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A cost-effective non-enzymatic isolation method for bovine adipose-derived mesenchymal stem cells: A preliminary study for tropical livestock applications

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ABSTRACT

Animal-derived mesenchymal stem cells (MSCs) have potential applications in livestock systems, particularly in regenerative medicine and reproductive biotechnology. In tropical settings, the development of cost-effective and enzyme-free isolation methods is important to support research and applications under limited laboratory resources. This study aimed to preliminarily evaluate bovine adipose-derived mesenchymal stem cells (AD-MSCs) isolated using a non-enzymatic method, focusing on in vitro morphology, marker expression, chromosomal profile, and post-cryopreservation viability. Adipose tissue from Ongole Grade cattle was processed using an explant culture approach. The isolated cells exhibited fibroblast-like morphology, expressed CD44 and CD166, and lacked expression of the hematopoietic marker CD45. Flow cytometry analysis further confirmed CD44 expression at the protein level, with a high proportion of CD44-positive cells. Karyotype analysis showed that most cells retained the normal diploid chromosome number (2n = 60), although minor variations in chromosome morphology were observed. Post-thaw viability remained above 90%, with no observable decline when evaluated using the same cell populations. These findings provide preliminary evidence that non-enzymatic isolation can support the establishment and maintenance of bovine AD-MSCs. This approach may serve as a cost-effective and practical alternative for MSC isolation and biobanking in tropical livestock research systems.

Keywords: Freezing thawing procedure, Karyotyping, Mesenchymal stem cells, Surface markers, Viability.

INTRODUCTION

Over the past decade, stem cell research has expanded rapidly due to its immense potential for therapeutic innovation (Hoang *et al.*, 2022; Ito and Suda, 2014). Stem cells are characterized by their ability to self-renew and differentiate into highly specialized cell types (Fujii and Miura, 2022). Among adult stem cells, Mesenchymal Stem Cells (MSCs) have been extensively studied. MSCs reside in various tissues and can differentiate into mesoderm-derived lineages, including adipogenic, chondrogenic, and osteogenic cells (Andrzejewska *et al.*, 2019; Dominici *et al.*, 2006). Their exceptional capacity for proliferation, multilineage differentiation, and immunomodulation has made them a cornerstone of regenerative medicine (Fujii and Miura, 2022; Mazini *et al.*, 2020).

In veterinary medicine and livestock production, MSCs present significant opportunities for disease management, genetic preservation, and advanced reproductive technologies, such as somatic cell nuclear transfer. Adipose tissue serves as an abundant, accessible, and minimally invasive source of MSCs compared to bone marrow or fetal annexes (L. L. Campos *et al.*, 2017; Sheykhhasan *et al.*, 2019). Traditional protocols for isolating adipose-derived MSCs (AD-MSCs) rely heavily on enzymatic digestion, such as collagenase treatment (Heldring *et al.*, 2015). However, non-enzymatic isolation methods are gaining attention as an alternative that reduces the risk of cell injury, preserves native cell surface receptors, and lowers processing costs (Al Naem *et al.*, 2020; De Francesco *et al.*, 2018; Sherman *et al.*, 2019). The primary objective of non-enzymatic approaches is to isolate a high yield of viable MSCs while preserving their intrinsic phenotypic and genetic properties (Bellei *et al.*, 2017).

Maintaining MSC characteristics such as specific membrane marker expression, robust proliferation capacity, and chromosomal stability is critical for their downstream therapeutic and biotechnological utility (Czerwińska *et al.*, 2022; Hosseini *et al.*, 2020; Ntege *et al.*, 2020). Specific surface markers, such as CD166, CD90, and CD44, are crucial for validating the identity of AD-MSCs (Dominici *et al.*, 2006; Pittenger *et al.*, 1999). Furthermore, ensuring that these cells possess resistance to freezing stress is essential for creating viable stem cell banks (C. L. K. Rebelatto *et al.*, 2023). Most importantly, confirming genetic stability through karyotyping guarantees that long-term culture and cryopreservation do not induce chromosomal abnormalities, which could compromise cellular function or induce unwanted post-transplantation effects.

While non-enzymatic isolation has been reported in human studies, its application in tropical livestock species remains limited. This study utilizes Ongole Grade (Peranakan Ongole) cattle, an important breed in tropical animal agriculture in Indonesia. This study aims to preliminarily evaluate the characteristics of bovine Adipose-derived Mesenchymal Stem Cells (AD-MSCs) isolated using a non-enzymatic method, focusing on morphology, marker expression, chromosomal profile, and cryopreservation response. The findings are expected to contribute to the development of a cost-effective and practical approach for cell isolation and biobanking in tropical livestock research systems.

MATERIALS AND METHODS

Isolation of MSCs With Non-Enzymatic Method

Bovine AD-MSCs were isolated using a modified non-enzymatic explant method, initially described for human tissues by Sherman *et al.* (2019). All procedures were approved under ethical clearance number 136/KE.02/SK/06/2023. Adipose tissue samples were obtained from the subcutaneous fat of 6 Ongole Grade bulls (aged 2 -6 years) at local abattoirs. Tissues were immediately transported to the laboratory. Samples were minced into fragments smaller than 1 mm and washed thoroughly to remove extraneous tissues. The fragments were placed in sterile 6-well culture plates. An initial minimal volume of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% Fetal Bovine Serum (FBS) and

1% penicillin-streptomycin was added, and plates were incubated at 37°C with 5% CO2 for 4 hours to promote tissue adherence. Subsequently, additional media was added to submerge the explants. Media was carefully replaced every 24 hours. Once robust cell outgrowths were observed (typically 7-10 days), the remaining tissue fragments were discarded.

In Vitro Culture of AD-MSCs

Cells migrating from the explants were cultured until colonies reached 70-80% confluence. Cells were then dissociated using 0.25% Trypsin -EDTA for 5 minutes at 37°C. The enzymatic reaction was neutralized using media containing 10% FBS. The cell suspension was centrifuged at 122 x g for 5 minutes. The resulting cell pellet was resuspended in fresh media and subcultured at a density of 2 x 10^5 cells/cm² (or a 1:3 split ratio).

AD-MSCs Cryopreservation

Following expansion, cells were harvested and centrifuged at 415 x g for 5 minutes. Cell concentration and viability were determined using a LUNA-II Automated Cell Counter. The cell pellets were resuspended in a cryopreservation solution containing 80% DMEM, 10% FBS, and 10% dimethyl sulfoxide (DMSO) (Irfan *et al.*, 2024). The suspension was transferred into 2 mL cryovials and subjected to controlled-rate freezing at 1°C/minute in a -80°C freezer before long-term storage.

Karyotyping Analysis

Karyotype analysis was carried out to determine normal chromosomes in AD-MSCs lines. Analysis was carried out using the protocol described previously (P. B. Campos *et al.*, 2009). The cultured cells were incubated in 0.1 g/ml KaryoMAX Colcemid Solution for 3 hours. The cells were separated into a single cell suspension using a 0.25% trypsin- EDTA solution. The spread of the metaphase phase was prepared by incubating a single cell suspension in a hypotonic solution (37°C) that has been warmed (KCl 75 mM) for 15 minutes andfixed in a 3:1 fixative solution (methanol: glacial acetic acid) one night at 4°C. Chromosomes were stained with Fluoromount-G mounting medium with DAPI (Invitrogen, USA) and observed under a fluorescence microscope. A total of 21 metaphase spreads were evaluated per sample to calculate the percentage of chromosomal abnormalities.

RT-PCR

To characterize AD-MSCs at the mRNA level, RT-PCR was carried out for positive markers CD44 and CD166 and negative marker CD45, according to a previously described method (Suyatno *et al.*, 2025). RNA was isolated from AD-MSCs using Agilent RNA Isolation Kits (Agilent, USA) according to the manufacturer's instructions. cDNA synthesis was carried out using ReverTra Ace cDNA synthesis kit (Toyobo, Japan) in the reaction of 1 µg of total RNA per 20 µL of PCR mixture. The collected cDNA was stored at -20° C or used directly for PCR reactions. PCR amplification was carried out using 1 µL cDNA per 20 µL PCR reaction mixture containing 2 mM MgCl2, 0.25 mM dNTPs, 1 x PCR buffer, 5 pmol of each primer and 1U Taq DNA polymerase. PCR products were separated and visualized on an agarose gel. The primer that has been prepared are listed in Table 1.

Flow Cytometry Analysis of CD44 Expression

Table 1. Primers to be Used for PCR Reactions

No.	Gen	Sequence (5'-3')
1	β-ACTIN (housekeeping)	Forward: TCCCTGGAGAAGAGCTACGA
		Reverse: ACATCTGCTGGAAGGTGGAC
2	CD44	Forward: CGGATACCAGAGACTACGGC
		Reverse: CCGCATAGGACCTGAGGTTG
3	CD166	Forward: GGCAGTGGAAGTGTCATAAACC
		Reverse: ACTTATCTCGTCTGCCTCATCG
4	CD45	Forward: CACCAGTTCAAGAAAGGACGC
		Reverse: CCACCTGAAGTCGGAGTAGAG

Cells at 70–80% confluency were harvested, washed, and resuspended in staining buffer (PBS supplemented with 3% FBS) at a concentration of 0.5–1.0 × 10^6 cells/mL. The cells were incubated with CD44-PE antibody (Novus Biologicals, USA) at a 1:50 dilution for 60 minutes at 4 °C in the dark. Following incubation, the cells were washed 1–2 times with PBS to remove unbound antibody. Prior to acquisition, cell suspensions were filtered through a 40 µm cell strainer to minimize cell aggregates. Flow cytometry analysis was performed using an Attune NxT Flow Cytometer (Thermo Fisher Scientific, USA). Unstained control samples were included to determine background fluorescence and to establish gating thresholds. Data acquisition was performed on the selected cell population, and the gating strategy included exclusion of debris based on forward scatter (FSC) and side scatter (SSC), as well as singlet discrimination (FSC-A vs FSC-H). CD44 expression was evaluated as the percentage of positive cells within the gated population. Data were analyzed descriptively.

RESULTS AND DISCUSSION

Isolation, Culture and Morphology of AD-MSCs

The non-enzymatic explant technique yielded AD-MSC-like cell populations. Cellular migration from tissue fragments was observed within 3 days of culture in six-well plates. By days 7-10, an adherent population with a distinct, spindle-shaped, fibroblast-like morphology emerged, consistent with standard MSC characteristics (Figure 1). The cells proliferated rapidly, achieving 80–90% confluence, and were successfully expanded up to passage 10 without apparent morphological senescence.

In recent years, alternative non-enzymatic methods for AD-MSC isolation have been sought to avoid tissue destruction and enzymatic toxicity (Ghorbani *et al.*, 2014). The yield and growth kinetics observed in this study align with reports by Lu *et al.* (2014a), where bovine MSCs demonstrated plastic adherence and achieved typical confluence timelines. This study prioritizes the development of a non-enzymatic isolation approach as a practical alternative; therefore, direct comparison with enzymatic methods was not included. These findings suggest that the non -enzymatic method can support the establishment of primary cultures from bovine adipose tissue under the conditions tested. This approach may be particularly relevant for tropical livestock systems where access to enzymatic reagents and advanced laboratory facilities is limited.

Gene and Protein Expression Analysis of MSC Markers

RT-PCR analysis was performed to assess MSC-associated marker expression. The isolated cells expressed CD44 and CD166, while CD45 expression was not detected across passages (Figure 2). β-actin was used as a housekeeping control. These expression patterns satisfy essen-

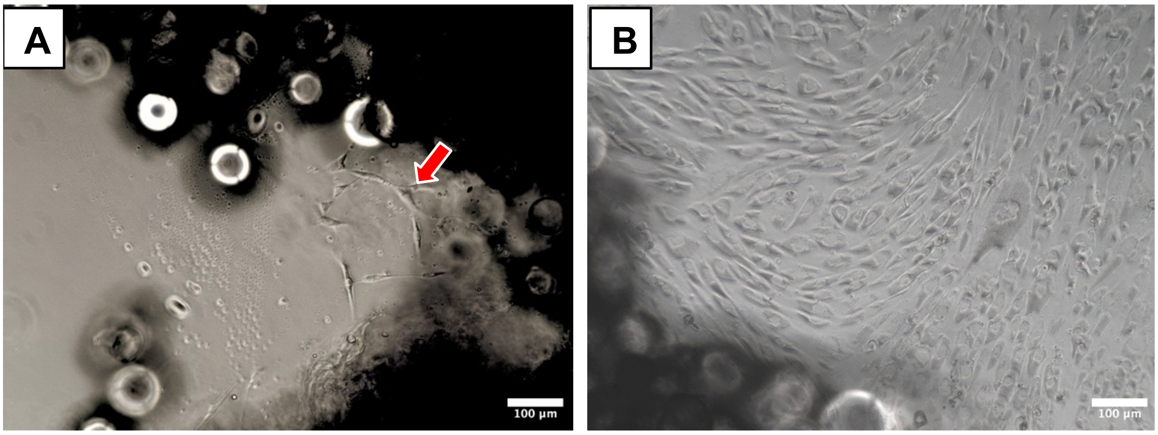

Figure 1. Small fragments of adipose tissue derived from bovine were placed into a tissue culture plate under usual conditions. By day 3 after seeding fibroblast-like cells (red arrow) formed around the tissue pieces (A), and initiated rapidly proliferation (B, day 9 post-seeding) and maintained over than 9 passages (P9).

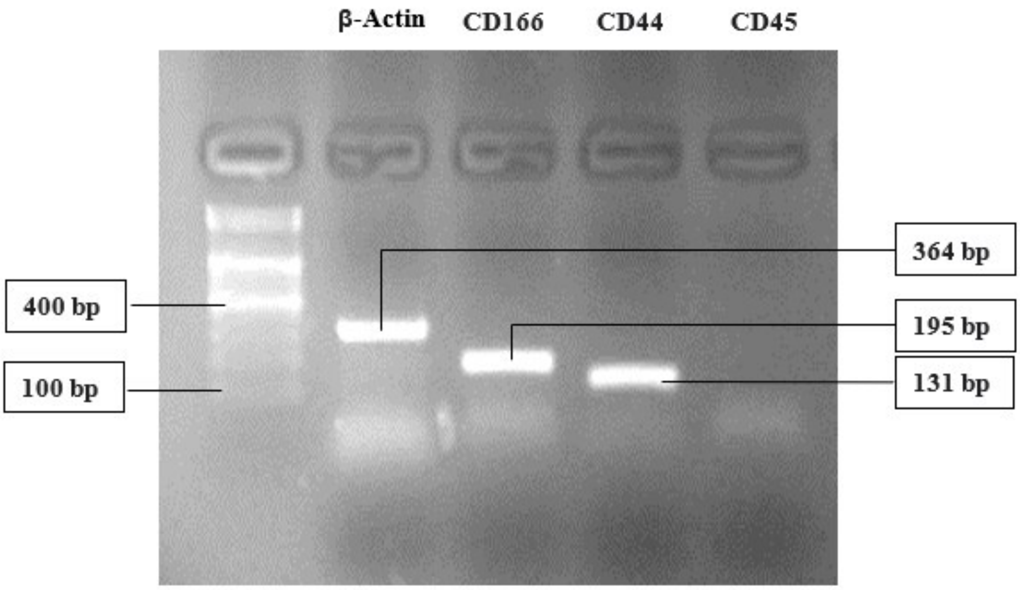

Figure 2. Surface marker identification of (AD-MSCs) at Passage (P8) using RT-PCR revealed the following expression pattern: isolated AD-MSCs expressed CD44 and CD166 as MSCs marker and negative for hematopoietic cell-surface marker CD45.

tial criteria for MSC identification. CD44 is a crucial cell surface glycoprotein involved in cellular adhesion, migration, and cell-cell interactions (Lu *et al.*, 2014b). Similarly, CD166 participates in signaling and intercellular adhesion, and its stable expression is a strong indicator of mesenchymal lineage (Rebelatto *et al.*, 2008).

To further validate marker expression at the protein level, flow cytometry (FACS) analysis was performed to assess CD44 expression in the isolated AD-MSCs (Figure 3). Gating was applied to the selected cell population (R3), representing approximately 24–26% of total events. The unstained control showed minimal background signal (0.75%), confirming the specificity of the staining. In contrast, the CD44-stained samples demonstrated a high proportion of positive cells, with approximately 86.62% of the gated population expressing CD44. These findings are consistent with the RT-PCR results and support the identification of the isolated cells as AD-MSCs populations. The high proportion of CD44 -positive cells suggests a relatively homogeneous cell population with mesenchymal characteristics. Due to the limited availability of bovine-specific antibodies, immunophenotypic characterization in this study was restricted to CD44. Therefore, further analysis using additional MSC markers (e.g., CD90, CD73, and CD105) is required to achieve comprehensive characterization according to established criteria.

Karyotyping Analysis

Karyotype analysis of the AD-MSCs at passage 9 demonstrated a normal diploid chromosome number (2n=60), comprising 58 autosomes and 2 sex chromosomes (Figure 4). These findings match the established normal karyotype for bovine species (Ahmad, 2004; Popescu, 1990; Woro *et al.*, 2012). Some metaphase spreads showed variations in chromosome number ($2n < 60$) and minor differences in chromosome morphology. The instances of reduced chromosome numbers ($2n<60$) are interpreted as technical artifacts specifically, chromosome scattering and loss caused by the hypotonic swelling and physical dropping techniques required during slide preparation (Ghorbani *et al.*, 2014) rather than true biological aneuploidy. Therefore, the results suggest that chromosomal integrity is generally maintained under the experimental conditions, although more rigorous quantitative and longitudinal analysis is required. This stability is critical for improving cattle production genetics and ensuring the safety of cells used in reproductive biotechnologies (Ciptadi *et al.*, 2017). The rele-

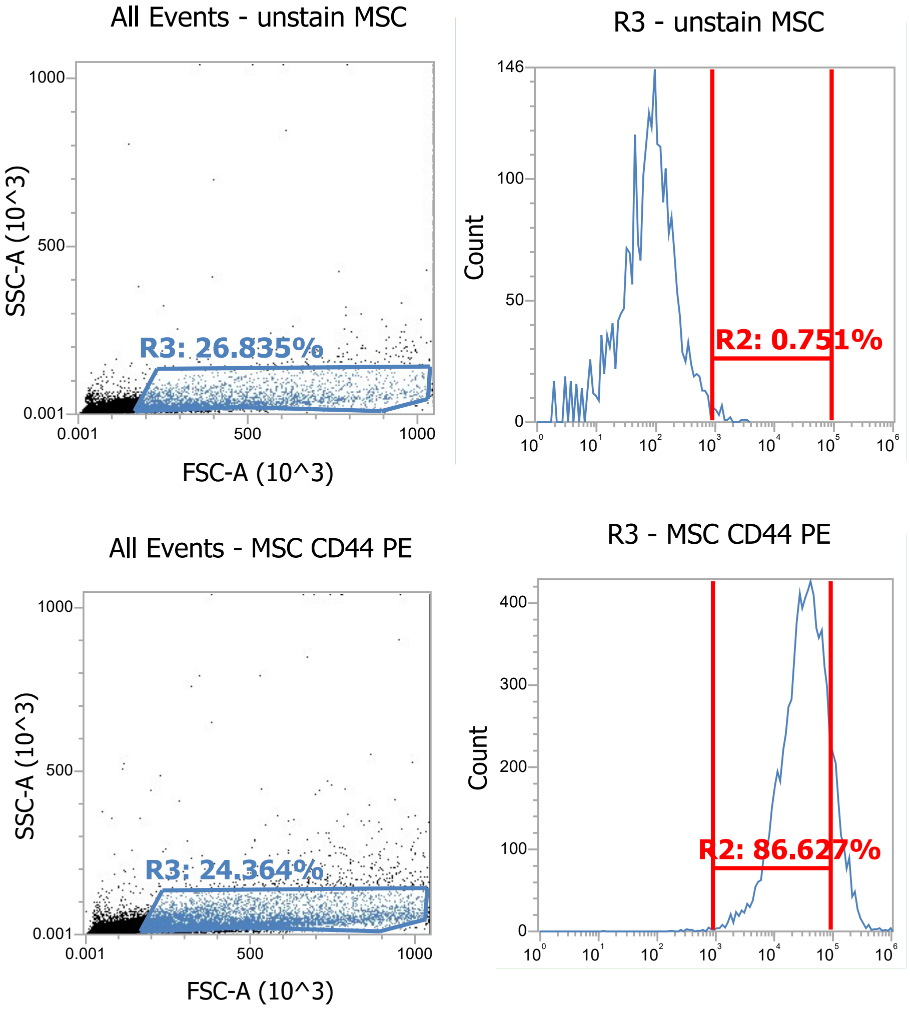

Figure 3. Flow cytometry analysis of CD44 expression in bovine AD-MSC. Histograms and dot plots show low background signal in unstained controls (0.75%) and high CD44 expression in stained cells (86.62%). Analysis was performed on gated cell populations (R3), indicating a predominantly CD44-positive population.

vance of chromosomal integrity is important for downstream applications, including reproductive biotechnology, but was not directly evaluated in this study.

Cryotolerance and Post-Thaw Viability

Post-thaw viability of AD-MSCs remained above 90% across passages (Figure 5). The post-thaw assessment was performed on the same cell populations prior to freezing, allowing direct paired observation of viability changes. Based on these observations, no apparent reduction in viability was detected following cryopreservation, although further studies with statistical analysis would strengthen this observation. The >90% viability is considered excellent for thawed MSCs (Antebi *et al.*, 2019). This suggests that the applied cryopreservation protocol including DMSO as cryoprotectant is capable of maintaining high cell viability under the tested conditions. DMSO effectively acts as an intracellular cryoprotectant by preventing severe ice crystal formation, while the high concentration of FBS provides crucial membrane stabilization during the thermal stress of freezing and rapid thawing at 37°C (Erol *et al.*, 2021; Linkova *et al.*, 2022). A fundamental requirement for establishing a stem cell bank is the ability of the cells to survive freezing and thawing protocols, and the results obtained here suggest that this method can support practical biobanking and subsequent use in reproductive biotechnology and cell-based appli-

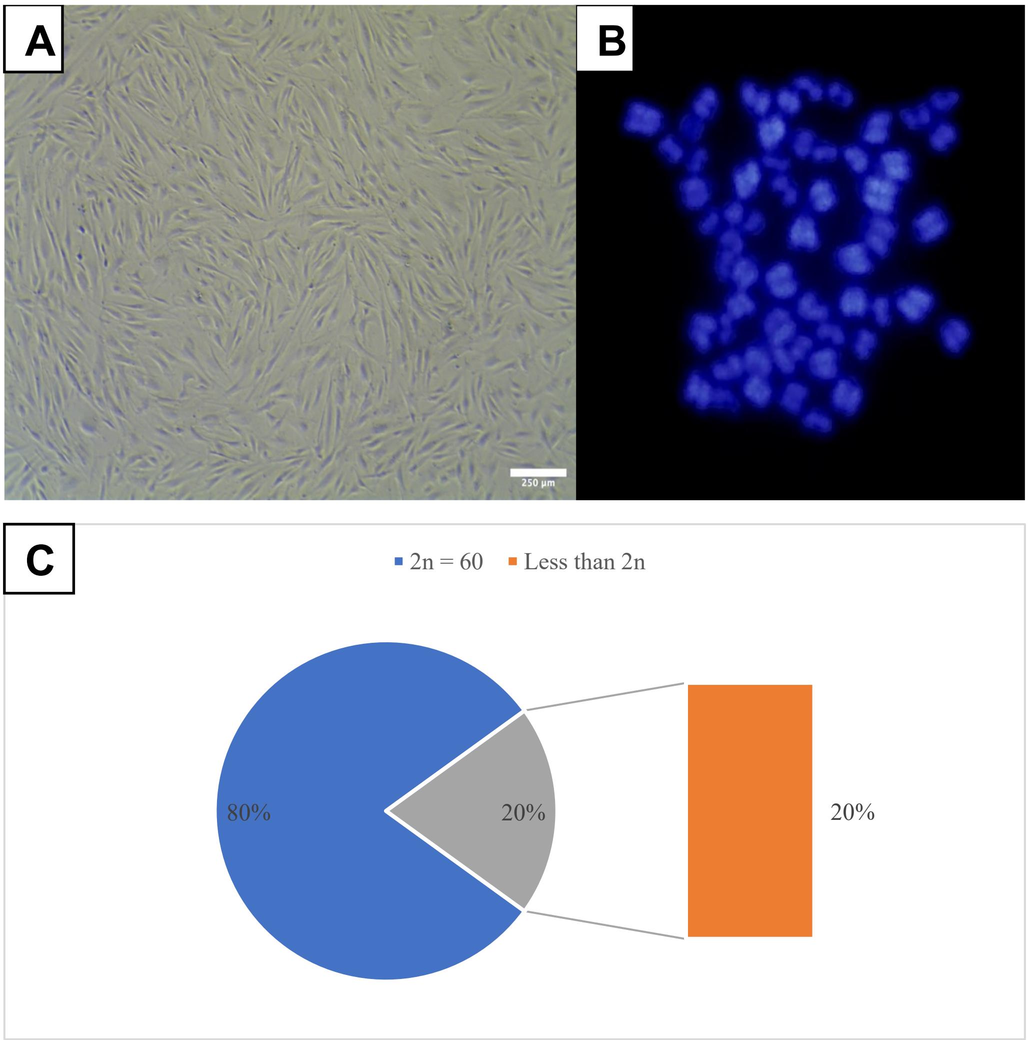

Figure 4. Karyotyping analysis of AD-MSCs: Colony morphology of AD-MSCs at passage 9 after cultured for 3 days (A), metaphase chromosome spread obtained from AD-MSCs at passage 9 (B), percentage of AD-MSCs chromosome number 2n=60 and 2n<60 (C).

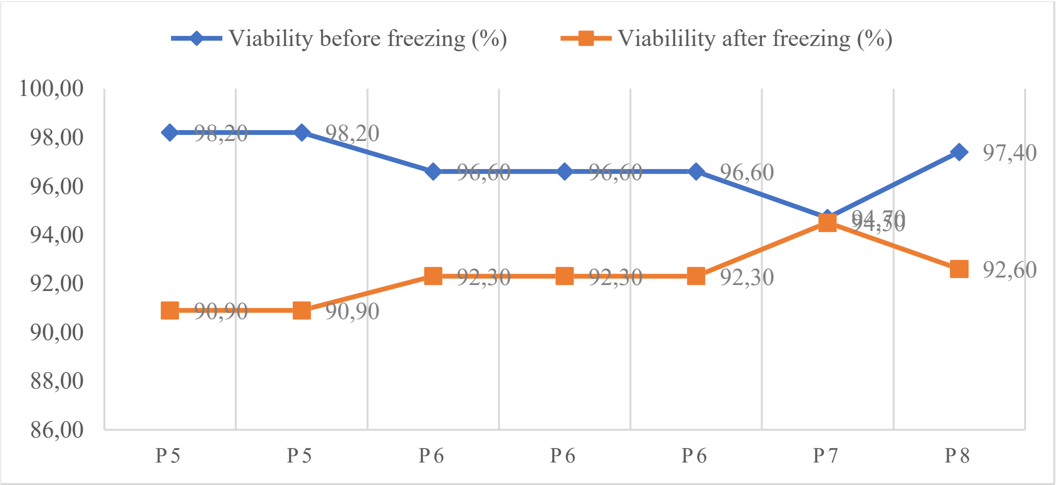

Figure 5. The effects of freezing and thawing on AD-MSC stability. The data show that cryopreservation at various passaging levels had no influence on MSC stability.

cations in tropical livestock systems.

CONCLUSION

The non-enzymatic isolation approach successfully generated AD-MSC populations from Ongole Grade bovine adipose tissue under the conditions tested. The isolated cells demonstrated characteristics consistent with bovine AD-MSCs, including stable chromosomal integrity, a high proportion of CD44-positive cells, and post-thaw viability above 90% following cryopreservation. These findings highlight the potential of the non-enzymatic method as a practical and cost-effective approach for bovine AD-MSC isolation and biobanking, particularly in resource-limited tropical livestock research settings. Further studies involving broader MSC characterization and functional evaluation may strengthen the application of this approach in bovine stem cell research.

CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest.

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