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Effect of Dimethyl Sulfoxide (DMSO) on Cryopreservation of Porcine Mesenchymal Stem Cells (pMSCs)

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Dimethyl sulfoxide (DMSO), a commonly used cryoprotectant in cryopreservation procedures, is detrimental to viability of cells. In this view point, a comparative study was carried out to evaluate the effect of DMSO on porcine mesenchymal stem cells (pMSCs). We compared the viability, colony forming unit-fibroblast (CFU-F) assay, expression of Bak and Bcl2 genes, Bcl2 protein antigen, and CD90 in pMSCs cryopreserved with 5%, 10%, and 20% DMSO. pMSCs isolated from bone marrow were characterized by alkaline phosphatase activity and the expression of transcription factors, such as Oct 3/4, Nanog, and Sox2. The cells were then cryopreserved by cooling at a rate of −1°C/min in a programmable freezer and stored in liquid nitrogen. The results of survival of pMSCs cryopreserved at 5% DMSO were comparable to control group (fresh pMSCs). The survival and the number of colonies formed in cryopreserved pMSCs were inversely proportional to the concentration of DMSO. The number of colonies formed in pMSCs cryopreserved with all concentrations of DMSO was significantly ($p < 0.05$) lower than the control group. An increased tendency for Bak and Bcl2 gene expression was noticed in cryopreserved pMSCs at 3 h postthawing compared to control group. There was a close resemblance in higher level of expression of CD90 between control and cryopreserved pMSCs. Because there was no considerable difference in the results of pMSCs cryopreserved at 5% and 10% DMSO, this study strongly suggests the use of 5% DMSO in cryopreservation of pMSCs as an alternative to conventional 10% DMSO.

Key words: Mesenchymal stem cells; Porcine; Cryopreservation; Dimethyl sulfoxide (DMSO)

INTRODUCTION

Although mesenchymal stem cells (MSCs) possess several benefits, cells under long-term ex vivo culture conditions give rise to unsatisfactory results such as genotypic drift, senescence, transformation, phenotypic instability, contamination, or incubator failure (14,35,36). To preserve their biological characteristics unaltered, MSCs should possibly undergo a minimal duration under ex vitro culture conditions. In addition, there is an urgent need for improvement in cryopreservation technology for the storage of isolated and cultured MSCs for the benefit of research and future clinical applications.

Cryopreservation of MSCs has several other advantages, such as saving of time and culture medium, protection from infection/contamination, genetic drift, and avoidance of immune rejection at autograft (20,36). However, the conventional slow freezing has always been associated with cell damage and mortality due to intracellular ice formation, cryoprotectant toxicity, and dehydration by rapid temperature change, especially at intermediate temperature zone, from −15°C to −60°C (5,7). The most commonly used permeating cryoprotective agent for the storage of MSCs, hematopoietic stem cells (HSCs), and embryonic stem cells is dimethyl sulfoxide (DMSO). The concentration of DMSO used in general cell cryopreservation is about 10% (v/v) of cryopreservation medium (1,2,12,19) with the cooling ramp adjusted to −1°C/min in a mechanical freezer or controlled rate freezing device (5). The successful storage of MSCs and HSCs with a relatively higher survival rate after thawing has been achieved by traditional cryopreservation method (12,18,19).

Recently, several side effects such as nausea, chills, hypotension, and cardiac arrhythmia have been reported in humans, especially in children submitted to infusion of HSCs cryopreserved with DMSO (9,34,38). Therefore, a complete removal of DMSO from cells is very

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important before transfusion to avoid the toxic side effects. Studies in humans have reported the reduced toxicity and a better performance of HSCs (12) and MSCs (7,29) with reduced concentration of DMSO. Once, the studies reported are limited to human model, there is a need for animal cell model for detailed comparison of toxic side effects of DMSO used in cryopreservation.

Due to the physiological and genetic similarities between pigs and humans, porcine skin has been used for transplantation in humans (13) and also as a biomedical model (24) for skin replacement therapy, including tissue-engineered skin substitutes (22). Further, porcine MSCs (pMSCs) have been used as an important medical model for the application in artificial bone and teeth regeneration for humans (11,26,28,32). Therefore, the present study investigated the effect of cryoprotectant DMSO on cryopreservation of pMSCs by evaluating the survivability, proliferation by colony forming unit-fibroblast (CFU-F) assay, expression of apoptosis-related genes, Bak and Bcl2, and the ability of pMSCs to maintain their immunophenotype by the expression of CD90.

MATERIALS AND METHODS

Chemicals and Media

All chemicals used in the present study were purchased from the Sigma Chemical Company (St. Louis, MO, USA) and media from Gibco (Life Technologies, Rockville, MD, USA) unless otherwise stated. For all the media, the pH was adjusted to 7.4 and the osmolality to 280 mOsm/kg.

Cell Extract and Culture

All experiments were authorized by the Animal Center for Biomedical Experimentation at Gyeongsang National University. pMSCs were isolated from bone marrow of living 3-week-old female pig as previously described with Ficoll-Paque Plus (Amersham Science, USA) (10,17). Isolated cells were cultured in advanced Dulbecco's modified Eagle medium (ADMEM) supplemented with 1 mM Na-pyruvate, 0.05 mM pyridoxine hydrochloride, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 10% fetal bovine serum (FBS) (17) at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Once confluent, cells were regrown for this study until five passages. Freshly isolated pMSCs, which were grown to 90% confluence and did not undergo the procedure of cryopreservation, were treated as control.

Cryopreservation and Thawing of MSCs

After dissociation with 0.25% (w/v) trypsin-EDTA solution, pMSCs were washed twice by centrifugation at $400 \times g$ for 10 min with ADMEM supplemented with 10% FBS. Followed by adjusting the cell density to 2×10^6 cells /ml, the 500 µl cell suspension was transferred to 1.5 ml cryovials and mixed with 500 µl of 40%, 20%, or 10% DMSO solution diluted with ADMEM solution (1:1, v/v) supplemented with 10% FBS and 1% penicillin-streptomycin (Pen-Strep) solution. Then the cryovials were transferred to the controlled rate programmable freezing device chamber (Kryo 360, planer 300, Middlesex, UK) set at 25°C. After freezing the cells at a cooling rate of −1°C/min from 25°C to −80°C, the vials were immediately plunged into liquid nitrogen (LN_2) and stored in LN_2 for less than a month.

The cryopreserved pMSCs were thawed by immersing in a circulating water bath at 37°C for 1 min and were washed twice by centrifugation with ADMEM supplemented with 10% FBS and 1% Pen-Strep at $300 \times g$ for 10 min.

Analysis of Alkaline Phosphatase (AP) Activity and Early Stem Cell Transcript Factors by Immunocytochemical Staining

For the analysis of AP activity and early stem cell transcript factors, the pMSCs were grown in a sic-well tissue culture plate (Nunctm, Danmark) for 2 weeks with regular change of a fresh culture medium at 3-day intervals.

Alkaline phosphatase activity was detected with AP chromogen kit (BCIP/NBT) (Abcam Inc., MA, USA) after fixing with 4% formaldehyde solution (17). The AP activity was recorded based on the development of purple brown color.

pMSCs transcript factors were analyzed by using goat polyclonal IgG octamer-binding transcription factor 3/4 (Oct 3/4) and Nanog, and rabbit polyclonal IgG Sox2 antibodies. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells fixed with 4% paraformaldehyde solution for 30 min were washed three times with D-PBS. The immunocytochemistry staining was performed according to the modified method of Carlin et al. (6). Briefly, samples were incubated overnight with Oct 3/4, Nanog, or Sox2 polyclonal antibodies (1:200 dilutions) at 4°C. The sections were washed three times with D-PBS. Then the sections were incubated with donkey anti goat IgG-fluorescein isothiocyanate (FITC) (Oct 3/4 and Nanog) or goat anti rabbit IgG-FITC [sex determining region Y (SRY)-box 2 (Sox2)] (1:200) antibodies at room temperature (RT) for 1 h. After washing three times with D-PBS the sections were counterstained with 1 µg/ml propidium iodide (PI) solution for 1 h. Precleaned slides mounted with Vectashield mounting medium (Vector Laboratories Inc., CA, USA) were covered with cover glass and observed under a confocal microscopy (Olympus, Japan). The green and yellow color staining indi-

cates the positive reaction of transcript factors and red color shows the nucleus.

Survival Rate of pMSCs by Trypan Blue Exclusion Test

Immediately after thawing, pMSCs were stained by 1:1 dilution of 0.4% trypan blue solution. A drop of solution containing stained pMSCs was transferred to hemocytometer, covered with a cover glass, and observed under a light microscopy. The numbers of dead and viable cells were recorded based on the development of blue color. Survival rate of pMSCs was calculated using the formula: number of blue positive cells/total cell number × 100.

Colony Forming Unit-Fibroblast (CFU-F) Assay of pMSCs

A concentration of 5×10^5 pMSCs was seeded on each well of a six-well tissue culture plate and grown for 2 weeks with regular change of a fresh culture medium at 3-day intervals. After removing the culture medium on the 14th day the tissue culture plate wells were air dried and fixed with 100% methanol for 5 min each. The pMSCs in the tissue culture plate wells were stained with 4% Giemsa solution for 5 min and washed with low stream tap water to remove the excess stain. Then the plates were observed under a light microscope for CFU-F colonies ranging from 1 to 8 mm in diameter. This assay was conducted in six replicates.

Analysis of Apoptosis-Related Genes, Bak and Bcl2, by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

pMSCs cryopreserved with various concentrations of DMSO were grown in the culture medium for 0 h, 3 h, and up to 90% confluence culture to evaluate the expression of apoptosis related genes. Following the extraction of total RNA from each pMSCs using RNeasy micro-Kit (Qiagen Valencia, CA) the cDNA was synthesised using an Omniscript RT kit (Qiagen Valencia, CA) and 10 μM Oligo-dT primer (Invitrogen, CA, USA) at 37°C for 60 min. The specific primers in the present study were used by targeting the amplicon size of 209 bp (Bak F: 5′-ACCGACCCAGAGATGGTCAC-3′ and Bak R: 5′-CAGTTGATGCCACTCTCGAA-3′), 196 bp (Bcl2 F: 5′-GAAACCCCTAGTGCCATCAA-3′ and Bcl2 R: 5′-GGGACGTCAGGTCACTGAAT-3′) and housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase primer targeting the amplicon size of 230 bp (GAPDH F: 5′-GGGCATGAACCATGAGAAGT-3′ and GAPDH R: 5′-AAGCAGGGATGATGTTCTGG-3′). PCR amplification was carried out in an automated thermal cycler (MJ Research Inc., USA) using the Maxime PCR premix Kit (iNtRON BIO, Korea) in 25 cycles with each cycle consisting of a initial denaturation step at 94°C for 2 min, annealing step at 55°C for 30 s, and elongation step at 72°C for 90 s, and 10 min final extension at 72°C after the 25 cycles. PCR product was mixed with gel loading dye (bromophenol blue and xylene cyanol) in 5: 1 ratio and loaded on to 1% agarose gel containing 1 μg/ml ethidium bromide and electrophoresed at 100 V for 20–25 min. Gel images were analyzed using zoom browser EX5.7 Software (Cannon, Korea) with Unok-8000 Gel Manger System Gel viewer 1.5 (Biotechnology, Korea).

Flow Cytometry Analysis of Bcl2 Antigen and Cell Surface Marker CD90

For the analysis of Bcl2 antigen the cryopreserved pMSCs were thawed and washed twice with PBS. After adjusting the cell density to 1×10^6 cells/ml the cell suspension was incubated either with 5 μl FITC-labeled anti-human Bcl2 (Caltag Lab., CA, USA) or FITC-labeled mouse IgG1 antibody (Caltag Lab.) as isotype-matched negative control at RT for 30 min. pMSCs of the control group were harvested by 0.25% (w/v) trypsin-EDTA solution and treated essentially as described above.

For the analysis of cell surface marker antigen CD90 (THY1) the cryopreserved pMSCs were thawed and grown to 90% confluence. After adjusting the cell density to 1×10^6 cells/ml the cell suspension was incubated either with 1:50 dilution of the FITC-labeled mouse anti-human CD90 (THY1; BD Bioscience, USA) or FITC-labeled mouse IgG1 (isotype matched negative control, Caltag Lab.) at RT for 30 min. FITC mouse anti-human CD90 used for this experiment has been known to cross-react with porcine antigens.

The above experiments were conducted in triplicate with 10,000 cells per sample using the Becton Dickinson FACS Calibur flowcytometer (BD Bioscience, Heidelberg, Germany) and data were analyzed by cell Quest Prosoftware.

Experimental Design

Experiment 1 was assessment of AP activity and stem cell transcript factors such as Oct 3/4, Nanog, and Sox2 by immunocytochemistry to confirm the presence of stem cells in the control group. Experiment 2 was estimation of survival rate among different groups (cryopreserved with 5%, 10%, and 20% DMSO) by exclusion test using 0.4% trypan blue solution in six replicates. Experiment 3 was confirmation of proliferation capacity among different groups by estimating the number of colonies using CFU-F assay. Experiment 4 was expression of apoptosis-related genes such as Bak and Bcl2 using RT-PCR following cryopreservation. Experiment 5 was

expression of Bcl2 antigen among different groups by flow cytometer. Experiment 6 was expression of CD90 to confirm the presence of MSCs among different groups.

Statistical Analysis

Differences among the groups were analyzed using one-way analysis of variance (ANOVA) by SPSS 12.0 (SPSS Inc. Chicago, IL, USA) of proportional data. Data were expressed as mean ± SD or percentage. Comparisons of mean values among treatments were performed using Duncan's and Tukey's multiple comparisons test. Differences were considered significant when $p < 0.05$.

RESULTS

General Observations and Expression of AP and Stem Cell Transcript Factors Such as Oct 3/4, Nanog, and Sox

Morphological appearance of cells when attached to the culture dish was similar to fibroblasts, and colonies were formed by overgrowth of cells (Fig. 1).

A strong alkaline phosphatase activity was noticed in cells, particularly by the colonies of cells (Fig. 1B). The transcript factors Oct 3/4, Nanog, and Sox2 were expressed both in nucleus and cytoplasm of the individual cells. However, the expression of transcript factors in individual cells was poor when compared to colonies (Fig. 1).

Survival Rate of pMSCs Cryopreserved With Different Concentrations of DMSO

The percentage of survival of pMSCs cryopreserved with different concentrations of DMSO is shown in Figure 2. The survival rates of pMSCs at concentrations of 5%, 10%, and 20% DMSO and the control group were 83.9 ± 5.8%, 77.6 ± 4.4%, 67 ± 7.9% and 92 ± 1.4%, respectively. In comparison to the control group, the higher percentage of pMSC survival was noticed in the 5% DMSO group, whereas the survival rate was significantly ($p < 0.05$) lower in the 10% and 20% DMSO groups.

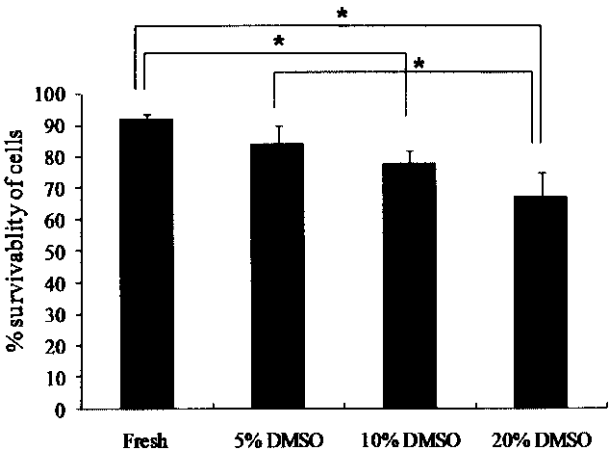

Figure 2. Survival rates of pMSCs cryopreserved with different concentrations of dimethyl sulfoxide (DMSO). Error bars indicate standard means ± SD (*$p < 0.05$).

CFU-F Assay of pMSCs Cryopreserved With Different Concentrations of DMSO

The results of CFU-F assay of pMSCs cryopreserved with different DMSO concentrations are shown in Figure 3. The number of colonies ranging from 1 to 8 mm in diameter was noticed in all groups of pMSCs. The numbers of colonies were 1087.0 ± 12.1, 1197.7 ± 2.1, and 1437.3 ± 113.2 in 5%, 10% DMSO, and control

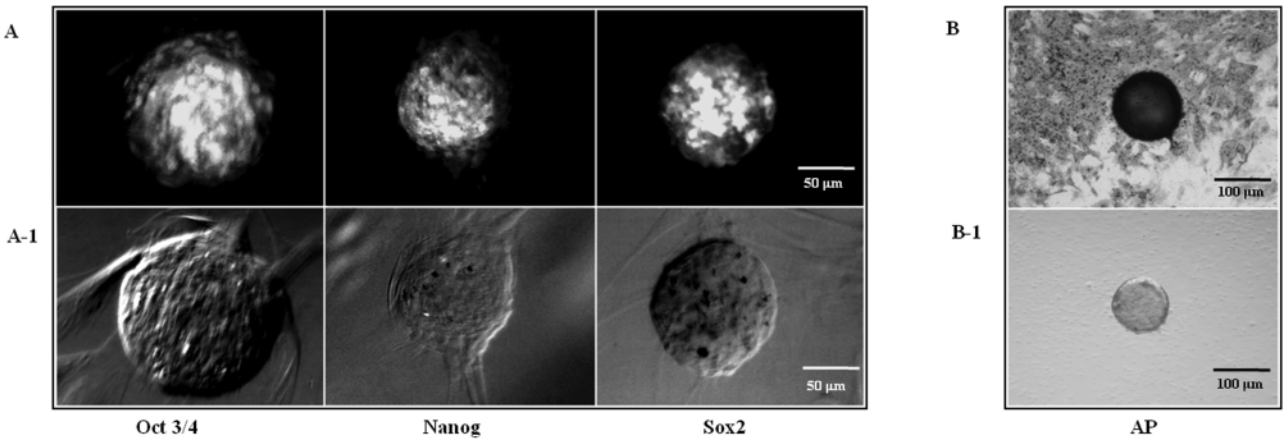

Figure 1. Expression of stem cell transcript factors, octamer-binding transcription factor 3/4 (Oct 3/4), Nanog, and SRY (sex determining region Y)-box 2 (Sox2), and alkaline phosphatase (AP) activity in porcine mesenchymal stem cells (pMSCs). (A) Colonies observed under a fluorescent microscope; green- and yellow-labeled cells indicate the transcript factors and red indicates the counterstaining of nucleus. (A-1) Colonies observed under a light microscope. Scale bars: 50 µm. (B, B-1) pMSCs and colony after and before AP staining, respectively. Scale bars: 100 µm.

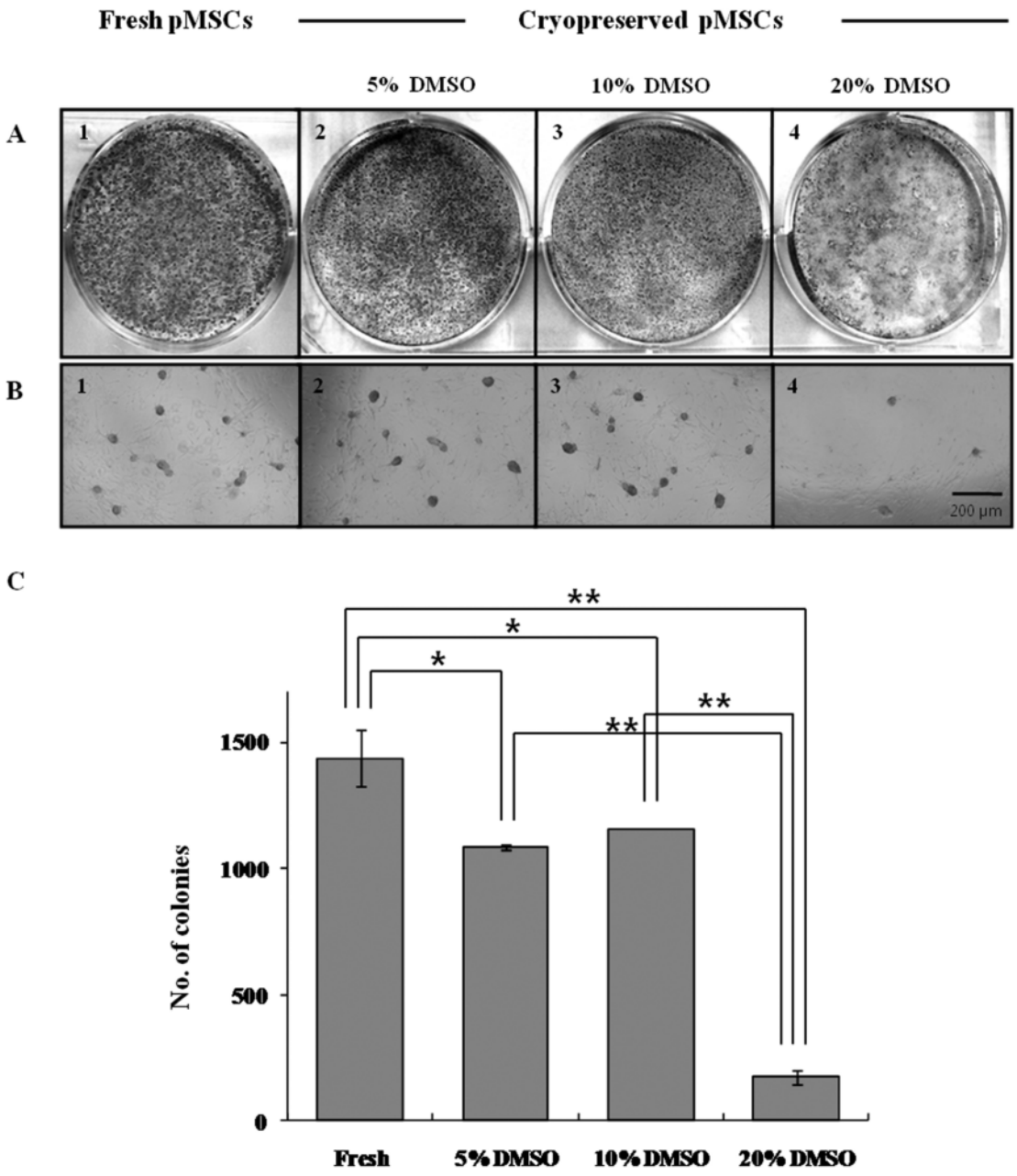

Figure 3. Colony forming unit-fibroblast (CFU-F) assay of pMSCs cryopreserved with different concentrations of DMSO. (A, B) Morphology of CFU-F assay after and before 4% Giemsa solution staining. 1, 2, 3, and 4 represent the control group (fresh pMSCs) and pMSCs cryopreserved with 5%, 10%, and 20% DMSO, respectively. Scale bar: 200 μm. (C) The number of colonies in pMSCs cryopreserved with different concentrations of DMSO. Error bars indicate standard means ± SD (*$p < 0.05$, **$p < 0.001$).

groups, respectively. However, the numbers (175.7 ± 27.6) in the 20% DMSO group were significantly ($p < 0.05$) lower compared to the others (Fig. 3). Although pMSCs of three cryopreserved groups showed significantly ($p < 0.05$) lower numbers of colonies compared to the control group, no difference was observed between the 5% and 10% DMSO groups (Fig. 3).

Expression of Bak and Bcl2 Genes in pMSCs Cryopreserved With Different Concentrations of DMSO Using RT-PCR

Expression patterns of Bak and Bcl2 genes in pMSCs cryopreserved with different concentrations of DMSO are shown in Figure 4.

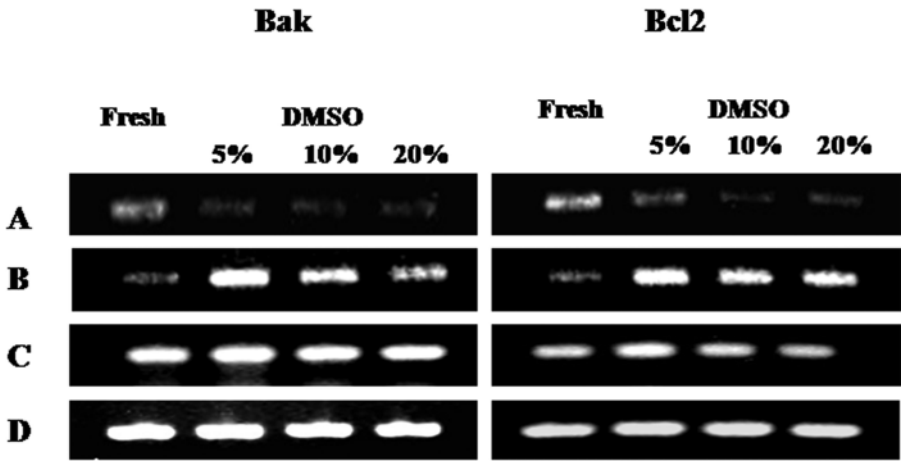

Figure 4. Expression of apoptosis-related genes, Bak and Bcl2, in pMSCs cryopreserved with different concentrations of DMSO using RT-PCR. A, B, and C represents the expression of Bak and Bcl2 genes at 0 and 3 h, and 90% confluence culture (C), respectively. D represents glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control.

At 0 h, although pMSCs in control expressed higher levels of Bak and Bcl2 than those in cryopreserved with different concentrations of DMSO, the levels did not differ among the cryopreserved pMSCs (Fig. 4A). At 3 h after thawing, although there was a slight difference in gene expression levels among the cryopreserved pMSCs groups, the levels of gene expression were noticeably high in all groups of cryopreserved pMSCs compared to the control group, especially the 5% DMSO group (Fig. 4B). The gene expression levels in all groups of cryopreserved pMSCs cultured to 90% confluence were comparable to the control group (Fig. 4C). GAPDH was used as a positive control to compare the gene expression levels in control and cryopreserved pMSCs (Fig. 4D).

Expression of Bcl2 Antigen in pMSCs Cryopreserved With Different Concentrations of DMSO Using Flow Cytometer

The expression pattern of Bcl2 protein assessed by flow cytometer is shown in Figure 5. Although Bcl2 antigen expression level was lower than that observed in pMSCs cryopreserved with 20% DMSO, a significant ($p < 0.05$) difference was not observed in the Bcl2 antigen expression level among cryopreserved pMSCs (96%, 95%, and 95% in 5%, 10%, and 20% DMSO groups, respectively) compared to the control group (97%) (Fig. 5).

Expression of CD90 in pMSCs Cryopreserved With Different Concentrations of DMSO Using Flow Cytometer

The expression pattern of CD90 by pMSCs is shown in Figure 6. The expression level of CD90 by pMSCs cryopreserved with 5%, 10%, and 20% DMSO and control group was 93.7%, 93%, 92.7%, and 93.3%, respectively (Fig. 6B–D). A significant ($p < 0.05$) difference was noticed in the expression pattern of CD90 among the cryopreserved pMSC groups as well as between the cryopreserved groups and control group.

DISCUSSION

The present study was conducted to evaluate the optimal concentration of cryopretectant DMSO for cryopreservation of pMSCs. We compared the lowest concentration of 5% and the highest 20% of DMSO with conventionally used concentration of 10% DMSO to minimize the toxicity on cryopreserved pMSCs. We have observed that the effect of 5% DMSO was similar to the conventional concentration of 10% with respect to the survivability, expression of apoptosis-related genes, proliferation capacity, and the maintenance of stemness by pMSCs in LN_2 for a month.

In general, the parameters such as AP activity (6,15) and the expression of early transcript factors (6) (Oct 3/4, Nanog, and Sox2) are used to confirm the presence of adult stem cells (16). In the present study, AP activity and the expression of early transcript factors in pMSCs revealed the characteristics that were similar to the observations made in the earlier reports (6,15). pMSCs isolated from bone marrow exhibited typical MSC characteristics of fibroblast-like structure and colonies on tissue culture plates. These findings are in accordance with previous observations (4,28,35). The results clearly showed the successful isolation and expansion of MSCs derived from pig bone marrow.

The success of clinical cell therapy largely depends

on the number of viable stem cells in the graft and hence the survival of stem cells following cryopreservation needs a special consideration (31,33). Therefore, an increase in cell survivability after cryopreservation using appropriate cryoprotectant and its optimal concentration is essential. Several authors have reported the successful cryopreservation of HSCs, MSCs, and neuronal stem cells using 10% DMSO (14,23,25). DMSO is not only responsible for exerting toxic side effects on transfusion but also promote the differentiation of cells in culture medium (14). Carvalho et al. (7) have reported the successful cryopreservation of cells at reduced concentration of DMSO. Although there are several attempts in reducing the concentration of DMSO in cryopreservation of cells, a direct comparison of effect of DMSO on survivability of pMSCs is limited. In the present study, the survivability of pMSCs was inversely proportional to the concentration of DMSO, although there were no significant ($p < 0.05$) differences in the survivability of pMSCs between 5% and 10% of DMSO concentrations. Similar observations were also made in the earlier reports on adult stem cells, such as HSCs and neural precursor cells in humans (39) and mice (23). Similarities in survivability of pMSCs cryopreserved at different concentrations of 5% and 10% DMSO suggest that 5% DMSO may also be an effective concentration for cryopreservation of pMSCs.

Proliferation capacity of pMSCs in ex vivo culture is an essential characteristic of stem cells and it is often tested by CFU-F assay (23,27). The numbers and size of the colonies in CFU-F assay are influenced by the serum concentration in cell culture medium (4). Bosch et al. (4) reported the formation of 60–70 colonies of 5 mm diameter by pMSCs (10 cells/cm^2 in 100×20-mm dish) in the culture medium supplemented with 10% FBS. However, we have recorded 1,087 colonies of less than 2 mm diameter in the present study (data not shown). The difference in the number of colonies might be due to different inoculation concentrations [5×10^5 cells/well of a six-well culture plate (52,083 cells/cm^2)]. Furthermore, formation of a very few numbers of colonies by pMSCs cryopreserved at 20% DMSO indicates the harmful effect of higher concentration of DMSO on propagation of cryopreserved pMSCs. However, no difference in the propagation of pMSCs cryopreserved in 5% and 10% DMSO was observed in this study.

In several types of mammalian cells, apoptosis is the major cause of cell death after cryopreservation (21,30). The principal proteins involved in regulating the apoptosis are Bcl2 family proteins, which are divided into two subgroups. Subgroup 1 includes Bcl-2, Bcl-w, Bcl-x_L, A1, and Mcl-1 proteins, which are antiapoptotic as well as promoting cell survival. Subgroup 2 includes Bax, Bak, Bok, Bik, Blk, Hrk, BNIP3, Bim, Bad, Bid, and Bcl-xs that are proapoptotic and accelerate the cell death under various internal and external conditions (8,37). Keeping this point in view, we have analyzed the expression of Bcl-2 and Bak genes before and after cryopreservation of pMSCs at different intervals of time. The low level of expression of Bcl-2 and Bak genes in pMSCs immediately after thawing indicates the detrimental effect of DMSO on cryopreserved cells. The expression level of both genes at 3 h after thawing was higher than fresh pMSCs. Further, the expression levels of genes among different groups were inversely proportional to the concentration of DMSO. However, the gene expression levels were similar to that of fresh pMSCs in the postthaw culturing of pMSCs up to 90% confluence (Fig. 5D). These results, therefore, suggested that cryopreservation and thawing procedures could induce the transitory silence and upregulation of genes responsible for survivability of pMSCs, which can be recovered by growing the cells in an appropriate medium for required duration.

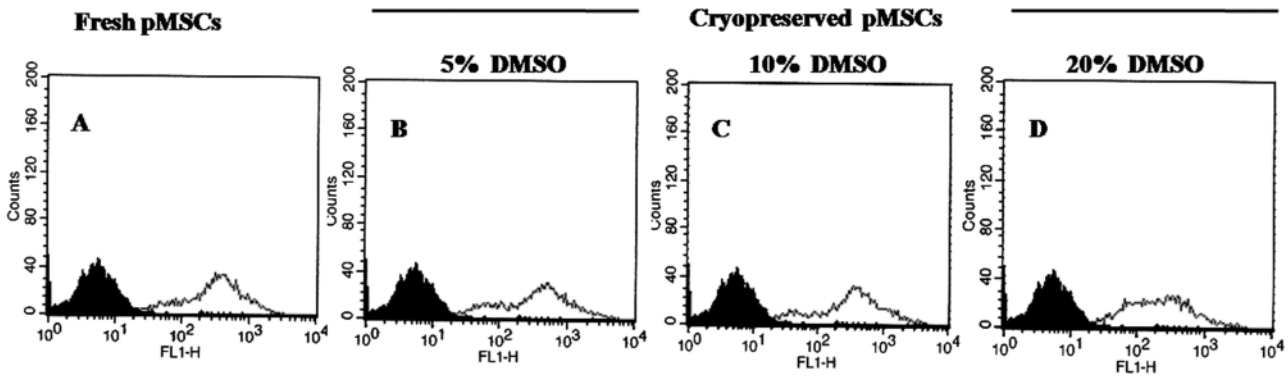

Figure 5. Expression of Bcl2 antigen in pMSCs cryopreserved with different concentrations of DMSO using flow cytometer. (A) The control group (fresh pMSCs) and pMSCs cryopreserved with (B) 5%, (C) 10%, and (D) 20% DMSO. Histogram marked in green represents the positive reaction and blue filled histogram represents the controls.

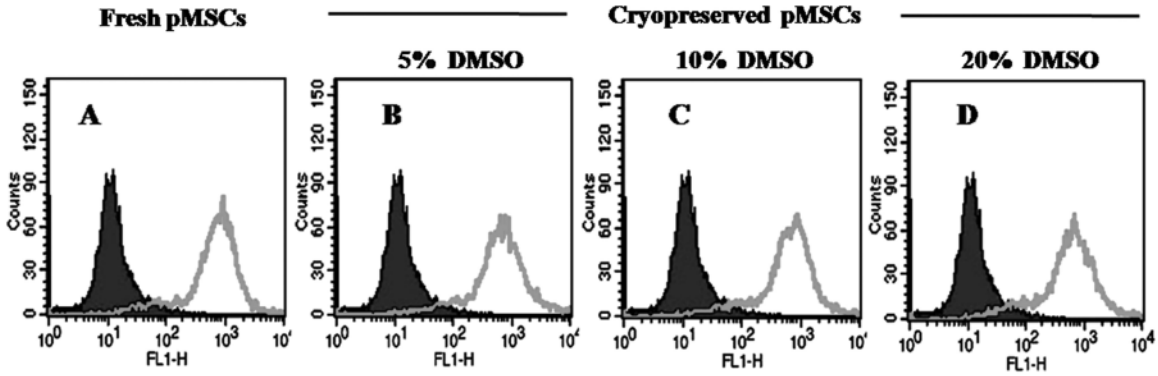

Figure 6. Expression of CD90 in pMSCs cryopreserved with different concentrations of DMSO using flow cytometer. (A) The control group (fresh pMSCs) and pMSCs cryopreserved with (B) 5%, (C) 10%, and (D) 20% DMSO. Histogram marked in green represents the positive reaction and blue filled histogram represents the controls.

CD29, CD90, and CD105 are the specific cell surface markers known to be expressed by pMSCs (4,35). Among these CD markers, CD90 has been shown to be expressed by cells derived from fetal liver, cord blood, and bone marrow apart from MSCs, HSCs, and proliferative cells with a colony-forming potential (3). This study analyzed the capacity of pMSCs in keeping their stemness during the pre- and postcryopreservation using CD90 marker. The similarity in expression of CD90 among pre- and postcryopreserved cells explains the insignificant effect of cryopreservation and DMSO concentrations on immunophenotypic characteristic of pMSCs. The results of our study are in agreement with the previous reports on the expression of cell surface markers CD90 (36), CD29, and CD105 (14) in human MSCs. These results indicate that the cryopreservation and cryoprotectant DMSO have no harmful effect on the maintenance of cell surface antigen profile of CD90 in pMSCs.

In conclusion, the cryopreservation of pMSCs with different concentrations of DMSO could induce not only cell death but also temporal reduction of proliferation capacity by suppressing the apoptosis-related genes, Bak and Bcl2. However, pMSCs cryopreserved with 5% and 10% DMSO showed similar results with respect to survivability, proliferation capacity, and the expression of apoptosis-related genes. Therefore, the present study strongly proposes the use of 5% DMSO instead of conventional 10% DMSO for the cryopreservation of pMSCs for minimizing the cryoprotectant toxicity on cells. The data acquired from our experiments could be used to advance the realization of MSC applications in clinical trials. However, it is noteworthy to mention that we have tried to evaluate the effect of DMSO at different concentrations on short-term storage of pMSCs in LN_2 (−196°C) and, hence, further research is needed to assess the effect of different concentrations of DMSO on long-term storage of pMSCs.

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REFERENCES

1. Areman, E. M.; Sacher, R. A.; Deeg, H. J. Cryopreservation and storage of human bone marrow: A survey of current practices. Prog. Clin. Biol. Res. 333:523–529; 1990.
2. Areman, E. M.; Sacher, R. A.; Deeg, H. J. Processing and storage of human bone marrow: A survey of current practices in North America. Bone Marrow Transplant. 6(3): 203–209; 1990.
3. Baum, C. M.; Weissman, I. L.; Tsukamoto, A. S.; Buckle, A. M.; Peault, B. Isolation of a candidate human hematopoietic stem-cell population. Proc. Natl. Acad. Sci. USA 89(7):2804–2808; 1992.
4. Bosch, P.; Pratt, S. L.; Stice, S. L. Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. Biol. Reprod. 74(1):46–57; 2006.
5. Buchanan, S. S.; Gross, S. A.; Acker, J. P.; Toner, M.; Carpenter, J. F.; Pyatt, D. W. Cryopreservation of stem cells using trehalose: Evaluation of the method using a human hematopoietic cell line. Stem Cells Dev. 13(3): 295–305; 2004.
6. Carlin, R.; Davis, D.; Weiss, M.; Schultz, B.; Troyer, D. Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells. Reprod. Biol. Endocrinol. 4:8; 2006.
7. Carvalho, K. A.; Cury, C. C.; Oliveira, L.; Cattaned, R. I.; Malvezzi, M.; Francisco, J. C.; Pachalok, A.; Olandoski, M.; Faria-Neto, J. R.; Guarita-Souza, L. C. Evaluation of bone marrow mesenchymal stem cell standard cryopreservation procedure efficiency. Transplant. Proc. 40(3):839–841; 2008.
8. Cory, S.; Adams, J. M. Matters of life and death: Programmed cell death at Cold Spring Harbor. Biochim. Biophys. Acta 1377(2):R25–44; 1998.
9. Davis, J.; Rowley, S. D.; Santos, G. W. Toxicity of autologous bone marrow graft infusion. Prog. Clin. Biol. Res. 333:531–540; 1990.

10. Faast, R.; Harrison, S. J.; Beebe, L. F.; McIlfatrick, S. M.; Ashman, R. J.; Nottle, M. B. Use of adult mesenchymal stem cells isolated from bone marrow and blood for somatic cell nuclear transfer in pigs. Cloning Stem Cells 8(3):166–173; 2006.
11. Fang, D.; Seo, B. M.; Liu, Y.; Sonoyama, W.; Yamaza, T.; Zhang, C.; Wang, S.; Shi, S. Transplantation of mesenchymal stem cells is an optimal approach for plastic surgery. Stem Cells 25(4):1021–1028; 2007.
12. Fleming, K. K.; Hubel, A. Cryopreservation of hematopoietic and non-hematopoietic stem cells. Transfus. Apher. Sci. 34(3):309–315; 2006.
13. Jiong, C.; Jiake, C.; Chunmao, H.; Yingen, P.; Qiuhe, W.; Zhouxi, F.; Xiangsheng, F. Clinical application and long-term follow-up study of porcine acellular dermal matrix combined with autoskin grafting. J. Burn Care Res. 31(2): 280–285; 2010.
14. Kotobuki, N.; Hirose, M.; Machida, H.; Katou, Y.; Muraki, K.; Takakura, Y.; Ohgushi, H. Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells. Tissue Eng. 11(5–6):663–673; 2005.
15. Kues, W. A.; Petersen, B.; Mysegades, W.; Carnwath, J. W.; Niemann, H. Isolation of murine and porcine fetal stem cells from somatic tissue. Biol. Reprod. 72(4):1020–1028; 2005.
16. Kuleshova, L. L.; Tan, F. C.; Magalhães, R.; Gouk, S. S.; Lee, K. H.; Dawe, G. S. Effective cryopreservation of neural stem or progenitor cells without serum or proteins by vitrification. Cell Transplant. 18(2):135–144; 2009.
17. Kumar, B. M.; Jin, H. F.; Kim, J. G.; Ock, S. A.; Hong, Y.; Balasubramanian, S.; Choe, S. Y.; Rho, G. J. Differential gene expression patterns in porcine nuclear transfer embryos reconstructed with fetal fibroblasts and mesenchymal stem cells. Dev. Dyn. 236(2):435–446; 2007.
18. Lee, M. W.; Choi, J.; Yang, M. S.; Moon, Y. J.; Park, J. S.; Kim, H. C.; Kim, Y. J. Mesenchymal stem cells from cryopreserved human umbilical cord blood. Biochem. Biophys. Res. Commun. 320(1):273–278; 2004.
19. Lee, M. W.; Yang, M. S.; Park, J. S.; Kim, H. C.; Kim, Y. J.; Choi, J. Isolation of mesenchymal stem cells from cryopreserved human umbilical cord blood. Int. J. Hematol. 81(2):126–130; 2005.
20. Liu, G.; Zhao, L.; Cui, L.; Liu, W.; Cao, Y. Tissue-engineered bone formation using human bone marrow stromal cells and novel beta-tricalcium phosphate. Biomed. Mater. 2(2):78–86; 2007.
21. Matsushita, T.; Yagi, T.; Hardin, J. A.; Cragun, J. D.; Crow, F. W.; Bergen, 3rd, H. R.; Gores, G. J.; Nyberg, S. L. Apoptotic cell death and function of cryopreserved porcine hepatocytes in a bioartificial liver. Cell Transplant. 12(2):109–121; 2003.
22. Middelkoop, E.; van den Bogaerdt, A. J.; Lamme, E. N.; Hoekstra, M. J.; Brandsma, K.; Ulrich, M. M. Porcine wound models for skin substitution and burn treatment. Biomaterials 25(9):1559–1567; 2004.
23. Milosevic, J.; Storch, A.; Schwarz, J. Cryopreservation does not affect proliferation and multipotency of murine neural precursor cells. Stem Cells 23(5):681–688; 2005.
24. Motlík, J.; Klíma, J.; Dvoránková, B.; Smetana, Jr., K. Porcine epidermal stem cells as a biomedical model for wound healing and normal/malignant epithelial cell propagation. Theriogenology 67(1):105–111; 2007.
25. Nicol, A.; Nieda, M.; Donaldson, C.; Denning-Kendall, P.; Truman, C.; Bradley, B.; Hows, J. Cryopreserved human bone marrow stroma is fully functional in vitro. Br. J. Haematol. 94(2):258–265; 1996.
26. Poncelet, A. J.; Vercruysse, J.; Saliez, A.; Gianello, P. Although pig allogeneic mesenchymal stem cells are not immunogenic in vitro, intracardiac injection elicits an immune response in vivo. Transplantation 83(6):783–790; 2007.
27. Price, E. M.; Prather, R. S.; Foley, C. M. Multipotent adult progenitor cell lines originating from the peripheral blood of green fluorescent protein transgenic swine. Stem Cells Dev. 15(4):507–522; 2006.
28. Ringe, J.; Kaps, C.; Schmitt, B.; Büscher, K.; Bartel, J.; Smolian, H.; Schultz, O.; Burmester, G. R.; Häupl, T.; Sittinger, M. Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. Cell Tissue Res. 307(3):321–327; 2002.
29. Rowley, S. D.; Feng, Z.; Chen, L.; Holmberg, L.; Heimfeld, S.; MacLeod, B.; Bensinger, W. I. A randomized phase III clinical trial of autologous blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethylstarch. Bone Marrow Transplant. 31(11):1043–1051; 2003.
30. Sarkar, S.; Kalia, V.; Montelaro, R. C. Caspase-mediated apoptosis and cell death of rhesus macaque CD4+ T-cells due to cryopreservation of peripheral blood mononuclear cells can be rescued by cytokine treatment after thawing. Cryobiology 47(1):44–58; 2003.
31. Sieburg, H. B.; Cho, R. H.; Müller-Sieburg, C. E. Limiting dilution analysis for estimating the frequency of hematopoietic stem cells: Uncertainty and significance. Exp. Hematol. 30(12):1436–1443; 2002.
32. Sonoyama, W.; Liu, Y.; Fang, D.; Yamaza, T.; Seo, B. M.; Zhang, C.; Liu, H.; Gronthos, S.; Wang, C. Y.; Shi, S.; Wang, S. Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS ONE 1:e79; 2006.
33. Theilgaard-Mönch, K.; Raaschou-Jensen, K.; Heilmann, C.; Andersen, H.; Bock, J.; Russel, C. A.; Vindeløv, L.; Jacobsen, N.; Dickmeiss, E. A comparative study of CD34+ cells, CD34+ subsets, colony forming cells and cobblestone area forming cells in cord blood and bone marrow allografts. Eur. J. Haematol. 62(3):174–183; 1999.
34. Thomé, S.; Craze, J.; Mitchell, C. Dimethylsulfoxide-induced serum hyperosmolality after cryopreserved stem-cell graft. Lancet 344(8934):1431–1432; 1994.
35. Vacanti, V.; Kong, E.; Suzuki, G.; Sato, K.; Canty, J. M.; Lee, T. Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. J. Cell. Physiol. 205(2):194–201; 2005.
36. Xiang, Y.; Zheng, Q.; Jia, B.; Huang, G.; Xie, C.; Pan, J.; Wang, J. Ex vivo expansion, adipogenesis and neurogenesis of cryopreserved human bone marrow mesenchymal stem cells. Cell Biol. Int. 31(5):444–450; 2007.
37. Yang, M. Y.; Rajamahendran, R. Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced in vitro. Anim. Reprod. Sci. 70(3–4): 159–169; 2002.
38. Zambelli, A.; Poggi, G.; Da Prada, G.; Pedrazzoli, P.; Cuomo, A.; Miotti, D.; Perotti, C.; Preti, P.; Robustelli della Cuna, G. Clinical toxicity of cryopreserved circulating progenitor cells infusion. Anticancer Res. 18(6B): 4705–4708; 1998.
39. Zhao, J.; Hao, H. N.; Thomas, R. L.; Lyman, W. D. An efficient method for the cryopreservation of fetal human liver hematopoeitic progenitor cells. Stem Cells 19(3): 212–218; 2001.