

Canine adipose-derived stromal cell viability following exposure to synovial fluid from osteoarthritic joints

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ABSTRACT

Introduction: Stem cell therapy used in clinical application of osteoarthritis in veterinary medicine typically involves intra-articular injection of the cells, however the effect of an osteoarthritic environment on the fate of the cells has not been investigated.

Aims and Objectives: Assess the viability of adipose derived stromal cells following exposure to osteoarthritic joint fluid.

Materials and Methods: Adipose derived stromal cells (ASCs) were derived from falciiform adipose tissue of five adult dogs, and osteoarthritic synovial fluid (SF) was obtained from ten patients undergoing surgical intervention on orthopedic diseases with secondary osteoarthritis. Normal synovial fluid was obtained from seven adult dogs from an unrelated study. ASCs were exposed to the following treatment conditions: culture medium, normal SF, osteoarthritic SF, or serial dilutions of 1:1 to 1:10 of osteoarthritic SF with media. Cells were then harvested and assessed for viability using trypan blue dye exclusion.

Results: There was no significant difference in the viability of cells in culture medium or normal SF. Significant differences were found between cells exposed to any concentration of osteoarthritic SF and normal SF and between cells exposed to undiluted osteoarthritic SF and all serial dilutions. Subsequent dilutions reduced cytotoxicity.

Conclusions: Osteoarthritic synovial fluid in this *ex vivo* experiment is cytotoxic to ASCs, when compared with normal synovial fluid. Current practice of direct injection of ASCs into osteoarthritic joints should be re-evaluated to determine if alternative means of administration may be more effective.

INTRODUCTION

The treatment of canine osteoarthritis (OA) with adipose-derived stromal cells (ASCs) has become prevalent in general practice following publication of studies indicating improved clinical symptoms following treatment (Black and others 2007, 2008, Guercio and others 2012). Treatment often involves the use of a stromal vascular fraction (SVF),

resulting from a fat sample that is processed to allow release and collection of nucleated cells from the stroma. Alternatively, some labs are offering culture expansion of the SVF to provide higher numbers of ASCs.

Previous reports described administration of autologous and allogeneic ASCs for the treatment of OA by intra-articular injection (Black and others 2007, 2008, Guercio and others 2012, Wong and others 2013, Ferris and others 2014, Jo and others 2014, Vangsness and others 2014, Koh and others 2015). This route is theorised to provide the most direct application to the area of disease. However, the osteoarthritic joint tends to be an unfavourable environment for local cellular health and viability. Synovial fluid contains mediators that promote inflammation, destroy cartilage, and/or induce apoptosis (Hay and others 1997, Amin and Abramson 1998, Fernandes and others 1999, de Bruin and others 2007, Xu and others 2009). The effect of this environment on local cells has been investigated *in vitro* and *in vivo* experiments and found to be detrimental to the health and viability of synoviocytes and chondrocytes (Vos and others 2000, Xu and others 2009, Bentz and others 2012, Huh and others 2012, Sunaga and others 2012). While there are much data demonstrating the immunomodulatory effects of ASCs, knowledge of the findings from these reports raises questions regarding the viability of transplanted cells into such an environment (Crop and others 2010, Kuo and others 2011, Melief and others 2013).

The purpose of this study was to investigate the viability of canine ASCs when exposed to osteoarthritic synovial fluid and to determine whether dilution of osteoarthritic synovial fluid altered cell viability. The authors tested the null hypothesis if canine ASCs are exposed to synovial fluid from an osteoarthritic joint and no difference cell viability would be

detected when compared with normal synovial fluid. Further, the authors tested the null hypothesis if cellular viability is reduced when ASCs are exposed to synovial fluid from an osteoarthritic joint and then dilution of the synovial fluid will not reduce the cytotoxic effect of the osteoarthritic synovial fluid.

MATERIALS AND METHODS

Informed owner consent was obtained and all procedures were performed in accordance with the University of Minnesota Institutional Animal Care and Use Committee.

Isolation of ASCs

Falciform adipose tissue was harvested at the time of surgery from five healthy dogs admitted to the University of Minnesota College of Veterinary Medicine for elective abdominal surgery unrelated to the study. Adipose tissue was processed according to previously reported protocols for isolation of the SVF (Black and others 2007, 2008). Cells were cryopreserved with 50 per cent keratinocyte N-acetylcysteine (KNAC) medium (Gibco, Life Technologies, Grand Island, New York, USA), 20 per cent fetal bovine serum (FBS; Hyclone, Thermo Fischer Scientific, Minneapolis, Minnesota, USA), 20 per cent Dulbecco's Modified Eagles Medium (Gibco, Life Technologies, Grand Island, New York, USA) and 10 per cent dimethyl sulfoxide (Sigma-Aldrich, St Louis, Missouri, USA), until all samples were collected. The nucleated cell fraction was placed into KNAC cell medium for ASC expansion consisting of modified MCDB153 medium (Keratinocyte-SFM; Gibco, Life Technologies, Grand Island, New York, USA), supplemented with: 2mM N-acetyl-L-cysteine (Sigma-Aldrich, St Louis, Missouri, USA), 0.2mM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St Louis, Missouri, USA), 0.09mM calcium and human recombinant epidermal growth factor (5 ng/ml; Gibco, Life Technologies, Grand Island, New York, USA), bovine pituitary extract (50 µg/ml; Gibco, Life Technologies, Grand Island, New York, USA), insulin (5 µg/ml; Sigma-Aldrich, St Louis, Missouri, USA), hydrocortisone (74 ng/ml; Sigma-Aldrich, St Louis, Missouri, USA), 5 per cent FBS and 1 per cent antibiotic (penicillin 10,000 IU/ml, streptomycin 10,000 µg/ml, amphotericin B 25 µg/ml; Mediatech, Corning, New York, USA) at 37°C in a humidified 5 per cent CO₂ atmosphere (Kang and others 2008).

Synovial fluid samples

Synovial fluid samples were collected from 10 dogs with pain and lameness secondary to OA and from seven normal dogs with no joint disease. The presence of OA was confirmed by owner history, orthopaedic exam, radiographic exam and when surgical intervention was indicated, visual identification of OA at the time of surgery. Normal synovial fluid was harvested from dogs that underwent euthanasia for an unrelated research

study. These dogs had no history of lameness, a normal orthopaedic examination and a visually normal joint assessed following synovial fluid collection. All synovial fluid samples were centrifuged at 400 g at 4°C for six minutes. The supernatant was aspirated from the pellet and subjected to three freeze-thaw cycles at -20°C to eliminate any cellular contamination of the joint fluid (Tansey 2006). They were stored at -80°C until all samples were collected. Because synovial volumes only ranged from 0.25 to 0.5 ml from normal joints, and 1 to 3 ml from joints with OA, multiple synovial samples were pooled to achieve enough volume for normal and OA synovial fluid testing conditions across multiple cell lines. Pooling samples also served to reduce variability within a group. Five separate pooled samples were prepared for OA synovial fluid, with two OA synovial fluid samples per each pooled sample. Three normal synovial fluid samples and four normal synovial fluid samples were pooled to provide two separate pooled normal synovial fluid samples of adequate volume to assess.

Cytotoxicity assay

The ASCs from passage 3 (a typical passage used for clinical use of culture expanded cells) of each of the five donors were plated at 10,000 cells per well in a 96-well plate in duplicate for each condition. Once the cells were confluent (within 24–48 hours), each cell line was treated with each of the following conditions in duplicate: 100 µl of normal synovial fluid, 100 µl of KNAC cell culture medium containing no synovial fluid and 100 µl of a specified dilution of synovial fluid from OA joints. Synovial fluid derived from OA joints were prepared and applied as no dilution or one of the following serial dilutions: 1:1 (one part synovial fluid, one part diluent), 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9 or 1:10 dilution, using KNAC growth medium as the diluent. Each cell line was tested with a pooled normal synovial fluid sample and each of the pooled osteoarthritic synovial fluid samples. Cells were placed in these conditions for 12 hours. The contents of each well was then aspirated and placed in a sterile centrifuge tube. The well was rinsed with phosphate buffered solution two times, and each rinse added to the aspirated well contents. Cells were then detached using Tryple-E (Invitrogen, Life Technologies, Grand Island, New York, USA), which was inactivated by the addition of KNAC growth medium after 10–15 minutes. The contents of the well were aspirated and added to the previous well contents. The well was rinsed two more times with phosphate buffered saline, and each rinse added to previous well contents. The accumulated well contents were centrifuged at 400g at 4°C for six minutes. The supernatant was aspirated and cells were resuspended in 500 µl of KNAC growth medium.

Viability of cells was counted using the trypan blue (Invitrogen, Life Technologies, Grand Island, New York, USA) exclusion method, with cells exposed to an equal volume of dye (1:1 dilution) at room temperature for five minutes before counting (Strober 2001; Louis and

Siegel 2011). Viable cells had no dye uptake while non-viable cells had dye uptake. A haemocytometer was used to count cells. The individual counting each treatment was blinded to group assignment. Per cent viability was calculated by dividing the number of viable cells (non-stained cells) by the total number of cells (stained and non-stained).

Statistical analysis

Data were analysed with the aid of StatPlus 2009 software. Viability of treatment conditions was analysed using Wilcoxon Signed-Rank tests for paired samples, with a $P < 0.05$ considered statistically significant. The data were found to violate the assumption of normal distribution, and the variances were unequal, making a non-parametric test preferable in place of a paired t test. The effect of serial dilutions on cell viability was analysed by generating a linear regression equation.

RESULTS

Within two hours of exposure to treatment conditions, cells were noted to lose adherence to plastic when treated with osteoarthritic synovial fluid, while control wells maintained adherence (Figure 1). Cells exposed to KNAC cell culture medium or normal synovial fluid had no significant difference in viability ($P = 0.7$). Cells treated with any dilution of osteoarthritic synovial fluid had significantly less viability than medium or normal synovial fluid ($P = 0.01 - 0.02$). A significant difference was found among many of the dilutions of osteoarthritic

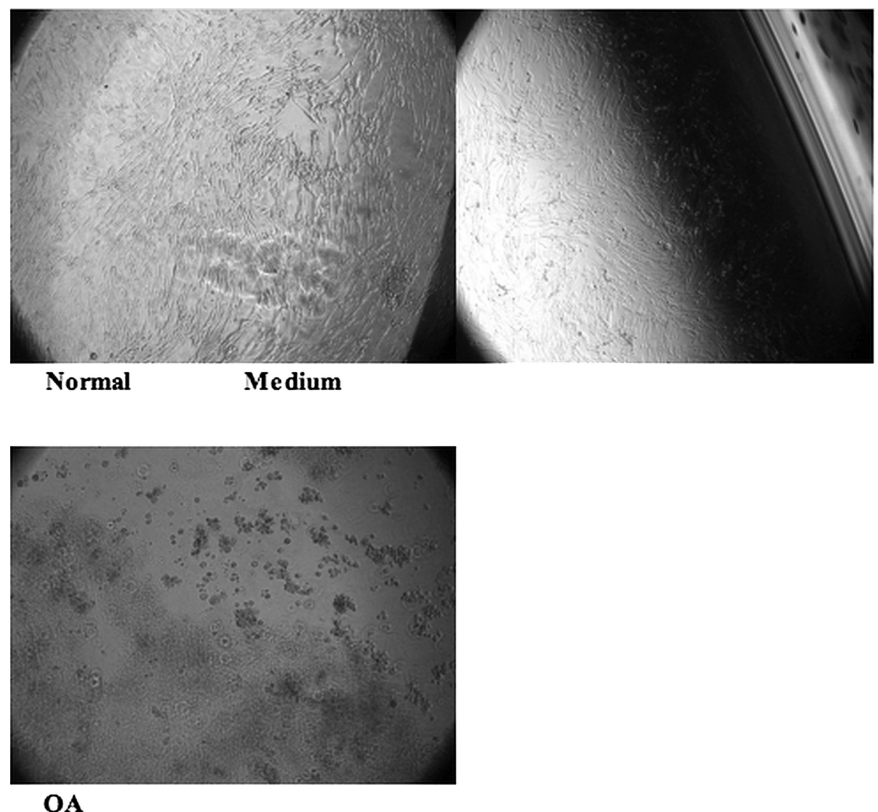
synovial fluid after a twofold to threefold dilution ($P = 0.01 - 0.04$; Figure 2 and Table 1). Linear regression described the relationship of the serial dilutions ($r^2 = 0.81607$, $y = 0.0465x + 0.1767$); residuals were estimated and a random pattern with alternating positive and negative values was observed, suggesting a good fit for the linear model to the data. The residual values were normally distributed with a mean of 0.0001, and no values were greater or less than 0.2. Extrapolation of the equation suggested that after a 1:16 dilution, the mean viability of cells exposed to OA synovial fluid would be equivalent to the mean viability of the control population.

DISCUSSION

The authors reject the null hypothesis and conclude that when canine ASCs were exposed to synovial fluid from an osteoarthritic joint, there were differences in cell viability that could be detected when compared with normal synovial fluid. The authors also reject the null hypothesis and conclude that when cellular viability was reduced by exposure of ASCs to synovial fluid from an osteoarthritic joint, then dilution of the synovial fluid did reduce the cytotoxic effect of the osteoarthritic synovial fluid in this ex vivo environment.

The loss of adherence to the cell culture dish is not a valid measure of cell viability; however, it does reflect disruption in culture homeostasis. Cells can be enzymatically disrupted from adherence to the culture surface, without losing viability (Kang and others 2008, Neupane

FIG 1: Images of cell culture wells two hours after exposure to control (medium or normal synovial fluid) or osteoarthritic (OA) synovial fluid. Note the rounded up detached appearance of cells in the OA-treated group, as apposed to the spindle shaped appearance of cells of medium or normal synovial fluid-treated wells. Microscopic magnification of x 10



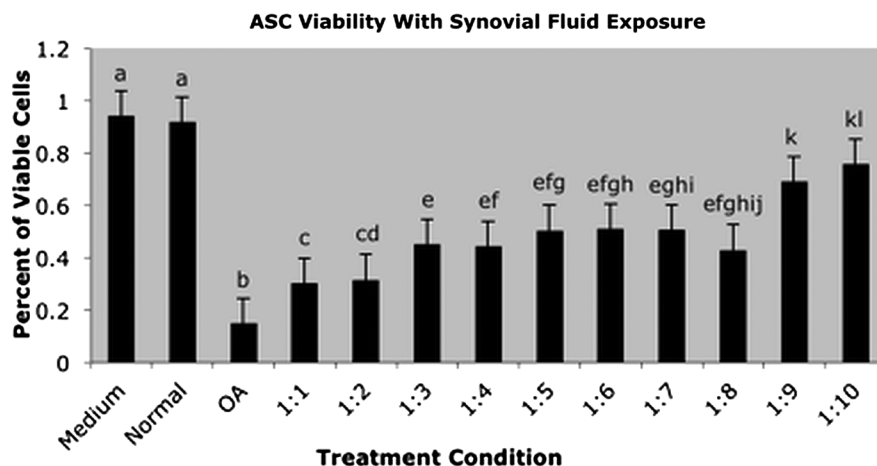


FIG 2: Viability of cells after exposure to osteoarthritic (OA) synovial fluid as an undiluted sample (OA) or an OA synovial fluid sample diluted from 1:1 to 1:10, or normal synovial fluid or medium alone. Values are expressed as the median of the percentage of viable cells (number of viable cells divided by the total number of cells in the sample). Each treatment condition on the x-axis was assessed with five cell lines in duplicate, and averaged. Error bars indicate the maximum value of each condition. Statistical significance set with Wilcoxon Signed-Rank test is set as $P < 0.05$. Conditions labelled with the same letter have no statistical difference, whereas those with different letters are statistically different. ASC, adipose-derived stromal cell.

and others 2008, Vieira and others 2010). Subjectively, this change was noted within a few hours after exposure to synovial fluid, which may be an indication that response to exposure to synovial fluid is rapid.

After 12 hours of exposure to normal synovial fluid, there was no significant difference in cell viability when compared with cells that remained within culture medium. Since the authors only made this assessment at a single time point after exposure, the authors cannot comment on the longevity of cells within normal synovial fluid beyond 12 hours. Longer exposure may result in lower viability in an *in vitro* environment, where synoviocytes or local stroma is not present to provide nutrients and metabolites necessary for normal cell physiology. After 12 hours of exposure, a significant difference was noted between any sample treated with osteoarthritic synovial fluid and normal synovial fluid or KNAC cell culture medium-treated cells. This suggests that osteoarthritic synovial fluid contains components that contribute to cytotoxicity. It would be of interest in a future study to assess the longevity of cell viability following exposure to synovial fluid, by evaluating viability at variable time points. Limited synovial fluid sample availability prevented this assessment in this study.

One possible explanation for reduced cell viability in osteoarthritic synovial fluid would be cell-to-cell interactions between ASCs and cells contained within the osteoarthritic synovial fluid. While this could occur with an intra-articular administration of an ASC treatment, the authors were interested in the cytotoxic effects of synovial fluid without cell-to-cell interactions. To minimise this possible scenario, synovial fluid samples were centrifuged and the supernatant was removed from any pellet produced. To further ensure no viable cells could mount a cytotoxic effect on ASCs through a direct cell-to-cell interaction, all synovial fluid samples went

through three freeze-thaw cycles (Tansey 2006). The presence of cells would be expected within a normal osteoarthritic joint environment, but their interaction with ASCs intra-articularly has not been characterised well *in vitro*. There is much evidence that ASCs have a potent immunomodulatory capacity, so cells from OA synovial fluid may not create much of a threat to ASC viability; however, the authors wished to focus on the soluble factors found within synovial fluid in this study (Götherström and others 2004, Kang and others 2008, Kuo and others 2011, Lee and others 2012, Melief and others 2013). A follow-up study investigating the effects of synovial fluid containing cellular components would be of interest, given the propensity of ASCs for trophic effects (Caplan and Dennis 2006, Potapova and others 2007).

In this study, the authors did not determine which factors, or combination of factors, contributed to cytotoxicity. Due to the small sample sizes, molecular characterisation of the synovial fluid groups was not possible.

A third possible explanation for reduced cell viability could be that in this *ex vivo* environment, the *in situ* cells and stroma that are expected to interact with ASCs may provide a cytoprotective effect, and their absence from the conditions within this experiment contributed to the loss of viability (Caplan and Dennis 2006, Potapova and others 2007). While eliminating the procedures to minimise cellular content within the synovial fluid in this project would provide some cellular interactions, there are a plethora of cells and cell types in the joint environment that are not present within the synovial fluid. Assessment of viability of ASCs *in vivo*, although challenging, will be necessary to answer many of these questions.

The length of time ASCs need to be present and viable at the site of injury has not been established, and it is

TABLE 1: Median, minimum, maximum values and sds for the per cent viable cells exposed to medium, normal and osteoarthritic synovial fluid at various dilutions

	Treatment condition			1:1	1:2	1:3	1:4	1:5	1:6	1:7	1:8	1:9	1:10
	Media	Normal	OA										
Median	0.928	0.928	0.143	0.329	0.297	0.463	0.407	0.502	0.516	0.525	0.458	0.752	0.786
Min-max	0.913, 0.987	0.843, 0.944	0.089, 0.203	0.193, 0.391	0.215, 0.445	0.306, 0.511	0.361, 0.626	0.382, 0.663	0.404, 0.574	0.378, 0.613	0.143, 0.581	0.605, 0.769	0.684, 0.795
sd	0.024	0.035	0.036	0.067	0.07	0.084	0.083	0.097	0.072	0.086	0.136	0.068	0.059
	P values	Normal	OA	1:1	1:2	1:3	1:4	1:5	1:6	1:7	1:8	1:9	1:10
Media		0.7	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>
Normal			<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>
OA				<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.02</i>
1:1					0.9	<i>0.02</i>	<i>0.02</i>	<i>0.02</i>	<i>0.01</i>	<i>0.02</i>	<i>0.04</i>	<i>0.01</i>	<i>0.02</i>
1:2						<i>0.03</i>	<i>0.02</i>	<i>0.01</i>	<i>0.01</i>	<i>0.01</i>	<i>0.04</i>	<i>0.01</i>	<i>0.02</i>
1:3							0.7	0.3	0.2	0.2	0.8	<i>0.01</i>	<i>0.02</i>
1:4								0.07	0.05	<i>0.04</i>	0.8	<i>0.01</i>	<i>0.02</i>
1:5									0.8	0.8	0.3	<i>0.01</i>	<i>0.02</i>
1:6										1	0.1	<i>0.01</i>	<i>0.02</i>
1:7											0.1	<i>0.01</i>	<i>0.02</i>
1:8												<i>0.01</i>	<i>0.02</i>
1:9													0.6

The P value for each pair-wise comparison is reported from multiple Wilcoxon Signed-Rank tests. Significance of P values was set at $P < 0.05$. Significant differences among treatment conditions are highlighted by bold italics. Conditions evaluated include growth medium alone (media), normal synovial fluid (normal), osteoarthritic synovial fluid (OA) or OA synovial fluid diluted with growth medium (1:1–1:10)

kely to be variable, dependent on disease state, individual patient response and therapeutic effect desired. Given the capacity for stem cells to provide trophic effects on their environment and local cells, it is plausible that it is not necessary for them to survive more than a few hours to have a positive therapeutic effect (Caplan and Dennis 2006; Potapova and others 2007).

It should be noted that the conditions of this study assessed the use of allogeneic cells, and this may elicit a different response than autologous cells. However, allogeneic cells have been assessed in vivo in many studies, and the immunomodulatory capacity of ASCs makes allogeneic cell use a likely option in the future of regenerative medicine (Kang and others 2008, Kuo and others 2011, Melief and others 2013, Vangsnes and others 2014). It was not possible to harvest fat from patients that donated normal or OA synovial fluid samples to allow autologous cell assessment in this study. Thus, culture expanded, allogeneic cells were used. Future investigations should assess the effect of OA synovial fluid on autologous cells in addition to allogeneic to determine whether this variable changes viability.

The authors used the nonparametric Wilcoxon Signed-Rank test because the assumptions required for parametric analysis were violated. With multiple comparisons being made, an alternative method for analysis would have been to use a Bonferroni correction where the level of significance is set at 0.05 divided by the number of comparisons (α/k). This adjusts for type I error associated with the analysis, although it is very conservative and has an increased rate of type II error. In this study, if the authors set the level of significance at α/k , none of the pairwise comparisons would be considered significant. Using the unadjusted P values, one would expect that about 5 per cent or one in 20 of them would be found to be significant by chance alone due to the sheer number of comparisons made.

Extrapolation from the linear regression model suggested that it would take approximately a 16-fold dilution to return to an equivalent viability as a healthy synovial fluid sample. To accomplish this dilution in an in vivo environment, the joint could be flushed with saline prior to administering the ASC treatment. Regardless, it would be prudent to document the necessity of flushing in an in vivo setting before translating these findings to patient care.

An alternative means of administration would be intravenous injection; however, this method is less used, as studies have demonstrated that the majority of intravenously administered stem and progenitor cells are filtered out by the lungs, liver and other peripheral organs (Aicher and others 2003; Fischer and others 2009; Pendharkar and others 2010). However, the requirement for site-specific location of stem cells and progenitor cells has not been established and may be cell or product dependent. The safety and efficacy of each means of administration have not been compared in dogs. The results of this study indicate that osteoarthritic

joint fluid has a cytotoxic effect on ASCs in an ex vivo environment. This suggests that the authors should re-evaluate if the current method of administration of ASCs is appropriate, and if revision of current protocols could improve therapeutic response.

The cytotoxic contents of OA synovial fluid and their destructive effects on the joint environment make the findings of this paper unsurprising. Nonetheless, reports of injecting cellular products into joints with OA are common practice. The need for viable cells to have a positive effect in the disease process has not yet been established; however, if the goal of the cellular product is to have viable cells transplantable within the joint, then placing them into this toxic environment may be counterproductive or minimise the potential effect of this therapy. Flushing an osteoarthritic joint prior to treatment may improve the viability of the administered cellular product. An in vivo assessment of intra-articular ASC administration with and without flushing prior to administration is indicated.

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