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Original Research Paper

# Adipose-Derived Canine Mesenchymal Stem Cells in PLAY® Media

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**Abstract:** In veterinary medicine, stem cell-based therapies present promise for addressing orthopedic and degenerative conditions in canines. Mesenchymal Stem Cells (MSCs) possess unique regenerative, immunomodulatory, and differentiation capabilities. However, their efficacy relies on optimal *in-vitro* conditions. Prolonged expansion over passages leads to a gradual loss of stem cell traits, highlighting the importance of optimized culture conditions and media supplements. This study focuses on optimizing culture conditions for adipose-derived canine MSCs using play®, an in-house product containing growth factors and bioactive modulators. Our study provides insights into cellular properties, growth kinetics, and differentiation ability aiming to enhance adipose-derived canine MSCs' therapeutic potential in the field of veterinary medicine. Adipose-derived canine MSCs (AD-cMSCs) were isolated from adipose tissue from a canine source with veterinary professionals at Cessna Lifeline Veterinary Hospital, with prior pet owner consent. AD-cMSCs were cultured in 10% PLAY® or 10% Fetal Bovien Serum-supplemented media. Population Doubling Time (PDT) assessed growth rates. Trilineage differentiation potential was evaluated with specific media and morphology was examined under microscopy. Pluripotency gene expression was performed using a semi-quantitative polymerase chain reaction. AD-cMSCs cultured with 10% play® showed accelerated growth, enhanced differentiation potential, and elevated pluripotency marker expression compared to those in 10% FBS. These findings underscore the efficacy of play® as a promising culture supplement for optimizing AD-cMSC expansion and advancing their therapeutic potential in veterinary regenerative medicine.

**Keywords:** Adipose-Derived Canine MSCs', Population Doubling Time, Regenerative Medicine, Stem Cells, Tri-Lineage Differentiation

## Introduction

### *Background: Rising Demand for Canine Stem Cell Therapy*

Over the last decade, interest in the field of stem cell therapies for regenerative medicine has exponentially increased, especially in the use of Mesenchymal Stem Cells (MSCs) (Voga *et al*., 2020). This approach holds great promise in veterinary medicine for its potential applications in treating osteoarthritis, spinal cord injury, myocardial infarction, etc., (Dias *et al*., 2019).

These advancements could potentially lead to the development of novel treatments in canine health care. (Prządka *et al*., 2021). Although the feasibility of stem cell therapy is greater now than it was 1 or 2 decades ago, comprehensive studies on characteristics of canine Mesenchymal Stem cells (cMSCs) needed for clinical use remain unexplored. Thus, our study bridges the existing research gap by providing a comprehensive report on the characteristics that cMSCs have while providing a standard for isolation, culturing, and *in-vitro* lineage maintenance using an innovative culture media ultimately successfully translating it for clinical applications.

Sources of cMSCs and their variability: Adipose tissue, bone marrow, synovial fluid, and other tissues can all be used for isolating canine mesenchymal stem cells (stromal cells) (Bearden *et al*.,2017). Adipose tissue is the

preferred site of extraction as it is relatively safer, easy to isolate, and a "procedurally" less invasive source of cMSCs for research and clinical applications (Humenik *et al*., 2022). Adipose-derived canine MSCs (AD-cMSCs) can have varying propensities to replicate depending on the age of the canine, location, and type of fat tissue (Rashid *et al*., 2021). Considering such variation in the propensity to replicate, the need to characterize *in-vitro* is imperative.

Importance of expansion of MSCs for clinical translation: A crucial step in the effective translation of MSC-based therapeutics to the clinic is the expansion of MSCs in enormous numbers (Fernández-Santos *et al*., 2022). Henceforth, in recent years researchers have been investigating different types of culture media and xeno-free media supplements alternative to Fetal Bovine Serum (FBS) that can be effectively used to expand and maintain the MSCs at *in-vitro* conditions (Subbiahanadar Chelladurai *et al*., 2021). One such alternative to FBS is play®, an in-house product of iCREST, International Stem Cell Services Limited. It is a human blood derivative, which is a cocktail of natural growth factors and cytokines that can promote MSC proliferation and differentiation. Our previous studies using play® in characterizing human umbilical cord-derived MSCs have outperformed FBS, by maintaining all the characteristics of the International Society for Cell and Gene Therapy (ISCT) criteria till passage 20 (Lakkhundi *et al*., 2023). Hence, we have extended our study in the characterization of adipose-derived canine MSCs aiming to enhance our understanding of their potential applications in regenerative veterinary medicine.

Research objectives: In this study, we aimed to isolate and characterize adipose-derived canine MSCs in the culture media supplemented with play®. We investigated the common properties of MSCs.

Morphological analysis of cMSCs supplemented with play® compared to CMSCs supplemented with FBS. Cell growth kinetics using Population Doubling Time (PDT) to understand their replicative potential and suitability for large-scale expansion in therapeutic applications. Tri-lineage differentiation potential into various cell lineages, such as adipocytes, chondrocytes, and osteoblasts which would provide insights into their regenerative capabilities. We also investigated pluripotency ability through SOX-2 and OCT-4 gene expression.

## Materials and Methods

### *Isolation and Culturing of Adipose-Derived Canine MSCSs*

Adipose tissue from canines was collected under veterinary doctors' supervision from Cessna Lifeline Veterinary Hospital with the prior consent of the pet owner. The adipose tissue collected was washed using Phosphate Buffer Saline (PBS) solution, minced in a sterile petri plate, and subjected to collagenase type 1 (Hi-media, Mumbai) treatment for 2 h at 37°C. The reaction was stopped using 10% FBS (fetal bovine serum) (Himedia, Mumbai) containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco, New York). Further, this digest was centrifuged for 10 min at 1500 rpm (REMI-8C) to obtain a cell pellet. The pellet was suspended in either 10% play® or 10% FBS-supplemented DMEM media and cultured in a T25 flask (Tarsons, India). The standard cell culture conditions were maintained (i.e., 37°C and 5% $CO_2$). The fresh media was added on every 3rd day and at 80% confluency cells were trypsinized using 0.25% trypsin-EDTA (Hi-media, Mumbai) and subcultured for future studies.

### *Population Doubling Time* (*PDT*)

The culture cells of both groups 10% play® and 10% FBS (used as control) across all the passages (P2 and 5) were plated at a concentration of 5000 cells/cm$^2$ tissue culture coated 6 well plates (Tarsons, India). On the 3$^{rd}$ day media was replaced with fresh media and on the 6 day, the cells were trypsinized and counted using the trypan blue dye using a hemocytometer.

The obtained cell numbers were used to calculate the PDT using Eq. 1:$(t\text{-}t_0)$ $*log^2/log\ Nh\text{-}logN$. Where "*t*" is the time of harvesting the cells, "$t_0$" is the initial time both represented in hours, "*Nh*" is the number of cells harvested at each passage, and "$N_0$" is the initial cell number seeded. Population Doubling Time (PDT) indicates the cell growth rate. It is the time required for a cell population to double in numbers. It is important for assessing the *in-vitro* expansion potential of stem cells. Expansion in sufficient numbers is crucial for clinical translation to achieve good therapeutic outcomes.

### *Trilineage Differentiation*

AD-cMSCs grown in media supplemented with 10% play® at P3 were cultured in tissue culture-coated 24-well plates (Tarsons, India) at a seeding density of 1000 cells/cm$^2$. After the cells have adhered, the respective differentiation media-osteogenesis (Hi Osteo XLTM osteocyte differentiation medium, Hi Media, Mumbai), chondrogenesis (Hi Chondro XLTM chondrocyte differentiation medium, Hi Media, Mumbai), and adipogenesis (Hi Adipo XLTM adipocyte differentiation medium, Hi Media, Mumbai) were added. The manufacturer's instructions were followed for the preparation of differentiation media. The cultures were maintained for 10 days for osteogenic and adipogenic differentiation and 21 days for chondrogenesis at 37°C and 5% $CO_2$. The media was replaced every 72 h. Further, both induced and non-induced cells were washed with 1× PBS solution, and fixation was done using 4% paraformaldehyde solution. The cells were stained with the following stains, respectively: Oil red stain (Hi media, Mumbai) for adipogenesis, alizarin red (Hi media,

Mumbai) for osteogenesis, and alcian blue (Hi media, Mumbai) for chondrogenesis.

The differentiation ability was assessed by staining for the morphological investigation using a phase-contrast inverted microscope (10× Olumpus Magnus).

### *Semi-Quantitative Polymerase Chain Reaction (PCR)*

Total RNA was isolated using the TRIZOL (Takara) method and was reverse-transcribed into cDNA by using the prime script™ cDNA synthesis kit (Cat. #6110A, Takara bio Inc., Shiga, Japan), and semiquantitative PCR was performed to measure the pluripotency gene expression levels using the below-mentioned primer sequences (applied biosystems).

### *Thermal Cycling Conditions*

The thermal cycling conditions used for the PCR amplification were as follows: 5 min of initial denaturation at 95°C; 30 cycles of denaturation at 95°C for 1 min; 30 seconds of annealing at 53.5°C (SOX-2), 57.5°C (Oct4) and 61.5°C (G3PDH); and 1 min of extension at 72°C. A last extension step was carried out for five minutes at 72°C.

### *Gel Electrophoresis and Quantification*

Two percent agarose gel was used to run the PCR product with 100 bp DNA (Takara, Japan) and separated bands were visualized using a UV transilluminator (Biobee tech). To analyze the desired gene expression, ImageJ software was used. Normalization was done using a housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH).

### *Statistical Analysis*

All the data represented are of 3 independent replicants. The data values are shown as mean ± S.D. An unpaired two-tailed t-test with degree of freedom 4 was performed to evaluate the statistical significance between the groups studied using graph prism software 10.0.2. (* $p<0.05$, ** $p<0.01$ and ***$p<0.001$) were considered significant.

## Results

### *Morphology of AD-cMSCs*

We examined the morphological features of AD-cMSCs cultured in DMEM medium supplemented with 10% play® and 10% FBS cells serving as the control. Microscopic images were taken at passages 1, 3, and 5 for both 10% play® and 10% FBS-supplemented cells (Fig. 1). In both groups, AD-cMSCs exhibited fibroblastic-like morphology, grew in a monolayer and were adherent to the culture dish. The notable differences observed are as follows: AD-cMSCs supplemented with 10% play® cells displayed a spindle-like and elongated structure, whereas cells supplemented with 10% FBS were more elongated and had polygonal morphology.

However, as the passage number increased (after P4), cells became less elongated, more flattened, and had polygonal morphology indicating that the cells were becoming senescent.

### *Population Doubling Time*

We evaluated the cell growth rate of AD-cMSCs at multiple passages for cells supplemented with 10% PLAY® or 10% FBS by finding the population Doubling Time (PDT). PDT is the time required for the population of cells to double in number calculated using Eq. 1:$(t\text{-}t_0)$ $*log2/logN_h–logN$.

As depicted in Fig. 2, AD-cMSCs supplemented with PLAY® consistently demonstrated a shorter PDT when compared to cells cultured in 10% FBS across all passages, suggesting efficient cellular growth and division. The difference was mathematically significant after passage 3 ($p<0.05$) and notably, at late passages, P5 and P6 a significant ($p<0.001$) difference was observed when compared to cells supplemented with 10% FBS. However, at an early passage P2, no significant difference was seen.

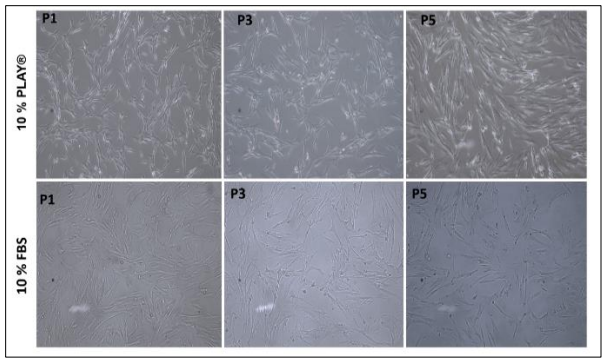


**Fig. 1:** Morphology of AD-cMSCs grown in either 10% PLAY® or 10% FBS. Representative inverted microscopic images (10×) displayed fibroblastic-like morphology of PLAY®-MSCs at passages P2, P3 and P5

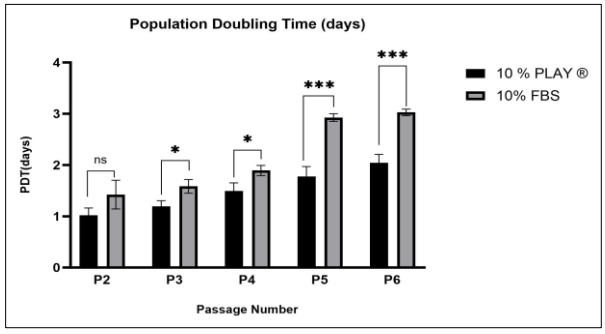


**Fig. 2:** Population doubling time (in days) of AD-cMSC supplemented with either PLAY® or FBS. (N = 3). The mean ± SD is used to represent data values. **$p<0.01$, ns indicates not significant, *$p<0.05$

These results imply that cells supplemented with 10% PLAY® exhibited superior cell growth kinetics, with a high proliferation rate and longer lifespans in *in-vitro* conditions. It also provides valuable information on how culture conditions influence cell behavior, crucial for optimizing cell culture strategies in various applications.

### *Trilineage Differentiation of AD-cMSCs*

We investigated the differentiation potential of AD-cMSCs supplemented with 10% PLAY® at $P_3$ into osteocytes (bone marrow cells), chondrocytes (cartilage cells), and adipocytes (fat cells)-critical for tissue regeneration and repair.

### *Osteogenesis*

Passage 3, cells of both the groups, 10% PLAY® and 10% FBS control groups were subjected to osteogenic differentiation, and cells were then stained with alizarin red to see calcium deposits. Both groups showed differentiation into osteoblasts as evidenced by the presence of calcium matrix mineralization (Fig. 3-A2) As shown in the graph 10% PLAY® cultured cells exhibited 96.25±1.086% and 10% FBS-cultured cells exhibited 93.65±1.58% differentiation (Fig. 4). Negative controls (non-induced P3 cells) validated staining protocol specificity (Fig. 3-A1).

The significant osteogenic potential of the cells in these findings shows promise for bone tissue engineering and regenerative medicine.

### *Chondrogenesis*

Cells cultured with 10% PLAY® and 10% FBS were directed to chondrogenic differentiation and assessed using alcian blue staining (Fig. 3-B2), a marker for proteoglycan-rich pellets in cartilage cells. As shown in Fig. 4, 10% PLAY® cells demonstrated significantly higher chondrogenic differentiation ($p<0.01$) compared to 10% FBS. Negative control (non-induced P3 cells) confirmed alcian blue staining specificity (Fig. 3-B1).

These findings highlight the robust chondrogenic differentiation capacity of cells cultivated with 10% PLAY® and underscore its potential in cartilage tissue engineering.

### *Adipogenesis*

To evaluate adipogenic differentiation, cells cultured in both 10% PLAY® and 10% FBS conditions at passage 3 were exposed to adipogenic differentiation media.

The assessment of adipogenic differentiation was carried out by observing lipid droplets within the cells, by Oil Red staining, as shown in Fig. 3-C2.

As observed in Fig. 4, PLAY® grown cells showed significantly ($p<0.05$) high adipogenicity when compared to FBS cells. Non-induced P3 cells showed the absence of lipid droplets when stained with oil red (Fig. 3-C1) thereby validating the experimental protocol.

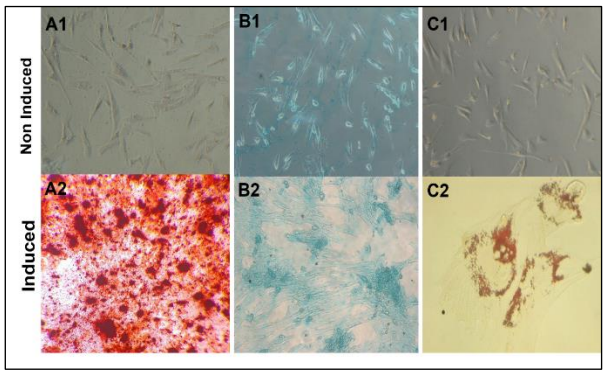


**Fig. 3:** Trilineage differentiation of AD-cMSC supplemented with PLAY® at passage 3. (N = 3). Representation of microscopic images was taken at 10X magnification; (A) Osteogenic differentiation; (B) Chondrogenic differentiation; (C) Adipogenicity differentiation respectively of induced and non-induced cells (negative control)

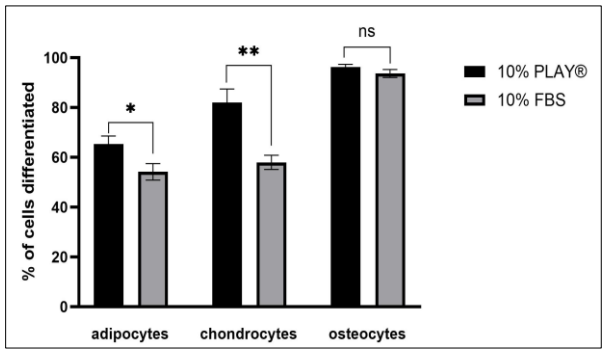


**Fig. 4:** Adipogenic, osteogenic, and chondrogenic differentiation of AD-cMSCs supplemented with PLAY®, compared with FBS cells at passage 3 expressed as a percentage (N = 3). The mean ± SD is used to represent data values. **p<0.01, ns indicates not significant, *p<0.05

Collectively, the trilineage differentiation results suggest that AD-cMSCs supplemented with 10% PLAY® possess enhanced regenerative capabilities, making them promising candidates for a range of therapeutic applications in regenerative medicine applications.

### *Pluripotency Markers*

To assess the stemness and degree of pluripotency, we evaluated key genes namely: SOX-2 and OCT-4 in AD-cMSCs at passage 3 using semiquantitative PCR. The gene expression was normalized with GAPDH.

As seen in Fig. 5, both the groups 10% PLAY® and 10% FBS showed the presence of bands for SOX-2 and OCT-4. Among both the groups, AD-cMSCs supplemented in 10% PLAY® media showed relatively high-intensity bands mainly for SOX-2 compared to FBS-grown cells.

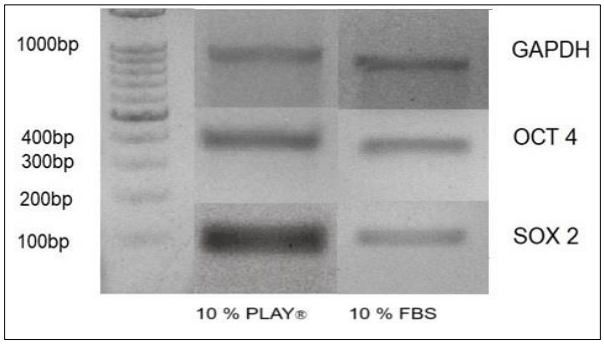


**Fig. 5:** Inverted image of agarose gel electrophoresis analysis of pluripotency gene bands normalized with GAPDH for AD-cMSCs at passage 3 (N = 3). The figure illustrates the presence and intensity of gene expression in cells cultured in media supplemented with 10% PLAY® (lane 2) and 10% FBS (lane 3) and lane 1 displays the DNA 1000 base pair ladder

These findings are significant as they confirm the stemness nature and self-renewing ability of AD-cMSCs, for evaluating their safety and efficacy before potential clinical applications. The robust expression of SOX-2 and OCT-4 underscores the potential of AD-cMSCs for regenerative medicine and therapeutic interventions, highlighting the importance of these pluripotency markers in assessing the therapeutic potential of MSCs.

## Discussion

Our comprehensive investigation of the characterization of adipose-derived canine MSCs grown in culture media supplemented with 10% PLAY®, a xeno-free media demonstrated superior cellular and functional compared to cells cultured in 10% FBS.

Morphological analysis of AD-cMSCs grown in PLAY® supplemented media exhibited fibroblastic-like morphology till P6, indicating the retention of phenotypic traits as per ISCT criteria.

AD-cMSCs grown in culture media supplemented with 10% PLAY® showed a lesser PDT of 1.01±0.14 days at P2 to 2.04±0.16 days at P6, whereas cells supplemented with 10% FBS had a higher PDT of 1.4±0.28 at P2 to 2.9±0.06 days at P6. Notably, as the passage number increased, cells in PLAY® media consistently showed significantly shorter doubling times compared to FBS. These findings, therefore suggest an increased cell proliferation rate and higher cell yield when grown in media supplemented with 10% PLAY®. To have a good therapeutic impact in clinical application, a substantial number of MSCs are necessary, henceforth understanding the cell growth kinetics *in-vitro* is crucial before scaling up. Our PDT results aligned with Russell *et al*. (2016) findings, ranging from 1.72±0.23 at P2 to 3.28±0.23 at P5 for the cells grown in 10% FBS supplement. In a study by Rashid *et al*. (2021), it has been shown that cellular and functional characteristics of AD-cMSCs depend upon the site of extraction of MSCs from adipose tissue, for example, infrapatellar fat pad-derived MSCs exhibited the highest cell proliferation rate, whereas omental-derived MSCs showed enhanced adipogenic and osteogenic differentiation abilities.

In our study, AD-cMSCs in 10% PLAY® exhibited superior trilineage differentiation (osteogenesis, chondrogenesis, adipogenesis) compared to 10% FBS, indicating enhanced multipotency. This criterion is considered to be crucial as the therapeutic potential of MSCs lies in their differentiation ability into various cell types for tissue repair and regeneration. The retention of trilineage differentiation potential enhances the translational value of the study's findings. Whereas, in a study Russell *et al*. (2015) found that platelet lysate from canine sources didn't show significant results when compared to FBS for chondrogenic differentiation in adipose and bone marrow-derived cMSCs, citing low proliferation and trilineage differentiation ability.

Similarly, Hagen *et al*. (2022) found that, unlike platelet lysate from an equine source supporting equine stem cells, platelet lysate from a canine source did not provide similar advantageous support for canine stem cell cultures. Contrastingly, Rashid *et al*. (2021), with autologous canine platelet lysate showed no significant differences in cellular behavior, including proliferation, viability, surface markers, PDGFRα expression, and adipogenic/osteogenic differentiation when compared to FBS. Considering these results, our findings have proved that AD-cMSCs grown in 10% PLAY® were superior to cells grown in 10% FBS in terms of cell growth kinetics and trilineage lineage differentiation ability.

Researchers are actively exploring diverse media supplements to optimize MSC expansion *in-vitro* while preserving stemness markers and maximizing cell yield. To date, species-specific culture media remains elusive, given the unique physiological and biochemical characteristics influencing cell growth and behavior across species. (Gharibi and Hughes, 2012; Nikolits *et al*., 2021; Jakl *et al*., 2023).

Although FBS has conventionally served as a widely used supplement due to its nutrient richness, it has limitations such as batch variability, ethical concerns about slaughtering bovine fetuses, and species compatibility issues, restricting clinical applications and species-specific studies like those involving dogs. High chances of adventitious agents/contaminants from animal sources can be introduced via FBS (Jochems *et al*., 2002; Lee *et al*., 2022). Canine platelet lysate limits itself due to the lack of a continuous source of canine blood and the requirement of consent from dog owners. PLAY®, a human blood derivative as a source of supplement for canine cell culture is more advantageous due to several

reasons including increased species compatibility as it is more closely related species to canine compared to the bovine source (Coelho *et al*., 2018), reduced the risk of xenogeneic contaminants and potential immune response in recipients (Saury *et al*., 2018) and standardized reproducibility, eliminating inter-batch variations commonly observed with FBS. Overall, PLAY® cultured AD-cMSCs exhibited robust proliferation and maintained their multilineage differentiation potential, indicating that PLAY® supports the expansion and maintenance of functional AD-cMSCs.

Though, PLAY® offers advantages over traditional supplements like FBS, developing species-specific culture media for canine MSCs is a complex challenge. This entails considering nutritional requirements, intricate signaling pathways, and unique microenvironmental factors. Future research should focus on refining tailored culture formulations to address challenges and enhance cell culture techniques for veterinary applications. The ongoing pursuit of species-specific media is crucial for advancing canine MSC research, necessitating a nuanced understanding of species complexities to optimize culture conditions for regenerative veterinary medicine.

### *Future Studies*

Our studies on AD-cMSCs cultured in PLAY® supplemented media would be extended to investigate the other functional characteristics apart from its differentiation capability including immunomodulatory properties and migration assay etc. Additionally, these cells will also be assessed for senescence markers, ultimately progressing towards translating the research to practical application in treating diverse veterinary conditions like osteoarthritis, spinal cord injuries, cardiac conditions, etc.

## Conclusion

We have successfully characterized AD-cMSCs in PLAY® supplemented media which outperformed cells supplemented with 10% FBS. Specifically, AD-cMSCs in PLAY®-supplemented media exhibited a decreased Population Doubling Time (PDT), with values ranging from 1.01±0.14 days at P2 to 2.04±0.16 days at P6. In contrast, cells receiving 10% FBS displayed a higher PDT, varying from 1.4±0.28 days at P2 to 2.9±0.06 days at P6. AD-cMSCs exhibited an enhanced trilineage ability, especially in chondrocytes and adipocytes, and relatively high expression of stemness genes. To the best of our knowledge, this is the first study where we have used PLAY®, a human derivative as the supplement replacing FBS for expanding AD-cMSCs representing a potential alternative for stem cell culture. Our study provides an understanding of cellular behavior and characteristics of AD-cMSCs, establishing a fundamental base for future clinical applications in regenerative medicine and stem cell-based therapies.

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## Author's Contributions

**Arjun Kullarni:** Involved in learning and conducted research experiments and manuscript preparation.

**Bhavana N. V.:** Involved in learned and conducting research experiments, data and statistical analysis, and manuscript preparation.

**Teena Mary Thomas:** Collaborated closely with Bhaana, offering their expertise in guided various research tasks and data analysis.

**Archana S.:** Collaborated closely with Bhaana, offering their expertise in guided various research tasks and data analysis.

**Mercy Jennis Pramod:** Involved in the preparation of PLAY@, an in-house product of iCREST, an international stem cell service ltd.

**Ramesh Jangra:** Contributed to the research paper by providing us with the canine adipose tissue samples to conduct these preliminary *in vitro* studies.

**Jyothsna A. Rao:** Research director and supervisor, provided guidance with her research expertise, and methodological expertise and reviewed the manuscript.

**Gururaj Rao:** The managed director offered invaluable research guidance and facilitated organizational support for the research endeavor.

## Ethics

This article is original and contains unpublished material. The corresponding author confirms that all the other authors have read and approved the manuscript and that no ethical issues are involved.

### *Conflict of Interest*

The authors declare that there is no conflict of interest.

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