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Safety of Intraperitoneal Injection of Adipose Tissue-Derived Autologous Mesenchymal Stem Cells in Cats

M. Parys, N. Nelson, K. Koehl, R. Miller, J.B. Kaneene, J.M. Kruger, and V. Yuzbasiyan-Gurkan

Background: Chronic inflammatory diseases are common in cats and mesenchymal stem cells (MSC) are a promising therapeutic approach for management of these disorders. The purpose of this study was to evaluate the safety of intraperitoneal injection of MSC in cats.

Hypothesis: Intraperitoneal injection of autologous MSC in cats is safe.

Animals: Ten healthy adult purpose-bred cats.

Methods: Mesenchymal stem cells were isolated from subcutaneous adipose tissue collected during ovariohysterectomy and characterized for expression of CD90, CD105 and CD44 and trilineage differentiation. Three weeks postoperatively a complete blood count, serum chemistry profile, urinalysis, and abdominal ultrasound were performed. Five cats then received 1 x 10^6 of autologous MSC/kg of body weight intraperitoneally with ultrasound guidance; 5 additional cats were sham injected. Cats were monitored for 6 weeks with daily physical examinations and weekly clinicopathological evaluations. Abdominal ultrasonography was repeated at weeks 1 and 5 after injection.

Results: Serious adverse effects were not observed in any MSC-injected cat. Two animals developed transient lethargy and decreased activity. Jejunal lymph node size was increased in MSC-injected cats compared to controls at weeks 1 (1.38 ± 0.25 versus 0.88 ± 0.25 cm^2; P = .036) and 5 (1.75 ± 0.82 versus 0.79 ± 0.12 cm^2; P = .047). A hyperechoic renal segmental cortical lesion was observed in 1 MSC-injected cat.

Conclusions and Clinical Relevance: Intraperitoneal MSC injection was well tolerated with only mild, self-limiting adverse effects being observed in 2 cats. This route provides a safe means of administration for cell-based treatment in cats.

Key words: Feline; Inflammatory disease; Regenerative medicine; Route of injection.

A
dministration of mesenchymal stem cells (MSC) has been recently proposed as an alternative therapeutic modality for management of chronic inflammatory conditions in cats and other species.\(^1\)-\(^3\) MSC express a myriad of factors such as IDO, IL10, HGF, PGE\(_2\), TGF-beta, IL6, PD-L1, FASL or HMOX\(_1\)\(^4\)-\(^15\) and affect the immunomodulating properties of many types of immune cells.\(^12\)-\(^16\)Interestingly, MSC are capable of inducing T-regulatory cells, which in turn can provide a long-lasting immunomodulation.\(^21\) In vitro experiments suggest that MSC need to be in close proximity to target cells and in sufficient quantity to induce a therapeutic effect.\(^11\),\(^13\),\(^14\),\(^18\),\(^22\) In addition, in vivo experiments also suggest paracrine effects of MSCs on other cells are also important.\(^3\),\(^5\),\(^23\),\(^24\)

Multiple routes of injection of MSC are described and include intravenous (IV), intraperitoneal (IP) as well as intra-articular, cardiac, hepatic or nasal.\(^1\),\(^2\),\(^4\)-\(^27\)

IV injection is a frequently reported route of MSC administration for immunomodulation.\(^1\),\(^2\),\(^15\),\(^22\),\(^24\)-\(^29\)

However, IV administration of MSC has potential limitations for treatment of inflammatory conditions localized to the peritoneal cavity. Exposure of IV administered MSC to plasma complement reduces cell survival.\(^30\) Retention of MSC in pulmonary vasculature reduces the number of cells reaching the target and increases the risk of pulmonary microthrombus forma-

Abbreviations:

MSC mesenchymal stem cells
IP intraperitoneal
IDO indoleamine 2,3-dioxygenase
IL10 interleukin-10
HGF hepatocyte growth factor
PGE\(_2\) prostaglandin E\(_2\)
TGF-beta transforming growth factor beta
IL6 interleukin-6
PD-L1 programmed death ligand 1
HMOX\(_1\) heme-oxygenase 1
IBD inflammatory bowel disease
CK creatine kinase
IBMR instant blood mediated inflammatory reaction
CBC complete blood count
APC allopurinol
PE phycoerythrin
FITC fluorescein isothiocyanate
FLR fluorescence intensity

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tion. Pulmonary thrombosis is a potential serious complication and might increase morbidity and mortality. Furthermore, instant blood mediated inflammatory reaction (IBMIR) occurs after injection of MSC and can be cause of decreased cell survival and increased risk of thromboembolism. Thus, IV injection can potentially limit the availability of MSC and their migration capabilities, decrease the overall cell survival, and increase risk of adverse effects.

Intraperitoneal injection is potentially a safer and more effective route of MSC administration for disorders within the abdominal cavity. IP-injected MSC are placed in close proximity to the target organ and could reach the target in greater numbers and subsequently effect a better outcome. IP injection is simple to perform and has the promise of widespread adoption in clinical settings and high impact on patient management. IP injection of MSC was significantly more effective than IV administration for ameliorating clinical signs in a rodent model of inflammatory bowel disease. MSC administered IP have been studied also for their regenerative potential and ability to deliver gene treatment.

The safety of IP injection of MSC has not been studied in the cats. This study was undertaken to test the hypothesis that IP injection is a safe route of autologous MSC administration in cats.

Materials and Methods

Animals

Ten purpose-bred mixed breed, intact female cats were obtained from the MSU Comparative Ophthalmology Cat Colony and from a licensed commercial vendor, 5 from each source. The mean age (±SD) at the time of injection of the cats was 20.7 (±5.7) months and the mean (±SD) weight was 3.79 (±0.5) kg. Animals were housed under standard conditions at the Michigan State University College of Veterinary Medicine Vivarium, fed a commercial diet and received environmental enrichment during the study period. Cats were determined to be healthy on the basis of physical examination. The study was approved by the Michigan State University Institutional Care and Animal Use Committee.

Isolation and Characterization of MSC

Subcutaneous adipose tissue (2–4 g) from the ventral midline abdomen was collected during ovariohysterectomy was placed in KNAC medium (Keratinocyte SFM medium supplemented with 2 mM of N-acetyl-L-cysteine and 0.2 mM of L-ascorbic acid 2-phosphate) with 5% of MSC-grade FBS in 50 mL tube for transfer to the laboratory for processing. Each tissue sample was finely minced with sterile scalpel blade and incubated in 1 mg/mL of Collagenase I d in HBSS for 2 hours at 37°C. FBS d and received environmental enrichment during the study period. Cats were determined to be healthy on the basis of physical examination. The study was approved by the Michigan State University Institutional Care and Animal Use Committee.

The result of IP injection of MSC was not studied in the cats. This study was undertaken to test the hypothesis that IP injection is a safe route of autologous MSC administration in cats.

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Adherent cells were characterized for cell surface expression of CD90, CD105, CD44, MHCII and for differentiation into osteocytes, adipocytes, and chondrocytes. Cell membrane marker expression was analyzed by flow cytometry and analyzed using commercial software. Differentiation of cells was achieved through use of commercial osteogenesis, adipogenesis, and chondrogenesis kits, according to manufacturer’s recommendations. For osteogenesis and adipogenesis experiments, 1 × 104 and 1 × 106 cells per well were plated in 24-well tissue culture plates containing KNAC medium with 5% FBS and incubated at 37°C for 24 hours to allow cells to attach. After incubation, the medium was changed to the specific differentiation medium and cultured for 3 weeks in 37°C with 5% CO2 with medium being changed twice a week. Chondrogenic differentiation was accomplished through culture of at least 1 × 103 cells in micromass. Cells were re-suspended in 1 mL of cell culture medium and spun down at 325 × g for 5 minutes. Subsequently, the medium was carefully exchanged with chondrogenesis medium, in order not to disrupt the pellet. Cells were then cultured for 3 weeks at 37°C with 5% CO2, with semiweekly medium changes. After 3 weeks of differentiation, the cells were stained with Alizarin Red to assess osteogenetic differentiation, Alcian Blue to assess chondrogenesis and Oil-o-Red stain to assess adipogenesis.

Procedures

All cats underwent a routine ovariohysterectomy, at which time a 2–4 g sample of ventral midline subcutaneous fat was obtained and processed for MSC isolation and propagation. For ovariohysterectomy, animals were sedated with acepromazine (0.05 mg/kg) and buprenorphine (10 mcg/kg), anesthetized with isoflurane (0.25–3% isoflurane in 100% oxygen in an induction chamber). For subsequent examinations (ultrasound, blood draw, cystocentesis), all cats were sedated or anesthetized for all procedures with either acepromazine (0.05 mg/kg) and buprenorphine (10 mcg/kg) or isoflurane (0.25–3% isoflurane in 100% oxygen in an induction chamber). Five cats (3 MSC-treated and 2 sham-injected) were sedated using acepromazine and buprenorphine and 5 cats (2 MSC-treated and 3 sham-injected) were anesthetized with isoflurane.

After a 3-week recovery period from ovariohysterectomy, all cats were evaluated at baseline (pretreatment) with a complete physical examination, a CBC, serum chemistry profile, urinalysis, complete organ evaluation. During each ultrasound evaluation, all cats were sedated or anesthetized for all procedures with either acepromazine and buprenorphine or isoflurane. CT/MR imaging was performed to assess organ status before and after treatment. CT/MR imaging was repeated at weekly intervals until complete organ evaluation.

Ultrasonographic examinations, cats were placed in dorsal recumbency and the abdomen was imaged using a linear array multifrequency transducer. The frequency used varied between 10 and 13 MHz, with the highest frequency chosen, which allowed complete organ evaluation. During each ultrasound evaluation,
the entire abdominal contents were surveyed, including the liver, spleen, kidneys, bladder, adrenal glands, pancreas, stomach and small intestines. The medial iliac and jejunal lymph nodes were also imaged at these time periods, and their long axis (cranial to caudal length) and short axis (dorsal to ventral height) were measured. For statistical comparisons, lymph node size was calculated as the multiplication of length times width. At 5 weeks after injection, fine needle aspirate biopsies of selected abdominal lymph nodes were obtained from 3 MSC-treated cats under ultrasound guidance using a 22 g 1.5 inch needle.

**Statistical Analyses**

Mean values with standard deviation were calculated for MSC-treated and control cats at each sampling time for data from physical examination, clinicopathological evaluations, and ultrasound measurements. Comparison between the before and after treatment results within each group were performed using paired \( t \)-test. Continuous quantitative variables that failed normality testing were evaluated with nonparametric analyses. Nonparametric Wilcoxon signed-rank tests were used for analyses of outcome variables for significant differences between treatment groups. In addition, multivariable ANOVA (MANOVA) was used to evaluate the linear changes over time. All data were analyzed with a commercially accessible statistical software package. Results with \( P < .05 \) were considered statistically significant.

**Results**

Each cell preparation displayed the expected MSC-phenotype. Cells underwent differentiation into adipocytes, chondrocytes, and osteocytes (Fig 1) and strongly expressed the surface markers CD44, CD90, CD105, while lacking expression of MHCII (Fig 2).

Severe adverse effects were not observed in any cat after injection of MSC. There were no significant differences in mean temperature, respiratory rate, heart rate, and body weight between groups over the course of the study. Two MSC-treated cats were lethargic and were less interactive with their caretakers immediately after injection.

**Fig 1.** Representative photomicrographs illustrating differentiation characterization of a mesenchymal stem cells cell line isolated from adipose tissue obtained from a 2 year old, female mixed breed cat (magnification 40×). (A) Lipid droplets in differentiated cells are stained red with Oil-o-red demonstrating adipogenesis, (B) Red-colored calcium deposits inside cells stained with Alizarin red stain demonstrating osteogenesis, (C) Blue-colored in glycosaminoglycan deposits in cells stained with Alcian Blue stain after chondrogenic differentiation. (D–F) Control photomicrographs after incubation of the same cell line in KNAC medium and stained with Oil-o-red (D), Alizarin red (E) and Alcian Blue (F). Note lack of uptake of the stains in both micrographs (D,E) and no blue staining extracellular matrix in the chondrogenic control stain (F).
injection. No pain was elicited by abdominal palpation in either cat. Both cats spontaneously recovered within 1 to 3 days after injection of cells.

Results of clinicopathologic evaluations revealed significant differences between study group for creatine kinase (CK) enzyme activity. CK activity was significantly higher in MSC-injected cats on weeks 1 (271.4 ± 154.36 IU/L versus 150.6 ± 54.79; \( P = .047 \), Wilcoxon) and 3 (196 ± 49.75 versus 125.8 ± 34.72; normal reference range = 59–354 IU/L; \( P = .047 \), Wilcoxon). Individual cat CK values were above the reference range at various times in 3 MSC-injected cats and 2 sham-injected cats. Abdominal ultrasonography revealed a significant increase in jejunal lymph node size in MSC-injected cats compared to controls at weeks 1 (1.38 ± 0.25 versus 0.88 ± 0.25 cm²; \( P = .036 \), Wilcoxon) and 5 (1.75 ± 0.82 versus 0.79 ± 0.12 cm²; \( P = .047 \), Wilcoxon) after cell injection (Fig 3). A significant increase in lymph node size over time was also identified in MSC-injected cats when comparing the before injection lymph node size with size at week 5 (1.1 ± 0.52 cm² before injection versus 1.75 ± 0.82 cm² after injection; \( P = .033 \)). A hyperechoic renal segmental cortical lesion consistent with a renal infarct was identified sonographically in 1 MSC-injected cat 1 week after injection. Cytologic

Fig 2. Flow cytometric analysis of surface markers expressed by a representative cell line from the same Animal 1 as in Fig 1. Gray lines represent the negative controls, while in black is the population of cells stained with antibody specific for each epitope. The X-axis represents the fluorescence intensity of the fluorophore (APC, PE or FITC) while the Y-axis represents the cell counts. Note strong expression of all markers (A) CD90, (B) CD105, (C) CD44 in each cell line (continuous line), compared to negative control (dashed line) and lack of expression of MHCII (D).
examined lymph node aspirates obtained from 3 MSC-injected animals revealed normal cell populations consisting primarily of small lymphocytes with low numbers of medium to large lymphocytes and occasional plasma cells and macrophages; there was no cytologic evidence of disease.

Discussion

This study evaluated the safety of IP injection of autologous MSC in cats. Our findings show that IP administration of autologous MSCs appears to be a safe and technically feasible approach to cell-based treatment in cats. Limited adverse effects were observed, manifesting as transient lethargy and decreased activity in 2 cats. The cause of these behaviors is uncertain, but might be related to discomfort associated with a host response to MSC or other components of the cell preparation. Although abdominal pain was not elicited during palpation, increased abdominal lymph node size in MSC-injected cats is consistent with a heightened host response to MSC injection. Alternatively, this reaction might have been caused by the sedative or anesthesia used. However, the fact that this behavior was observed only after cell injection and not at other times the cats underwent anesthesia, suggests that an adverse anesthetic reaction is less likely the cause of lethargy.

All clinicopathological results were similar between MSC-injected and sham-injected animals with the exception of CK. Importantly, CK values were only mildly outside of the reference ranges. Activity of this enzyme can be induced by multiple factors. Cats in this study received intramuscular injections of sedatives, which can increase the CK values. Some animals required additional physical restraint, which could result in increase in CK as well.

A hyperechoic renal segmental cortical lesion was observed in 1 MSC-injected cat 1 week after injection, which was assumed to be a renal infarct. These changes are incidentally encountered in cats in the clinical practice setting and have been observed more frequently in Ragdoll cats and cats with cardiomyopathy. In the MSC-injected cat with the renal segmental cortical lesion in this study, no heart murmur was identified on cardiac auscultation. An echocardiogram was performed to investigate for cardiomyopathy, but no cardiac abnormalities were noted. The animal developed other similar infarcts in the other kidney at the 1 year follow-up, which suggests that this animal is prone to this kind of changes and suggest that the infarcts were not related to the injected MSCs.

We also observed a significant increase in the size of jejunal lymph nodes in MSC-injected cats over the course of the study. We speculate that secretion of IL6 by MSC, which is known to cause T-cell expansion, may be responsible for the differences in lymph node size observed between the 2 groups. Cytological examination of lymph nodes of a limited number of MSC-injected animals did not reveal any pathological process. However, proof of this hypothesis would require histopathological evaluation of the jejunal lymph nodes. Necropsy examinations were not part of the experimental design, so it could not be assessed. Another explanation might be a complement-mediated reaction to injected MSCs similar to IBMIR reaction to injected cells and subsequent activation of innate immune system. Elements of complement are constitutively expressed by mesothelial cells. C3 complement component expressed by peritoneal mesothelial cells has been previously implicated to be responsible for IBMIR reaction after MSC IV injection in vivo and subsequent activation of innate immune system.

Interestingly, the complement binding also enhances the immunomodulatory properties of MSCs. Regardless, follow-up ultrasonograms performed on MSC-injected cats approximately 12 months after injection, revealed that all abdominal lymph nodes had returned to pre-injection size (data not shown). These observations suggest that abdominal lymphadenopathy associated with MSC injection is transient in nature. Further studies are needed to determine the precise cause of the increased lymph node size observed in MSC-injected cats.

A limitation of this study is that only a single dose of MSC was evaluated. Responses to IP MSC injection may vary by dose and frequency of administration, as suggested by in vitro studies in other species. Future studies evaluating a higher dose of MSC than the one evaluated in this study may be necessary to fully evaluate the safety profile of the IP injection in cats.

Although the efficacy of IP MSC injection has been previously studied in a variety of species and disease models, there have been few studies reported that comprehensively investigate the safety of IP administration of MSC in healthy control animals. In one study...
evaluating the safety and distribution of amniotic fluid stem cells injected IP in neonatal rats, no adverse effects were noted in MSC-injected rats during the 21-day after injection observation period. However, neither lymph node size nor behavioral changes were investigated in the rat study.

We conclude that IP injection of autologous MSC in cats is overall safe and is associated only with mild, self-limiting, and short-lasting adverse effects and provides a safe alternative route of administration for cell-based treatment in cats. Given the apparent safety of IP administration of MSC and the potential of IP administered MSC to reach intra-abdominal target sites, the effectiveness of the IP route for MSC-based treatment in cats warrants further investigations.

Conflict of Interest Declaration: Authors disclose no conflict of interest.

Off-Label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

References


Footnotes

a Liberty Research, Inc., Waverly, NY
b Adult Optimal Care, Hill’s Pet Nutrition, Inc., Topeka, KS
c Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY
d Sigma-Aldrich, St. Louis, MO
e Fetal Bovine Serum-mesenchymal stem cell-qualified, Gibco, Grand Island, NY
f Falcon Cell Strainers, Thermo Fisher Scientific Inc, Waltham, MA
g Anti-human HLA-DR, clone Tu39, BD Biosciences
h Gibco, Life Technologies, Thermo Fisher Scientific Inc
i Anti-human CD90, clone 5E10, BD Biosciences, San Jose, CA
j Anti-human CD105, clone SN6, Invitrogen, Grand Island, NY
k Anti-mouse CD44, clone IM7.8.1, Invitrogen
l Anti-human HLA-DR, clone Tu39, BD Biosciences
m BD Biosciences
n BD Medical, Franklin Lakes, NJ
o FlowJo, LLC, Ashland, OR
p StemPro Chondrogenesis, Adipogenesis, Osteogenesis Differentiation Kit, Gibco
q Boehringer Ingelheim, Ridgefield, CT
r Reckitt Benckiser, Hull, England
s IsoFlo, Abbott Laboratories, Abbott Park, IL
t Dulbecco Phosphate Buffered Saline, Sigma-Aldrich
u Countess, Life Technologies, Thermo Fisher Scientific Inc
v DCPAH, Michigan State University, East Lansing, MI
w Veterinary Medical Center, Michigan State University, East Lansing, MI
x GE Logiq 9, General Electric, Princeton, NJ

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