

Conditioned media and DMSO enhance the cryopreservation of bovine adipose tissue-derived mesenchymal stem cells

S. Irfan¹, S. Suyatno^{2*}, H. Zulfihar¹, D. A. Lestari¹, A. Hafid², T. Kostaman², H. Herdis²,
T. P. Priyatno², P. I. Sitaresmi², M. F. Hudaya², F. B. I. Lupitasari², and M. Pangestu³

¹Department of Animal Science, Faculty of Animal and Agricultural Sciences,
Universitas Diponegoro, Semarang 50275, Indonesia

²Research Center for Animal Husbandry, National Research and Innovation Agency,
Cibinong Science Center, Jalan Raya Jakarta-Bogor, Bogor, 16915, Indonesia

³Education Program in Reproduction and Development, Department of Obstetrics and Gynecology,
Monash Clinical School, Monash University, Clayton, VIC, 3168 Australia

*Corresponding E-mail: suya017@brin.go.id

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ABSTRACT

Mesenchymal stem cells derived from adipose tissue (AD-MSCs) show great potential for reproductive biotechnology in the livestock sector. However, enzyme-based isolation of MSCs is expensive and time-consuming, so it is still rarely done, especially for applications in the livestock sector in developing countries. So, MSCs must be cryopreserved with an efficient cryoprotective agent to be stored and reproduced in various laboratories after isolation. This study was aimed to optimize the cryopreservation media for adipose-derived MSCs in cattle. This study evaluated the viability, proliferation, and morphology of AD-MSCs. The results of this study indicate that a combination of 10% DMSO, 45% DMEM, and 45% conditioned media significantly improves post-thaw viability, proliferation, and survival as compared to other mediums. Furthermore, AD-MSCs cryopreserved in this medium exhibit similar morphology as fresh cells. These findings suggest that the optimized cryopreservation medium can enhance the quality and safety of AD-MSCs for clinical applications in the livestock industry.

Keywords: Adipose Tissue, Cryopreservation, Cryoprotective agents, Livestock, Mesenchymal Stem Cells, Proliferation, Viability

INTRODUCTION

In recent years, mesenchymal stem cells (MSCs) have become therapeutic agents for livestock in a variety of regenerative medicine applications (Harness *et al.*, 2022; Xu *et al.*, 2023). One of the abundant sources of MSCs is adipose tissue, which in humans can be isolated using non-enzymatic methods (Sherman *et al.*, 2019). The International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) have clas-

sified adipose-derived stem cells with fibroblast-like morphology and adhering to plastic culture plates, which are the same as MSC characteristics (Khazaei *et al.*, 2022). Multilineage differentiation potential is seen in cattle MSCs, which express CD105, CD90, and CD73 but not CD45 (Harness *et al.*, 2022). However, research on MSCs in developing countries is still limited, with a particular focus on animal models and humans. There is a notable absence of reports on locally produced cell lines from bovine MSCs, which hinders their potential applications in the

country's livestock industry. The isolation and culture process of MSCs in developing countries is relatively rare and expensive due to the extensive use of enzymes in the isolation process (Han *et al.*, 2019).

In vitro adipose-derived stem cell (ASC) growth may take too long, even when demanding applications require numerous cells (Yong *et al.*, 2015). The most crucial stage in the successful application of mesenchymal stem cells in a therapeutic setting is cryopreservation. Cryoprotective agents (CPAs) like glycerol and DMSO are commonly mixed with FBS or proteins like albumin for cryopreservation (Matsumura and Hyon, 2009). The role of CPAs in preserving cell viability is crucial. As a key sign of cryopreservation success, a post-thaw viability threshold of >70% is widely acknowledged and used in clinical applications (Di Bella *et al.*, 2021). Most stem cell cryopreservation procedures use 10% DMSO and 10%–90% FBS. In freezing solutions, FBS helps stabilize cell membranes and adjust osmotic pressure. Due to its xenobiotic origin, it always risks infection. At room temperature, DMSO is toxic to cells, and its removal from tissue requires washing and centrifugation (Crowley *et al.*, 2021). Despite its widespread application as a cryoprotectant, DMSO can affect the characteristics of cells and has been associated with *in vivo* and *in vitro* toxicity (Erol *et al.*, 2021).

Clinical trials have shown glycerol to be safe and non-toxic. Glycerol, the backbone of triglycerides found in adipose tissue, protects cells from freezing injury by minimizing intracellular ice crystal formation and osmotic pressure differences (Lebeck, 2014; Zhang *et al.*, 2022). This makes it beneficial for cryopreservation of adipose-derived stem cells. At low concentrations (<20%), glycerol may not completely prevent crystallization, but it can protect some cells from apoptosis (Erol *et al.*, 2021). The precise nature and extent of cryoprotection provided by these compounds to a particular cell type are still speculative and inadequately understood. Nevertheless, variations in cryopreservation efficiencies due to changes in protocol levels result in variations in the quality of cryopreserved cell-based products.

MSCs produce protective bioactive substances known as the secretome, which are crucial for tissue healing and regeneration. These

molecules, also known as conditioned media, facilitate cell-to-cell communication and mediate biological functions between cells and tissues (Smolinská *et al.*, 2023). The use of conditioned media for mesenchymal stem cells has a beneficial effect on cryopreservation because it activates protective mechanisms carried out by soluble MSC factors. Reusing culture media as conditioned media can also replace FBS and make cryopreservation of MSCs more efficient. According to Maia *et al.* (2017), conditioned media supported cryopreservation and better protected the biological characteristics of fresh cells. The factors present in an MSC-conditioned medium preserves cell viability and functionality during the freezing and thawing process. Studies by Youssef *et al.* (2017) show that MSC-conditioned media containing insulin-like growth factor-1 (Igf-1) promote cell proliferation and survival. One study conducted by Ramasamy *et al.* in 2012 shows that the basic fibroblast growth factor enhances the proliferation capacity of MSCs. Kim *et al.* (2018) found that platelet-derived growth factor (PDGF) in MSC-CM promotes cell proliferation. However, combinations of different cryoprotectants have been evaluated for AD-MSCs, but the comparative efficacy of conditioned media remains unexplored, especially in the livestock industry. This study was aimed to optimize the cryopreservation protocol to ensure MSC quality and safety for future therapeutic applications in the livestock industry.

MATERIALS AND METHODS

Collection of Adipose Tissue

At the Cibirong abattoir, we collected adipose tissue samples from the subcutaneous parts of Peranakan Ongole (PO) cattle aged 2–6 years. The tissue samples were washed with phosphate-buffered saline containing 2% of *Penicillin streptomycin* (Invitrogen Gibco, USA) to remove excess blood before being placed in a 50-ml centrifuge tube containing medium containing DMEM (Dulbecco's Modified Eagle's Medium) and 2% *Penicillin streptomycin* (Invitrogen Gibco, USA) and immediately transported to the lab for processing.

Isolation and Culture of AD-MSCs

The isolation method for MSCs from bovine adipose tissue was a non-enzymatic tech-

nique based on a modified approach from human adipose tissue isolation (Sherman *et al.*, 2019) at the Stem Cells Laboratory, Genomics Building, Cibinong Science Center. After collection, the tissue was meticulously sectioned into pieces smaller than 1 cm³, which were placed in a 90mm dish. Then the tissue was dipped in 1% povidone-iodine solution and washed with PBS containing 2% *Penicillin streptomycin*. In the subsequent step, a 90-mm petri dish was utilized to contain 100 µl of phosphate buffer solution (PBS). Each tissue sample, weighing approximately 0.2g, was then washed with the PBS solution. Following this initial washing, the tissue underwent a fine-mincing process. The finely mincing tissue was distributed to each well, with a spacing of approximately 5mm between each piece. Subsequently, 100 µl of DMEM with 20% FBS and 1% *Penicillin streptomycin* was gently placed in each well during the culture initiation phase. Then, the tissue was incubated in an incubator to allow the tissue to adhere to the culture plate. After approximately 4 hours, an equivalent volume of tissue culture medium (DMEM with 20% FBS and 1% *Penicillin streptomycin*) was added to fully submerge the tissues. At 37°C, these prepared petri plates were placed in an incubator with 5% CO₂. Continuous observations were carried out to monitor cell migration and colony formation. Routine media replacement occurs every 24 hours to facilitate optimal cell growth and colony development. Over time, cells migrated from within the tissue to the surface of the petri dish. Upon observing an adequate number of cell colonies on the dish or after 10 days, any remaining tissue pieces were carefully removed.

MSCs that were obtained in isolation were cultivated in six-well plates using a specifically prepared medium of DMEM with 10% FBS and 1% penicillin-streptomycin. The initial culture phase allowed the cells to proliferate until the cell colonies reached 70-80% confluence. Colonies demonstrating this growth were promptly dissociated by employing 0.25% trypsin-EDTA to facilitate subculturing. At a density of 5 x 10⁴ cells per square centimeter, the cells possessing the capacity for subculturing were shifted into a new culture vessel at a ratio of 1:3. to assess cell concentration, a trypan blue exclusion test was performed.

Preparation of Conditioned Medium

The MSCs were isolated at the 5th passage to prepare conditioned media, indicating optimal secretome activity at this passage (Sagaradze *et al.*, 2019). After the 5th passage, MSCs were cultured, and the media was removed when the cells reached 60–70% confluency. The MSCs were then washed twice with PBS and incubated in non-phenol red DMEM (PAN-Biotech, German) serum-free media for 24 hours. The conditioned medium was collected and centrifuged at 300g for 10 minutes to remove cell debris. A 0.22µ filter was then used for filtering the medium. Subsequently, it was stored at -80°C.

Cryopreservation of AD-MSCs

For cryopreservation, AD-MSCs were cryopreserved at a concentration of 1.0 × 10⁵ cells/cryotube with four different mediums, as described below.

Medium1 (M1): 80% DMEM + 10% FBS (100 ul/ml) + 10% DMSO (100 ul/ml).

Medium2 (M2): 70% DMEM + 10% FBS (100 ul/ml) + 20% glycerol (200 ul/ml).

Medium 3 (M3): 45% DMEM + 45% conditioned media (450 ul/ml) + 10% DMSO (100 ul/ml).

Medium 4 (M4): 40% DMEM + 40% conditioned media (400 ul/ml) + 20% glycerol (200 ul/ml).

Cryopreservation of Cells and Thawing

Approximately 1.0 × 10⁵ AD-MSCs were resuspended in 1 ml CPAs and immediately transferred into cryovials for storage. The cryovials were placed in a standard container at a temperature of -80°C for a month to initiate the controlled freezing process. The temperature decrease was carefully managed at an approximate rate of 1°C per minute. This gradual cooling preserves the integrity of the cells during the freezing process by preventing the formation of damaging ice crystals. Once the controlled freezing was completed, the cryovials were moved to liquid nitrogen for one week.

For thawing, the cryovials were removed from the freezer, placed immediately in a water bath (37 °C), and shaken to ensure the sample was defrosted. The thawed sample in the tube was filled with 500 ul of DMEM. The sample was then centrifuged at 415 rpm for 5 minutes at 4 °C. Following centrifugation, the pellet was

resuspended with DMEM for further assessment after the supernatant was removed.

Cell Viability Assessment

Trypan blue staining was used to determine cell viability before and after freezing, as per standard protocol (Dar *et al.*, 2021). Ten microliters of resuspended AD-MSCs were combined with an equivalent proportion of trypan blue and left for two minutes. The viability and cell number were calculated using a LUNA-II Automated Cell Counter after the stained cell suspension was transferred to the cell counting slide.

Cell Proliferation Assessment

The proliferation rate of cryopreserved MSCs was determined by seeding the thawed AD-MSCs onto a 24-well plate (2.0×10^4 /well) and incubating them for 72 hours at 37 °C. The number of cells obtained was compared to that of fresh-thawed MSCs proliferation rate estimation. We assessed cell viability using the Trypan blue exclusion method and calculated it using the LUNA-II Automated Cell Counter.

Cell Morphology Assessment

The morphology of AD-MSCs (2.0×10^4) in the various cryopreservation mediums was observed under an inverted microscope both before cryopreservation and 72 hours after the thaw culture.

Statistical Analysis

The data is presented as the mean \pm standard deviation (SD). The statistical analysis used SPSS (IBM, Version 27) to perform a one-way ANOVA with Duncan's post hoc test. The significance level was set at $p < 0.05$.

RESULTS

Morphology of the Isolated AD-MSCs

After 24 hours, the predominant morphology of isolated adherent adipose-derived mesenchymal stem cells was flat. Then, after 3 to 5 days, spindle-shaped fibroblast-like cells appeared, which is similar to the characteristic morphology of these cells as shown in figure 1A, and after 10 days, as shown in figure 1B, the cells gained confluency of 70–80%.

The Post-thaw Viability of Adipose-derived Mesenchymal Stem Cells (AD-MSCs)

A research study was conducted to determine how the viability of AD-MSCs was affected by the use of various cryopreservation media. In Figure 2, it was observed that the viability of post-thaw AD-MSCs that were preserved in M3 was significantly higher than the viability of those preserved in other cryopreservation mediums. To determine the decrease in MSCs viability during the cryopreservation process. We compared viability before and after freezing. The results in Figure 3 indicate that the viability of post-thawed AD-MSCs preserved in M3 was comparable to that of cells preserved in M1 and

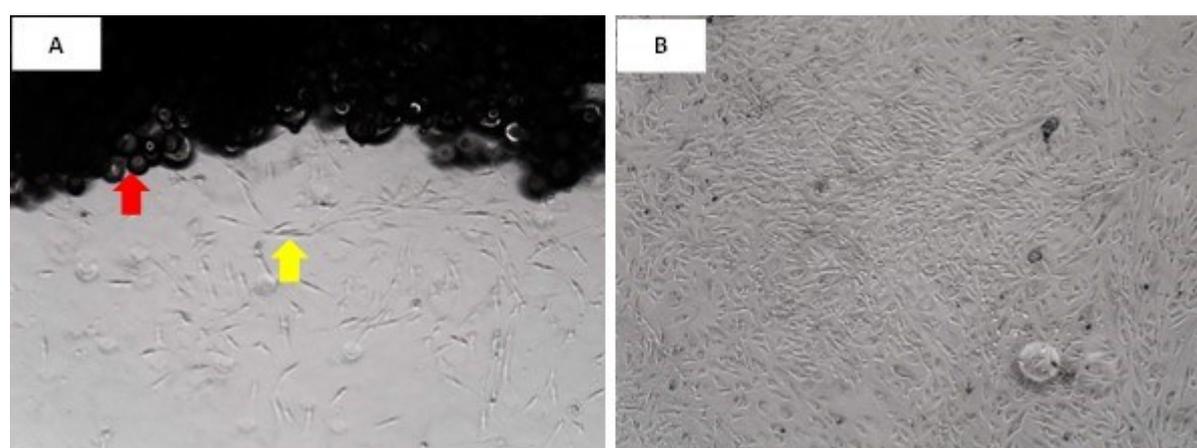


Figure 1. Morphology of the colonies at the start of culture, cells begin to appear on the day 3 and continue to grow on day 6 (A) and can be passaged after 10 day of culture (B). Red arrow indicates adipose tissue and yellow arrow indicates MSC-like colonies that have appeared.

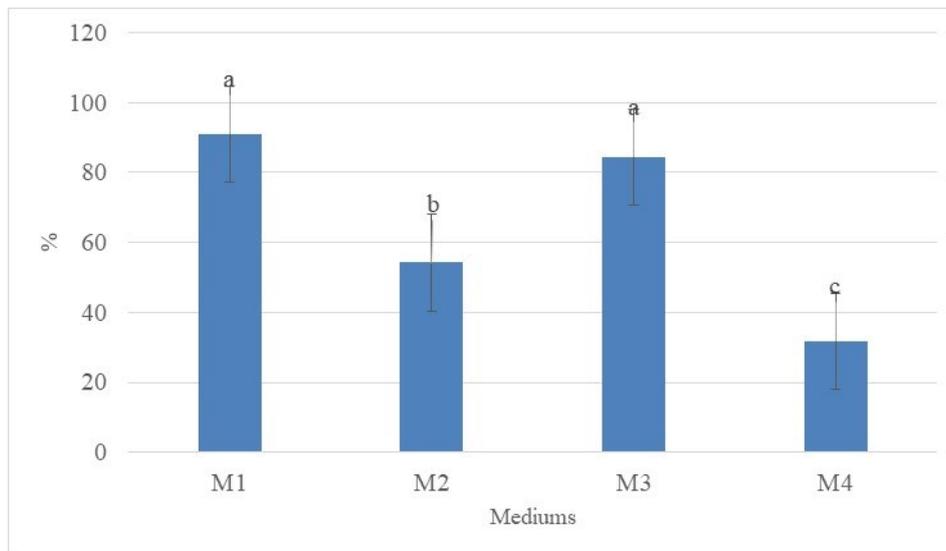


Figure 2. The post-thaw viability of adipose-derived mesenchymal stem cells (AD-MSCs) cryopreserved with different combinations of various cryopreservation mediums. Data are represented as mean \pm SD with a significance level of $p < 0.05$.

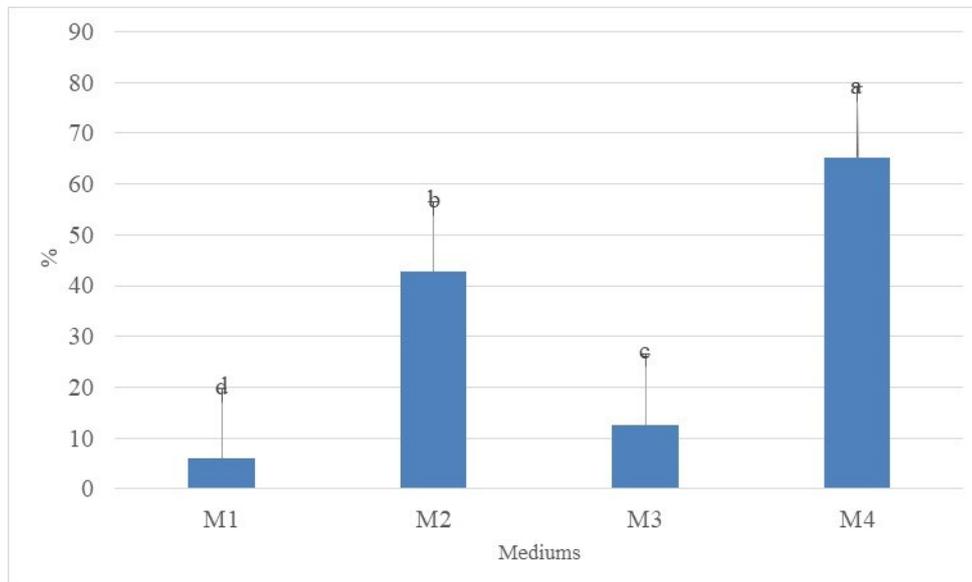


Figure 3. Decreased cell viability of AD-MSCs cryopreserved with M1 = 10% DMSO + 10% FBS + 80% DMEM, M2 = 20% glycerol + 10% FBS + 70% DMEM, M3 = 10% DMSO + 45% DMEM + 45% conditional media, and M4 = 20% glycerol + 45% DMEM + 45% conditional media. The graph is made with this formula. δ viability = before cryopreservation - after cryopreservation.

substantially greater than that of M2 and M4.

The Effect of Different Cryopreservation Media on post-thawing AD-MSCs Proliferation

Figure 4 shows that after culturing for 72 hours, M1 and M3 showed almost the same colony morphology and cell confluence. The proliferation of post-thaw AD-MSCs that were preserved in M1 and M3 was statistically significantly higher than that of cells preserved in M2

and M4 solution (Figure 5). However, M3 was higher than that of cells preserved in M2. The results show that the morphology of M3 is similar to M1 (Figure 4).

DISCUSSION

The use of cryopreservation has been essential to recent research that has demonstrated

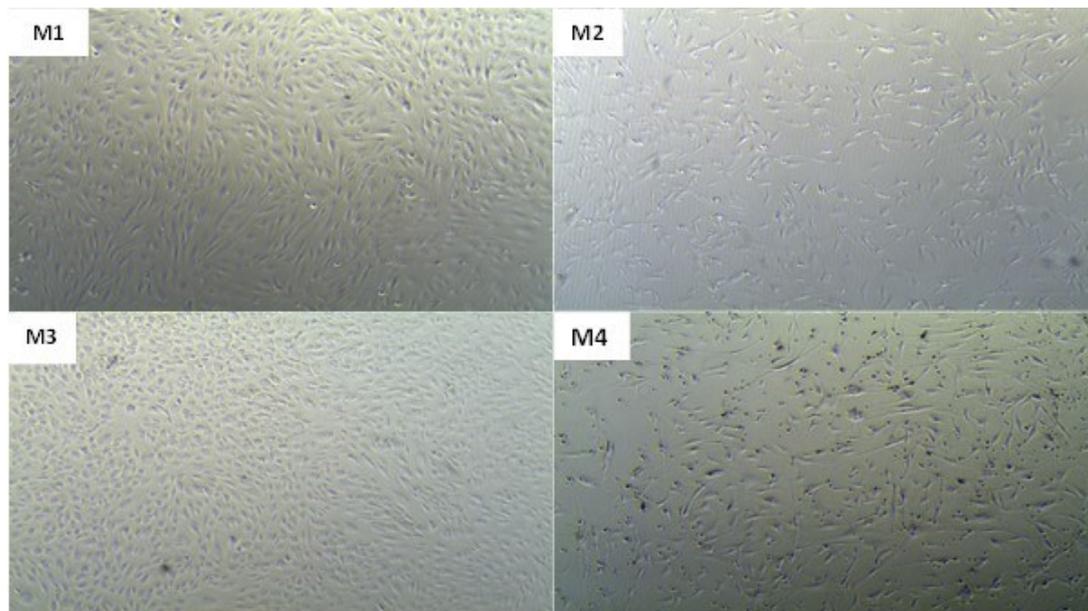


Figure 4. The impact of various cryopreservation mediums on the morphology of AD-MSCs after 72 hours of culture in all mediums. In this figure, M3 has a similar morphology to M1.

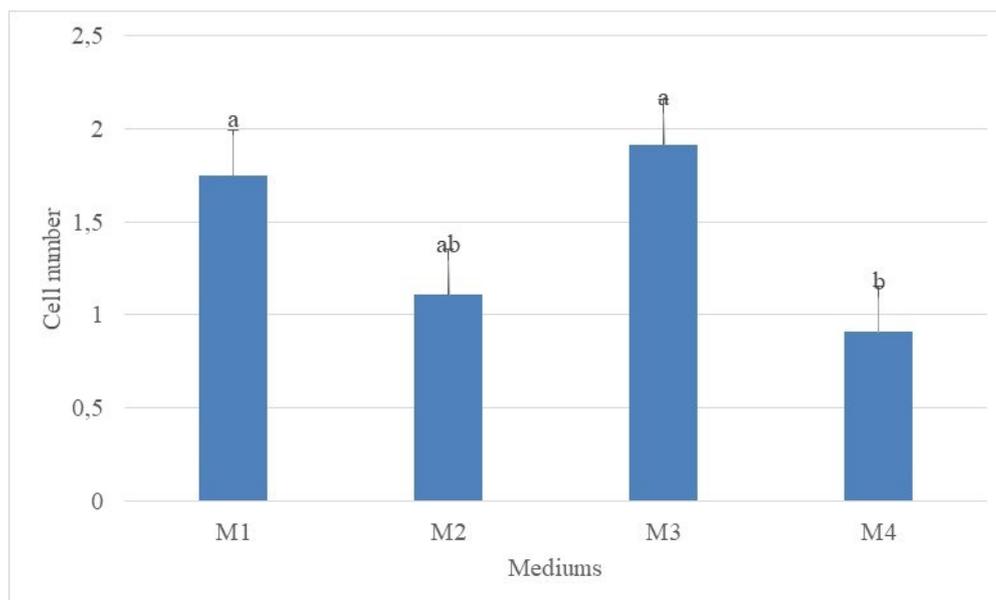


Figure 5. The proliferation of AD-MSCs cryopreserved with various cryopreservation mediums after 72 hours of thawing culturing. The data is indicated as mean \pm SD with a significance level of $p < 0.05$.

promise for the development of cellular therapies in cattle, especially in bovine using MSCs (Oyarzo *et al.*, 2021). One of the most important factors is to consider the source of MSCs. Evidence suggests that adipose-derived mesenchymal stem cells (ASCs) can acquire the characteristics of subcutaneous adipose tissue with relative ease; therefore, cryopreservation is currently

the standard method for acquiring large quantities of ASCs in a safe manner (Miyagi-Shiohira *et al.*, 2015). Stem cell therapy has gained attention in the scientific community as a potential treatment for a wide range of animal diseases. Autologous or allogenic therapy with MSC is possible following fresh isolation or thawing of a previously frozen culture. Even though MSCs

have a long history of applications in treating companion and sport animals, their biotechnological and clinical possibilities in the expanding livestock business remain largely unexplored (Hill *et al.*, 2019). The majority of ASCs are cryopreserved in 10% DMSO in FBS or other pooled animal serum, which supplies nutrients and chemicals that improve cell functionality, membrane integrity, antioxidant defense, shear stress reduction, and buffer capacity (López *et al.*, 2016). However, the therapeutic utility of MSCs is hindered by using these two components in cryosolution (Shivakumar *et al.*, 2016). Thus, the primary objective of this research is to enhance the combination of cryopreservation mediums for MSCs obtained from adipose tissue in bovine to achieve the standards of clinical application.

The current study primarily focuses on two approaches. The first approach is to perform a combination of cryopreservation mediums such as glycerol and DMSO to evaluate the post-thaw viability and stemness characteristics of cryopreserved MSCs. After 72-hour thawing culture, Table 1 shows the data on post-thaw viability that was cryopreserved using four cryopreservation mediums. The results show that post-thaw viability varied among cryopreservation mediums. Our findings indicate that M3 significantly maintains better viability than M2 and M4 and is close to the M1. This could be because of dimethyl sulfoxide (DMSO), which has been used as a cryoprotectant agent at a concentration of 10% for many years. DMSO is able to penetrate cells and inhibit the formation of intracellular ice crystals and membrane rupture (Gao *et al.*, 2020). When compared to DMSO, glycerol has lower viability for the cryopreservation adipose-derived from MSCs. Research indicates that cryopreservation in DMSO results in greater cell viability than glycerol (Van Velthoven *et al.*, 2021). One

probable explanation is that DMSO is known to penetrate cells faster than glycerol, which may contribute to its increased viability. However, DMSO has been linked to in vivo and in vitro toxicity, whereas glycerol appears to be less toxic. Despite studies indicating that DMSO can affect cell properties, it remains the CPA of choice in both research and clinical settings (Erol *et al.*, 2021). The cells cryopreserved in M3 and M1 media have similar fibroblast-like morphologies to the fresh ones, as shown in Figure 4. The author of this study observed that the viability of M4 is lower than that of M2. It means that glycerol alone does not work effectively for cryopreservation. According to study by Zhang *et al.* (2020), cells preserved in glycerol alone had lower viability than cells preserved in DMSO and FBS. However, when glycerol was combined with trehalose, the viability of post-thaw ADSCs significantly increased compared to using either agent alone. The same could be possible in our study, as FBS is composed of a wide range of components, including carbohydrates, lipids, cytokines, hormones, proteins, vitamins, minerals, growth factors, non-protein nitrogen, and inorganic substances. Hormone factors and transport proteins are essential for regulating pH changes in cell culture and influencing protease inhibition. These effects are crucial for maintaining cell viability (Lee *et al.*, 2022).

In cryopreservation, the conditioned media from mesenchymal stems obtained from adipose-derived cells can be used as a substitute for FBS. This is because the conditioned medium contains secreted growth factors and bioactive molecules that are capable of promoting cell growth and enhancing cell survival. Using FBS raises several ethical concerns. As a result, the International Society for Cellular Therapy and several other regulatory organizations have published position statements providing guidance against the use of

Table 1. Growth in the number of AD-MSCs cryopreserved with various cryopreservation media after thawing and cultured for 72 hours.

Cryopreservation medium	Post-thaw viability (%)	Number Post-thaw cell seeding/well	Number of living cells	Number of cells obtained after culture	Number of fold (%)
M1	91 ± 7.7	20,000	18200 ± 3.7	17480 ± 0.7	96.04 ± 0.7
M2	54 ± 19.4	20,000	10800 ± 2.1	11070 ± 0.6	102.5 ± 0.3
M3	80 ± 3.6	20,000	16000 ± 1	19140 ± 0.2	119.63 ± 0.2
M4	32 ± 6.7	20,000	6400 ± 1.8	9092 ± 0.7	142.06 ± 0.8

FBS in MSC for therapeutic purposes (Pezzanite *et al.*, 2021). Using conditioned media is also more cost-effective than FBS because it reuses the previously used medium. Furthermore, the conditioned media produce soluble factors that help control the immune system and promote cell growth, communication, repair, and regeneration (Maia *et al.*, 2017). Our findings are comparable with those of Maia *et al.* (2017), who reported that conditioned media improves viability and proliferation rate during cryopreservation. According to the data in Table 1, it is evident that M3 exhibits a higher proliferation rate compared to other media due to the presence of growth factors in the conditioned medium. Furthermore, the fold increase in cell number, indicated as a percentage, provides an additional perspective on each medium. Interestingly, M4 yielded the lowest number of cells after culture, but it exhibited the highest fold increase as compared to other mediums. In this study, Tsuji *et al.* (2014) documented that ASCs secrete a variety of growth factors, including insulin-like growth factor 1, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)- β 1 and basic fibroblast growth factor (bFGF). In addition to secreting growth factors, ASCs respond to them, notably bFGF and platelet-derived growth factor (PDGF), which promote proliferation. In contrast, FBS is abundant in growth factors and has low levels of γ -globulins, which can inhibit cell growth (Lee *et al.*, 2022).

The effectiveness of cryoprotectants was examined in this study. Permeable cryoprotectants like dimethyl sulfoxide (DMSO) and glycerol inhibit the formation of ice crystals during freezing. However, both have limitations. Glycerol is considered less effective as a cryoprotectant compared to DMSO because of its lower cryoprotective efficiency. The lower cryoprotective efficiency of glycerol could be due to its limited ability to remain intact with the cell membrane and protect it from freezing damage (Rajan *et al.*, 2016). Moreover, glycerol may not be as efficient as DMSO in preventing ice crystallization, resulting in cell death in cryopreservation. However, DMSO is still commonly utilized as a cryoprotectant in many biological processes due to the lack of an effective substitute. Overall, while

DMSO has been shown to have higher viability compared to glycerol after cryopreservation of ADSCs, the use of alternative cryoprotective agents and optimization of cryopreservation protocols are still being explored to improve the safety and efficacy of cryopreservation for clinical use.

CONCLUSION

The study highlights the importance of optimizing cryopreservation media for adipose-derived mesenchymal stem cells (AD-MSCs) from cattle adipose tissue. The combination of 10% DMSO, 45% DMEM, and 45% conditional media effectively maintains post-thaw viability, proliferation rate, and morphology. The use of conditioned media can replace FBS, making MSC cryopreservation more efficient. However, the study also highlights the potential drawbacks of glycerol, which may pose a risk for AD-MSCs. The livestock industry could use AD-MSCs for various therapeutic purposes, which has implications for cellular therapy.

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