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RESEARCH ARTICLE

Isolation and Characterization of Adipose-Derived Mesenchymal Stem Cells (ADSCs) from Sheep and Goats

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ABSTRACT

Recent therapeutic approaches in animal diseases involve stem cell-based therapies which are showing promising results, particularly with the use of mesenchymal stem cells (MSCs). Our study aimed at isolating and characterizing adipose tissue-derived stem cells (ADSCs) in goats and sheep, evaluating their characteristics mesenchymal nature, and adipogenic, chondrogenic, and osteogenic differentiation potentials. For this purpose, subcutaneous adipose tissues were collected from the inguinal region under sterile conditions from five healthy adult sheep and five goats each slaughtered at a slaughterhouse. MSCs were isolated, cultured, and differentiated into adipogenic, chondrogenic, and osteogenic lineages, followed by respective histochemical staining to confirm differentiation. In passage 3 (P3), the surface markers CD44, CD90, CD105, and CD45 were analyzed using flow cytometry to characterize mesenchymal properties. The cells expressed CD44, CD90, and CD105 but did not express hematopoietic marker CD45, confirming their mesenchymal nature. This study successfully identified ADSCs from sheep and goats as mesenchymal stem cells and characterized their strong trilineage differentiation potential, highlighting their strong therapeutic capabilities and revealing interspecies differences in MSCs properties. These findings provide valuable insights for future MSCs-based therapeutic applications in veterinary regenerative medicine, particularly for economically and clinically important species.

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INTRODUCTION

Stem cells hold significant importance for therapeutic applications across various clinical domains and can easily be characterized by their self-renewing capacity and differentiation potential (Gattegno-Ho *et al*., 2012). Among various stem cell types, mesenchymal stem cells (MSCs) are greatly studied for their strong regenerative capabilities in both medical and veterinary sciences. As a result, both basic and applied research on MSCs is rapidly advancing with the growing need for alternative therapeutic sources, with significant progress being made in the area of various stem cell sources (Gugjoo *et al*., 2016; 2017; 2018). MSCs can be isolated from diverse tissue sources such as adipose tissue, bone marrow, dental pulp, cord blood, Wharton's jelly, and amniotic fluid (Özen and Sancak, 2014). Among these, adipose tissue (AD) and bone marrow (BM) remain popular due to their abundance and ease of extraction. The tissue source significantly impacts the concentration, proliferation, and differentiation properties of MSCs (Ribitsch *et al*., 2017; Sasaki *et al.*, 2018;).

Adipose-derived mesenchymal stem cells (ADSCs) are considered important due to their ease of isolation, high yield, and differentiation potential, making them potent sources in tissue engineering and regenerative therapies (Nıcpoń *et al*., 2014; Stachura *et al*., 2021). These cells, exhibit similar morphology to fibroblasts and express specific mesenchymal markers including CD90, CD73, and CD105 while lacking hematopoietic markers like CD45, CD34, and HLA-DR, as standardized by the International Society of Cellular Therapy (ISCT) (Karaöz and İnci, 2014). Preclinical studies involving domestic animals like dogs, pigs, sheep, and goats have shown promising translational results, offering closer parallels to human clinical outcomes compared to laboratory rodents (Hotham and Henson, 2020; Williams *et al*., 2024). Furthermore,

advancements in gene editing and sequencing for farm animals have further facilitated their use in biomedical research (Polejaeva *et al*., 2016). Sheep, in particular, is considered an ideal model for studying human systemic conditions, including cardiovascular, skeletal, and neurological disorders, due to their physiological resemblance to humans (Divincenti *et al*., 2014). ADSCs derived from sheep and goats have demonstrated significant potential in addressing conditions such as bone injuries, cartilage damage, and skin injuries, with region-specific advantages highlighted by studies on infrapatellar fat pads (Vahedi *et al*., 2016).

The current study aims to isolate and characterize ADSCs from both sheep and goats, focusing on their proliferation and tri-lineage differentiation potential (adipogenic, chondrogenic, and osteogenic). By establishing their distinguishing features, this research work seeks to explore their potential for futuristic clinical applications in veterinary medicine.

MATERIALS AND METHODS

Ethical statement: The current study was conducted following ethical approval from the Animal Experiments Local Ethics Committee of Firat University, Türkiye (Protocol no: 2019/l07, Decision No. 161, dated 04.09.2019). All procedures opted for during the study complied with the ethical guidelines of the committee. The study was funded by the Firat University Scientific Research Projects Unit under grant number VF-20.08.

Isolation and culture of adipose stem cells (ADSCs) from adipose tissue: Adipose tissue samples were aseptically collected from five healthy sheep and five goats (1.5 years old, 45–50 kg) at the ELET A.S. slaughterhouse by a modified as described by Ozden Akkaya *et al*., 2023. Inguinal subcutaneous fat tissue was dissected using a sterile scalpel, placed in Hank's Balanced Salt Solution (HBSS) supplemented with 2% penicillin-streptomycin and 0.2% amphotericin B, and transported to Firat University Stem Cell Laboratories within one hour. In the laboratory, tissues were washed thrice with sterile phosphate buffer saline (DPBS) supplemented with 2% penicillin-streptomycin and 0.2% amphotericin B, minced, in a laminar airflow cabinet flow (Biosafety Cabinet class II) and digested with Type I collagenase (10 mg/10 ml HBSS with 1% penicillin-streptomycin and 0.1% amphotericin B) for 2 hours at 37°C in a water bath with continuous stirring. The digested material was filtered through 40µm strainers, and cells were centrifuged, washed with DPBS, and resuspended in a sterile complete medium (88% RPMI medium+10% FBS+1% Penicillin/Streptomycin+0.1% Amphotericin B). Cells were seeded in T25 flasks and incubated at 37°C with 5% CO2 to establish primary adipose-derived stem cell cultures**.** Adipose tissue cells reaching 85–90% confluence were passaged by detaching with Trypsin/EDTA, neutralizing with complete medium, centrifuging, and counting before reseeding into new flasks. Cells were cultured till passage 3 before the characterization.

Characterization of ADSCs by flow cytometry analysis: Flow cytometric analyses were conducted following the techniques described by Carroll *et al*. (2007). Briefly, 5µL of flow cytometry dye and 20µL of anti-CD44, CD90, CD105, and CD45, antibodies (BD-Biosciences) were added separately to a tube containing 100µL of adipose tissue cell mixture suspended in DPBS. The mixture was vortexed, incubated at room temperature for 20 minutes, and then supplemented with 1.5mL of isoflow fluid. After centrifugation at 1500rpm for 5 minutes, the supernatant was discarded, and the pellet was resuspended in a few drops of isoflow fluid to detect and quantify the expression of markers of interest.

Differentiation and histochemical analysis of ADSCs: At the end of the third passage, the cells were trypsinized and induced for osteogenic, chondrogenic, and adipogenic differentiation. Von Kossa, Alcian blue, and Oil red O staining protocols were applied respectively for histochemical analysis.

Osteogenic differentiation: ADSCs from passage 3, were cultured in a CO_2 incubator for four weeks in an osteogenic differentiation medium (StemPro Osteogenesis Kit, Gibco) supplemented with 1% penicillin/streptomycin at a density of 3000 cells/cm² in culture dishes. At the end of the differentiation period, cells were assessed for osteogenesis using Von Kossa staining. For this purpose, cells were fixed with 4% paraformaldehyde for 30 minutes, washed with distilled water, and treated with silver nitrate under UV light for 60 minutes. After a subsequent wash, 1% sodium thiosulfate was applied to remove excess silver, and differentiation was visualized using an inverted microscope (Olympus CKX53) (Kibria *et al*., 2020).

Adipogenic differentiation: ADSCs were seeded at a density of 3000 cells/cm² in culture plates and cultured for three weeks in adipogenic differentiation medium (StemPro Adipogenesis Kit, Gibco) supplemented with 1% penicillin/streptomycin. The differentiation was estimated microscopically by detecting the presence of intracellular lipid vacuoles using Oil Red O staining (Sigma Aldrich). For this purpose, briefly, the cells were fixed with 4% paraformaldehyde, rinsed thrice with distilled water, and washed with 50% alcohol. Subsequently, the cells were stained with Oil Red O (0.1% in 60% isopropanol)(5 mM) for 20 minutes, followed by decolorization with 50% alcohol and a final rinse with distilled water. Lipid vacuoles were visualized using an inverted microscope (Olympus CKX53) (Nawaz *et al*., 2020).

Chondrogenic differentiation: For chondrogenic differentiation, ADSCs were cultured for three weeks in a chondrogenic differentiation medium (StemPro Chondrogenesis Kit, Gibco) supplemented with 1% penicillin/streptomycin at a seeding density of 3000 cells/cm² in 6 well culture plates. The medium was replaced with freshly prepared media every 3rd day. Chondrogenic differentiation was assessed microscopically using Alcian Blue staining. At the end of the culture period, the cells were fixed with 4% paraformaldehyde for 30 minutes, washed with distilled water, and stained with Alcian Blue for 30 minutes. After staining, the cells were rinsed with distilled water and passed through graded alcohols (80%, 96%, and 100%) for dehydration. Chondrogenic differentiation was confirmed using an inverted microscope (Olympus CKX53).

RESULTS

Culture of ADSCs and morphology: Images of MSCs were taken at 24 hours post-culture, both before and after washing (Fig. 1a-d). By 48 hours, cells had adopted a spindle-shaped morphology. On day 3, asymmetrical division (3 cells) was observed, a characteristic feature of stem cell division (Fig. 2a-b). After approximately one week, stem cell foci (fibroblast colony-forming units, CFU-f) were evident (Fig. 2c-f). Cells reached 85-90% confluence by two weeks, at which point passaging was performed, and morphological images were taken with an inverted microscope (Fig. 3). Goat-derived MSCs exhibited faster proliferation and confluence compared to sheep-derived MSCs.

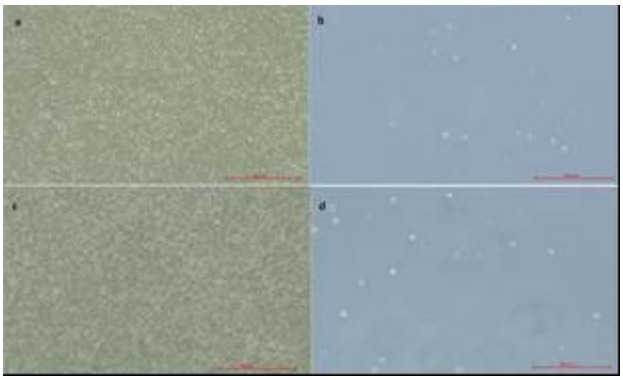

Fig. 1: Inverted microscope images (10X) of sheep primary culture cells for the first 24 hours before (a) and after (b) washing. Goat Primary culture cells for the first 24 hours before (c) and after (d) washing (10X) (Scale bars: 80µm).

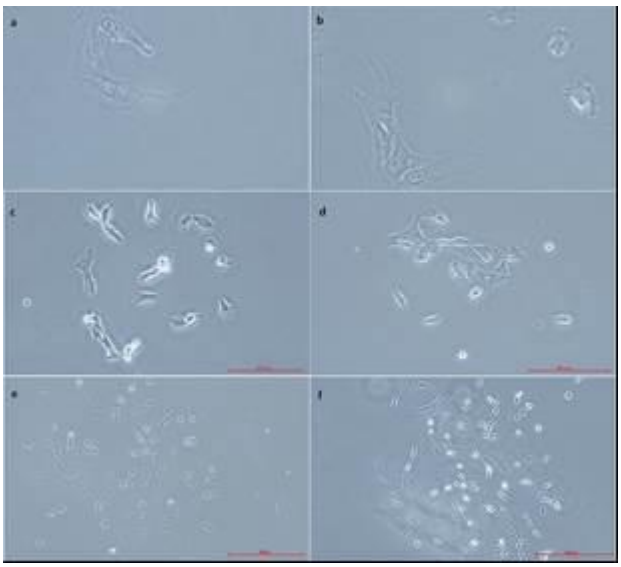

Fig. 2: Asymmetric cleavage, representing stem cell-specific form of cell division (a-b). Fibroblast colony forming (CFU-f) image of sheep MSCs 20X(c) 10X(e), fibroblast colony forming (CFU-f) image of goat MSCs 20X(d) 10X(f). (Scale bars: 80µm).

Flow cytometric analysis: MSCs isolated from sheep and goat adipose tissues were analyzed for surface markers using four antibodies. Forward Scatter Chanel (FSC) and Side Scatter Chanel (SSC) expansion, along with gating, were performed (Fig. 4). Both sheep and goat MSCs expressed CD44, CD105, and CD90, while CD45 was not detected. This immunophenotyping confirmed the identity of MSCs from both species (Fig. 5-6).

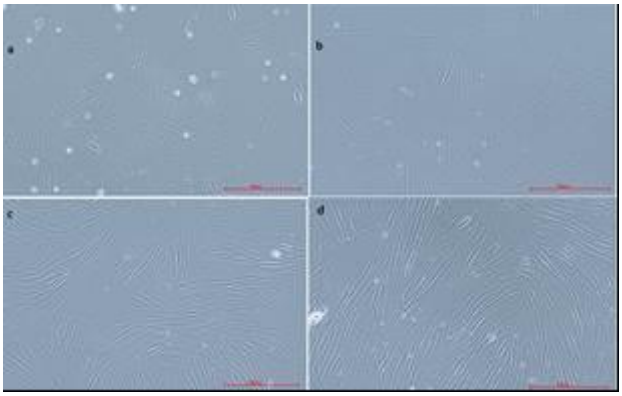

Fig. 3: Adhesion image of sheep MSCs after 3rd passage (P3) 10X(a), image of 85-90% confluency 10X(c), attachment image of goat MSCs after 3P 10X(b), image of 85-90% confluence 10X(d). (Scale bars: 80µm).

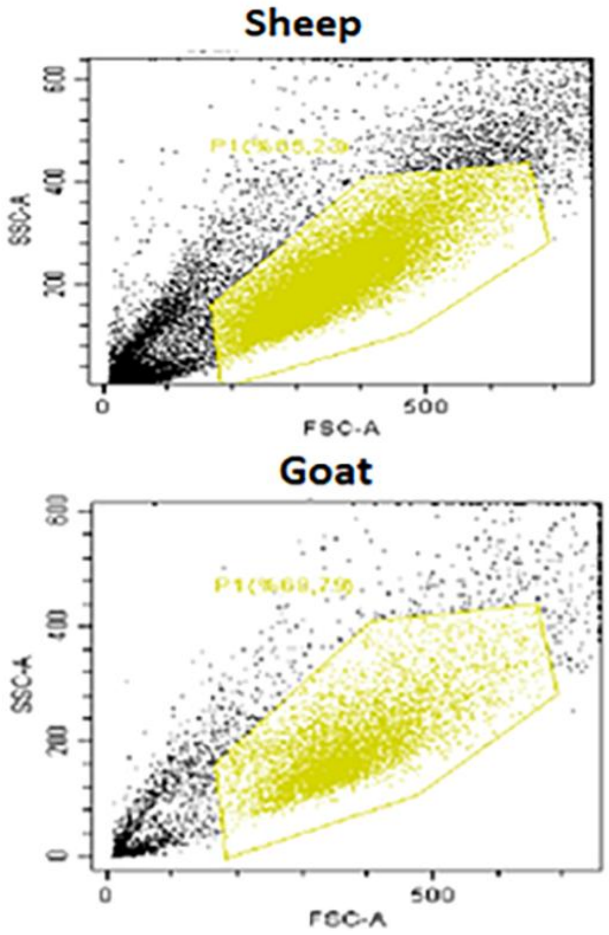

Fig. 4: Flow cytometric analysis of Sheep (P1=65.23%) and Goat (P1=69.79%) MSCs, display of FSC and SSC opening, gating process.

Osteogenic differentiation: MSCs were cultured in an osteogenic medium, and after 9-10 days, both control and osteogenic cultures reached near-confluence. Calcium deposits, indicated by Von Kossa staining, were observed after 21 days (Fig. 7-8), confirming effective osteogenic differentiation in both species.

Adipogenic differentiation: After reaching 60-70% confluence in passage 3, MSCs were cultured in an adipogenic medium. Fat vacuoles were visible by day 14,

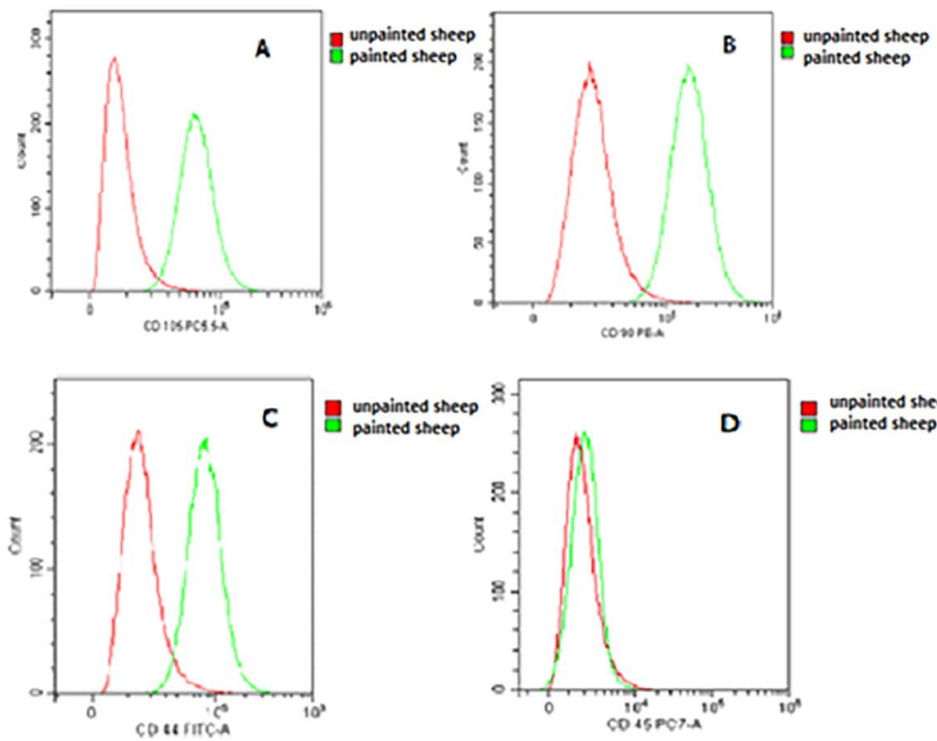

Fig. 5: CD105(A), CD90(B), CD44(C) positivity histogram, CD45(D) negativity histogram status of mesenchymal stem cell markers of MSCs isolated from sheep adipose tissue. Red peak: unstained control, green peak: belongs to the corresponding antibody.

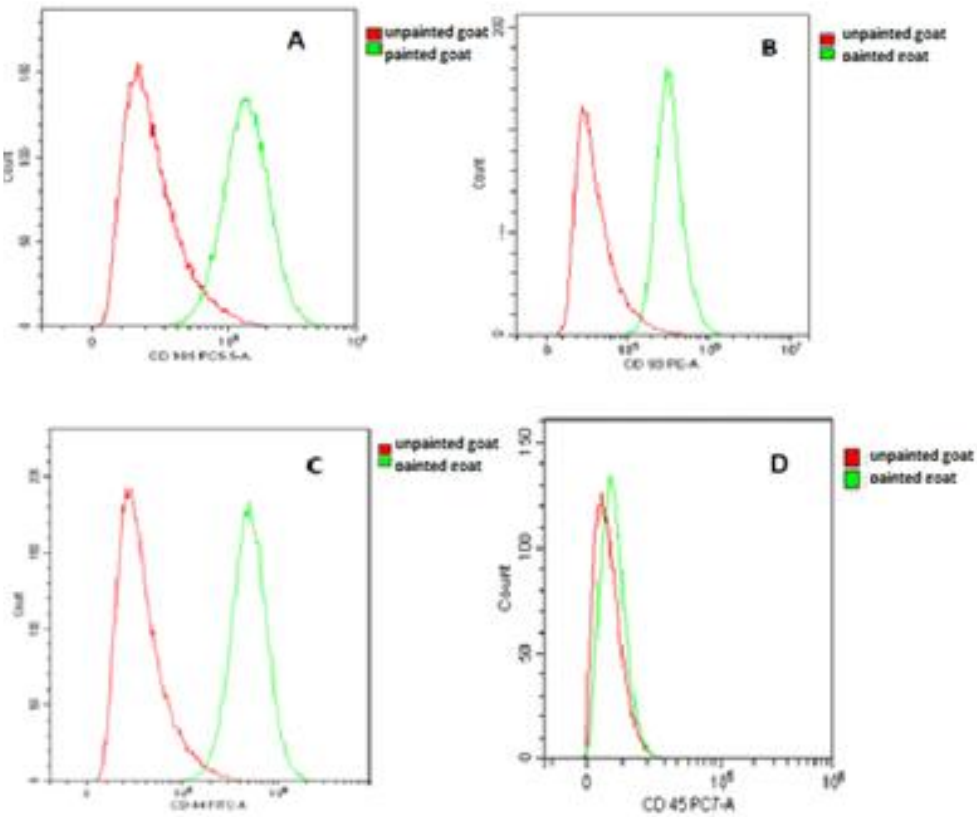

Fig. 6: CD105(A), CD90(B), CD44(C) positivity histogram, CD45(D) negativity histogram status of mesenchymal stem cell markers of MSCs isolated from the fat tissue of goat. Red peak: unstained control, green peak: belongs to the corresponding antibody.

and Oil-red O staining confirmed adipogenic differentiation (Fig. 9-10). However, osteogenic differentiation was more efficient than adipogenic differentiation in both sheep and goats.

Chondrogenic differentiation: MSCs cultured in a chondrogenic medium showed morphological changes by day 15, and proteoglycan-secreting foci were detected on day 19. Alcian blue staining on day 21 highlighted the mineralized intercellular matrix, a characteristic of cartilage (Fig. 11-12). These results suggest the potential use of these MSCs for cartilage regeneration in the future.

DISCUSSION

The choice of mesenchymal stem cell (MSC) origin is crucial in stem cell research, as it affects the suitability, accessibility, and applicability of various studies. Different studies have explored MSCs derived from multiple species and tissues, horses (bone marrow) (Özen *et al*., 2013),

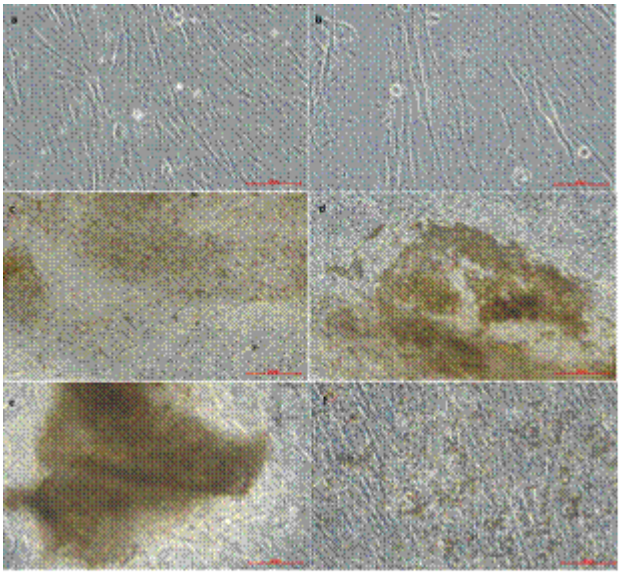

Fig. 7: Sheep MSCs control group Von-Kossa negative 20X (a) 40X (b), osteogenic differentiation group Von-Kossa positive 10X (c), 20X (d), 20X (e), 40X (f). (Scale bars: 60µm).

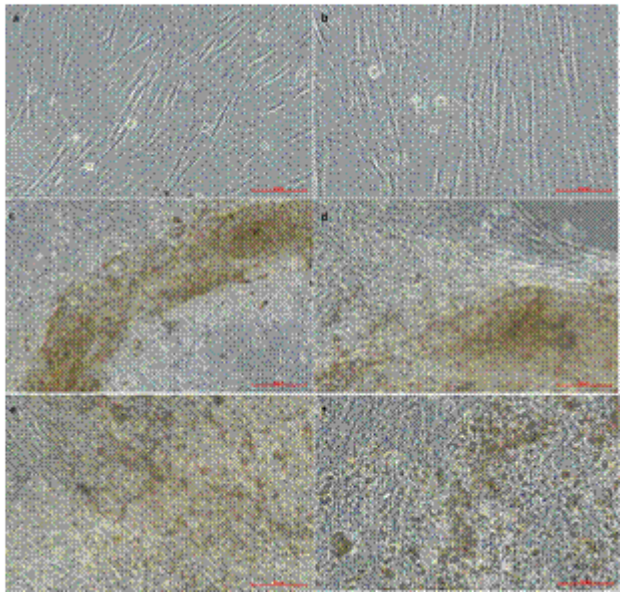

Fig. 8: Goat MSCs control group Von-Kossa negative 20X (a) 40X (b), osteogenic differentiation group Von-Kossa positive 10X (c), 20X (d), 20X (e), 40X (f). (Scale bars: 60µm).

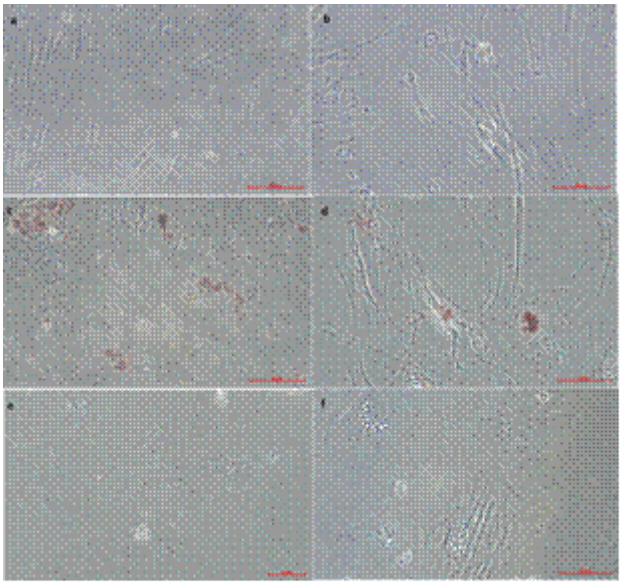

Fig. 9: Sheep MSCs control group Oil Red-O staining negative 20X (a), 40X (b), adipogenic differentiation group Oil Red-O positive 20X (c), 40X (d), adipogenic differentiation group morphological 20X (e), 40X (f). (Scale bars: 60µm).

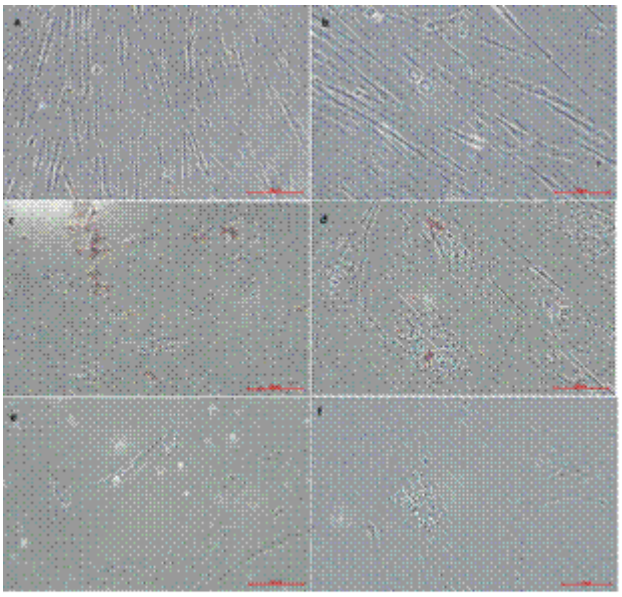

Fig. 10: Goat MSCs control group Oil Red-O staining negative 20X (a), 40X (b), adipogenic differentiation group Oil Red-O positive 20X (c), 40X (d), adipogenic differentiation group morphological 20X (e), 40X (f). (Scale bars: 60µm).

humans (cord blood, adipose tissue) (Nguyen *et al*., 2022; Solali *et al*., 2024), and porcine peripheral blood (Shradhanjali *et al*., 2022), as well as from amniotic sac (Nawaz *et al*., 2020; Li *et al*., 2022) and bovine cord blood (Raoufi *et al*., 2010). This diversity reflects the broad range of MSCs sources available for research. However, the choice of proper tissue and stem cell sources should consider various factors like tissue accessibility and contamination risks, particularly in veterinary species.

In our study, adipose tissues from the inguinal region of sheep and goats were selected, as they offer advantages in ease of access and high cell yield, as reported in previous studies (Wu *et al*., 2001; Arrigoni *et al*., 2009; Özgenç, 2019). Despite potential contamination risks in slaughterhouse settings, steps were taken to ensure sample sterility, including disinfection and short, controlled handling procedures. The use of antibacterial and antifungal solutions, as well as cell isolation in a sterile biosafety cabinet, was implemented to mitigate this risk. Ren *et al.* (2012) also focused on the isolation, expansion, and differentiation of goat ADSCs and implemented protocols compatible with our study.

The enzymatic method (type 1 collagenase) was used for cell isolation, which is commonly preferred for MSC extraction due to its higher cell yield compared to non-enzymatic methods (Conde-Green *et al*., 2016; Şahin, 2017). This method was consistent with the literature, and the timing of MSC confluency in the present study (around two weeks) aligns with findings in similar research (Bhurmann *et al*., 2007; Özgenç, 2019). However, differences in confluency times may be attributed to factors such as animal breed, age, and adipose tissue location.

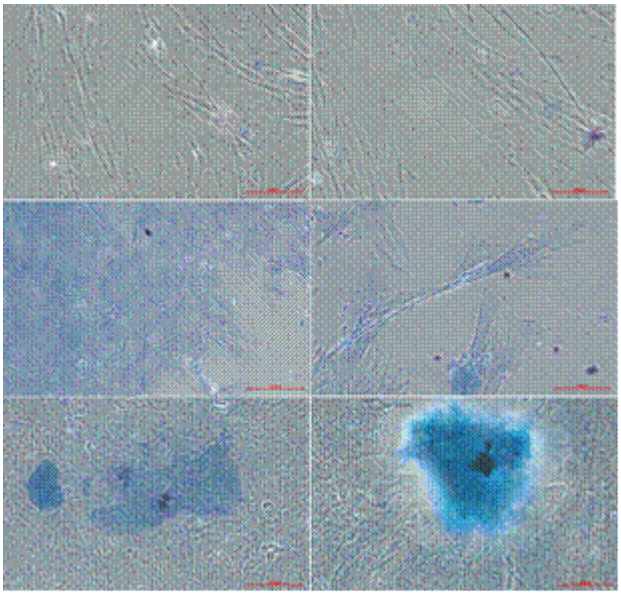

Fig. 11: Sheep MSCs control group alcian blue negative 20X (a), 40X (b), chondrogenic differentiation group alcian blue positive 10X (c), 20X (d), 20X (e), 40X (f). (Scale bars: 60µm).

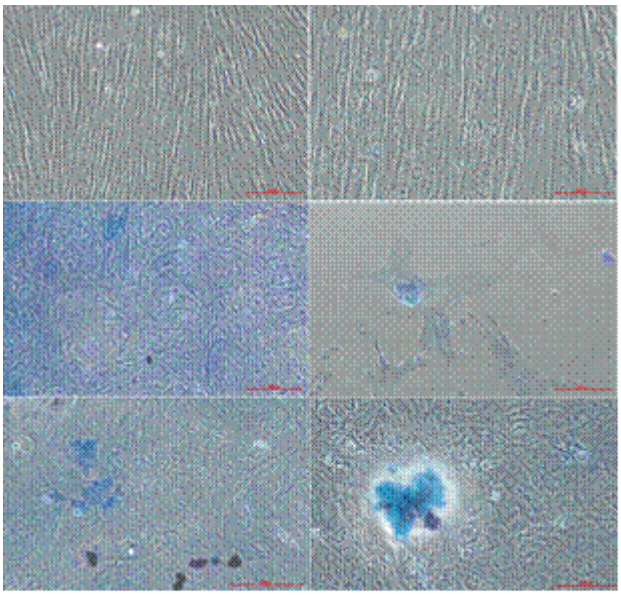

Fig. 12: Goat MSCs control group alcian blue negative 20X (a), 40X (b), chondrogenic differentiation group alcian blue positive 10X (c), 20X (d), 20X (e), 40X (f). (Scale bars: 60µm).

In a study on goats (Abraham *et al*., 2024), four media types including MEM, DMEM/F12, D-LG, and D-HG (Gibco) supplemented with 10% FBS were used in a humidified 5% CO_2 environment at 37°C. In contrast, our study utilized a complete medium (88% RPMI, 10% FBS, 1% Penicillin/Streptomycin, and 0.1% Amphotericin B) under similar conditions, achieving comparable cell densities indicating an ease of ADSCs culture under varied conditions.

The cells exhibit fibroblastic morphology which was consistent with the findings of Algorta *et al.* (2024). Flow cytometric analysis was performed to characterize the surface markers of the MSCs. Positive markers included CD44, CD90, and CD105, while CD45 was negative, in line with the International Society for Cell & Gene Therapy (ISCT) guidelines for MSC identification (Dominici *et al*., 2006; Gratwohl *et al*., 2006). The CD90, CD44, CD105, and CD45 antibodies used for flow cytometric identification were anti-human and, as reported in previous studies (Mastrangelo *et al*., 2019; Zhang *et al*., 2020; Akpinar *et al*., 2021), also performed effectively in sheep and goats. Can (2014) identified CD44, CD90, CD105, CD9, CD10, w4a5, and STRO-1 as positive markers and CD45, CD4, CD11a, and CD11b as negative markers in mesenchymal MSCs. Çoban *et al*. (2016) observed substantial expression of CD105, CD90, and CD105 genes in early culture of MSCs, with reduced expression in later stages, while negative markers CD34, CD45, and CD11b showed no expression at either stage. Similar to our findings, Flow cytometric studies on MSCs by Marx *et al*. (2015), Villatoro *et al*. (2015), and Özgenç (2019) identified CD44, CD73, CD81, and CD90 as positive markers and CD34 and CD271 as negative.

The differentiation potential of MSCs was also investigated, with cells differentiating into bone, cartilage, and adipose tissue, consistent with previous studies (Bhurmann *et al*., 2007; Park *et al*., 2009; Patrikoski *et al*., 2013). The study confirmed that differentiation into bone occurred by day 21, adipogenesis by day 14, and chondrogenesis by day 19. These timeframes are comparable to those reported in other studies on MSCs from different species.

The findings in this study are consistent with research on the differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages (Dar *et al*., 2021), where osteogenic differentiation was observed through calcium deposition by day 21, adipogenic differentiation through fat vacuoles by day 14, and chondrogenic differentiation through proteoglycan secretion by day 19. Likewise, Ceylan *et al.* (2017) conducted their study using MSCs derived from the umbilical cord stroma of rats, and these cells were differentiated into osteogenic, chondrogenic, and adipogenic differentiation, with the findings revealed by histochemical staining.

The results indicate that irrespective of the species, ADSCs possess a huge potential for therapeutic applications in various lineages. A study by Malik *et al.* (2014) on the generation of handmade cloned embryos from adipose tissue-derived MSCs in goats provided insights into the potential of ADSCs in reproductive cloning, an area closely related to tissue engineering and regenerative applications. This particular study paves the way toward the strong potential of ADSCs as an alternative therapeutic source for future applications in domestic animal species. Through, more detailed characterization is needed to establish the true potential of ADSCs. We assume that future research should include in vivo experiments to validate the therapeutic potential of ADSCs and expand the scope to explore the molecular mechanisms involved in their extensive differentiation potential. Investigating long-term stability and functionality across species would also enhance translational applications.

Conclusions: In conclusion, the present study has successfully established and characterized adipose tissue-derived mesenchymal stem cells from sheep and goats, confirming their ability to differentiate into bone, cartilage, and adipose tissue. This study advances the understanding of the biology of MSCs of veterinary species and establishes

grounds for use in future studies involving regenerative medicine, therapies, and tissue engineering.

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Data availability: N/A

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Conflict of ınterest: The authors declare no potential conflicts of interest concerning the research, authorship, and publication of this article.

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