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## Non-enzymatic method of isolation of canine mesenchymal stem cells from Wharton jelly and its characterization

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### Abstract

Wharton jelly is touted as another heavy source of the human stem cells. Considering the promising therapeutic values of stem cells in canines, this has led to the investigation of other alternative sources relevant in the extraction and identification of canine stem cells. The process of making mesenchymal stem cells (MSCs) traditionally employed in clinical apps entails collagenase-rich Wharton jelly (WJ), which has been time as well as resource-consumptive. Besides, it can lead to the presence of mechanical stress in cell isolation, and using bacterial enzymes creates a risk of contamination with endotoxins and xenoantigens. Thus, during the current research, we chose explant culture method as a more efficient and easier yet effective method of isolating MSCs in canine Wharton jelly. The cells were isolated and adherent to plastic and they had a fibroblastoid shape. Larger studies which have evaluated the adult mesenchymal stem cells *in vitro* conditions that allow trilineage differentiation show homogeneous trend of the gene-expression profiles they are analysing. The results of the present study help to determine that this patient demonstrates positive phenotype condition (manifestation of CD44, CD73, non-ticketed CD90 and incapability of presenting edging CD34). All these data help in the definition of mesenchymal stem cells. This was further confirmed by immunofluorescence labelling, which pointed at strong expression of MSC specific markers. Our findings show that explant culture-method allows the easy isolation of canine WJ-MSCs without affecting their morphology and multipotensoriness.

**Keywords:** Canine Wharton jelly, explant culture, mesenchymal stem cells, gene expression, Immunostaining

### 1. Introduction

The stem cells are very adaptable due to their multipotent nature, and they can be used to see to it that various damaged tissues are regenerated. The capacity of stem cells to renew itself is vital in effecting a sufficient number of a therapeutic outcome. They also have multipotency which makes them regenerate and rebuild numerous types of damaged tissues. Adult progenitor cells are so-called, and in particular in the form of MSCs, reside at the stromal niche of most body organs, and have been methodically probed, with respect to their regenerative potential, both in the biomedical and veterinary sectors. The MSCs of the bone marrow have been the canonical population of cells, but recent evidence indicates a variety of extramedullary sites have similar and multipotent stromal cells. These are lipocytes, the cords of Wharton's jelly, the umbilical cord matrix or cord blood, synovial membranes, dental pulp, corneal tissue, amniotic fluid, placenta and menstrual blood (Meirelles *et al.*, 2006) <sup>[1]</sup>. The findings confirm the opinion that stromal multipotency is not restricted to bone marrow rather to a wide variety of tissues.

Mesenchymal stem cells form a linchpin of the modern-day cell-based treatment and tissue engineering developments, with a significant potential towards therapy and treatment, especially as applied to canine-centric veterinary medicine.

With the wide and well-documented arguments that companion animals now enjoy a longer lifespan as it was in bygone years, diseases associated with old age among them osteoarthritis and other forms of degenerative illness are increasingly becoming common place. This means that veterinary medicine will need to invest in research and subsequent implementation of new forms of therapy and prevention measures aimed to maximise the prevention of these health conditions. Canines are also used as translational models to various human disorders and they present an avenue to determine therapeutic effects and safety profile of stem cell-based interventions (Hayes *et al.*, 2018) [2].

They are multipotent, immunomodulatory and have paracrine signalling support, thus it would be logical to say that MSCs are a promising candidate to a regenerative therapy (Arthur *et al.*, 2009) [3]. Mesenchymal stem/stromal cells can specify several kinds of tissues, namely, bone, cartilage, adipose tissue, skeletal muscle, structural connective tissues, blood vessels, ligaments, and tendons under normal *in vitro* conditions (Ullah *et al.*, 2015) [4]. Following the definitions of "International Society for Cellular Therapy (IST)", MSCs should possess a number of defining features. Cultured cells need to demonstrate an adherent phenotype with the growth on a plastic tissue culture substrate and show positive immunophenotype expression of the cell-surface antigens CD44, CD73, CD90, and CD 105. The induction capabilities of mesenchymal stem cells (MSCs) result in the differentiation of MSCs into the three main mesodermal lineages of the osteogenic, chondrogenic and adipogenic lineages (Dominici *et al.*, 2006) [5]. This plasticity of committing itself to various specialized cells as well as the ability of controlled differentiation is one of the driving factors behind the increasing interest in their clinical application. The qualities of stem cells, mesenchymal, or MSCs, these being able to develop into many different cell lineages, as well as the possibility of being manipulated into developing to any of the cell lineages, allow their use in the development of therapeutic activities in a more complete way. They have found application in most therapy procedures and the curing process of various diseases. Dompe *et al.* (2019) [6]. In majority of studies, both experimental and clinical, of MSCs in dogs are used, bone marrow-derived and adipose-tissue-derived populations are utilized because they are the most well characterized and share a high degree of similarity (Takemitsu *et al.* 2012) [7]. The MSC-like cell was acquired by both enzymatic digestion and explant method and both cell types produced colonies in the form of spindle shaped fibroblast-like cells with high levels of alkaline phosphatase (Eswari *et al.*, 2016) [8]. MSCs of the canine origin may be acquired through a wide range of anatomical selection including adipose, bone marrow, umbilical cord, and synovial fluid (Vieira *et al.*, 2010; Sasaki *et al.*, 2018) [9,10].

The canine umbilical cord consists of Wharton jelly, which is an ideal provider of allogeneic MSCs, also because it may be obtained non-invasively during parturition or caesarean section without damaging the source. WJ-MSCs present an easy isolation and culture and can proliferate robustly, maintain their stemness *in vitro* have low immunogenicity, as well as non-tumorigenic potential, making them a more consistent and multipotent substitute to the tissue-derived adult MSCs (Bonso *et al.*, 2018) [11].

Standard methods of MSCs separation tend to rely on enzymatic digestion and centrifugation, which can be resource involved and can give rise into contaminants such as bacterial enzymes, endotoxins or xenogeneic proteins and additionally

present mechanical pressure to the cells. The current study hypothesizes that use of explant culture technique represents an easy and effective way to isolate mesenchymal stromal cells of the canine Wharton jelly located in the umbilical cord.

### 3. Materials and methods

#### 3.1 Isolation of canine Wharton jelly

The study protocol (synopsis) was given ethical clearance by the Institutional Animal Ethics Committee on approval number 17/SA/IAEC/2024 dated 28.02.2024 of the Chairman, IAEC, Madras Veterinary College and Chennai. Dogs between the ages of 1 to 3 years, without clinical problems, and exposed to experimental surgical procedures irrelevant to this study were used. Samples of umbilical cord tissues were obtained during the caesarean sections or natural birth of bitches of any breeds at the Teaching Veterinary Clinical Complex, Madras Veterinary College in Chennai. Routine operations followed institutional and governmental guidelines of ethically using animals. Umbilical cords of 2-4 cm were harvested under the aseptic conditions, put in the good sterile transport medium of clinical sample to use is phosphate-buffered saline solution with 1% antibiotics added. Then transferred to the Stem Cell Laboratory for further processing.

#### 3.2 *In vitro* culture of MSCs

Umbilical artery and vein sections were emptied and carefully removed so as to extract the Wharton jelly and then into the phosphate buffer saline (PBS, Gibco). The isolated tissue was finely chopped into small fragments approximately 1-2 mm in size. These tissue explants were placed in 6-well plates containing high-glucose Dulbecco modified Eagles Medium (DMEM) enriched with 10% foetal bovine serum (FBS) and 1% Penicillin-Streptomycin (10,000 U/mL and 10,000 g/mL respectively). The culture plates were then incubated at 37°C and 5 percent CO<sub>2</sub> and high humidity, without any disturbance of the explants to 72 hours in order to allow them to adhere by explants and to expand. When the migrating cells of the given explants had reached an approximate confluency, media were removed, and then the culture surface was gently washed using dPBS. The TrypLE (Gibco, USA) was added, and cells were incubated at 37°C to encourage cells detachment within 5 minutes. The enzymatic activity was neutralised with equivalent volume of pre-warmed culture media. To pellet the cells; the solution resulting during centrifugation at 1,500 rpm and 15 minutes was pelleted and again resuspended in 1000 µl culture media which was new in this case. In the current analysis, an evaluation of the proportion of viable cell concerning total cell number was done through the counting of 0.1 trypan blue stained cells in a hemocytometer. The viable cells were then sub-cultured *in vitro* in flasks of cultures and were allowed to attain passage 3 thereby completing the establishment of the culture.

#### 3.3 Evaluation of cell morphology

Cells were seeded in DMEM media supplemented with 10% foetal bovine serum (FBS) and antibiotic antimycotic solution with a concentration of  $1 \times 10^5$  cells in 35 mm normal culture dish. The cultures were then grown in a humidified 5% CO<sub>2</sub> at 37°C till an 80 percent confluence was achieved. When the necessary confluency was attained, the culture media was removed and extremely careful rinsing of the monolayer with PBS was carried out. Then the fixed cells were incubated in 95 percent methanol at room temperature within a period of five minutes. Methanol was removed followed by addition of stain (0.1% Giemsa) into the cell

layer and incubation at room temperature of 30 minutes. Dye removal was carried out by disposing the chromophore since after stain, the cells were washed using distilled water to remove residual dye and insoluble materials. Assessment of the samples was done visually using the inverted phase-contrast microscope.

### 3.4 Tri-lineage differentiation potential of canine WJ-MSCs.

Wharton jelly derived Mesenchymal Stem Cells (MSCs) at passage 3 were utilized to investigate tri-lineage differentiation. The WJ-MSCs were grown in specific induction media of adipogenic, osteogenic and chondrogenic differentiation (StemPro, Gibco Life Technologies and USA). The Passage 3 cells were seeded on six-well plates at a density of 1,000 cells/cm<sup>2</sup> in a medium composed of appropriate differentiation medium and kept at 37°C with media change every three days over a period of 21 days. After the induction phase was completed, the culture media was discarded and the cells washed very carefully with phosphate-buffered saline (PBS). To confirm lineage-specific differentiation, the differentiated cells were stained as follows; Adipocytes stained with EZ Stain™ kits: Oil red O (Himedia), osteocytes with Alizarin red (Himedia), and chondrocytes with Alcian blue (Himedia).

### 3.5 Immunofluorescence staining

The MSC specific surface markers were visualised by performing immunofluorescence labelling of the wharton jelly origin MSCs (WJ-MSCs) by incubation with specific primary antibodies and fluorescein isothiocyanate (FITC) conjugated secondary antibodies. The MSCs, prepared in passage 3, were grown in glass chambers (Ibidi, Germany) until they reached approximately 80 per cent confluence. Culture media was subsequently aspirated followed by washing of the cells with PBS. The cells were then fixed in 4% paraformaldehyde at room temperature for 20 min and washed three times in 1X DPBS. To permeabilize the cells, they were incubated with a 0.2 percent solution of Tween 20 followed by incubation in a

5 percent BSA diluted in DMEM to prevent non-specific binding after 1 hour. After additional DPBS rinses, cells were left to incubate overnight at 4 °C with a 1:100 dilution of the following primary antibodies; anti-CD44, anti-CD34, anti-CD73, and anti-CD90 (Abcam, UK). The cells were later washed with PBS and labelled with a 1:1000 dilution of FITC-conjugated secondary antibodies in PBS and were finally incubated to a time range of one hour. The slides were then counterstained with DAPI (1 g/ml) placed in Vectashield mounting media after the last addition of DPBS and then mounted on slides. The labelled cells were visualized using an inverted fluorescent microscope, Nikon Eclipse.

### 3.6 RNA extraction and real time PCR analysis

In the given study, mesenchymal stem cells (MSCs) were derived *in vitro*, through canine tissues. RNA was then isolated by use of RNeasy Mini kit (Qiagen, Germany) following the manufacturer's instructions. NanoDrop Lite Plus spectrophotometer (Thermo Scientific, USA) was used to determine the levels of the purity and the concentration of RNA. The iScript cDNA synthesis Kit (Bio-Rad, Hercules, and CA) was used in the synthesis of complementary DNA. Briefly, cDNA was synthesized in a volume of 20µl which was composed of the following constituents: reverse transcriptase 1µl, 5X reaction buffer (that contained dNTPs), 4µl nuclease free water and template RNA 2µl (2g). The thermal cycling protocol included a starting step at 25 °C, 10 minutes which was followed by reverse transcription which took place at 42 °C, 60 minutes and lastly enzyme deactivation at 85 °C, for 10 minutes.

To determine the levels of marker genes CD44, CD73, CD90 and CD34, GAPDH as housekeeping gene expression was determined by quantitative PCR (qPCR) using 50ng ds cDNA. The volumes of reaction that were used to perform each qPCR experiment were 20µl of reaction mixture that included: 50ng of cDNA, 10 mM/µl forward and reverse primers, ultra-pure water, and 10µl SYBR Green Supermix (Bio-Rad). The table 1 shows the specific primer strands used to amplify.

**Table 1:** Primer sequence for real time PCR analysis

| Gene  | Primer sequence (5'-3')                                  | Annealing temperature (°C) | Gene Bank accession number |
|-------|----------------------------------------------------------|----------------------------|----------------------------|
| CD 34 | F: TCAGGGCCCCGACATCTC<br>R: TCTCTGCTCACCCCTCTGGAAAAA     | 65                         | NM_001003341.1             |
| CD 73 | F: CTCCAACACATTCTTTACAC<br>R: ACTCAACCTTCAAATAGCCT       | 61                         | XM_038684083.1             |
| CD 90 | F: CAGCATGACCCGGGAGAAAAAG<br>R: TGGTGGTGAAGCCGGATAAGTAGA | 63                         | XM_844606.2                |
| CD 44 | F: CTTCTGCAGATCCGAACACA<br>R: GAGTAGAAGCCGTTGGATGG       | 60                         | XM_038423375               |
| GAPDH | F: ACTTATGACCACTGTCCACGC<br>R: CACAACAGACACGTTGGGAGT     | 58                         | NM_001034034.2             |

### 3.7 Statistical analysis

The distribution of the data that did not exhibit a normal distribution was log transformed to obtain the normal distribution. Once they were designated to be normal, a statistical test was done on them, and the result of the test was presented as a graph of the average value of these restructured figures.

## 4. Results

**4.1 Isolation and *in vitro* culture of canine Wharton jelly derived MSCs:** This research case used umbilical cords in parturition or caesarean parturition of pregnant bitches and the

Wharton jelly was extracted manually. Kept at *in vitro* condition, with an approximate amount of 3-4 tissue explants uniformly added to each well of a six-well culture dish. The tissue explants started growing within 3 and 4 days, with migration of the round-shaped cells on the surface of the culture plate (Figure 1A). These migrating cells revealed a normal spindle shape and further proliferated even in the presence of the removal of the explants and this process usually occurred 5 to 7 days after initiating culture (Figure 1B). Its cultures were kept running around 8 to 12 days or up to the point that the cells became confluent (Figure 1C). Under Giemsa stain by 0.1% stain of canine MSCs, the

characteristic appearance of the fibroblast-like or spindle shape was found. The cells under the inverted phase-contrast microscope appeared to have well-defined nuclei (dark-

stained) and light stained cytoplasm which is prototypical of mesenchymal stem cells (Figure 1D).

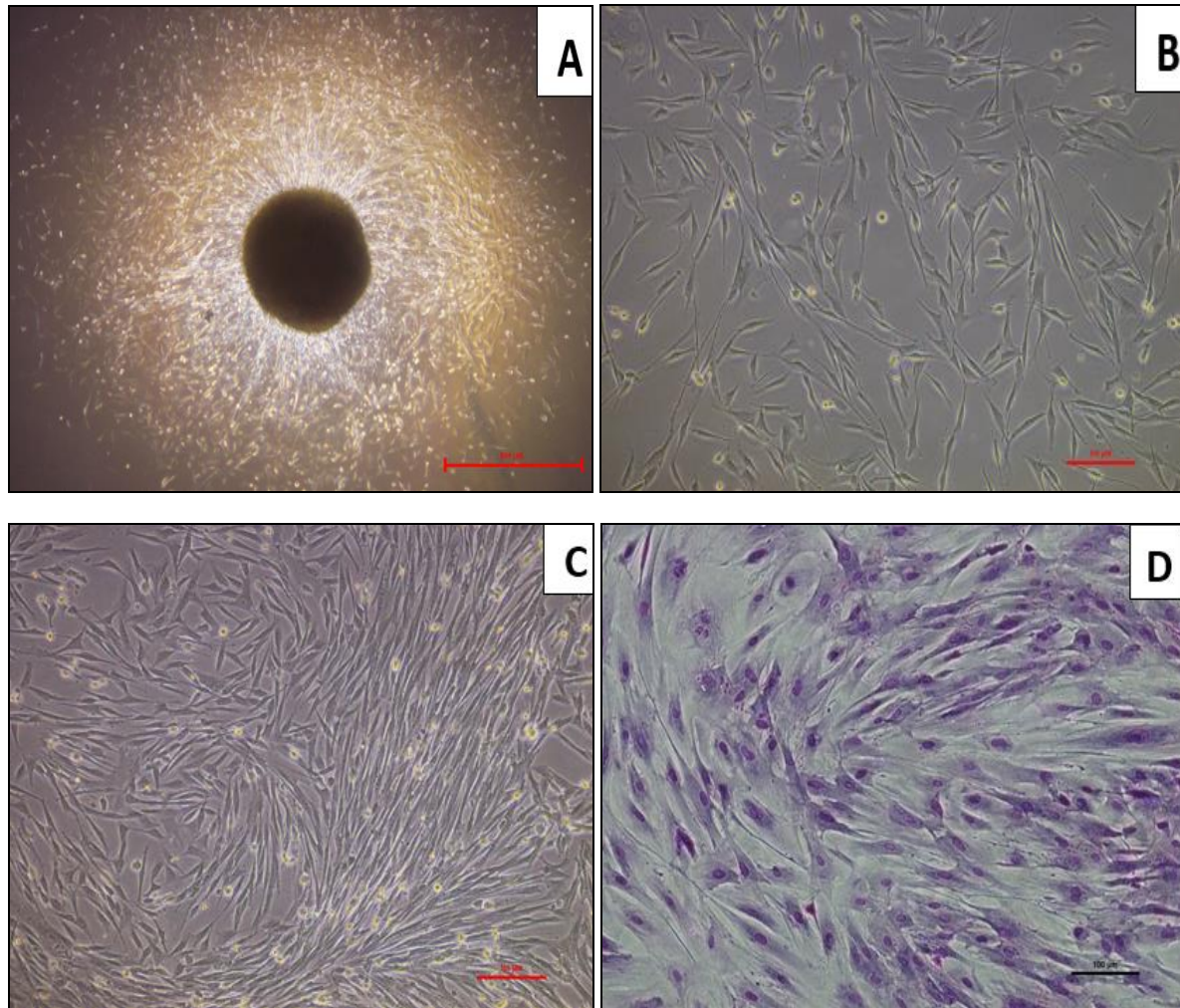


**Fig 1:** Canine WJ-MSCs isolation through explant method. Migration of round cells of the WJ explants on day 3 (A). The MSCs originating in explant displayed spindle shaped morphology on day 6 (B). 70-80 percent of MSC culture with confluent rate at passage one (C). MSCs in normal morphology stained by Giemsa (D).

#### 4.2 Tri-lineage differentiation potential of canine WJ-MSCs

To perform the breeding experiment, the stem cells obtained from canine Wharton jelly (WJ-MSCs) were investigated to determine the presence of the multipotent differentiation potential of adipogenic, osteoblast guidance, and chondrogenic edges by evaluating them in passage 3.

- **Adipogenic differentiation:** Canine WJ MSCs were induced into adipogenic differentiation shown by the production of intracellular lipid vacuoles, a characteristic of adipocytes that were coloured red using Oil Red O. Morphologically, differentiated cells used grew in a rounded shape and had conspicuous lipid inclusions of cytoplasm (Figure 2A).
- **Osteogenic differentiation:** When the WJ-MSCs were isolated and cultured in osteogenic induction medium after 21 days, extracellular calcium deposits were begun in the medium. This osteogenic activity was observed in Alizarin Red staining that stains calcium crystal indicating that osteoblast-like cells have been

successfully differentiated (Figure 2B).

- **Chondrogenic differentiation:** After culturing the WJ-MSCs in the chondrogenic induction medium 21 days later, cell culture response evidence was observed showing the chondrogenic differentiation of the WJ-MSCs in terms of the synthesis of sulphated glycosaminoglycans. The finding of these matrix components was proven through the Alcian Blue staining, which proved the formation of the cartilage-like tissue (Figure 2C).

#### 4.3 Immunofluorescence detection of surface markers of canine WJ MSCs

Monoclonal antibodies that target CD44, CD73, CD90, and CD34 were used in immune-cytochemical labelling to locate mesenchymal stem cell surface markers in canine WJ-MSCs. They grew these cells *in vitro* in which findings revealed the MSC phenotype: the highly expressed markers CD44, CD73 and CD90 the hematopoietic marker CD34 was not extracted.

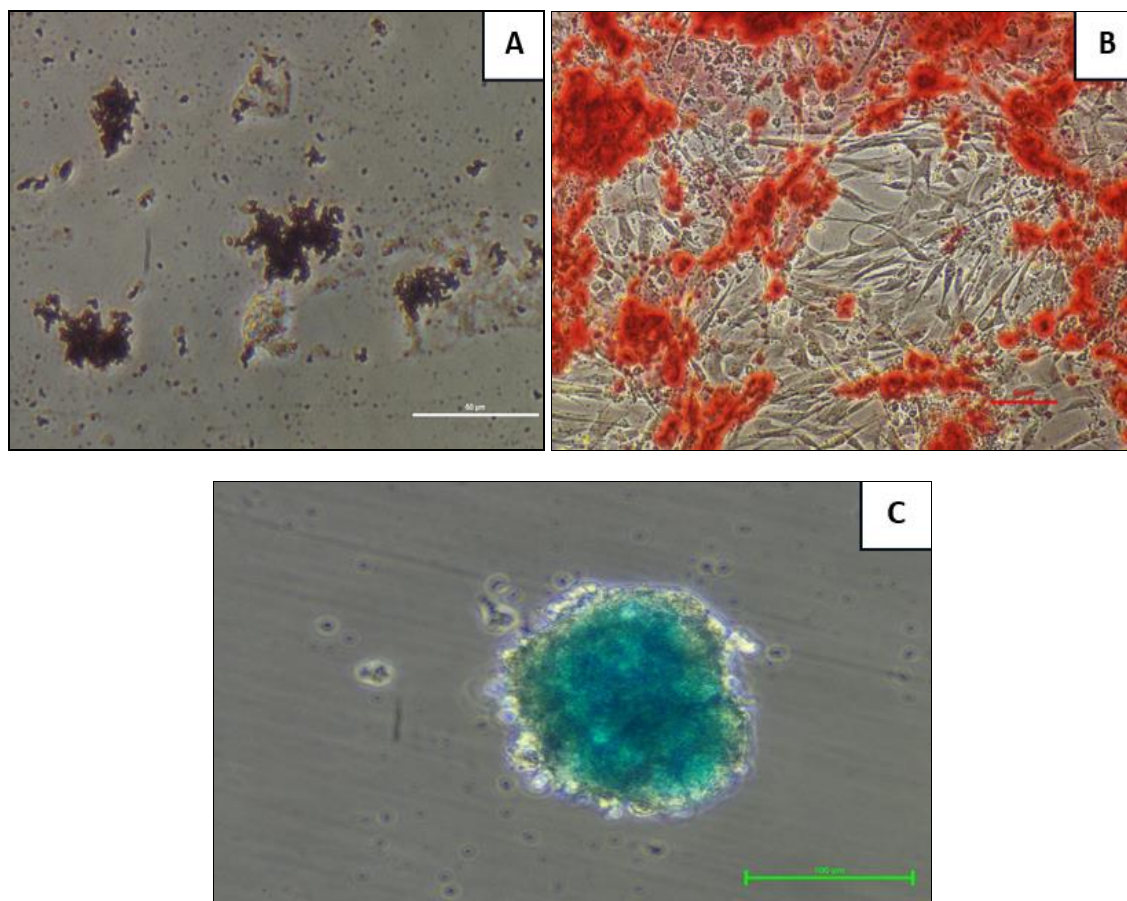


**Fig 2:** Canine WJ-MSCs differentiation potential along tri-lineage. Adipogenic mechanism of MSCs stained with Oil red O (A), Staining of osteogenically induced MSCs by alizarin red S (B), induced chondrocyte from MSCs stained with Alcian blue (C)

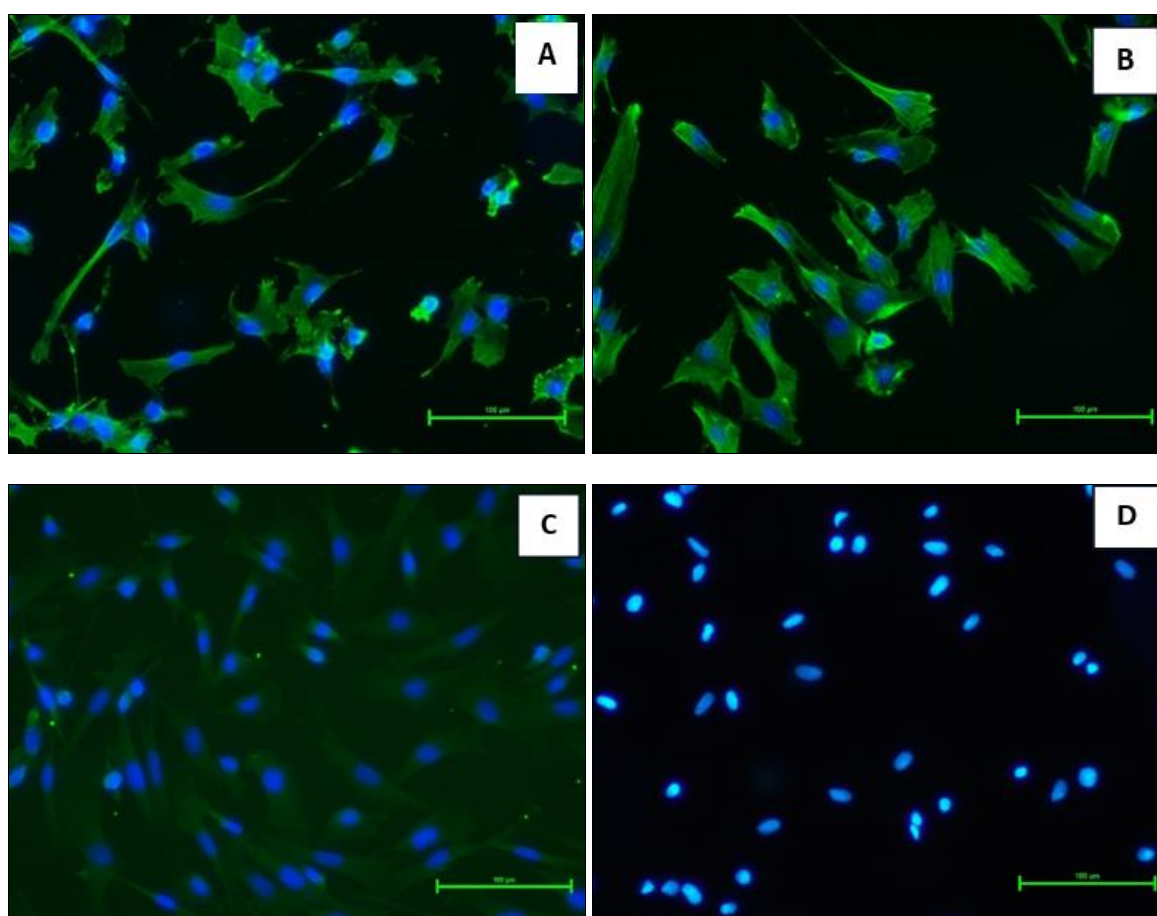


**Fig 3:** Canine WJ-MSCs in passage 3 stained with immunofluorescence. The immuno-staining fluorescent images depicted canine WJ-MSC phenotypic labellers. They had (A) CD44, (B) CD73, (C) CD90, (green fluorescence) and the lack of CD34. They also had nuclei as blue colour. Scale bar, 100 nm. CD, cluster of differentiation.

#### 4.4 Gene expression of MSC specific markers

Passage 3 canine WJ-MSCs expression of MSC-specific marker genes was assessed by analysis of gene expression. Three positive markers, CD44, CD73 and CD90, were examined and one marker, CD34 was negative. The expression data were obtained in a form of log FC in a bar

graph to be able to better demonstrate the direction and magnitude of change in gene expression (Figure 3). The result of the analysis showed that the CD44, CD73, and CD90 positive MSC markers were intensively upregulated, and the hematopoietic marker (CD34) had been lower in the passage 3 WJ-MSCs (Figure 3).

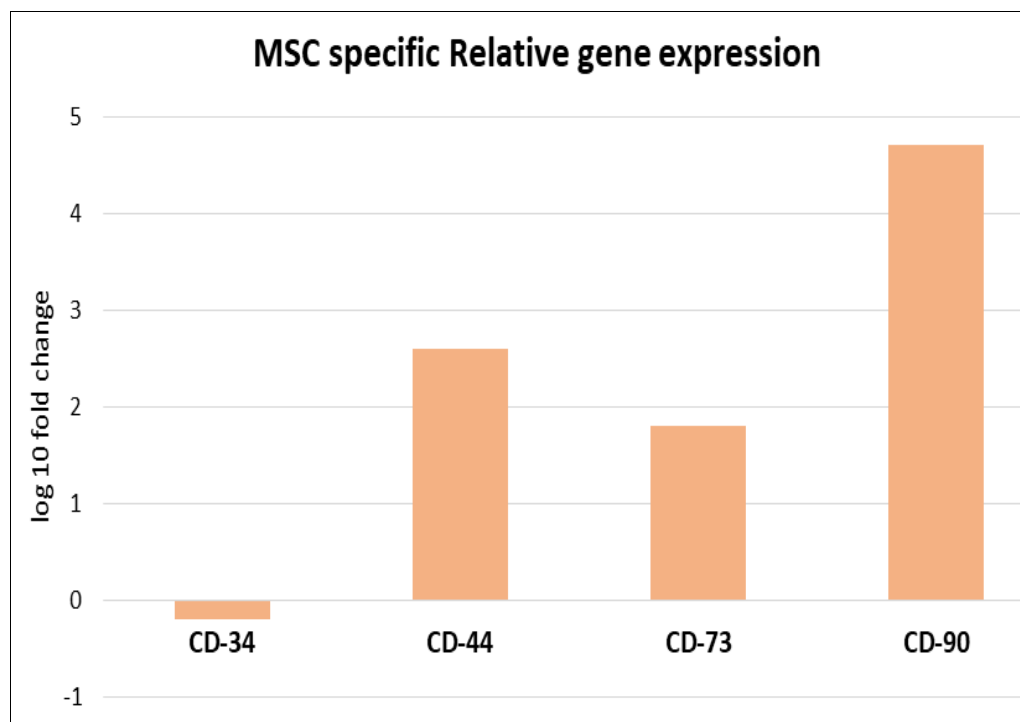


**Fig 4:** The qPCR study of canine Wharton jelly-derived MSC indicated that there is a considerable change in expression of MSC markers genes. Samples were positive in terms of the MSC markers, CD44, CD73, and CD90, and were negative with regards to the haematopoietic stem cell marker, CD34. The expression values were reported as log10 fold change

#### 5. Discussion

In the earlier studies, the mammalian literature has found out populations of stem cells in several different sources such as bone marrow, umbilical cord blood, adipose, placenta and various organ contexts (Rocca *et al.*, 2009) [12]. In particular, the isolation and characterisation of human umbilical-cord matrix (Wharton jelly) mesenchymal stem cells (WJ-MSCs) published recently by Rehman *et al.* (2010) and Procopi *et al.* (2011) [13, 14] is interesting in the context of the current report. Wharton jelly is routinely discarded during Whelping, making it an easily accessible source of raw materials for stem-cell research. In the current study, attempts were made to define canine Wharton-jelly-derived mesenchymal stem cells, although an essentially analogous explant-culture strategy was adopted to that which is known about human WJ-MSCs. Of the six attempts of canine Wharton jelly, 0 was failed in culture expansion; thus, the isolation rate was 100% successful. Isolated cells were adherent and spindle-shaped, and only consistently positive with markers expressed by the human mesenchymal stem cells (Troyer *et al.*, 2008) [15], negative with lineage-specific markers. These data therefore provide evidence that canine Wharton-jelly derived mesenchymal stem cells do exist providing an easily obtained source of MSCs in veterinary and comparative use.

To verify the mesenchymal characteristic of canine WJ-MSCs, we also performed various differentiation stains. The WJ MSCs of the dogs were differentiated into adipocyte, osteocyte and chondrocyte at varying conditions. They have discussed multi lineage differentiation potential as the significant aspect of mesenchymal stem cells. Other experiments described in the past were unsuccessful in

adipogenic differentiation of canine MSCs in human media (Neupane *et al.*, 2008) [16]. When the canine WJ-MSCs were cultured under osteogenic induction media, drastic change in appearance was observed. Oil Red O staining was used to confirm the adipogenic induction. By day 21 differentiation of the cell to adipocytes was successful as stained by oil red O stain, where the fat droplets appeared clear. The canine WJ-MSC was seen to form aggregates and there was positive staining of cells with alcian blue stain after 3 weeks of induction in the chondrocyte induction medium because of the presence of glycosaminoglycans. The canine WJ-MSCs had positive alizarin red staining of calcium crystal deposition after 21 days of osteogenic induction medium incubation, which is also consistent with the findings of Rani *et al.* (2017) [17] and closely aligned to the findings of Seo *et al.* (2012) [18] and Rashid *et al.* (2021) [19].

Immuno-phenotyping of canine WJ MSCs indicated that they were CD44, CD73 and CD90 positive and CD34 negative in characteristic of the mesenchymal stem cell. In line with previous reports that used MSCs based on *ex vivo* adipose tissue of canine, bovine and human donors, the current results revealed positive expression of CD44, CD73, and CD90 and negative expression of CD34 in all the canine WJ MSCs (Vieira *et al.*, 2010; Sasaki *et al.*, 2018; Sandhu *et al.*, 2017) [9, 10, 20]. The qPCR findings of the fact that canine MSCs marker genes expression was up regulated CD44, CD73 and CD 90 and down regulated CD34 that is haematopoietic marker. There is similar expression of markers in WJ-MSC in ovine (Eswari *et al.*, 2016) [8] in bovine (Sreekumar *et al.*, 2014 and Venugopal *et al.* 2011) [21, 22] as well as in porcine (Sing

*et al.*, 2021)<sup>[23]</sup> and canine adipose tissue (Rashid *et al.*, 2021)<sup>[19]</sup>.

## 6. Conclusion

In our results, it was established that canine Mesenchymal Stem Cells (MSCs) obtained by explant culture of Wharton's jelly have similar phenotypic and functional features with those described of MSCs in other organisms. Since explant culture is simple, economic and minimally involved method, it can serve as a viable alternative to the enzymatic digestion approach to the isolation of MSCs of canine Wharton's jelly (especially when it needs to be used in the field of tissue engineering and regenerative bio medicine). Further on, the canine WJ-MSCs acquired during the explant culture are of great importance in terms of developing the science of stem cells and could potentially be used as a stem cell surplus in Veterinary Medicine preclinical studies. To sum up, the method of explant culture also has all the benefits of processing small amounts of Wharton's jelly, therefore, it is most appropriate to use in cases where only partial pieces of umbilical cord tissue can be obtained.

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## Conflict of Interest

Not available

## Financial Support

Not available

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