

Supplementation freeze-thawed media with selenium protect adipose-derived mesenchymal stem cells from freeze-thawed induced injury



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ABSTRACT

Successful freeze-thaw of adipose-derived mesenchymal stem cells (ADMSCs) could be a major step in regenerative medicine as well as in the cloning of animal breeds. The aim of this study was to evaluate the efficacy of selenium on the optimizing of freeze-thaw media in the ADMSCs. ADMSCs were extracted from NMRI mice and purified with positive selection Monoclonal CD105 Antibody (PE) and negative selection Monoclonal CD31 and CD45 Antibody using MACS method as well as differentiation to adipose and bone tissue. ADMSCs were divided into four groups. ADMSCs were freeze-thaw under standard condition with or without the addition of 5 ng/ml selenium to both the cryopreservation and thawing solutions. Frozen cells were thawed after four months and viability and cytotoxicity of the cells were analyzed by the Trypan blue test and MTT assay respectively. RNA was extracted and cDNA was synthesized and the expression of apoptotic genes (P53, Fas, Bax, Caspase3, and Bcl2) was examined using Real time-PCR Rotor gene 2009. This study compares slow and rapid methods of cryopreservation. After thawing, viability of the cells treated with selenium was higher than the control group in rapid and slow cryopreserved ADMSCs. Also, the percentage of living cells in the slow cooling method was considerably more than with the rapid cooling method. After analysis of the results using Real time-PCR, the Bcl2 gene was shown to be expressed in both the rapid and slow cooling methods. In the rapid cooling group in addition to the BCL-2 gene, p53 was also expressed. It appears that selenium prevented the apoptotic genes from expression due to its anti-apoptotic effects. The slow cooling method is better and more optimized for ADMSCs protecting them from oxidative damage to a greater extent compared to the rapid cooling method.

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1. Introduction

Cell and gene therapy are two powerful strategies for the treatment of inherited and acquired disorders. For this type of treatment a reliable cell source is necessary. A stem cell is considered as an undifferentiated cell with the capability of proliferation, self-renewal and production of different cell lines [21]. Stem cells are divided into two groups, embryonic stem cells and adult stem cells. Tumorigenesis, immunorejection and ethical issues are hampering the development of embryonic stem cells for clinical

applications. In contrast, adult stem cells are an available resource and are a good alternative [27]. Among tissue stem cells, ADMSCs are adult's multi-potent cells with characteristics very similar to bone marrow. The isolation of stem cells from adipose tissue is much easier than bone marrow and does not involve painful procedures and also contains more stem cells than bone marrow [6] which compared to other adult stem cells, have a higher rate of division and self-renewal [9]. Recent studies show that these cells inhibit the immune system and prevent the proliferation of T-cells. Therefore the clinical use of these cells in regenerative medicine is promising a bright future [1]. Collecting stem cells in the early stages of treatment without the urgent need to do cell transplants is becoming a common practice and most cells are collected and

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stored for several years [26]. The ability of mesenchymal stem cells to survive long-term storage and maintain their phenotype is critical. Cold storage causes physical changes that lead to molecular changes in the cell structure. To protect the cells from damage during the freeze-thaw process, cryoprotectant mediums are used. The concentration of the cryoprotectants added to the frozen cells is one of the primary factors for their survival [5]. The theory of protecting the cells and tissue by adding anti-freeze before freezing has been investigated for decades. A sufficient concentration of anti-freeze protects cells from oxidative stress [10]. Furthermore the optimization of the freezing-thawing by antioxidant substances can reduce the harmful and fatal effects of the freezing-thawing process to a minimum. Among the most well-known oxidants, selenium can be considered. Most texts and tests have proven the protective, antioxidant and anti-apoptotic effects of selenium. For example glutathione peroxide is a powerful antioxidant and a selenoenzyme. Selenium is a trace element of defense in preventing damage to cells and cell membrane that is caused by hydrogen peroxide and other peroxides [16]. Furthermore selenium is related to the growth, survival and toxicity of cells and also the ability to change the shape of redox regulation, chemical toxicity and transcriptional regulation of genes [29]. The purpose of this study was to evaluate the efficacy of selenium in decreasing oxidative damage to ADMSCs in both slow and rapid freeze-thawing procedures.

2. Materials and methods

2.1. Isolation and culture of ADMSCs

Adipose tissues of NMRI mice were isolated and digested at 37 °C in phosphate buffer saline (PBS), 2% bovine serum albumin and 2 mg/ml collagenase, for 15–20 min. After filtration through 40 µm nylon filter mesh (BD falcon) and centrifugation, isolated cells were re-suspended in medium, counted with a hemocytometer, plated at 5×10^6 cells/ml on culture plates, and cultured in the presence of DMEM containing 20% fetal bovine serum (FBS). The cells were observed daily under an inverted phase-contrast microscope and were passaged after 80% confluence. The culture media was changed every 2–3 days.

2.2. Separation and purification ADMSCs with MACS

Adipose mesenchymal stem cells were purified with positive selection Monoclonal CD105 Antibody (PE) and with negative selection Monoclonal CD31 and CD45 Antibody; the procedure was performed according to the manufacturer's instructions (Miltenyi Biotec).

2.3. Multipotential differentiation of ADMSCs

Passage 3 ADMSCs were cultured to confluence in DMEM containing 20% FBS, and then were induced to differentiate using appropriate media to determine the multipotential differentiation capability. For adipogenic differentiation, 100% confluent ADMSCs were cultured in 10% FBS-containing DMEM, supplemented with 100 nM dexamethasone (Sigma) and 50 µl/ml indomethacin(Sigma). After 3 weeks, the cells were stained with oil red O and examined by phase contrast microscopy. For osteogenic differentiation, 80% confluent ADSCs were cultured in 10% FBS-containing DMEM supplemented with 50 µg/ml ascorbic acid 3-phosphate and 10 mM β-glycerophosphate and 10 nM dexamethasone(Sigma) for 3 weeks, and then the cells were stained with Alizarin red S and examined by phase contrast microscopy (Fig. 4).

Table 1
Primers feature used for Real-time PCR.

Primer name	Prime sequence
GAPDH	F:CAA TGT GTC CTG CGT GGA TCT R:GTC CTC AGT GTA GCC CAA GAT
FAS	F:GAG AAT TGC TGA AGA CAT GAC AAT cc R:GTA GTT TTC ACT CCA GAC ATT GTC C
P53	F:GTT TCC TCT TGG GCT TAG GG R:CTT CTG TAC GGC GGT CTC TC
BAX	F :CGA GCT GAT CAG AAC CAT CA R:GAA AAA TGC CTT TCC CCT TC
BCL-2	F:TAA GCT GTC ACA GAC GGG CT R:TGA AGA GTT CCT CCA CCA CC
CASPASE3	F:CAG CAC CTG GTT ACT ATT CTT R:GTT AAC GCG AGT GAG AAT GTG

2.4. Freeze-thaw procedure

The freezing solution contained DMEM supplemented with 10% (v/v) FBS, 10% (v/v) dimethyl sulfoxide (DMSO) and 5 ng/ml Selenium. The cells were divided into two groups, a rapid-cooling group and the second group for slow-cooling. For each group, a control group containing DMEM supplemented with 10% (v/v) FBS, 10% (v/v) dimethyl sulfoxide (DMSO) was considered. Rapid-cooling cryotubes were transferred into the liquid nitrogen tank (−196 °C) directly. But the slow-cooling cryotubes were kept at −4 °C for an hour, 2 h at −20 °C, overnight at −80 °C and finally, transferred into the liquid nitrogen tank. After four months cryotubes were immersed in 37 °C water bath for 1 min. Immediately after thawing, the cells were carefully aspirated, mixed with an equal volume of DMEM containing 15% (v/v) FBS.

2.5. Cell viability assay

Cell suspensions (20 µL) were mixed with trypan blue (20 µL) to obtain a 1:1 dilution factor, and the mixtures were transferred to a

Table 2
Percentage of cell viability of non-cryopreserved and two different cooling methods by the trypan blue staining test.

Groups	Cryopreservation	Vitrification
Control group	80%	35%
Experimental group	89%	45%

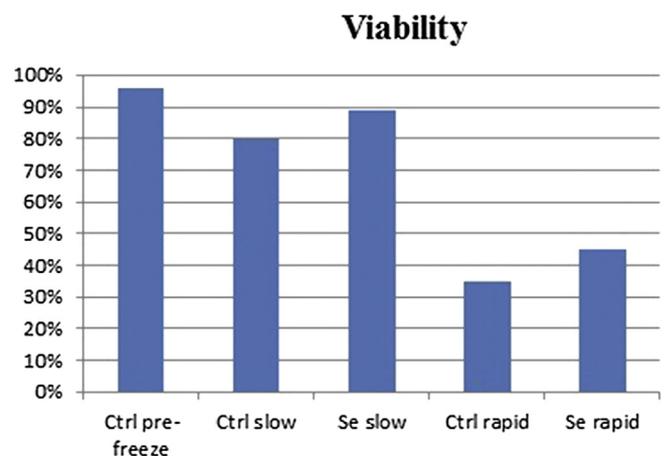


Fig. 1. Percent of MSCs viability before after thaw.

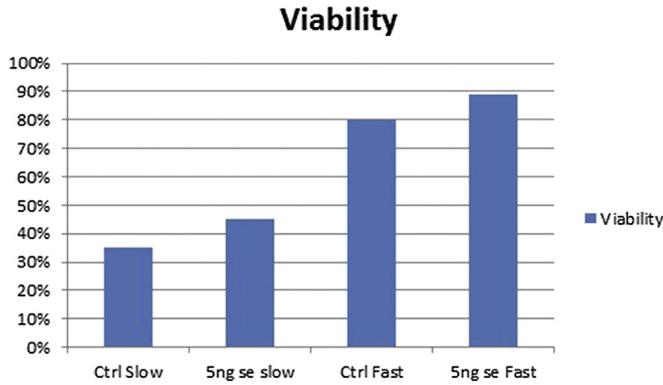


Fig. 2. Gene expression levels chart in fast-freezing procedure. The group has demonstrated significant expression in P53 and Bcl-2.

hemocytometer. A light microscope was used to determine the number of viable and non-viable cells to yield the percentage cell viability.

2.6. The cytotoxicity assay

For evaluating cytotoxicity of selenium, the MTT assay was performed. Cells were seeded into 96-well culture plate at density 500 cells/well and grown in DMEM supplemented with 10% FBS. Cytotoxicity was measured using MTT reagent (Sigma) dissolved in PBS (5 mg/ml). After 48 h, medium was carefully replaced on fresh DMEM+10% FBS with diluted MTT (1:10, 10%MTT), and incubated for 4 h at 37 °C. After removing incubation medium, formazan

crystals were dissolved in 200 µl solution of DMSO. MTT reduction was quantified by measuring the light absorbance at 560 nm using the ELx800 absorbance microplate reader (BioTek Instruments, VT, USA).

2.7. RNA isolation

RNA isolation was performed with the Jena Bioscience kit (Bioscience, Cat. no.#PP-210S) by following the manufacturer's instructions included in the kit.

2.8. cDNA synthesis

cDNA synthesis was performed using the Bioneer kit (Bioneer, Cat.no.k-2046) according to manufacturer's instructions.

2.9. RT real-time PCR

Real-time PCR was performed using a Qiagene rotor gene 6000. Forty reactions were considered each reaction cycle was performed for 15 s at 95 °C and 45 s at 60 °C. Primers are listed in (Table 1).

2.10. Statistical analysis

The Real-time PCR results were analyzed using the Rest-RG 2009 software, and its charts were drawn. Viability and MTT results were analyzed by the U Mann Whitney test, SPSS 16 software.

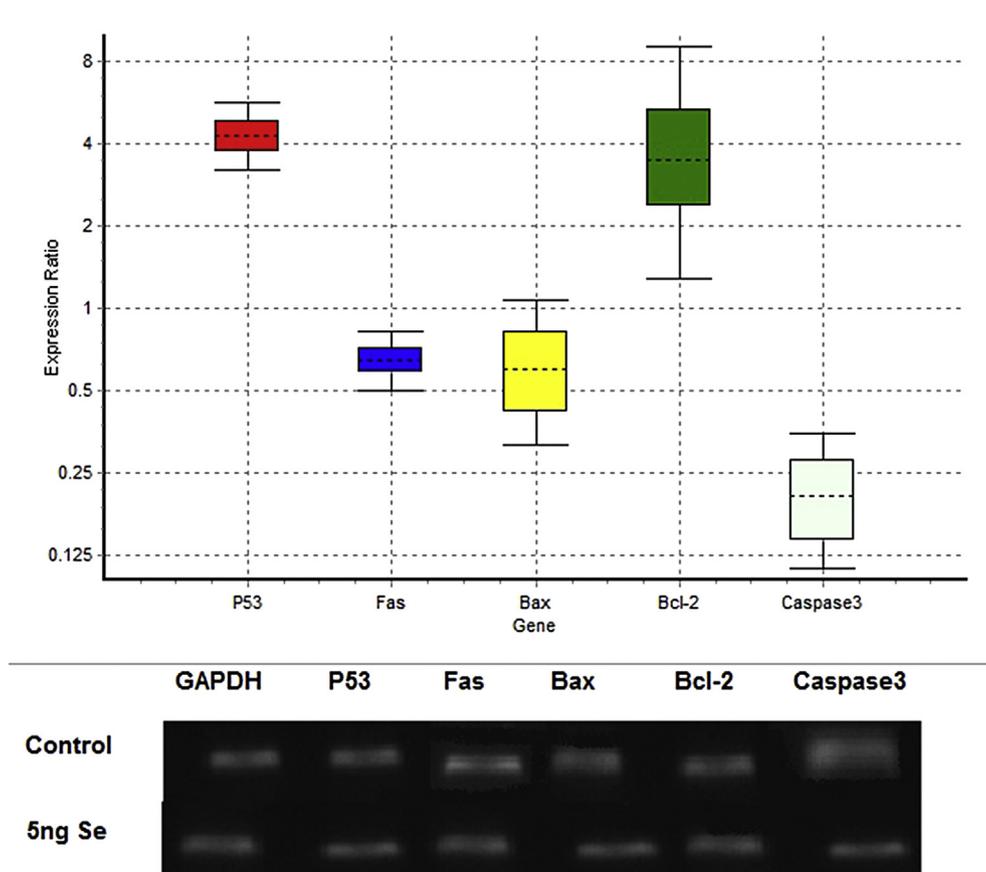


Fig. 3. Gene expression levels chart in fast-freezing procedure. Selenium increased expression of Bcl-2.

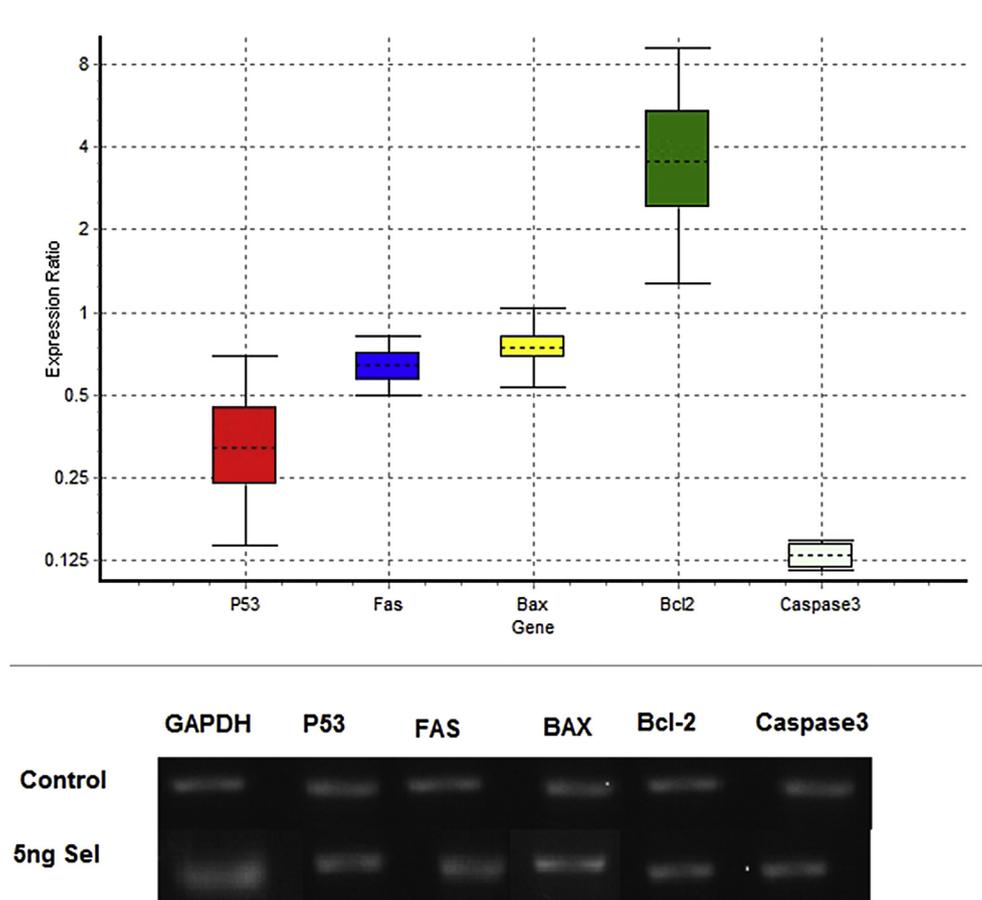


Fig. 4. (A) in Adipogenic differentiation stem cells After 21 day's oil spots in most of cytoplasm cells can be seen. (B) after staining with oil red O, oil spots will gain the red color.(C) in osteogenic differentiation stem cells after 21 days extracellular matrix were calcified,(D) after staining with Alizarin red S the calcified region will gain the red color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Cytotoxicity assay

Cytotoxicity assay results not shown that selenium has no effect on the ADMSCs.

3.2. ADMSCs viability

Viability of ADMSCs was determined by the trypan blue staining test. After the isolation of ADMSs the viability was assessed as fresh group, and 96% of cells were viable. After thawing, viability in rapid and slow cooling control groups were respectively 35% and 80% and Viability in rapid and slow cooling groups treated with 5 ng/ml Selenium were respectively 45% and 89%. Viability in rapid cooling procedure was significantly less compared to slow cooling ($P < 0.002$), also viability in treated groups in both procedures was higher compared to control ($P < 0.043$) (Table 2 and Fig. 1).

3.3. Apoptotic gene expression

Expression levels of apoptotic genes (P53, Fas, Bax, Caspase3, and Bcl2) were determined using Real time-PCR. The results were analyzed with Rest-RG software. Expression levels of Fas, Bax, and Caspase3 in rapid cooling group did not change compared to the control group. However expression level of P53 and Bcl-2 increased in comparison to the control group (Fig. 2). In slow cooling only Bcl-2 gene expression level increased compared to the control group

and P53, Fas, Bax and Caspase3 did not show significant changes (Fig. 3). The comparison between the rapid-cooling group and the slow-cooling group has shown that Bcl-2 gene was increased in both procedures compared with their control groups and P53 increased only in rapid cooling procedure group.

4. Discussion

In recent years, changes have been made to the tissue engineering strategy towards the use of stem cells for plastic surgery procedures and regenerative medicine. So the development of safe reproducible protocols for the extraction of stem cells derived from adipose tissue allowing its storage in liquid nitrogen for future use is needed [19]. However any freezing method isn't completely safe for the cells as it puts severe stress on them. Induction of apoptosis by the freezing-thawing process causes damaged to cell membranes and DNA, as well as the production of reactive oxygen [11–13,23]. It has also been shown that DMSO is toxic to the cells [28]. In this study it was clearly shown that the percentage of living cells in the slow cooling method was greater than that in the rapid cooling method and also that the viability in groups that contained selenium in both methods was higher than the control groups. BCL-2 and P53 were expressed in the rapid cooling group while only BCL-2 was expressed in the slow cooling group. Our study indicates that the increase of expression of BCL-2 gene in groups containing selenium can be an antioxidant defense mechanism; preventing stress induced cell apoptosis. The two major pathways for apoptosis are the inner and outer pathway [17]. Intrinsic apoptosis pathway is

not dependent on the external death stimuli. The inner cell stimuli like DNA damage and oxidative stress cause apoptosis which is induced by the BCL-2 family proteins that are present in the outer membrane of mitochondria. The BCL-2 families are divided in two groups with conflicting activities: a group of BCL-2 proteins which inhibit apoptosis and the other which strengthens it [4,24]. Many studies have shown the mitochondrial release of cytochrome C and apoptotic factors are controlled by the BCL-2 [25]. Selenium works as a cofactor for the intracellular glutathione antioxidant system. Glutathione peroxidase, are a group of antioxidant enzymes that are necessary for the protection of body cells from damage and free radicals [14,31]. Since the enzyme glutathione peroxidase is a selenoenzyme, it can be said that selenium works as a defensive factor in preventing damage to cell membranes caused by hydrogen peroxide and other peroxides and is believed to have an antioxidant role [14,16] and molecular targets associated with selenium-induced growth inhibition in human breast cells, have been reported [7]. Research has shown that treating neuroblastoma cell lines with selenium, can lead to an increasing the expression of BCL-2 gene and a reduction of caspase-3 activity [22]. Antioxidant effects of selenium can reduce cadmium-induced apoptosis and reduce the expression of the bax and bak proteins and also increased BCL-2s expression [3]. In other studies the anti-apoptotic effect of selenium has been documented [20,30] while it has also been shown that temperature shocks can activate p53 [8]. One of the important functions of glutathione peroxidase is preventing membrane lipid peroxidation [18], it is the first level of stem cells membrane protection against peroxidation and antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX). Antioxidants may reduce the production of free radicals and neutralize them by increasing the expression of genes responsible for DNA damage and cell death during the frozen storage process [15]. The dose and duration of treatment with antioxidants are important to the extent that even inappropriate doses may have the opposite effect. Use of antioxidants importance should always be given to the dose amount. For example, in many of the articles the dose of selenium has been mentioned as one of the critical factors influencing the antioxidant in a way that a high dose of it could cause adverse effects and damage [2].

5. Conclusion

In conclusion, data from this study indicates that supplementation of the freeze-thaw media with 5 ng/ml selenium increased Bcl-2 expression and antiapoptotic effects in both groups, as well as the increase of ADMSCs viability in groups containing selenium in both slow and rapid procedures. And slow cooling procedure is safer for cryopreservation of ADMSCs.

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