THE EFFECTS OF CRYOPRESERVATION ON THE BIOLOGICAL EFFICACY OF HUMAN ADIPOSE STROMAL CELLS: A PRELIMINARY STUDY

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**ABSTRACT**

Cryopreservation is a common method used for cells storage. However, cryopreservation has been reported to affect the biological function of cells, which leads to a need to improve the current cryopreservation methods. In this preliminary study, we aim to evaluate the effects of cryopreservation using DMSO as a cryoprotectant agent on the biological function of human adipose stromal cells (ASC) in relation to the growth and differentiation abilities of non-cryopreserved and cryopreserved ASC. ASC growth was evaluated by the time that the cells took to migrate and proliferate in the gap created from the scratch test method, which can be used as an indication of ASC ability in wound healing injury. Differentiation ability was evaluated by analyzing the percentage of coverage area by the differentiated cells during adipogenic and osteogenic induction, which were stained using Oil Red O and Alizarin Red, respectively. Both cryopreserved and non-cryopreserved ASC showed a similar growth as the average time duration needed to reach 80% to 90% confluence in the gap was 14 days. Both groups also showed similar adipogenic differentiation ability but the cryopreserved group showed a decrease in osteogenic differentiation ability compared to the non-cryopreserved group. In conclusion, ASC differentiation ability was affected by cryopreservation. However, we have yet to determine whether this is due to the cryopreservation process or the cryoprotectant agent, DMSO used in the study. Further investigation is needed to improve cryopreservation of stem cells in order to maintain their biological function and efficacy for cell-based therapy.

**1.0 Introduction**

Stem cells have the potential to be used in the treatment of several diseases such as heart disease and neurodegenerative disease due to their self-renewal ability and the ability to differentiate into multiple cell lineages. However, despite their potential for cell-based therapy, the availability of stem cells still remains a challenge. Embryonic stem cells have been shown to have much success in cell-based therapy but due to ethical and tumorigenic issues, most investigators have turned to adult stem cells as an alternative. Adult stem cells can be found in many parts of the body, for example bone marrow, adipose tissue and placenta. However, which of these sources can yield the amount of stem cells needed for success in clinical procedure such as transplantation is yet to be established. One of the stem cells sources that have the potential to produce a high yield of stem cells is the adipose tissue, which can be found in abundance and is accessible [1]. Stem cells isolated from adipose tissue were found to be multipotent and have low risk of tumorigenicity [2]. Although certain types of stem cells source have been found to have certain advantages in terms of stem cells yield, there is a need for the stem cells isolated to be stored for urgent clinical usage. Cryopreservation of stem cells is an important method for cells storage and banking which is a process to
maintain biological function by freezing and storing cells samples below -80°C, typically at or near the temperature of liquid nitrogen (-196°C) [3]. The greatest challenge in cryopreservation is the lethality of the cooling and warming processes [4]. However, cryoprotectant agents such as dimethyl sulfoxide (DMSO) have been commonly used to protect against the danger of the cooling and warming processes. On the other hand, the cryopreservation method itself poses a threat to cellular biological function particularly with the use of cryoprotectant agents, which are known to have cytotoxic effects [5]. Cryopreservation effects on stem cells particularly on adipose derived stem cells have not been well established. Therefore, this study aims to study the effects of cryopreservation on the differentiation ability of human adipose stromal cells. This study is a preliminary study where aspectsof growth, differentiation ability i.e. adipogenic and osteogenic and morphological changes of non- cryopreserved and cryopreserved ASC were evaluated.

2.0 Materials and Methods

2.1 Isolation of ASC

Human adipose tissues (n = 6) were collected following C-section procedure at the University Malaya Medical Centre (UMMC) after obtaining informed consent from female patients (age 25 – 35 years old). All procedures performed in this study were reviewed and approved (Reference number: 943.28) by the Medical Ethics Committee University Malaya Medical Centre. The adipose tissues were digested in 0.3% Collagenase Type I solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at 37°C. The digested tissue was centrifuged at 1200 rpm for 10 min to obtain the cell pellet. The cells were resuspended in medium consisting of equal volumes of Ham’s F12 and Dulbecco’s modified Eagle medium (DMEM/F12; Gibco-Invitrogen, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen), 1% glutamax (Gibco-Invitrogen), 1% antibiotic-antimycotic (Gibco-Invitrogen) and 50µg/mL Vitamin C (Sigma-Aldrich). The resuspended cells were cultured in T25 flasks (Falcon; BD Biosciences, San Jose, CA, USA) and maintained at 37°C with 5% CO₂. The culture medium was changed every 3 days. When the primary cells (P0) reached 80% - 90% confluence in the culture, the cells were trypsinized with 0.125% trypsin-ethylene diamine tetra acetic acid (EDTA) (Gibco-Invitrogen) and expanded into the next passage with expansion degree of 1:4 under the same culture condition with the seeding density of 2500 cells/cm² in each culture. The cells were divided into two groups: 1) Non-cryopreserved cells which was passaged until passage 3 (P3) where they underwent proliferation and differentiation evaluation and 2) Cryopreserved cells which was cryopreserved at the end of P2 for 7 days before undergoing for proliferation and migration as well as differentiation evaluation at P3.

2.2 Cryopreservation of ASC

At the end of P2, cells were trypsinised and suspended in 1 ml of 10% (v/v) of DMSO in 90% fetal bovine serum (FBS) prior to freezing in order to prevent crystallisation and dehydration of the cell cytoplasm. They were then transferred into sterile cryogenic vials (Nalgene, Thermo Fisher Scientific Inc, USA). These vials were kept at -80 °C for overnight. Then these vials were taken out and kept in liquid nitrogen (-196 ºC) for 7 days. The cells contained in the cryogenic vials were revived by quickly thawing them in a water bath at 37 °C. The cell suspension was then washed twice before culturing in 5 ml of cell culture medium and centrifuged at 1000 rpm for 10 minutes. The supernatant was then discarded and the cell pellets were resuspended with 5 ml of cell culture medium. Cells were then seeded in a petri dish for analysis and incubated at 37 °C in an atmosphere of 5% CO₂.

2.3 Scratch Test

Both non-cryopreserved and cryopreserved ASCs at P3 were subjected to scratch test when they reach 80% to 90% confluence to evaluate the cells migration and growth. A 0.6mm scraper (TPP, Switzerland) for cell harvest was used to scratch a straight line across the petri dish which created a gap (total surface area of approximately 294.25mm²) in the middle of the petri dish. Cells morphology and progression of cells toward 80% - 90% confluence in the gap were observed every day. The time and the coverage rate that is needed for the cells to cover the gap area were evaluated. The percentage of cells coverage of the gap was analyzed using microscopic imaging software (Scopeimage Advanced). The surface area covered by cells in the gap indicates the cells migration and growth through the evaluation of proliferation ability of the cells and the duration that they need to reach 80% - 90% confluence within the gap was observed. The percentage of surface area covered by the cells in the gap was calculated using the formula as follow:

\[
\text{Surface area covered by cells in the gap (\%) } = \frac{\text{Total surface area with cells}}{\text{Total surface area of the gap}} \times 100
\]

The coverage rate (per day) of the cells in the gap was calculated using the formula as follows:

\[
\text{Coverage rate (mm²/day)} = \frac{\text{Total surface area with cells}}{\text{Total days needed to reach confluence}}
\]
2.4 Differentiation ability

2.4.1 Adipogenic differentiation

The adipogenic differentiation capability of both non-cryopreserved and cryopreserved ASCs at P3 was tested using the adipogenic differentiation medium: growth medium was added with 200 μM indomethacin (Sigma-Aldrich), 10 μM insulin (Sigma-Aldrich), 0.5mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich) and 1μM dexamethasone (Sigma-Aldrich). Cells were seeded at 2500 cells/cm² in a 9 cm² petri dish and maintained in 5% CO₂ incubator at 37°C. The cells were initially culture in growth medium for 3 days before alternately changed with adipogenic differentiation medium for every 3 days. The ASCs underwent adipogenic induction for 3 weeks. Adipogenic cells were identified by the formation of lipid droplets in the cell cytoplasm. The lipid droplets were stained red with Oil Red O staining (Sigma-Aldrich). After adipogenic induction, the cells were fixed with 10% (v/v) formalin and stained with Oil Red O for 15 minutes. The stained cells were observed under the inverted contrast phase microscope for evaluation (Nikon Eclipse E100). The positive stained area was quantified using Scopeimage Advanced software.

2.4.2 Osteogenic differentiation

The osteogenic potential of RFMSCs at P3 was tested using the osteogenic differentiation medium: growth medium was added with 1 x 10⁻⁷ M dexamethasone (Sigma), 10mM β-glycerophosphate (Sigma) and 50ug/ml ascorbate-2-phosphate (Sigma) for 3 weeks. Cells were seeded at 3500 cells/cm² in a 9 cm² petri dish and maintained in 5% CO₂ incubator at 37°C. The morphological changes were observed under the inverted contrast phase microscope (Nikon Eclipse E100) and the medium was changed every 3 days. Osteogenic cells can be evaluated by the formation of calcium deposition in cells and stained using Alizarin Red staining solution. The positive stained area was quantified using Scopeimage Advanced software.

2.5.3 Statistical analysis

Data was analyzed by using SPSS 15.0 (SPSS Inc., Chicago, IL) and subjected to two sample t-test to determine significance difference (P < 0.05) of growth and differentiation abilities (adipogenic and osteogenic) of non-cryopreserved and cryopreserved ADSCs at P3. Data was presented as mean ± standard error of mean (SEM).

3.0 Results

3.1 Morphology of cryopreserved ADSCs

The morphology of non-cryopreserved and cryopreserved ASCs at P3 showed similar fibroblastic features i.e. spindle-like (Figures 1A and 1B) after one week in culture.
3.2 ASC growth

The average day for ASC to reach 80% to 90% confluence in the gap created from the scratch test method is 14 days for both non-cryopreserved group and cryopreserved group (Figure 2). ASCs progress was observed throughout the experiment and noted on days 1, 5, 7, 9 and 13 for both non-cryopreserved (Figure 3) cells and cryopreserved cells (Figure 4). The average surface covered by both non-cryopreserved and cryopreserved ASCs were approximately $260.7 \pm 3.04 \text{ mm}^2$ and $258.6 \pm 2.2 \text{ mm}^2$ and with the coverage rate of $18.6 \pm 4.4 \text{ mm}^2/\text{day}$ and $18.5 \pm 3.25 \text{ mm}^2/\text{day}$, respectively.

3.3 Differentiation ability of cryopreserved ADSCs

3.3.1 Adipogenic differentiation ability

Both non-cryopreserved and cryopreserved ADSCs have the ability to undergo adipogenic differentiation under the current prescribed condition (Figure 5). There was no significant difference between non-cryopreserved and cryopreserved ADSCs in terms of the area covered by the stained differentiated cells. Non-cryopreserved ADSCs have the percentage coverage of $7 \pm 1.2\%$ while the cryopreserved ADSCs have the percentage coverage of $6.6 \pm 1.06\%$ (Figure 6a).

![Fig.3 Proliferation ability of ADSCs. The progression of non-cryopreserved ADSCs at (a) 1, (b) 5, (c) 7, (d) 9 and (e) 13 in the gap created from scratch test method](image)

![Fig.4 Proliferation ability of ADSCs. The progression of cryopreserved ADSCs at (a) 1, (b) 5, (c) 7, (d) 9 and (e) 13 in the gap created from the scratch test method](image)
3.3.2 Osteogenic differentiation ability

Both non-cryopreserved and cryopreserved ASC have the ability to undergo osteogenic differentiation under the current prescribed condition (Figure 7). However, there was a significant (P<0.05) difference between non-cryopreserved and cryopreserved ASC in terms of the area covered by the stained differentiated cells whereby the non-cryopreserved group showed a higher coverage percentage (3.3 ± 0.3 %) compared to the cryopreserved ASC (2.2 ± 0.24 %) (Figure 6b).

Fig.5 Adipogenic differentiation of ADSCs at P3. The ability of adipogenic differentiation for both non-cryopreserved and cryopreserved ADSCs was similar as evaluated from the surface area coverage by the formation of lipid droplets stained with Oil Red O

4.0 Discussion and Conclusion

The emerging need to store clinically valuable stem cells for transplantation in the treatment of a growing number of human diseases has increased efforts to improve the cryopreservation method which include the use of cryoprotectant agents to protect the cells from the freezing and thawing effects. However, these agents have been found to have detrimental effects on the biological functions of stem cells. The cryoprotectant agents such as DMSO, have to be removed during the thawing process before cryopreserved cells can be administered in vivo [6]. Although cryoprotectant agents are commonly used in the cryopreservation of stem cells, their effects on the biological function and efficacy of the cells are relatively unknown.

In this preliminary study, we found that both non-cryopreserved and cryopreserved ASC have similar growth which reflects its migration and proliferation ability as indicated from the scratch test hence, the wound healing ability. The scratch test method may serve as wound healing injury model to evaluate the time duration that the cells need to migrate and proliferate in order to close up the gap (wound) created from this method. From here, we can elucidate that cryopreserved ASC do have the ability to migrate and proliferate similar as non-cryopreserved ASC but further investigation is needed to evaluate particularly, the migration mechanism. However, from the morphological observation, the cryopreserved ASC appeared smaller and rounder compared to the non-cryopreserved ASC, which have a typical fibroblastic morphology i.e. spindle-like shape [7]. The changes in the morphology could be due to cryopreservation effects. However, we have yet to determine whether it is due to the effects of cryoprotectant agent (DMSO) or the freezing and thawing processes. The cells may try to assume a smaller morphology to decrease the surface area of the cell that was exposed to the environment during cryopreservation.

On the other hand, study on the adipogenic differentiation ability of ASC showed that there was no significant difference between non-cryopreserved and cryopreserved ASC. However, there was a significant decrease in osteogenic differentiation ability of cryopreserved ASC in terms of the surface area covered by stained differentiated cells. The hallmark of osteogenic differentiation is cell mineralization, which was significantly reduced in cryopreserved ASC during induction. In osteogenic differentiation, the main factors behind the mineralization of calcium matrix are the bone morphogenetic proteins [8] and this is accompanied by the increased expression or activity of the osteoblast-associated proteins, osteocalcin and alkaline phosphatase. The increase activity of these two proteins is associated with the osteoblast phenotype, the production of enzyme alkaline phosphatase and secretion of osteocalcin during cell mineralization [9]. Cryopreservation may have decreased or down regulated the osteocalcin and alkaline phosphatase genes expression leading to the decrease of ASC osteogenic differentiation ability. In addition, viscosity of the cryoprotectant agent or medium may also play a role where viscosity inhibits protein folding and enzyme activity especially in the case of trehalose [10]. Thus, this property of cryoprotectant agent could inhibit the mineralization process by inhibiting the formation of alkaline phosphatase and osteocalcin proteins which give rise to low mineralized calcium matrix.
From this study, cryopreservation affects the biological function hence the efficacy of ASC. This may reduce the potential of cryopreserved ASC to be used in cell-based therapy.

On the contrary, Liu et al. (2008) showed that cryopreservation has no effect on the phenotype, proliferation or osteogenic differentiation of human ASCs [11] which reflects that further investigation is needed to establish a better, safer and a more standardized cryopreservation method to maintain the biological function and efficacy of ASCs during cryopreservation. In reality, cryopreservation period of cells are longer, which can be years before they are finally thawed and used in clinical settings. This may be impossible to do in research as it will involve a lot of resources to cryopreserve cells for a long period of time and investigators would have to wait for a long time for the outcome of the research.

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