

Transplantation of amniotic membrane-derived multipotent cells ameliorates and delays the progression of chronic kidney disease in cats

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Contents

Chronic kidney disease (CKD) is a common clinical condition in domestic cats, characterized by tubulointerstitial, vascular and glomerular inflammation and severe fibrosis. Studies in rodent model of induced CKD have shown a decrease and stabilization of the clinical condition. In this study was evaluated the safety and effect of intrarenal and intravenous infusion of allogeneic mesenchymal stem cells (AMSCs) derived from feline amniotic membrane in cats with naturally occurring CKD. Cat AMSCs were harvested after mechanical and enzymatic digestion of amnion. A healthy cat received intrarenal injection of AMSCs guided by ultrasound in both kidneys (5×10^5 cells/kidney). Nine cats with CDK received repeated intravenous infusions of AMSCs (2×10^6 cells \times 2 treatments). The clinical parameters of healthy cat did not change, but sedation and general anaesthesia was required. The number of interventions stressed the animal, and he developed transient haematuria after AMSC injection. Cats with CDK registered a significant improvement of renal function (decrease in serum creatinine and urine protein concentrations and increase in urine specific gravity). The kidney architecture and morphology did not change following the treatment. The feline AMSCs have a renoprotective effect and improve renal function in cats with naturally occurring CKD, stabilizing the clinical condition and disease progression. Thus, intravenous injection of AMSCs may be an important tool to provide welfare in cats with chronic kidney disease.

1 | INTRODUCTION

Chronic kidney disease (CKD) is a common clinical condition in domestic cats, responsible for a high rate of mortality and morbidity in elderly animals. It is defined as structural and/or functional impairment of one or both kidneys, which persists for long periods, up to 3 months (Kuwahara, Ohba, Kitoh, Kuwahara, & Kitagawa, 2006; Lawson, Elliott, Wheeler-Jones, Syme, & Jepson, 2015; Polzin, 2011). There is no definitive treatment to restore the kidney function in cats with CKD. The first step for CKD manipulation would be the identification and treatment of the major cause of the disease. It is almost impossible because the CKD is usually a silent condition and symptoms are observable when more than 75% of the nephrons have been lost. In some cases, pre-renal and post-renal disorders may worsen the

CKD condition (Bartges, 2012; Quimby, 2015a). Therefore, the management of metabolic disorders and secondary impairments caused by renal function failure appears to be the best alternative to improve welfare of affected cats. The administration of special feed has shown significant benefits and has guaranteed a survival of CKD cats; however, the progression of disease to terminal stages is painful and inevitable (Elliott, Rawlings, Markwell, & Barber, 2000; Polzin, 2011).

Recently, the therapeutic benefits of MSCs have been shown in normal and transgenic rodents as a model of chronic or degenerative diseases. Beneficial results in rodents encouraged several pilot studies on models of naturally occurring diseases in domestic animals. Despite government restrictions and the absence of clear regulations, with increasing demand, veterinarians offer unproven cell-based therapies (Cyranoski, 2013).

The potential MSC therapy for CKD has been reported in numerous studies of induced CKD in rodents (Cavaglieri, Martini, Sogayar, & Noronha, 2009; Kirpatovskii et al., 2007; Lee et al., 2010; Ninichuk et al., 2006; Semedo et al., 2009; Villanueva et al., 2011, 2013). These studies have shown a decrease and stabilization of the clinical condition, evidenced by renal function improvement and by inflammation and renal fibrosis reduction (Cavaglieri et al., 2009; Lee et al., 2010; Ninichuk et al., 2006; Semedo et al., 2009; Villanueva et al., 2011). Quimby, Webb, Gibbons, and Dow (2011) evaluated the effect of intrarenal application, and recently, Quimby, Webb, Habenicht, and Dow (2013) and Quimby et al. (2015) evaluated the safety and efficacy of intravenous application of MSCs in cats with naturally occurring CKD. The results of these series of pilot studies showed modest improvement on renal function, but not in the same degree of efficacy reported in rodents.

The mechanisms by which MSCs exert the therapeutic effects have not been elucidated. Researchers have attributed these effects to intense paracrine activity through MSC-derived microvesicles, exosomes and growth factors. Therefore, intravenous route for stem cells delivery is the most important route because it induces the chemotactic, renotropic and paracrine effects (Togel et al., 2005, 2007). The activation of these mechanisms plays a key role on cell migration, homing and differentiation (Sohni & Verfaillie, 2013).

The primary aim of this study was to evaluate the safety of bilateral intrarenal injection of AMSCs in cats. Due to significant adverse effects associated to intrarenal MSC delivery, the second aim was to evaluate the effect of repeated intravenous injection of AMSCs in cats with naturally occurring CKD. We previously showed the high plasticity and safety of feline AMSCs (Vidane et al., 2014), and other studies have shown that these cells have immunomodulatory, anti-inflammatory and antifibrotic properties (Barboni et al., 2014; Lange-Consiglio et al., 2013; Mihai, Ciuca, Soritau, Susman, & Mihai, 2009).

In this study was observed that repeated intravenous administration of AMSCs in cats with naturally occurring CKD resulted in significant improvement of renal function and delays the disease progression.

2 | MATERIALS AND METHODS

All experiments and procedures were performed in accordance with the Ethical Principles in Animal Research (COBEA), and this study was approved by the Faculty of Veterinary Medicine and Animal Science Ethical Committee, number: 3240290115.

2.1 | Isolation of cat AMSCs

AMSCs were isolated from amniotic membranes collected from 30 to 35 pregnant cats after routine castrations. The amnion was carefully washed with sterile phosphate-buffered solution (PBS) and transported in sterile standard collection containers. Pieces of amnion were minced and subjected to enzymatic digestion with 0.1% collagenase (Gibco by Life Technologies, NY, USA) at 38.5°C. The product of digestion was passed through a 40-µm nylon filter (BD-Falcon, cell

strainer nylon). The cell solution was centrifuged at 303 g for 5 min, room temperature. Each supernatant was discarded, and the cell pellet was suspended with 1 ml of the culture medium DMEM/Ham's F12 solution (Gibco by Life Technologies) supplemented with 10% of Fetal Bovine Serum (LGC Biotecnologia, SP, Brazil), 1% of non-essential amino acids, 1% of L-glutamine and 1% of penicillin and streptomycin (Gibco by Life Technologies, NY, USA). The isolated cells were seeded in cell culture dishes with DMEM F12 culture medium supplemented as described above, at 38.5°C and 5% of CO₂. The culture medium was replaced 24 hr after the initial plating to remove the non-adherent cells. When plated cells reached 80%–90% confluence, the culture was recovered using trypsin 0.25% (Gibco by Life Technologies) and reseeded for each subsequent passage.

2.2 | Cryopreservation

The cat AMSCs were frozen at P2-P3 in DMEN supplemented with 45% of FBS and 10% of DMSO (Sigma-Aldrich, SP, Brazil). The cells were frozen for 24 hr at –80°C using the freezing container (Nalgene, Mr Frosty; Sigma-Aldrich), and then were transferred and stored in liquid nitrogen until trial assays.

2.3 | Flow cytometry

Cat AMSCs at passage P3 (3×10^4 cells/mL) were blocked with 2% bovine serum albumin (BSA) for 45 min, washed with 0.2% BSA and labelled with primary antibodies for CD73, CD90, CD34, CD45 and CD79 (Santa Cruz Biotechnology Inc., CA, USA) in 0.2% BSA (1:300) overnight at 4°C. Controls were incubated with 0.2% BSA instead of primary antibodies. After that, the cells were washed in PBS and incubated with secondary conjugated antibodies (1:300) (Alexa Fluor-488, Thermo Fisher Scientific Inc., USA) for 45 min at room temperature in the dark. Labelled cells were transferred to Falcon tubes and analysed by flow cytometry (FACSAria BD Biosciences).

2.4 | Cell preparation

After thawing at room temperature, the cell solution was centrifuged and the cell pellet was recovered and cultured in the similar conditions described above. When plated cells reached 80%–90% confluence, the culture was recovered using trypsin 0.25% and centrifuged, and the cell pellet was suspended with 1 ml of 0.9% sodium chloride solution (normal saline). The isolated cells were counted using a Neubauer chamber, and cell viability was checked using trypan blue dye method (1:1). Cell viability was expressed as a percentage of unstained cells (living cells).

2.5 | Study animals

Twenty-seven intact or castrated cats (male and female), with suspicion of chronic kidney disease, were enrolled from different veterinary care units. Recruitment was based on two repeated blood biochemical (20 days apart) and ultrasound evidence of morphological changes of CKD. Eighteen cats with infectious diseases or concurrent diseases

not associated with renal failure or in the end stage of CKD were excluded from the study. Before any procedure, a survey of medical history was performed and the complete blood count, blood biochemistry, blood gases, urinalysis and ultrasound to determine the degree of renal injury in experimental patients were evaluated. The administration of palliative therapies was allowed as no changes were guaranteed during the trial period. One healthy cat was used for pilot study of bilateral intrarenal injection of AMSCs. The all owners of the cats signed the consent forms for animal experimentation, designed by the local ethical committee. The summary description of cats included in the study is shown in Table 1.

2.6 | Intrarenal injection of AMSCs

A healthy cat was used to assess the feasibility of bilateral intrarenal injection of AMSCs. The cat was sedated using intramuscular administration of methadone 0.3 mg/kg, midazolam 0.2 mg/kg and ketamine 6 mg/kg, IM. The general anaesthesia was induced and maintained using intravenous continuous infusion of propofol 5 mg/kg. The animal was placed in lateral recumbency, trichotomized in the lateral abdomen, followed by local antiseptics. Under ultrasound guidance, a 27-gauge sterile needle attached to a 1-ml syringe was stuck into the kidney cortex. About 1×10^5 AMSCs suspended in 150 μ l normal saline were slowly injected into one site of each kidney cortex using the freehand technique. The animal was monitored for possible side effects and cortical haemorrhage 1, 5, 24 hr and 7 days post-injection.

2.7 | Repeated intravenous infusion of AMSCs

For intravenous injection, about 2×10^6 at P3/P4 were suspended in 3–4 ml of normal saline and transferred to 5-ml syringe. Nine cats with naturally occurring CKD received slowly allogeneic intravenous infusion (cephalic vein) of AMSCs, for 15 min using the freehand technique. Each cat received two cell administrations, 21 days apart. The intravenous administration of normal saline was maintained for about 30 min while was monitored for possible side effects of AMSC injection.

2.8 | Clinical monitoring

At day 0 (D0), immediately prior to AMSC injection, was performed a survey of medical history, physical examination and sample collection for complete blood count, blood biochemistry, blood gases and urinalysis. The kidney dimensions, architecture and morphology were assessed by ultrasound imaging. Each cat underwent clinical monitoring at D7, D30 and D60 after cell administration through physical examination and sample collection for routine blood count, blood biochemistry, blood gases and urinalysis. At D60 was performed the ultrasound imaging to assess the kidney architecture and morphology evolution following AMSC therapy. Similar procedures were performed for healthy cat (intrarenal pilot study); however, cats were evaluated at D0 and D7 only.

2.9 | Laboratory analysis

All analysis and procedures were performed at the Faculty of Food Engineering and Animal Science Teaching Hospital, University of São Paulo (UDCH-FZEA-USP). Samples were collected after a minimum 8 hr fast and transported under refrigerated conditions at 4°C.

Haematological analysis was carried out using an automated hematology analyzer (BC 2800 Vet, Mindray, China), and differential leucocyte count was assessed using Rosenfeld-stained blood smears.

Estimation of serum biochemistry parameters (blood urea, creatinine, total serum protein, ALT, AST, alkaline phosphatase, albumin, globulin and cholesterol) was assessed using automated biochemical analyzer (BS 120, Mindray, China) and commercial kits (Labtest, Brazil).

Urine samples were collected by cystocentesis. Physical examination was performed to estimate colour, odour, brightness, consistency and urine specific gravity (using refractometry method). Chemical analyses were performed using colorimetric test strips (Labtest, Brazil) for semiquantitative determination of urobilinogen, glucose, ketones, bilirubin, protein, pH and occult blood. Urine creatinine and protein were estimated using automated biochemical analyzer (BS 120, Mindray) and commercial kits (Labtest, Brazil).

TABLE 1 Summary description of cats included in this study

Cat	Description	Blood urea and creatinine (mg/dl)	IRIS stage	Group	Treatment
1	4 years FS DSH	U 44.4 C 1.35	N/A	Health + AMSCs	1×10^5 AMSCs injected into the cortex of both Kidneys
2	12.5 years FS DSH	U 96.5 C 3.7	III	CKD + AMSCs	2×10^6 AMSCs intravenously \times 2 treatments
3	12 years FS DSH	U 63.5 C 2.4	II	CKD + AMSCs	2×10^6 AMSCs intravenously \times 2 treatments
4	8 years MC DSH	U 42.9 C 2.0	II	CKD + AMSCs	2×10^6 AMSCs intravenously \times 2 treatments
5	11 years FS DSH	U 55.4 C 1.85	II	CKD + AMSCs	2×10^6 AMSCs intravenously \times 2 treatments
6	13 years FS DSH	U 67.2 C 2.29	II	CKD + AMSCs	2×10^6 AMSCs intravenously \times 2 treatments
7	10 years FS DSH	U 64 C 1.61	II	CKD + AMSCs	2×10^6 AMSCs intravenously \times 2 treatments
8	16 years MC DSH	U 119.9 C 3.15	III	CKD + AMSCs	2×10^6 AMSCs intravenously \times 2 treatments
9	10 years MI DSH	U 186.9 C 3.03	III	CKD + AMSCs	2×10^6 AMSCs intravenously \times 2 treatments
10	14 years MC DSH	U 40.4 C 1.78	II	CKD + AMSCs	2×10^6 AMSCs intravenously \times 2 treatments

FC, female castrated; MC, male castrated; MI, male intact; DSH, domestic shorthair; IRIS, International Renal Interest Society; U, blood urea; C, creatinine.

Determination of blood pH, pO₂ (oxygen partial pressure), pCO₂ (partial pressure of carbon dioxide), serum bicarbonate, sodium, potassium and ionized calcium was carried out using hand-held blood gas analyzer (i-STAT® System, USA) and EG7+ or CG8+ kits (Tecno4, Brazil).

2.10 | Statistical analysis

Changes in clinical parameters data (blood count, blood biochemistry, blood gases and urinalysis) over the time (before and after cell injection) were evaluated by ANOVA. For all variables were evaluated the variances homogeneity (Levene's test) and residual normality. When the residual normality was not attended, the outliers were excluded and the analysis was repeated. When significant differences were observed, the means between the groups were compared by Tukey's test. Changes of clinical parameters data before (D0) and after (D7) intrarenal AMSC injection were evaluated by paired *t* test. Statistical analysis were performed using Statistical Analysis System Software, version 9.3 (SAS, 2012), and means were considered statistically different for $p < .05$.

3 | RESULTS

3.1 | Isolation of cat AMSCs

After 48 hr in culture medium DMEN-F12, the cat amniotic mesenchymal-like cells demonstrated plate adherence and reached 70%–80% of confluence. The cell morphology was similar to fibroblastic cells, indistinguishable from bone marrow-derived mesenchymal stem cells, and rapidly cell clusters were observed (Figure 1a,b). The culture reached the sufficient level of homogeneity at passage 3 (P3) without any morphology visible changes. Cell viability before cryopreservation was up to 90%; however, after 1 year stored in liquid nitrogen, the cell viability decreased to 83%, without depreciation in the morphology and proliferation ability.

Cat AMSCs at passage three, collected by trypsinization, expressed high levels of MSCs surface markers, CD73 (89.5%) and CD90 (91.9%), but the hematopoietic-specific markers CD34 (47%), CD45 (1.7%) and CD79 (29.1%) were not expressed (Figure 1c–h). The immunophenotypic profile was identical to that recently reported in feline amniotic membrane-derived stem cells (Vidane et al., 2014) and cat bone marrow-derived MSCs (Martin, Cox, Hathcock, Niemeyer, & Baker, 2002).

3.2 | Effects of allogeneic intrarenal injection of AMSCs

The feasibility of bilateral intrarenal injection of AMSCs was assessed in a healthy cat. The animal was monitored for possible side effects and cortical haemorrhage 1–5 hr, 24 hr and 7 days post-injection through physical examination to assess cardiac and respiratory frequency, mucous colour and abdominal discomfort. During the procedure, the cat was stressed; thus, multiple sedations and general

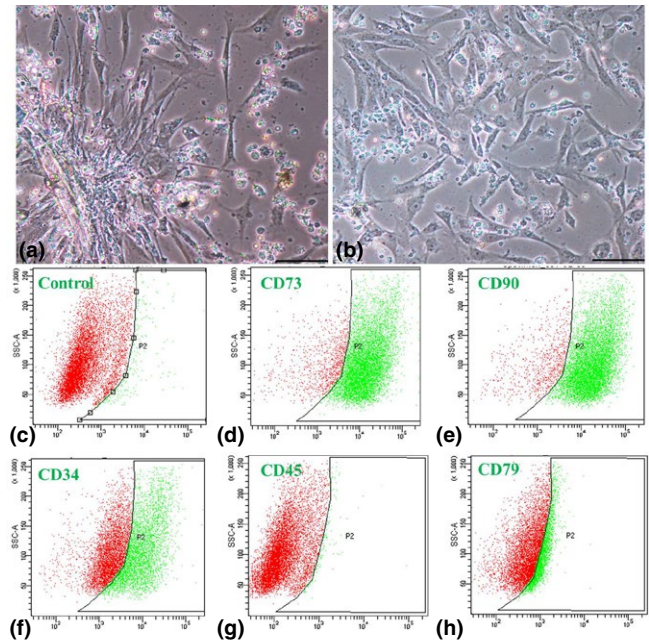


FIGURE 1 Photomicrography of feline amniotic membrane-derived MSCs and histogram overlays of immunophenotyping assays. (a, b) Cell morphology: fibroblastic-like cells adhering to plastic culture dishes (P0). Primary culture, a small cell clusters were observed. Cat AMSCs expressed high MSCs surface markers CD73 and CD90 but did not express hematopoietic markers CD34, CD45 and CD79 (c–h). The dot plots represent the number of cells relative to the fluorescence intensity of the control cells. (a, b) 20 μ m (20 \times)

anaesthesia were required. There were no significant changes of physical examinations parameters, and we did not notice renal discomfort through abdominal palpation. By ultrasound imaging, we did not observe haemorrhage in the cortical site of AMSC injection in both kidneys; however, we observed hyperechoic particles without acoustic shadowing in the bladder, not observed prior cell injection. Few hours later, we observed transient haematuria but the animal was not clinically affected.

The cat was monitored for 1 week, and at D7, samples were collected for routine blood count, blood biochemistry, blood gases and urinalysis. The paired *t* test showed no significant changes of clinical parameters data ($p = .339$) before (D0) and after (D7) intrarenal AMSC injection. Besides sedation and general anaesthesia, in this procedure was required up to five persons (one ultrasound operator, one auxiliary for cell injection, two anaesthetists, one readiness surgeon for intervention in case of possible complication and one assistant for animal restraint).

3.3 | Effects of allogeneic intravenous injection of AMSCs

Due to adverse effects and complications associated with the intrarenal AMSC injection, the cats with naturally occurring CKD received slowly allogeneic intravenous infusion of AMSCs. Each cat underwent physical examination and sample collection for routine blood count, blood biochemistry, blood gases and urinalysis, prior AMSC injection

(D0) and at D7, D30 and D60. During and immediately after infusions, we did not observe adverse effects. The physical examination revealed that the cats were clinically normal and stable. Only one cat experienced vomiting during the procedure, but the other parameters appeared clinically normal. The vomiting condition in this cat was present even before the manipulation and the owner reported that it ceased after the return home.

The overall clinical condition of the cats showed considerable improvement. Owners reported improvements in animal welfare, food intake and social behaviour. On the other hand, weight did not show significant change following treatment (Figure 2).

3.4 | Blood count

Relevant blood count parameters did not change significantly at different stages of treatment, and the mean values were within normal standards for domestic cats. The mean values of platelets were below the recommended standards for cats, and we observed a significant increase in D7 after first AMSC administration and then were stable in

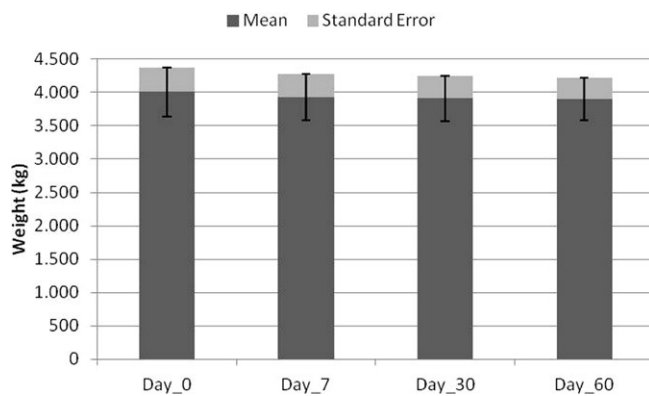


FIGURE 2 Weight values (means and standard error) of treated cats in different stages of treatment. No significant changes were observed following treatment

the following days. The differential white blood counts did not change significantly; however, decrease in the lymphocyte count above the normal values was observed (Table 2).

3.5 | Blood biochemistry

Serum creatinine concentration decreased significantly ($p = .028$) in CKD cats treated with AMSCs (Figure 3). The change was evident shortly after first application (D7) and more pronounced after the second application of AMSCs (D60). The serum albumin also experienced significant variation between D7 and D60 (p -value = .004), although the variation occurs within the cat normal range, and therefore, we believe that the variation has no significant relevance. Blood urea, cholesterol and AST enzyme did not change significantly but remained above normal standards for domestic cats. We did not observe substantial changes in remaining biochemical parameters (Table 3).

3.6 | Urinalysis

We did not observe significant changes in relevant urinary parameters following treatment (Table 4). However, the urine protein-to-creatinine (UPC) ratio decreases following AMSC administration ($p = .131$), and we believe that the variation has clinical relevance (Figure 4). This scenario results from a gradual reduction of protein loss in the urine (decrease in proteinuria) and increase in creatinine excretion throughout different stages of treatment. On the other hand, the urine specific gravity improved following treatment.

3.7 | Blood gases

The blood gases and electrolytes in general did not change following AMSC injection, and the values varied within normal limits for domestic cats (Table 5). Blood potassium and ionized calcium did not change significantly but remained above normal standards for domestic cats during all experimental periods.

TABLE 2 Blood count means (\pm SE) of treated cat in different stages of treatment (before and after)

	Reference	Stage			
		DO	D7	D30	D60
Leucocytes ($\times 10^3/\mu\text{l}$)	5.5–19.5	9.17 ± 1.36^A	9.89 ± 1.25^A	9.35 ± 2.39^A	11.19 ± 3.12^A
Erythrocytes ($\times 10^6/\mu\text{l}$)	5.4–10.7	7.70 ± 0.31^A	7.71 ± 0.14^A	7.55 ± 0.19^A	7.60 ± 0.30^A
Haemoglobin (g/dl)	9–15	12.15 ± 0.88^A	12.42 ± 0.29^A	11.65 ± 0.53^A	11.27 ± 0.62^A
Haematocrit (%)	30–47	34.33 ± 2.05^A	32.90 ± 2.21^A	33.49 ± 1.58^A	34.24 ± 1.54^A
MCV (fL)	41–51	47.15 ± 1.23^A	45.92 ± 1.13^A	46.07 ± 1.32^A	47.83 ± 1.63^A
MCH (pg)	13–18	16.53 ± 0.56^A	16.19 ± 0.33^A	15.99 ± 0.52^A	15.85 ± 0.41^A
MCHC (g/dl)	31–35	35.14 ± 0.69^A	36.09 ± 0.58^A	34.77 ± 0.58^A	33.96 ± 0.82^A
Platelets ($\times 10^3/\mu\text{l}$)	300–800	111.78 ± 23.22^B	171.44 ± 25.71^A	147.44 ± 26.53^{AB}	147.99 ± 27.27^{AB}
Segmented Neutrophils (%)	35–75	60.22 ± 4.61^A	68.00 ± 4.62^A	73.00 ± 3.87^A	73.83 ± 4.09^A
Lymphocytes (%)	20–55	19.67 ± 3.93^A	18.78 ± 3.05^A	19.78 ± 2.92	14.71 ± 2.85^A

Means followed by the same upper-case letters on a line do not differ significantly by Tukey's test at 5% of error probability.

3.8 | Ultrasound

By ultrasound, kidney architecture and morphology did not change 60 days post-AMSC therapy. In general, all initial morphological and structural patterns were maintained. The main initial changes observed in CKD cats included changes in shape, reduction in size, irregular borders, atrophy, loss of corticomedullary definition and the presence of hyperechoic spots related to calcification or severe fibrosis.

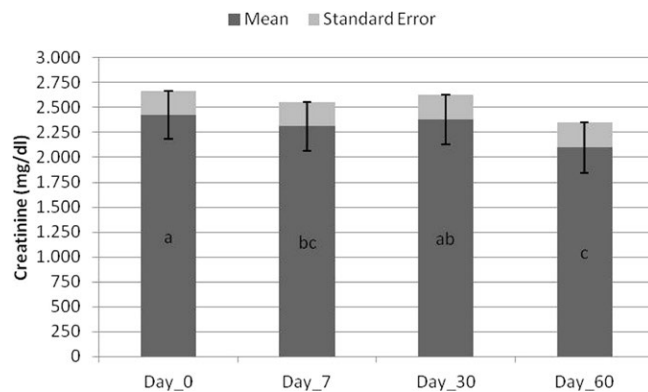


FIGURE 3 Serum creatinine values of treated cats in different stages of therapy. Serum creatinine concentration decreased significantly following treatment

4 | DISCUSSION

4.1 | Isolation of cat AMSCs

Our research group has recently isolated and characterized the mesenchymal stem cells derived from cat amniotic membrane (Vidane et al., 2014).

In this study, we successfully isolated and expanded the MSCs derived from cat amniotic membrane. These cells adhered to the culture dishes (24 hr after culture) showed fibroblastic-like morphology and high self-renewal capability. These culture characteristics are similar to those described in cat bone marrow-derived MSCs (Martin et al., 2002; Webb, Quimby, & Dow, 2012) and are in accordance with MSC description by the International Society for Cellular Therapy (Dominici et al., 2006). In our primary cultures, we also observed rapidly formation of cell clusters, suggesting a cell-cell contact and a very strong paracrine signalling. The cell-cell contact and paracrine signalling plays a key role for MSCs commitment, proliferation and migration (Vidane et al., 2013).

The culture was expanded until passages two and three where the sufficient level of homogeneity was reached. Therefore, the AMSCs were frozen in these passages and administered in passages three and four. Recently, Vidane et al. (2014) reported that AMSCs exhibit maximum proliferation rate and plasticity in this phase. These data are consistent with previous studies in foetal membranes from dogs (Filioli

TABLE 3 Blood biochemistry means (\pm SE) of treated cat in different stages of treatment (before and after)

	Reference	Stage			
		DO	D7	D30	D60
Urea (mg/dl)	38.6–70.1	81.86 \pm 15.59 ^A	81.69 \pm 15.75 ^A	82.03 \pm 14.08 ^A	82.59 \pm 13.76 ^A
Creatinine (mg/dl)	0.7–1.8	2.43 \pm 0.24 ^A	2.31 \pm 0.24 ^{BC}	2.38 \pm 0.25 ^{AB}	2.10 \pm 0.25 ^c
Protein (g/dl)	5.7–7.9	7.36 \pm 0.14 ^A	7.43 \pm 0.13 ^A	7.31 \pm 0.16 ^A	7.46 \pm 0.17 ^A
Albumin (g/dl)	2.3–3.4	2.52 \pm 0.07 ^{AB}	2.62 \pm 0.06 ^A	2.78 \pm 0.25 ^{AB}	2.35 \pm 0.10 ^B
Globulin (g/dl)	2.6–4.5	4.80 \pm 0.17 ^A	4.81 \pm 0.16 ^A	4.52 \pm 0.38 ^A	5.14 \pm 0.24 ^A
ALT (UI/L)	30–100	44.50 \pm 5.08 ^A	48.12 \pm 3.98 ^A	53.75 \pm 5.77 ^A	51.40 \pm 9.16 ^A
AST (UI/L)	12–56	35.13 \pm 1.34 ^A	36.13 \pm 4.67 ^A	36.63 \pm 6.92 ^A	38.24 \pm 4.11 ^A
Alkaline Phosphatase (UI/L)	15–92	27.22 \pm 2.03 ^A	26.56 \pm 2.75 ^A	28.67 \pm 5.26 ^A	24.64 \pm 3.50 ^A
Cholesterol (mg/dl)	38–186	196.10 \pm 13.65 ^A	193.94 \pm 11.69 ^A	193.99 \pm 17.60 ^A	159.48 \pm 19.10 ^A

Means followed by the same upper-case letters on a line do not differ significantly by Tukey's test at 5% of error probability.

TABLE 4 Relevant urinary parameters means (\pm SE) of treated cat in different stages of treatment (before and after)

	Reference	Stage			
		DO	D7	D30	D60
Urine specific gravity	1.02–1.04	1.029 \pm 0.005 ^A	1.033 \pm 0.005 ^A	1.028 \pm 0.004 ^A	1.031 \pm 0.005 ^A
pH	4.5–7.0	5.89 \pm 0.34 ^A	5.67 \pm 0.28 ^A	5.72 \pm 0.29 ^A	5.90 \pm 0.33 ^A
Creatinine		156.44 \pm 38.61 ^A	173.58 \pm 37.58 ^A	175.77 \pm 37.68 ^A	238.14 \pm 63.72 ^A
Protein		44.77 \pm 10.33 ^A	47.91 \pm 10.43 ^A	34.32 \pm 6.57 ^A	33.95 \pm 6.73 ^A
UPC ratio	<0.2	0.28 \pm 0.05 ^A	0.25 \pm 0.04 ^A	0.25 \pm 0.06 ^A	0.2 \pm 0.06 ^A

Means followed by the same upper-case letters on a line do not differ significantly by Tukey's test at 5% of error probability.

Uranio et al., 2011) and recent trials involving feline foetal fluids and membranes (Iacono et al., 2012). Demerdash et al. (2015) reported that MSCs exhibit high expression of pluripotency genes in early passages, and this expression decreases gradually with increasing passages.

The AMSCs were isolated from cat amnion from approximately 35-day pregnant embryos, estimation based on Miglino et al. (2006), Martins et al. (2011) and Pieri et al. (2015). The morphology and functional properties of amniotic membrane-derived cells depend on embryos age. With pregnancy progression, the expression of pluripotency markers and plasticity reduce and the DNA methylation increase (Barboni et al., 2014). On the other hand, amniotic cells derived from early pregnancy exhibit high expression of pluripotency markers, and their use in cell transplantation is questionable. When undifferentiated AMSCs were injected into immunodeficient (BALB/c-Nu) mice, no teratomas were observed by 4 weeks post-injection, and the transplanted animals survived without difficulty (Vidane et al., 2014). We hypothesize that the amniotic membrane-derived MSCs would be more efficient in improving renal function than adipose-derived MSCs used in previous studies. Amnion is formed during gastrulation and

is believed that these cells retain high plasticity as a memory of their progenitor cells. Because the amnion differentiates from the epiblast at a time when it retains pluripotency, it is reasonable to speculate that amniotic cells may have escaped the specification that accompanies gastrulation and may preserve some of the original characteristics. The pluripotency of amniotic cells has been demonstrated “in vitro” in different experiments, and we believe that it represents a good source of MSCs than the MSCs found in adult adipose tissue, used in several experiments for CKD cats before. A tissue bank of these cells will be an option for veterinary centres, which uses stem cells therapy.

4.2 | Effects of allogeneic intrarenal injection of AMSCs

A healthy cat was used to assess the feasibility of bilateral intrarenal injection of AMSCs. During the cell injection and immediately after, the animal was perturbed and stressed; therefore, multiple sedations and general anaesthesia was required. Transient haematuria was observed; however, the physical examination parameters were clinically stable. Similar findings were reported after bone marrow-derived MSC transfer by intrarenal route in CKD cats (Quimby et al., 2011). We conclude that intrarenal route for AMSC delivery is not suitable for routine application in CKD cats due to several side effects observed, sedation and anaesthesia. We think it is important to demonstrate and discuss this issue. To find a best route for stem cell delivery for specific is in many cases the key point in cell-based therapies.

The absence of significant clinical changes between D0 and D7 stages suggests that intrarenal transfer of AMSCs did not affect the renal function in healthy cat. Although this procedure was feasible in this pilot study, routine approach in veterinary practice for CKD cats is questionable. Sedation and anaesthesia required to implement are not indicated for CKD cats and may adversely affect renal function and therefore complication of disease condition. In previous study was also suggested that intrarenal intervention may precipitate progression of disease and decline renal function (Quimby et al., 2011).

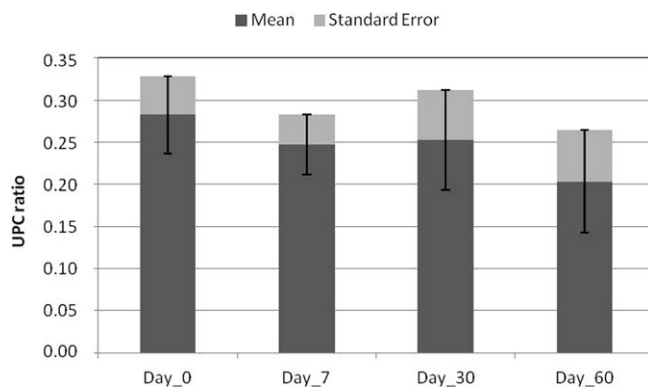


FIGURE 4 Urine protein-to-creatinine (UPC) ratio values of treated cats in different stages of treatment. Modest decrease, but not statistically significant of UPC ratio was observed

TABLE 5 Blood gases and electrolytes means (\pm SE) of treated cat in different stages of treatment (before and after)

	Reference	Stage			
		D0	D7	D30	D60
pH	7.24 to 7.40	7.30 \pm 0.02 ^A	7.28 \pm 0.02 ^A	7.31 \pm 0.02 ^A	7.28 \pm 0.03 ^A
pCO ₂ (mmHg)	32.7 to 44.7	40.51 \pm 1.69 ^A	41.70 \pm 2.35 ^A	39.89 \pm 3.02 ^A	42.74 \pm 4.71 ^A
pO ₂ (mmHg)	47.9 to 56.3	40.78 \pm 2.32 ^A	39.22 \pm 2.41 ^A	39.22 \pm 2.71 ^A	36.42 \pm 1.93 ^A
BE (mmol/L)	-9.8 to -2	-5.89 \pm 0.56 ^A	-7.44 \pm 1.14 ^A	-6.11 \pm 0.56 ^A	-7.11 \pm 0.73 ^A
HCO ₃ ⁻ (mmol/L)	17 to 21	20.00 \pm 0.52 ^A	19.34 \pm 0.92 ^A	19.90 \pm 0.62 ^A	19.74 \pm 0.68 ^A
TCO ₂ (mmol/L)	18 to 23	21.56 \pm 0.56 ^A	21.02 \pm 0.66 ^A	21.11 \pm 0.73 ^A	21.44 \pm 0.58 ^A
SO ₂ (%)	-	65.89 \pm 3.02 ^A	64.22 \pm 3.91 ^A	65.56 \pm 3.44 ^A	59.55 \pm 3.52 ^A
Na (mEq/L)	151 to 158	152.11 \pm 0.45 ^A	151.89 \pm 0.89 ^A	150.00 \pm 0.85 ^B	150.57 \pm 0.72 ^{AB}
K (mEq/L)	4.0 to 5.3	3.77 \pm 0.19 ^B	3.98 \pm 0.21 ^A	3.80 \pm 0.25 ^{AB}	3.64 \pm 0.20 ^B
iCa (mEq/L)	3.6 to 6.0	1.31 \pm 0.03 ^A	1.31 \pm 0.02 ^A	1.30 \pm 0.02 ^A	1.27 \pm 0.02 ^A

Means followed by the same upper-case letters on a line do not differ significantly by Tukey's test at 5% of error probability.

Despite possible benefits of AMSC delivery in the interest site, the paracrine mechanisms, microvesicles/exosomes secretion and growth factors are activated through intravenous MSC injection. These mechanisms are also responsible for mobilization and activation of further progenitor cells as well as immunomodulatory properties activation, providing the benefits of cell-based therapies (Quimby, 2015b; Semedo et al., 2009; Togel et al., 2005, 2007).

Finally, we conclude that intrarenal route for AMSC delivery is not suitable for routine application in CKD cats; therefore, we adopted in this study intravenous injection of AMSCs in our experimental animals.

4.3 | Effects of allogeneic intravenous infusions of AMSCs

Disadvantages of intravenous repeated infusion of MSCs include pulmonary thrombosis and embolism (especially in rodents) due to quick injection of large quantities of cells, required in this procedure (Deak et al., 2010; Quimby et al., 2013). However, mechanisms involved in cell-based therapies (paracrine, renotropic, immunomodulatory, anti-inflammatory and antifibrotic activity) are activated after intravascular MSC infusion. In this study, CKD cats with no concurrent illness received two applications of AMSCs (slowly infusions 21 days apart).

No apparent adverse effects were observed after AMSC infusion, and animals recovered immediately after manipulation. The cats were clinically normal, and sedation or anaesthesia was not necessary. One cat experienced vomiting and nausea, but this condition was also reported prior AMSC infusion; however, physical examination parameters were normal and stable. Following second AMSC infusion, this cat did not experience adverse effects.

The general clinical condition of these animals improved greatly. The relevant blood count and blood gases parameters did not change following treatment. Serum creatinine concentration decreased significantly (p -value = .0280) following treatment. The change was evident shortly after first application (7 days) and more pronounced after second AMSC infusion. On the other hand, the UPC ration decreased; although it was not statistically significant, we believe that the variation has clinical relevance. Despite significant reduction in serum creatinine in our study, this parameter is not a very sensitive or specific indicator of renal function or renal impairment. Thus, it is commonly used as measure of kidney function in veterinary practice (Quimby, 2015a). Creatinine is a product of creatinine phosphate catabolism in muscle fairly produced in constant rate. Creatinine values may alter depending on muscle mass, muscle function, muscle composition, activity, diet, dehydration and health status (Gowda et al., 2010; Quimby, 2015a). Moreover, kidney impairment may occur without necessarily change in serum creatinine levels, although this observation is no longer true after the animals reach moderate or severe levels of renal dysfunction (Kerl & Cook, 2005; Quimby, 2015a). Our experimental cats were at II or III stage of CKD, and no significant change in weight was observed. Concurrent improvement in urinary parameters (reduction in proteinuria and increase in urine specific gravity) suggests improvement in renal function. Loss of protein in urine is one of the earliest indicators of almost all renal diseases, and the use of protein/creatinine

ratio has been recommended as an index of quantitative estimation of proteinuria (Gowda et al., 2010). Thus, identification of biomarker for feline CKD that would allow detection of CKD prior serum creatinine and feasibility in daily veterinary practice are recommended (Quimby, 2015a).

Previous studies of MSC application in rodent model of induced CKD reported renal function improvement through intrarenal fibrosis and inflammation reduction (Cavaglieri et al., 2009; Kirpatovskii et al., 2007; Lee et al., 2010; Ninichuk et al., 2006; Semedo et al., 2009; Villanueva et al., 2011, 2013; Wang, He, Pei, & Zhao, 2013). Our findings are as satisfactory as those found in rodent model of induced CKD; however, despite using MSCs with similar therapeutic potential, findings in previous studies in cats with naturally occurring CKD did not report significant variations (Quimby et al., 2011, 2013, 2015).

Cell migration, homing and differentiation are the key steps on cell-based therapy. In rodent models of induced diseases, the period between disease induction and MSCs administration is relatively short and the renal injury is thereby acute not chronic (Quimby & Dow, 2015; Quimby et al., 2013; Semedo et al., 2009). In such condition, inflammation is exacerbated and the changes are not similar with those present in naturally occurring CKD condition (months or years) (Quimby et al., 2013). Severe fibrosis in CKD cats (Lawson et al., 2015; Quimby, 2015b) may greatly hinder cell diffusion and cell homing, limiting their interaction with extra cellular matrix and therefore the differentiation and tissue repair.

Migration of transplanted stem cells towards the lesion site is the first challenge. Authors suggest that renotropic and migration mechanisms of MSCs are activated by proinflammatory cytokines produced by injured tissues and also by intense paracrine activity (Togel et al., 2007). In general, inflammation enhances the process of cell migration.

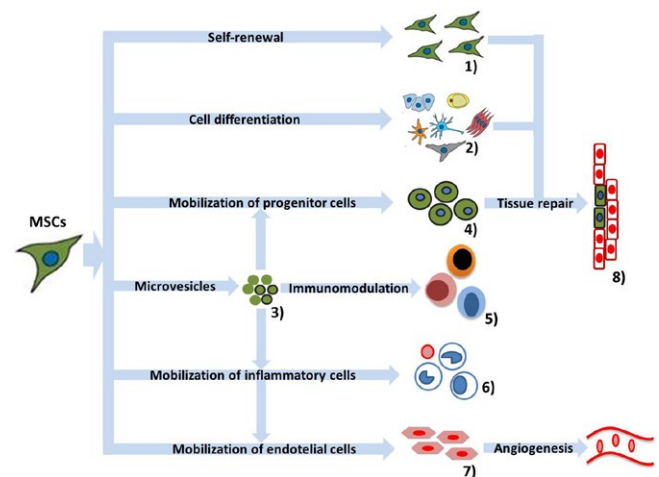


FIGURE 5 Mechanism of MSCs action for tissue repair. MSCs have the ability of self-renewal (1) and ability to differentiate into specialized cell lineages (2) that can directly act on tissue repair. Microvesicles/exosomes transport mRNA, microRNA, protein, membrane receptors, that are important mediators of cell-to-cell communication (3). MSCs activate regulatory progenitor cells (4) immune cells (5), blood cells (6) and endothelial cells (7), which mediated tissue repair (8)

The presence of inflammatory cytokines in CKD cats was observed, and it suggests the presence of intrarenal inflammation, fibrosis and vascular damage in these animals (Habenicht, Webb, Clauss, Dow, & Quimby, 2013; Lawson et al., 2015). Herrera et al. (2007) reported that expression of hyaluronan in injured kidneys might favour cell migration by regulating cell adhesion, proliferation and survival.

Cell adhesion and homing into injured site (kidney) is the second step after MSC infusion. Several forms of environmental signalling and interaction with ECM guide this stage. The end stage (cell differentiation) depends on overall environment signalling, cell–cell contact and paracrine interaction between different cell types (Sohni & Verfaillie, 2013; Vidane et al., 2013). Migration and adhesion of MSCs into injured kidneys in rodents were observed (Semedo et al., 2009); however, severe fibrosis in CKD cats may significantly decline these events.

The route of MSCs delivery is not less important, particularly depends on injury localization and clinical condition of the patient (Quimby & Dow, 2015). In CKD cats, intravenous infusion is considered the eligible route, and it allows interaction between MSCs and endogenous growth factors that favour activation of different mechanisms involved in tissue repair.

By ultrasound, we did not observe changes in kidney architecture and morphology during the evaluation period. Ultrasound examination is not a suitable method to assess kidney morphology compared with histopathology. Nevertheless, we conclude that clinical condition and renal function improvement may not occur following structural modeling, but also secondary mechanisms of MSC actions (Figure 5). Morigi (2004) observed that MSCs migrate towards kidney, adhere and differentiate into tubular epithelial cells, after intravenous infusion. The immunomodulatory activity and immune tolerance are important characteristics attributed to MSCs. It has been reported that MSCs improve kidney function by regulating T cells (Hu et al., 2013), B cells, monocytes, macrophages and other immunomodulator mediators (Insausti, Blanquer, García-Hernández, Castellanos, & Moraleda, 2014; Lange-Consiglio et al., 2013; Sohn & Verfaillie, 2013). Differential count of white blood cells revealed no statistically significant changes of lymphocytes; however, a modest reduction throughout treatment was observed. The lymphocyte reduction was pronounced after second infusion of MSCs. This information is not sufficient for a substantial conclusion, but this observation suggests immunomodulatory activity of AMSCs.

The major finding of our study of AMSC therapy was that clinical parameters showed most significant improvement after second administration of AMSCs. We conclude thereby that AMSCs have a long-term action. Long-term (1-year) follow-up of two experimental animals we observed that renal serum urea and serum creatinine values were significantly lower than values observed before AMSC therapy (animal 3: urea = 61.4 and creatinine = 1.81; animal 4: urea = 45.8 and creatinine = 1.76). All study cats will have a follow-up to evaluate the long-term effects of MSCs on disease progression. Individual analysis revealed that cats in II stage of CKD had more significant improvement than cats in III CKD stage. Thus, we have concluded that it is far better to treat the condition sooner rather than later.

In this study, we evaluated the effects of undifferentiated cultured AMSCs in larger number of experimental cats, and long-term follow-up. However, the use of heterogeneous group and the absence of placebo-controlled group due to patient's scarcity is the weakness of our study. Control groups and repeated infusions are recommended in future trial studies involving AMSC transplantation for CKD in cats.

In this study, we observed that MSCs derived from amniotic membrane of domestic cats have a renoprotective effect and improve renal function in cats with naturally occurring CKD, stabilize the clinical condition and disease progression. The major finding from our study was that AMSCs have a long-term action and repeated infusions (minimum follow-up = 1 year) may be required. Thus, intravenous injection of AMSCs may be an important tool to provide welfare in cats with chronic kidney disease. In contrast, bilateral intrarenal injection of AMSCs for CKD therapy in cats is unlikely to be a suitable option due to certain complications caused by sedation, anaesthesia, haemorrhage and stress.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

AS Vidane, AO Pinheiro and CE Ambrósio involved in experimental conception and design, technical procedures, acquisition of data, analysis and interpretation of data, manuscript production and critical revision of manuscript. D Passarelli and MCFNS Hage involved in technical procedures, analysis and interpretation of data, and critical revision of manuscript. JB Casals involved in technical procedures and acquisition of data. DS Martins involved in experimental conception and design, and critical revision of manuscript. RS Bueno involved in analysis and interpretation of data and critical revision of manuscript.

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