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Cell density, DMSO concentration and needle gauge affect hydrogel-induced BM-MSC viability

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Abstract

Mesenchymal stromal cells (MSCs) have shown potential therapeutic benefits for a range of medical disorders and maintain a focus of intense scientific investigation. Transplantation of MSCs into injured tissue can improve wound healing, tissue regeneration and functional recovery. However, implanted cells rapidly lose their viability or fail to integrate into host tissue. Hydrogel-seeded BM-MSCs offer improved viability in response to mechanical forces caused by syringe needles, cell density and dimethylsulfoxide (DMSO) concentration, which in turn, will help to clarify which factors are important for enhancing biomaterial-induced cell transplantation efficiency and provide much needed guidance for clinical trials. In this study, under the control of cell density (lower than 2×10^7 cells/ml) and final DMSO concentration (lower than 0.5%), hydrogel-induced BM-MSC viability remained over 82% following syringe needle passage by 25 or 27 gauge needles, providing improved cell therapeutic approaches for regenerative medicine.

Keywords

Cell density; cell transplantation; cell viability; cryopreserved cells; DMSO; hyaluronan hydrogel; MSCs

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Introduction

Stem cell therapy is the introduction of progenitor cells into a tissue or organ as a means to offer treatment for various diseases and injuries [1]. Mesenchymal stromal cells (MSCs) have become a popular source for stem cell therapy because they have differentiation and immunological features [2], and offer feasibility and safety in clinical trials [1]. Enhancement of both autologous and allogenic transplanted cell survival has become a vital and rapidly expanding area of investigation [3], with good manufacturing practices (GMPs) required to offer optimal defined quality and safety in cell transplantation.

Furthermore, enriching stem cell therapy is the use of biomaterials as a vehicle for implantation of cells into local tissue for regenerative purposes [4]. Within the past four decades, chemically and physically diverse hydrogels have emerged to become standard materials in regenerative biology due to their unique biocompatibility. Hyaluronan (HA) hydrogels have been found to be a synthetic biomaterial [5] that can protect encapsulated cells from inflammation and surrounding macrophages [6], and provide a biocompatible environment for cell attachment, survival, migration, growth and proliferation [7]. In addition to the mechanical protection provided by encapsulated hydrogels, other factors for optimal cell viability outcomes involve cell seeding density, as well as, cryopreservation techniques.

Proper freezing, transport and cryopreservation of stem cells are crucial for viability, safety and efficiency in cell therapy [8], with recent cell treatments more often involving cryopreserved cells due to the ease of storage, transportation and large-scale supplement. Dimethyl sulfoxide (DMSO) has widespread applications as a drug injectate solvent, cryoprotectant, and differentiating agent [9]; however economical, has been shown to adversely affect cell viability, morphology, differentiation, and gene-expression in a dose-dependent manner [10, 11]. Furthermore, numerous studies have contested the GMP of conventional 10% DMSO for long-term cell storage, elucidating a safe alternative at 5% [12, 13]. DMSO concentration in freezing medium, cell freezing procedures and period of cell storage time [14] have all been extensively scrutinized, however, to date, no studies have reported on the effects of DMSO on MSC viability when used as an injectable solvent for cell transplantation following clinically relevant delivery models.

Previous investigations have provided initial insight into important biological concerns for enhancing the efficiency of cell therapy, however further study is required to identify important parameters for enhancing biomaterial-induced cell transplantation efficiency and provide current GMPs for clinical trials. The goal of the present study was to evaluate the effects of several cell delivery factors on the survival of clinical grade cGMP MSCs for hydrogel-induced cell treatment after passage through clinically relevant 25 or 27-gauge syringe needles (25G, 27G). These parameters included: (1) cell seeding density, and (2) concentration of DMSO in final hydrogel-cell solution. Results of the present study should provide clinically relevant and necessary benchmarks for cell transplantation clinical trials.

Methods

Hyaluronan hydrogel preparation

HyStem-C is a low salt hyaluronan-gelatin hydrogel (Biotime Inc., Alameda, CA), which was obtained by mixing 1ml 1.4% (w/v) Glycosil with 75ul 1.0% (w/v) Gelin-S and cross-linking this mixture with 8.2% (w/v) Extralink (PEGDA). The final concentration of HyStem-C is 1.2% Glycosil, 0.06% Gelin-S and 0.8% PEGDA. All components were dissolved in Lactated Ringer's solution (pH 7.3 to 7.4) in a cell culture hood to ensure sterility. At room temperature, HyStem-C casts in about 5 minutes.

Cells and three dimensional cell culture

Clinical grade primary human BM-MSCs were produced, expanded and tested by the Waisman Biomanufacturing at the University of Wisconsin-Madison, using a manufacturing process and quality control test methods that are similar to those used for cGMP protocols for human clinical trials. Bone marrow aspirates were obtained from a 22-year-old female donor, and BM-MSCs were isolated by the Ficoll-Paque gradient method as previously described [15]. Once isolated, MSCs were cultured in AlphaMEM medium supplemented with 10% fetal bovine serum (FBS) and 1× Glutamax. Cell cultures were maintained at 37°C in a humidified incubator with 5% CO₂ atmosphere and medium was changed once every two days; cell growth was monitored under phase-contrast light microscopy. Cells positively expressed common MSC specific cell surface markers CD105, CD73 and CD90, and were negative for CD34, CD45, CD19 and CD14 [29]. Once the cells reached sub-confluence, cells were harvested with TrypLE, and expended into new flasks. For cryopreservation, BM-MSCs were re-suspended in cold freezing medium containing PlasmaLyte, 10% serum albumin, and 2.5% DMSO, aliquoted into cryovials and subsequently frozen by steps involving slowly decreasing temperatures to a final freeze point of -196°C. Cryopreserved cells were stored in liquid nitrogen. Passage 6 cells were used in all experiments.

NIH 3T3 cells, used as controls, initially emerged from a cell line established in 1962 at the New York University School of Medicine Department of Pathology, and have since become a widely accepted and standard fibroblast cell line. NIH 3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% calf bovine serum (CBS), 1× non-essential amino acid (all from Sigma, St. Louis, MO), and cryopreserved for long-term storage in DMEM containing 10% CBS and 5% DMSO. Before use, cryopreserved cells were quickly thawed in a 37°C water bath with gentle agitation, and then mixed with hydrogel dressing.

For 3D culture, cell suspensions were mixed with HyStem-C at one of three cell densities -- 2×10^6 , 4×10^6 , 1×10^7 , and 2×10^7 cells per milliliter (ml). These cell densities were chosen as they represent densities that have been reported in the literature for tissue specific injections. Cell-gel mixture (0.5 ml) was placed into the individual wells of a 6-well plate with transwell permeable inserts (0.4µm membrane pore size, Millipore Inc. Billerica, MA) by pipette, 25-gauge (25G), 27-gauge (27G) standard syringe needles, and 27-gauge longer ENT (27G ENT) needle. These gauges were chosen as they are common gauges used for injection of biomaterials. After gelation (gel thickness was approximately 0.5 mm), cell

culture medium (DMEM-10% FBS or DMEM-10% CBS) was added above and below the gel. Cell plates were kept in an incubator at 37°C and 5% CO₂. Use of transwell plates allowed for completion of the cell viability assays. In order to test DMSO of frozen media on cell viability, DMSO concentration in final cell-gel mixture was controlled at 0.1, 0.5 and 1.0%.

Cell viability assay

Cell survival rates in HyStem-C were analyzed by the LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA), which is based on a cell-permeable dye for staining of live cells and a cell-impermeable dye for staining of dead and dying cells, subsequently characterized by compromised cell membranes. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity as determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to calcein, which displays as an intense, uniform green fluorescence in live cells (ex/em 495 nm/515 nm). The red component (ethidium homodimer-1, EthD-1) is cell-permeant and therefore only enters cells with damaged membranes. In dying and dead cells, a predominant unclear red fluorescence (ex/em 570 nm/602 nm) is generated upon binding to DNA, which is a strong indicator of cell death and cytotoxicity. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually non-fluorescent before interacting with cells. After 48 hour cultures, inserts with cells and gel were washed three times in 1× PBS, pH 7.4, then incubated with the staining solution (2 μM calcein AM and 4 μM EthD-1) in 1× PBS, pH 7.4, for 30 minutes at room temperature. After incubation, samples were washed again three times in 1× PBS, and then covered in 1× PBS. Cells were imaged with a Nikon E600 fluorescence microscopy (Nikon Instruments Inc., Melville, NY) equipped with an Olympus DP71 CCD (Olympus America Inc., San Jose, CA) at 10× magnification by common green and red imaging filters stained with FITC and Texas Red. Percentage of live and dead cells was determined with MetaMorph software for three images randomly selected for each condition.

Statistical analysis

Percentage of cell survival rates was expressed as mean ± standard deviation. Analysis of variance (ANOVA), with adjustments for multiplicity of testing, was performed with the goal of determining differences in cell viability using multiple needle types for the following experimental conditions: (1) DMSO concentration (2) cell density and (3) cell delivery tool. All analyses were performed using the procedure PROC MIXED from SAS/STAT software (version 9.3), with statistical significance assigned at P<0.05.

Results

Cell viability in response to cell density

In order to test delivered cell viability in response to cell density, we compared cell survival of cryopreserved BM-MSCs to NIH 3T3 cells, which served as controls. Given varying cell densities of 2×10^6 , 1×10^7 , and 2×10^7 cells/ml, and utilizing the delivery methods of 25G/27G needle and pipette control (Figure 1), viability of BM-MSCs displayed over 82% survival rate, and overall, had higher viability than relative cryopreserved NIH 3T3 cells

after 48-hours of incubation in HyStem-C. In particular, at 2×10^7 cells/ml density, the viability of BM-MSCs by needle-delivery was maintained over 85% after passing through all conditions; significantly greater than NIH 3T3 cells at the same densities and passage conditions ($p < 0.05$). In addition, NIH 3T3 cells at the largest dose, 2×10^7 cells/ml, had the lowest viability percentages and were significantly different from 2×10^6 when compared across pipette and 27G conditions ($p < 0.05$).

Cell viability in response to final DMSO concentration in gel-cell solution

In order to test the effect of DMSO on cell survival of HyStem-C seeded cells, we tested a range of DMSO concentrations (0.1, 0.5, and 1.0% in final gel-cell solution) for cryopreserved NIH 3T3, as well as, BM-MSCs delivered by 25G/27G syringe needles and pipette control. As displayed in Figure 2, with final DMSO solvent concentrations at 0.1, 0.5, and 1.0%, cryopreserved BM-MSCs exhibited higher survival rates than fibroblasts, ranging from 92.3% to 83.6% when utilizing pipette or 25G syringe needle delivery methods. However, when DMSO concentration was increased to 1.0% and delivered with a 27G needle, BM-MSCs displayed a significantly lower survival rate (77.7%), compared to 0.1% DMSO (87.6%) ($p < 0.05$). In addition, NIH 3T3 cells exhibited similar findings, with significant reductions in viability at 1.0% DMSO (74.9%), as compared to 0.1% DMSO (85.3%), following 27G needle passage ($p < 0.05$). Overall, BM-MSCs displayed trends of reduced viability while utilizing 27G needle with cell densities at 2×10^7 and at 1.0% DMSO concentration.

BM-MSC viability in response to syringe-needle type and gauge size when controlling for optimal seeding density and DMSO concentration

At present, longer and finer needles are required for specific surgical procedures in order to reach from point of entry (i.e. skin) to injectable destination (i.e. larynx, ear, sinuses). These needles can be as long as 9.8 inches or more, about 6–7 times the length of standard syringe needles. Given the overall viability of the biomaterial embedded BM-MSCs, we additionally tested their ability to withstand a longer needle length and found no significant differences after 48h incubation, following passage through either pipette, 25G, 27G standard syringe, and longer 27G ENT needle when controlling for cell density at 4×10^6 and in 0.5% DMSO (Table 1).

Discussion

Based on the unique characteristics of differentiation and immune-regulation [16, 17], MSCs are considered an ideal source for clinical stem cell therapy. However, poor survival of implanted stem cells (0.2% to 5% viability) has been observed one month after injection [18, 19]. These findings raised several important questions regarding survival, location and differentiation of stem cells as well as their effects on ECM and endogenous cells. Previous findings have reported that implanted cells in local tissue rapidly lose their viability or fail to integrate into host tissue because of inflammatory responses [3]. Biomaterials can provide a biocompatible environment for cell attachment, growth and proliferation and instruct cell function [20, 21]. They can also protect encapsulated cells from inflammation and surrounding macrophages [6], and improve the acute cell viability of stem cells [22]. In

preclinical and clinical studies, MSC transplantation with biomaterials have shown greater benefits than without biomaterials in skeletal and bone tissue regeneration [23]. HyStem-C is a hyaluronic acid (HA)-based injectable biomaterial, which has been shown to approximate the biomechanical properties of the tissue and encourage ECM regeneration [7, 24–26]. Over the last decade, this hydrogel-induced cell treatment was applied in animal studies, and demonstrated certain functional recover in injured tissue [24, 26]. However, our understanding and knowledge of cell viability after delivery with HyStem-C is limited. Therefore, in this study we used BM-MSCs seeded in this hydrogel to test the survival rates in clinically relevant conditions.

Considering the clinical need for minimal cell infusion volume, we tested cell viability using a relatively high cell density and combined the cells with a biocompatible biomaterial. We evaluated maximum cell densities which has been reported in the literature to have been used to test for safety and efficiency [27]. Rates of cell viability were cell specific; cryopreserved BM-MSCs were not highly sensitive to higher cell density concentrations when compared to cryopreserved frozen NIH 3T3 cells. Cryopreserved NIH 3T3 cells displayed significant reduction in viability with cell concentrations increasing from 2×10^6 to 2×10^7 cells/ml. Possible explanations for this phenomenon may be that cells may have a more limited supply of nutrients from medium at these higher concentrations, within a solution. Also at higher cell densities, protection provided by HA-based hydrogel particularly when passed through smaller gauge needles may be insufficient to prevent cell apoptosis and death. Similar results have also been reported in 3D cultured bovine nucleus pulposus cells in alginate beads at a range of cell densities (1.25×10^5 to 1.25×10^6 cells/ml) [28], where cell proliferation was inversely related to cell seeding density, and the number of apoptotic cells was positively correlated to cell seeding density. We expected to find parallel results with our BM-MSCs, given previous investigations where displayed MSCs seeded at lower densities exhibited faster rates of proliferation and achieved increasingly larger numbers of adherent cells than those seeded at higher concentrations [29, 30]. However, human BM-MSCs did not show any viability decrease when cell density ranged from 2×10^6 to 2×10^7 cells/ml. Their viability remained high (82.5 to 88.8%) with all delivery tools (e.g. needles or pipet). This phenomenon may be explained by a glucagon-like peptide-1 agonist, Exendin-4, which has been shown to promote BM-MSC proliferation and migration, as well as, reduce apoptosis through specific cell pathway signaling [31, 32]. These results suggest that, at least for hydrogel-induced BM-MSC therapy, the maximum and safe cell density could extend up to 2×10^7 cells/ml in as small as 27 gauge needles. In order to enhance cell transplantation efficiency, both cell type and cell concentration should be equally considered.

For cell therapy, stem cells could be delivered either fresh or cryopreserved. In this study, we investigated cryopreserved BM-MSCs due to the vast utility of these technique. As a cryoprotective agent, DMSO is added in certain quantities to cell freezing medium to prevent the formation of ice crystals during the freezing process; otherwise cells would be destroyed and expire. To prevent this phenomenon, 5–10% DMSO is commonly used when cell banking, and has exhibited low toxicity to cells. In this study, the safety range of DMSO in cell therapy was tested for clinical grade human BM-MSCs and NIH 3T3s. We found that when the final DMSO concentration for injecting was increased to 0.5%, cell survival rates

continued to remain over 80%. Although, it was reported that 1.4% DMSO in cell culture medium did not affect cell growth [33]; most studies demonstrated that DMSO substantially altered the morphology and attachment of cultured cells in concurrence with a significant reduction in cell viability in a dose-dependent manner by permeabilization [34]. Furthermore, it has been reported that a maximum DMSO concentration should not be over 0.5% [10]. Our results revealed that 1% DMSO showed some cytotoxicity for our seeded cells (both types) after passage through a 27G syringe needle, but not with larger gauge needles.

Prior studies have shown that the gauge and length of various syringe needles affect cell viability during cell delivery [9, 11, 12, 14, 35], likely due to the mechanical forces imposed by narrow-bore needles resulting in cell membrane damage and ultimate cell death. We examined two clinically relevant cell delivery systems (25G or 27G), and found no meaningful effect with hydrogel-induced human BM-MSCs and NIH 3T3 cell viability in regular cell density (2×10^6 to 1×10^7 cells/ml) and DMSO concentration (0.1 to 0.5%). The longer 27G needle likely prolonged cell exposure to physical shearing forces, however, no statistically significant differences were observed with regard to cell viability 48hrs after injection utilizing the various delivery method conditions.

Conclusion

The present study was designed to scrutinize the following conditions during clinical cell encapsulated transplantation: (1) quantity of cells, (2) DMSO concentration, and (3) injection needle on cell viability of clinical grade human BM-MSC with NIH 3T3 cells utilized as controls. Our results demonstrated that BM-MSC survival rates can be maintained at about 85 to 88%. Hydrogel-induced cell viability can be affected by needle gauge and length, while the viability of injected hydrogel-cells is more likely affected by cell density and DMSO concentration. Under the control of cell density (lower than 2×10^7 cells/ml) and final DMSO concentration (lower than 0.5%), hydrogel-induced BM-MSC viability could remain over 82% post-injection with clinically relevant needles (25G or 27G), whereby improving cell therapeutic approaches for regenerative medicine. Although our study investigated the viability of BM-MSCs in response to various injection concerns, future projects could provide additional benefit for clinical trials by investigation of delivered cell differentiation and function.

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Figure 1.
Cryopreserved Cell Viability of 3T3 and BM-MSCs in Response to Cell Density.

Figure 2.
Cryopreserved Cell Viability of 3T3 and BM-MSCs in Response to DMSO Concentration

Table 1BM-MSC Viability in Response to Syringe-Needle Type and Gauge Size in 0.5% DMSO and 4×10^6 cells/ml

Name	Internal diameter	Length	Cell Survival Rate (%)
1000 μ l pipet tip	0.830 mm	50.8 mm	86.5+/-3.0
25 gauge needle	0.260 mm	38.1 mm	86.3+/-2.9
27 gauge needle	0.210 mm	31.75 mm	84.9+/-4.2
27 gauge ENT needle	0.210 mm	248.92 mm	82.8+/-4.0