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Research paper

Safety and immunomodulatory properties of equine peripheral blood-derived mesenchymal stem cells in healthy cats

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ABSTRACT

Objective: Due to the immunomodulatory properties of mesenchymal stem cells (MSCs) through stimulation of endogenous immune cells by paracrine signals and cell contact, they have been proposed as alternative treatment option for many inflammatory and immune-mediated diseases in veterinary medicine. However, the long-term cultivation possibilities of feline MSCs are currently compromised due to a restricted proliferation capacity. Therefore, the xenogeneic use of equine peripheral blood-derived MSCs (ePB-MSCs) would present an interesting alternative thanks to their superior cultivation properties. To the authors' knowledge, there are currently no safety reports concerning the xenogeneic use of ePB-MSCs in cats. Therefore, the overall goal of this preliminary study was to investigate if ePB-MSCs can safely be administered in healthy cats and by extension evaluating their immunogenic and immunomodulatory properties.

Methods: Ten healthy cats were intravenously (i.v.) injected with 3×10^5 ePB-MSCs at three time points (T_0, T_1, T_2). All cats were daily inspected by the caretaker and underwent a physical examination with hematological and biochemical analysis at day 0 (T_0), week 2 (T_1), week 4 (T_2) and week 6 (T_3) by a veterinarian. Furthermore, a modified mixed lymphocyte reaction (MLR) was performed at T_0 and T_3 for each cat in order to evaluate immunogenic and immunomodulatory properties of the ePB-MSCs

Results: No adverse clinical effects could be detected following repeated i.v. administration of ePB-MSCs in all cats. Significant lower protein (T_1: P-value = 0.002; T_2: P-value > 0.001; T_3: P-value = 0.004) and albumin levels (T_1: P-value = 0.003; T_2: P-value = 0.001) were seen after repeated administration of ePB-MSCs, compared to T_0. However, all biochemical and hematological parameters stayed within clinical acceptance level. In addition, the repeated injections did not induce a cellular immune response before and after repeated ePB-MSCs administration. Furthermore, convincing immunomodulatory properties of ePB-MSCs on feline peripheral blood mononuclear cells were confirmed in the MLR-assay

Conclusion: This preliminary study demonstrates that ePB-MSCs can safely be administered in healthy cats and provide a promising alternative for the treatment of various inflammatory diseases in cats.

1. Introduction

In recent years, mesenchymal stem cells (MSCs) have been proposed as an alternative treatment for many inflammatory and immune-mediated diseases in both human and veterinary medicine (Webb et al., 2012). MSCs are plastic-adherent cells with high proliferation capacity

Abbreviations: 7-AAD, 7-aminoactinomycine D; AB, antibiotics; AM, antimycotica; BM, bone marrow; BME, β-mercapto-ethanol; CFSE, carboxyfluorescein succinimidyl ester; CKD, chronic kidney disease; ConA, concanavalin A; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethylsulfoxide; ePB-MSCs, equine peripheral blood-derived MSCs; FBS, fetal bovine serum; FCGS, feline chronic gingivostomatitis; FFV, feline foamy virus; MLR, mixed lymphocyte reaction; MSCs, mesenchymal stem cells; PBMCs, peripheral blood mononuclear cells; SDMA, serum symmetrical dimethylarginine; SPF, specific pathogen free

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which are able to differentiate in various mesenchymal cell types, including osteocytes, chondrocytes, adipocytes and myocytes. Due to their immunomodulatory properties, the use of MSCs has also been focused on their ability to modulate inflammation rather than regeneration of damaged tissues (Murray and Péault, 2015; Clark et al., 2017).

In cats, the first isolation and characterization of bone marrow (BM) derived MSCs was described in 2002, by Martin et al. Martin et al. (2002) and were shown to present comparable phenotype and immunomodulatory profile to human MSCs (Clark et al., 2017). Although BM represents an excellent source of MSCs, the invasive harvesting process often requires anesthesia. Therefore, similar to other species, less invasive tissue sources of MSCs have been investigated in cats. Adipose tissue (Webb et al., 2012), fetal fluids and membranes (Iacono et al., 2012) and peripheral blood (Sato et al., 2016), all showed similar morphology to BM derived MSCs (Sato et al., 2016). However, the use of feline autologous MSCs has proven to be difficult because up to 50 % of the feline MSCs develop into giant foamy multinucleated cells in later passages (from P5), leading to proliferation arrest. Arzi et al. (2015) hypothesized this was due to an infection with the feline foamy virus (FFV), which is present in 20–80 % of the cats without clinical signs (Arzi et al., 2015). Next to this safety issue concerning the use of cell lines with an active viral infection, autologous MSCs comprise the need of harvesting tissue of an already compromised patient. Therefore, the use of allogeneic MSCs derived from specific pathogen free (SPF) cats presented a better option. Moreover, as in other veterinary species, feline MSC quality declines with age (Zajic et al., 2017), so the use of allogeneic MSCs allows a strict donor selection to ensure high quality stem cells are being selected and can be used immediately "off-the-shelf" (Quimby and Borjesson, 2018).

To date, the therapeutic use of allogeneic MSCs has shown promising results in several feline inflammatory diseases. In this regard, the i.v. injection of allogeneic adipose-derived MSCs in cats diagnosed with feline chronic gingivostomatitis (FCGS) has shown to result in complete clinical and histological resolution or reduction in clinical disease severity and immune modulation in most cats (Arzi et al., 2017). Furthermore, the potential use of allogeneic MSCs as treatment strategy for chronic kidney disease (CKD) has been investigated by several research groups. Vidane et al. (2017) described a positive effect on the symptoms and progression of CKD using allogeneic amniotic membrane-derived MSCs (Vidane et al., 2017), however, other studies using allogeneic adipose-derived MSCs did not support these findings (Rosselli et al., 2016; Quimby et al., 2016). Pilot studies of Trzill et al. (2015) and Webb et al. (2015) with repeated administration of allogeneic adipose-derived MSCs, have described promising results in the treatment of asthmatic cats and chronic enteropathy, respectively (Trzil et al., 2014; Webb and Webb, 2015). Finally, allogeneic adipose-derived MSCs have been shown to be safe and effective in the treatment of feline eosinophilic keratitis (Villatoro et al., 2018).

Nevertheless, the use of allogeneic feline MSCs is still restricted because of their limited proliferation capacity compared to equine and human MSCs and the financial and practical challenge of finding and housing SPF cats (Clark et al., 2017; Arzi et al., 2017). Therefore, the xenogeneic use of equine peripheral blood-derived MSCs (ePB-MSCs) would present an interesting treatment alternative for above mentioned diseases, as tissue harvesting from easily available healthy donor horses provides an effective manner to produce MSCs. Furthermore, xenogeneic MSCs have the advantage of being free of transferrable highly virulent species-specific pathogens. A recent study of Daems et al. (2019) described already the feasibility and safe use of xenogeneic ePB-MSCs in the treatment of naturally occurring osteoarthritis in dogs (Daems et al., 2019). However, to the authors' knowledge, there are currently no safety reports concerning the xenogeneic use of ePB-MSCs in cats.

The overall goal of this preliminary study was to investigate the safety of repeated administrations of ePB-MSCs in healthy cats. Therefore, effects on general physical condition and the hematological parameters were evaluated. Furthermore, their immunogenicity and immunomodulatory properties before and after multiple i.v. administrations were examined using a modified mixed lymphocyte reaction (MLR) assay.

2. Materials & methods

2.1. Animals

This animal study was approved by the ethics committee of Global Stem cell Technology (approval number EC: 2018_001, permit number: LA1700607). All animal handlings were conducted according to European, national and regional regulatory requirements and in compliance with Directive 2010/63/EU. Ten healthy privately-owned cats of different breeds (European shorthair, European longhair and Maine Coon) were included in this study (mean age: 6 ± 4 years; 4 males, 6 females). The sample size was calculated based on previous MLR experiments by our research group (data not published) using G*power software, resulting in an effect size (f) = 2.86. Assuming $\alpha = 0.05$ and $1\text{-}\beta = 0.95$ a sample size of 9 was needed. Taking into account the potentially loss of one blood sample a sample size of 10 cats was necessary for the safety evaluation. Blood samples of the cats were collected by a licensed veterinarian and an informed consent was signed by the owner. During the study, all cats stayed with the owner under its care and protection.

2.2. Isolation and cultivation of equine peripheral blood-derived mesenchymal stem cells (ePB-MSCs)

The ePB-MSCs were produced according to GMP-guidelines in a GMP-certified site (number: BE/GMP/2018/123). They were isolated from venous blood collected from the *vena jugularis* of one single donor horse (approval number EC: EC_2012_001 and 2016_003) according to previously described methods (Broeckx, 2012). Serum was tested on multiple transmittable diseases by Böse laboratory before culturing (Harsum, Germany). Subsequently, the ePB-MSCs were cultured until passage (P)5 and thoroughly characterized (i.e. viability, morphology, presence of cell surface markers, and population doubling times) as previously described (Spaas et al., 2013), prior of being frozen as intermediate cell stock. After characterization, cells were thawed and further cultivated until P10. Consequently, the ePB-MSCs were trypsinized, resuspended at final concentration of 3×10^5 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) low glucose with 10 % dimethylsulfoxide (DMSO) and stored at −80 °C in cryovials until further use. The ePB-MSCs were immunophenotypically characterized by evaluating the presence (CD29, CD44 and CD90) and absence (MHC II and CD45) of specific cell surface markers using flow cytometry as previously described (Spaas et al., 2013). The cell viability was assessed using trypan blue before freezing the cells. Sterility of the final product was tested by the absence of aerobic bacteria, anaerobic bacteria, fungi, endotoxins and mycoplasma.

2.3. Injection, general physical examination and hematology

All cats underwent a general physical examination by a veterinarian at day 0 (T_0), week 2 (T_1), week 4 (T_2) and week 6 (T_3) consisting of the assessment of rectal temperature, heart rate, respiratory rate, mucosal membranes appearance and capillary refill time. At the same time, blood samples (3 mL) were collected for hematological and biochemical analysis. At T_0 and T_3, a larger blood volume (6 mL) was collected to perform the MLR assay. At T_0, T_1 and T_2, after the general physical examination and blood collection, cats were intravenously (i.v.) injected with 3×10^5 ePB-MSCs. After thawing the cryovial in the palm of a hand, the content was checked for transparency and clearness and the cell suspension was immediately injected using a 22 G i.v. catheter.

Table 1
Hematological and biochemical parameters at T_0, T_1,T_2 and T_3 together with their reference interval.

Parameter	Unit	Reference interval	T_0 (mean ± SD) (min-max)	T_1 (mean ± SD) (min-max)	T_2 (mean ± SD) (min-max)	T_3 (mean ± SD) (min-max)
Erythrocytes	million/μL	5.00 – 10.00	8.38 ± 1.06 (5.96–10.63)	7.91 ± 1.20 (6.35–10.34)	8.12 ± 1.28 (6.62–10.41)	7.79 ± 1.08 (6.26–9.80)
Hemoglobin	g/dL	8.3 – 17.3	12.0 ± 1.2 (7.8–14.6)	11.0 ± 1.6 (9.3–13.8)	11.2 ± 1.6 (9.5–14.4)	8.70 ± 0.44 (8.70–12.8)
Hematocrit	%	27.0 – 47.0	37.6 ± 5.71 (23.0–49.3)	32.7 ± 4.8 (27.0–41.0)	33.6 ± 4.6 (29.0–43.0)	32.2 ± 4.4 (26.0–39.0)
MCV	fL	39.0 – 55.0	45.2 ± 6.8 (36.4–61.5)	41.5 ± 2.7 (37.1–45.0)	41.6 ± 2.9 (36.7–46.8)	41.6 ± 3.4 (36.7–46.7)
MCH	g/dL	12.0 – 18.0	14.4 ± 0.9 (12.3–15.6)	14.0 ± 1.0 (12.1–15.3)	13.9 ± 1.0 (12.3–15.4)	13.8 ± 1.0 (12.3–15.4)
MCHC	g/dL	30.0 – 36.0	32.2 ± 3.4 (23.3–33.9)	33.8 ± 0.5 (32.8–34.4)	33.3 ± 0.3 (32.8–33.9)	33.2 ± 0.4 (32.8–33.8)
Leukocytes	μL^{-1}	5000 – 19500	10283 ± 2794 (4060–13740)	9208 ± 2638 (5880–13910)	9216 ± 1952 (6580–12030)	8406 ± 1843 (5950–10620)
Neutrophil bars	Abs.	< 300	0	37 ± 74 (0–229)	0	0
Neutrophil segments	Abs.	2500 – 12500	5829 ± 2663 (1624–11365)	5227 ± 2445 (2799–10850)	5191 ± 1217 (4248–8180)	4068 ± 1340 (2530–6546)
Eosinophils	Abs.	< 1500	343 ± 247 (81–748)	416 ± 348 (96–1006)	512 ± 625 (0–2148)	614 ± 670 (0–2198)
Basophils	Abs.	< 100	0	0	0	0
Lymphocytes	Abs.	1500 – 7000	4293 ± 2113 (2031–8107)	3665 ± 1552 (1947–5959)	3527 ± 1330 (1513–6021)	3566 ± 1279 (893–5700)
Monocytes	Abs.	< 850	224 ± 111 (41–445)	185 ± 138 (59–497)	253 ± 160 (90–602)	157 ± 93.3 (60.0–304)
Platelets	x 1000/μL	180 – 430	304 ± 182 (38–525)	338 ± 182 (35–554)	414 ± 157 (160–627)	297 ± 188 (34–606)
Creatinine	mg/dL	0.20 – 1.40	1.59 ± 0.36 (1–1.96)	1.43 ± 0.34 (0.96–1.97)	1.52 ± 0.28 (0.89–1.87)	1.37 ± 0.27 (0.91–1.67)
Urea	mg/dL	40.0 – 70.0	52.6 ± 8.6 (43.2–64.7)	49.6 ± 10.4 (39.3–69.1)	51.0 ± 7.0 (40.4–60.1)	50.1–7.3 (40.3–63.5)
Total protein	g/L	60 – 85	71 ± 4 (64–76)	68 ± 3* (60–73)	67 ± 2* (64–70)	67 ± 3* (61–70)
Albumin	g/L	23.6 – 46.6	33.7 ± 3.1 (26.4–39.3)	32.7 ± 3.6* (25.1–36.9)	32.4 ± 3.1* (25.9–36.5)	33.5 ± 4.0 (26.0–39.1)
Total bilirubin	mg/dL	< 0.40	< 0.15	< 0.15	< 0.15	< 0.15
Bilirubin directly	mg/dL	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
Bilirubin indirectly	mg/dL	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
GOT (AST)	U/L	< 83	43 ± 13 (27–68)	30 ± 7 (19–39)	34 ± 11 (21–57)	27 ± 6 (19–37)
GPT (ALT)	U/L	< 91	45 ± 8 (31–68)	41 ± 9 (31–54)	48 ± 13 (33–70)	44 ± 18 (29–77)
Gamma-GT	U/L	< 10	< 3	< 3	< 3	< 3
Alkaline phosphatase	U/L	17 – 63	28 ± 18 (5–69)	30 ± 14 (13–51)	30 ± 13 (14–48)	29 ± 15 (14–53)

*significant difference compared to T_0 ($P < 0.05$).

After injection all cats were clinically monitored for any adverse events (such as fever, increased respiratory rate, reduced feed uptake, vomiting, diarrhea or other signs of illness) by an experienced veterinarian for 30 min. Consequently, the cats were handed over to the caretaker and were monitored on a daily basis. An adverse event form was present for documenting adverse events and the veterinarian would be contacted to discuss the plan of action.

2.4. Modified mixed lymphocyte reaction (MLR)-assay

The modified MLR assay was used to investigate whether or not the ePB-MSCs induce a cellular immune response (immunogenicity) in the cats and by extension to evaluate their immunomodulatory properties by co-incubating the ePB-MSCs with stimulated responder peripheral blood mononuclear cells (PBMCs). Therefore, a modified MLR assay was performed using PBMCs isolated from the fresh blood of each cat at T_0 and T_3.

For the MLR experiment, ePB-MSCs from the same batch as the ones that were used for injection, were seeded at 2×10^4 MSCs/well in a 96-well plate in a humidified incubator at 37 °C with 5 % CO_2. PBMCs were isolated from 6 mL venous blood from each cat and diluted with Hank's Balanced Salt Solution (HBSS). The diluted blood was layered on an equal volume of Percoll and the interphase was collected after gradient centrifugation. After washing the cells with HBSS, the PBMCs were diluted in HBSS to a final concentration of 1×10^6 cells/mL. Consequently, the PMBCs were labelled with carboxyfluorescein succinimidyl ester (CFSE) according to manufacturers instructions (CFSE, Life Technologies) in order to evaluate cell proliferation. Finally, the PBMCs were diluted in MLR medium (culture medium supplemented with (fetal bovine serum (FBS), antibiotics/antimycotica (AB/AM) and β-mercapto-ethanol (BME)) to a final concentration of 2×10^6 cells/mL and 100 μL was added to the designated wells of the 96-well plate at a ratio of 1:10 MSCs/PBMCs ($= 2 \times 10^4/2 \times 10^5$) to test the immunogenicity of the MSCs. Immunomodulatory properties of the ePB-MSCs were assessed by co-incubating them with stimulated PBMCs. The PBMCs stimulation was performed using concanavalin A (ConA, 5 μg/mL, Sigma Aldrich). As positive control, PMBCs stimulated with ConA without addition of ePB-MSCs were added to the 96-well plate. The negative control sample consisted of PMBCs alone. Finally, the 96-well plate was incubated for 4 days at 37 °C with 5 % CO_2 containing MLR medium.

After incubation, the PBMC proliferation (%) was measured using flow cytometry analysis (BD FACSCanto II, BDbiosciences, US). All samples were transferred from the 96-well plate to FACS tubes and pelleted before staining for cell viability with 7-aminoactinomycine D (7-AAD) at 1:100 (BioLegend, US). Viable PBMCs were gated on 7-AAD after forward-, side scatter and double doublet discrimination. Consequently, proliferation (%) of the viable PBMC population for every FACS tube was evaluated using the CFSE staining.

2.5. Statistical analysis

In order to compare hematological and biochemical blood levels, a mixed effects model with cat as random effect and time as categorical fixed effects factor was used. Time points T_1, T_2 and T_3 were compared with time point T_0 using Dunnett's multiple comparisons technique. Log transformed data were used for cell counts, and normal scale for others. Significance level was set at 0.05. PBMC proliferation (%) was analyzed using the non-parametric Wilcoxon signed rank test as data were not normally distributed. A Bonferroni adjustment was applied to adjust for multiple comparisons, setting the significance level at $0.05/3 = 0.0167$.

3. Results

3.1. Physical examination and hematology

Based on the above described physical assessments, no abnormalities could be noticed by the veterinarian or the caretaker of the cats after the injection with ePB-MSCs at all time points (daily examination or T_1, T_2 and T_3). Furthermore, no adverse events related or unrelated to the injections were reported during the study.

The effects of the i.v. injections with ePB-MSCs on the hematological and biochemical parameters at each time point are summarized by

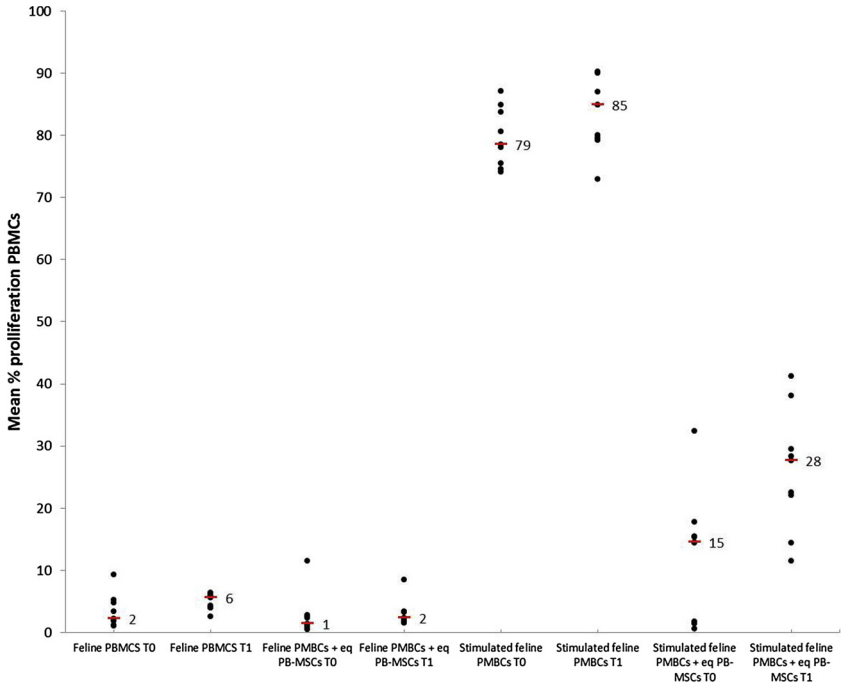

Fig. 1. Mixed lymphocyte reaction (MLR) assay at T_0 and T_3. Individual PBMC proliferation (%) derived from 9 of the 10 cats (black dots) and median value (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

mean ± SD and min-max values in Table 1. All parameter means were within the reference interval except for the creatinine level. Mean creatinine values were higher than the reference value (< 1.4 mg/dL) at each time point during the study, however, creatinine values did not increase significantly for any patient at all time point post-injection compared to T_0. When considering the individual values of all other parameters, values were within clinically acceptable limits. Pairwise comparisons showed that the total protein level was significantly lower at T_1 (p-value = 0.002), T_2 (p-value > 0.001) and T_3 (p-value = 0.004) compared to T_0. Moreover, significant lower albumin levels were seen at T_1 (p-value = 0.003) and T_2 (p-value = 0.001) compared to T_0. Other hematological and biochemical parameters did not change significantly after i.v. injections with ePB-MSCs at T_1, T_2 and T_3 (Table 1).

3.2. MLR assay

Individual results of the MLR assay are shown in Fig. 1 as PBMC proliferation (%). Due to an extreme low number of events ($< 1 \times 10^3$) during the FACS analysis of the samples of one cat at T_0, the results of this particular cat were excluded from the data analysis for each time point. Therefore, the results of the MLR assay were based on nine cats instead of ten.

3.2.1. Immunogenicity

The addition of ePB-MSCs to feline PBMCs did not lead to a significant increase in PBMC proliferation (median (min-max)) at T_0 (1.4 % (0.5–11.5 %)) and T_3 (2.3 % (1.6–8.5 %)) compared to the negative control at T_0 (2.2 % (1.0–9.3 %)) (p-value = 0.343) and T_3 (5.6 % (2.5–6.4 %)) (p-value = 0.05). For both time points, the co-culture of ePB-MSCs and feline PBMCs was significantly lower than the associated positive control at T_0 (78.6 % (74.1–87.2 %)) (p-value = 0.008) and T_3 (85.0 % (73.0–90.3 %)) (p-value = 0.008). Interestingly, when co-culturing ePB-MSCs with feline PBMCs of cats who received three i.v. injections with ePB-MSCs (T_3) (2.3 % (1.6–8.5 %)), no significant increase in mean proliferation could be seen compared to T_0 (1.4 % (0.5–11.5 %)) (p-value = 0.109) (Fig. 1).

3.2.2. Immunomodulatory

At T_0, the proliferation of the co-culture of ePB-MSCs with stimulated feline PBMCs was not significantly different compared to the associated negative control (2.2 % (1.0–9.3 %)) (p-value = 0.05). In contrast, at T_3 (27.7 % (11.5–41.2 %)) a significant difference was found compared to the associated negative control (5.6 % (2.5–6.4 %)) (p-value = 0.008). Moreover, the proliferation of the co-culture was significantly lower than the positive control at T_0 (78.6 % (74.1–87.2 %)) (p-value = 0.008) and T_3 (85 % (73.0–90.3 %) (p-value = 0.008)). No significant difference in mean PMBC proliferation could be found in the co-culture of ePB-MSCs with stimulated feline PBMCs at T_3 (27.7 % (11.5–41.2 %)) compared to T_0 (14.6 % (0.6–32.4 %)) (p-value = 0.017) (Fig. 1).

4. Discussion

To the best of our knowledge, this is the first study to describe the effects of repeated injections of equine PB-derived MSCs on the hematological status of cats and to assess their immunogenicity and immunomodulation properties before and after repeated injections.

During the study all cats were in good health and at all time points no adverse effects on their physical condition could be detected following the injections with ePB-MSCs. Furthermore, after communication with the caretaker of the cats, no adverse effects were noticed during the entire study. Concerning the hematological and biochemical parameters, all means of the blood parameters were within the reference interval except for the mean creatinine levels, which were higher than the reference interval at each time point. Nevertheless, this mildly increased creatinine concentration was already present at baseline condition (T_0) and did not significantly increase for any cat at all post-treatment time points. Therefore this could not be assigned to the administration of the ePB-MSCs and was an existing abnormality in the cat population. This could potentially be caused by dehydration or an early stage renal insufficiency. However, because no clinical signs of kidney problems were detected, we can assume this increase was not clinically relevant. In order to improve determination of the feline renal status and exclude potential chronic kidney disease, urinalysis and

determination of serum symmetrical dimethylarginine (SDMA) could be added in future studies.

Even though all other investigated parameters were within clinically acceptance level, a significant decrease in total protein was found at T_1, T_2 and T_3, as well as for albumin levels at T_1 and T_2, compared to T_0. To the best of our knowledge, findings of lower protein and albumin levels in blood after MSCs therapies have never been reported in cats. However, these results were found in healthy cats and the impact of ePB-MSCs on total protein and albumin levels in blood should be further investigated in future studies using cats with different inflammatory diseases. Furthermore, a trend in lymphocyte decrease and platelet increase could be seen at T_1 and T_2 compared to T_0, but missed significance due to high variability. The lymphocyte decrease is in line with the *in vitro* immunomodulatory findings, which are also more pronounced when lymphocytes are stimulated. Further research in patients with increased lymphocyte levels should confirm this finding. The platelet increase is in accordance with a previous reported study by Broeckx et al. (2013) 24 h after a single injection with allogeneic ePB-MSC in horses, and was assigned to bone marrow activation within the acceptor horses after allogeneic ePB-MSC administration (Broeckx and Forier, 2013). However, the current feline blood collection was performed two weeks after each MSCs therapy and could explain why significance was missed. Therefore, a blood collection 24 h post-injection of ePB-MSCs should be added in future feline studies. Overall, from a clinical and hematological point of view, repeated injections (i.v) of xenogeneic ePB-MSCs in cats can be considered safe and potentially effective for patients with inflammatory diseases.

In the modified MLR assay, immunogenicity of the ePB-MSCs in cats was tested using feline PBMCs derived before (T_0) and after repeated injections (T_3). When co-culturing ePB-MSCs with feline PBMCs, no cellular immune response was detected. This was demonstrated by a comparable mean proliferation of the negative control sample. These results clearly indicate that ePB-MSCs can be repeatedly injected without inducing a cellular immune response.

Furthermore, various *in vitro* immunomodulatory properties of MSCs have been described in literature, such as suppression of the immune system and lymphocytes regulation (Kode et al., 2009). Therefore, the immunomodulatory properties of ePB-MSCs on stimulated feline PBMCs were also investigated. The co-culture of ePB-MSCs with stimulated feline PBMCs resulted in a large immunosuppression before and after repeated injections, which was demonstrated by a significant lower mean proliferation of the co-culture compared to the positive control at both time points. To date, numerous feline studies have already demonstrated the immunomodulatory properties of both autologous and allogeneic feline MSCs and showed similar immunomodulatory phenotype to human-, equine- and canine MSCs (Clark et al., 2017; Zajic et al., 2017; Arzi et al., 2017; Parys et al., 2017; Arzi et al., 2016). However, to the best of our knowledge, this is the first study to describe the immunomodulatory properties of xenogeneic ePB-MSCs in cats after repeated injections. Results of the current study showed superior immunomodulatory properties of xenogeneic ePB-MSCs on feline PBMCs proliferation compared to previously reported results with allogeneic feline MSCs (Clark et al., 2017; Zajic et al., 2017). Clark et al. (2017) and Zajic et al. (2017) have reported a median decrease in PBMC proliferation of 50 % (positive control (100 %) – co-culture (50 %)) and 47 % (100 % - 53 %), respectively, after co-culturing stimulated feline PBMCs with the MSCs. In contrast, our study showed a median decrease in PBMC proliferation of 64 % (79 % - 15 %) at T0.

Their safe use, based on clinical and blood parameters, along with the significant immunomodulatory properties, provides us with a strong rationale for the xenogeneic use of ePB-MSCs in the treatment of various infectious diseases in cats and other species for that matter. This would serve as a more efficient and potentially effective alternative compared to the currently used allogeneic MSCs in cats. Nevertheless, future studies using these ePB-MSCs in feline infectious diseases will have to be performed to confirm their treatment efficacy.

In conclusion, to the authors' knowledge, this is the first study to evaluate the safety and immunomodulatory properties of xenogeneic ePB-MSCs in cats before and after repeated administration. No adverse clinical, hematological and biochemical effects could be detected. In addition, the repeated injections did not induce a cellular immune response. Furthermore, strong immunomodulatory properties of the xenogeneic ePB-MSCs were confirmed in the MLR-assay. These promising results prove that the xenogeneic use of ePB-MSCs in cats provides a very interesting and cost-effective alternative for the treatment of various feline infectious diseases.

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Declaration of Competing Interest

The author JHS declares competing financial interests as shareholder in Global Stem cell Technology (GST) NV. ED, GP, CB, LVH, LT and JHS are all employed by GST. JHS is an inventor of a pending patent covering the described immunomodulating technology owned by GST (EP19162270.3). The other authors declare no competing interests. The content of this manuscript contains a stem cell product under development owned by GST. YD is the owner of 'Veterinary Dental Services', a Veterinary practice specialized in dentistry. LV is the owner 'Dier en tand', a Veterinary clinic specialized in dentistry.

Author contributions

Authors YD, LVH and JHS conceived the study and planned the design. MSCs administration, animal handling and blood sampling was performed by YD and CB. The modified MLR was conducted by ED and LT. Statistical analysis was done by LD and GP. YD and GP managed the literature research and wrote the first draft of the manuscript. LV, JS and JHS provided supervision and critical review of the manuscript. All authors contributed and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2020.110083.

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