

Human amniotic fluid stem cells (hAFSCs) expressing p21 and cyclin D1 genes retain excellent viability after freezing with (dimethyl sulfoxide) DMSO

Shiva Gholizadeh-Ghaleh Aziz¹, Zahra Fardiyazar^{2,3}, Fatima Pashaei-Asl⁴, Mohammad Rahmati-Yamchi⁵, Khodadad Khodadadi⁶, Maryam Pashaiasl^{2,3,7*}

¹Department of Biochemistry, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran, ²Department of Molecular Medicine, School of Advanced Medical Science, Tabriz University of Medical Science, Tabriz, Iran, ³Women's Reproductive Health Research Center, Tabriz University of Medical Sciences Tabriz, Iran, ⁴Molecular Biology Laboratory, Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran, ⁵Department of Clinical Biochemistry, Tabriz University of Medical Science, Tabriz, Iran, ⁶Murdoch Childrens Research Institute, Royal Children's Hospital, Melbourne, Australia, ⁷Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

ABSTRACT

Human amniotic fluid stem cells (hAFSCs) have features intermediate between embryonic and adult SCs, can differentiate into lineages of all three germ layers, and do not develop into tumors *in vivo*. Moreover, hAFSCs can be easily obtained in routine procedures and there is no ethical or legal limitations regarding their use for clinical and experimental applications. The aim of this study was to assess the effect of slow freezing/thawing and two different concentrations of DMSO (10% DMSO + 90% fetal bovine serum [FBS] and 5% DMSO + 95% FBS) on the survival of hAFSCs. hAFSCs were obtained from 5 pregnant women during amniocentesis at 16–22 weeks of gestation. The expression of pluripotency markers (Octamer-binding transcription factor 4 [Oct4] and NANOG) by reverse transcription polymerase chain reaction and cell surface markers (cluster of differentiation [CD31], CD44, CD45, and CD90) by flow cytometry was analyzed before and after the slow-freezing. Cell viability was assessed by trypan blue exclusion or MTT assay. Quantitative mRNA expression of Oct4, NANOG, cyclin D1 and p21 was determined by real-time PCR before and after the slow-freezing. Pluripotency of hAFSCs was confirmed by *NANOG* and *POU5F1* (Oct4) gene expression before and after slow-freezing. All hAFSC cultures were positive for CD44 and CD90. A higher viability of hAFSCs was observed after freezing with 90% FBS + 10% DMSO. There was increased expression of *NANOG* and decreased expression of *POU5F1* gene after freezing, compared to control cells (before freezing). DMSO and the process of freezing did not significantly change the expression of p21 and cyclin D1 genes in hAFSCs. Overall, our results indicate the applicability of slow-freezing and DMSO in cryopreservation of SCs.

KEY WORDS: Human amniotic fluid stem cells; hAFSCs; dimethyl sulfoxide; DMSO; cryopreservation; biopreservation; cell viability; p21; cyclin D1; Oct4 (*POU5F1*); *NANOG*; pluripotency

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INTRODUCTION

Stem cells (SCs) have the ability to self-renew for long periods and to differentiate into specialized cells, through symmetric and asymmetric cell divisions [1]. SCs are classified into two broad types: embryonic stem cells (ESCs) and tissue-specific (adult or somatic) SCs. ESCs are pluripotent cells, found only in the inner cell mass (ICM) of the blastocyst during early development, and are capable of differentiating into all

derivatives of the germ layers, i.e., ectoderm, mesoderm and endoderm. On the other hand, somatic SCs are more specialized compared to ESCs, they remain in the body throughout life, and are specific to the tissue or organ in which they occur. Due to their multipotentiality as well as immunosuppressive properties in some cases, adult SCs are regarded as promising candidates in regenerative cell-based therapies and other clinical and experimental applications.

Mesenchymal stromal cells (MSCs), also named mesenchymal stem cells, are a type of adult SCs that are able to differentiate into various cell types, including chondrocytes, osteocytes, adipocytes, and myocytes. Up until now, bone marrow (BM) has been the most common source for MSCs. However, isolation of MSCs from BM is an invasive,

*Corresponding author: Maryam Pashaiasl, Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Golgasht Street Iran. Phone: +984133342086; Fax: +984133342086. E-mail: pashaim@tbzmed.ac.ir

time-consuming, and expensive procedure, painful for the patient. Also, preservation and banking of BM-derived MSCs (BM-MSCs) has a number of obstacles, such as the use of adequate cryopreservation protocols. In addition, the number and proliferation potential of BM-MSCs diminish with age [2,3].

Among the alternative sources of SCs that have been explored is the human umbilical cord (HUC). MSCs may be derived from different compartments of the HUC, including Wharton's jelly, cord lining, and the perivascular region. Some advantages of HUC- over BM-derived MSCs are abundant supply and painless collection of the cells [4]. However, the tissue processing and extraction procedures from the UC need to be optimized, to meet the specific conditions of birth environment [4].

Another potential source of SCs is amniotic fluid (AF). Human AF stem cells (hAFSCs) can be easily and safely obtained during amniocentesis, which is routinely performed at the second trimester to screen for genetic abnormalities of the fetus or to determine other characteristics, such as sex [5-7]. An additional advantage of human hAFSCs is that these SCs have a lower risk of tumorigenicity compared to ESCs [8,9].

Octamer-binding transcription factor 4 (Oct4) and NANOG are transcription factors required for self-renewal of undifferentiated ESCs and maintenance of their pluripotency, and the expression of these SC markers has also been demonstrated in hAFSCs [10], suggesting the potential of these cells in cell-based transplantation therapies [11,12].

Although a group of transcription factors is required for the establishment and maintenance of the ESC pluripotent state, Oct4 has the central role in this process [13,14]. Together with other core transcription factors (e.g., Sox2 and NANOG) or transcriptional repressive factors, Oct4 may respectively activate or suppress other protein-coding genes and related noncoding RNAs [15]. Moreover, in studies on induced pluripotent SCs (iPSCs) using bovine adult fibroblasts, the ectopic expression of Oct4, SOX2, KLF4, and c-MYC alone was not sufficient for stable induction of pluripotency in these cells and the additional expression of NANOG was critical for the production of stable bovine iPSCs, as confirmed by *in vitro* and *in vivo* experiments [16,17]. NANOG expression is observed in the ICM of human and mouse blastocysts, and is restricted to the pluripotent SCs and ES-like cells of the ICM in cattle. Downregulation of NANOG in human and mouse ESCs leads to their differentiation to extraembryonic lineages [18,19].

hAFSCs represent a valuable source of pluripotent SCs, as they possess characteristics intermediate between ESCs and adult SCs, can differentiate into lineages representative of all three germ layers, and do not develop into tumors in *in vivo* models. Moreover, hAFSCs can be easily obtained in routine procedures and there is no ethical or legal limitations regarding their use for clinical and experimental applications [20,21].

Nevertheless, the molecular profile of hAFSCs needs to be comprehensively investigated and compared to that of BM-MSCs, to understand the full therapeutic potential of these cells [22].

One of the most important issues in SC research is finding a suitable method for the preservation and maintenance of SCs over a long period of time, that preserves the multipotency and unique properties of these cells [23,24] necessary for their use in clinical applications and cell-based therapies [25].

Dimethyl sulfoxide (DMSO) has numerous applications in cell biology, among others, it is used as a cryoprotective agent in freezing-thawing of tissues and cells. Moreover, DMSO is a well-known inducer of cell differentiation. The positive and negative cell cycle regulators (such as cyclin D1 and p21) have been used as markers of DMSO effect on cell cycle arrest in the G₁-phase [26-28].

The aim of this study was to assess the effect of slow freezing/thawing and two different concentrations of DMSO (as a cryoprotectant) on the survival of hAFSCs. The cells were obtained from pregnant women during amniocentesis at 16–22 weeks of gestation. To determine the potency of the cells after a long period of cryopreservation, we analyzed the expression of pluripotency markers (Oct4 and NANOG) and cell surface markers (cluster of differentiation [CD31], CD44, CD45, and CD90), before and after the slow-freezing. Cell viability was analyzed by trypan blue exclusion or MTT assay. The effect of cryopreservation on cell cycle of hAFSCs was evaluated by determining the quantitative mRNA expression of p21 and cyclin D1.

MATERIALS AND METHODS

Materials

Dulbecco's modified eagle medium (DMEM), FBS, non-essential amino acids, 2-mercaptoethanol, and recombinant human basic fibroblast growth factor (bFGF) were purchased from GIBCO BRL Invitrogen Corp. (San Giuliano Milanese, Milan, Italy). Real-time PCR reagents were purchased from Takara Shuzo (Kyoto, Japan). Antibodies for fluorescence-activated cell sorting (FACS) analysis and immunocytochemistry (ICC) were acquired from BD Pharmingen (San Jose, CA, USA) or Abcam (Cambridge, MA, USA), and MTT powder was obtained from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade and used according to the instruction given by the manufacturer.

Isolation of hAFSCs

AF samples (5 mL) were obtained from 5 pregnant women (age range: 35–42 years; 16–22 weeks of gestation) undergoing amniocentesis at the Tabriz University of Medical Sciences

Al-Zahra Teaching Hospital, as described previously [10]. Written informed consent was obtained from patients prior to the procedure, according to the ethics committee guidelines (registration number 5.4.753 at ethic committee of TUMA). Cases with abnormal karyotype or malformations detected by ultrasound were excluded from the study.

Samples were centrifuged at 1500 rpm for 10 minutes, after which the cell pellets were washed twice using PBS. Then, the pellets were resuspended in AmnioMAX II Complete Medium 1X (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA, cat# 11269) in 6-well plates for 2–3 weeks, and incubated in a humidified environment with 5% CO₂ at 37°C. The medium was changed twice a week. After 3 weeks in the primary culture, the cells with 90% confluency were subcultured at 1:2 to 1:4 with trypsin-EDTA [0.25%] (Gibco). The cells were then re-seeded in DMEM containing 15% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1 mM of nonessential amino acids, 0.55 mM of 2-mercaptoethanol, and 10 ng/mL bFGF. The 3rd–5th passages of cells were used for further experiments, as recommended previously [10,29–31].

Cryopreservation method (slow freezing/thawing)

The hAFSC lines were frozen with a slightly modified previously described method [23]. We assessed the effect of two different concentrations of DMSO on the survival of hAFSCs after slow-freezing. The following groups were analyzed:

1. A solution of DMSO and adipose tissue-derived MSCs as control group [32].
2. A Solution of 10% (v/v) DMSO and 90% FBS.
3. A Solution of 5% (v/v) DMSO and 95% FBS.

Following trypsinization, the cells were harvested and centrifuged at a room temperature for 5 minutes at 400 g. Then, the supernatant was discarded, the cell lumps were resuspended, counted using a hemocytometer, and 1×10^6 cells were prepared per cryovial. After adding each cryoprotectant solution to the cell pellets, cell suspension was transferred immediately to -20°C for a short duration, and then to -70°C for 24 hours. The samples were then stored in a liquid nitrogen tank for one year until further analysis.

To thaw cryopreserved cells, the cryovials were quickly dipped in a water bath set at 37°C. Then, the cells were transferred into a 15 mL falcon tube, and 10 mL hAFSC medium was gradually added. The supernatant was discarded after centrifugation at room temperature for 3 minutes at 400 g. The cell pellet was resuspended in hAFSC medium and the numbers of cells were calculated by trypan blue exclusion. After thawing, cell survival and colony morphology was compared between the two cryopreservation protocols. The experiment was repeated three times (triplicate) and statistical analysis was carried out.

FACS analysis and ICC

In addition to ICC, FACS analysis was used to evaluate the phenotypic profile of hAFSCs, as described previously [10]. The cells were first stained with monoclonal antibodies and analyzed using BD FACSCalibur Flow cytometer (Ref: 342976, BD Biosciences, Bedford, MA, USA) [a total of 10,000 events for each sample]. Next, the samples were prepared for flow cytometry. Briefly, the cells were trypsinized and washed twice in FACS wash buffer. Supernatant was completely removed and the cell pellets were incubated with phycoerythrin (PE)-conjugated monoclonal antibodies (1:30 dilution) for 20 minutes on ice. Then, 100 µL of FACS wash buffer containing propidium iodide (PI) was added to the cells. Approximately 5×10^4 cells were labeled with conjugated monoclonal antibodies against MSC markers CD90 (BD Pharmingen, cat# 560977) and CD44 (BD Pharmingen, cat# 555596), and against hematopoietic markers CD31 (PECAM-1, cat# FAB3567c) and CD45 (Abcam, cat# ab65952). The cells were centrifuged at 450 g for 3 minutes, and washed twice with FACS wash buffer. Detection of antibodies was performed on the FACS Calibur analyzer.

MTT assay

MTT assay was used to assess hAFSC viability before and after freezing for one week. One million cells were exposed to the two different freezing methods. In the first group, the cells were frozen with a combination of 90% FBS and 10% DMSO, while a mixture of 95% FBS and 5% DMSO was used in the second group. The freezing time was one week for both groups. Untreated cells were used as control.

Trypan blue was used to calculate the number of living and dead cells. In a 96-well plate, 10,000 cells/well were seeded and stored for 24 hours in a CO₂ incubator, to stimulate cell attachment. After 24 hours, the medium was substituted with 200 µL fresh media and left for 24 hours. The cells were then incubated with 50 µL of 2 mg/mL MTT solved in PBS, for 4 hours. For this step, the plates were covered with aluminum foil. Supernatant was removed from the wells, and 200 µL of pure DMSO and 25 µL of Sorensen's glycine buffer were added to each well. Finally, the absorbance at 570 nm was measured on an ELISA reader with a reference wavelength at 630 nm. The cell viability was calculated using the following formula: Mean optical absorbance of treated cells $\times 100$ / Mean optical absorbance of control cells.

Analysis of hAFSC pluripotency by reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using the RNX-Plus kit (CinnaGen, Tehran, Iran, cat# RN7713C) from 5×10^5 – 6×10^5 undifferentiated hAFSCs (3th–5th passages), following the

manufacturer's protocol. Complementary DNA (cDNA) was synthesized from the total RNA using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA, cat# K1621) and an oligo-dT primer, in a 20 µL reaction mixture. The cDNA synthesis was carried out in a thermal cycler (PeQLab). The *NANOG* and *POU5F1* (*Oct4*) genes were analyzed as markers of pluripotency in hAFSCs cells, and the respective primers are given in Table 1. Beta-actin gene was used as an internal control.

The *NANOG* and *POU5F1* gene expression was detected using 2 µL of single stranded cDNA and the HyperScript RT master mix (GenAll, cat# 601-710). Initial denaturation was performed at 94°C for 4 minutes, followed by amplification at 94°C for 30 seconds. The annealing temperature was 56°C for 30 seconds and 72°C for 30 seconds, and extension was set at 72°C for 5 minutes for a total of 35 cycles. Amplified PCR products were separated on an agarose gel (2%) by electrophoresis and the resulting bands were visualized using a dye and photographed with a gel doc (Quantum-ST4, 1100/20M, France).

Quantitative analysis of gene expression in hAFSC lines by real-time PCR

RNA extraction from hAFSC lines and cDNA synthesis were carried out as described above. The primers for *POU5F1* and *NANOG* genes are provided in Table 1. β-actin gene was used as an internal control (147 bp, forward primer: 5'-CCCTTCCTTCCTGGGCATG-3', reverse primer: 5'-TCCTGTCTGGCAATGCCAG-3'). To assess the effect of freezing and DMSO on cell cycle arrest in the G₁-phase, the mRNA levels of cell cycle regulators p21 (*CDKN1A*) and cyclin D1 (*CCND1*) were analyzed using the following primers: *CDKN1A* (forward primer 5'-GCTTCATGCCAGCTACTTCC-3', reverse primer:

5'-CCCTTCAAAGTGCCATCTGT-3') and *CCND1* (forward primer: 5'-GCGGAGGAGAACAAACAGAT-3', reverse primer: 5'-TGAACTTCACATCTGTGGCA-3').

Real-time PCR procedures were carried out using the SYBR® Premix Ex Taq™ II (Tli RNase H Plus, Takara Biotechnology Co., Ltd. Dalian, China) [Cat # RR820] in a Rotor Gene 6000 (Corbett, USA). The PCR cycling conditions are given in Table 2. Relative gene expression was calculated using the 2^{-ΔΔCt} method [33]. The amount of target gene was normalized to an endogenous reference gene and relative to a calibrator, as follows: ΔCt = Ct target gene - Ct endogenous reference gene and ΔΔCt = ΔCt sample of interest - ΔCt calibrator. All experiments were performed in triplicate [34].

Statistical analysis

SPSS for Windows, Version 15.0. (SPSS Inc., Chicago, IL) was used for statistical analysis. Results were expressed as mean ± standard error (SE). The groups were compared using analysis of variance (ANOVA) with post hoc Bonferroni correction. The significance level was set at *p* < 0.05.

RESULTS

Isolation and characterization of hAFSCs

HAFSCs were successfully isolated from pregnant women and the morphology of cells in each passage was analyzed using an inverted microscope. In the primary passage, a heterogeneous population of cells was observed; the cells were round and showed the formation of spherical colonies. Fibroblast-like morphology or shape of the spindle was observed in cells after the 3rd passage (Figure 1). Based on RT-PCR results for *NANOG* and *POU5F1* genes, pluripotency of hAFSCs was confirmed in 3rd-5th passages, both before and after slow freezing (Figure 2).

TABLE 1. Primers used for reverse transcription polymerase chain reaction (RT-PCR)

	Gene name	Forward (F) and reverse (R) primers	PCR product size
1	Oct4 (<i>POU5F1</i>)	F 5' CCATGCATTCAAACTGAGGT 3' R 5' CCTTTGTGTTCCCAATTCCT 3'	146 bp
2	<i>NANOG</i>	F 5' AGTCCCAAAGGCAAACAACCCACTTC 3' R 5' TGCTGGAGGCTGAGGTATTCTGTCTC 3'	161 bp
3	Beta actin	F 5' GGCACCCAGCACAATGAAGA 3' R 5' CGACTGCTGTACCTTCACC 3'	342 bp

Oct4: Octamer-binding transcription factor 4

TABLE 2. Cycling protocol for real-time polymerase chain reaction (PCR) analysis

Cycle	Cycle Point
Hold at 95°C, 5 minutes 0 seconds	
Cycling (40 repeats)	Step 1 at 95°C, hold 15 seconds Step 2 at 60°C, hold 35 seconds, acquiring to Cycling A (SYBER Green) Step 3 at 72°C, hold 15 seconds, acquiring to Cycling B (SYBER Green)
Melt (72-95°C), hold seconds on the 1 st step, hold 5 seconds on next steps, Melt A (SYBER Green)	

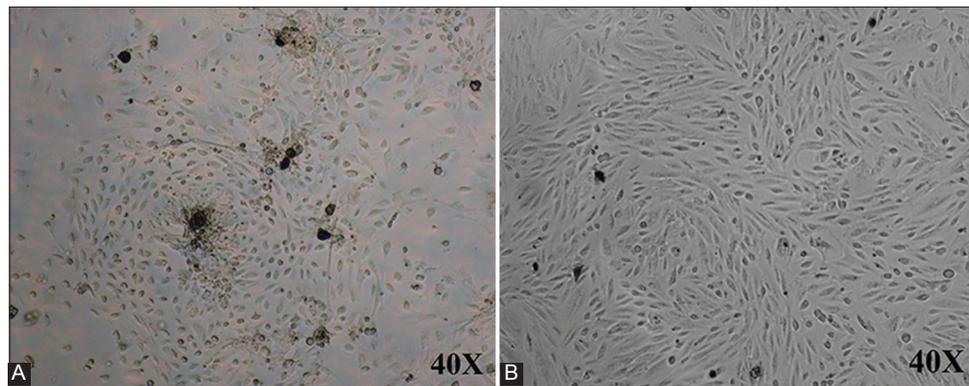


FIGURE 1. Cell morphology in cultured human amniotic fluid stem cells (hAFSCs). (A) In the primary passage, a heterogeneous population of cells was observed; the cells were round and showed the formation of spherical colonies (40x magnification). (B) After the 3rd passage, a more homogenous population of cells with fibroblast-like morphology or shape of the spindle was observed (40x magnification).

hAFSC viability after slow freezing/thawing

A total of 1×10^6 cryopreserved cells for each cryovial were thawed in two different media containing 5% and 10% DMSO, respectively, and the cell viability was determined by trypan blue exclusion. The results showed that up to 90% of the cells (9×10^5 of 10^6 primary cells) frozen with 10% DMSO were viable, but the viability of cells frozen with 5% DMSO was only 60% [65×10^4 of 10^6 primary cells] (Figure 3).

Flow cytometry

Cell surface antigen expression at 3rd–5th passage was detected in hAFSCs using monoclonal antibodies and flow cytometry, before and after slow freezing. All cultures were positive for MSC markers CD44 (85%) and CD90 (90–99%), and negative for CD45 and CD31 (Figure 4A and 4B).

MTT assay

Cell viability of hAFSCs treated with one of the cryopreservation protocols (95% FBS + 5% DMSO or 90% FBS + 10% DMSO) for one week was evaluated using the MTT assay. Untreated cells were used as a control group. The viability was higher in cells treated with 90% FBS + 10% DMSO (84% vs. 71% for 95% FBS + 5% DMSO group), indicating that this method is more suitable for cryopreservation of hAFSCs (Figure 5).

Quantitative gene expression analysis

Real-time PCR results showed that the expression of *NANOG* gene increased in hAFSCs at 3rd–5th passage after slow freezing compared to control cells (before freezing). Compared to control group and *NANOG* gene expression, mRNA level of *POU5F1* was decreased in hAFSCs after freezing (Figure 6).

On the other hand, the expression of cell-cycle regulators p21 (*CDKN1A*) and cyclin D1 (*CCND1*) was not significantly

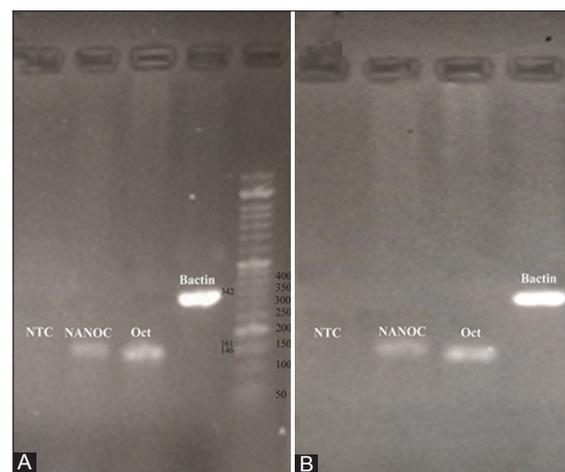


FIGURE 2. Gene expression of pluripotency markers in human amniotic fluid stem cells (hAFSCs) before (A) and after (B) cryopreservation with dimethyl sulfoxide (DMSO). Beta-actin (342 bp) was used as an internal control. The expression of Octamer-binding transcription factor 4 gene (*POU5F1*) [161 bp] and *NANOG* (146 bp) was confirmed by reverse transcription polymerase chain reaction and electrophoresis in agarose gel (2%).

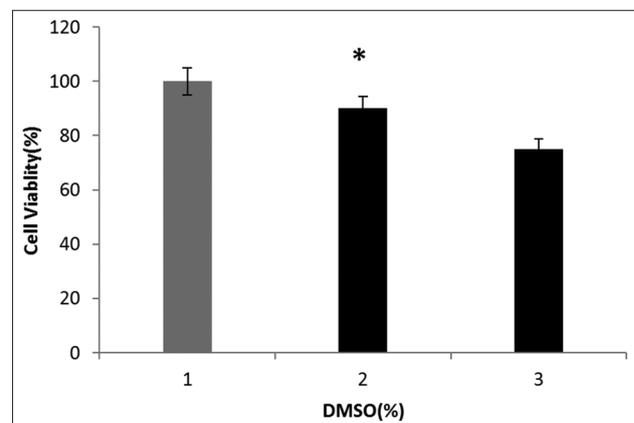


FIGURE 3. Cell viability determined by trypan blue exclusion in untreated, control human amniotic fluid stem cells (hAFSCs) [1], hAFSCs treated with 10% dimethyl sulfoxide (DMSO) [2], and 5% DMSO [3]. Up to 90% of the cells (9×10^5 of 10^6 primary cells) frozen with 10% DMSO were viable, while the viability of cells frozen with 5% DMSO was only 60% [65×10^4 of 10^6 primary cells]. The *p* values for 5% DMSO, control, and 10% DMSO were 0.00, 0.013 and 0.003 (**p* < 0.05 for 2 vs. 1 [control]), respectively.

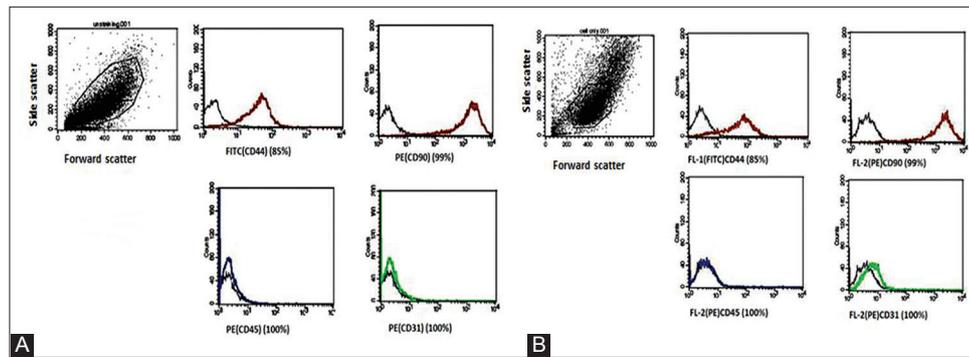


FIGURE 4. Cell surface antigen expression at 3rd–5th passage was detected in human amniotic fluid stem cells (hAFSCs) using monoclonal antibodies and flow cytometry, before (A) and after (B) cryopreservation with dimethyl sulfoxide (DMSO). All cells were positive for cluster of differentiation (CD)44 (85%) and CD90 (90–99%), and negative for CD45 and CD31.

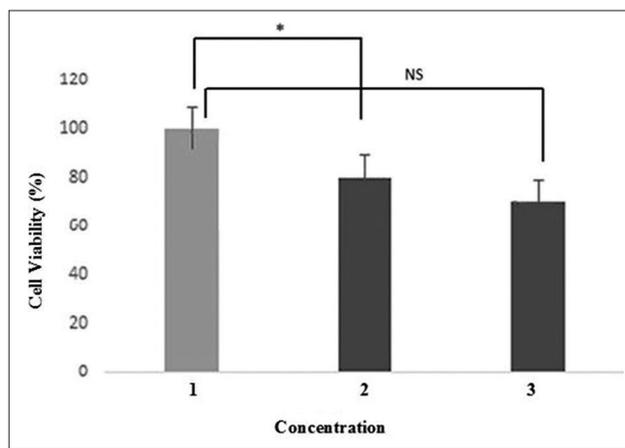


FIGURE 5. Normalized MTT assay data before and after freezing of human amniotic fluid stem cells (hAFSCs) for one week. 1 (control = 10% dimethyl sulfoxide [DMSO] and 90% fetal bovine serum [FBS] + adipose tissue mesenchymal stem cells), 2 (10% DMSO + 90% FBS + hAFSCs), and 3 (5% DMSO + 95% FBS + hAFSCs). The viability was higher in cells treated with 90% FBS + 10% DMSO (84% vs. 71% for 95% FBS + 5% DMSO group) [$*p < 0.05$ for 2 vs. 1 (control)]. NS: non significant.

different in the cells before and after slow freezing, indicating that the cryopreservation process did not negatively affect the cell cycle of hAFSCs (Figure 7).

DISCUSSION

hAFSCs were obtained from pregnant women who underwent amniocentesis for karyotyping. Amniocentesis is a safe procedure and routinely used for prenatal diagnosis, during which fetal stem cells can be isolated without harm to the fetus or mother (the risk of miscarriage is $>1\%$).

We observed a higher viability of hAFSCs after freezing with 90% FBS + 10% DMSO compared to 95% FBS + 5% DMSO. *NANOG* and *POU5F1* gene expression was detected by RT-PCR before and after slow freezing, confirming the pluripotency of hAFSCs. According to flow cytometry, all hAFSC cultures were positive for MSC markers CD44 and CD90. Real-time PCR results showed increased expression of *NANOG*

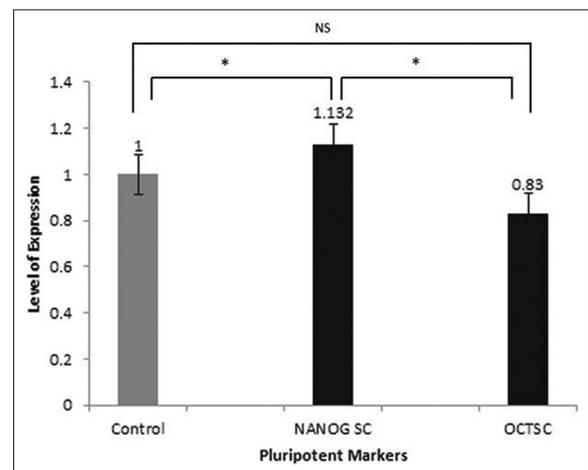


FIGURE 6. Quantitative mRNA expression of Octamer-binding transcription factor 4 (Oct4), and *NANOG* was determined by real-time polymerase chain reaction (PCR) in human amniotic fluid stem cells (hAFSCs) before and after slow freezing. The mRNA expression of *NANOG* increased in hAFSCs at 3rd–5th passage after slow freezing compared to control cells before freezing ($*p < 0.05$). The mRNA level of Oct4 was decreased in hAFSCs after slow freezing compared to control group (NS: non significant), and compared to *NANOG* expression (1.132 vs. 0.83, respectively, $*p < 0.05$).

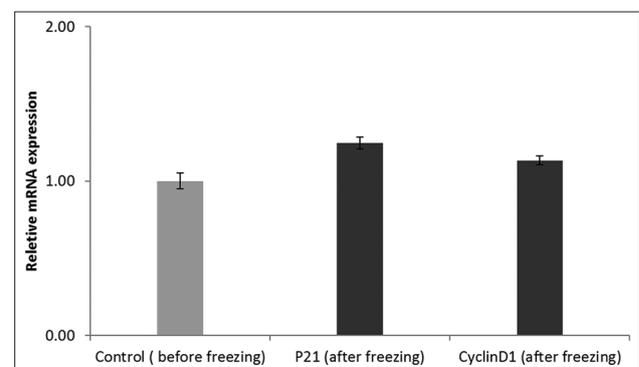


FIGURE 7. Quantitative mRNA expression of cyclin D1 and p21 was determined by real-time polymerase chain reaction (PCR) in human amniotic fluid stem cells (hAFSCs) before and after slow freezing. The expression of cell cycle regulators was not significantly different in the cells before and after freezing ($p > 0.05$).

gene in hAFSCs and decreased expression of *POU5F1* after slow freezing, compared to control cells (before freezing).

Oct4, Sox2 and NANOG are transcription factors essential in maintaining pluripotency and self-renewal of ESCs. Oct4 exerts its function as a transcriptional regulator by binding to the classical octamer DNA motif, and in ESCs, it acts together with Sox2 which binds to sox element [35,36]. NANOG modulates the levels of Oct4 and Sox2 to sustain the undifferentiated state of ESCs. In turn, Oct4 and Sox2 control the transcription of downstream genes involved in maintaining pluripotency or inhibiting differentiation of ESCs. *NANOG* also modulates molecular effectors of ES cell fate, e.g., Foxd3 (transcriptional repressor) and Setdb1 (histone H3 Lys9 methyltransferase) [37,38]. Other examples of genes regulated by *NANOG* and *POU5F1*, that are important for pluripotency and self-renewal of ESCs, include *Myc* [39] and downstream target genes *Esrrb* and *Rif1* [40-42].

Additional mechanisms may modulate the activity of Oct4 in ESCs [43]. For example, stable levels of Oct4 in totipotent cells may be associated with the establishment of active chromatin rather than the activity of transcription activators [44]. Ben-Shushan et al. showed that the extinction of *POU5F1* gene activity in stem cell-fibroblast hybrid cells was accompanied by fast methylation of regulatory sequences, such as proximal and distal enhancers in the *POU5F1* promoter/enhancer region [45,46]. The expression of Oct4 and/or NANOG as markers of pluripotency was also demonstrated in BM-MSCs and in human multipotent adult SCs generated *in vitro* from adult human liver, heart, and BM [47,48]

Previously, it was shown that DMSO can cause arrest of cells in the G1 phase, a process that is regulated by p21 and cyclin D1 cell cycle regulators [26-28]. In this study, DMSO and the process of freezing did not significantly change the expression of p21 and cyclin D1 genes in hAFSCs, indicating the applicability of this cryopreservation protocol.

CONCLUSION

A higher viability of hAFSCs was observed after freezing with 90% FBS + 10% DMSO, suggesting that is method is convenient for longer-term storage of SCs. *NANOG* and *POU5F1* gene expression was detected before and after slow freezing, confirming the pluripotency of hAFSCs. Moreover, hAFSCs were positive for MSC markers CD44 and CD90, and negative for CD45 and CD31. The observed viability of hAFSCs as well as unchanged expression of p21 and cyclin D1 regulators indicate that the cryopreservation protocols did not have harmful effects on the cell cycle of SCs. The importance

of cryopreservation methods that allow efficient storage of hAFSCs without affecting their potency is highlighted, as these cells are easily obtainable and have a potential for numerous therapeutic applications in regenerative medicine.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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