (F), CD73 (G), CD 105 (H) in BM-MSCs. Flow cytometry analysis of WJ-MSCs were done on three independent experiments and their averages were indicated in diagram (E).

of lipid components, carbohydrates and proteins which are secreted by somatic cells and the female germ cells [17]. FF can play an important role in providing a suitable environment for primordial germ cells (PGCs) to develop into the functional oocytes [18]. Cumulus cells are a group of cells associated granulosa cells that surround and feed oocytes. Previous studies have shown that cumulus cells contribute to oocyte maturation and fertilization [19]. Cumulus cells may secrete some factors that have a crucial role in the process of oogenesis and differentiation of PGCs [20]. Some effective components in follicular fluid, such as VEGF (Vascular endothelial growth factor), IL (Interleukin), EGF (Epidermal growth

factor), TGF (Transforming growth factor beta), and BTC (Betacellulin) are related to cumulus cell secretions [21]. So, to better understand the differentiation ability of WJ-MSCs into OLCs and GCLs, in this study, we used FF and CCM as the inducers.

2. Materials and methods

We conducted the procedure in accordance with the declaration of Helsinki following approval by the Ethical Committee of Royan Institute

(IR.ACECR.ROYAN.REC.1398.195) and informed consent was obtained from all patients for the experiment.

2.1. Isolation and expansion of WJ-MSCs

Umbilical cord samples under a sterile condition were taken from 10 full-term and healthy female newborns by cesarean section in Arash hospital (Tehran, Iran). All samples were obtained from women who had caesarean section. The umbilical cords were transferred to the laboratory (Figure 1 A) in phosphate buffered saline (PBS, MP Biomedicals, 2810305, France) on ice. The blood cells, arteries, vein and amniotic membrane (Figure 1 D) were removed from the umbilical cords (Figure 1 B) and the remaining Wharton's jelly were minced into small pieces (Figure 1 C). The Wharton's jelly (WJ) fragments were cultured in the α -MEM (Gibco, 12571063, USA) supplemented with 10% FBS (Gibco, 10270106 USA), 1% pen-strep (Gibco, 15070063, USA), 1% L-glutamine (Gibco, 25030024, USA), and 2 ng/mL Bfgf (Sigma-Aldrich, F0291, USA) and maintained in 37 °C humidified incubator with 5% CO₂, until the cells migrated out from the explants. The attached cells were dissociated by 0.25% trypsin-EDTA (Gibco, 25300054, USA) when reached to 80% confluency. The morphologically evaluation was done with inverted phase contrast microscope (Olympus CKX-41).

2.2. Flow cytometry assay

For cell surface antigen phenotyping, third-passage cells were detached by 0.25% Trypsin-EDTA and centrifuged at 1200 rpm for 5 min. At least 10⁵ cells per each marker resuspended in 100 μ l PBS. Then, the cells were incubated for 30 min at 4 °C with anti-human antibodies against CD73-PE (2:100 μ l, BD bioscience, 550257, USA), CD105-PE (2:100 μ l, BD bioscience, 560839, USA), CD90-FITC (2:100 μ l, BD bioscience, 555595, USA) and human leucocyte antigen CD45/CD34-FITC/PE (2:100 μ l, BD Biosciences, 341071, USA). Isotype antibodies conjugated to FITC or PE were used for cell surface staining. Finally, the cells analyzed with FACS Calibur (BD Biosciences) and Flowing software (Version 2.5.1). The human bone marrow mesenchymal stem cells (BMSCs) were considered as a positive control for expressing the mesenchymal surface markers.

2.3. Adipogenic differentiation of WJ-derived cells

3rd passage cells were treated with adipogenic medium for three weeks and the medium was refreshed every 3 days. The adipogenic medium composed of α -MEM containing 10% FBS, 50 μ g/ml

indomethacin (Sigma-Aldrich, I2280, USA) 50 μ g/ml ascorbic acid-2 phosphate (Sigma-Aldrich, A5960, USA) and 100 nM dexamethasone (Sigma-Aldrich, D2915, USA). Adipocyte differentiation was tested by oil-red O staining (Sigma-Aldrich, O0625, USA). Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, 30525-89-4, USA) and stained with 0.5% oil-red O solution in isopropyl alcohol (Sigma-Aldrich, 563935, USA) for 10 min. Finally, stained lipid droplets were observed by an inverted phase contrast microscope (Olympus CKX41).

2.4. Osteogenic differentiation of WJ-derived cells

3rd passage cells were treated with osteogenic medium for 3 weeks with medium changes twice weekly. The osteogenic differentiation medium contained of α -MEM supplemented with 10% FBS, 10 mM β -glycerophosphate, 10 nM dexamethasone and 50 μ g/ml ascorbic acid-2 phosphate, 2.1604 g/l 6-glycerol phosphate (all purchased from Sigma-Aldrich). The WJ-MSCs potential for osteoblast differentiation was evaluated by Alizarin-red S staining. Briefly, cells were fixed with methanol and stained with 1% Alizarin-red S solution (Sigma-Aldrich, A5533, USA) in water for 2 min. Finally, the extracellular staining was observed by an inverted phase contrast microscope.

2.5. Cumulus cells culture and procurement of conditioned medium

Cumulus cells were obtained from the healthy women under in vitro fertilization at Royan IVF center (Tehran-Iran). Collected cumulus cell samples were centrifuged at 1500 rpm for 5 min to remove red blood cells. Cumulus cells were seeded in culture plates containing α -MEM medium supplemented with 10% FBS, 1% L-Glutamine, 0.5% bFGF. The supernatant culture medium of cumulus cells collected from the cells at the first passage with 80% confluency (Figure 1 E-G). The medium collection was done every 48 h and stored at -20 °C to use as cumulus cells conditioned medium (CCM).

2.6. Follicular fluid purification

The human follicular fluid (FF) samples also prepared from healthy women by IVF (in vitro fertilization) at Royan IVF Center (Tehran, Iran). The samples centrifuged at 4000 rpm for 5 min to separate the cell pellet from the clear supernatant fluid. After taking the supernatant fluid and discarding the cell pellet, the fluid was filtered (0.22 μ m pore size). For inactivation of the unnecessary enzymes, the filtered FF was incubated for 30 min at 56 °C in water bath and finally stored at -20 °C.

Table 1. List of primers used in qPCR (annealing: 60 °C and cycle citation: 40 °C).

Gene Name	Primer Sequences	Product Length (bp)
<i>Gapdh</i>	F: CTCATTTCCTGGTATGACAAC R: CTTCTCTTGTGCTCTTGCT	122
<i>Vasa</i>	F: TGAAATTCTGCGAACATAGGG R: TCCCGATCACCATGAATACITG	129
<i>Zp-1</i>	F: ACACCTTTCAGTCGCACTA R: GCAGAACAAGTAAACAGTCCCT	102
<i>Zp-2</i>	F: AGCATTGAGGTTGGTATGG R: ACTGTTACCTTGACATAGTGA	153
<i>Zp3</i>	F: GCCAGATACACTCCAGTTCAC R: AGATGTCAGCCGAGCCTT	185
<i>Gdf-9</i>	F: TAGAAGTCACCTCTACAACACTG R: GGTAGTAATGCGATCCAGGTTA	130
<i>C-kit</i>	F: ATTGTTCTGTGACCCAGGAG R: GGTGTTGTGACATTTGCTG	362
<i>Syp3</i>	F: TTACGAGAGCCTATGACTTTGAG R: ATGTCAACTCCAACCTCTTCC	181

Table 2. The list of antibodies in immunofluorescence staining.

Primary Antibodies				
Antibodies	Clone	Catalog No.	Manufacturer	Dilution
DDX-4 (VASA)	Rabbit	ab13840	Abcam	1:200
ZP3	Rabbit	sc2802	Santacruz	1:200
GDF-9	Goat	sc7407	Santacruz	1:200
SYCP3	Rabbit	NB300-232	Novusbio	1:200
Secondary Antibodies				
Anti-rabbit	IgG	A21266	Invitrogen	1:500
Anti-goat	IgG	A11055	Invitrogen	1:500

2.7. Differentiation induction of WJ-MSCs into oocyte-like cells and germ cell-like cells

In order to induce differentiation into oocyte-like cells (OLCs) and germ cell-like cells (GCLs), WJ-MSCs at 3rd passage trypsinized and 6×10^3 cells/well were plated in 24-well culture plates (BD Biosciences, 354723, USA). In this study, we selected and used two differentiation media after a pilot study as follows: we evaluated various concentrations of FF and CCM before selecting the final concentration (10%). We examined the effects of 5%, 10%, 20% and 50% FF and 5%, 10%, 20%, 50%, 100% CCM on WJ-MSCs culture. In our preliminary assessments, the highest concentrations for FF (50%) and CCM (50–100%) observationally were extremely deleterious for the cells. So they were omitted from the study and we focused on the lower concentrations (5, 10 and 20%). The results derived from the cultured cells, based on oocyte-like cell diameter, in three selected concentrations of 5%, 10% and 20% of FF and CCM showed that morphologically and statistically, 10% concentration of both FF and CCM is the optimum dose (HYFigure 5A). Thereafter, the first differentiation medium contained α -MEM, 10%

FF, 1% L-glutamine, 1% Pen-Strep, and 2 ng/ml bFGF (FF group). The second differentiation medium consisted of α -MEM supplemented with 10% CCM, 10% FBS, 1% L-glutamine, 1% Pen-Strep, 2 ng/ml bFGF (CCM group). WJ-MSCs cultured in α -MEM, 10% FBS, 1% pen-strep, 1% L-glutamine and 2 ng/ml bFGF were used as a control. All samples were incubated under the same conditions (97% humidity, 21% O₂, 5% CO₂ and 37 °C) for 21 days. The culture medium was replaced every 3 days. Cellular morphology was examined and images were captured using an inverted microscope (Olympus CKX41) at days 0, 7, 14 and 21 after differentiation.

2.8. Transcriptional profiling of germ and oocyte lineage-associated genes by RT-qPCR

Total cellular RNA was isolated using a RNeasy micro kit (Qiagen, 74004, USA). Subsequently, the total RNA was reverse transcribed into cDNA using a cDNA synthesis kit (TAKARA, 6130, Japan) in accordance with the manufacturer's instructions. RT-qPCR was performed using the SYBR-Green mix kit (Applied Biosystems, 4364344,

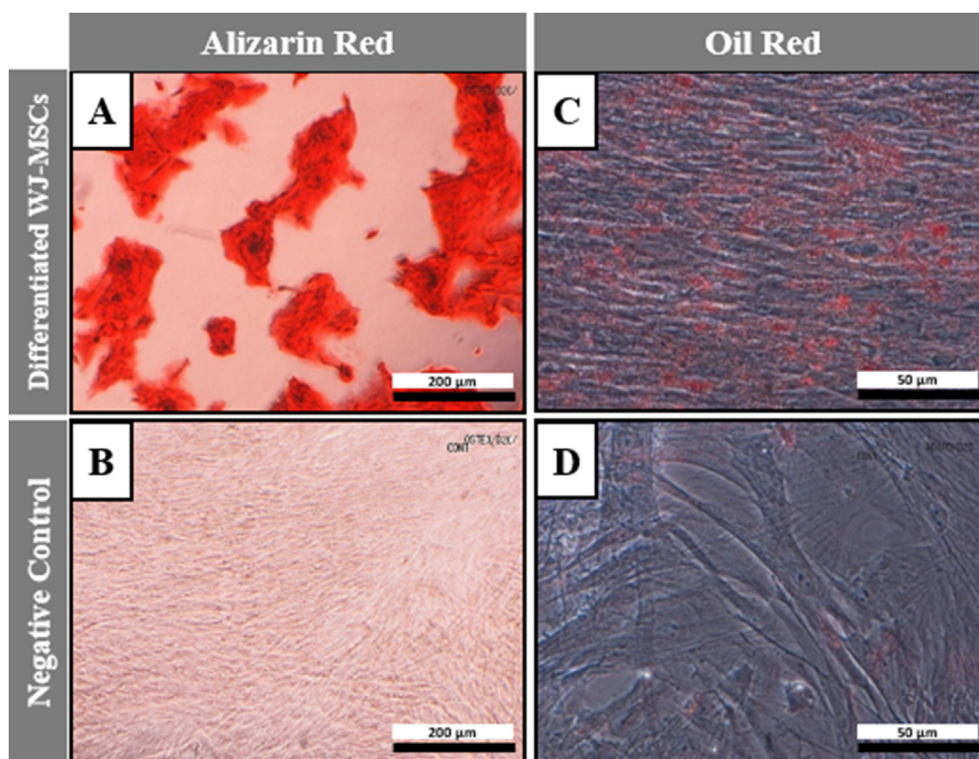


Figure 3. Osteogenic and adipogenic differentiation of WJ-MSCs. Calcium deposition was observed after staining with Alizarin Red on day 21 of induction (A) and there was no calcium deposition in control undifferentiated cells (B). Oil-Red staining on day 21 of induction into adipocytes indicated fat globules (C) while fat globules were not observed in control undifferentiated cells (D).

USA). A total of 2.0 μ l (12.5 μ g) cDNA was added to 5 μ l SYBR-Green mix with 1 μ l each of the forward and reverse primers. Water was added to produce a final volume of 10 μ l and reaction was carried out for 40 cycles. The primer sequences were Synaptonemal Complex Protein 3 (*Sycp3*), Growth and Differentiation Factor 9 (*Gdf9*), Zona Pellucida 1, 2 and 3 (*Zp1*, *Zp2*, *Zp3*), Dead box Polypeptide 4 (*Vasa* also known as *DDX4*) and Tyrosine-Protein Kinase Kit (*C-kit*) listed in Table 1. Glyceraldehyde-3-Phosphate Dehydrogenase (*Gapdh*) expression used as an internal control in Real-Time PCR. The mean expression of each target gene was normalized against the geometric mean of corresponding threshold cycles obtained for the endogenous control. The relative expression of germ cell and oocyte lineage associated genes in the differentiated cells was calculated using $2^{-\Delta\Delta Ct}$ method, with the results being presented as the fold change in the expression of the genes in the cells induced to differentiate relative to the undifferentiated cells.

2.9. Immunofluorescence staining for germ cell and oocyte specific proteins

The cells that were treated for 21 days with or without the differentiation medium were washed 3 times with PBS and fixed with 4% paraformaldehyde for 30 min. Then, the cells were washed 2 times with 0.05% PBS-Tween (PBST) and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, T9284, USA) for 10 min. The common antigens between both species were blocked with 10% secondary host serum for 1 h. Next, the cells were washed 2 times with PBST and incubated with the primary antibodies at 4 °C for an overnight. The source and dilution information of the primary and secondary antibodies are listed in Table 2. Following 3 washes with PBST, the cells were incubated with FITC-conjugated donkey anti-rabbit or donkey anti-goat antibodies in the dark at 37 °C for 1 h. After 2 washes with PBST, the nuclei were stained with DAPI (Abcam, ab104139, United Kingdom) for 1 min and visualized under a

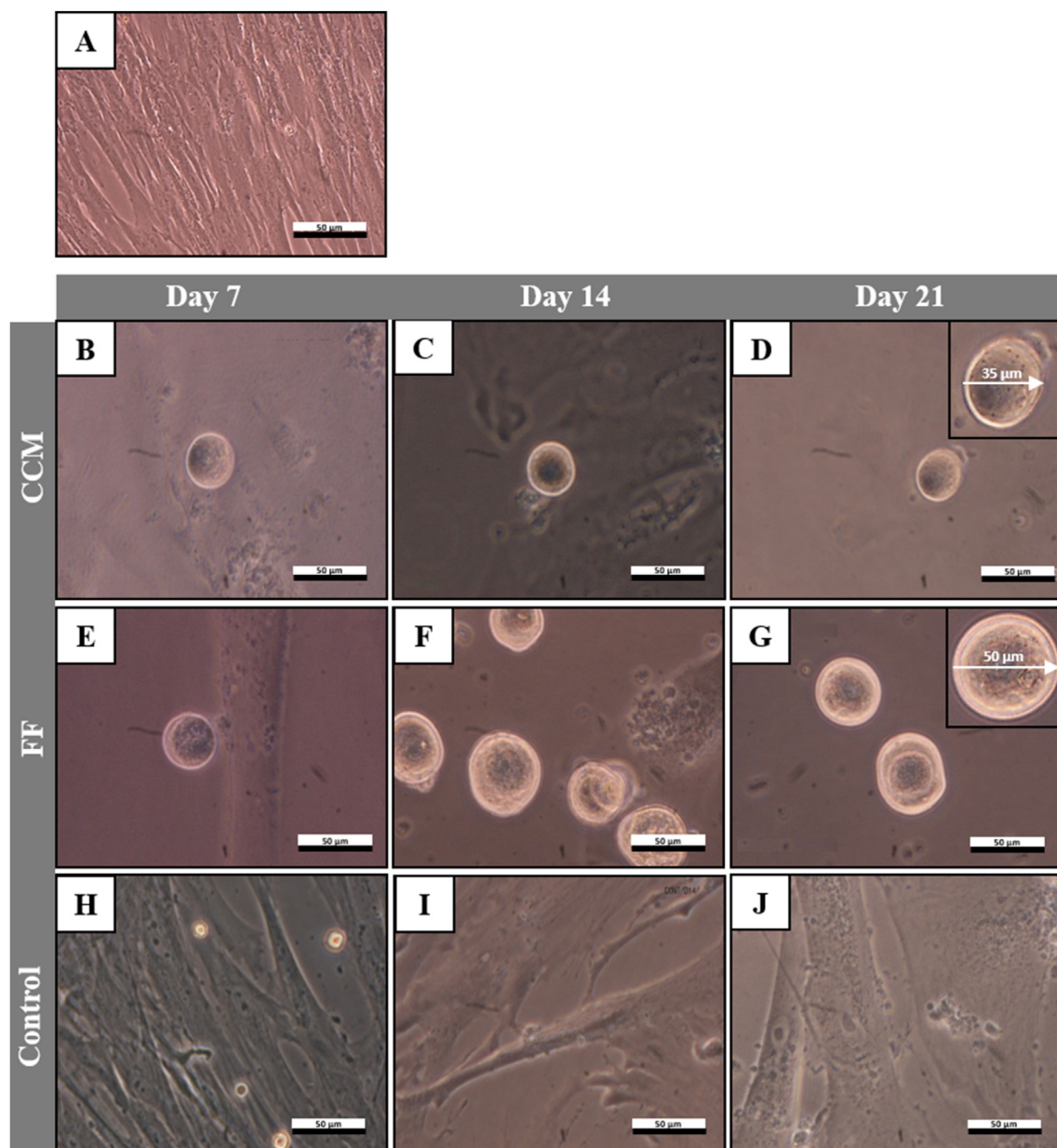


Figure 4. WJ-MSCs differentiation into oocyte-like cells. OLCs induced by CCM on days 7, 14 and 21 (B, C and D). OLCs induced by FF on days 7, 14 and 21 of culturing (E, F and G). WJ-MSCs appearances in control un-induced group on day 7, 14 and 21 of culturing (H, I and J). Day 0 of differentiation (A).

fluorescence microscope. Negative controls were performed by the elimination of primary antibodies.

2.10. Statistical analysis

In this study, although, all statistical analysis was performed using the SPSS18 software package (SPSS Inc, USA) and A value of $P < 0.05$ was considered to indicate a statistically significant difference and all experiments were performed in triplicate, data for oocyte diameter and protein expression were analyzed using GLM procedure and LSMEANS statement was used for multiples comparisons. The last mentioned analyses were conducted in SAS version 9.4 (SAS Institute Inc., Carry, NC, USA) and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Isolation and characterization of WJ-MSCs

Primary cultures were established using Wharton's jelly explants in 24-well culture plates. Isolated cells from the fragments grew adherent and started to migrate out from the explants after 5–6 days. The isolated cells reached to 50% and 80% confluency respectively in 2nd and 4th weeks of culturing prior to passaging. These cells exhibited spindle shape along with colony forming units similar to BMSCs (Figure 1 N). The morphological characteristics of these cells were maintained for several passages (Figure 1 H–M).

3.2. Immunophenotypes of WJ-MSCs

To determine whether WJ-MSCs have multipotent capacity, WJ-MSCs at third passage were assessed for the expression of markers associated with mesenchymal stem cells using flow cytometric assay. As shown in Figure 2, 94%, 92% and 78% of WJ-MSCs respectively were positive for CD105, CD73 and CD90 surface markers and had no expression against hematopoietic cell markers CD34/CD45 (Figure 2). BMSCs applied as a positive control and mesenchymal and hematopoietic markers expression in WJ-MSCs were similar to the positive control (BMSCs).

3.3. Osteogenic and adipogenic differentiation

Furthermore, multipotency potential of WJ-MSCs in passage 3 was evaluated by differentiating them into osteocytes and adipocytes. After 21 days, the WJ-MSCs treated with osteogenic induction medium stained positive for mineral nodules with Alizarin Red-S (Figure 3A). The cells treated with adipogenic induction medium demonstrated lipid vacuoles that were stained by Oil Red-O (Figure 3C). In addition, morphological changes were detected corresponding to differentiated cell phenotype. As a negative control, cells were cultured in medium without induction factors, containing only α -MEM supplemented with 10% FBS (Figure 3B, D).

3.4. Morphological changes of WJ-MSCs into oocyte-like cells

WJ-MSCs at 3rd passage were induced to oocyte-like cells using media containing 10% FF or 10% CCM for 21 days. The cellular morphology was investigated at 0, 7, 14 and 21 days of induction. During this time, a subpopulation of large and round cells up to 50 μm and 35 μm in diameter was observed for the cells cultured in 10% FF and 10% CCM, respectively (Figure 4). Morphologically, the cells appeared similar to oocytes. Hereafter, we called these cells oocyte-like cells (OLCs). OLCs were not found in control group. Diameter of oocyte-like cells was larger in FF 10% group than FF 5%, FF 20%, CCM 5% and CCM 20% groups ($P < 0.001$); moreover, diameter of oocyte-like cells was larger in FF 5% and FF 20% groups compared with CCM 5% group ($P < 0.01$). Besides, size of oocyte-like cells was larger in CCM 10% group than CCM 5% and CCM 20% groups ($P < 0.01$) (Figure 5A).

Additionally, in our microscopic investigation, it looked like the number of OLCs produced by FF is more than OLCs in CCM group. OLCs were seen from the 7th day of culture in both media, but the number and diameter in some of them gradually increased after 14 and 21 days of differentiation. The morphology changes first occurred in attached cells and then with completion of the differentiation process, the cells became suspended and floated in conditioned medium (Figure 4). In FF 10% group, size of oocyte-like cells increased from day 7–14 ($P < 0.0001$), but it did not change between days 14 and 21 ($P > 0.05$). In CCM 10% group, diameter of oocyte-like cells did not change between days 7 and 14 ($P > 0.05$), yet it dwindled from day 14–21 ($P = 0.018$). Furthermore, diameter of oocyte-like cells was greater in FF 10% than CCM 10% group on days 7, 14 and 21 ($P < 0.05$) (Figure 5B).

3.5. Expression of germ cells and oocyte lineage genes

Transcriptional profiling was done by RT-qPCR for oocyte-associated genes (*Zp1-Zp2*, *Zp3* and *Gdf9*) and germ cell lineage markers (*Vasa*, *C-kit* and *Sypc3*) at 0, 7, 14 and 21 days of induction by 10% FF and 10% CCM. The group that was treated by α -MEM supplemented with 10% FBS is

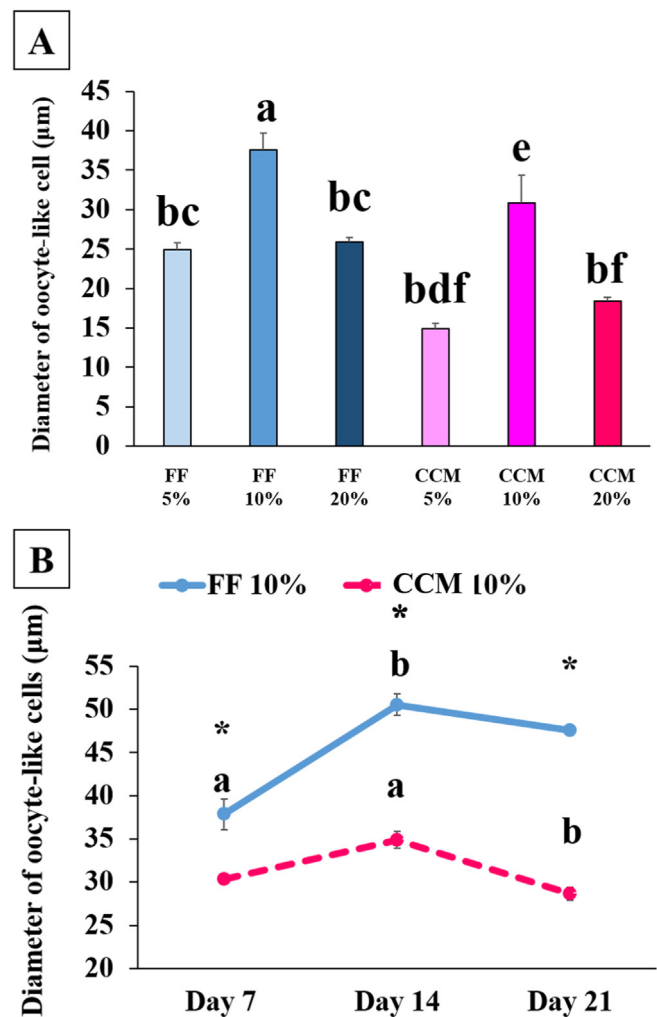


Figure 5. A) Diameter of oocyte-like cells in FF 5%, FF 10%, FF 20%, CCM 5%, CCM 10% and CCM 20% groups. Different letters (a, b, c and d) indicate significant difference among various experimental groups ($P < 0.05$). B) Diameter of oocyte-like cells in FF 10% and GCM 10% groups on days 7, 14 and 21 of culture. Different letters (a, b) indicate significant difference among various time points within the specified group ($P < 0.05$). Asterisk (*) denotes significant difference between two experimental groups at the specified time point ($P < 0.05$).

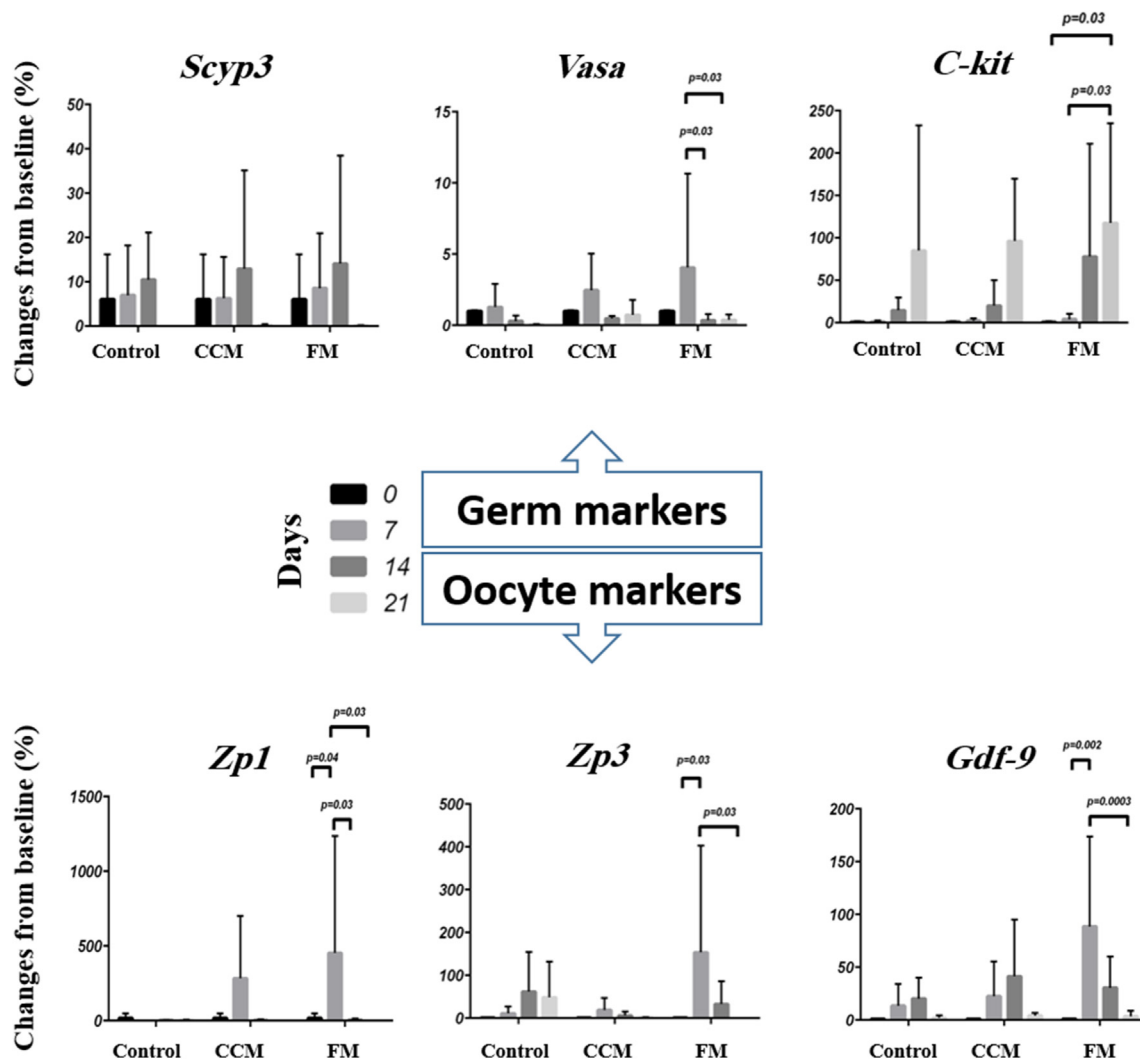


Figure 6. Germ and oocyte genes expression after induction of WJ-MSCs with CCM and FF on days 0, 7, 14 and 21.

considered the control group. The changes in the relative mRNA levels were evaluated using *Gapdh* as an inner control and amplified products for each of the markers compared with day 0. As shown in Figure 6 for *Zp1*; the highest expression belonged to day 7 of induction by FF and CCM. Also, a significant increase in FF group was shown at day 7 compared to days 0, 14 and 21 (P -Value < 0.05). *Zp2* expression was not detected in any of the groups and did not achieve to statistical threshold. The highest expression of *Zp3* was at day 7 of induction by FF and it was significant compared to days 0 and 21 in FF group. The expression of *Gdf-9* increased in all groups (FF, CCM and FBS), but the highest belonged to day 7 of induction by FF, similar to *Zp1* and *Zp3*. *Syp3* showed an increasing trend until day 14 of culture with FF, CCM and FBS, but a sharp decrease occurred from day 14–21. The highest levels of expression for *Syp3* were at day 14 of culture in all groups (not significant). The highest expression of *Vasa* was at day 7 of induction by FF and CCM, while it was significant in FF group compared to days 14 and 21. For *C-kit*, the highest expression was shown at day 21 in all groups, while it was significant in FF group compared to days 0 and 7.

3.6. Immunofluorescence for germ cell and oocyte lineage proteins

We performed immunofluorescence staining for the induced and control cells on day 21st of culture. The expression of specific markers related to germ cell formation (*VASA*, *SYCP3*) and oocyte development (*ZP3*, *GDF9*) were investigated. All the markers were detected in FF and

CCM groups. *GDF-9* protein was detected in the induced cells with less fluorescent intensity than the other proteins and almost was not detected in the control group. Except *GDF-9*, the rest of markers were detectable in the control group. The expressed markers were more positively in WJ-MSCs cultured in FF compared to CCM and control groups on 21st day of culture. In detail; *VASA* expression was greater in FF and CCM groups than the control group ($P < 0.05$). Expression of *SYCP3* did not differ among various groups ($P > 0.05$). *ZP3* and *GDF9* expression was higher in FF group as compared with the control and CCM groups ($P < 0.01$). No signal was detected in the negative controls with only the anti-rabbit and anti-goat secondary antibodies (Figure 7).

4. Discussion

Production of germ cells from stem cells is a very interesting and promising area in the field of reproductive medicine. It has been shown that stem cells derived from different regions, such as menstrual blood, amniotic fluid, lung, skin and pancreas, have the capacity to differentiate into germ cell-like cells [22, 23, 24]. MSCs can be isolated from various sources especial from neonatal tissues, such as placenta, umbilical cord and Wharton's jelly may offer certain advantages. These include their non-invasive and ethically non-problematic availability. Moreover, MSCs from birth-associated tissues have increased proliferative capacity in vitro, in comparison to some MSCs derived from adult tissues [25]. On the other hand, MSCs of human umbilical cords can be obtained from

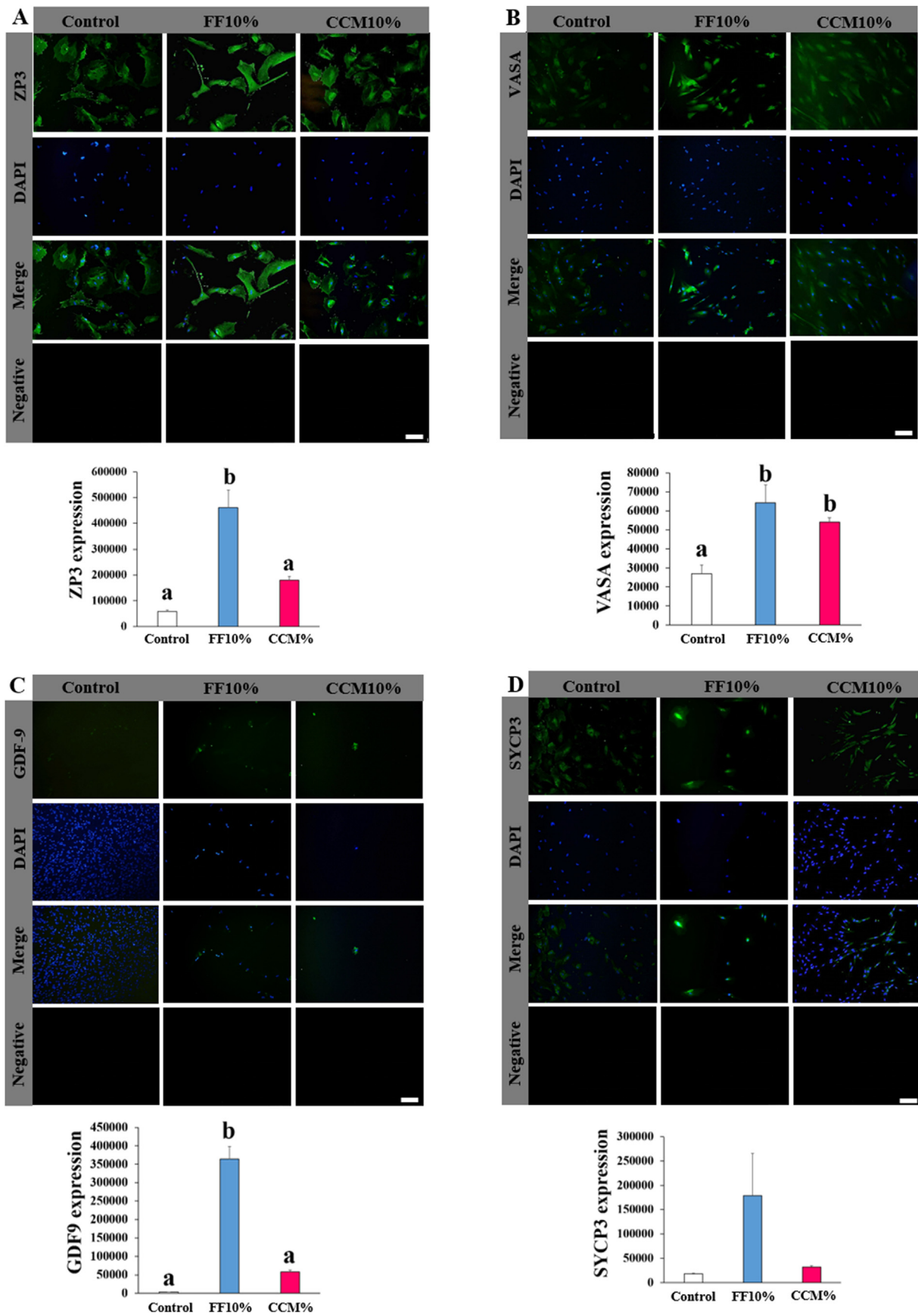


Figure 7. Immunofluorescent staining of WJ-MSCs induced by CCM and FF. ZP3 (A), VASA (B), GDF-9 (C) and SYCP3 (D) expression after 21 days in induced and control groups. Scale bar in all figures: 100 μ m.

Wharton's jelly (WJ). UC-MSCs contain heterogeneous populations of MSC [26], more than WJ-MSCs. Recently, several studies have reported that germ cell-like cells can be produced from WJ-MSCs [27, 28, 29, 30]. WJ-MSC are maintained in a very early embryological stage and retain some of their primitive stemness properties. These cells are slightly easier to harvest compared to other MSCs, with a greater proliferative capacity and ability to differentiate into the germ cell lineages [31]. The cells derived from Umbilical cord or Wharton's jelly have a same origin with primordial germ cells during the embryogenesis and develop from the proximal epiblast [32, 33]. Therefore, WJ-MSCs may have the capacity to differentiate into germ cell-like cells, so we used them in the present study for producing female germ-like cells or oocyte like-cells. For the purpose of female germ cell production by WJ-MSCs, we cultured Wharton's jelly tissue explants from female umbilical cords. Qiu et al. (2013) indicated that MSCs from female umbilical cords more easily differentiated into oocyte-like cells (OLCs) compared to male umbilical cord MSCs [28]. In the primary culture, isolated cells indicated characteristics such as colony-forming units, plastic adherence and heterogeneous morphology. The morphology of the cells isolated from Wharton's jelly at the third passage was similar to that of MSCs derived from bone marrow and other tissues [34, 35, 36]. MSCs have a fibroblast-like morphology, are self-renewing and able to differentiate into mesoderm lineages including osteocytes, adipocytes and chondrocytes [37, 38]. Sabapathy et al. (2014) showed that WJ-MSCs efficiently differentiate into ectoderm (neural cells), mesoderm (adipocyte, osteocyte, chondrocyte, smooth muscles etc.) and endoderm (Pancreatic progenitors) lineages [39]. In this study, fibroblast-like cells isolated from WJ tissue were capable to differentiate into osteocytes and adipocytes at the third passage. Additionally, these cells were characterized by evaluating their mesenchymal and hematopoietic surface markers according to the previous studies [40, 41]. MSCs derived from WJ dominantly expressed CD73, CD90 and CD105 like BMSCs (positive control) but not CD34/CD45 at the third passage. Ali et al. (2017) and Ayatollahi et al. (2016) demonstrated that WJ-MSCs express CD73, CD90 and CD105 in vitro [42, 43]. The morphology, potential of differentiation into lineages of the mesoderm, and the cell surface markers indicated that the cells from WJ have consistent features with the other MSCs.

Germ cell production has decades of history, yet there is a lack of a standard model and appropriate inducers to produce mature gametes in vitro. As the components of follicular fluid (FF) and cumulus cells have been shown to play key roles during oogenesis and stimulation of oocyte (43) in recent years, many researchers have used FF and cumulus cells conditioned medium (CCM) as inducers to make OLCs. De Souza et al. (2016) produced OLCs in a medium supplemented with FF by the germ line stem cells from bovine ovaries [44]. Qiu et al. (2013) revealed that OLCs could be differentiated from MSCs derived from human umbilical cord cultured in 20% FF during 7–14 days of induction [28]. Shah et al. differentiated buffalo embryonic stem cells into OLCs in 20–40% CCM [45]. In the current study, FF and CCM were compared for development of OLCs from WJ-MSCs in vitro. OLCs were characterized based on morphologic changes and marker expression profiles at the mRNA and protein levels. After induction of WJ-MSCs with 10% FF and 10% CCM, some large and round cells appeared that were morphologically similar to oocytes, which we called OLCs, that were not observed in the control group. OLCs developed with culture in both media at 7–21 days after the induction, however the number and diameter of some OLCs gradually increased until day 21. The size of OLCs developed by FF and CCM reached up to 50 μm and 35 μm in diameter respectively. Hu et al. (2015) reported MSCs derived from human first-trimester umbilical cords after induction by 25% FF differentiated into OLCs which varied in size from 50 to 120 μm at days 21–24 [30]. We used lower amount of FF (10%) during 21 days and the umbilical cord was derived from full term newborns, but we observed a similar result in morphology. Mirzaeian et al. (2018) achieved a same result when they induced mouse peritoneum mesenchymal stem cells with 10% human FF or 50% human CCM, resulting in round OLCs with 50 μm diameter at day 21 of differentiation

[46]. On the basis of the results; the generated OLCs in FF group were larger than the CCM group. Furthermore, intensity of immunofluorescence for oocyte and germ cell markers was higher in the cells which were under treatment of FF. In addition, gene expressions indicate FF have a significant impact for inducing the MSCs toward OLCs. Since *Sycp3* is a meiotic marker and it belongs to late stages of a germ cell development, so the expression of *Sycp3* ensue later. In the present study, the expression level of *Zp1*, *Zp3*, *Gdf-9*, *C-kit*, *Vasa* and *Sycp3* as oocyte and PGC specific markers were clearly increased in FF compared to CCM and control groups, while *Zp2* gene was not expressed in any of groups. Immunocytochemistry results on day 21st of differentiation indicated that cells in FF and CCM groups expressed VASA, SYCP3, ZP3 and GDF9 proteins. However, the expressions were more positively in FF group compare to CCM and control group. In addition, VASA, SYCP3 and ZP3 were seen at low levels in control group. Our study revealed that WJ-MSCs in control group weakly express germ cell markers at the protein and mRNA levels, indicating WJ-MSCs have a germ cell memory. Asgari et al. (2015) also had a similar result when they used a co-culture system of placental cells with WJ-MSCs to induce OLCs differentiation. They found that WJ-MSCs, after 14 days of co-culture with placental cells, strongly presented positive signal for PGCs and oocyte markers. Correspondingly with our results, the cells in their control group expressed the markers with weaker signals [16].

5. Conclusion

This study demonstrated WJ-MSCs have an intrinsic ability to differentiate into oocyte-like cells using FF and CCM, albeit FF had a greater impact. Moreover, WJ-MSCs without induction could express the germ cell related genes at low levels, while after induction, these genes were expressed increasingly. Hence, WJ-MSCs have a germ cell memory and Wharton's jelly tissue is a favorable source of mesenchymal stem cells to produce germ cells like-cells.

Declarations

Author contribution statement

R. Fathi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Zolfaghar: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

B. Beiki, A. Moini, P. Eftekhari-Yazdi and A. Vernengo: Contributed reagents, materials, analysis tools or data.

L. Mirzaeian, T. Naji and V. Akbarinejad: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

- [1] G.D. Adamson, et al., World collaborative report on in vitro fertilization, 2000, *Fertil. Steril.* 85 (6) (2006) 1586–1622.
- [2] K. Hubner, et al., Derivation of oocytes from mouse embryonic stem cells, *Science* 300 (5623) (2003) 1251–1256.
- [3] A.I. Marques-Mari, et al., Differentiation of germ cells and gametes from stem cells, *Hum. Reprod. Update* 15 (3) (2009) 379–390.
- [4] M. Imamura, et al., Generation of germ cells in vitro in the era of induced pluripotent stem cells, *Mol. Reprod. Dev.* 81 (1) (2014) 2–19.
- [5] I. Ullah, R.B. Subbarao, G.J. Rho, Human mesenchymal stem cells-current trends and future prospective, *Biosci. Rep.* 35 (2) (2015), e00191.
- [6] X. Wei, et al., Mesenchymal stem cells: a new trend for cell therapy, *Acta Pharmacol. Sin.* 34 (6) (2013) 747–754.
- [7] S. Roura, et al., The role and potential of umbilical cord blood in an era of new therapies: a review, *Stem Cell Res. Ther.* 6 (2015) 123.
- [8] B. Beiki, B. Zeynali, E. Seyedjafari, Fabrication of a three dimensional spongy scaffold using human Wharton's jelly derived extra cellular matrix for wound healing, *Mater. Sci. Eng. C Mater. Biol. Appl.* 78 (2017) 627–638.
- [9] L.P. Kamolz, M. Keck, C. Kasper, Wharton's jelly mesenchymal stem cells promote wound healing and tissue regeneration, *Stem Cell Res. Ther.* 5 (3) (2014) 62.
- [10] R. Carlin, et al., Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells, *Reprod. Biol. Endocrinol.* 4 (2006) 8.
- [11] L.R. Gao, et al., Common expression of stemness molecular markers and early cardiac transcription factors in human Wharton's jelly-derived mesenchymal stem cells and embryonic stem cells, *Cell Transplant.* 22 (10) (2013) 1883–1900.
- [12] S.J. Greco, K. Liu, P. Rameshwar, Functional similarities among genes regulated by OCT4 in human mesenchymal and embryonic stem cells, *Stem Cell.* 25 (12) (2007) 3143–3154.
- [13] J. Hua, et al., Multipotent mesenchymal stem cells (MSCs) from human umbilical cord: potential differentiation of germ cells, *Afr. J. Biochem. Res.* 5 (4) (2011) 113–123.
- [14] M. Latifpour, et al., Differentiation of human umbilical cord matrix-derived mesenchymal stem cells into germ-like cells, *Avicenna J. Med. Biotechnol.* 6 (4) (2014) 218.
- [15] L. Xie, et al., Sertoli cell-mediated differentiation of male germ cell-like cells from human umbilical cord Wharton's jelly-derived mesenchymal stem cells in an in vitro co-culture system, *Eur. J. Med. Res.* 20 (2015) 9.
- [16] H.R. Asgari, et al., Human Wharton's jelly-derived mesenchymal stem cells express oocyte developmental genes during co-culture with placental cells, *Iran. J. Basic Med. Sci.* 18 (1) (2015) 22.
- [17] S. Fahiminiya, N. Gerard, Follicular fluid in mammals, *Gynecol. Obstet. Fertil.* 38 (6) (2010) 402–404.
- [18] L. Basuino, C.F. Silveira Jr., Human follicular fluid and effects on reproduction, *JBRA Assist. Reprod.* 20 (1) (2016) 38–40.
- [19] C.-J. Zhou, et al., The beneficial effects of cumulus cells and oocyte-cumulus cell gap junctions depends on oocyte maturation and fertilization methods in mice, *PeerJ* 4 (2016), e1761.
- [20] S.M. Shah, et al., Cumulus cell-conditioned medium supports embryonic stem cell differentiation to germ cell-like cells, *Reprod. Fertil. Dev.* (2015).
- [21] S. Fahiminiya, N. Gerard, Follicular fluid in mammals, *Gynecol. Obstet. Fertil.* 38 (6) (2010) 402–404.
- [22] D. Bhartiya, et al., Making gametes from alternate sources of stem cells: past, present and future, *Reprod. Biol. Endocrinol.* 15 (1) (2017) 89.
- [23] J. Hua, et al., Characterization of mesenchymal stem cells (MSCs) from human fetal lung: potential differentiation of germ cells, *Tissue Cell* 41 (6) (2009) 448–455.
- [24] S. Danner, et al., Derivation of oocyte-like cells from a clonal pancreatic stem cell line, *Mol. Hum. Reprod.* 13 (1) (2006) 11–20.
- [25] R. Hass, et al., Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue-derived MSC, *Cell Commun. Signal.* 9 (1) (2011) 12.
- [26] C. Mennan, et al., Mesenchymal stromal cells derived from whole human umbilical cord exhibit similar properties to those derived from Wharton's jelly and bone marrow, *FEBS Open Bio* 6 (11) (2016) 1054–1066.
- [27] D. Dissanayake, H. Patel, P. Wijesinghe, Differentiation of human male germ cells from Wharton's jelly-derived mesenchymal stem cells, *Clin. Exp. Reprod. Med.* 45 (2) (2018) 75–81.
- [28] P. Qiu, et al., Gender depended potentiality of differentiation of human umbilical cord mesenchymal stem cells into oocyte-like cells in vitro, *Cell Biochem. Funct.* 31 (5) (2013) 365–373.
- [29] R. Ghaem Maghami, T. Mirzapour, A. Bayrami, Differentiation of mesenchymal stem cells to germ-like cells under induction of Sertoli cell-conditioned medium and retinoic acid, *Andrologia* 50 (3) (2018), e12887.
- [30] X. Hu, et al., Stem cells derived from human first-trimester umbilical cord have the potential to differentiate into oocyte-like cells in vitro, *Int. J. Mol. Med.* 35 (5) (2015) 1219–1229.
- [31] N. Watson, et al., Discarded Wharton jelly of the human umbilical cord: a viable source for mesenchymal stromal cells, *Cytotherapy* 17 (1) (2015) 18–24.
- [32] A.S. Ansari, et al., Osteogenic induction of Wharton's jelly-derived mesenchymal stem cell for bone regeneration: a systematic review, *Stem Cell. Int.* 2018 (2018).
- [33] Y. Moodley, et al., Human umbilical cord mesenchymal stem cells reduce fibrosis of bleomycin-induced lung injury, *Am. J. Pathol.* 175 (1) (2009) 303–313.
- [34] R. Gonzalez, et al., A putative mesenchymal stem cells population isolated from adult human testes, *Biochem. Biophys. Res. Commun.* 385 (4) (2009) 570–575.
- [35] M.F. Pittenger, et al., Multilineage potential of adult human mesenchymal stem cells, *Science* 284 (5411) (1999) 143–147.
- [36] O.K. Lee, et al., Isolation of multipotent mesenchymal stem cells from umbilical cord blood, *Blood* 103 (5) (2004) 1669–1675.
- [37] S. Bobis, D. Jarocha, M. Majka, Mesenchymal stem cells: characteristics and clinical applications, *Folia Histochem. Cytobiol.* 44 (4) (2006) 215–230.
- [38] N. Kim, S.G. Cho, Clinical applications of mesenchymal stem cells, *Korean J. Intern. Med.* 28 (4) (2013) 387–402.
- [39] V. Sabapathy, et al., Human Wharton's jelly mesenchymal stem cells plasticity augments scar-free skin wound healing with hair growth, *PLoS One* 9 (4) (2014), e93726.
- [40] C.-S. Lin, et al., Commonly used mesenchymal stem cell markers and tracking labels: limitations and challenges, *Histol. Histopathol.* 28 (9) (2013) 1109.
- [41] A. Lange, et al., Marrow cells cultured in MSC medium expand to CD73, CD90 and CD105 cells of fibroblast-like morphology, *Am Soc Hematology* (2005).
- [42] F. Ali, F. Aziz, N. Wajid, Effect of type 2 diabetic serum on the behavior of Wharton's jelly-derived mesenchymal stem cells in vitro, *Chron. Dis. Transl. Med.* 3 (2) (2017) 105–111.
- [43] M. Ayatollahi, T. Talaei-Khozani, M. Razmkhah, Growth suppression effect of human mesenchymal stem cells from bone marrow, adipose tissue, and Wharton's jelly of umbilical cord on PBMCs, *Iran. J Basic Med. Sci.* 19 (2) (2016) 145.
- [44] G. de Souza, et al., Bovine ovarian stem cells differentiate into germ cells and oocyte-like structures after culture in vitro, *Reprod. Domest. Anim.* 52 (2) (2017) 243–250.
- [45] S.M. Shah, et al., Cumulus cell-conditioned medium supports embryonic stem cell differentiation to germ cell-like cells, *Reprod. Fertil. Dev.* 29 (4) (2017) 679–693.
- [46] L. Mirzaeian, et al., Induction of mouse peritoneum mesenchymal stem cells into germ cell-like cells using follicular fluid and cumulus cells-conditioned media, *Stem Cell. Dev.* 28 (8) (2019) 554–564.