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Impact of cell seeding density and serum starvation on VEGF expression in canine adipose tissue derived mesenchymal stromal cells

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Abstract
Optimization of cell culture protocols in terms of serum percentage as well as seeding density can affect the angiogenic potential of mesenchymal stromal cells. Present study demonstrates the impact of seeding density and serum starvation on the expression of *VEGF* gene in canine adipose tissue derived mesenchymal stromal cells (cAD-MSCs). Canine adipose tissue derived mesenchymal stromal cells were successfully isolated and expanded *in vitro* demonstrating typical sigmoid pattern of growth curve. The population doubling time of *in vitro* expanded cAD-MSCs was found to be 40.04 hrs. and around 40-45% of initial MSCs were observed to show clonogenicity in colony forming unit assay. We demonstrated that higher seeding density i.e. $6x10^4/cm^2$ showed significantly ($P = 0.005$; N=4) better expression (2.495±0.35fold) of *VEGF* in cAD-MSCs compared to the initial seeding of $1x10^4/cm^2$ (1.000±0.00) and $3x10^4/cm^2$ (1.393±0.30) cells in the *in vitro* cAD-MSCs culture. Further, we could not find any statistically significant change in the relative expression of *VEGF* in cAD-MSCs while serum depriving the culture for 12hrs (1.11±0.43), 24hrs (1.64±0.53) and 36hrs (0.83±0.58) compared to serum rich media (1.000±0.00). Further research is required to optimize the cell culture protocols thereby creating rapid expansion of MSCs without compromising the cell characteristics and making cell-based therapies more cost effective.

Keywords: canine adipose tissue, mesenchymal stromal cells, vascular endothelial growth factor, serum starvation, cell seeding density

1. Introduction
Adipose tissue derived mesenchymal stromal cells (AD-MSCs), categorized under multipotent stem/stromal cells which are isolated from fat tissues enzymatically are considered to be most commonly used in the clinical applications in recent years. Adipose tissue has the advantage in the harvesting, isolation, and expansion of stromal cells, especially an abundant amount of stromal cells compared to the bone marrow (Chu *et al.*, 2019) [6]. *In vitro*, AD-MSCs can be differentiated into osteoblasts, chondroblasts, adipocytes, myocytes, and cardiomyocytes in suitable conditions (Rogne *et al.*, 2018; Chu and Tao, 2017; Chu *et al.*, 2016) [30, 7, 8]. The cell yield in adipose tissue is reportedly 100–500 folds higher than that of bone marrow, which makes it an attractive cell source for human and veterinary therapeutics (Chu *et al.*, 2019) [6].
It is widely accepted that paracrine factors and cytokines from adipose tissue-derived stem cells (ASCs) can promote repair of injured tissue and/or improve the quality of tissues that are regenerated (Yoshida *et al.*, 2016) [38]. Vascular endothelial growth factor (VEGF), originally known as vascular permeability factor is such a paracrine factor being produced by cells including adipose tissue-derived stem cells that stimulates the formation of blood vessels. *VEGF's* normal function is to create new blood vessels during embryonic development, new blood vessels after injury, muscle following exercise, and new vessels (collateral circulation) to bypass blocked vessels (Senger *et al.*, 1983) [33]. It can contribute to even in disease/ cancer metastasis.
Factors affecting optimization of MSCs for therapy can be grouped into donor factors such as age and gender, as well as cell culturing factors such as seeding density, serum concentration, growth factors used etc. The details such as the cell passage number, confluence status, attachment factors, and stimulation times had some influence in gene expression but were not as critical as cell density and serum starvation. Serum deprivation believed to promote *VEGF* expression by the cells and which in turn promote angiogenesis (Page *et al.*, 2014) [28].

Tratwal *et al.* (2015) [36] demonstrated that expressions of more than 190 genes were altered in AD-MSCs by serum deprivation compared to those grown in serum rich media. Different studies have provided compelling evidence that *VEGFR-2* signaling is affected by environmental conditions, in particular by cell density (Dejana, 2004) [10] which is known to discriminate and characterize different functional aspects of the vasculature. Indeed, it was previously reported that confluent cells in comparison to sparse cells show a reduction in proliferative responsiveness to *VEGF* (Lampugnani *et al.*, 2003) [21]. McBeath *et al.* (2004) [25] demonstrated that high seeding density led MSCs toward adipogenesis, whereas low seeding density promoted osteogenesis demonstrating the influence of seeding density in deciding the signaling pathway for their ultimate fate.

Very few reports have been so far published regarding the impact of serum starvation and cell seeding density on the expression of *VEGF* by the mesenchymal stromal cells which in turn may have a noteworthy influence on their therapeutically potential. Hence, the present study was designed to study the impact of serum deprivation and cell seeding density on the *VEGF* mRNA expression level *in vitro* expanded canine adipose tissue derived mesenchymal stromal cells.

2. Materials and Methods

2.1 Collection of canine adipose tissue

The adipose tissue of mature dog was collected as medical waste. Approximately 5gm of subcutaneous adipose tissue from canine patient going under regular surgical procedure was received from the Teaching Veterinary clinical complex, College of Veterinary and Animal sciences, Pookode and transported in 1X Phosphate Buffered Saline (PBS) with 1% antibiotic-antimycotic solution (HiMedia Laboratories Pvt. Ltd., India).

2.2 Isolation, *in vitro* culture and expansion of cAD-MSCs.

For the isolation of canine adipose tissue derived mesenchymal stromal cells (cAD-MSCs), tissue was processed as per the procedure done by previously descripted methods (Ayala-Cuellar *et al.*, 2019). To briefly summarize, tissue pieces were minced with surgical scissors and transferred to a 15ml tube (Tarsons Products Pvt. Ltd. India). Minced tissue was washed thoroughly with 1X PBS and debris formed underneath was removed with a pipette. After removal of debris, 1ml of 0.2% Collagenase Type III (*C. histolyticum*) (Abnova; Antibody Innovation, Taiwan) solution per gm of tissue was added for tissue digestion. The mixture was incubated at 37 °C, 5% CO_2 in tubes loosely capped, for 3hrs while shaking intermittently in every 30 min. To stop enzyme activity, the complete media containing 15% fetal bovine serum (HiMedia Laboratories Pvt. Ltd., India) in Dulbecco's Modified Eagle's Media (HiMedia Laboratories Pvt. Ltd., India) was added. The mixture was pipetted up and down for further adipose tissue disintegration. Lysed tissue sample solution was filtered using 40μm cell strainer (HiMedia Laboratories Pvt. Ltd., India) to eliminate undigested tissue clumps. Digested and filtered cell suspension was centrifuged at 2,000 rpm for 5 min. The supernatant was removed through aspiration without disturbing the pellet. The cells were washed again with 1X PBS and lastly centrifuged at the same condition previously mentioned. After discarding the supernatant, the pellet was resuspended in 1ml of complete media containing Dulbecco's modified Eagle's media (DMEM) fortified with 15% FBS 200mM L-Glutamine (HiMedia Laboratories Pvt. Ltd., India) and 1% antibiotic-antimycotic solution (HiMedia Laboratories Pvt. Ltd., India) and seeded in same. The cells were seeded initially in 24 well cell culture plates incubated in in a humidified atmosphere with 5% CO_2 at 37 °C. The media were changed 24hrs later and all non-adherent cells were removed. Thereafter, the medium was replaced every 3-4days. Confluent, adherent cells were designated P0. At 80–90% confluence, the cells were detached with Accutase (HiMedia Laboratories Pvt. Ltd., India) and passaged repeatedly. Passaging was performed in T25 cell culture flask (Tarsons Products Pvt. Ltd., India)using complete media fortified with 10% FBS.

2.3 Growth Kinetics

Canine adipose tissue derived mesenchymal stromal cells (cAD-MSCs) from the third passage *in vitro* culture once attained 80-90% confluence were harvested and resuspended in complete media. Representative samples were counted using 0.4% trypan blue (HiMedia Laboratories Pvt. Ltd., India) and Neubauer chamber (Rohem Instruments Pvt. Ltd., India) and diluted at the rate of $1X10^4$ cells/ml and seeded in 24-well plates. At every 48hrs, cells from three culture wells were collected by Accutase® treatment and cell number was counted excluding the dead cells by 0.4% trypan blue staining using Neubauer counting chamber from day 1 to 14. All the experiments were performed in triplicate at each time point to draw cell growth curve. Semilog curve of the increase in cell concentration was plotted and population doubling time (PDT) was calculated using the following equation: PDT = t log2/log(Nt/N0), where t = culture period in hours, N0 = initial cell number and Nt = cell number at culture period (Pratheesh *et al.*, 2013) [29].

2.4 Colony forming unit assay

For measuring colony forming units, third passage cAD-MSCs (~100 cells) were plated in three 100x20 mm cell culture petri dishes (Sigma-Aldrich Chemicals Pvt. Ltd., India) for a period of 14 days and incubated at 37 °C in a humidified atmosphere having 5% CO_2. At the end of incubation period, cells were washed twice with 1X PBS followed by staining with 1% crystal violet (Sigma-Aldrich Chemicals Pvt. Ltd., India) for 15 min in each dish and counted under Carl Zeiss microscopy GmbH (Germany). The colonies consisting of around 15-20 nucleated cells were counted and recorded.

2.5 Experimental design to investigate the impact of serum starvation and cell seeding density on *VEGF* expression in cAD-MSCs.

Forth passage canine adipose derived mesenchymal stromal cells were utilized for the experiment. A total of 12 treatment groups having a combination of four serum starvation periods (0hr, 12hrs, 24hrs & 36hrs) and three different cell seeding density ($1x10^4/cm^2$, $3x10^4/cm^2$ & $6x10^4/cm^2$) have prepared in triplicates (X, Y & Z).

Cells were seeded in six well cell culture plates (Cole-Parmer, India) at respective concentration by 0.4% trypan blue and using Neubauer chamber technique and grown for 72hrs (pre-incubation) in normal serum rich (10% FBS) complete media. After pre-incubation, the normal serum rich media replaced with either serum free media (- ; 0% FBS) or serum rich media (+ ; 10% FBS) in every 12hrs interval for a total period

36hrs as given in the Table-1. The procedure was repeated in triplicates (X, Y & Z). At the end of incubation period 36hrs adherent cells were harvested from each well and isolated Total RNA.

2.6 Real time PCR

The total RNA was isolated from canine adipose tissue derived mesenchymal stromal cells after treatment using commercial Total RNA Isolation Kit (Catalogue No.ODP419, M/S Origin labs, India) as per the manufacture's instructions. A total of 0.1µg messenger RNA (mRNA) was quantified with a Nanodrop™ spectrophotometer 2000C (M/s Thermo scientific, USA) and reverse transcribed into complementary DNA (cDNA) using verso cDNA synthesis kit (AB-1453/A, M/s Thermo Scientific, USA) as per manufacturer's protocol. Briefly, cDNA was synthesized in a total of 20µl reaction volumeand was reverse-transcribed using Verso RT enzyme by incubating at 42^0C for 30 minutes followed by enzyme inactivation at 95^0C for 2 minutes. The purity of RNA and the quality of cDNA was assessed by putting an amplification reaction for a housekeeping gene canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplified product was resolved on 2.0% agarose gel (HiMedia Laboratories Pvt. Ltd., India). Then reverse transcription reaction product was stored at -80^0C for future use.

The relative expression of mRNA was determined by qPCR using SYBR Green Technology (Maxima SYBR green/ROX qPCR Master Mix (2X)-Thermo scientific, USA) and canine-specific primers for *VEGF* (Sense:5'GCAACTCCGGCAGAAGCAT 3'; Antisense:5'TGGCGATCCAATTCCAAGAG 3') and *GAPDH* (Sense: 5'GATGGGCGTGAACCATGAG 3'; Antisense:5'TCATGAGGCCCTCCACGAT 3'). A 65bp fragment of the *VEGF* and 131bp fragment of *GAPDH* were chosen as target and housekeeping gene respectively for comparing the *VEGF* level in canine adipose derived MSCs in various treatment groups. Cycling condition followed in qPCR was: 1 cycle, 9 °C for 5 min; 40 cycles, 94 °C for 30s; 56 °C for 30s; 72 °C for 30s; 1 cycle, 64 °C for 5; 1 cycle. A final melting curve profile at 60 °C for 30s was set for fluorescence acquisition and reaction specificity. The qPCR products were separated by electrophoresis on 2.5% agarose gel, stained with 1% ethidium bromide and visualized under UV light. Digital images were captured in gel documentation system GBOX F3 (Syngene, U.K.). Values were normalized to *GAPDH* mRNA levels. Relative gene expression was quantified with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) [23].

2.7 Statistical analysis

One-way ANOVA was carried out for comparing between groups. Pairwise comparison was done using Duncan Multiple Range Test.

3. Results

After enzymatic digestion from the canine adipose tissue, round transparent single cell suspension of adipose cells (Figure **1**a) were isolated and were seeded in 24 well cell culture plates. After 24hrs small adherent cells were noticed and the rest of floating cells were washed with PBS. After 72hrs, fibroblastic adherent cells were observed (Figure **1**b), thus obtaining cAD-MSCs. Primary culture of cAD-MSCs obtained 80-85% confluency by 12-13 days post seeding (Figure **1**c). Calibrated growth curve plotted using culture period against cell concentration for the third passage canine adipose mesenchymal stromal cells followed typical sigmoid curve (Figure 1d). Calculations showed that the population doubling time of canine adipose mesenchymal stromal cells is 40.04hrs based on the exponential growth phase. On colony forming units assay, the mean rate of colony formation of canine adipose derived mesenchymal stromal cells was found to be 40-45% of the starting MSCs population (Figure 1e). Therefore cAD-MSCs showed high clonogenicity thereby indicating the existence of high cell subpopulation with self-renewing capacity *in vitro.*

In this study, the impact of seeding density and serum starvation on the expression of *VEGF* in canine adipose derived mesenchymal stromal cells has studied by SYBR green based real time PCR. Grossly we did not observe any difference in adherent cell density and morphology after 72hrs pre-incubation period and 36hrs experimental incubation time especially for those treated with serum free(-) medium for different periods (supplementary data). In addition to melting curve analysis, to confirm the specificity of the product we have amplified in real time PCR, the Real time PCR products we obtained in all the three replicated experiments were run on 2.5% agarose gel and we found the single specific bands for our PCR products of *VEGF* (65bp) and *GAPDH* (131bp) in parallel to the respective standard marker bands (supplementary data).

In order to find the impact of seeding density on the expression of *VEGF* in cAD-MSCs, serum starvation period has kept constant and the average of ΔCt value obtained for $1x10^4/cm^2$ seeding density was taken as control while ΔΔCt computation. We observed that there is a linear relationship between the initial cell seeding density and *VEGF* expression by cAD-MSC in *in vitro* culture. Further demonstrated that irrespective of the serum starvation period, higher seeding density i.e. $6x10^4/cm^2$ (2.495±0.35) showed significantly (P = 0.005; N=4) better expression of *VEGF* in cAD-MSCs compared to the initial seeding of $1x10^4/cm^2$ (1.000±0.00) and $3x10^4/cm^2$ (1.393±0.30) cells in the *in vitro* expanded cAD-MSCs culture (Figure 2a).

Further we analyzed the impact of serum starvation on *VEGF* expression by keeping the cell seeding density as constant factor and 0hr serum starvation period in each group as control for calculating the ΔΔCt value. Based on the mean value obtained, there is a non-significant (P =0.384; N=3) increase in *VEGF* expression after serum starving for 24hrs (1.64±0.53) than 0hr (1.000±0.00) and 12hrs (1.11±0.43) serum starvation and there was a decline in expression when we continue to serum starve the cells for 36hrs (0.83±0.58). But, we did not find any statistically significance (P =0.384; N=3) in this change in the relative expression of *VEGF* while increasing the serum starvation period (Figure 2b).

Table 1: Pre-incubation period and Serum starvation period assigned for each treatment groups at different time point

Serum starvation period	Seeding density	Pre-incubation 72 hrs.	Experimental incubation after preincubation			
			0h	12hrs.	24hrs.	36hrs.
0hr. (Serum rich)	1 x 10^4/cm^2 3 x 10^4/cm^2 6 x 10^4/cm^2	+	+	+	+	Harvested the cells and isolated Total RNA
12 hrs.	1 x 10^4/cm^2 3 x 10^4/cm^2 6 x 10^4/cm^2	+	+	+	_	
24 hrs.	1 x 10^4/cm^2 3 x 10^4/cm^2 6 x 10^4/cm^2	+	+	_	_	
36hrs.	1 x 10^4/cm^2 3 x 10^4/cm^2 6 x 10^4/cm^2	+	_	_	_	
+ Using serum rich media (10% FBS) - Using Serum free media (0% FBS)						

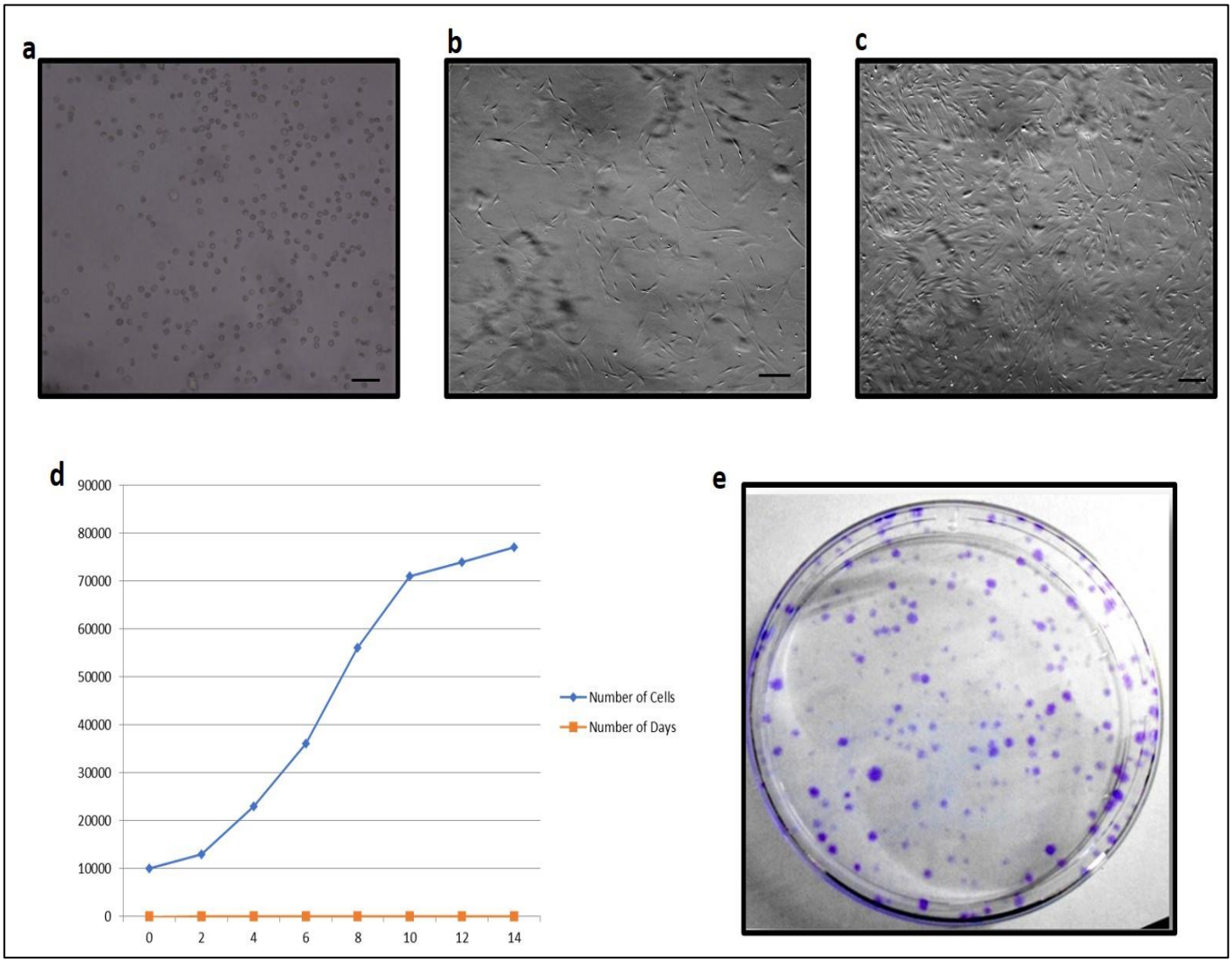

Fig 1: Growth characteristics and growth kinetics of canine adipose tissue derived mesenchymal stromal cells. Isolated single round transparent floating canine adipose tissue derived stromal cells on the day of seeding (20x) (Figure **1**a). Fibroblastic adherent cells were observed in the primary culture After 72hrs of cell seeding (20x) (Figure **1**b). Primary culture of cAD-MSCs obtained 80-85% confluency by 12-13 days post seeding (20x) (Figure **1**c). Calibrated growth curve plotted using culture period against cell concentration for the third passage canine adipose mesenchymal stromal cells followed typical sigmoid curve (Figure **1**d). On colony forming units assay, the mean rate of colony formation of canine adipose derived mesenchymal stromal cells was found to be 40-45% of the starting MSCs population. Photograph was taken after staining with 1% Crystal violet (Figure **1**e).

Fig 2: Effect of cell seeding density and serum starvation on *VEGF* expression in cAD-MSCs. Fold change expression of *VEGF* in canine adipose tissue MSCs at different seeding densities showing a significant (P<0.05) increase in *VEGF* level at higher seeding density of $6x10^4/cm^2$ compared to lower seeding densities (Figure 2a). Fold change expression of *VEGF* in canine adipose tissue MSCs after exposing to various serum starvation period showing a non- significant (P>0.05) increase in *VEGF* level at 24 hrs. (Figure 2b)

4. Discussion

Mesenchymal stromal cells (MSCs) have shown great capabilities for use in clinical application; however, as they are found in very low numbers in adult tissue, expansion *in vitro* is required to reach the desired numbers before their use in clinical application. We have yet to develop a clear understanding of how to optimize MSC expansion efficiently without compromising on their characteristics and therapeutic potential. Factors affecting optimization of MSCs can be grouped into donor factors such as age and gender, as well as cell culturing factors such as serum percentage and seeding density used. The amount of serum present in the culture media affects stem cell behavior. MSC are generally cultured in 10–20% serum which contains numerous factors that may not be present in the tissues where these cells reside. In the present study we looked at how the cell seeding density and serum starvation have impact on the expression of vascular endothelial growth factor (VEGF) in *in vitro* expanded canine adipose tissue derived mesenchymal stromal cells(cAD-MSC) which in turn can possibly increase their therapeutic potential in veterinary regenerative medicine.

Going through the aforesaid hypothesis we isolated mesenchymal stromal cells from canine adipose tissue and *in vitro* cultured and expanded using specific cell media. We followed the enzymatic digestion method using collagenase enzyme for the isolation and used 15% FBS in primary culture and 10% FBS for further passages. Similar protocols for isolation and culture of canine adipose derived stromal cells were described by (Vieria *et al.*, 2010) [37] where they used 0.075% collagenase type I for 15 min (Fischer *et al.*, 2009) [12] isolated the adipose cells using 4mg/g Collagenase type I for 1hr and (Ryu *et al.*, 2009) [31] used 0.2% collagenase type I and incubated for 2hrs for the single cell separation. In our study, 13-14th day post seeding cell growth continued and attained 70-75% confluency. Similarly, has been reported by (Beaulah *et al.*, 2017) [5] and (Adolfsson *et al.*, 2020) [2] where they found cells are plastic adherent 24 hrs post seeding and 70-80% confluency was attained on day 14 by using 10% FBS.

In the current study, the population doubling time of canine adipose mesenchymal stromal cells was found to be 40.04hrs based on the exponential growth phase. Our results are comparable with reports from (Taguchi *et al.*, 2019) [35] who demonstrated that the doubling time of adipose tissue cells isolated from young (38.3 ± 1.3hrs) and old dogs (42.9 ± 3.3hrd, p =0.6) did not differ at first passage but in later passages doubling time of cells from young dogs were shorter than the old dogs. In mice, the population doubling time value for ADSCs (39.88±4.4hrs) appeared to be significantly lower than that for the bone marrow derived MSCs (49.9±4.2hrs) indicating the more rapid rate of proliferation of ADSCs (Lotfy *et al.*, 2014) [24]. The population doubling time for canine ASCs was found to be shorted (48 ± 4hrs) when cultured in FBS than those expanded in commercial serum-

free media (68 ± 17hrs) like Ultra Culture medium (Schwarz *et al*, 2012) [32].

In the present study, individual colonies were generated, and the mean rate of colony formation of canine adipose derived mesenchymal stromal cells was found to be 40-45% of the starting MSC population. Present study clearly shows that the canine subcutaneous fat-derived cells have the ability for proliferation and the clonogenicity. In a recent study comparing the effect of subcutaneous and visceral fat-derived ASCs on cardiac infarction, the authors revealed a higher proliferation rate and CFU ability of the subcutaneous tissue derived cells compared to visceral fat-derived cells (Arnhold *et al.*, 2019; Devireddy *et al.*, 2019) [3, 11]. Likely the doubling time, age also has a negative effect on the number of colony forming units (CFU) in cAD-MSCs (Taguchi *et al.*, 2019) [35]. Moreover Lotfy *et al.* (2014) [24] showed that ADSCs are expanding more rapidly than BMSCs which recommends adipose tissue derived MSCs as a potential agent for veterinary and human regenerative medicine.

4.1 Impact of seeding density on *VEGF* expression by cAD-MSCs

Finding the optimum seeding density for maximal cell expansion is useful in both laboratory investigations as well as potential clinical applications as the cell culturing procedure can be less time consuming, decreasing the risk of cell culture contamination, infection or loss of characteristics in cell culture, in addition to making the process more cost effective. Kim *et al.* (2014) [18] reported that proliferation-related genes were highly expressed in all MSCs harvested at low density, whereas genes associated with the cell's therapeutic functions were highly expressed in all MSCs harvested at high density. In general, MSCs at a lower density have a faster proliferation rate than those seeded at a higher density. Higher growth potential at lower seeding densities may be due to more availability of nutrients per cell (Fossett *et al.*, 2012) [14]. Following confluence, cells enter a quiescent growth phase that is associated with alterations in expression of cell surface receptors, transcription factors, cytochemical enzymes, oncogenes, and growth factors (Singh *et al.*, 1996) [34].

We hypothesize that increased seeding density triggers certain signaling mechanisms that may up-regulate expression of *VEGF* in cAD-MSC. Like the results obtained by the (Abdalla *et al.*, 2015) [1] in human pancreas cancer cell line (PC-3M), grossly we did not observe any significant difference in the growth rate and phenotype of cAD-MSCs seeded at high and low seeding density. This study clearly demonstrates that alteration of seeding density results in significant changes in the mRNA expression and secretion of paracrine factors specifically vascular endothelial growth factor *VEGF* by cAD-MSC. Average of ΔCt values obtained for $1x10^4$ cells/cm^2 seeding density was taken as control and we demonstrated that irrespective of the serum starvation period, higher seeding density i.e. $6x10^4/cm^2$ showed better expression of *VEGF* in cAD-MSCs compared to the initial seeding of $1x10^4/cm^2$ and $3x10^4/cm^2$ cells in the *in vitro* expanded cAD-MSCs culture. Corroborating with our results, northern blot analysis by (Liu and Ellis, 1998) [22] demonstrated that *VEGF* mRNA expression in confluent human umbilical vein endothelial cells was more than two fold greater than in sparse cells. Later, by combining biologic experiments, theoretical insights, and mathematical modeling, (Napione *et al.*, 2012) [27] found that cell density influences *VEGFR*-2 protein level, as receptor number is 2 fold higher in long-confluent cells than in sparse cells and also the cell density affects *VEGF*R-2 activation by reducing its affinity for *VEGF* in long-confluent cells. Mukhopadhyay *et al.* (1998) revealed that *VEGF* mRNA levels were four to eight folds higher in human glioblastoma cells (U87) and human fibrosarcoma cells (HT1080) seeded at high density compared to cells seeded at low density. Koura *et al.* (1996) [19] reported *VEGF* mRNA increased >2fold in human colon cancer cell lines grown as confluent monolayer or spheroids compared with cells grown as sparse monolayers. Later Kuhn *et al.* (2004) [20] reported that *VEGF* gene expression increased inconfluent slow growing human colon carcinoma cells (H460 cells) compared with sparse fast growing cells. In contrast to what we have obtained in our study, (Abdalla *et al.*, 2015) [1] reported in human prostate cancer cell line (PC-3M), that the *VEGF* levels was increased when seeded at the low density ($1.5x10^5$cells/well).

4.2 Impact of serum starvation on *VEGF* expression by cAD-MSCs

Serum starvation is one of the most frequently performed procedures in molecular biology and there are literally thousands of research papers reporting its use. Serum starvation probably induces a fast and active response, which exhibits apparent qualitative and quantitative differences across different cell types and experimental conditions in spite of certain unifying features. Serum starvation reportedly activates several mitogen-activated protein kinases, but activation of Erk-1/2 is critical for the up-regulation of *VEGF* mRNA in colon carcinoma cells (Jung *et al.*, 1999) [17]. Unlike the reports from (Abdalla *et al.*, 2015) [1] in pancreatic cancer cell line, grossly we could not observe any significant differences in the adherent cell density for those treated with serum free medium for 12hrs, 24hrs and 36hrs fold change in expression level of the *VEGF* was compared when cAD-MSCs were grown in serum-starved conditions at 0h, 12hrs, 24hrs and 36hr. We observed that there is a non-significant ($P>0.05$) increase in *VEGF* expression when the cells were serum-starved for 24hrs but afterwards there is a sharp decline in expression by cAD-MSC when starved for 36hrs. Early in 2007, (Gimble *et al.*, 2007) [15] reported that ASCs increased production of paracrine growth factors in stressful environments like low serum concentration, which leads to reduction in nutrient and growth factors needed by the cells, could potentially cause stressful events in the culture. Later, it was reported that human ASCs cultured in low serum (2%) secreted higher levels of vascular endothelial growth factor (VEGF) than human ASCs cultured in 20% serum (Iwashima *et al.*, 2009) [16]. It was demonstrated that most angiogenic gene's expression including that of vascular endothelial growth factor (VEGF) along with angiopoetin-1 (*Ang-1*) and basic fibroblast growth factor (*bFGF*) were increased significantly when human ASCs at P6 were cultured in medium with reduced serum (2% FBS) (Chua *et al.*, 2013) [9]. Serum-deprived medium significantly increased transcription receptor *VEGF*R-1 compared with ASCs from complete medium (Follin *et al.*, 2013) [13]. Tratwal *et al.* (2015) [36] demonstrated that in comparison to ASCs in complete medium, 190 genes were significantly altered by serum deprivation. In contrast to aforementioned statements, (Abdalla *et al.*, 2015) [1] reported in pancreatic cancer cell line (PC3M) that 24hrs serum starvation has down regulated the expression of *VEGF*. Similarly reports from Page *et al.* (2014)

[28] demonstrated that serum deprivation from 20% to 5% caused significant decrease in the expression for *VEGF* by murine bone marrow derived MSCs. Secretion of *VEGF* protein by cells was also significantly reduced under serum deprived condition as evidenced by specific ELISA technique. They also observed that serum reduction causes a decline in murine bone marrow derived MSCs proliferation probably due to lack of protein and signaling growth factors.

In our study, although there is an increase in the mean value of *VEGF* expression by cAD-MSCs at 24hrs serum starvation confirming its impact on the *VEGF* expression, we could not find any statistical significance in the result obtained. It is unclear why lowering serum did not significantly alter *VEGF* expression in our study while others found it to be elevated, but this could be attributed to high standard error between the experimental replicas. The observed impact of serum-deprivation in our study is consistent with (Jung *et al.*, 1999) [17] who has reported no significant change in *VEGF* mRNA expression in colon carcinoma cells after completing the first 24hrs of serum-free conditions. However, unlike the reports from our study they observed that *VEGF* mRNA increased abruptly more than 4-fold between 24 and 48hrs of growth in serum-free medium.

5. Conclusion

In fact, the present study demonstrated that the MSCs, a population of potent multipotent cell precursors used in regenerative medicine, cultured and expanded in laboratories, can be readily isolated from adipose tissue of the mature dog. Further, we verified that the cell seeding density but not serum starvation has impact on the expression of vascular endothelial growth factor (VEGF) in *in vitro* expanded canine adipose tissue derived mesenchymal stromal cells (cAD-MSC) which in turn can possibly increase their therapeutic potential in human and veterinary regenerative medicine. Collectively we demonstrated that, cell therapy using stromal cells derived from canine subcutaneous adipose tissue, which are regulated by high seeding density, may be a novel therapeutic option to enhance angiogenesis thereby provide therapeutic benefits in pathological vascular conditions including wound repair.

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7. Declarations

7.1 Conflict of interest

The authors declare no competing interests.

7.2 Availability of data and materials

The data and materials are available when the request is reasonable.

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