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Original Article

Early Passage Characterization of Canine Synovial Fluid-Derived Stem Cells Isolated from Stifle Joint

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ABSTRACT

Synovial Fluid-Derived Stem Cells (SFSCs) have emerged as a promising source of mesenchymal stem cells, offering a minimally invasive way of obtaining cells with high proliferative capacity and robust multilineage differentiation potential. Originating from the synovial membrane, SFSCs are believed to retain a cellular bias towards musculoskeletal tissue repair, positioning them as a valuable tool in treating musculoskeletal injuries and morbidities. **Objective:** To portray SFSCs differentiation behavior at early passage (P2) by evaluating their growth dynamics, immunophenotypic profile, and ability to differentiate into multilineages. **Methods:** In this experimental study, a typical MSC-like proliferation pattern was seen with distinct phases of lag, exponential and plateau growth curve. Immunohistochemistry revealed that CD73+, CD90+, and CD105+ lacking hematopoietic markers, further validated their MSC like nature. **Results:** SFSC showed bi-lineage differentiation into adipocytes and osteocytes validated by Oil O Red and Alizarin Red S staining respectively. **Conclusions:** In conclusion SFSC's possesses regenerative potential, which could be a future of regenerative medicine to repair bones and soft tissues. These findings contribute to MSC biology and its implementation as therapeutic role via SFSCs in musculoskeletal disorders.

INTRODUCTION

Synovial Fluid-Derived Stem Cells (SFSCs) have attracted attention in the field of regenerative medicine due to their undeniable ability of self-renewal, differentiation, and immune modulation [1]. These cells are found in the synovial joint milieu, offer a conveniently accessible and less intrusive source for tissue engineering and therapeutic applications, particularly for cartilage regeneration leading to osteoarthritis treatment [2]. SFSC's are more tolerant to mechanical stress contrasted to other Sources of Stem Cells (SCs), (bone marrow and adipose tissue) make them a viable approach among SC based therapies [3]. The ability of SCs to develop into many mesodermal lineages, including osteogenic, chondrogenic, and adipogenic, has resulted in promising results in regenerative therapies. Nevertheless, information is insufficient regarding the functional capacity and differentiation potential of SFSCs at early passages into osteogenic and adipogenic lineages [4]. Understanding the phenotypic stability and multipotency of SFSCs at passage 2 is crucial, since subsequent passages may result in senescence and may diminish its functionality, hence early passages are often preferred for therapeutic applications [5]. This study was conducted to describe the morphology, immunophenotype, proliferation capacity, and differentiating of SFSCs at P2. By focusing on P2, we want to know whether SFSCs at this early passage

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retain their stemness and ability to differentiate into osteogenic and adipogenic lineages since these properties are critical to use in regenerative medicine.

METHODS

Isolation and Culturing of SFSCs

This experimental study was conducted following ethical approval from the institutional ethical committee. Synovial fluid samples were collected (n = 3) from the stifle joints of dogs. After pelleting the cells at 1500 rpm for 10 minutes, they were resuspended in Dulbecco's Modified Eagle Medium (DMEM; Gibco) with 10% FBS (Invitrogen) and 1% penicillin-streptomycin. Seeded cells were incubated at 37°C in 5% CO_2 in 25 cm^2 culture flasks. Media was replaced after every 48 hours until cells achieved 80% confluence. Adherent cells were trypsinized and passaged at P0 using 0.25% trypsin-EDTA (Gibco). P2 cells were characterized further [6].

Cellular Viability Analysis

Cellular viability assay was conducted with trypan blue exclusion solution (Thermo Fisher Scientific) to stain the viable cells. The cell culture was pooled in 1/1 ratio with a 0.4% trypan blue working solution. Following the mixing process, the solution was allowed to rest for one minute at ambient temperature. To enumerate viable and non-viable cells, 10µL of solution was introduced into the Neubauer Improved Chamber. Viable cells appeared white under the microscope due to intact membranes, but dead cells exhibited a blue coloration resulting from membrane breakdown. In this way we confirmed cells viability percentage [7, 8].

Cellular Proliferation Analysis

The Cells at P2 were seeded on 96 well plates at a density of $5x10^3$ cells in each well and incubated for 24h, 48h, and 72h. MTT assay was conducted with MTT reagent (Sigma-Aldrich), 20 µL was added to each well and incubated for 4h and after blue formazan crystals, they were dissolved in 100 µL DMSO. To check the proliferation activity of cells at P2, absorbance was measured at 570 nm via microplate reader (BioTek 800TS, Linden Ave N Shoreline, WA, USA) [9, 10].

Growth Curve Analysis

To evaluate the proliferation behavior of SFSCs at P2, the growth curve was calculated. In 6-well cell culture plates (Costar®, USA), SFSCs were collected at a density of 1×10^4 cells/cm^2, using 2 mL of DMEM per well. The plates were kept in a humidified atmosphere with 5% CO2 at 37°C during the incubation process. For a total of 14 days, cells were trypsinized with 1 mL of Caisson Laboratories Inc., USA's 0.06% trypsin (in HBSS) every other day, and then neutralized with 2 mL of DMEM. Cell counting was done as described earlier. Throughout the experiment, the culture medium was changed every third day to guarantee that the SFSCs were growing in the best possible circumstances [11].

Immunophenotyping

SF-MSCs were cultured in 6-well plates with DMEM and 10% FBS at P2 until 80% confluent, with 5×10^4 cells per well on sterile 6-mm coverslips. The cells were given a quick was with DPBS following half-hour in 4% formaline and allowed to permeabilize for 15 minutes using 0.3% Triton X-100. Primary antibodies (CD73, CD90, CD105, FABP4, osteopontin; all diluted 1:50) were applied for one hour at 37°C and then incubated overnight at 4°C after 30 minutes of blocking with 10% normal goat serum. Secondary goat anti-rabbit IgG (1:100) was added and allowed to sit in the dark for an hour after being cleaned with DPBS. Under a fluorescence microscope, coverslips were coated with antifade medium, and nuclei were counterstained with DAPI (1:500) for five minutes [1, 12].

Differentiation Assay

P2 cells evaluated bi-lineage differentiation by stimulating them to differentiate into the osteogenic and adipogenic lineages. Osteogenic differentiation was induced by culturing the cells for 21 days in DMEM medium (Sigma Aldrich, Germany) enriched with 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 µM ascorbic acid. Calcium nodules were detected with Alizarin-Red staining as a marker of osteogenic differentiation [13]. Adipogenic differentiation was induced while culturing the cells for 14 days in DMEM medium (Sigma Aldrich, Germany) added with 1 µM dexamethasone, 0.5 mM IBMX, 200 µM indomethacin, with 10 µg/mL insulin and lipid droplets were marked with oil red O staining as an identifier of successful adipogenic differentiation [12, 14]. All experiments were performed in triplicate, and results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. A p-value < 0.05 was considered statistically significant [15].

RESULTS

At P2 stage, SFSCs exhibited elongated spindle-shaped cells adherent to the culture surface (Figure 1A).

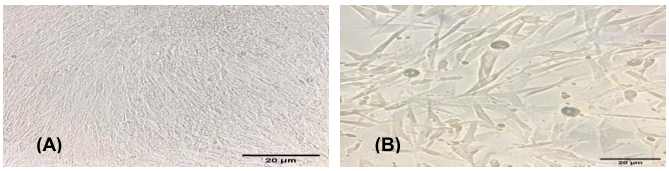

Figure 1A: Isolated Synovial Fluid-Derived Stem Cells (A) 10 X (B) 40 X Scale Bar= 20µm

Cells maintained uniform morphology without signs of senescence. The MTT assay demonstrated that P2 SFSCs proliferated actively over time, with a significant increase in cell viability from 24 to 72 h ($p < 0.05$). The absorbance values at 72 hours were 1.3-fold higher than at 24 hours, indicating robust proliferative capacity (Figure 1B).

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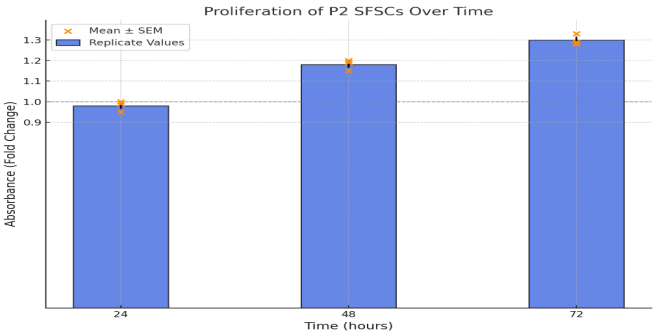

Figure 1B: Proliferation of passage 2 (P2) synovial fluid-derived stem cells (SFSCs) over time. Absorbance (fold change) was measured at 24, 48, and 72 hours to assess cell proliferation. Bars represent mean values of replicate measurements, while orange crosses indicate the mean ± standard error of the mean (SEM). The increase in absorbance over time reflects the proliferative potential of SFSCs.

The growth curve of SFSCs at passage 2 exhibited a typical sigmoidal proliferation pattern. An initial lag phase (Days 0–2) was followed by a rapid exponential growth phase (Days 2–8), where cell numbers increased significantly. By Day 10, the growth rate slowed, reaching a plateau phase at approximately 12.1 × 10^4 cells/cm^2, indicating contact inhibition or nutrient limitations figure 2.

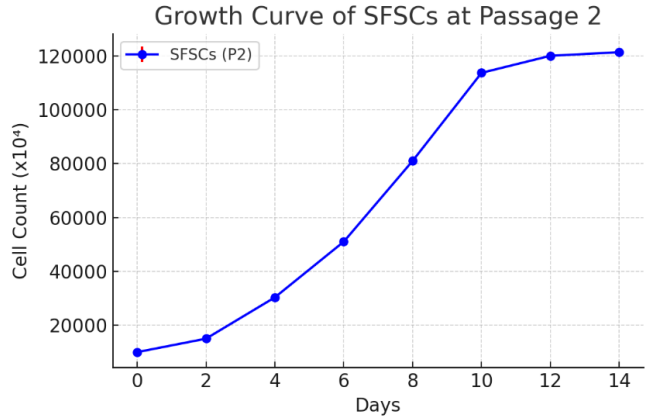

Figure 2: Growth curve of synovial fluid-derived stem cells (SFSCs) at (P2). The proliferation pattern was monitored over 14 days. An initial lag phase (Days 0–2) was followed by an exponential growth phase (Days 2–8), reaching a plateau phase after Day 10. Cell numbers were counted using a Neubauer Improved hemocytometer, and data presented as mean ± SEM (n=3)

SFSCs at P2 demonstrated a high clonogenic potential, forming visible colonies after 14 days in culture. The colony-forming efficiency was 15 ± 1.8%, indicating that a significant proportion of P2 cells retained their capacity for self-renewal and clonal expansion. Cell surface marker analysis confirmed that SFSCs at P2 retained the characteristic MSC immunophenotype. Most cells expressed CD73 (98.2% ± 0.6), CD90 (97.8% ± 0.7), and CD105 (95.6% ± 1.2), while negative for hematopoietic markers CD34 (1.2% ± 0.3) and CD45 (0.9% ± 0.2) (Figure 3).

These results confirm the mesenchymal origin of the isolated cells and their consistency at P2.

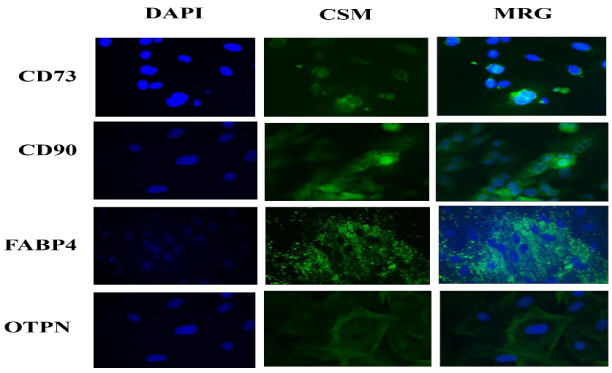

Figure 3: Expression of MSCs markers (CD73, CD90) on the synovial fluid derived stem cells and expression of special markers on differentiated cells (FABP4= Adipocytes, OTPN= Osteocytes). However, OTPN= Osteopontin, DAPI= 4′,6-diamidino-2-phenylindole., CSM=Cell Surface Marker, MRG= Merged.

SFSCs at P2 were differentiated into osteoblasts in a 3-week culturing in the osteogenic induction medium, evident by positive Alizarin Red staining for calcium deposits (Figure 4). Quantification of mineralized matrix formation showed a significant increase ($p < 0.01$) in osteogenic differentiation compared to the control group, confirming the osteogenic potential of P2 SFSCs. At P2, SFSCs successfully differentiated into adipocytes after 14 days of adipogenic induction confirmed by Oil Red O staining which indicated a cytoplasm full of with numerous lipid droplets of differentiated cells (Figure 4). Quantification of Oil Red O-positive areas showed a significant increase ($p < 0.01$) in lipid accumulation, indicating successful adipogenic differentiation of SFSCs at P2. Histological images reveal adipogenic lipid accumulation in AI and mineralized matrix in OI, confirming lineage-specific differentiation (Figure 4).

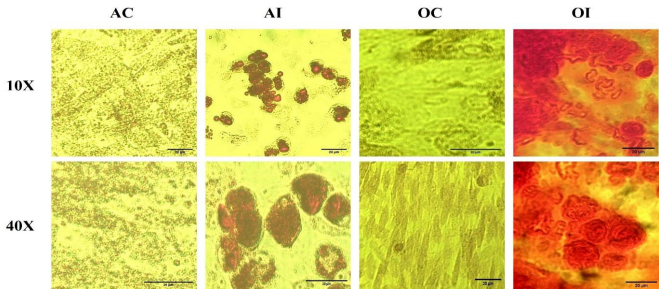

Figure 4: Representative microscopic images showing histological differences among the four experimental groups: AC (control adipogenic), AI (induced adipogenic), OC (control osteogenic), and OI (induced osteogenic). Images were captured at 10X and 40X magnifications. Lipid droplets (stained red) are prominent in the AI group, indicating successful adipogenic differentiation, whereas the OI group shows distinct mineralized extracellular matrix deposition. Scale bars = 20 µm

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DISCUSSION

This study aimed to characterize synovial fluid-derived stem cells (SFSCs) at early passage from canines and evaluate their potential for use in regenerative medicine. This study's findings indicate that SFSCs at P2 maintain their mesenchymal characteristics, which includes multipotent differentiation potential along with, proliferation making them a promising regenerative approach in tissue repair, particularly for osteoarthritis. In P2, SFSCs exhibited a spindle-shape morphology typical of stem cells derived from various sources, such as bone marrow and adipose tissue [16, 17]. The MTT values were indicated a substantial increase in proliferation of SFSC's at P2 with time. Similar results were previously described by Nantavisai *et al.*, where a significant increase in proliferative activity was seen in early stages of stem cells differentiation, making them suitable agents to be used in cell growth in clinical setting due to their increased differentiation or stemness as senescence was induced at later stages [18, 19]. The growth kinetics of SFSCs showed a sigmoidal growth pattern at P2 where a lag phase was observed at 0-2d moved to exponential growth phase (2-8d) and then decline in growth after 10 days which could be limited due to nutrients/growth factors deficiency or may their contact inhibition align with previous results reported by Garcia *et al.*, and Walczak *et al* [20, 21]. The small standard deviation across replicates reflects consistent proliferation patterns, reinforcing the reliability of these results. Such reproducibility is crucial for the scalability of SFSCs in therapeutic applications. Overall, these findings support the suitability of SFSCs for in vitro proliferation and their potential use in regenerative medicine, particularly for cartilage repair [20, 22]. Phenotypic study verified that P2 SFSCs exhibit essential MSC cell surface receptor CD73+, CD90+, and CD105+ which indicated SFSC's mesenchymal nature and not haematopoietic confirmed by lacking specific cell surface receptors CD34- and CD45-. This immunophenotypic profile aligns with the minimal criteria established by the International Society for Cellular Therapy (ISCT) for designating mesenchymal stem cells (MSCs) [23]. The higher proportion of positive cells for these markers at P2 substantiates the stemness and reliability of SFSCs, signifying their suitability for therapeutic applications where maintenance of an MSC phenotype is crucial for efficacy [11]. The colony-forming unit (CFU) assay indicated that SFSCs at P2 have a colony-forming efficiency of roughly 15%. The finding suggests that a significant fraction of the cell population maintains the capacity for self-renewal, a characteristic of MSCs. Prior studies indicated that the clonogenic capacity of MSCs diminishes with consecutive passages; nonetheless, early passages such as P2 retain a robust ability for self-renewal, hence endorsing its application in clinical studies [15, 24]. These findings indicated that SFSCs at P2 effectively differentiated into (i) osteogenic lineage validated by calcium nodules in differentiated osteocytes, and (ii) adipogenic lineages as validated lipid deposition in differentiated adipocyte cells which highlights multipotency of SFSCs at P2, makes their use in regenerative medicine. Differentiation capacity of SFSC's is crucial as osteocytes and adipocytes lineages could be a promising approach for cartilage and bone regeneration, rendering SFSCs a compelling option for addressing problems like osteoarthritis. These study stated that osteocyte lineage differentiation ability of SFSC's could be a promising option in the treatments of osteoarthritis by regenerating cartilage and bones. Previous studies also reported parallel findings of regenerating bone and cartilage using alternative sources of stem cells i.e. bone marrow due to their regenerative abilities at joint intrinsically subjected to mechanical stimuli [21, 25]. On the other hand, the adipogenic differentiation highlights their potential use in soft tissue regeneration. Jorgenson *et al.*, reported similar findings where synovial fluid derived mesenchymal stem cells showed multipotency at P2 [26]. This study provides valuable insights into the distinct ability of SFSCs at early passages. The use of canin derived SFSC's may not be directly relevant to human derived MSC's. This highlight a future research on human derived SFSC's to further verify the findings and to assess their therapeutic potential in pre-clinical models. As senescence induced at later passages would also provide forecast to study the stemness of SFSC's comprehensively at later stages. In conclusion, SFSC's possesses MSC's like characteristics of self-renewal, growth and differentiation at early passages which make them a suitable candidate in regenerative therapies.

CONCLUSIONS

SFSC's possesses regenerative potential, which could be a future of regenerative medicine to repair bones and soft tissues. These findings contribute to MSC biology and reinforce the therapeutic promise of SFSCs in musculoskeletal disorders.

Authors Contribution

Conceptualization: MUS
Methodology: US, HMA
Formal analysis: TI
Writing, review and editing: US, HMA, TI, RK, MA

All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

All the authors declare no conflict of interest.

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