Ovine Fetal Adnexa-Derived Mesenchymal Stem Cells exhibit Stemness and Pluripotency with Distinction in Growth Kinetics and Clonogenic Potential

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Abstract

Fetal adnexa derived Mesenchymal Stem Cells (MSC) offer all in one solution in therapeutics. The present study was designed to isolate, cultivate, characterize and to evaluate the growth kinetics of ovine fetal adnexa-derived Mesenchymal Stem Cells (MSCs). The gravid uteri of ewes were collected from a local abattoir. The four cell lines viz. Wharton's Jelly (oWJ), Cord Blood (oCB), Amniotic Fluid (oAF), and Amniotic Sac (oAS) were isolated and expanded *in vitro* and characterized for surface markers (CD73, CD90 & CD105) and pluripotency markers (SOX2 & OCT4) at 3rd passage. The growth kinetics of MSCs was investigated at 3rd and 5th passages. Similarly, the colony forming unit (CFU) Assay was performed at 3rd passage. The fetal adnexa derived Ovine MSCs showed the amplification of CD73, CD90 & CD105, whereas, negative marker CD34 was not amplified. Similarly, the MSCs also qualified for the amplification of Pluripotency markers (OCT4, SOX2). The MSCs in culture showed a typical growth curve with initial lag phase, an exponential phase, a plateau phase and a decline phase. The proliferation potential of MSCs was higher at P5 than P3. The overall growth rate was significantly ($p < 0.05$) highest in oAF, followed by oCB, oWJ and oAS. The population doubling time (PDT) was significantly ($p < 0.05$) highest in oAS (87.28 ± 3.24 hr) and least in oAF (39.75 ± 1.09 hr). The MSCs showed a significant ($p < 0.05$) difference in their clonogenic potential. The colony number was highest in oAF (53.67 ± 4.06) and least in oAS (22.0 ± 2.08). The study reveals that in terms of growth kinetics and clonogenic potential, oAF-MSCs were superior which outperformed other MSCs indicating that oAF derived MSCs could be utilized for regenerative medicine.

Introduction

Sheep is one of the important farm animals and has many biophysical and biochemical similarities to humans. Of its many unique features, its size, character, and similarities to humans makes it a reasonable tool for preclinical evaluation and optimization of extensive biotechnological developments (Scheerlinck et al. 2008). J & K's standing at 6th place for sheep population makes it a viable source for stem cell research as the samples for stem cell isolation are readily available. Mesenchymal stem cells (MSCs) are considered the most promising cell populations for regenerative medicine and tissue engineering as the cells carry all in one therapeutic solution for diverse clinical disorders, attributable to their characteristic properties such as self-renewal, multiplication, immune-modulation and multi-lineage differentiation potential (Gugjoo et al. 2017). MSCs in body tissues are very limited and as such their culture expansion becomes imperative (Gugjoo et al. 2020). Unlike Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), MSCs are free of ethical and teratogenic concerns and demonstrate low immunological rejection. The fetal adnexal tissue has many advantages over other sources in being ethically acceptable, easily available, non-invasive nature of isolation and greater tissue mass for effective harvesting of MSCs with efficient differentiation potential and no immunogenicity (Cremonesi et al. 2011; Fernandes et al. 2012; Iacono et al. 2015). The bovine MSCs show immunosuppressive attributes, mainly due to their lower MHC-I/MHC-II expression (Cardoso et al. 2017). The population and proliferation capability of MSCs depends on the tissue source. Due to homing ability,

MSCs in sheep have been introduced through parenteral route (Gugjoo 2018). The differentiation potential of Ovine Mesenchymal stem cells (oMSCs) varies depending on the tissue source (Ribitsch et al. 2017; Burk et al. 2017). Many other factors like the food standard, the age of the animal, and the disease in an animal also influence the cellular features of MSCs. Based on the sources and passage number, MSCs may exhibit bias in proliferation potential and self-renewal capabilities (Lu et al. 2014). Senescent cells seem vacuolated and show karyopyknosis morphologically, but their growth kinetics reveal significantly reduced or no growth at all. The evaluation of karyotypic characteristics is desired to maintain track of cellular activity (Colleoni et al. 2005).

Owing to the ease of harvesting, more acceptability, little tissues rejection and non-ethical issues associated with mesenchymal stem cell therapy, besides very scanty research available in sheep fetal mesenchymal stem cells, the present study was designed to establish (including isolation, expansion and characterization) ovine fetal adnexa based MSC culture. Simultaneously, the growth kinetics and clonogenic potential of the MSCs was compared.

Materials And Methods

Chemicals and Reagents

The chemicals/reagents were mainly procured from Thermo Scientific Pvt. Ltd., USA; Himedia Laboratories, India; Sigma-Aldrich, St. Louis, Missouri, United States; Life Technologies Carlsbad, California, United States; unless otherwise indicated. The plastic ware was chiefly purchased from Tarson, India; Genetix Biotech Asia Pvt. Ltd., India and Jet Biofil Guangzhou, China.

Experimental Animals & Sampling

The Gravid Uteri (N = 22) of pregnant ewes (*Ovis aries*) containing 2–3 month old fetuses were collected from the local abattoir located in Srinagar, J & K. The samples were transported to the laboratory in an insulated box containing warm 1X buffered saline (8.5 g of NaCl + 1.365 g of Na_2HPO_4 + 0.243 g of NaH_2PO_4 in 1000ml of distilled water) fortified with antibiotics (100 IU Penicillin, 100 µg Streptomycin) within 2 hours of slaughter. The study was granted Institutional Ethical Clearance Vide No. AU/FVS/PS-57/21/5402, dated: 12/08/2021.

Isolation and Culture of MSCs from Ovine Fetal Adnexa

The gravid uterus was washed a few times with luke warm buffered saline fortified with antibiotics to remove debris, if any. It was followed by dissection of the gravid horn to expose fetal adnexa. The fetal adnexa served as a source of four cell lines of MSCs viz., oWJ, oAS, oAF, oCB and were isolated while following a standard protocols for each cell type (Plate 1). The oAF from the amniotic cavity was collected in a 50 ml Falcon tube (Genetix Biotech Asia Pvt. Ltd., India) using a sterile syringe and centrifuged at 1500 rpm for 15 minutes. The cell pellet was washed twice by 1X DPBS (TS1006, Himedia Laboratories, India) and suspended in the culture medium Dulbecco's Modified Eagle Medium (AL149,

Himedia Laboratories, India) containing 15% FBS (RM9955, Himedia Laboratories, India) along with 2 mM L-Glutamine (25030149, ThermoFisher Scientific, Brazil), 50µg/ml Gentamycin (15750037, ThermoFisher Scientific, Brazil, USA) and 250 µg/ml Amphotericin (15290018, ThermoFisher Scientific, Brazil, USA) and later seeded in the wells of a 12-well culture plate (980020, Tarsons, India) which was placed in a CO_2 incubator (New Brunswick Scientific Co Galaxy 170S, San Diego, USA) maintained at 37°C with 5% CO_2. The oWJ and oAS were separated from the fetus, excised into small 1–2 mm pieces, washed thoroughly and cultured *in vitro* using explant method. Similarly, mononuclear MSCs from oCB were separated by density gradient centrifugation method using LSM-1077 (LS001, HiMedia Laboratories Pvt. Ltd, India). The oCB-MSCs were cultured in a similar pattern as that of oAF.

The explants of oWJ and oAS were removed as soon as the growth around them was visible (Plate 2). The media in the culture wells was changed after every 3–4 days. The cells were harvested for sub-culturing at 70–80% confluence using 0.25% Trypsin (TCL006, HiMedia Laboratories Pvt. Ltd, India) and seeded at 2:1 ratio. The cells were sub-cultured upto P3 and P5 for all the experiments.

RNA isolation and cDNA synthesis from MSCs

The MSC (oWJ, oAS, oAF & oCB) monolayer was washed with ice cold DPBS (TS1006, Himedia Laboratories, India). The RNA was isolated from the cells by TRIZOL method (Rio et al. 2010) at P3 and P5. The purity and concentration of isolated RNA was examined in Nanodrop Spectrophotometer (Thermo Scientifc, USA). The purity ($\lambda_{260}/\lambda_{280}$) of 1.8-2.0 was considered as good for RNA. Agarose gel (1.5%) (RM201, HiMedia Laboratories Pvt. Ltd, India) electrophoresis was performed to access the integrity of RNA.

The RevertAid First Strand cDNA synthesis kit (K1622, Thermo Scientific, Lithuana) was used to reverse transcribe total RNA (500 ng) into cDNA as per the manufacturer's guidelines using a Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Singapore). The cDNA formed was stored at -20^{o}C for various downstream processes.

Amplification of cDNA and Agarose Gel Electrophoresis

The Ovine specific primers for GAPDH, Stem cell surface markers (CD34, CD73, CD90 & CD105) and Pluripotency markers (SOX2 & OCT4) were selected from the published literature (Tables 1 & 2). The fragment size of PCR amplified products was confirmed by Agarose gel (1.8%) electrophoresis against a Gene ruler, 50 bp DNA ladder (SM0371, Thermo Scientific, USA).

Table 1
Gene transcripts, primer sequences and resulting fragment size of stem cell markers

Gene	Primer sequence	Accession number	Product size (bp)	Annealing Temperature (°C)
CD73	**f** - TGGTCCAGGCCTATGCTTTTG	BC114093	115	57
	r - GGGATGCTGCTGTTGAGAAGAA			
CD90	**f**- CAGAATACAGCTCCCGAACCAA	BC104530	97	58
	r - CACGTGTAGATCCCCTCATCCTT			
CD105	**f** - CGGACAGTGACCGTGAAGTTG	NM-001076397	115	59
	r - TGTTGTGGTTGGCCTCGATTA			
CD34	**f**- TGGGCATCGAGGACATCTCT	AB021662	107	58
	r - GATCAAGATGGCCAGCAGGAT			
GAPDH	**f** - TGACCTATGGCAACCGATACAA	AJ507200	76	58
	r - CCGCAAAAGACATCCAGGAT			

Table 2
Gene transcripts, primer sequences and resulting fragment size of pluripotency markers

Gene	Primer sequence	Accession number	Product size (bp)	Annealing Temperature (°C)
Sox2	**f -** CATGAACGGCTCGCCCACCTACAG	XM-004003838.1	267	57
	r - TCTCCCCCGCCCCCTCCAGTTCAC			
Oct4	**f -** GATCGGGCCGGGGGTTGTGC	XM-004018968.1	235	58
	r - TCGGCTCCAGCTTCTCCTTGTCCA			

For amplification of cDNA, the 20 µl reaction mixture (1 µl template cDNA, 1 µl forward primer, 1 µl reverse primer, 7 µl nuclease free water and 10 µl Dream Taq Green PCR Master Mix) were incubated in a Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Singapore). The program for amplification was set as initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, 57°C for

CD73 & SOX2; 58^{o}C for GAPDH, CD34, CD90 & OCT4; 59^{o}C for CD105 for 30 seconds and 72^{o}C for 30 seconds and final extension at 72^{o}C for 5 minutes and then holding temperature was 4^{o}C. All these samples were examined on Agarose gel (1.8%) by following the standard procedure given by Sambrook (1989). The gels were subsequently photographed by Bio Rad Gel Doc System (Bio-Rad laboratories Inc., USA).

Growth Kinetics and Population Doubling Time (PDT)

The growth kinetics at P3 and P5 for all the four types of MScs was determined by seeding 10,000 viable cells per well and incubated in the culture medium which was changed after every 4th day. The cells were harvested and counted after every 2 days until day 12. The viable cells were counted by Trypan Blue Dye Exclusion Method (Tolnai 1975) using the formula;

No. of Cells/µl = (N/4) x 10 x DF

Where, DF is the dilution factor.

The growth curve was plotted between the culture time along X-axis against the change in the number of cells along Y-axis.

PDT was calculated using the formula put forth by Pratheesh et al (2013);

PDT (in hours) = t x log(2)/log (N_t/N_0)

Where, t is culture period (in hours) at which cells were harvested and subsequently counted, N_t is the number of cells harvested at a particular time and N_0 is the number of cells seeded at day 0 (i.e., 10,000).

Colony Forming Unit (CFU) assay

The colony forming unit (CFU) assay was performed at P3 by seeding 100 viable MSCs in the wells of a 12 well culture plate (980020, Tarsons, India) and incubated for 3 weeks at 37^{o}C in a humidified CO_2 incubator (New Brunswick Scientific Co Galaxy 170S, San Diego, USA). The medium was replaced with fresh one after every 4th day. At the end of incubation, cells were washed with DPBS (TS1006, Himedia Laboratories, India), fixed with 4% formaldehyde followed by staining with 1% crystal violet (V5265, Sigma-Aldrich, India) solution for 20 minutes. The wells were observed under inverted Microscope (CKX53, Olympus, Japan), clusters with > 20 cells were considered as clones and were scored as per the method of Gade et al (2013).

Statistical Analysis

The data for growth kinetics and colony forming unit (CFU) assay were analyzed by three way and one way ANOVA, respectively using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) Statistical Software. The data presented as mean ± SE were considered significant at $p \leq 0.05$.

Results

Molecular Characterization (Stem cell markers & Pluripotency markers)

The Ovine Fetal derived MSCs viz oWJ, oAS, oAF, oCB showed the expression of positive cell surface markers (CD73, CD90 & CD105) at passages 3 and 5 whereas, negative marker CD34 was not amplified (Plate 3). Similarly, the cultured MSCs derived from fetal adnexa also qualified for the amplification of pluripotency markers like SOX2 and OCT4 (Plate 4).

Growth Kinetics and PDT

The plate 5 represents the microscopic (20X) view of stem cell population at P3 and P5 when cultured for evaluation of growth kinetics. The growth curve of oWJ, oAS, oAF, oCB was plotted at P3 and P5. All the MSCs showed an initial lag phase of 48 hours, followed by an exponential phase of 6–10 days at P3 and P5. It was followed by a plateau phase with declined growth rate (Fig. 1). Each MSC type showed a better growth rate at P5 than P3 and the difference was significant ($p < 0.05$) in oWJ (59660 ± 5935.38 cells/ml at P3 vs 86837 ± 8926.72 at P5) whereas, it was non-significant for rest of the sources (Fig. 2). The overall growth rate was significantly ($p < 0.05$) highest in oAF (193515 ± 15604.58 cells/ml), followed by oCB (144413 ± 11918.13 cells/ml), oWJ (73248 ± 5532.39 cells/ml) and oAS (44812 ± 2940.71 cells/ml), which showed the least growth rate (Fig. 3). The PDT was lesser at P5 than P3 as is shown in the Fig. 4. The MSCs showed a significant ($p < 0.05$) difference in PDT with oAF (39.75 ± 1.09 hours) showing the least value followed by oCB (47.21 ± 1.34 hours), oWJ (72.77 ± 5.69 hours) and oAS (87.28 ± 3.24 hours) where PDT was highest or the growth rate was the least (Fig. 5).

Colony Forming Unit (CFU) assay

The CFU assay at P3 showed that there was a significant ($p < 0.05$) difference in the number of round colonies (Plate 6). The colony number followed the order (from increasing to decreasing); oAF (53.67 ± 4.06), oCB (32.00 ± 2.08), oWJ (28.00 ± 2.65) and oAS (22.00 ± 2.08) (Fig. 6).

Discussion

Despite the significance of sheep as an animal model for conditions like bone degeneration and regeneration thereof, encephalopathies (Dias et al. 2018), reproductive disorders (Andersen et al. 2018), the same haven't been explored fully vis-à-vis mesenchymal stem cell characterization and growth kinetics. MSC proliferation and clonogenic potential may or may not be affected by age (Han et al. 2010). Breed diversity had no effect on the proliferation of BM-MSCs in sheep, unlike in dogs (Rhodes et al. 2004). The passaging increases the overall cell number, however phenotypic alterations occur during extended passaging, resulting in diminished cell proliferation. The early passaged cells are clinically superior to widely passaged cells in terms of effectiveness (Textor et al. 2018). Dar et al (2021) showed that the tissue type and physiological status of donor animal affect the MSC characteristics. Moreover,

the ambient temperature affects the recovery of the cells from any tissue. García-Muñoz and Vives (2021) ensured cost efficient and large-scale expansion of WJ-MSCs without altering their biological properties

In the present study, we successfully isolated, cultivated, characterized and then evaluated the growth kinetics of MSCs from multiple sources of ovine fetuses. All the four types of oMSCs were $CD73^+$, $CD90^+$ and $CD105^+$ but lack hematopoietic markers. The lack of species-specific antibodies, as well as variances in the types of tissue sources and harvesting methods, are blamed for the heterogeneity in marker expression (Gugjoo et al. 2020). The CD90 was better amplified in oWJ showing a superior glycoprotein content in the MSC membrane and hence a better ability for cell adhesion, cell-cell and cell-matrix interactions (Somal et al. 2016; Moraes et al. 2016). The oWJ shows a brighter band of CD105 upon gel electrophoresis which suggests that it has a higher angiogenic potential than rest of the sources (Duff et al. 2003). In yet another study, Cleary et al (2016) demonstrated that BMSC CD105 expression does not associate with a chondroprogenitor phenotype. Colosimo et al (2013) in ovines and Deedwania et al (2020) in humans demonstrated that isolated oAF-MSCs and hAF-MSCs were positive for all surface markers CD29, CD73, CD90, and CD105, but negative for CD34/CD45 (hematopoietic markers), representing characteristic phenotypes of MSCs either in hAF or in oAF and such reports were also confirmed in present study. The source and isolation procedures influence MSC surface markers, resulting in substantial variation (Wagner et al. 2006).

The pluripotency markers like OCT4 and SOX2 got amplified in all the four types of oMSCs. Our results coincide with those of Ma et al (2017) wherein MSCs were shown to exhibit pluripotency markers such as OCT4 and SOX2. These cells confirmed their ability of self-renewal by expressing SOX2 gene and their properties of pluripotency and plasticity by expressing OCT4 (Eswari et al. 2016). Campbell et al (2007) proposed OCT4 gene as a master regulator of pluripotency of MSCs. The early transcription factors were expressed in MSCs derived from subcutatneous and visceral adipose tissue in human (Potdar and Sutar 2010), sheep bone marrow and adipose tissue (Heidari et al. 2013), canine umbilical cord (Uranio et al. 2011), bovine umbilical cord blood (Raoufi et al. 2011), buffalo amniotic fluid (Dev et al. 2012), buffalo fetal skin derived fibroblast cells (Yadav et al. 2012) and fetal adnexa derived caprine MSCs (Somal et al. 2016). Our findings that oMSC are $OCT4^+$ and $SOX2^+$ are in agreement with those of Pratheesh et al (2013) and Choi et al (2013) who conducted the experiments in ovine and canine, respectively.

The MSCs in culture showed a typical growth curve with initial lag phase, an exponential phase, a plateau phase and a decline phase which determines the general behavior of cellular growth (Colter et al. 2001). The oAF-MSC and oAS-MSC exhibited the longest and the shortest exponential phase, respectively. Whereas, caprine Wharton's jelly derived stem cells show faster growth rate as compared to cCB-MSC, cAF-MSC and cAS-MSC (Somal et al. 2016), our study is the first of its kind to show that oAF-MSC have the highest proliferation rate and hence the least population doubling time (PDT) out of the four sources. Our study is agreement with the one conducted by Corradetti et al (2013) which showed a greater proliferation potential of cattle amniotic fluid MSC as compared to amniotic membrane upto P10. Later on, the vitality of both the cell lines diminishes. Our findings are similar to those of Filioli Uranio et al (2014) wherein AF- MSCs showed faster growth rate than the other fetal adnexa MSCs in dog. But, other

findings suggest that our results are contrary to those of Karahuseyinoglu et al (2007) and Iacono et al (2012) who conducted the study on humans and equines, respectively. Thus, the proliferation potential of MSCs reveals a species-specific growth rate. Our study showed a better growth rate of MSCs at P5 than P3 as against the findings of Rizal et al (2019) in human's, wherein the proliferation rate of hWJ-MSCs was better at lower passage (P6) than at higher passage (P12).

The CFU assay at P3 revealed the highest clonogenic potential of oAF-MSC followed by oCB-MSC, oWJ-MSC and oAS-MSC. Previous studies by Somal et al (2016) revealed that the caprine Wharton's Jelly had the highest clonogenic potential and caprine Amniotic sac the least as against our findings. Similarly, Pratheesh et al (2013) revealed an excellent clonogenic potential of cAF-MSCs. The CFU indicates the proliferation potential of the MSCs. As the number of cells increase, the colonies become irregular with few gaps between them (Orozco-Fuentes et al. 2019). Along the path of protracted passaging, their colony-forming unit (CFU) potential declines (Lu et al. 2014).

Conclusions

The current study reveals ovine fetal adnexa as an abundant source of Mesenchymal Stem Cells expressing characteristic stem cell and pluripotency markers. The study is the first of its kind which reveals that oAF-MSCs in sheep outperformed the rest of the sources vis-à-vis growth kinetics and clonogenic potential.

Declarations

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Authorship Contribution

AAS: Investigation, Data compilation; **AAS:** Investigation, Data curation, Writing - Original draft; **DH, FS and OA:** Supervision; **RAS and SBA:** Methodology; **SM:** Data analysis; **ZAP:** Supervision, Conceptualization, Funding acquisition.

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Data Availability

The manuscript covers all the data of the experiment.

Conflict of Interest/Competing Interests

The authors don't have competing interests with any person or organization.

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Plates

Plates 1 to 6 are available in the Supplementary Files section

Figures

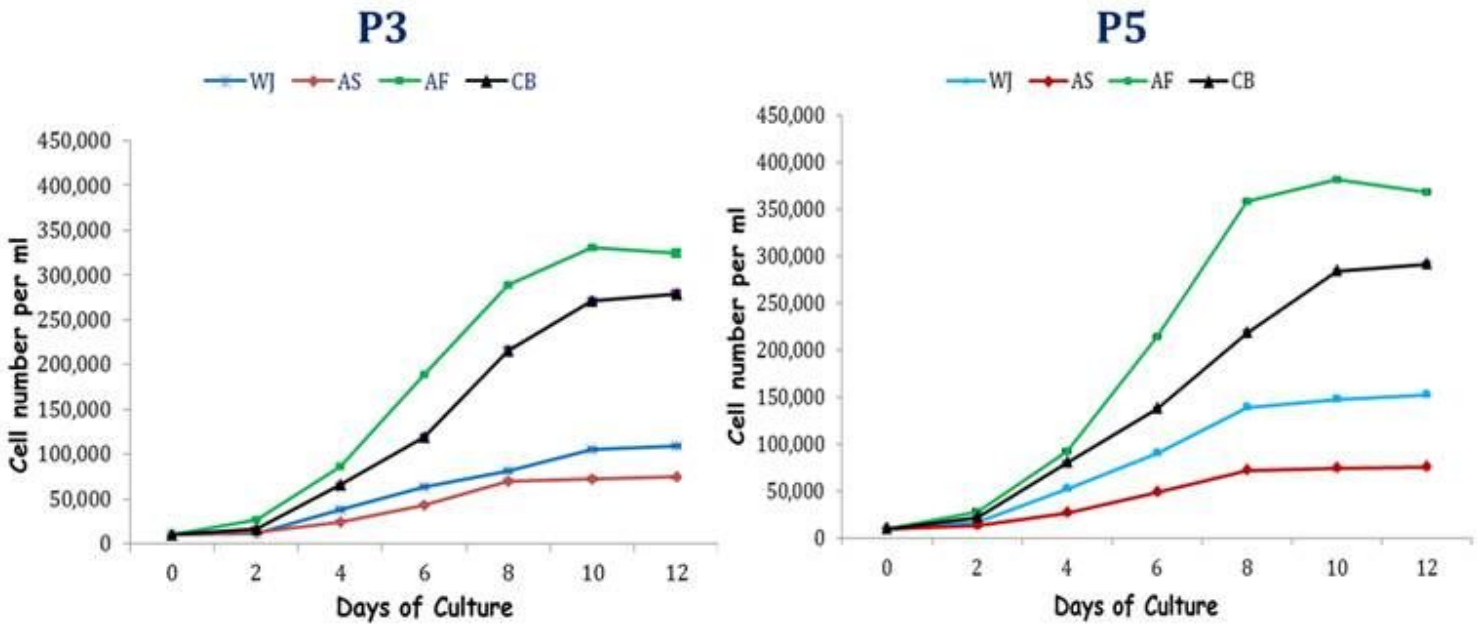

Figure 1

Growth Kinetics of Ovine fetal adnexa derived MSCs at two passages (P3 & P5) is time dependent. The cells (oWJ, oAS, oAF & oCB) increase in number with the passage of time. The growth curve shows well defined initial lag phase followed by an exponential phase, a plateau phase and a phase of declined growth.

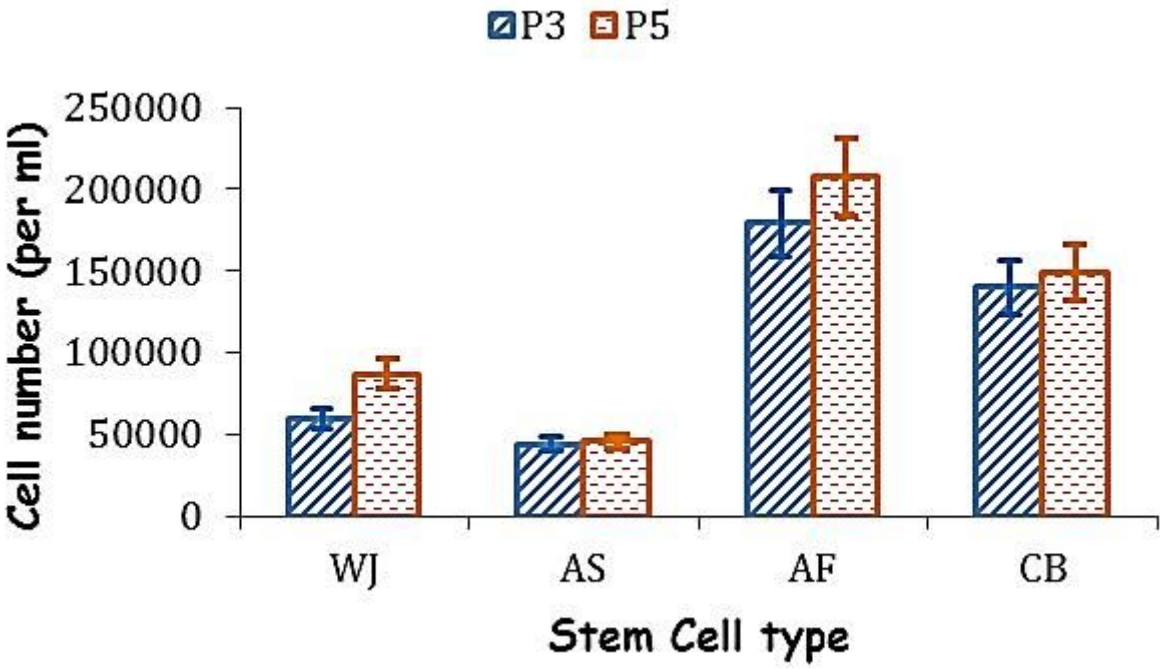

Figure 2

Growth Kinetics of Ovine fetal adnexa depends upon cell type and the passage number. The figure depicts highest cellular proliferation in oAF and least in oAS. The growth rate was more at P5 than P3.

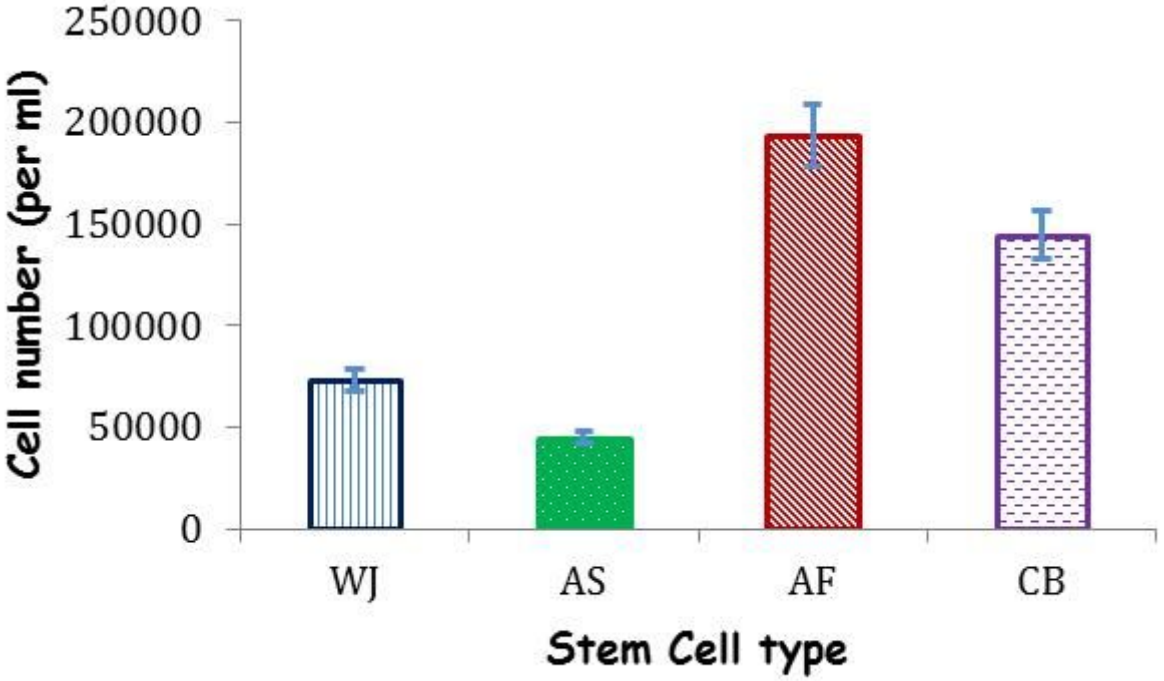

Figure 3

The figure represents an overall growth kinetics of MSC (independent of time). The MSC isolated from oAF showed highest growth rate (193515 ± 15604.58 cells/well) as compared to the rest of the sources.

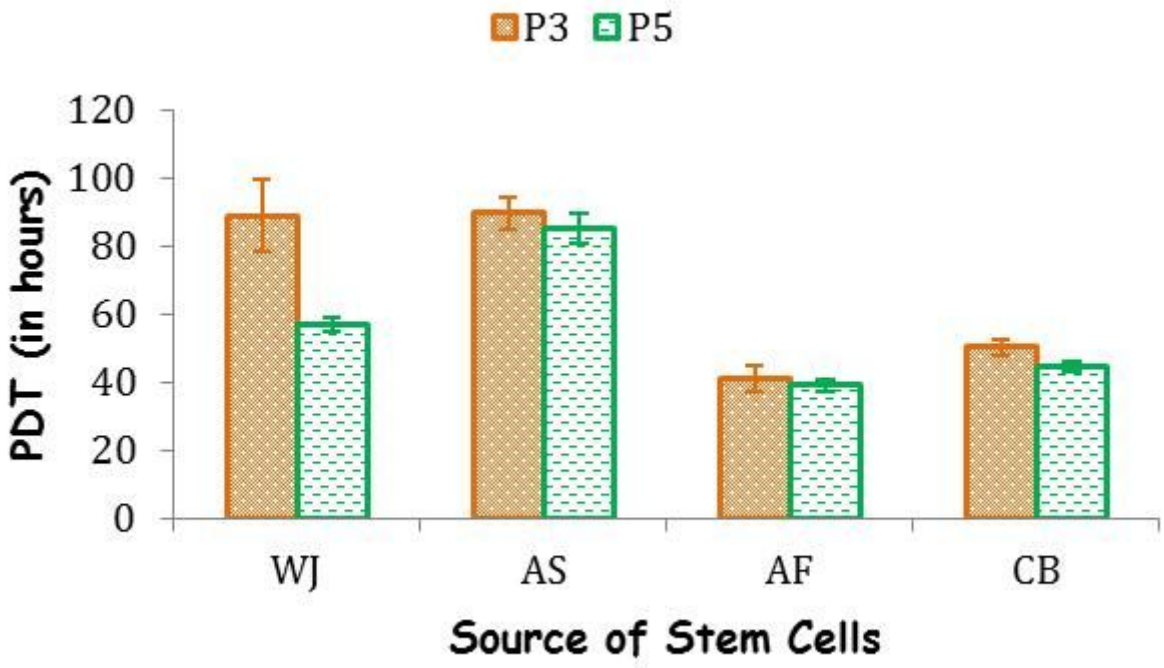

Figure 4

Population doubling time (in hrs) varied with the MSC source and the passage number. The oAF-MSC showed highest proliferation and hence time less time to double their population i.e. the PDT of oAF is less than the rest of the sources. The PDT was less at P5 as compared to P3.

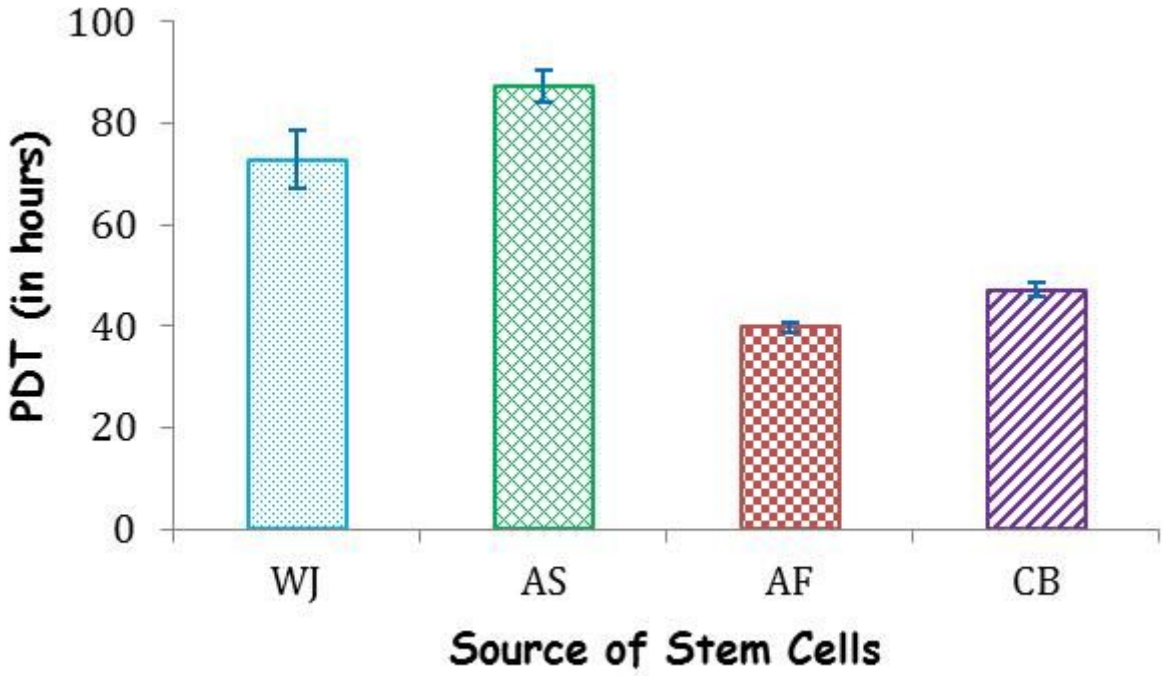

Figure 5

Population doubling time (in hrs) of Ovine fetal adnexa derived MSCs independent of passage number.

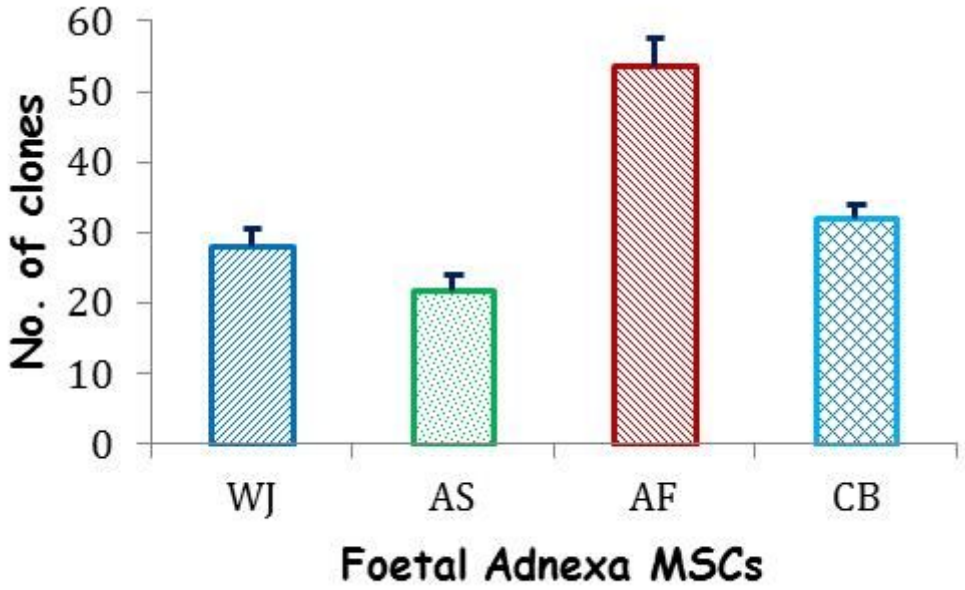

Figure 6

Colony Forming Unit (CFU) assay of Ovine fetal adnexa derived MSC. The oAF-MSC formed significantly more colonies followed by oCB-MSC, oWJ-MSC and oAS-MSC. The CFU portrays a viable cell count in a colony and is an indicator of healing potential of a particular stem cell type.

Supplementary Files

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