Safety and Complications Reporting on the Re-implantation of Culture-Expanded Mesenchymal Stem Cells using Autologous Platelet Lysate Technique

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Abstract: Mesenchymal stem cells (MSCs) hold great promise as therapeutic agents in regenerative medicine. Numerous animal studies have documented the multipotency of MSCs, showing their capabilities for differentiating into orthopedic tissues such as muscle, bone, cartilage, and tendon. However, the complication rate for autologous MSC therapy is only now beginning to be reported.

Methods: Between 2005 and 2009, two groups of patients were treated for various orthopedic conditions with culture-expanded, autologous, bone marrow-derived MSCs (group 1: n=45; group 2: n=182). Cells were cultured in monolayer culture flasks using an autologous platelet lysate technique and re-injected into peripheral joints (n=213) or into intervertebral discs (n=13) with use of c-arm fluoroscopy. While both groups had prospective surveillance for complications, Group 1 additionally underwent 3.0T MRI tracking of the re-implant sites.

Results: Mean follow-up from the time of the re-implant procedure was 10.6 +/- 7.3 months. Serial MRI's at 3 months, 6 months, 1 year and 2 years failed to demonstrate any tumor formation at the re-implant sites. Formal disease surveillance for adverse events based on HHS criteria documented 7 cases of probable procedure-related complications (thought to be associated with the re-implant procedure itself) and three cases of possible stem cell complications, all of which were either self-limited or were remedied with simple therapeutic measures. One patient was diagnosed with cancer; however, this was almost certainly unrelated to the MSC therapy.

Conclusions: Using both high field MRI tracking and general surveillance in 227 patients, no neoplastic complications were detected at any stem cell re-implantation site. These findings are consistent with other reports that also show no evidence of malignant transformation *in vivo,* following implantation of MSCs that were expanded *in vitro* for limited periods.

Keywords: Mesenchymal stem cell, mesenchymal stem cells, platelet lysate, complications, safety, orthopedic, orthopedics, culture expansion.

INTRODUCTION

Mesenchymal stem cells (MSCs) hold great promise as therapeutic agents in regenerative medicine [1-5]. These adult stem cells are readily isolated from many sources in the body [1], and numerous animal studies have demonstrated their multipotency in terms of differentiating into muscle, bone, cartilage, tendon, and various cells of internal organs [13].

The complication rate for autologous MSC therapy is only now beginning to be reported, and limited information is available on the safety of this application. Because MSCs are multipotent, one issue that needs to be addressed is the potential of these cells, when implanted, to form neoplasms [6,7]. Lending credence to this concern are reports of chromosomal abnormalities in MSCs that have been cultured *in vitro* for extended periods of time [8,9]. However, other investigators have noted telomere shortening in cultured primary MSCs and observed that MSCs cultured *in vitro*, for less than approximately 60 days (equating to approximately 10 culture passages), poses no detectable risk of cell mutation or neoplasm formation [10,11].

Animal studies that utilize MSCs for orthopedic treatment, also indicate no associated neoplasm [1, 12-19], and the few early human clinical trials completed thus far support the conclusion that MSCs are safe as a therapeutic agent [20-24]. To date, however, we are unaware of any published, large human clinical trials that have tested the safety of re-implantation of autologous MSCs. Here we report on the results of a prospective study aimed at examining the safety profile of culture-expanded MSCs in human orthopedic applications.

METHODS

Group 1 (2006-2007) patients (n=45) were followed with the use of high field MRI of the MSC implant sites. Once a general safety profile for implantation was established, we then followed a second, larger patient cohort (Group 2, 2007-2009) (n=182) with the use of a formal disease and complications surveillance program. For Group 1 patients, MSC transplant protocols and therapy were approved by a non-profit, IRB (Spinal Injury Foundation-IRB00002637) that is registered with the Department of Health and Human Services.

Patients

Inclusion Criteria for Group 1 Patients

1. 18-65 years of age.
2. Chronic or degenerative disc disease causing significant functional disability.
3. Failure of conservative management.
4. Unwillingness to pursue surgical options.

Exclusion Criteria for Group 1 Patients

1. Active inflammatory or connective tissue disease (i.e. lupus, fibromyalgia, RA).
2. Active non-corrected endocrine disorder potentially associated with symptoms (i.e. hypothyroidism, diabetes).
3. Active neurologic disorder potentially associated with symptoms (i.e. peripheral neuropathy, multiple sclerosis).

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Note: Dr. Marasco is a consultant for and has equity ownership in NeoStem. Dr. Centeno, Dr. Schultz, Michelle Cheever, and Brent Robinson have equity ownership in Regenerative Sciences, LLC (RS). Dr. Centeno and Schultz as well as Brent Robinson act as consultants for RS, while Michelle Cheever is an RS employee.

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4. Pulmonary cardiac disease.
5. Pulmonary disease requiring medication usage.
6. History of active neoplasm within the past 5 years.
7. Anemia.

Blood Work for Group 1 Patients

Complete Blood Count (CBC), electrolytes, Liver Function Tests, and Creatinine were done within 3 months of procedure and at 1 and 3 months post procedure. Anemia was ruled out by evaluating patients' hematocrit before each bone marrow draw.

Inclusion Criteria for Group 2 Patients

The same as for Group 1 patients.

Exclusion criteria for Group 2 patients

1. Medical condition precluding the injection procedure
2. History of active neoplasm within the past 5 years
3. Anemia

Blood Work for Group 2 Patients

Anemia was ruled out by evaluation of patients' hematocrit before each bone marrow draw.

Imaging

Group 1 patients underwent MRI surveillance using a GE 3.0 Tesla Excite HD to image the injection site. Patient follow-up was done as follows:

1. Within 3 months prior to the MSC injection procedure.
2. At 12 weeks post procedure.
3. At 6 months post procedure.
4. At 1 year post procedure.
5. At two years post procedure.

Coronal as well as sagittal proton density weighted images, with and without fast spin sequences, were used; the TR/TE and imaging planes were matched exactly for all images on each patient. This unique protocol reduced the likelihood of errors in interpretation of serial images in the same patient. All images were independently read by two board certified physicians (authors CJC and JRS), who reviewed the images for evidence of new tumors, lesions, or abnormal growth at the injection site.

The Effects of Omnipaque Contrast on MSCs

Use in patients: We used fluoroscopy to accurately place stem cells into various areas of the musculoskeletal system. Radiographic contrast is frequently used with fluoroscopy to ensure proper localization of injectates. Omnipaque (GE Healthcare NDC 0407-1413-50) is a non-ionic contrast agent that is commonly used in making fluoroscopically-guided injections into the spine and peripheral joints; we used this agent to ensure accurate targeting (i.e. intra-articular, intra-discal) of the stem cells. The contrast agent was first diluted (1:1 or 1:2) with phosphate buffered saline (PBS) and then mixed with cells prior to injection. To our knowledge no previously published data are available on the effect of radiographic contrast agents on MSCs.

Omnipaque's active ingredient is Iohexol. Edwards *et al*. determined that Iohexol is eliminated from peripheral joints after approximately 3 hours [20]. We expected that a localized injection of Iohexol into the intra-articular knee space would also undergo dilution via the normal circulation of synovial fluid. When synovial fluid is obtainable from the knees of patients via aspirate, the average volume is 2.3 ml [21]. Based on our clinical experience with visualizing synovial fluid on high field 3.0T MRI, we also estimate that 1-3 ml of synovial fluid is a typical volume for aspirates (this is a conservative estimate: some patients with moderate to severe osteoarthritis suffer from knee effusions). We thus anticipated that an Omnipaque injection into the knee would, on average, become diluted in about 2 ml of synovial fluid, plus in the volume of the injectate (autologous platelet product) that is inserted into the peripheral joint (usually an additional 2 ml). Therefore, in a large peripheral joint such as the knee, we started with a maximum of 1 ml of Omnipaque (200 mg/ml), which would then become diluted on average with 2 ml of native synovial fluid plus 2 ml of injectate, resulting in an estimated final Omnipaque concentration of 40 mg/ml. Cells would thus be initially exposed to 40 mg/ml Omnipaque, and with the rapid clearance (assuming a linear clearance rate), this cell exposure would fall to approximately 20 mg/ml Omnipaque within 1.5 hours. This was believed to be the minimum exposure of cells to Omnipaque in terms of concentration and time. We also found no published data for Omnipaque clearance from the intervertebral disc: our clinical experience, however, suggests a longer half-life than for the knee, as patients are routinely imaged several hours after lumbar discography with contrast still visible on CT scan [22]. Since the IVD has very little native fluid that can be aspirated, no dilution by native fluids was factored: average injectate into the disc was 1 ml of platelet products with average Omnipaque volume to be used at 0.25 to 0.5 ml. Thus we used 0.5 ml of Omnipaque (200 mg/ml), which was diluted to 67 mg/ml by the injectate – this results in a higher conservative estimate of cell exposure to Omnipaque, at a maximum of 80 mg/ml for 4 hours.

Use in culture: MSC suspensions (1 X 10^6 cells/mL) were incubated in the presence of 80, 40, 20, 10, 1, or 0 mg Omnipaque/mL in plating media for 1.5, 3, and 4 hours. Plating media consisted of alpha-modified eagle medium (A-MEM-GibCo 12571) supplemented with 10% platelet lysate (v/v), 5ug/mL doxycycline (Bedford Labs-NDC 55390-110-10), and 2 I.U./mL of heparin (Heparin Sodium-Abraxis, NDC 63323-047-10). At the various time points, a 1ml aliquot of the cell suspension was removed, and cell viability was evaluated with use of Trypan Blue (MP Biomedials-Catalogue #1691049): the cell suspension sample was centrifuged and resuspended in fresh plating media, transferred into one well of a 6-well culture plate, and incubated 37^{o}C in 5% CO_2. Cell viability was determined by trypsinizing and counting cells on day 0, day 3, and day 5.

The Platelet Lysate (PL) Technique

Fetal Calf or Bovine Serum is commonly used for cell culturing. To reduce the risks of possible disease transmission from animal-based serum, several authors have published on the use of platelet lysate (PL) for expanding bone marrow derived MSCs in culture [23-25]. However, these reports either do not specify the donor source, or use healthy volunteers as donors. In light of the report by Murphy that cultured MSCs from patients with osteoarthritis have a decreased capacity for proliferation [26], we were interested in determining if the PL technique could be applied for the culture-expansion of bone marrow derived MSCs from patients who were diagnosed with moderate to severe osteoarthritis (OA). Ten patients were actively referred for study from a private medical practice, based on their diagnosis of either OA or traumatic arthropathy that was causing significant ongoing pain and disability, and on their willingness to proceed with the study. These patients were divided into two groups:

Group A: Patients' bone marrow MSCs were expanded in serum free monolayer culture, supplemented with either 5% or 10% PL.

Group B: Patients' bone marrow MSCs were expanded in serum free monolayer culture supplemented with either 10% or 20% PL.

Inclusion/exclusion criteria for this study were the same as noted above for Group 1 patients. Methods used to culture-expand their MSC are detailed below. Cell counts were obtained after colony formation and with each culture passage.

Isolation and Expansion of MSCs

Patients in Groups 1 and 2 were restricted from taking corticosteroids or NSAIDs for one week prior to the marrow harvest procedure. Coincident with marrow harvesting, heparinized IV venous blood was drawn to be used for PL; the lysate was prepared by centrifuging the blood at 200g, at 6 minutes to separate platelet rich plasma (PRP) from the red blood cells (RBCs). PRP was drawn off and stored at -20°; platelet bodies were centrifuged at 1000g at 6 minutes, and the supernatant drawn off to produce the PL. Cell culture media was prepared at 10-20% solution of platelet lysate with A-MEM.

Each patient was placed prone on an operating room (OR) table and the area to be harvested was anesthetized with 1% Lidocaine. A sterile disposable trocar was used to draw 10 cc of marrow blood from the right Posterior Superior Iliac Spine (PSIS) area, and 10cc from the left PSIS area, into heparinized syringes. To ensure accurate placement of the trocar for the aspirate into the bone marrow cavity, C-arm fluoroscopy was used for all bone marrow draws. From 1-3 sites on each side were used to draw the a whole marrow aspirate (see Fig. **1**).

This aspirate was next transferred to the lab and centrifuged at 200g for 4-6 minutes to separate nucleated cells (from the RBCs). The nucleated cells were placed in a separate 50ml conical centrifuge tube, and pelleted by centrifuging at 1000g for 6 minutes. The pellet was washed once in PBS, cells were counted, and then re-suspended in Dulbecco's modified eagle medium (DMEM-GibCo, 11885) with 10-20% PL, 5ug/mL doxycycline, and 2IU/mL heparin. Nucleated cells were seeded in a tissue culture flask at $1x10^6$ cells/cm^2, and incubated at 37℃/5% CO/5-17% $O2_2$ in a humidified environment. Culture medium was changed after 48-72 hours, removing the majority of the non-adherent cell population. MSC colonies that developed after 6-12 days in culture were harvested with use of an animal origin-free trypsin like enzyme (TrypLE Select-Gibco, 12563): this procedure detached only the colony-forming MSCs. To expand the MSCs, cells were re-plated at a density of 6,000-12,000 cells/cm^2 in A-MEM with 10-20% PL, 5ug/mL doxycycline, and 2IU/mL of heparin, and grown to near confluence. Primary cells derived from the bone marrow were designated as passage 0, and each subsequent subculture of MSCs was considered one further passage.

After MSCs had been sub-cultured to the 2^{nd}- 7^{th} passage, they were harvested, washed, and suspended in 20% PL in PBS, in readiness for injection. With the patients' written consent, and under fluoroscopic guidance, MSCs were inserted percutaneously into either a peripheral joint or an intervertebral disc. Prior to MSC injection, contrast (Omnipaque 300 mg/ml-NDC 0407-1413-51; diluted 1:1 or 1:2 with PBS) was injected to ensure proper targeting of the injectate. MSCs were injected with either autologous PL 10-20% (as described above) or with conditioned serum (below).

Conditioned serum was prepared from the PRP by activation with $CaCL_2$ 2.86-14.3 mg/ml (American Regent, Inc, NDC 0517-2710-25) and human Thrombin 28.6-142.8 IU/ml (Johnson and Johnson Inc, NDC 63713-460-05) and was incubated at 37℃/5% CO in a humidified environment for 1 hour to 6 days. Platelets were then pelleted at 2000 g and the supernatant drawn off for re-injection with MSCs.

Frequently, MSCs for re-injection were cryogenically preserved prior to their clinical use; they were first grown to ~80% confluence, harvested as described above, and suspended at a density of 1 x 10^6 - 3 x 10^6 cells/ml in either 90-95% autologous PL or in platelet poor plasma (PPP). Cells were frozen to -80°C in a controlled rate freezing device (5100 Cryo 1C Freezing Container, "Mr. Frosty", Nalgene), and 24 to 72 hours later, they were transfered to a -150 C freezer or into liquid nitrogen storage. For re-injection, cells were thawed rapidly in a 37°C water bath, then transferred into warmed PL/A-MEM and plated as described. Cells were re-covered by incubating through at least one passage before being injected. Cell counts of MSCs placed back into monolayer culture after using the above cryogenic storage process routinely documented 85-95% viability. Not every sample was tested for viability testing prior to re-implantation; however, all samples were actively growing in culture at the time of re-implantation, and transport times from culture to syringe were short (generally less than 2 hours). Agashi has shown more than 80% cell viability rates within this 0-2 hour window [27].

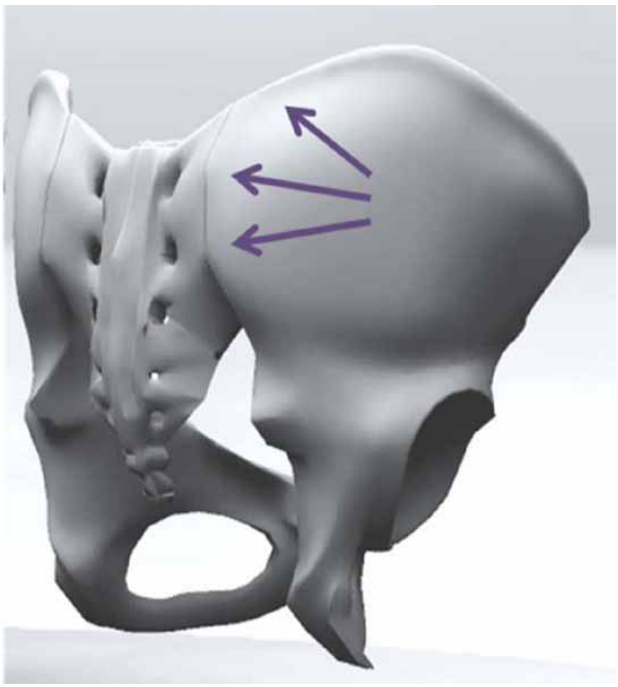

Fig. (1). A 3-D model of the human pelvis demonstrating the bone marrow aspirates sites near the PSIS (blue arrows). These sites were confirmed on AP fluoroscopy before the aspiration of marrow.

Follow-up Questionnaire

To enhance surveillance of treated patients, Group 2 patients were provided with a specific set of follow-up questions, which were presented to patients via phone, e-mail, or postal mail at the endpoints listed above (Table **1**). A "Yes" or "Maybe" answer to any question was considered a complaint. Two authors (CJC and JRS) were tasked with following up on patient complaints with the database coordinator and if necessary with patient.

Complaints Reporting, Tracking, and Adjudication System

Inclusion Criteria for Adjudicated Complication

1. Either the patient reported a complaint on the tracking questions at a routine follow-up time point, or the complaint was patient-initiated. For inclusion in the list of complications, the complaint had to be received prior to 4/15/09.
2. The complaint was sent for adjudication to one of the treating physicians (CJC or JRS), who responded that either the complaint was clearly not a complication (i.e. was clearly an unrelated complaint) or that the complaint could be a complication (e.g., a treated knee was severely swollen immediately after the knee injection). If the complaint was considered to be a possible complication, it was adjudicated further by either treating physician.
3. Additional data were gathered via phone, e-mail, or office visit. If the complaint was clearly not a complication, then the file was closed. If the complaint was considered a possible or likely complication, it was graded using the conventions below.

Table 1. The Follow-Up Disease Surveillance Questionnaire Used in Group 1 and 2 Patients

At the outcome endpoints listed above, patients in both groups 1 and 2 were asked	
1	Did you experience any complications you believe may be due to the procedure (i.e. infection, illness, etc.)? If yes or maybe, please explain. Yes No Maybe
	a. Comments:
2	Have you been diagnosed with any new illness since the procedure? If yes, please explain. _____Yes _____No _____Maybe
	a Comments:

Relationship to Treatment: The relationship of the complaint to the treatment was broken down into procedural or stem cell-related complications. The grading scale we used (see below) was adapted from the Adverse Event Reporting system used by the Department of Health and Human Services Office of Human Research Protections (OHRP-http://www.hhs.gov/ohrp/). *Unlikely*: Little or no chance that the complaint is related to the procedure; more likely that complaint can be attributed to another condition (concurrent illness, progression of disease, medication related, etc.) *Possible:* Association between treatment and complaint unknown, but complaint cannot readily be attributed to another condition. *Probable:* Reasonable temporal sequence between complaint and treatment, association seems likely based on physician's experience/medical knowledge.

If the complaint was felt to represent a probable complication, then that complaint was assigned an Intensity grade by the adjudicating physician.

Intensity of Condition: This grading scale was adapted from the Adverse Event Reporting system used by the Department of Health and Human Services Office of Human Research Protections (OHRP). *Mild:* Requires no treatment, does not interfere with ADL's *Moderate:* Low level of inconvenience, may interfere with ADL's, treated with simple measures *Severe:* Interrupts patient's ADL's, requires ongoing systemic drug therapy or other invasive surgical measures to treat condition.

MSC Phenotyping and Quality Assurance

MSCs were counted after every passage. Cells were consistently observed by microscopy during their culture process , and were graded using the morphology scale published by Katsube [28]. The goal of the cell culture was to keep cells in Katsube grades A or B (thin cells that are spindle- like and not bloated). If MSCs tended toward Katsube grade C (larger bloated cells), the treatment protocol was to use cells from an earlier passage for re-injection, due to concerns about excessive cell stress in culture. To determine the phenotype of the cultured adherent cells that were being injected, random cryogenically preserved quality assurance samples were taken from 10 patients, incubated with fluorescently labeled, monoclonal antibodies (mAbs) directed against known stem cell surface markers, and the expression level of the cell surface markers on the cultured cells was analyzed using an Accuri C6 flow cytometer (Accuri Cytometers-Ann Arbor, MI).

RESULTS

Patient Demographics and Areas Treated

A total of 227 patients was treated (Groups 1 and 2), with 14 lost to follow-up. The mean age was 52.8 +/- 13.5 years; 141 were males and 86 females.; 224 were Caucasian and 3 were African American. Some patients underwent more than one procedure. Mean follow-up time from procedure was 10.6 +/- 7.3 months, with 235 procedure follow-up contacts occurring at 3 months or more, 180 at 6 months or more, 96 contacts at 12 months or more, and 19 contacts at more than 24 months (Fig. **2A**). Patients underwent 118 knee procedures, 78 hip procedures, 13 disc procedures, 10 ankle/foot procedures, 10 shoulder procedures, 6 hand/wrist procedures; 9 received various other site treatments (Fig. **2B**)

For Group 1, 45 patients underwent 49 MRI surveillance procedures. On three of these patients, we were unable to obtain imaging follow-up due to non-compliance. One patient failed to get a pre-op MRI, but a post-op MRI was obtained at 3 months. Eight patients had follow-up MRIs after their procedure at various time points, but dropped out of the study; 6 of these dropped out of the study due to

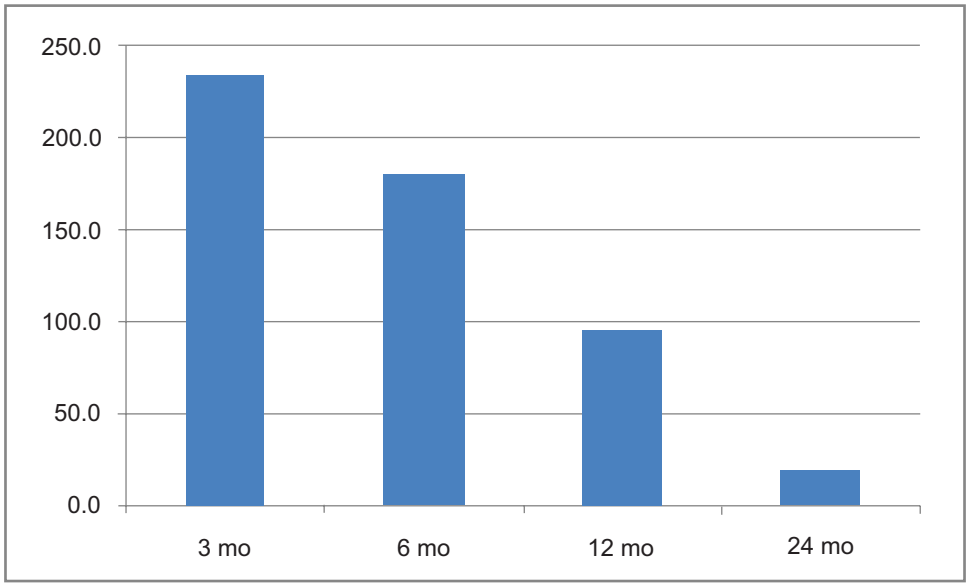

Fig. (2a). Number of follow-up contacts at each end-point (groups 1 and 2).

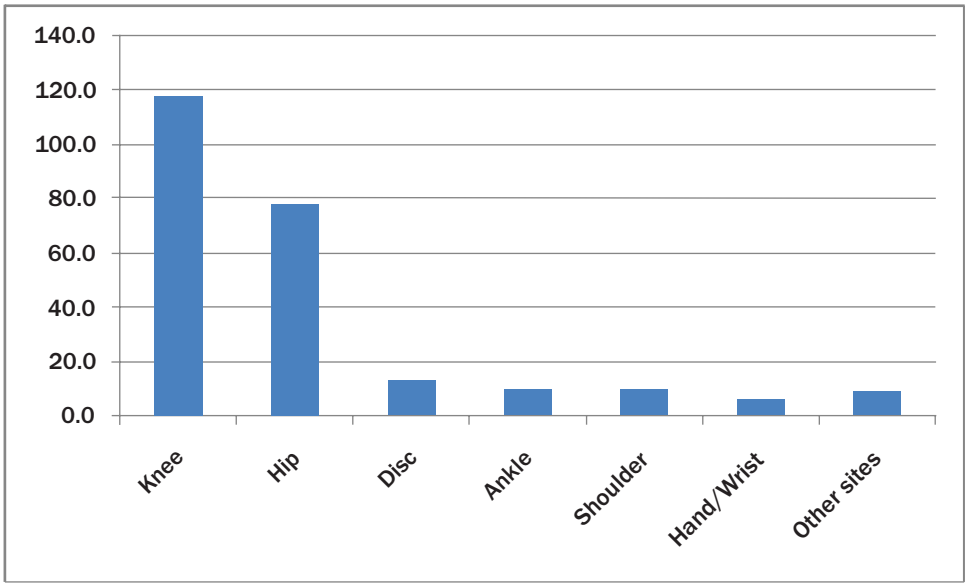

Fig. (2b). Frequency of sites treated in all patients (groups 1 and 2).

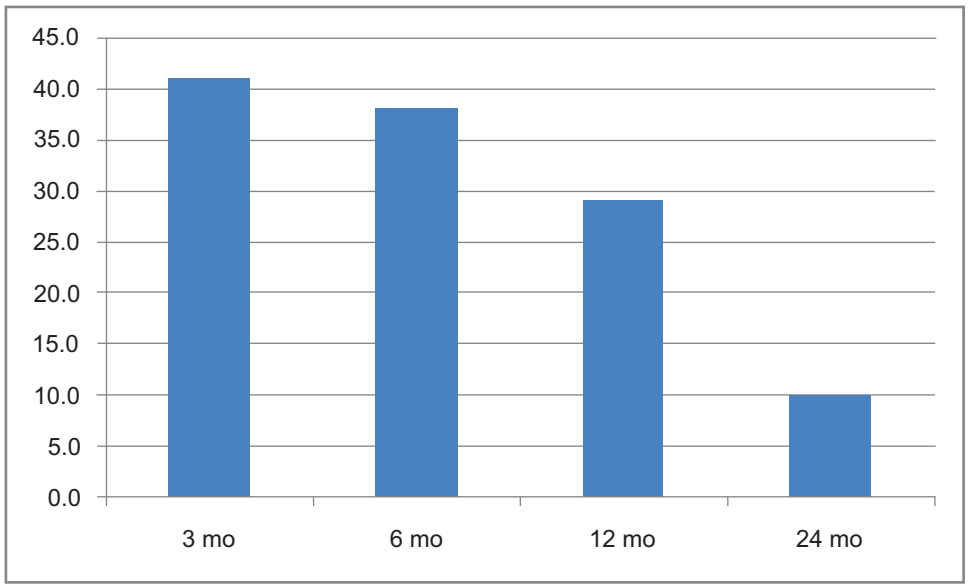

Fig. (2c). Number of negative MRI follow-ups at each end-point (group 1).

a decision to have surgery on the re-implant site, while 2 could no longer be contacted. Median time for last MRI follow-up since the procedure was 15.4 +/- 9.1 months. Median age for Group 1 patients studied was 45.6 +/- 13.8 years with 31 males and 14 females. All had negative MRIs (as read by both examiners) for any evidence of tumor formation at all measured imaging outcome endpoints. Due to some patients receiving more than one procedure, of the 45 patients, 41 MRIs were read 3 months after the procedure, 38 MRIs were read at 6 months, 29 MRIs at 12 months, and 10 MRIs at 24 months. As a result, a total of 118 MRIs were evaluated (Fig. **2C**).

Effects of Radiographic Contrast on MSCs

Live/dead cell analysis of the 1.5, 3, and 4 hour MSC exposure groups at various concentrations of Omnipaque and after 3 days in culture is shown in Fig. (3A). Note that viability decreases only for the 80 mg/ml Omnipaque concentration. Fig. (**3B**) shows the same data, but with all culture conditions compared (Day 0, Day 3, and Day 5): for the day 0 culture group (no recovery time), cell viability with both exposure conditions (1.5 hour and 4 hours) increases with increasing concentrations of Omnipaque, while the data for Day 3 and Day 5 show slight declines in viability with increasing Omnipaque concentrations (>20mg/ml).

Viability of Platelet Lysate Technique Experiments

Demographic and diagnosis data from the patients used for the PL experiments are listed in Table **2**. Fig. (**4**) reveals that average fold increase in number of cells per passage varies by PL concentration. Patients with osteoarthritis generally benefited from an increase in PL concentration, over the 5% PL that has been recommended in earlier studies. However, the benefit observed is greater for middle-aged and older patients ((Gi, Re, Ed, De, and Pr) Note: patient IDs are not based directly on the patient's name, but are assigned according to a HIPPA compliant patient identifier.) Only one older patient did not show a significant benefit from 20% PL over 10% PL (Ca). Of the two younger patients without osteoarthri-

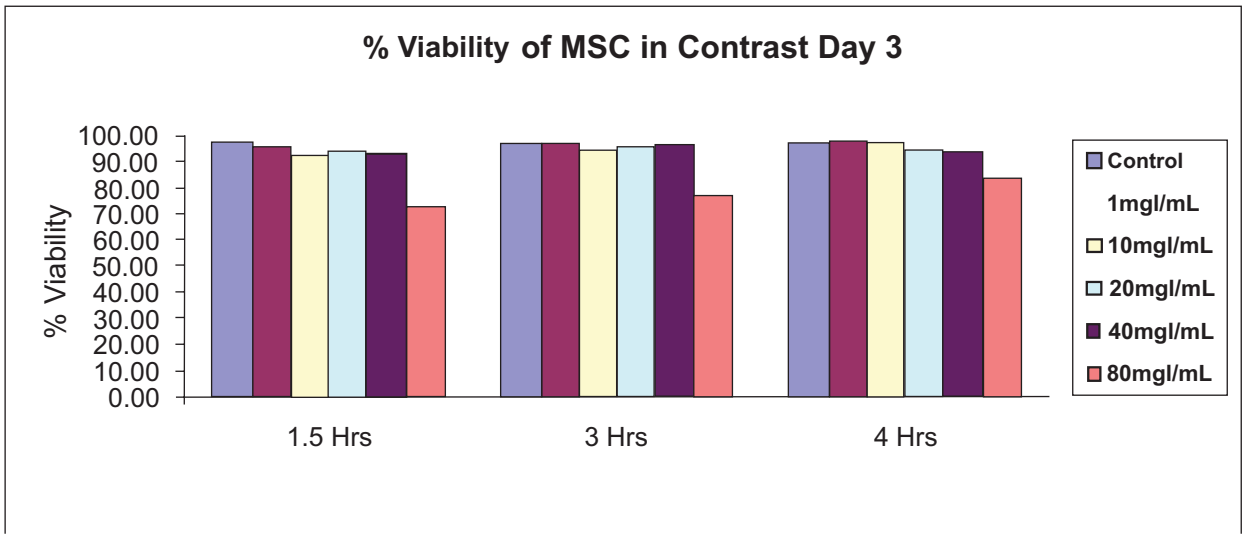

Fig. (3a). Pervent of viable MSC's on culture day 3 after omnipaqque exposure at various concentrations and durations (mgI=mg of Iodine).

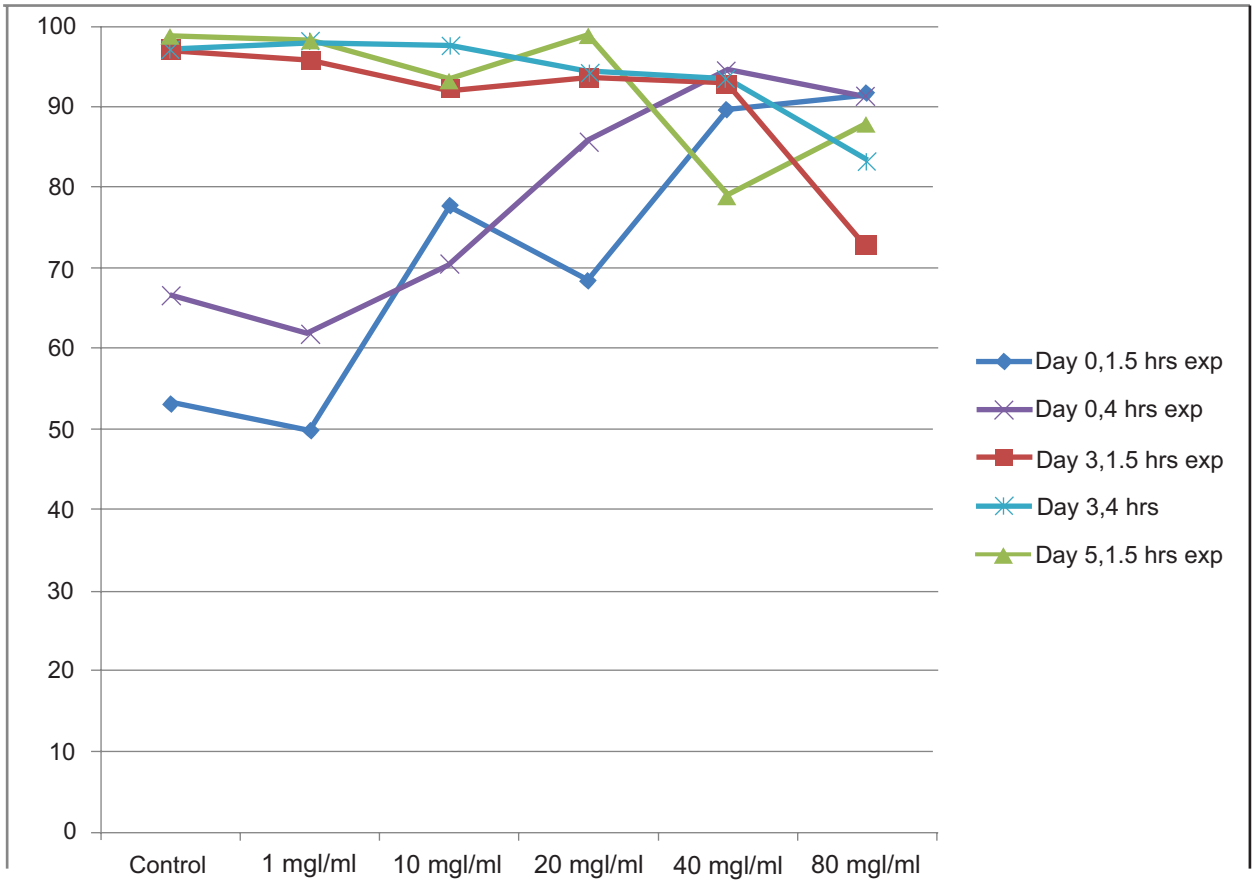

Fig. (3b). Percentage of MSC's live after 1.5 and 4 hours exposure and after 0, 3, and 5 days in culture (mgI=mg of Iodine).

tis (both with traumatic arthropathy), only one showed a modest benefit from 20% PL vs. 10% PL (Ve), whereas the other patient (Sl) showed only a benefit from 10% PL over 5% PL, but no benefit from 20% PL over 10% PL.

Culture Results

The mean volume +/- SD of marrow drawn was 51.3 ml +/- 12.7. Number of nucleated cells obtained from the marrow aspirate was 5.72 X 10^8 +/- 3.75 X 10^8, the average time in culture was 17.3 days +/- 2.30, and the average number of MSCs injected was 19.79 X 10^6 +/- 18.13 (Table **3**). Cultured cells were spindle-shaped (Fig. **5**), which is typical of MSCs in monolayer culture**.** Flow cytometry was performed on cultured MSCs from 10 randomly chosen patients. At time of testing, average +/- SD of days in culture was 14.4 +/- 2.2, with all samples except one (passage 4) at passage 1 or 2. Cultured cells (% positive +/- SD) were CD29+ (95.3 +/- 3.2), CD44+ (96.% +/- 2.37), CD59+ (98.3 +/- 1.37), CD73+ (96.7 +/- 2.34), CD90+ (97.9 +/- 1.24), CD105+ (98.1 +/- 1.35), CD166+ (93.5 +/- 5.4), CD31- (5.78 +/- 2.93), CD34- (2.52 +/- 1.44), and weakly positive for CD14 (20.1 +/- 8.81), CD45 (15.2 +/- 11.7),

Table 2. Demographic Data on Patients Used for Platelet Lysate Experiments

Patient	Age	Race/Sex	Diagnosis
Gi	54	WM	Hip OA
Re	46	WM	Knee OA
Ve	33	WM	Traumatic Knee Arthropathy
Ca	59	WM	Shoulder OA
Ed	57	WF	Hip Bursitis/OA
De	41	WM	Knee OA
Pr	40	WM	Hip Osteonecrosis/OA
Sl	24	WM	Traumatic Spinal Arthropathy

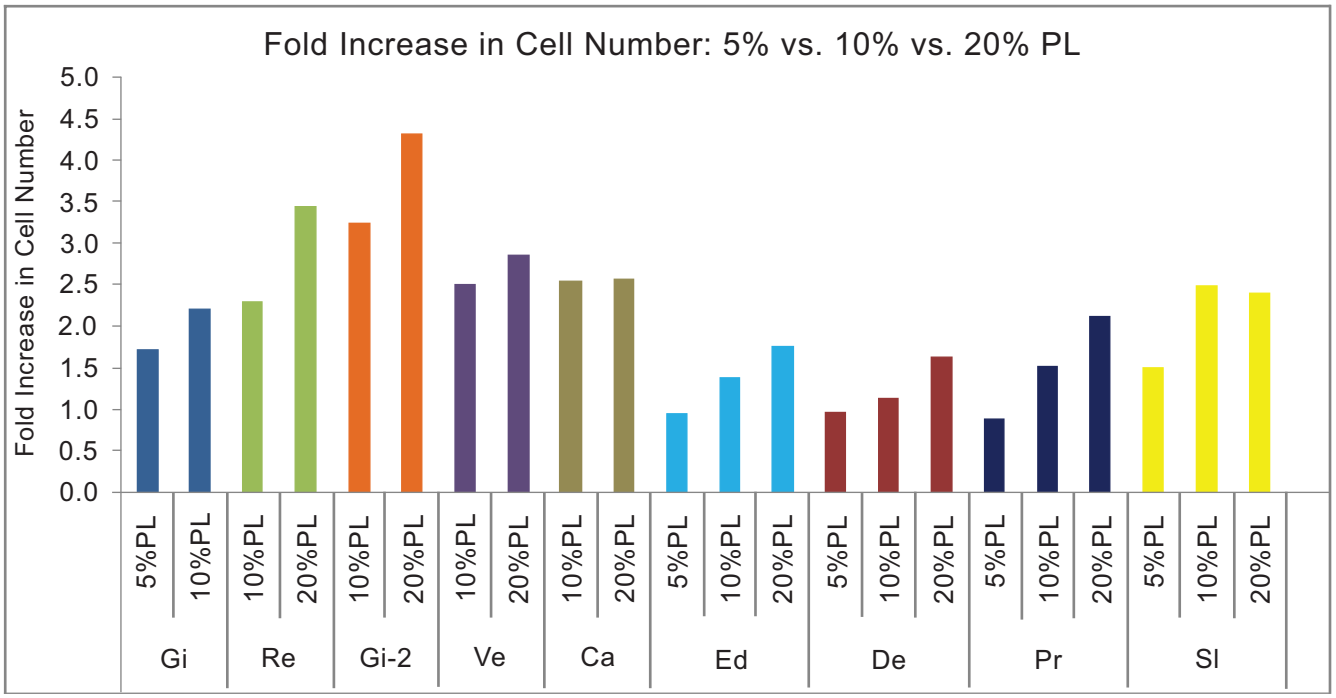

Fig. (4). Average Cell Fold Increase per passage: data presented by patient. Note that Gi-1 and Gi-2 are the same patient who underwent two subsequent expansions approximately 6 months apart. PL=platelet lysate.

CD106 (29.6 +/- 9.6) (Fig. **6**). These findings were consistent with the reported morphology and cell surface phenotype of MSCs grown in monolayer culture [29,30].

Complications Reporting

Reported complications are listed in Table **4** (summary) and Table **5** (more detailed narrative summary). The seven major complications can be summarized as follows:

1. Increased pain and swelling: Less than 5% of the patients treated (n=9) reported increased pain and swelling that was adjudicated by a physician as likely to be related to the procedure. Four of these patients reported self limited symptoms. Three of these patients had their knee effusions drained via arthrocentesis (NM1, LR1, BJ2). Two eventually reported some improvement from the procedure and one went on to total knee arthroplasty (BJ2). Three patients had their knees aspirated and all had clear serous fluid showing no microscopic signs of infection. Two patients who were not drained were told they were candidates for joint arthroplasty before the procedure, and they eventually went on to undergo this procedure due to the increased pain/swelling (CJ5 and HK1).
2. Infection: Two patients reported infection at the marrow draw site. One was unconfirmed by his treating physician and not adjudicated as a possible complication. The second patient (PD2) is listed as a likely procedural complication, and was treated with oral antibiotics (unconfirmed on exam, but history deemed likely accurate).
3. Neurologic: One patient with mild neurologic complaints had a history of MS seen on pre-procedure brain/c-spine MRI (BJ2); complaint was adjudicated as due to a pre-existing disease process. BD1 had a history of type 2 diabetes mellitus with pre-procedure work-up of dizziness and a pre-operative normal brain MRI, and after undergoing a disc procedure reported a general worsening of neurologic symptoms in his legs (which were present before procedure). A follow-up MRI showed no change in his structural disc bulge at L5-S1, a normal

Table 3. Cell Culture Data Summary

	Nucleated Cells (1 x 10 exp 8-Mean +/- Std Dev)	Days in Culture (Mean +/- Std Dev)	MSCs Injected (1×10^6)
Average	5.72 +/- 12.7	17.3 +/- 2.3	19.8 +/- 18.1
Min	13.1	9	0.05
Max	3070.4	26	160.8

EMG/NCS, and a normal post-op skin biopsy revealed no small fiber neuropathy. Follow-up brain MRI was also normal. It was thought that his complaints were musculoskeletal in origin. WE2 reported multiple complaints, but conversations with one of the authors (JS) lead to the realization that her complaints could not be verified via history or via additional follow-up testing.

4. Spine: One patient (MJ4) reported a herniated disc (requiring surgery) some 8 months after a successful disc treatment. Post-surgical pathology demonstrated normal morphology and expected tissue types. This was adjudicated as potentially being related to the procedure, perhaps due to mechanical unhealed needle trauma at the posterior disc annulus.
5. Tumor: One patient (KR2) had the incidental finding of a lumbar spine tumor some 8 months after his procedure. The patient had a long history of low back and hip pain. On later work-up of his chronic low back pain, a benign Schwannoma from T12-L2 was identified; this was surgically removed, leading to resolution of much of his pre-existing back pain. Since the tumor was not at the site of injection, and since his low back pain pre-existed the stem cell procedure, this tumor was thought to be unrelated. To gather additional data, his cryogenically preserved QA sample was sent for cytogenetic analysis (metaphase spreads) and analyzed after approximately 30 days in culture (exceeding our average culture time by almost two times). Only one in 20 MSC's examined displayed trisomy 5 (non-clonal), a genetic abnormality commonly associated with osteoarthritis [31-33]. A search of the literature on chromosome patterns associated with Schwannoma uncovered no published data linking Schwannoma with 5+ [34-36]. In addition, to better replicate the culture conditions prior to his re-injection of cells, he was asked to return for a new diagnostic marrow draw and the therapeutic culture process was repeated to a similar time in culture. The resultant cells were sent for cytogenetic analysis and found to have normal cytogenetics with no clones detected.
6. Hepatic: Several patients reported self-limited elevation of hepatic enzymes on routine blood work (DE1, GJ1).
7. Skin, GI, Other: One patient (MR1) was diagnosed with dermatomyositis 6 months after the injection of his hip; no temporal association was found. One patient (HP2) developed the onset of skin rashes and GI symptoms several months after the procedure and was eventually diagnosed with Barrack's disease; no temporal association was found. One patient (BJ1) reported the detection of methicillin-resistant Staphylococcus aureus (MRSA) (she had reported a prior confirmed and treated MRSA dermal infection) one year after the procedure; no temporal association was found. One patient reported the onset of a pulmonary embolism two weeks after the marrow draw (no stem cell therapy was ever initiated in this patient) and was hospitalized and discharged without sequelae. And lastly, one patient (AJ3) was being concurrently treated by an alternative health practitioner for multiple unconfirmed pathogens that were thought to be causing pre-procedure pain in various areas. We could find no connection between the procedure and these diagnoses.

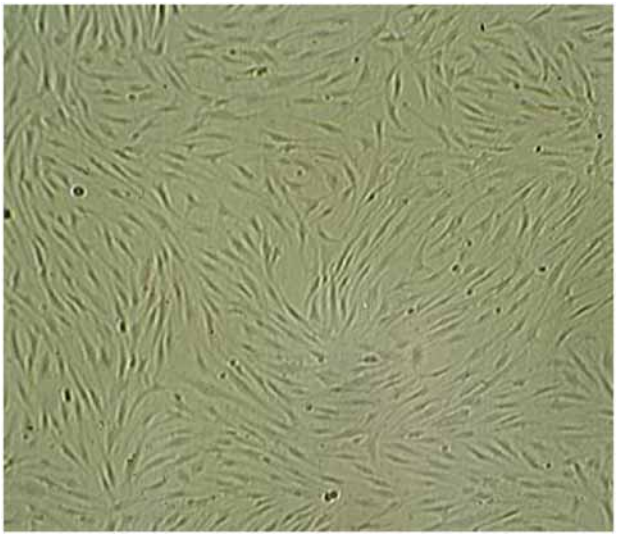

Fig. (5). Exemplar of MSC's grown with this technique in monolayer culture.

DISCUSSION

Based on the exposure data from use of the contrast agent Iohexol (Omnipaque), joint and disc exposures (minimum and maximum expected exposures, concentrations, and times) had a minimal kill effect on the population of injected MSCs. These levels of exposure to the contrast agent *in vitro* were generally much higher than the levels used by us in the present clinical study. For example, the 80 mg/ml concentration of Iohexol was approximately twice the final concentration we estimated for the knee joint. As a result, it is unlikely that in our study, the contrast agent had serious adverse effects on the viability of implanted MSCs. In fact, for the Day 0 culture condition, cells in both the 1.5 and 4 hour exposure groups displayed greater viability as the concentration of Omnipaque was increased. This might be due in part to an initial supportive effect of the contrast agent on the cells. Romano *et al.* determined that ascorbic acid has a cell protective effect when used with contrast agents [37]. The Omnipaque also contains a small amount of hydrochloric acid as an inactive ingredient (acidifying agent), but all of our culture conditions included use of a pH buffering solution. The culture conditions with 3 days or 5 days in the contrast agent would have exposed cells to a neutral pH for significantly longer periods of time, perhaps explaining why the supportive effect was not seen in the longer term cultures.

For the PL technique that we developed for this study, we were able to reliably culture-expand MSCs to total yields of 10^6-10^8. The rates of MSC expansion were influenced by variations in PL concentration, but considerable inter-patient variability was also recorded, even when the same concentration of PL was used. Moreo-

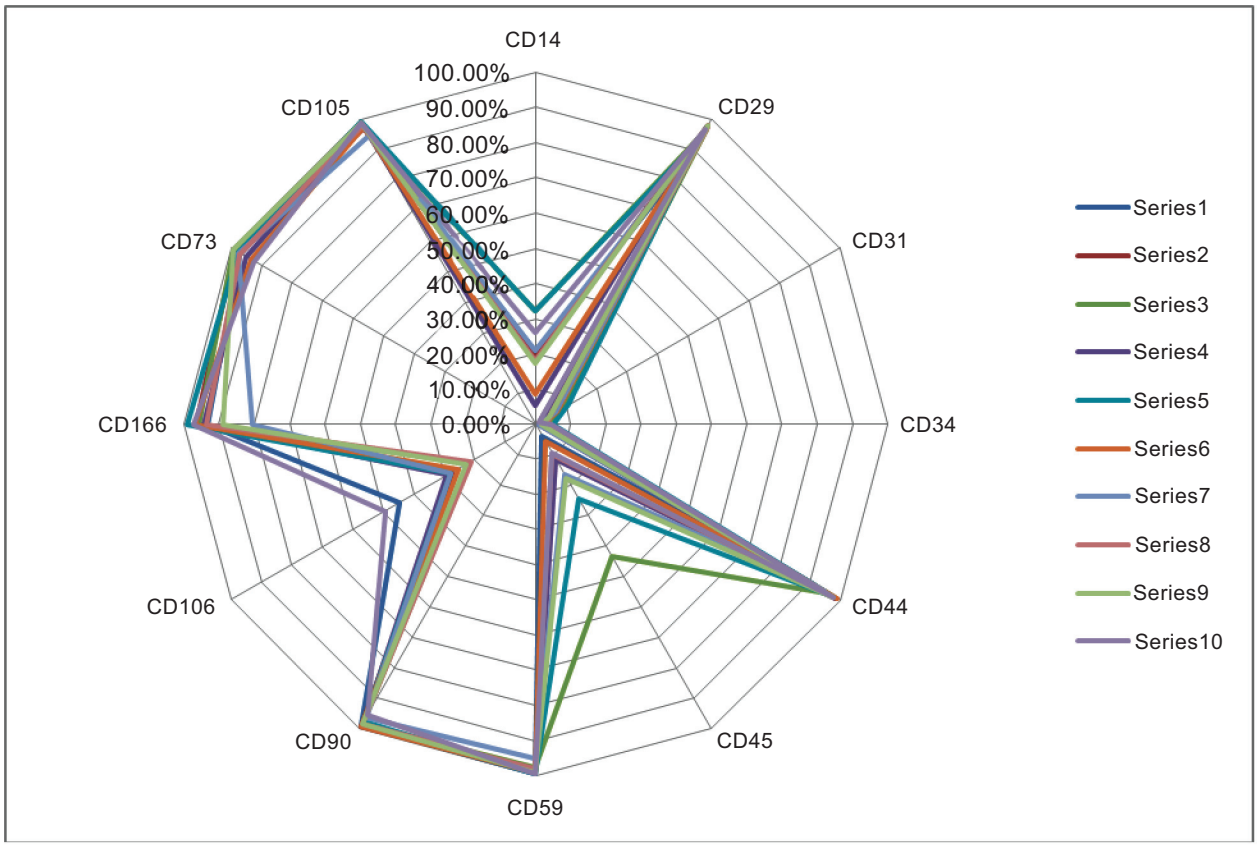

Fig. (6). Flow cytometry of 10 random cryo-preserved MSC samples cultured with the PL technique. At time of testing, average +/- SD of days in culture was 14.4 +/- 2.2 with all samples except one (passage 4) at passage 1 or 2.

ver, older patients tended to show improved cell culture proliferations at PL concentrations of up to 20%. These differences among patients could be related to variable concentrations of growth factors in platelets, or to individual differences in the MSCs. The data published by Martineau clearly showed variable concentrations of growth factors available in platelet products when different donors were compared [38]. In addition, MSCs from some osteoarthritis patients also have variable functionality [39,40]. It should be noted that other authors have reported on adequate culture expansion of MSCs with lower concentrations of PL than used in this study (generally 5-10% while our group used 10-20%); however these authors did not compare their MSC yields with use of higher concentrations of PL (namely 10%-20%), and they also generally used younger patients without osteoarthritis, whereas our trial generally used older patients with osteoarthritis [23-25].

Flow cytometry demonstrated that the randomly sampled MSCs that were isolated and culture-expanded using this technique exhibit the hallmark antigens of MSCs. In particular, classic cell surface markers for hematopoietic stem cells, such as CD34, were not expressed [41] of the 12 cell surface markers tested, only 4 showed some variable expression between the 10 subjects tested (CD14, CD45, CD106, and CD166).

No MRI evidence of tumorigenesis, or of significant complications, was observed at the re-implant sites. The adjudicated complaints list showed that based on HHS criteria for adverse event reporting, 7 cases of probable procedure-related complications were noted (thought to be related to the re-implant procedure itself), all of which were either self-limited or were remedied with simple therapeutic measures. Based on the same criteria, three possible stem cell complications were also reported. These were again either self-limited or were remedied with simple therapeutic measures. There was one report of cancer in this group of 227 patients; however this was almost certainly unrelated to the MSC therapy. The National Cancer Institute, Surveillance Epidemiology and End Results (SEER) program estimates the annual incidence of cancer for all sites at approximately 0.5% of the US white population (using year 2000 data) [42]. With the 224 Caucasian patients followed in the present study, it's not surprising that our dataset would have at least one or more patients with cancer detected over the surveillance period. Moreover, this patient had no tumor detected at the re-implant site, even on serial MRIs (through 1 year). Neither the patient's cryo-preserved quality assurance sample, nor an exemplar sample cultured for a similar period of time that was used for his re-implantation procedure, demonstrated any evidence of transformation on cytogenetic testing, as defined by the International System for Human Cytogenetic Nomenclature-2009 [43]. In addition, recent research by Horie *et al*., demonstrates that MSCs implanted in a joint remain localized to the transplant site [17]. Furthermore, based upon this premise, we conclude that the tumor in this patient is highly unlikely to be correlated to the MSC procedure.

The complete absence of re-implant site tumor formation among these 227 patients is consistent with findings of other authors who report a lack of neoplasia with use of MSCs that have been cultured for short periods and that have some expected telomere shortening [10]. Additionally, for this specific culture technique (i.e., with PL used as an animal serum substitute in the culture expansion of MSCs), prior reports of animal testing for tumorgenicity after 90 days post-reimplantation revealed no tumor formation [25].

Table 4. Summary of Adjudicated Complications Reported Prior to 4/15/09

Patient ID	Procedure Complication Probable	Procedure Complication Possible	Procedure Complication Unlikely	Stem Cell Complication Probable	Stem Cell Complication Possible	Stem Cell Complications Unlikely	If complaint is probable complication, intensity?
AL1			X			X	
AJ3			X			X	
BJ1			X			X	
BJ2			X	X			Mild-Inc Pain/Swelling
BW2			X			X	
BP2			X	X			Mild-Inc Pain/Swelling
BW3	X					X	Mild-Bloodwork
BD0	X						Mild-Inc Pain/Swelling
BD1			X			X	
BA2			X			X	
CA1			X			X	
CJ5	X					X	Moderate-Inc Pain/Swelling
DE1			X			X	
FR1			X			X	
FE1		X				X	
GK1	X					X	Moderate-Allergic Reaction
GJ1			X			X	
HK1			X		X		Moderate
HP2			X			X	
KJ4	X					X	Mild-Bloodwork
KR2			X			X	
LR1			X				Moderate-Inc Pain/Swelling
LS3	X					X	Mild-Inc Pain/Swelling
MR1		X				X	
MR1			X			X	
MF1			X			X	
NM1			X	X			Moderate-Inc Pain/Swelling
PD2	X					X	Moderate-Infection
VI1			X			X	

Table 5. More Detailed Narrative Summary of Complications Reported Prior to 4/15/09

Patient ID	Report Class	Description
AL1	Increased Pain/Swelling	Patient reported increased pain/swelling with post op infrared treatment. The physician determined the patient was using the treatment to excess and it was discussed with him during office follow-ups to d/c use.
AJ3	Other	The patient reported the ongoing diagnosis of multiple pathogens by another physician believed to be causing some of her musculoskeletal symtoms.
BJ1	Other	The patient reported MRSA diagnosed some 1 year after procedure. Also generalized fatigue.
BJ2	Neurologic/Increased Pain Swelling	The patient had a cervical spinal lesion (likely MS) identified on imaging prior to stem cell procedure. No correlation to stem cell procedure identified. Ongoing leg pain and knee swelling. Knee drained , no evidence of infection on post-op imaging nor on joint aspirate performed after injection.
BW2	Cardiac	The patient reported increased pain and swelling after several procedures, this was elf-limited. The patient had a long and significant cardiac history with ongoing work-up during his procedures, this included the development of ongoing arrthymias felt unrelated in timing to any given stem cell related procedure.

Table 5. Contd….

Patient ID	Report Class	Description
BP2	Increased Pain/Swelling	The patient reported self-limited increased pain from the procedure that took several months to resolve.
BW3	Bloodwork	Routine CBC revealed a a transitory and self limited drop in WBC's after bone marrow aspiration.
BD0	Increased Pain/Swelling	The patient reported increased pain at the bone marrow aspirate site.
BD1	Neurologic	The patient had a history of type 2 DM with peripheral neuropathy before procedure. He reported continued deterioation of neuropathic symptoms in lower extremities following procedure. Post procedure EMG/NCS was WNL and already planned biopsy of skin showed mild drop out of small fibers c/w DM. Follow-up low back MRI's failed to show structural worsending of his disc bulge at L5-S1. The patient reported unusual facial symptoms and had brain MRI prior to the procedure which was WNL.
BA2	Cardiac	Reported ongoing cardiac problems related to general medical concerns (history of cardiac problems). Did not correlate timing with stem cell procedure.
CA1	Endocrine	The patient reported an incidental finding on adrenal galnd found on unrelated lumbar MRI. The patient followed up with an endocrinologist and reported back that she was cleared for any concerns regarding neoplasm.
CJ5	Increased Pain/Swelling	The patient reported significant pain with all injections into the involved hip, stem cell or otherwise. He reported increased pain after the procedure which did not resolve after several months, so the patient proceeded with planned hip arthroplasty. He reported good success with his hip arthroplasty.
DE1	Hapatic	The patient had elevated LFT's several months after the procedure and was later diagnosed with gall ballder disease. This was treated with cholecystectomy and the LFT's returned to normal. Thought unrelated to stem cell injection as no mechanism defined.
FR1	Bloodwork	The patients post procedural CBC identified increased WBC's and eosinophils, ultimatley belived related to allergic rhinitis.
FE1	Pulmonary	The patient reported that she suffered a pulmonary embolus approximately 2 weeks after the bone marrow aspirtae procedure before the inititaion of any stem cell therapy. She was treated in hospital and released without known sequela. Due to the timing, relatedness to the bone marrow aspiration was thought unlikely.
GK1	Allergic Reaction	The patient reported generalized hives and was treated for an allergic reaction thought secondary to radiographic contrast.
GJ1	Hepatic	Self limited elevation of LFT's.
HK1	Increased Pain/Swelling	The patient reported insidious onset of knee swelling 2 weeks after procedure and ultimately decided to end treatment and move toward knee replacement. This was adjudicated as likely meeting the temporal association criteria.
HP2	Gastrointestinal/Skin	The patient reported the onset of esphageal symptoms and skin rashes several months after the procedure. Was diagnosed with new onset excema and Barrack's disease. After discussion with the patient, thought that these dianoses did not meet the temporal association criteria.
KJ4	Bloodword/Gastrointestinal	The patient reported self-limited elevated CPK levels post procedure, thought secondary to trauma from Injection. The patient reported new onset GERD after the procedure that was later diagnosed as due to hiatal hernia. We failed to find a mechanisim that would expalin new onset hiatal hernia as being casued by the knee stem cell injection.
KR2	Tumor	The patient had a long history of low back pain as well as hip osteoarthritis. Stem cell injections were intra-articular hip. On later work-up of his chronic low back pain, a benign Schwannoma from T12-L2 was later identified. This was surgically removed with resolution of much of his back pain. Since the tumor was not at the site of injection and his low back pain pre-existed the stem cell procedure, this tumor was thought to be unrelated.
LR1	Increased Pain/Swelling	The patient had immediate onset of pain and significant joint effusion after the stem cell injection. Knee was drained several times and fluid was clear of infection, with only increased PMN's showing inflamation. The swelling later resolved and the knee improved.
LS3	Increased Pain/Swelling	The patient reported immediate increased pain after the stem cell injection that improved, but baseline pain still above pre-injection. Due to it's occurance immediately upon injection, it was felt that his increased pain was from the procedure itself of percutaneous implantation.
MR1	Neurologic	The patient had a history of prior herpes zoster and reported a recurence after the bone marrow aspiration. It was thought this could have been due to transient immunosupression from the bone marrow aspirate. The condition was self-limited.
MR1	Skin	The patient was developed dermato-myositis 6 months after a stem cell injection of his hip. No temporal association.
MF1	Skin	The patient had a long history of venous stasis in lower extremities. The patient reported development of a venous stasis ulcer several months after an injection of stem cells into the hip. No temporal assocation, pre-existing condition.
NM1	Increased Pain/Swelling	The patient had an increase in an existing knee effusion after stem cell injection that needed drainage and ultimately an injection of corticosteroids. Swelling resolved.
PD2	Infection	Likely infection of marrow draw site sucessfully treated with oral antbiotics.
VI1	Bloodwork/Gastrointestinal	The patient with a history of GERD reported an attack of espohagitis several months after the stem cell injection. The patient reported a transiently elevated TSH.

The follow-up period in the present study ranged from approximately 3 to 32 months. Although not ideal, we included cases with shorter 3 month follow-ups, since previous *in vivo* studies for tumorgenicity have used 90 day terms [25]. While it is possible that tumors may still form at some time beyond the average follow-up period represented in our data, this possibility likely decreases at a geometric rate. MSCs replicate every 2-4 days in culture and if that growth were to continue at a similar pace following implantation, a small tumor would be discernable on high field MRI within just a few weeks to months.

Our study does not address the question of tumor formation beyond our surveillance period. Another limitation of this study is that the sample size is not large enough to detect a very low prevalence of tumor formation, however overall new onset malignancy reporting in this group was equal to or less than that generally reported in the U.S. Caucasian population. Additionally, another study weakness is that the number of patients that were tracked declines with longer follow-up periods.

CONCLUSIONS

The present study demonstrated no evidence of neoplastic complications in any re-implant site in 227 patients, who were monitored with high field MRI tracking or via general surveillance. These findings are consistent with those of other authors who have failed to find evidence of malignant transformation in MSCs culture-expanded for limited periods [15]. In summary, based on this longitudinal case series, MSC related complications were generally infrequent, transient, or able to be remediated with simple therapeutic measures.

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