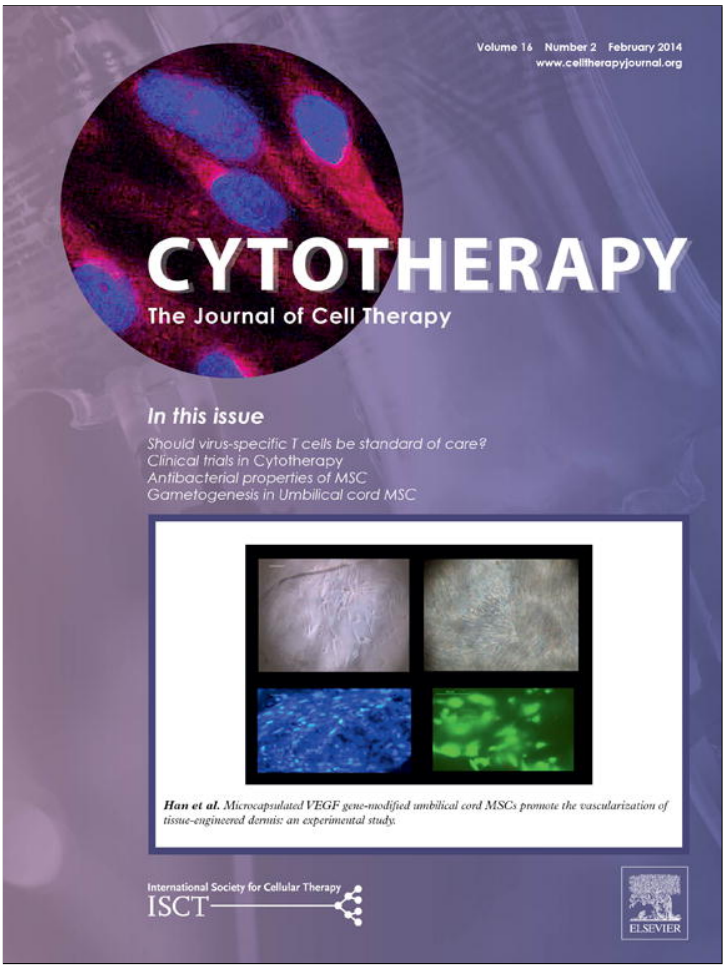

Cytotherapy, 2014; 16: 203–212

International Society for Cellular Therapy
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Evaluation of gametogenic potential of vitrified human umbilical cord Wharton's jelly–derived mesenchymal cells

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Abstract

Background aims. Vitrification as an advanced cryopreservation method is recommended for cell storage toward future applications. The purpose of this report was to appraise whether gametogenic potential of these cells is altered by vitrification. *Methods.* A two steps method was applied for hUCM cells vitrification. An n-hUCM group of hUCM cells served as control. In order to differentiation of hUCM cells into male germ cells, the cells were induced by retinoic acid, testosterone and testicular-cell-conditioned medium. To evaluate induced hUCM cells toward germ cells, we used immunocytochemistry and karyotyping methods. *Results.* v-hUCM cells similar to n-hUCM cells formed flat cells after gametogenic induction, and showed protein expression of germ-cell-specific markers DAZL, VASA (DDX4) and SCP3. Karyotyping pattern remained unchanged in the either groups. *Conclusions.* The analysis of these results demonstrates that vitrification does not alter differentiation potential of hUCMs to male germ like cells. These results may set an in vitro pattern to study germ-cell formation from hUCM cells and also as a potential source of sperms for male infertility.

Key Words: *gametogenic potential, mesenchymal cells, umbilical cord, vitrification*

Introduction

Human umbilical cord matrix–derived mesenchymal (hUCM) cell culture has advanced in recent years. Proliferative capacity, multipotentiality and noninvasive procedure to access and isolate hUCM cells are among the advantages of these cells (1). Invasive procedures for harvesting of bone marrow stem cells (BMSCs) (2) and adipose tissue–derived MSCs (3), the low rate of successfully isolating umbilical cord blood mesenchymal stromal cells (MSCs) (4), reduction of the quantities, differentiation potential and frequency of BMSCs with aging (2,5,6) and ethical reasons regarding the use of of embryonic stem cells (ESCs) (7) create limitations in the use of these stem cells. In contrast, MSCs isolated from Wharton's jelly of the human umbilical cord is closely lacking of imperfections, as previously mentioned (8).

To bank cells for future applications in clinics and laboratories, cryopreservation is critical (9). Vitrification (glass-like solidification of a solution) is more desirable than is conventional slow freezing. In vitrification, the defects of slow freezing such as damage caused by cytoplasmic ice crystal formation (10), the requirement of an expensive programmable freezer and time-consuming procedures are eliminated (11). As a cryopreservation method, vitrification has been successfully applied for human cord blood hematopoietic progenitor cells (12), human embryonic stem cells (hESCs) (13,14) and human amnion-derived MSCs (15). We have previously reported on the vitrification of hUCMs (16).

Trials for application of stem cells in reproductive medicine have been an important concern for many years. In the past decade, researchers have reported *in vitro* differentiation of ESCs (17–22) and adult stem cells such as BMSCs (23), fetal porcine skin stem cells (24) and clonal pancreatic stem cells (25) into germ line

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(Received 28 July 2013; accepted 27 October 2013)

ISSN 1465-3249
http://dx.doi.org/10.1016/j.jcyt.2013.10.015

cells. One study was carried out wherein hUCM cells were induced toward male germ cells (26). Human UCM cells are being considered as a non-immunogenic (27) source of stem cells capable of differentiating into various cell types suitable for experimental (28,29) and clinical studies, especially regenerative medicine (30). Whether vitrification would alter differentiation capacity of hUCM cells has not been investigated. Regardless of existent results, there is long way to achievement of final goals. Therefore, in the present study, we evaluate the gametogenic potential of hUCM cells after vitrification.

Methods

Isolation and culture of hUCM cells

All materials were purchased from Sigma-Aldrich Co (Sigma-Aldrich, St. Louis, MO, USA) except for the cases mentioned in the text. Ethics approval was obtained from the ethics committee at Kerman University of Medical Sciences, Kerman, Iran. After informed consent was given by mothers in the Afzalipour hospital gynecology ward in Kerman University of Medical Sciences, fresh human umbilical cords (UCs) from normal, full-term newborn infants were obtained during cesarean section. UCs were transferred to the laboratory in Hanks' balanced salt solution supplemented with 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate, and tissue processing was performed in less than 24 h from acquisition. After removal of blood vessels, the Wharton's jelly was scraped from the amnion and apportioned to 2- to 3-mm pieces. These pieces were explanted onto 35 × 10 mm petri dishes (Falcon BD, Franklin Lakes, NJ, USA) and cultured in a general culture medium of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum (PA Biologicals, Sydney, Australia), 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate in a humidified 37°C incubator with 5% CO_2 in air. After removal of Wharton's jelly pieces subsequent to cell bud appearance, cell culture was continued to >80% confluence.

Flow cytometry analysis

For evaluation of surface antigen expression, approximately 1×10^5 4th-passage viable hUCM cells were suspended in washing buffer containing 2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS). After centrifugation, 4% paraformaldehyde was added and samples were incubated for 15 min at 4°C. Subsequent to washing, the cells were suspended in 10% normal goat serum in PBS. Samples were then incubated for 15 min at 4°C. After cell washing, the cells were labeled with the following antibodies: CD44–fluorescein-isothiocyanate (FITC), CD90-FITC, CD45-FITC and CD34-FITC for 1 h at 4°C. Finally, the cells were suspended in 4% paraformaldehyde and the examination was conducted. A fluorescence-activated cell sorter (FACS Calibur; Becton Dickinson, San Jose, CA, USA) equipped with Cell Quest software was used for antibody binding evaluation.

Evaluation of the differentiation potential of hUCM cells

Cultured cells (at passages 4–5) at a density of 1×10^3 cells/cm^2 on glass coverslips were incubated in adipogenic differentiation medium, osteogenic differentiation medium and chondrogenic differentiation medium kits (Invitrogen, Carlsbad, CA, USA). Each medium was changed every 3–4 days. After completion of the differentiation period, histochemical staining was performed. Cells induced with the osteogenic, adipogenic and chondrogenic formulas were stained with alizarin red S and alkaline phosphatase, oil red O and toluidine blue, respectively, to reveal these differentiations.

Vitrification of hUCM cells

Vitrification was performed by application of a two-step exposure to vitrification solution on hUCM cells at passages 2–4, described previously (15). Initially, a pellet of 1×10^6 hUCM cells was mixed with 50 µL of equilibration solution including 20% ethylene glycol (EG), which was based on DPBS (Dulbecco's phosphate-buffered saline) containing 20% FBS for 5 min. Afterward, the cell pellet was suspended in 500 µL of vitrification solution, including 40% EG, 18% Ficoll 70 and 0.3 mol/dm^3 of sucrose, which was based on DPBS containing 20% FBS for 40 seconds. Finally, suspended hUCM cells were immediately loaded into 1.5-mL cryovials (Nunclon, Roskilde, Denmark) and plunged directly into liquid nitrogen. After 24 h (31), the cryovial was submerged in a 37°C water bath. The warmed cells were suspended serially in 0.5, 0.25 and 0 mol/L sucrose in DPBS containing 20% FBS. The cells were plated at a density of 3×10^3 to 5×10^3 cells/cm^2 in a culture flask and subcultured to >80% confluence.

Testicular cell–conditioned medium preparation

Testicular cell cultures (TCCs) were prepared according to a previously reported protocol (20). Briefly, 10 testes from 1- to 3-day-old newborn mice were ripped into pieces in trypsin–ethylenediaminetetraacetic acid (EDTA) solution. Subsequent to 5-min trypsinization, the suspension was centrifuged for 3 min at 300*g*. The sediment was mixed with 1 mL of 10% FBS in DPBS and was incubated at room temperature for 10 min. The top 0.8 mL was then

removed and mixed with 11 mL of DMEM medium supplemented with 10% FBS, 1% nonessential amino acids and 1% penicillin/streptomycin solution. The suspension was allocated into a six-well culture dish (31). TCCs were incubated in 5% CO_2 at 37°C to >80% confluence. The harvested cells were diluted at 1:2 ratio and replated into new wells. Ten days after cultures were started, conditioned medium was collected from cultures with evident germ cell proliferation. TCC was collected every 3 days for 30 days from the start of the culture, filtered and stored at −20°C for further use.

Gametogenic induction of hUCM cells

Non-vitrified hUCM (n-hUCM) and vitrified hUCM (v-hUCM) cells at the 4th passage were induced by differentiation medium containing HDMEM (high glucose Dulbecco's modified Eagle medium) supplemented with 5% FBS, 50% filtered testicular cell−conditioned medium, 2 mmol/L retinol acetate and 1 mmol/L testosterone. During the 21-day differentiation period, half of the medium was exchanged every 3−4 days. n-hUCM and v-hUCM cells at the 4th passage were considered as the control group and were cultured in the general culture medium.

RNA isolation, complementary DNA synthesis and SYBR green real-time reverse transcriptase−polymerase chain reaction

At days 7 and 21 of gametogenic induction, total RNA was extracted from n-hUCM and v-hUCM cells. RNA extraction was performed with the use of the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Crawley, UK). After RNA integrity was checked by means of denaturing agarose gel electrophoresis and ethidium bromide staining, complementary DNA (cDNA) was synthesized by use of the Omniscript RT Kit (Qiagen).

Quantitative polymerase chain reactions (qPCR) were performed in duplicate on each sample of cDNA. Aforementioned reactions were accomplished with the use of the Maxima SYBR green/ROX qPCR Master Mix (Fermentas, Burlington, Canada) in the presence of forward and reverse primers. SYBR green PCR amplifications were initiated at 95°C for 10 min followed by 40 cycles of 95°C for 15 seconds for denaturation and 60°C for 60 seconds for annealing/extension.

We aimed to analyze qPCR on each sample with the use of the Rotor Gene 6000 machine (Qiagen) for *YWHAZ, GAPDH, VASA, DAZL* and *SCP3* genes.

For SYBR green, equal reaction efficiencies were confirmed by serial dilution of cDNA. Negative controls (no cDNA) were considered in all experiments.

Detection of housekeeping gene expression in hUCM cell culture after different treatments

After approval of reverse transcriptase (RT)-PCR efficiency, the cycle threshold (Ct) values for *GAPDH* and *YWHAZ* genes were determined after different treatments on hUCM cell culture. In this step, it was revealed that the expression of the above-mentioned genes was affected after different treatments, including vitrification and germ cell differentiation.

Immunocytochemistry

To determine germ cell protein localization under various culture conditions established on glass coverslips, hUCM cultures were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 30 min. After three times of washing in PBS, the cells were incubated in PBS with 0.1% Triton X-100, 1% bovine serum albumin and 10% normal goat serum for 45 min. Finally, the harvested cells were incubated with anti-*VASA*, anti-*DAZL* and anti-*SCP3* for 60 min at room temperature or overnight at 4−8°C. Afterward, the cells were washed in PBS for 10 min and incubated with anti-rabbit immunoglobulin G−FITC for 1 h at room temperature. Last, the cells were washed three times with PBS and mounted with the use of p-phenylenediamine as an anti-fading agent. The cells were then observed under a fluorescence inverted microscope (IX71; Olympus, Tokyo, Japan) equipped with a digital camera.

Karyotyping

To determine n-hUCM and v-hUCM cell karyotype in different groups, cells were detached from culture flasks with the use of 0.25% trypsin-EDTA. The harvested cells were incubated for 45 min in 2 mL of DMEM with 15% FBS and 100 μL colchicin (Gibco-BRL, San Francisco, CA, USA). After centrifugation and removal of supernatant, the cells were lysed by means of incubation in warm hypotonic 0.075 mol/L KCl for 20 min at 37°C. The smears were washed in cold Carnoy's fixative solution three times. The slides were stained with a 1:1:8 Giemsa:H_2O:PBS solution for 10 min at room temperature, washed with tap water and left to dry. Chromosome sets in differentiated n-hUCM and v-hUCM cells were carefully examined at least three times individually. In each smear, at least 25 giemsa-stained chromosomeal plates were evaluated

with the use of a light microscope (×1000 magnification). The model number of chromosomes was concluded through analysis of 23 metaphases by karyotyping technique.

Statistical analysis

For comparison of post-thaw immunostaining rate, statistical analysis by means of the χ^2 test was performed. Statistically significant values were considered as $P < 0.05$.

Results

Features of hUCM cells

Ten days after the Wharton's jelly pieces were cultured, spindle-shaped, fibroblast-like cells were observed at the periphery of the explants. Flow cytometric analysis demonstrated that the cells expressed mesenchymal cell markers (CD44 and CD90) but did not express hematopoietic lineage markers (CD34, CD45) (Figure 1).

Osteogenic differentiation of hUCM cells was examined after 2–3 weeks with alizarin red S and alkaline phosphatase staining (Figure 2A,B). Calcium phosphate mineral accumulation and formation of alkaline phosphatase–positive aggregates were observed in cells. Chondrogenic differentiation of hUCM cells by means of toluidine blue staining of chondrogenic matrices was attained 3 weeks after incubation in chondrogenic medium (Figure 2C). Adipogenic differentiation was achieved after 10 days as accumulation of lipid-rich vacuoles in cell cytoplasm (Figure 2D).

Testicular cell culture

Cultivation of testicular cells resulted in two phenotypes. Some of the harvested cells showed a fibroblast-like phenotype with high affinity to attach to the substratum. Others were round germ cells that loosely attached to the substratum (Figure 3). Germ cells evidently proliferated at 5–10 days after beginning of the culture. These cells were single or clustered and propagated rapidly.

Morphological changes in n-hUCM and v-hUCM cells after treatment

The cell morphology was monitored alternatively under a phase contrast microscope. In the control groups of untreated n-hUCM and v-hUCM cells, the cell density was higher, with typical mesenchymal cell features on day 14 (Figure 4A,B). Subsequent to gametogenic induction of n-hUCM and v-hUCM cells, the cells appeared as flat cells with few cytoplasmic extensions after 2 weeks (Figure 4C–F). In the differentiation groups, the cell density was lower than in the control groups.

Evaluation of germ cell–specific gene expression

Evaluation of RNA integrity was performed on the extracted RNA. The high quality of RNA was confirmed by the appearance of 28S and 18S ribosomal bands on the staining gel.

In the next step, cDNA was synthesized. To follow the method of qPCR, the efficiency of the system was investigated with *GAPDH* and *YWHAZ* as housekeeping genes. Standard curves for two housekeeping

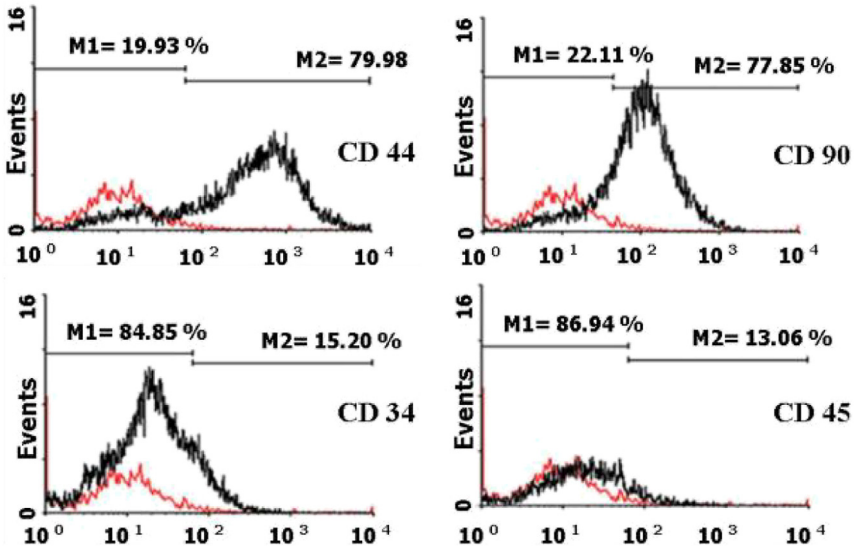

Figure 1. Flow cytometric analysis of hUCM cells. Red histograms show the isotype control-stained cells; black histograms show the antibody-stained cells.

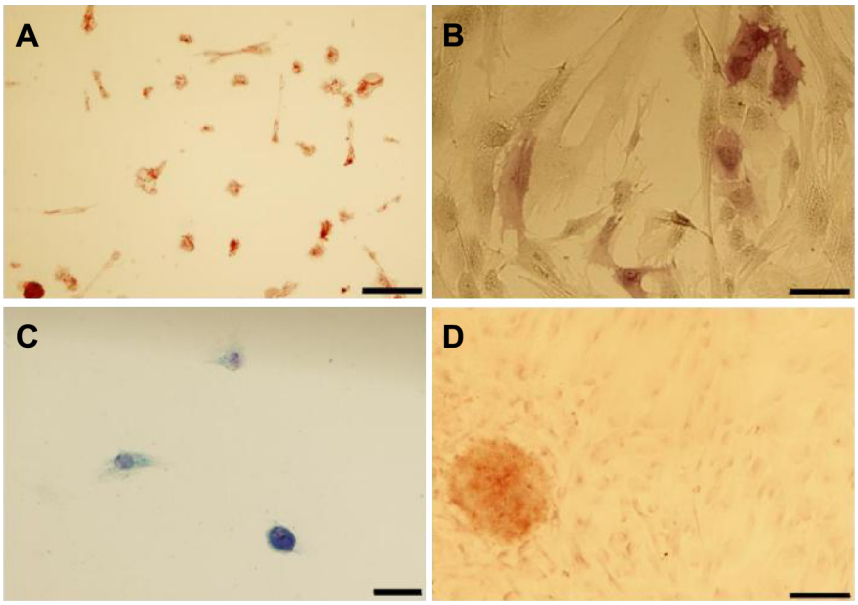

Figure 2. Osteogenic, chondrogenic and adipogenic differentiation of umbilical cord mesenchymal cells. In osteogenic induction, formation of calcium phosphate mineral accumulation and alkaline phosphatase–positive aggregates by means of alizarin red S staining (A) and alkaline phosphatase staining (B), respectively, is visible. During chondrogenic differentiation, extracellular matrix rich in sulfated proteoglycans is shown by toluidine blue staining (C). Subsequent to adipogenic induction, oil red O staining (D) shows lipid droplets in cytoplasm (scale bars: A: 40 μm; B, C and D: 20 μm).

genes, including *GAPDH* and *YWHAZ*, were generated from the mean of Ct values, and the data were fitted in a linear relationship with 10-fold serially diluted cDNA. The slopes of the plots and R^2 values for *GAPDH* and *YWHAZ* were $y = 3.284x + 18.626$, $R^2 = 0.9939$, and $y = 3.753x + 11.87$, $R^2 = 0.990$, respectively.

Detection of housekeeping gene expression in various treatments on hUCM cells

After efficiency definition, Ct values were determined for various treatments with the use of *GAPDH* and *YWHAZ* to discover appropriate housekeeping genes (Figures 5 and 6). In this step, it was revealed that these genes are affected by various treatments including vitrification and germ cell differentiation and therefore could not be used as housekeeping genes in this experiment.

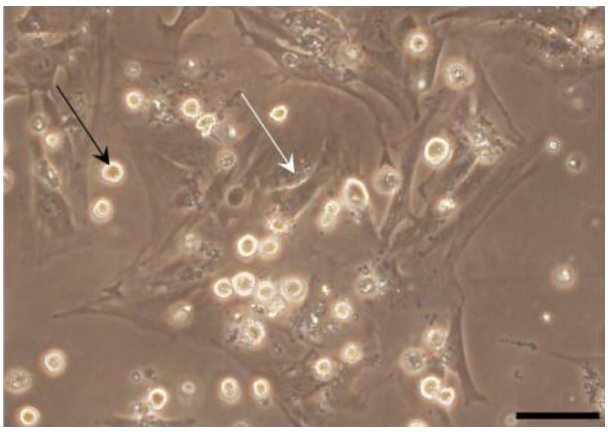

Figure 3. Testicular cells at day-5 cultivation. Germ cells indicated by black arrows show a round morphology; somatic cells indicated by white arrows appear as fibroblast-like cells (scale bar: 20 μm).

At day 21 after incubation with differentiation media, the presence of germ cell–specific proteins including DAZL, VASA and SCP3 in differentiated n-hUCM and v-hUCM cells were detected by immunohistochemical analysis (Figure 7). Under differentiation conditions, 58% ± 2.8% and 53% ± 3.5% of the n-hUCM and v-hUCM cells expressed DAZL protein, respectively. However, 31.5% ± 0.7% and 26% ± 23.3% showed VASA-positive staining, and after staining of hUCM cells with SCP3 antibody, 29.5% ± 21.2% and 29% ± 7.1% of the induced cells were positive.

There was no statistical difference between the two groups of cells. Negative controls, in which the primary antibodies were removed, showed negligible fluorescence.

Karyotyping study

After cytologic analysis of differentiated n-hUCM and v-hUCM cells, we determined that the cells retained the diploid state and had not entered meiosis cell division (Figure 8).

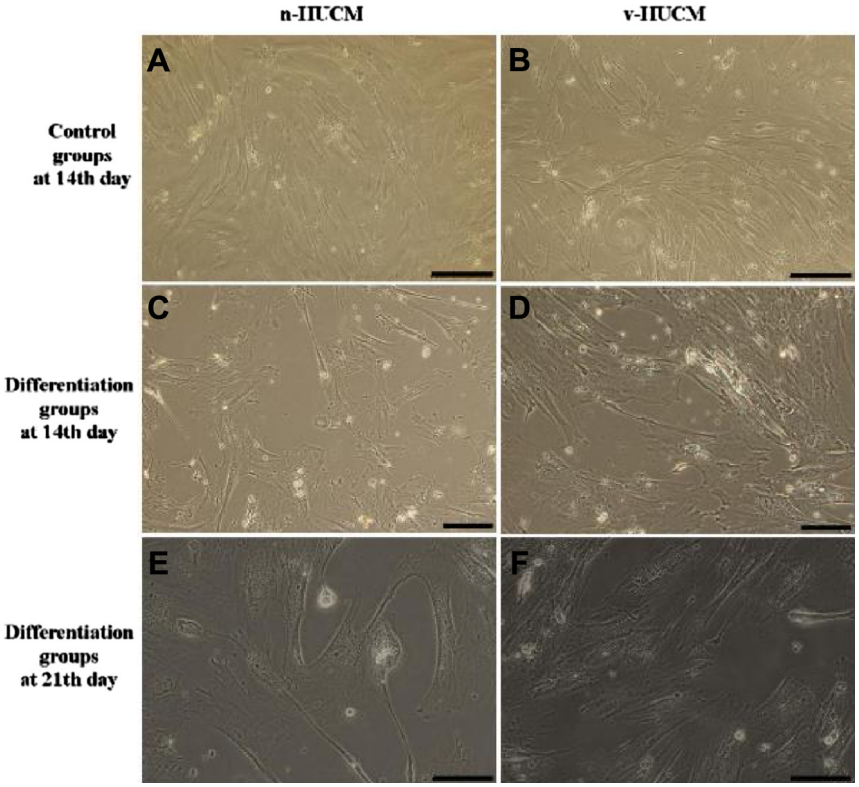

Figure 4. Morphological changes in different groups of cultured hUCM cells. Differentiated cells (C–F) are compared with undifferentiated cells (A,B) (scale bars: A, B, E and F: 50 μm; C and D: 20 μm).

Discussion

Our research attempts to evaluate whether differentiation potential of hUCM cells toward male germ cells is affected by vitrification. To achieve this aim, a two-step vitrification protocol that was based on ethylene glycol as a cryoprotectant and rapid warming was used.

Our criterion for the evaluation of hUCM cells was provided by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy guidelines (32). Adipogenic, osteogenic and chondrogenic differentiation potential and surface antigen expression (positive for CD44 and CD90; negative for CD34 and CD45) of hUCM cells indicated mesenchymal characteristics of these cells, and our previous research has shown that vitrification exerts no significant effect on these features. The absence of a significant difference between differentiation potential of v-hUCM and n-hUCM into male germ-like cells indicates that vitrification is an effective and recommendable method for cryopreservation of hUCM cells. Success in cryopreservation of hUCM cells is probably the result of the use of vitrification solution containing EG as a permeable cryoprotectant. The use of EG has been recommended because of low toxicity and high permeability properties (33). The use of cryoprotectants with low toxicity is an essential element in all vitrification solutions (10). In the current study, the vitrification solution EFS40, containing 40% EG, was used; this has been

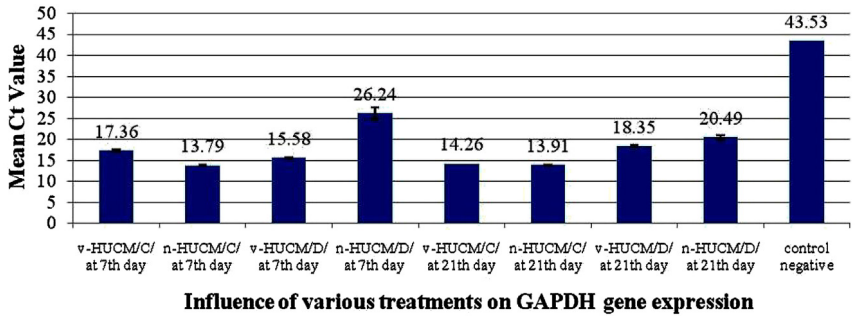

Figure 5. Evaluation of Ct values in various treatments with the use of *GAPDH* gene primers. D indicates differentiation group.

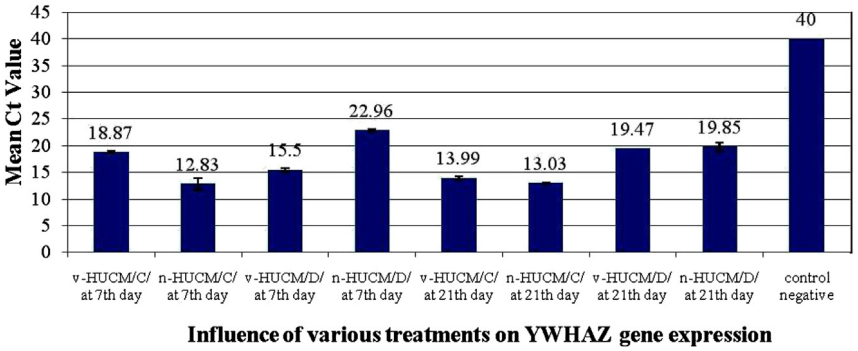

Figure 6. Evaluation of Ct values in various treatments with the use of *YWHAZ* gene primers. D indicates differentiation group.

extensively applied in the successful vitrification of mouse embryos (34,35), human blastocysts (36) and embryonic stem cells (14). Compared with other reports on the traditional slow cryopreservation of MSCs, the cryopreservation protocol used in this study has advantages. Complete removal of damage caused by intracellular ice crystal formation, which is the main source of damage and disruption of cytoplasm, appears to be difficult in the slow-freezing method (37). Cryopreservation of stem cells by means of the traditional slow-freezing method is time-consuming and requires a programmable temperature container, but these difficulties have been overcome by vitrification. In the vitrification procedure, after the use of high concentrations of cryoprotectant and rapid cooling, glass-like freezing occurs without ice crystal formation (33). Kuleshova and Lopata (38) in 2002 have shown vitrification advantages compared with the previous freezing techniques. These advantages are (i) control of cryoprotectant permeation and dehydration rate, (ii) prevention of long-term thermal shock and damage caused by ice crystal formation and (iii) affordable equipment.

In the present study, the effects of vitrification on the differentiation potential of hUCM cells to the male germ cells have been explored. Although the

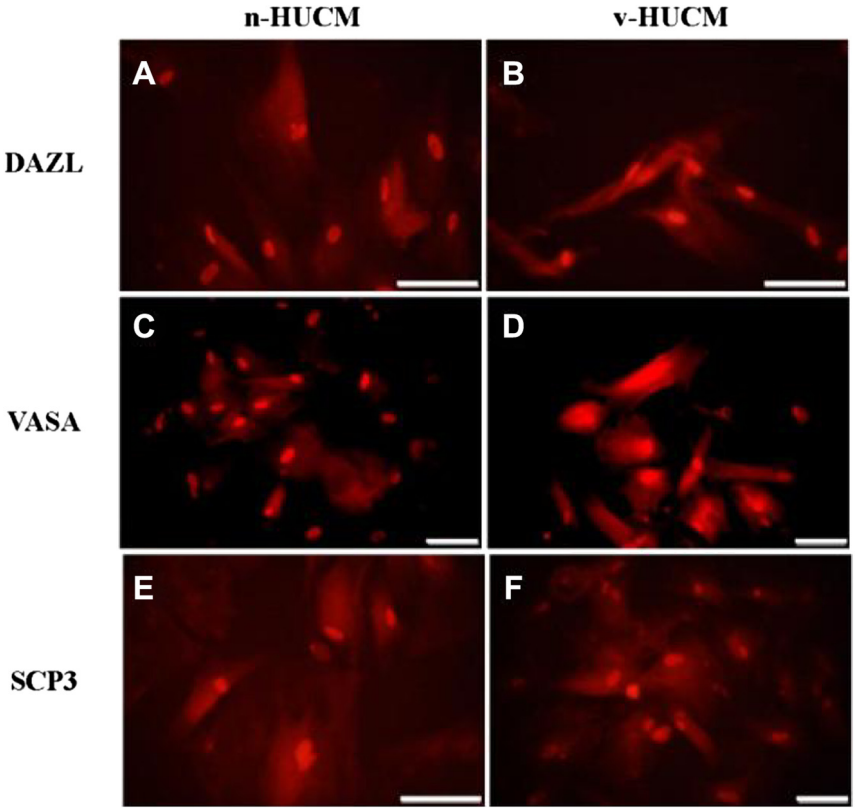

Figure 7. Immunofluorescence of germ cell–specific markers. *DAZL* is mainly expressed in the nuclei and in some cases in the cytoplasm. *VASA* expression is mainly observed in the cytoplasm and in some nuclei. *SCP3* is expressed in both cytoplasm and nuclei (scale bars: 20 μm).

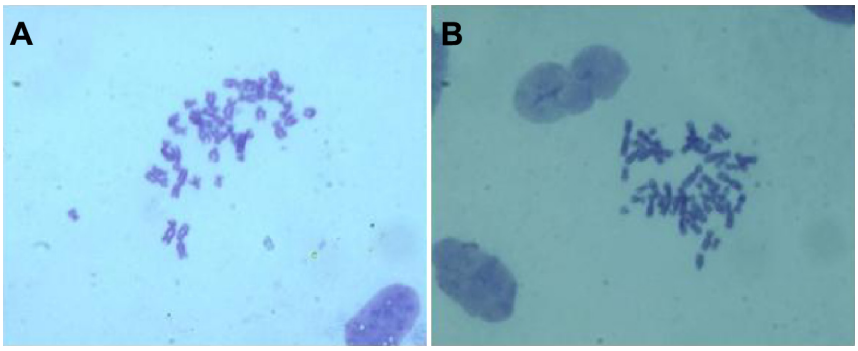

Figure 8. Diploid karyotype of treated n-hUCM (A) and v-hUCM (B) to germ-like cells.

gametogenic potential of embryonic stem cells and somatic stem cells such as BMSCs, pancreatic stem cells and porcine skin stem cells have been studied (17–25), it appears that hUCM cells have preference because of characteristics such as the lack of ethical prohibition, easy access and proliferation capacity (1). Huang *et al.* (26) in 2010 attempted to produce male germ cells with the induction of hUCM cells in differentiation medium for 14 days; they showed expression of germ cell markers Oct4, Ckit, CD49f, Stella and Vasa in the induced cells. In the present study, we showed through the use of immunocytochemistry a comparable expression of the gametogenic markers as well as messenger RNA in vitrified and non-vitrified cells after 21-day induction. We observed some morphological changes in n-hUCM and v-hUCM cells after induction. We examined the pre-meiotic germ cell marker DAZL and later germ cell markers such as VASA and SCP3. Expression of VASA *in vivo* and *in vitro* represents an irreversible commitment toward germ lineage cells (39). It has been reported that VASA is not only in the cytoplasm but also is in the nucleus of some germ cells (40). In the present study, the VASA protein was expressed in the nucleus and cytoplasm of each group of cells without significant differences between groups. Gene family members deleted in azoospermia *(DAZL)* is exclusively expressed in generating cells; DAZL protein, which is one of them, is present in most of the life period of germ cells and is necessary for differentiation and maturation of germ cells (41,42). In this study, DAZL protein was present both in the cytoplasm and the nucleus of n-hUCM and v-hUCM cells similarly at day 21. Synaptonemal complex proteins such as SCP3 are meiosis-special structural proteins that synapse homologous chromosomes. Synaptonemal complex formation begins during the leptoten stage of meiosis prophase; this protein locates in the nucleus. The cytoplasmic expression of SCP3 indicates recently synthesized protein that subsequently migrates to the nucleus (43). In the present study, SCP3 protein was detected in the cytoplasm and the nucleus of each group of cells at day 21. In addition to cellular SCP3 expression (as a meiosis marker), this issue indicated that the induced hUCM cells have entered a more advanced meiosis stage compared with Huang et al. study.

RT-qPCR analysis to determine messenger RNA expression is widely used in many studies conducted thus far on MSCs. However, because of different methods of data analysis and normalization, RT-qPCR studies on MSC *in vitro* and *in vivo* can be difficult (44). We selected and evaluated two housekeeping genes, *GAPDH* and *YWHAZ*, to achieve a suitable reference gene. Our results show that the expression of these genes was influenced by the processes of vitrification and germ cell differentiation. We may conclude that *GAPDH* and *YWHAZ* genes as housekeeping genes have little relevance to real-time RT-PCR in the process of gametogenic induction of hUCM cells, vitrified and non-vitrified. However, Barber *et al.* (45) have shown that *GAPDH* and *YWHAZ* are stable reference genes for hUCM cells at the 7th passage. A review of studies on BMSCs shows that *GAPDH* is one of the most commonly used genes for normalization of data in RT-qPCR, whereas the high levels of variability in expression of this gene were seen in homogeneous populations of human MSC such as MIAMI (marrow-isolated adult multilineage inducible) cells derived from the bone marrow, in contrast to its common use in research of human MSCs (46–49). Researchers have shown that *GAPDH* is not reliable gene, and *EF1a, RPL13a* and *YWHAZ* are more suitable genes for data normalization in BMSC studies (44). Reports have shown that the stability of gene expression for the reference genes to specific tissues (eg, cell lines) and different experimental treatments must be examined and approved before real-time RT-PCR analysis is started (50–52). According to the results of the present study showing high variability of expression of these genes, it is necessary to evaluate a panel of different genes to find the best housekeeping gene whose expression is constant in the various treatments on hUCMs.

A karyotyping study on n-hUCM and v-hUCM cells showed that these cells have not entered the meiosis stage but have arrested in the pre-meiotic stages. Nayernia *et al.* (21) first demonstrated that mouse embryonic stem cell–derived gametes can successfully fertilize mouse oocytes to create live mice. However, under these conditions, the morphology of the sperm was abnormal and the mouse size was smaller or larger than the control mice and had died between 5 days to 5 months after birth (21). In the other study that carried out on the differentiation of human embryonic stem cells to embryonic-like body; gene expression showed that meiotic development markers (such as VASA, SCP1 and SCP3) and specific male gamete gene (TEKT1), were expressed in the 3 days embryonic body and reached their maximum level of expression in the 14 days old embryonic bodies (17). Kee *et al.* (53) have shown that BMP (bone morphogenic protein) factors are involved in germ cell formation from human embryonic stem cells; they showed that by the use of these factors, *VASA* and *SCP3* gene expression would have been significantly increased. However, to our knowledge, no report has already been published on human ESC differentiation to post-meiotic germ cells.

Although this study shows that the human umbilical cord Wharton's jelly mesenchymal cells *in vitro* can be induced toward the germ cell differentiation, the process of vitrification did not alter this process, but, to ensure progression of these cells in the meiotic cell route and obtaining the functional sex cells, further investigation is needed.

Acknowledgments

This work was supported by Physiology Research Centre of Kerman University of Medical Sciences. The authors thank the Yazd Genetic Laboratory of Research and Clinical Centre of infertility staff for their valuable assistance in the karyotyping study.

Disclosure of interests: The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

References

1. Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, et al. Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. Stem Cells. 2007;25:319–31.
2. Bieback K, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells. 2004;22:625–34.
3. Goh BC, Thirumala S, Kilroy G, Devireddy RV, Gimble JM. Cryopreservation characteristics of adipose-derived stem cells: maintenance of differentiation potential and viability. J Tissue Eng Regen Med. 2007;1:322–4.
4. Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, et al. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. Cell Physiol Biochem. 2004;14:311–24.
5. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem. 1997;64:278–94.
6. Cui XD, Gao DY, Fink BF, Vasconez HC, Pu LL. Cryopreservation of human adipose tissues. Cryobiology. 2007;55:269.
7. Eggan K, Jurga S, Gosden R, Min IM, Wagers AJ. Ovulated oocytes in adult mice derive from non-circulating germ cells. Nature. 2006;441:1109–14.
8. Mareschi K, Biasin E, Piacibello W, Aglietta M, Madon E, Fagioli F. Isolation of human mesenchymal stem cells: bone marrow versus umbilical cord blood. Haematologica. 2001;86:1099.
9. Hanna J, Hubel A. Preservation of stem cells. Organogenesis. 2009;5:134–7.
10. Kasai M, Mukaida T. Cryopreservation of animal and human embryos by vitrification. Reprod Biomed Online. 2004;9:164–70.
11. Karlsson JO. Cryopreservation: freezing and vitrification. Science. 2002;296:655–6.
12. Kurata H, Takakuwa K, Tanaka K. Vitrification of hematopoietic progenitor cells obtained from human cord blood. Bone Marrow Transplant. 1994;14:261–3.
13. Reubinoff BE, Pera MF, Vajta G, Trounson AO. Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. Hum Reprod. 2001;16:2187–94.
14. Fujioka T, Yasuchika K, Nakamura Y, Nakatsuji N, Suemori H. A simple and efficient cryopreservation method for primate embryonic stem cells. Int J Dev Biol. 2004;48:1149–54.
15. Moon JH, Lee JR, Jee BC, Suh CS, Kim SH, Lim HJ, et al. Successful vitrification of human amnion-derived mesenchymal stem cells. Hum Reprod. 2008;23:1760–70.
16. Ezzatabadipour M, Kaviani M. Vitrification of human umbilical cord Wharton's jelly-derived Mesenchymal stem cells. Cryo Letters. 2013;34:471–80.
17. Clark AT, Bodnar MS, Fox M, Rodriquez RT, Abeyta MJ, Firpo MT, et al. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. Hum Mol Genet. 2004;13:727–39.
18. Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature. 2004;427:148–54.
19. Hubner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, et al. Derivation of oocytes from mouse embryonic stem cells. Science. 2003;300:1251–6.
20. Lacham Kaplan O, Chy H, Trounson A. Testicular cell conditioned medium supports differentiation of embryonic stem cells into ovarian structures containing oocytes. Stem Cells. 2006;24:266–73.
21. Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, Drusenheimer N, et al. In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. Dev Cell. 2006;11:125–32.
22. Toyooka Y, Tsunekawa N, Akasu R, Noce T. Embryonic stem cells can form germ cells in vitro. Proc Natl Acad Sci U S A. 2003;100:11457–62.
23. Nayernia K, Lee JH, Drusenheimer N, Nolte J, Wulf G, Dressel R, et al. Derivation of male germ cells from bone marrow stem cells. Lab Invest. 2006;86:654–63.
24. Dyce PW, Wen L, Li J. In vitro germline potential of stem cells derived from fetal porcine skin. Nat Cell Biol. 2006;8:384–90.

25. Danner S, Kajahn J, Geismann C, Klink E, Kruse C. Derivation of oocyte-like cells from a clonal pancreatic stem cell line. Mol Hum Reprod. 2007;13:11–20.
26. Huang P, Lin LM, Wu XY, Tang QL, Feng XY, Lin GY, et al. Differentiation of human umbilical cord Wharton's jelly derived mesenchymal stem cells into germ like cells in vitro. J Cell Biochem. 2010;109:747–54.
27. Cho PS, Messina DJ, Hirsh EL, Chi N, Goldman SN, Lo DP, et al. Immunogenicity of umbilical cord tissue-derived cells. Blood. 2008;111:430–8.
28. Latifpour M, Nematollahi-Mahani SN, Deilamy M, Azimzadeh BS, Eftekhar-Vaghefi SH, Nabipour F, et al. Improvement in cardiac function following transplantation of human umbilical cord matrix-derived mesenchymal cells. Cardiology. 2011;120:9–18.
29. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells. 2004;22:1330–7.
30. Lindenmair A, Hatlapatka T, Kollwig G, Hennerbichler S, Gabriel C, Wolbank S, et al. Mesenchymal stem or stromal cells from amnion and umbilical cord tissue and their potential for clinical applications. Cells. 2012;1:1061–88.
31. Kader A, Agarwal A, Sharma R, Falcone T. Vitrification of isolated mice blastomeres using a closed loading device. Reprod Biol Endocrinol. 2009;7:17.
32. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, et al. Minimal criteria for defining multipotent mesenchymal stromal cells: the International Society for Cellular Therapy position statement. Cytotherapy. 2006;8:315–7.
33. Chian RC, Kuwayama M, Tan L, Tan J, Kato O, Nagai T. High survival rate of bovine oocytes matured in vitro following vitrification. J Reprod Dev. 2004;50:685.
34. Kasai M, Komi JH, Takakamo A, Tsudera H, Sakurai T, Machida T. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J Reprod Fertil. 1990;89:91–7.
35. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. Reprod Biomed Online. 2006;12:779–96.
36. Cho HJ, Son WY, Yoon SH, Lee SW, Lim JH. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. Hum Reprod. 2002;17:2419–22.
37. Youssry M, Ozmen B, Zohni K, Diedrich K, Al-Hasani S. Current aspects of blastocyst cryopreservation. Reprod Biomed Online. 2008;16:311–20.
38. Kuleshova LL, Lopata A. Vitrification can be more favorable than slow cooling. Fertil Steril. 2002;78:449–54.
39. Pirouz M, Valadbeigy T, Shahverdi A, Baharvand H. In vitro production of germ cells from stem cells: hypes and hopes. Iran Anatomical Sci. 2009;6:591–626.
40. Anderson RA, Fulton N, Cowan G, Coutts S, Saunders PTK. Conserved and divergent patterns of expression of DAZL, VASA and OCT4 in the germ cells of the human fetal ovary and testis. BMC Dev Biol. 2007;7:136.
41. Cauffman G, Van de Velde H, Liebaers I, Van Steirteghem A. DAZL expression in human oocytes, preimplantation embryos and embryonic stem cells. Mol Hum Reprod. 2005;11:405–11.
42. Rocchietti-March M, Weinbauer GF, Page DC, Nieschlag E, Gromoll J. Dazl protein expression in adult rat testis is upregulated at meiosis and not hormonally regulated. Int J Androl. 2000;23:51–6.
43. Pousette, Leijonhufvud P, Arver S, Kvist U, Pelttari J, Hg C. Presence of synaptonemal complex protein 1 transversal filament-like protein in human primary spermatocytes. Hum Reprod. 1997;12:2414–7.
44. Curtis K, Gomez L, Rios C, Garbayo E, Raval A, Perez-Pinzon M, et al. EF1a and RPL13a represent normalization genes suitable for RT-qPCR analysis of bone marrow derived mesenchymal stem cells. BMC Mol Biol. 2010;11:61.
45. Barber RD, Harmer DW, Coleman RA, Clark BJ. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genom. 2005;21:389–95.
46. Block GJ, Ohkouchi S, Fung F, Frenkel J, Gregory C, Pochampally R, et al. Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1. Stem Cells. 2009;27:670–81.
47. Gang EJ, Darabi R, Bosnakovski D, Xu Z, Kamm KE, Kyba M, et al. Engraftment of mesenchymal stem cells into dystrophin-deficient mice is not accompanied by functional recovery. Exp Cell Res. 2009;315:2624–36.
48. Leonardi E, Ciapetti G, Bagl SR, Devescovi V, Baldini N, Granchi D. Osteogenic properties of late adherent subpopulations of human bone marrow stromal cells. Histochem Cell Biol. 2009;132:547–57.
49. Lu H, Kawazoe N, Tateishi T, Chen G, Jin X, Chang J. In vitro proliferation and osteogenic differentiation of human bone marrow-derived mesenchymal stem cells cultured with hardystonite (Ca2ZnSi 2O7) and beta-TCP ceramics. J Biomater Appl. 2009;25:39–56.
50. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol. 2000;25:169–93.
51. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation: strategies and considerations. Genes Immunity. 2005;6:279–84.
52. Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. Biotechniques. 2005;39:75.
53. Kee K, Gonsalves JM, Clark AT, Pera RA. Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells. Stem Cells Dev. 2006;15:831–7.