



A Comparative Analysis of the Age-dependent Features of Mesenchymal Stem Cells from Buffalo Adipose Tissue

Mohamed Hasif¹, Manjinder Sharma¹, Milindmitra K. Lonare², Digvijay Singh¹

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ABSTRACT

Background: Mesenchymal stem cells are a potential candidate for regenerative medicine. Many factors may influence the physiological characteristics of stem cells that may lead to the alteration in their potential.

Methods: The impact of age on mesenchymal stem cell characteristics was investigated in older buffaloes (aged over 10 years) and compared to younger counterparts (aged less than 2 years). Following isolation through enzymatic digestion, the mesenchymal stem cells were cultured in DMEM containing 12.5% FBS and antibiotics. Different variables indicating stem cell characteristics were evaluated at subsequent cultures.

Result: The results revealed both groups displayed positive staining for alkaline phosphatase (AP) and expressed stem cell markers, including CD73, CD105 and OCT4, while remain negative for CD34. Furthermore, both sets of stem cells demonstrated the ability to differentiate into adipogenic, osteogenic and chondrogenic lineages. There were no notable variations observed between the two groups in terms of the cell yield, percentages of senescent cells, necrotic cells, dead cells and the apoptotic index. Expression of the P53 gene in RT-PCR amplicon in both groups confirms that stem cells are dividing normally as healthy cells and not tumor cells. Consequently, it was concluded from the present investigation that the age of the animal does not influence the characteristics of mesenchymal stem cells derived from adipose tissue. Thus, the stem cells derived from older and younger buffaloes are suited equally as a cell source for regenerative therapeutics.

Key words: Apoptosis, Cell viability, Cell yield, Differentiation, Mesenchymal stem cell, Older, Younger, Population doubling time, Senescence.

INTRODUCTION

Therapeutic potential of adult mesenchymal stem cells (MSCs) for treating various degenerative diseases is gaining ground in veterinary practice. Autologous or allogenic cell-based therapies are currently being in use for treating chronic wounds, cardiovascular, musculoskeletal and neurological conditions in animals (Textor *et al.*, 2018). The therapeutic potential of MSCs is either because of their immunomodulatory (Shi *et al.*, 2011) and paracrine effects (Gnecchi *et al.*, 2008) or their differentiation capability (Hepsibha *et al.*, 2011) into a particular lineage cell type which need to be replaced. Furthermore, one of the most important criteria for the effectiveness of these cell-based treatments is the precise selection of MSCs donors. MSCs can be readily obtained from most animal tissues (Gade *et al.*, 2012) and among these, bone marrow and adipose tissues are the main sources of adult MSCs. Adipose tissue-derived MSCs are preferred over bone marrow-derived MSCs for clinical use as it is easier to harvest adipose tissue-derived stem cells with a high yield compared to mesenchymal stem cells isolated from bone marrow and other sources (Locke *et al.*, 2009).

Undeniably, stem cells in many adult tissues have been found to undergo reflective changes with age and become nonresponsive to tissue injury, reduced proliferative activities and functional capacities. All these changes in stem cells reduce the effectiveness of cell tissue regeneration *in vivo* in old age (Oh *et al.*, 2014). *In*

¹Department of Veterinary Physiology and Biochemistry, College of Veterinary Sciences, Ludhiana-141 004, Punjab, India.

²Department of Veterinary Pharmacology and Toxicology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Rampura Phul, Ludhiana-141 004, Punjab, India.

Corresponding Author: Milindmitra K. Lonare, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Rampura Phul, Ludhiana-141 004, Punjab, India.

Email: milindmitra@gmail.com

ORCID ID: <https://orcid.org/0000-0003-3983-2237>.

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vitro studies conducted over the past few years suggested that the age of the animal from which the MSCs are collected also affects various MSC properties such as the initial cell recovery, proliferation rate, population doubling time and differentiation potential which diminished with the age of donor/sourced animal (Schultz and Sinclair, 2016; He *et al.*, 2020; Babenko *et al.*, 2021). MSC properties are quite variable and changes with donor, therefore, experimental approaches are often not consistent and it becomes difficult to co-relate trends in different age groups to make a reliable

and accurate conclusion. However, the age of the buffalo has not been taken into consideration for adipose tissue collection for cell isolation and characterization and no data is available. Therefore, it becomes important to understand the effect of animal age on the mesenchymal stem cell characteristics in farm animals before collecting the tissue sample for effective therapeutic use. Moreover, highlighting differences in MSCs characteristics which may be affected by age could help the clinicians to identify an optimal cell source for cell-based therapies in animals. Hence, the study was planned to compare the growth pattern, differentiation potential and senescence in MSCs derived from adipose tissue of two different age groups of buffalo.

MATERIALS AND METHODS

Collection and transportation of adipose tissue samples

Laboratory research work of the present study was done in Tissue Culture Laboratory of Department of Veterinary Pharmacology and Toxicology, while tissue samples were collected from Teaching Veterinary Clinical Complex, College of Veterinary Science, Ludhiana, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. The subcutaneous adipose tissue was collected (approximately 2-4 gm) from apparently healthy buffalo of less than two years (<2 years) and more than ten years (>10 years) of age which were presented to the Veterinary Hospital, College of Veterinary Science, Ludhiana for abdominal surgeries during the period of March, 2021 to June, 2022. These collected tissues were transported in PBS immediately to the cell culture lab for further processing. Stem cells were isolated by the enzymatic digestion method using Collagenase type I enzyme @ 2 mg/ml. Isolated cells were then serially washed, counted in the Neubauer chamber and finally transferred into 25 cm² culture flask containing DMEM and 12.5% FBS and incubated in CO₂ incubator (37°C with 5% CO₂ level). The growth pattern was studied from day 0 to day 28. Cells were subcultured using 0.25% (w/v) Trypsin-EDTA solution at 70-80% confluency.

Cell viability

The viability of these isolated stem cells was checked using the trypan blue dye exclusion method by counting the live and dead cells where live cells appeared transparent and dead cells take up the stain. The live cells were counted using the formula

$$\text{Viable cell/ml} = \frac{\text{Total viable cells}}{4} \times \text{Dilution factor} \times 10^4$$

Characterization of mesenchymal stem cells

Characterization of MSCs was done after 3rd passage. The cells were seeded in 35 mm culture dishes and allowed to grow in the growth medium and then monitored for 70-80% confluency. The growth media was removed and washed with PBS thrice and fixing of the cells was done using 4%

formalin followed by PBS washing thrice and stained for alkaline phosphatase and immunostaining for surface markers.

Alkaline phosphatase staining

The fixed cells were stained using AP stain (25 mM Tris-HCl, 150 mM NaCl, 8 mM MgCl₂, 0.4 mg/ml Naphthol AS-MX Phosphate and 1 mg/ml FastRed TR salt) for 1 hr at 37°C. Stained MSCs were then subjected to nuclear staining with Hoechst 33258 (1µg/ml) for 5 min to make the nucleus visible. Dual stained cells were observed under bright and fluorescent fields of microscope (Singh *et al.*, 2023).

Immunostaining

Polyclonal antibodies specific for the MSCs (CD73 and OCT4) were used to characterize the cells as per our laboratory protocol (Singh *et al.*, 2023). After fixation, antigen retrieval was done using 0.01 M sodium citrate for 20 min at 37°C followed by PBS wash thrice. Then nonspecific binding was blocked by donkey serum for 30 min at 37°C followed by washing with PBS thrice. Further, these cells were incubated with primary antibody for 24 hrs at room temperature followed by washing and incubation with a secondary antibody conjugated with Texas red for 1 hr at 37°C. These stained cells were visualized in a phase contrast inverted microscope under bright and fluorescent fields. Further confirmation was done using molecular marker (RT-PCR amplicons) for CD105 as positive marker and CD44 as negative marker.

Population doubling time (PDT)

The 3rd passaged cells were seeded into 96 well plates of each group in triplicate and allowed to grow until 70-80% confluency. Cells were then subjected to cell counting kit (CCK-8) as per manufacturer's instructions for PDT. Briefly, 10 µl of reagent was added after 6 hrs of initial seeding and incubated for 4 hrs and OD was observed at 450 nm in Multiplate Reader (initial reading). The same procedure was repeated 48 hrs from initial seeding and was taken as the final reading. The total population doubling time was calculated in both the groups using the formula (Singh *et al.*, 2022).

Differentiation potential

The 5th passaged MSCs were differentiated into adipogenic, osteogenic and chondrogenic lineage in this study. 70,000 cells were cultured in culture dish and grown upto 70-80% confluency. Thereafter, growth medium was replaced with 10% differentiation supplements in DMEM for adipogenic and osteogenic differentiation. Cultures were maintained for 12 days by changing differentiation media after every 2 days. Differentiation was confirmed by staining the culture with Oil Red O stain (Adipogenic lineage) and Alizarin Red (Osteogenic lineage). Chondrogenesis was induced in pellet culture by replacing growth medium with 10% differentiation supplements in

DMEM and cultured for 21 days. Confirmation of differentiation was done using Chondrocyte staining kit. The pellets were further confirmed by sectioning and staining with Hematoxylin and Eosin staining (Luna 1968).

Senescence-associated β -galactosidase activity

Senescent cells were counted in 5th passaged MSCs using a cell senescence detection kit as per the manufacturer's instructions (EZdetect™ Cell Senescence Detection Kit; HiMedia Laboratories).

Apoptosis

Apoptosis was assessed in 5th passaged MSCs using acridine orange/ethidium bromide (AO/EB) double staining as per the method of earlier workers (Singh *et al.*, 2023).

Statistical analysis

Data obtained in the present study were analyzed by using IBM SPSS Statistics 26.0 statistical software and Microsoft Excel and were expressed as Mean $\pm$ SEM. All the data were compared at a 5% level of significance by employing an independent sample t-Test. A p-value of <0.05 was considered significant statistically.

RESULTS AND DISCUSSION

Mesenchymal stem cells were successfully isolated in the current study using enzymatic digestion from subcutaneous adipose tissue from buffalo of two different age groups (<2 years and >10 years). Adipose tissue-derived MSCs isolated from young and adult group showed non-significant

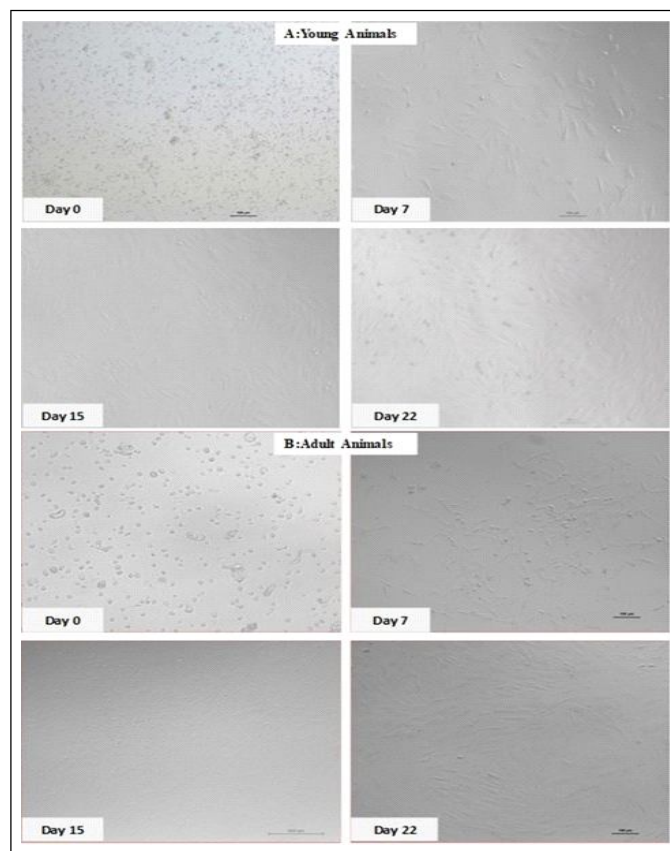


Fig 1: Cell morphology with day wise growth pattern from day 0, day 7, day 15 and day 22 for AD-MSCs young (A) and adult (B) buffaloes.

Table 1: Mean $\pm$ SEM of Initial cell yield, Cell viability, Percent attachment, Morphometry, Population doubling Time (PDT) of AD-MSCs from Young and Adult buffaloes.

Parameter	Young group (Mean $\pm$ SEM)	Adult group (Mean $\pm$ SEM)
Initial cell yield per gram (Million)	0.27 $\pm$ 0.01	0.28 $\pm$ 0.02
Cell viability (Million)	0.65 $\pm$ 0.12	0.62 $\pm$ 0.11
Percent attachment	54.97 $\pm$ 1.41	55.97 $\pm$ 1.40
Morphometry (μ m)	20.28 $\pm$ 0.40 ^a	21.60 $\pm$ 0.52 ^b
Population doubling time (hrs)	37.14 $\pm$ 1.18	36.29 $\pm$ 0.83

Value (Mean $\pm$ SEM, n=5) bearing different superscripts in the same row differ significantly ($p \leq 0.05$) from each other in an independent sample t-Test.

($p>0.05$) changes in initial cell yield per gram and cell viability (Table 1). Similarly, Hendawy *et al.*, 2021 assessed cell yield in canine adipose tissue derived MSCs and found high cell yield using enzymatic digestion of fat tissue with collagenase. In our study, adipose tissues collected from subcutaneous region were digested with collagenase enzyme for MSCs isolation in both the groups. As same protocol is followed in both the groups which might be the possible reason for non-significant changes in cell yield and cell viability.

Maximum attachment was seen after 72 hrs of initial seeding in culture flask in young ($54.97\pm1.41\%$) as well as adult ($55.97\pm1.40\%$) buffalo MSCs. However, no significant difference was observed for percent attachment in two groups. Earlier also, an average time of attachment of the cells to the culture flask was approximately 72 hrs after initial seeding as recorded by the researchers (Prichard *et al.*, 2007; Wadkin *et al.*, 2019). Cell attachment is the initial step to cellular processes such as cell proliferation and differentiation (Ruoslahti, 1996) and without attachment, the cell eventually undergoes apoptosis (Ruoslahti and Reed, 1994). In present study, we attained $>50\%$ attachment in

both the groups after 72 hrs which was quite considerable for further growth and proliferation of cells. Cell morphology was observed with differences in their shape from polygonal to spindle cells even though all species have the same fibroblast-like larger morphology as their common characteristics of MSCs. In our current study, it was found that on day 0 (primary culture) after isolation and seeding, these MSCs were found to have non-fibroblastic and round morphology (Fig 1). After 72-96 hr of initial seeding, the attached MSCs were visualized as spindle and round-shaped cells. Symmetrical colonies were formed by day 15 from the adherent cells which further grew as fibroblast-like cells on day 22 in both the groups (Fig 1). There was significant ($p\leq0.05$) increase in the cell size in adult group as compared to the young group (Table 1 and Fig 2). Similar pattern of cell attachment, morphometric and morphological changes corresponding to the days from cell isolation to cell attachment, cell growth and multiplication of MSCs of different species and different tissue origin such as ovine and porcine adipose tissue and canine ovarian surface epithelium have been reported (Beaulah Violet *et al.*, 2017; Singh *et al.*, 2022; Jha Kumar *et al.*, 2022). Further,

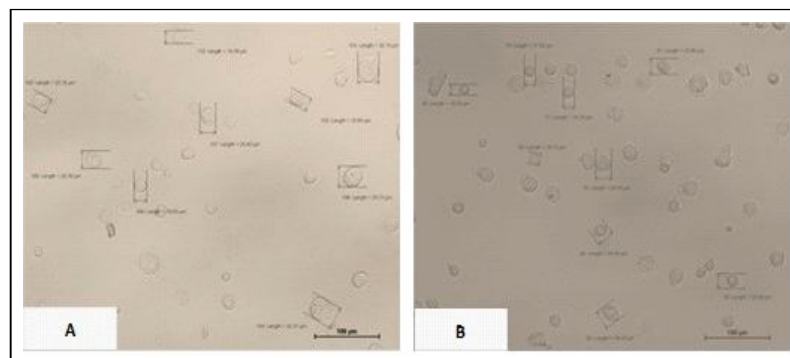


Fig 2: Representative AD-MSCs morphometry of (A) young and (B) adult buffaloes.

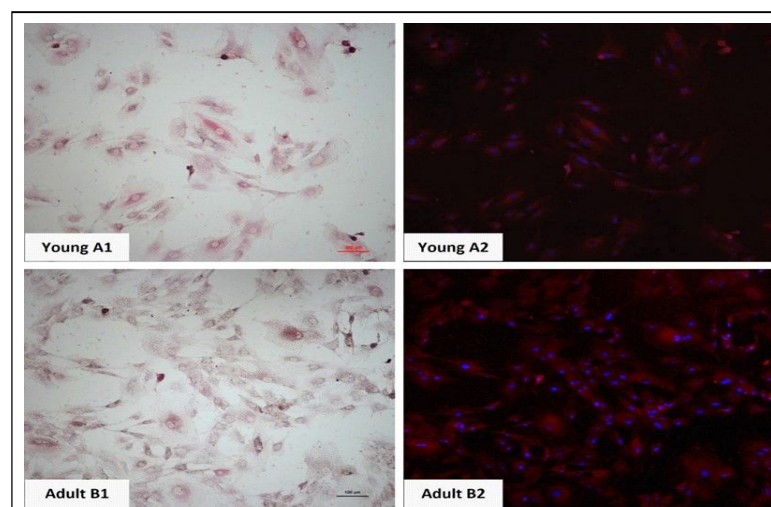


Fig 3: AP staining of AD-MSCs from (A) young and (B) adult buffaloes (1: bright field and 2: fluorescent field).

investigation conducted in rat MSCs of young and aged groups was reported to have morphological changes but, they also reported no functional changes and secretory growth factors (Mantovani *et al.*, 2012). Another study reported in human MSCs that increase in size of the cells did not have any alternation in their cell function and changes in apoptosis activity (Nicolay *et al.*, 2013). These finding support our study as we also did not find any change

in the differentiation potential, senescence status and apoptosis of these stem cells from two different age groups.

Population doubling time (PDT) of AD-MSCs from Young and Adult buffaloes calculated after passage 3 using CCK8-Kit was 37.140 ± 1.18 and 36.292 ± 0.834 for young and adult groups, respectively which showed non-significant change ($p > 0.05$) in the two age groups (Table 1). Population

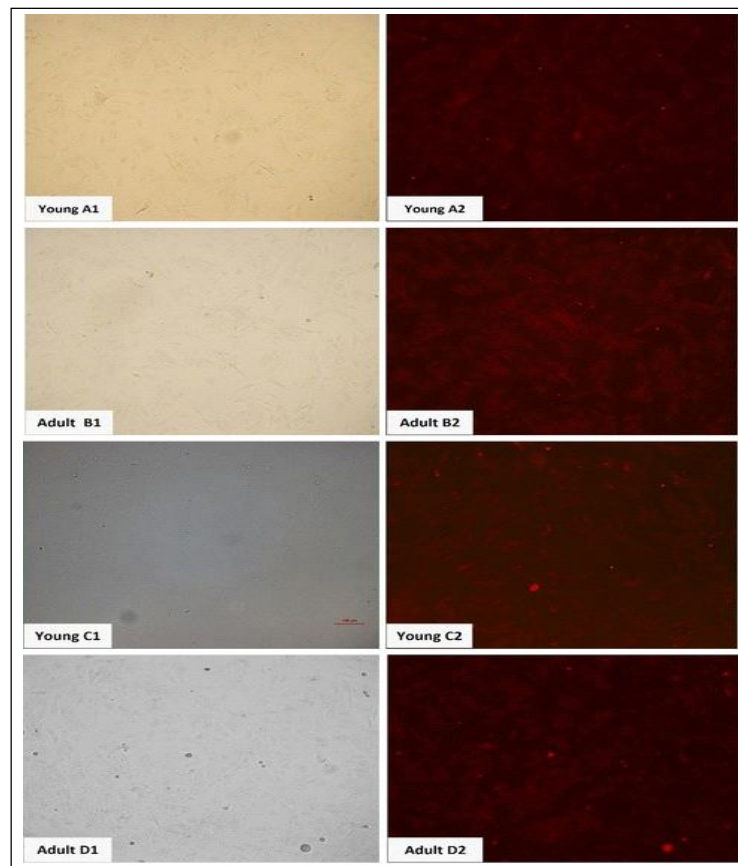


Fig 4: Representative photomicrograph of surface markers CD73 in young (A1-2) and Adult (B1-2) and OCT4 in young (C1-2) and Adult (D1-2) of AD-MSCs (1: bright field and 2: fluorescent field).

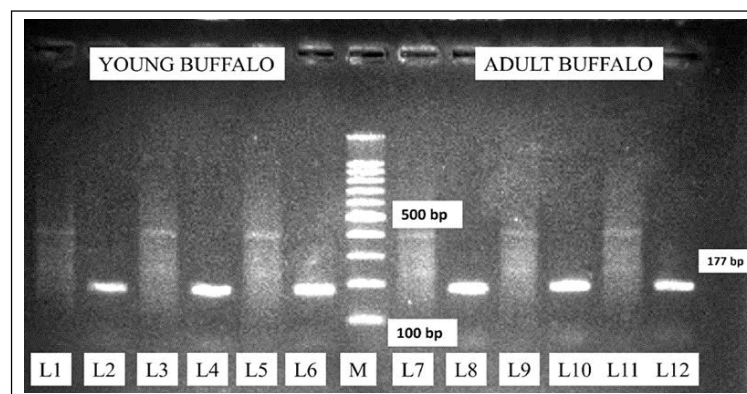


Fig 5: Expression profile of gene CD105 (177bp) and CD34 (314 bp) in AD-MSCs from young and adult buffaloes. M: 100 bp DNA ladder. L:1,3,5,7,9,11: CD34 (negative marker); L: 2,4,6,8,10,12: CD105 (positive marker).

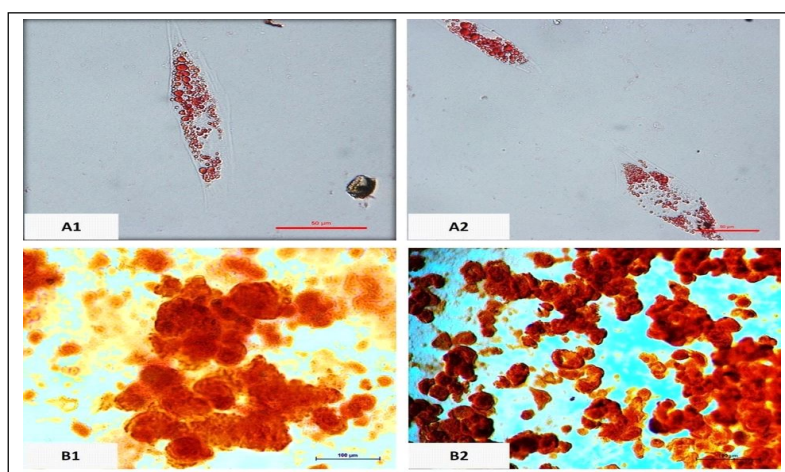


Fig 6: Representative photomicrograph of Adipogenic (A1-A2) and Osteogenic (B1-B2) differentiation of AD-MSCs from young (A1 and B1) and adult (A2 and B2) buffalo.

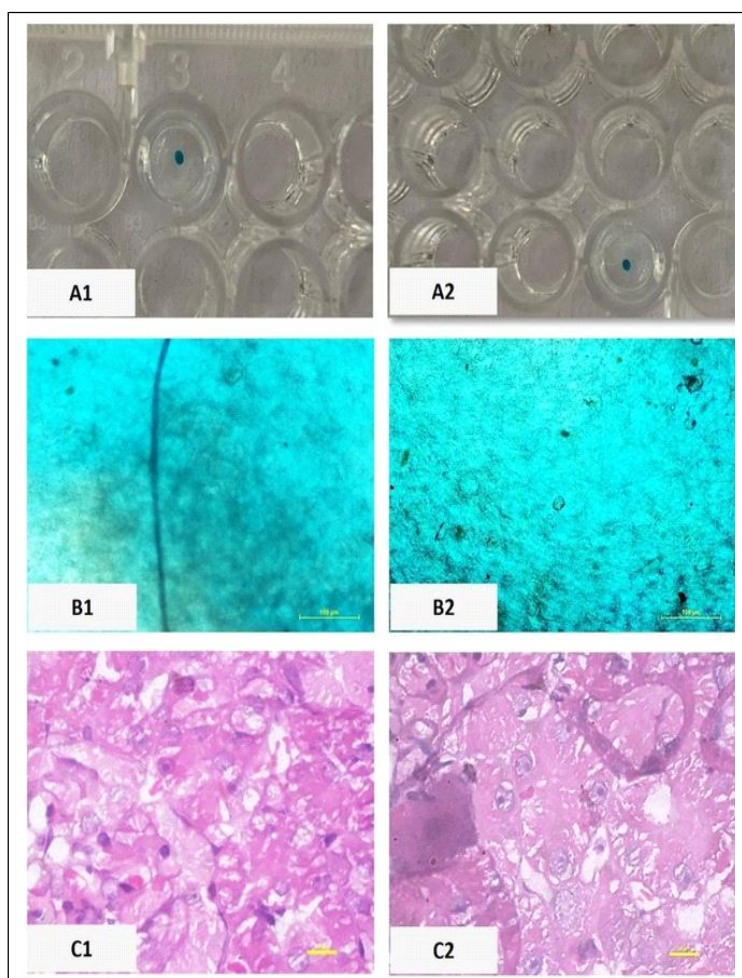


Fig 7: Representative photomicrograph of chondrogenic differentiation [A1-2: spheroid, B1-2: spheroid micrograph; C1-2: histomicrograph, young (A1, B1 and C1) and adult (A2, B2 and C2) buffalo, respectively] of AD-MSCs.

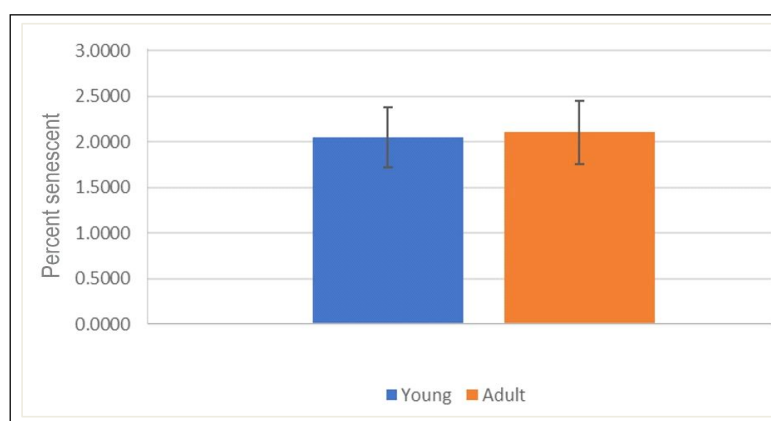


Fig 8: Bar diagram showing percent senescent cells of adipose tissue-derived MSCs from young and adult buffalo (Mean $\pm$ SEM; student t-test).

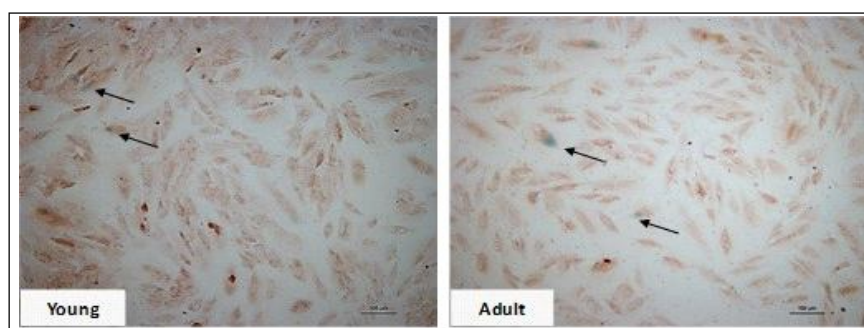


Fig 9: Representative photomicrograph of senescence associated β -galactosidase activity indicated by arrow mark by blue colored cells with senescence and red cells remains healthy from AD-MSCs of young and adult buffaloes.

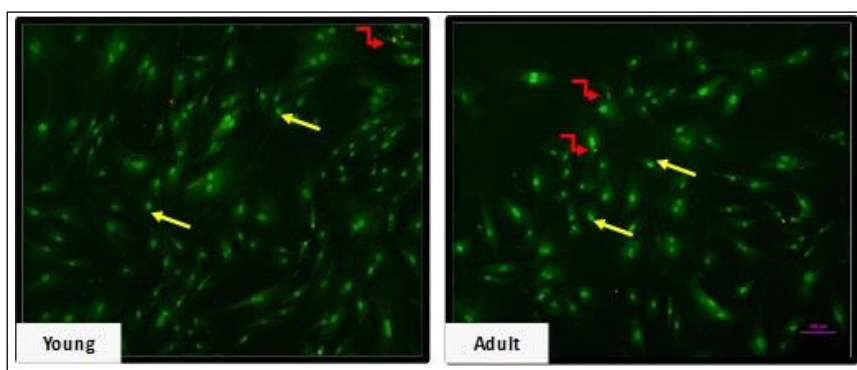


Fig 10: Representative photomicrograph of apoptosis indicated by red arrow mark with red colored nucleus under senescence and green nucleus remains healthy from AD-MSCs of young and adult buffaloes.

Table 2: Mean $\pm$ SEM of per cent senescent cells, per cent necrotic cells, per cent dead cells and apoptotic index of AD.

Parameter	Young group (Mean $\pm$ SEM)	Adult group (Mean $\pm$ SEM)
Per cent senescent cells (%)	2.05 $\pm$ 0.33	2.11 $\pm$ 0.35
Per cent necrotic cells (%)	0.794 $\pm$ 0.380	1.612 $\pm$ 0.580
Per cent dead cells (%)	3.305 $\pm$ 0.882	4.501 $\pm$ 0.761
Apoptotic index	2.72 $\pm$ 0.830	3.074 $\pm$ 0.988

doubling is defined as a time at which cell population size doubles with time. Population doubling time was reported as an important factor influencing the concentration of the cells as well as adhesion property of the cells (Hata *et al.*, 2004). Similar PDT of 36.6 hr in BM-derived buffalo MSCs (Gade *et al.*, 2013) and higher PDT of 45 hr in buffalo amnion MSCs has also been reported (Gugjoo *et al.*, 2019).

Adipose tissue derived MSCs from young and adult group were found positive for alkaline phosphatase staining appeared as red (Fig 3) under a fluorescence microscope while nucleus of the cells reflected as blue fluorescence. Alkaline phosphatase (AP) is a common enzyme secreted by all the living individuals which are used to access the establishment of cells during their growth study. A higher level of AP is associated with the process of undifferentiated cells status rather than with stemness (Stefkova *et al.*, 2015). Positive AP activity showed that cells were in an actively growing state, expressed phenotypically and yet not differentiated (Wu *et al.*, 2013). MSC-specific positive markers CD73 and OCT4 were used to characterize the cells by immunostaining. The adipose tissue derived MSCs in both the groups showed positive expression as revealed by red fluorescence for CD73 and OCT4 (Fig 4). Molecular marker CD105 (positive marker) by reverse transcriptase amplicon cDNA (Fig 5) of RNA isolated from the adipose-derived MSCs using gel electrophoresis was expressed in young and adult groups, while negative marker (CD34) did not appear in both the groups. CD73 and OCT4 were commonly used to characterized the undifferentiated MSCs. CD105 is an endoglin which was defined as a glycoprotein in cell surface which has a main function of endothelial cell proliferation indicator as well a standard marker for converting long term repopulating hemopoietic stem cells from Receptor-Targeted gene transfer (Fonsatti and Maio., 2004; Kays *et al.*, 2015). CD34 is defined as a transmembrane phosphor-glycoprotein encoded by the gene CD34 (Cluster of differentiation-34) which defined the differentiated stage of the hemopoietic stem. As per ISCT (International Society for Cellular Therapies), MSCs must express CD 105, CD 73 and CD 90 and lack expression of CD45, CD34, CD14 or CD11b, CD79 alpha

or CD19 as minimum criteria (Dominici *et al.*, 2006). In the current study, the adipose tissue-derived MSCs in both the groups showed positive expression for CD73 and OCT4 and negative expression for CD34 fulfilling the criteria of characterization of stem cells as per ISCT.

MSCs derived from adipose tissue in both the groups differentiated into mesodermic (adipogenic, osteogenic and chondrogenic). Adipogenic differentiation was revealed as lipid vacuolation after staining with Oil red O (Fig 6) while osteogenic differentiation was observed as extracellular calcium deposits stained as red with Alizarin Red stain (Fig 6) in both the young and adult AD-MSCs. Chondrogenic differentiation was confirmed by staining of proteoglycans into blue with Alcian Blue stain. These proteoglycans are the proteins that are heavily glycosylated and secreted by the chondrocytes (Fig 7). Further, the histological sectioning of chondrocyte spheroid revealed the presence of binucleated chondrocytes surrounded by cartilage (Fig 7). Synergistic results were also observed by other research workers for adipogenic, osteogenic and chondrogenic differentiation of the mesenchymal stem cells (Deng *et al.*, 2018; Wang *et al.*, 2021; Singh *et al.*, 2022; Jha Kumar *et al.*, 2022).

A complex stress response leading to irreversible loss of proliferation capacity and becoming resistant to stimuli promoting growth along with the enormous change in the gene expression is called cellular senescence (Gorgoulis *et al.*, 2019; Campisi and D'Adda Di Fagagna, 2007). Due to stress, when these cells are grown in *in-vitro* conditions may undergo senescence with varying levels according to the type of stress even though they contain endogenous germline and somatic cells irrespective of embryonic or induced pluripotent stem cells (Mistriotis *et al.*, 2016). Our results revealed no significant difference ($p > 0.05$) in percent senescent cells between the young and adult buffalo (Fig 8) clearly indicating that senescence in stem cells is not related to the age of animal. Senescence cells appeared blue in colour under bright microscopy indicative of increased β -galactosidase activity in aged cells while healthy cells were colorless and appeared red after counter staining with neutral red (Fig 9). Small proportion of senescent cells appeared which confirm normal senescent

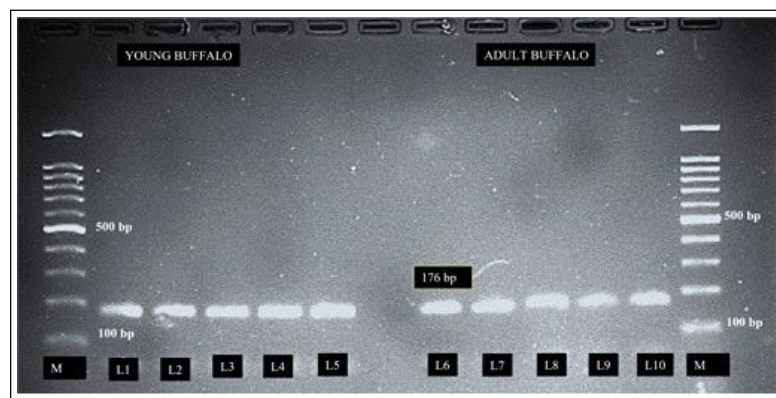


Fig 11: Expression profile of gene P53 (176 bp) in AD-MSCs from young (L1-5) and adult (L6-10) buffaloes.

process occurring in AD-MSCs in both the groups. Study of senescence in adipose tissue derived MSCs was reported in species like humans (Rouault *et al.*, 2021), canine (Teshima *et al.*, 2019) and equine (Vidal *et al.*, 2012) also.

Apoptosis is a programmed cell death where proteolytic enzymes such as caspases initiates and execute the process of cell death. Apoptosis can be physiological or pathological and often eliminates the abnormal or unwanted cells. In the current study, non-significant difference ($p>0.05$) was observed between the two age groups (Fig 10) for per cent necrotic cells, percent dead cells and apoptotic index (Table 2). Apoptosis study of AD-MSCs in bovine, canine (Haussler *et al.*, 2013) and equine (Carrade Holt *et al.*, 2014) was also reported. Further confirmation of apoptosis was done using the P53 gene in the reverse transcriptase amplicon of the cDNA from the RNA isolated from adipose-derived young and adult buffalo MSCs. The PCR product of the P53 gene is then run into gel electrophoresis (Fig 11). Hence, P53 expression indicates these cells are not tumor cells but normal healthy cells. Being a key role of P53 as a tumor suppression protein in regulating the cell cycle which acts as a transitional factor in regulating apoptosis in different physiological conditions (Valadbeygi *et al.*, 2016; Rezaei-Tazangi *et al.*, 2022). It also controls the proliferation and differentiation capacity in stem cells (Solozobova *et al.*, 2011). This supported our findings also as in the current study, MSCs showed differentiation potential and low apoptotic index in both the groups which might be related to expression of P53 gene in stem cells.

CONCLUSION

From the present investigation, it is concluded that age doesn't make any difference in cell characteristics in terms of cell yield, cell viability, population doubling time (PDT), differentiation pattern, senescence and apoptosis. Therefore, adipose-derived MSCs can be taken from buffaloes of any age group as a cell source for regenerative therapeutics.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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