

Characterization of teratogenic potential and gene expression in canine and feline amniotic membrane-derived stem cells

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Contents

The biosafety of innovative procedures that utilize stem cells in regenerative medicine has been addressed in several studies. Previous work has showed no tumour formation following the use of feline and human amniotic membrane-derived stem cells (AMSCs). In contrast, tumour formation was observed when canine AMSCs were utilized. These findings suggested that feline and human, but not canine, AMSCs are suitable for cell transplantation trials. This study aimed to further evaluate the feasibility of utilizing canine AMSCs for transplantation purposes as well as for felines. We tested teratoma formation following cell injection into BALB/c nude mice and then assessed expression of haematopoietic, mesenchymal, tumorigenic, pluripotency and cellular regulation markers using flow cytometry and qPCR. The use of canine AMSCs did not result in macroscopic tumour formation as determined 60 days after transplantation. The immunophenotypic characterization by flow cytometry revealed expression of mesenchymal markers (CD73 and CD90) and expression of the pluripotent marker OCT4 and SOX2. Quantitative PCR analysis revealed that there were no differences in the patterns of gene expression (CD34, CD73, OCT4, CD30 and P53) between canine and feline AMSCs, with the exception of the expression of SOX2 and CD90.

1 | INTRODUCTION

Mesenchymal stem cells have been characterized from feline, canine, equine and human amniotic membrane tissues, which are reported to be good sources of mesenchymal-like stem cells (Bačenková, Rosocha, Tóthová, Rosocha, & Šarisský, 2011; Park, Seo, Kim, & Kang, 2012; Urânio et al., 2011; Vidane et al., 2014; de Vita et al., 2012). These cells of amniotic membranes have been used for wound and corneal reconstruction in animals and humans with favourable results (Moreira & Oliveira, 2000; de Vita et al., 2012). As amniotic membranes are discarded after birth, mesenchymal-like stem cells can be obtained from this source in convenient and non-invasive fashion (Park et al., 2012; Urânio et al., 2011; de Vita et al., 2012). Canine amniotic membrane

stem cells have high regenerative and proliferative capacities and can differentiate into distinct cell lineages (i.e. adipocyte, bone, neural, cartilage) in vitro (Park et al., 2012; Urânio et al., 2011; Urânio et al., 2014). For these reasons, diverse studies and specific tests are being developed to optimize the use of these cells for clinical applications.

A previous study showed that feline mesenchymal stem cells (MSCs) derived from amniotic membranes (AMSCs) did not form tumours after transplantation into immunosuppressed mice (Vidane et al., 2014). In contrast, canine AMSCs formed embryonic carcinomas 30 days after injection into nude mice (Lima et al., 2009). Despite this observation, additional studies centred on canine AMSCs appear warranted. First, these cells have high proliferative potential and high plasticity. Second, domestic carnivores are generally considered as

important animal models of human diseases owing to their genetic similarities (Urânio et al., 2011).

Most processes involving cell differentiation are subject to potential errors that may result in somatic and/or germ line mutations, functional disorders and tumour formation (Bapat, 2007). A key feature that affects the utility and safety of cell-based therapies is the extent to which these approaches effectively guide cell differentiation and ensure that no tumours are formed (Bapat, 2007). Therefore, it is essential to assess tumour formation before conducting clinical trials using stem cells. Studies in canine experimental models are a step forward towards development of clinical trials in humans. The objective of this study was to characterize the expression of mesenchymal, haematopoietic and pluripotency markers in canine AMSCs. In addition, we sought to evaluate the levels of regulatory and tumorigenic genes, with the goal of ensuring the biological safety of cells used in clinical trials. The results were compared to those obtained using feline AMSCs; all experiments were performed under the same experimental conditions.

2 | MATERIALS AND METHODS

2.1 | Laboratories, material collection and procedures

The study protocol was approved by the Faculty of Animal Science and Food Engineering Research Ethical Committee (13.1.2823.74.2). The canine and feline AMSCs used in this study were isolated from foetal membranes collected after routine ovariohysterectomy in cats and dogs at veterinary clinics in Pirassununga City, SP, Brazil. The embryos were analysed by measuring the crown-rump (CR) length, following previously described methodology (Evans & Sack, 1973; Miglino et al., 2006; Pieri et al., 2015). Pregnant uteri (35–45 days) were collected and foetal membranes were separated and washed using sterile

phosphate-buffered saline (PBS) (Figure 1a,b). The amnion was mechanically separated from the allantoic sac, washed repeatedly with sterile PBS and then minced using a scalpel blade. Subsequently, the minced pieces of amnion were plated in 35-mm culture dishes containing with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin (alpha-MEM; Life Technologies) and incubated at 38.5°C with 5% of CO₂ and 85% relative air humidity. The culture medium was replaced 48 hr after initial plating and, when the cells reached 80% of confluence, they were harvested using 0.25% trypsin (Tryple Express; Invitrogen, Carlsbad, CA, USA). This protocol was based on previous work using canine and feline AMSCs (Lima et al., 2009; Urânio et al., 2011 and Vidane et al., 2014), with some modifications (e.g., no enzymatic cell dissociation, lower concentration of foetal bovine serum (10%)). Cells were subcultured three times (passage three cells, P3) due to the optimal growth and differentiation potential exhibited by these cells (Lima et al., 2009; Urânio et al., 2011; Vidane et al., 2014).

2.2 | Immunophenotypic characterization by flow cytometry

Flow cytometry was used to characterize the expression of mesenchymal (CD73, CD90 and CD105), haematopoietic (CD34 and CD45), tumorigenic (CD30), pluripotency (OCT4, NANOG and SOX2) and regulatory (C-myc) marker. Approximately 1×10^5 P3 canine and feline AMSCs were utilized in these studies. The cells were washed using 1 ml of PBS and FACS (fluorescence-activated cell sorting) buffer containing 0.1% BSA and were then centrifuged at $6000 \times g$ for 8 min. Then, we incubated the cells for 1 hr at room temperature with primary antibody diluted 100-fold in 0.1% BSA (Table 1). The cells then were washed once with 1 ml of FACS buffer to remove excess antibodies and were then incubated for 30 min with secondary antibody diluted 300-fold in 0.1% BSA (Table 2). The cells were washed

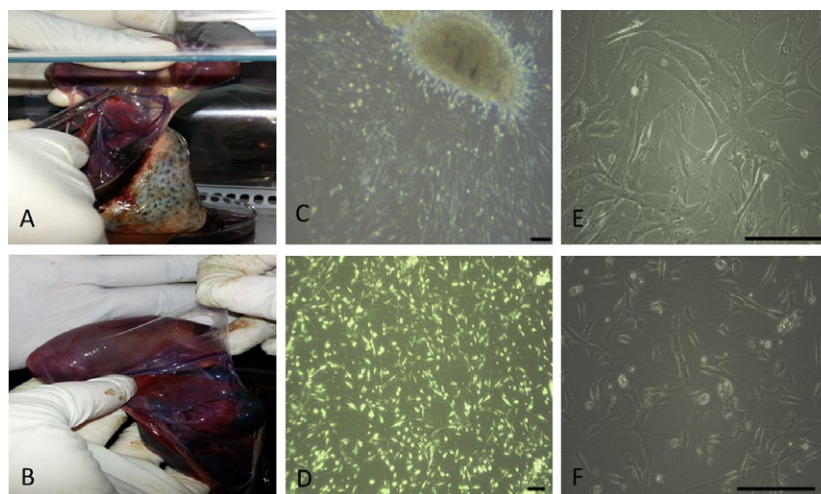


FIGURE 1 Preparation and characteristics of canine and feline amniotic membrane-derived cells. (a) Separation of canine amniotic membranes from other placental membranes; (b) canine amniotic membrane; (c) canine amniotic membrane-derived cells, at passage zero (P0) at 72 hr in culture; (d) cells at passage three (P3) in 72 hr of culture; (e) feline amniotic membrane-derived cells, at passage zero (P0) in 120 hr of culture; (f) cells at passage three (P3), in 72 hr of culture. Note the elongated spindle, fibroblast-like morphology and the ability of the cells to adhere to plastic. These are specific characteristics of mesenchymal stem cells. Bars: 50 µm

TABLE 1 Primary antibodies used in flow cytometry analyses of canine- and feline-derived AMSCs

Antibodies	Isotype	Company	Catalogue number	Species	Monoclonal/polyclonal	Specificity
Endoglin (CD105)	IgG2a	Santa Cruz	sc-71042	Rat	Monoclonal	Mouse
CD34	IgG	Santa Cruz	sc-7045	Goat	Polyclonal	Human/mouse/rat
Thy-1 (CD90)	IgG	Santa Cruz	sc-6071	Goat	Polyclonal	Human/mouse/equine/canine
CD45	IgG2a	Santa Cruz	sc-101839	Mouse	Polyclonal	Cow
CD73	IgG	Santa Cruz	sc-14682	Goat	Polyclonal	Human/mouse
CD30	IgG1	Santa Cruz	sc-46683	Mouse	Monoclonal	Human/mouse
Oct-4	IgG	Abcam	Ab18976	Rabbit	Polyclonal	Mouse, rat, human
Cmyc	IgG	Santa Cruz	sc-788	Rabbit	Polyclonal	Human
Nanog	IgG	Abcam	Ab80892	Rabbit	Polyclonal	Mouse, monkey, human,
Sox-2	IgG	Abcam	Ab97959	Rabbit	Polyclonal	Sheep, horse, cow, pig

Antibodies	Company	Catalogue number	Isotype	Species	Monoclonal/polyclonal	Specificity
Goat anti-rabbit IgG (FITC)	Abcam	ab6717	IgG	Goat	Polyclonal	Rabbit
Goat anti-Mouse IgG2b (FITC)	Abcam	ab97249	IgG2b	Goat	Polyclonal	Mouse
Alexa Fluor 488 (FITC)	Invitrogen	A21210	IgG	Rabbit	Polyclonal	Rat
Alexa Fluor 488 (FITC)	Invitrogen	A11078	IgG	Rabbit	Polyclonal	Goat

TABLE 2 Secondary antibodies used in flow cytometry analyses of canine- and feline-derived AMSCs

again with FACS buffer and were then fixed using 2% buffered paraformaldehyde. Samples were analysed using a flow cytometer (BD FACSAria). Cell populations were estimated by evaluating the percentage of cells expressing each marker relative to the total number of cells. Negative controls consisted of incubations in which the primary antibody was replaced with PBS for each secondary antibody (rabbit, rat, goat, mouse) utilized.

2.3 | Real-time polymerase chain reaction (qPCR)

RNA was isolated from each AMSC sample in triplicate. Total RNA was isolated from purified cellular samples using a DNA/RNA Mini Kit (QIAGEN-80204) according to the manufacturer's instructions. RNA concentrations were determined using UV spectrophotometry at 260 nm; the cDNA High-Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) was used for cDNA synthesis using random hexamers for total RNA conversion into cDNA. Gene expression was assessed by quantitative PCR (StepOne Real-Time PCR Systems; Life Technologies, Carlsbad, CA, USA). The reactions were performed using a commercial assay system (SYBR® Green PCR Master Mix; Life Technologies); CD90, CD73, OCT4, SOX2, P53, CD30 and CD34 were the target genes. The levels of 18S RNA were used for normalization purposes. The sequences of the primers used are shown in Table 3. A total of 40 cycles of amplification were conducted, using an annealing temperature of 60°C. The levels of CD90, CD73, OCT4, SOX2, P53, CD30 and CD34 were normalized to 18S

RNA levels using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001, Tables 3 and 4).

2.4 | Teratoma formation assays in nude mice

A total of 1×10^6 P3 canine and feline AMSCs were resuspended in PBS (Invitrogen) and injected via intramuscular, subcutaneous and intraperitoneal routes into four immunodeficient (BALB/c nude) mice (two mice received canine AMSCs and two mice received feline AMSCs). Teratoma formation was evaluated every week for a total of 60 days following injection. At this point, the animals were euthanized and samples of organs (i.e. muscle, lung, heart, liver, intestine, spleen, pancreas and kidney) were collected and fixed in 4% paraformaldehyde for subsequent histopathological analyses. After 72 hr, the samples were dehydrated in increasing concentrations of alcohol and were then embedded in paraffin. Histological slices (5-µm-thick sections) were cut using a microtome (LEICA 2165) and were then stained with haematoxylin and eosin (H&E).

2.5 | Statistical analysis

For the qPCR analysis, results were analysed first using analyses of variance (PROC ANOVA), and to compare the means, a Student's *t* test was used. The level of significance was taken as 5% ($p < .05$) for all experiments.

TABLE 3 Primer sets used for qRT-PCR of canine-derived AMSCs

Gene symbol	Gene name	Accession no.	Primer	Sequence (5'–3')
CD34	<i>Hematopoietic progenitor antigen</i>	NM_001003341.1	Forward	CCAAGTACCATCAAGGGAGA
			Reverse	TTGGGTCAGTTTTCTTCGT
CD73	<i>NT5E-5'Nucleotidase ecto</i>	XM_532221.4	Forward	CAACCTGATTGTGATGCAA
			Reverse	TGGATTCCATTGTTGCGTTC
CD90	<i>Thy-1</i>	XM_844606.3	Forward	CTGTGCTCAGAGACAAACTG
			Reverse	TTAGCCAACCTCAGAGAAAGTAGG
P53	<i>Tumor protein</i>	NC_006587.3	Forward	GAAGAAGCCACTAGATGGAG
			Reverse	TTCTGAACATCTCATAGCG
CD30	<i>TNFSRF8</i>	XM_005617984.1	Forward	GATTCAGCAGAAGCTGCAC
			Reverse	TCGACCACCGATATACTCTT
OCT4	<i>POU5F1</i>	XM_538830.1	Forward	GCAGTGACTATTGCGAACGA
			Reverse	ATTTGAATGCATGGGAGAGC
SOX2	<i>SRY</i>	XM_005639752.1	Forward	CCCACCTACAGCATGCTCTA
			Reverse	GGAGTGGGAGGAGGAGGTAA
18S	<i>18S Ribosomal RNA</i>	XM_541299.4	Forward	CCTGCGGCTTAATTTGACTC
			Reverse	CTGTCAATCCTGTCCGTGTC

TABLE 4 Primer sets used for qRT-PCR of feline-derived AMSCs

Gene symbol	Gene name	Accession no.	Primer	Sequence (5'–3')
CD34	<i>Hematopoietic progenitor antigen</i>	NC_018739.2	Forward	ACCATCAAGGGAGAAATCA
			Reverse	GTCAGTTCTCCCCATTAC
CD73	<i>NT5E-5'Nucleotidase ecto</i>	XM_011282497.1	Forward	AACCTGATTGTGATGCCA
			Reverse	TAATTGTGCCGTTGTTCCG
CD90	<i>Thy-1</i>	XM_003992443.3	Forward	CTGCAGCAGCAGAGGACCCTGCA
			Reverse	AGACTGTCTCAGCAA
P53	<i>Tumor protein</i>	NM_001009294.1	Forward	CCGAACCTCACTTCCTAAAA
			Reverse	CAGGGAACAGACCTTGATAG
CD30	<i>TNFSRF8</i>	XM_011284600.1	Forward	CCATCTCCTTCCTCTGT
			Reverse	ATATGCAACTCCTCAAGGC
OCT4	<i>POU5F1</i>	NC_018727.2	Forward	TGACAACAACGAAAATCTGCTCG
			Reverse	GTTCTCGATACTTGTTTC
SOX2	<i>SRY</i>	NM_001173447.1	Forward	GAAACCAGAGGAAAGGGTG
			Reverse	GATTCTCTAGAGCCACCTG
18S	<i>18S Ribosomal RNA</i>	XM_006939041.2	Forward	CCTGCGGCTTAATTTGACTC
			Reverse	CTGTCAATCCTGTCCGTGTC

3 | RESULTS

3.1 | Cell culture

Canine and feline AMSCs in culture formed polygonal colonies with fibroblast-like morphologies that adhered to the plastic culture surface. The period between each passage was approximately 4 and 6 days for feline and canine AMSCs, respectively (Figure 1c–f).

3.2 | Flow cytometry

To perform the characterization of canine and feline AMSCs, immunophenotyping technique was performed and analysed using a panel of nine antibodies: CD73, CD90, CD105, CD34, CD45, OCT4, NANOG, SOX2 and C-Myc. The analyses revealed expression of mesenchymal markers in canine AMSCs (CD90 and CD73) and low expression of CD105. In the feline AMSCs, the expression of mesenchymal markers (CD90 and CD73) increased when

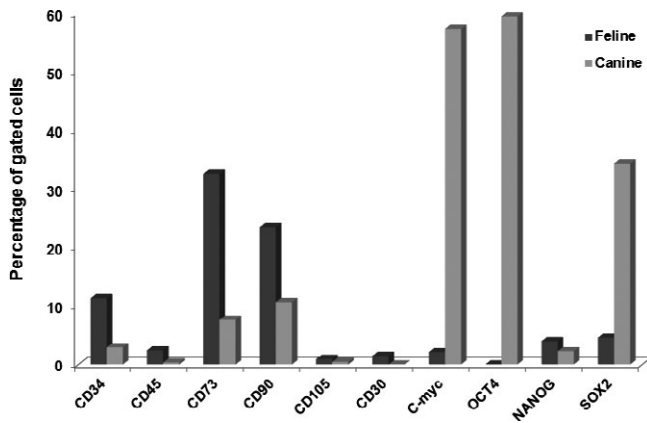


FIGURE 2 Bar graphs illustrating the canine (light grey bar) and feline (dark grey bar) AMC immunophenotyping analyses

compared with canine and had a minimum expression of CD105. CD45 (haematopoietic marker) was not detected in canine and low for feline AMSCs. The CD34 was expressed in the canine AMSCs and had higher expressed in feline AMSCs. However, high expression of pluripotency markers (OCT4 and SOX2) and low expression of the NANOG were observed in canine AMSCs. In feline AMSCs, the expression of these markers when compared with canine data was very low. In addition, c-Myc was expressed in canine AMSCs but few feline AMSCs expressed c-Myc. The tumorigenic marker CD30 was not detected in canine and feline AMSC (Figure 2).

3.3 | Teratoma formation assay

The canine and feline AMSCs were injected into immunodeficient (BALB/c nude) mice (two animals for each cell lineage). We observed no teratomas at 60 days after the injections in any of the examined regions or organs. Histopathologic analyses of the brain, lung and liver fragments revealed no morphologic changes (Figure 3).

3.4 | Real-time polymerase chain reaction (qPCR)

The comparison of the expression of CD34, CD73, OCT4, CD30 and P53 revealed that there were no differences in the patterns of gene expression between canine and feline AMSCs. However, there was a small difference in expression of SOX2 and CD90 when compared among the species (Table 5).

4 | DISCUSSION

Cultured feline and canine AMSCs showed characteristics similar to those of MSCs, including fibroblast-like morphology and adherence to plastic tissue culture plates (Bydlowski, Debes, Maselli, & Janz, 2009). Flow cytometry revealed that the mesenchymal markers (CD90 and CD73) had expression in the felines AMSCs and no expression of CD45 in accordance with a previous report in felines (Vidane et al., 2014). In contrast, the canine AMSCs revealed expression to markers CD73 and CD90 and low expression to CD105. These results are

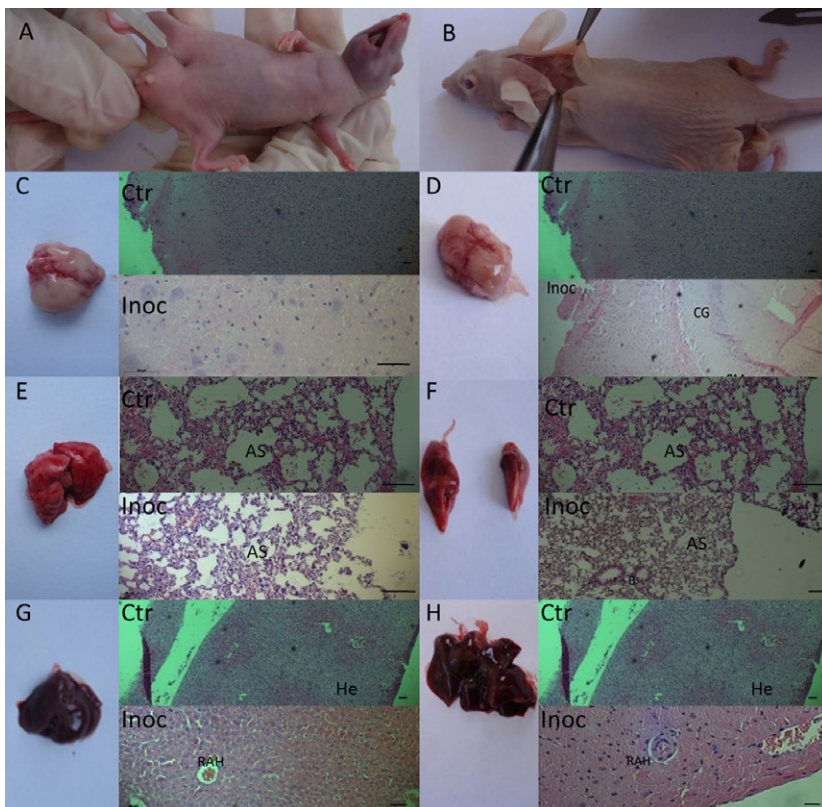


FIGURE 3 Tumour formation assays: An intraperitoneal injection of MSCs (10^6 cells/site) into immunodeficient (BALB/c nude) mice; (b) organ collection from euthanized nude mice; (c, d) brain photomicrographs of nude mice, 60 days after injection of feline (left) and canine (right) MSCs injection, showing the granular layers (CG); (e, f) lung photomicrographs showing terminal bronchioles (TB) and alveolar sacs (AS); (g, h) liver photomicrographs showing the hepatic artery branches (RAH) and hepatocytes (HP). Bars: 50 μ m. Stain: haematoxylin and eosin (H&E)

TABLE 5 Mean \pm SD of $2^{-\Delta Ct}$ of mesenchymal, hematopoietic, pluripotency and tumorigenic gene expression of canine and feline amniotic membrane stem cells

Groups	CD34	CD73	CD90	OCT4	SOX2	CD30	P53
Canine	1.28 \pm 0.40	1.22 \pm 0.75	1.33 \pm 0.14*	1.31 \pm 0.16	1.29 \pm -0.09*	1.23 \pm 0.33	1.05 \pm 0.01
Feline	1.31 \pm 0.14	1.03 \pm 0.08	1.12 \pm 0.14*	0	1.23 \pm 0.04*	1.30 \pm 0.07	1.07 \pm 0.15

*Significant difference.

in contrast to those of Park et al. (2012) who reported expression of these markers.

Cluster of differentiation 90 (CD90) is expressed in the mesenchymal cells as well as haematopoietic cells, myeloid cells and primitive erythrocytes (Oliveira, 2006). Park et al. (2012) reported that canine AMSCs express CD90. Our analysis revealed the expression of CD90 in both canine and feline AMSCs, suggesting that these are mesenchymal cells. The genes CD34, CD73, CD90, OCT4, SOX2, CD30 and P53 were all detected in the canine and feline AMSCs by real-time PCR. The marker CD45 and CD34 were less expressed in the canine and feline AMSCs similar to findings of Park et al., (2012). We may suggest these types of cells are real mesenchymal lineage.

The genes OCT4 and SOX2 are important to maintenance of cell pluripotency during embryonic development (Beyer & da Silva Meirelles, 2006). Park et al. (2012) found high expression of pluripotency markers OCT4 and SOX2 and SOX2 genes in canine AMSCs. In the current study, we have demonstrated that feline and canine AMSCs express SOX2 genes as well as protein markers, whereas only canine and not felines AMSCs express OCT4. This represents the most significant molecular difference between AMSCs from different species.

The results of the current work demonstrate that despite similarities in animal age, size and gestational stage, there is great heterogeneity among individuals and their foetal adnexa, suggesting the possibility of variations at the genetic level. The present findings may provide a key explanation to account for reports of tumorigenic (Lima et al., 2009) versus non-tumorigenic (Winck, 2012) effects. The expression of the tumorigenic CD30 marker has not previously been reported, and in the current study, this marker was not expressed in flow cytometry analysis. The current study was based on a previous report in human tissue tumours (Preda, Dulcey, & Nogales, 2012) and was the first to investigate the expression of CD30 in canine cells. The considerable variation in gene expression we observed was unexpected, considering that the foetuses were derived from the same pregnancy and were collected at the same gestational age (40 days). These observations demonstrate that the two species under consideration (human and canine) are distinct and that the behaviour of cells isolated from their foetal membranes is unique to each species. Despite the low expression levels of genes detected in feline cells, there was good agreement among the samples analysed as relatively small standard deviations were observed. These observations demonstrate that feline cells show little animal-to-animal variation in gene expression. In contrast, we found that canine cell gene expression varied considerably among individual siblings, as reflected by the relatively high standard deviations observed.

The main factor that calls into question the biological safety of canine cells is related to their great capacity to proliferate and differentiate into multiple lineages and their potential to form tumours (Bapat, 2007; Lakshmipathy & Verfaillie, 2005). According to Miki, Lehmann, Cai, Stolz, and Strom (2005), human amniotic membrane epithelial cells also have pluripotent features as demonstrated by OCT4 and NANOG expression, but these cells are not tumorigenic. Canine-derived MSCs did not form teratomas at 60 days post-injection into nude mice. Similar findings were reported using the same MSC lineage (Winck, 2012). However, our results differ from those reported by Lima et al., 2009; who observed embryonic carcinoma formation 4 weeks following injection of canine AMSCs into immunodeficient mice (BALB/c nude). The use of feline AMSCs did not result in tumour formation 60 days after injection into nude mice, in agreement with findings reported by Vidane et al., 2014 in feline AMSCs.

In conclusion, AMSCs are a readily available source of stem cells that can be used for transplantation without the risk of tumour formation.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

MTC executed the main experiment as master degree thesis working on results and writing. Martins, D.S. and Ambrosio, C.E. helped in technique performance and are PI of the group. AOP, ASV, JBC, VCO and NJNG helped with techniques and also wrote and designed the final manuscript.

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