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Title: Impact of concurrent pressure and shear on MSC viability and differentiation during simulated intervertebral disk needle injection.

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Corresponding Author: Dr. Jean. F. Welter, MD, PhD

Corresponding Author's Institution: Case Western Reserve University

First Author: J. David Prologo, MD

Order of Authors: J. David Prologo, MD; Lori Duesler, BA; Jim A Berilla; Harihara Baskaran, PhD; Mark D Schluchter, PhD; Jean. F. Welter, MD, PhD

Abstract: Purpose: Imaging-guided mesenchymal stem cell (MSC) injection is a promising therapy for intervertebral disk (IVD) degeneration, a major cause of chronic disability, and has provided functional restoration in animal models. However, injection of MSCs into the IVD occurs against considerable backpressures, and the effects of combined shear and pressure on the MSCs during injection is unknown. We evaluated these effects in an in vitro model. Materials & Methods: MSC (2M or 4M cells/ml) were studied either before or after passing them through a needle (25 and 22 Ga, 7" spinal needles), into vials without and with backpressures (30 or 60 PSI). We solved Navier-Stokes equations to estimate peak shear forces. Cells were subject to live-dead assays, or chondrogenic aggregate cultures. Results: Peak shear stress (143-247 Pa), insufficient to cause cell death, occurred at the transition from the hub to the needle. The % dead cells remained unremarkably low at <11. At 21 days, chondrogenic differentiation was evident in all aggregate sections, but the cross-sectional area of the proteoglycan-positive fraction was lower in those from the no-injection groups (57% and 74% for the 2M and 4M cells/ml groups, respectively) compared to 100% in all other groups. Conclusion: Peak pressure and shear levels achieved during injection did not alter cell viability. All sheared groups had better differentiation than controls, which may represent a beneficial effect of needle delivery.

Suggested Reviewers: Jack W Jennings MD, PhD
Assistant Professor, Musculoskeletal Section, Radiology, Mallinckrodt Institute of Radiolog, Washington University
Jenningsj@mir.wustl.edu
Expertise in spine intervention

John F Cardella MD
Director of interventional radiology, Radiology, Geisinger Health System
jfcardella@geisinger.edu
Active in this field, has related publications

David C Madoff MD
Chief of interventional radiolog, Radiology, Weill Cornell Medical Cente
dcm9006@med.cornell.edu
Performs related research

Cover Letter

CASE WESTERN RESERVE UNIVERSITY EST. 1826

Cleveland, January 9, 2014

Editorial Office
Journal of Vascular and Interventional Radiology
Society of Interventional Radiology
3975 Fair Ridge Drive
Suite 400 North
Fairfax, VA 22033

To whom it may concern,

I am attaching a manuscript entitled "*Impact of concurrent pressure and shear on MSC viability and differentiation during simulated intervertebral disk needle injection*" for consideration for publication in the regular issue of *Journal of Vascular and Interventional Radiology*.

My co-authors and myself do not have any financial conflict of interest to declare. The study was funded in part by funds from the NIH. Merrit Medical donated or lent some of the equipment used, however they were not involved in the study design or interpretation.

Please let me know if I can supply any additional information. As corresponding author, I can be reached using the contact information on this page.

With kind regards,

Jean Welter

JEAN F. WELTER, M.D., Ph.D.
RESEARCH ASSOCIATE PROFESSOR
DEPARTMENT OF BIOLOGY, SKELETAL RESEARCH CENTER

MAILING ADDRESS:
Case Western Reserve University
2080 Adelbert Road
Cleveland, OH 44106-7080

VISITORS AND DELIVERIES:
Millis Science Center
Room 112A

Phone: 216-368-1333
Fax: 216-368-4077
e-mail: jfw2@cwru.edu

Impact of concurrent pressure and shear on MSC viability and differentiation during simulated intervertebral disk needle injection.

[1]J. David Prologo, MD, [2]Lori Duesler, BA, [3]Jim A. Berilla, [4]Harihara Baskaran, PhD, [5]Mark Schluchter, PhD, and [1,2]Jean F. Welter, MD, PhD

[1]National Center for Regenerative Medicine and the Departments of [2]Biology, [3]Civil Engineering, [4]Chemical Engineering, and [5]Epidemiology & Biostatistics, Case Western Reserve University, Cleveland, OH 44106

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Please address correspondence to:

Jean F. Welter

Department of Biology, Skeletal Research Center

Millis Building, room 112A

2080 Adelbert Road, Cleveland, OH 44106

T. : +1(216) 368-1333, F. : +1(216) 368-4077, Email : jean.welter@case.edu

Abstract

Purpose: Imaging-guided mesenchymal stem cell (MSC) injection is a promising therapy for intervertebral disk (IVD) degeneration, a major cause of chronic disability, and has provided functional restoration in animal models. However, injection of MSCs into the IVD occurs against considerable backpressures, and the effects of combined shear and pressure on the MSCs during injection is unknown. We evaluated these effects in an in vitro model. Materials & Methods: MSC (2M or 4M cells/ml) were studied either before or after passing them through a needle (25 and 22 Ga, 7" spinal needles), into vials without and with backpressures (30 or 60 PSI). We solved Navier-Stokes equations to estimate peak shear forces. Cells were subject to live-dead assays, or chondrogenic aggregate cultures. Results: Peak shear stress (143-247 Pa), insufficient to cause cell death, occurred at the transition from the hub to the needle. The % dead cells remained unremarkably low at <11. At 21 days, chondrogenic differentiation was evident in all aggregate sections, but the cross-sectional area of the proteoglycan-positive fraction was lower in those from the no-injection groups (57% and 74% for the 2M and 4M cells/ml groups, respectively) compared to 100% in all other groups. Conclusion: Peak pressure and shear levels achieved during injection did not alter cell viability. All sheared groups had better differentiation than controls, which may represent a beneficial effect of needle delivery.

Introduction

Back pain is a major public health issue affecting up to a third of the industrialized world population, and is strongly associated with intervertebral disk (IVD) degeneration [1]. Patients experience pain-related quality of life deterioration, loss of productivity, and psychological distress [2]. Lumbar IVD degeneration, in particular, begins early, with clear changes found in adolescence that accumulate over the life of the patient [3]. By age 50, IVD degeneration reaches a prevalence of 88%. Costs are estimated at 50 billion dollars annually in the United States and 12 billion in the United Kingdom, either through loss of wages, medical costs, or disability benefits [2, 4] .

There are few effective therapies for symptomatic degenerated discs. Conservative interventions such as intradiscal electrotherapy or nucleoplasty produce inconsistent and temporary results, similar to placebo [5-7]. Spinal fusion improves back pain, however, patient satisfaction following surgical fusion is low at 65%. Fusion (and prosthetic disc replacement) reduces spinal flexibility, and overloads adjacent segments, leading to additional surgery in 30% [8, 9].

A treatment which preserved the physiological function of the IVD would be preferable, and cell-based therapy is being explored as an option. Several groups have established methods of isolating, characterizing, expanding, labeling, and delivering adult bone marrow derived mesenchymal stem cells (MSCs) to degenerated or damaged articular surfaces [10-15]. MSCs are multipotent adult stem cells with the capacity to differentiate into several tissue types, that, when implanted into the IVD, secrete ECM constituents and growth factors. The effectiveness of this technique has been demonstrated in vitro and in animals [12, 13, 16-21], using auto- [22, 23] or allo-transplantation [24]. In early

work this was done though open grafting of scaffold-MSC composites [23] as engineered tissue. The interventional radiology technique of fluoroscopically guided percutaneous needle access to the IVD is potentially a less invasive approach to delivering MSC to the IVD [22, 25].

Infusion of cells through a needle or catheter is well-established, but, in general, these infusions take place into a low backpressure environment such as the peripheral venous circulation in blood transfusions [26]. The effects of injection against a higher pressure head have been speculated on but remain unstudied. In particular, the impact of combined pressure and shear on the injected cells is not known; in this study, we sought to determine whether MSCs can be injected against backpressures typical of an IVD NP, without adverse effects.

Materials and Methods

Materials

Integra spinal needles (25 Ga × 7” and 22 Ga × 7”, Integra Life sciences, Salt Lake City UT). Intellisystem 25 syringes were used for injection, as were the Intellisystem display and printer. These devices were loans or gifts from Merit Medical Systems (South Jordan, UT).

Cell culture medium (Dulbecco’s Modified Eagle’s Medium with either 4.5 g/l – DMEM-HG or 1.5 g/l glucose – DMEM-LG), trypsin, L-glutamine, antibiotic/antimycotic (Penicillin G, streptomycin sulfate, and amphotericin B), non-essential amino acids, and sodium pyruvate, as well as the Live-dead assay kit were obtained from Invitrogen (Carlsbad, CA). A lot of fetal bovine serum (FBS) was also obtained from Invitrogen,

after screening as described by Lennon et al. [27]. Recombinant human transforming growth factor beta -1 (TFG-β1) was from Peprotech (Rocky Hill, NJ); recombinant human fibroblast growth factor-2 (FGF-2) was generously provided by the Biological Resources Branch of the National Cancer Institute. ITS$^+$ Premix™ was from Collaborative Biomedical Products (Collaborative Research, Bedford, MA), while ascorbate-2-phosphate was from Wako (Richmond, VA). Bovine calf serum was from Hyclone (Logan, UT).

All cell culture plasticware was from BD Biosciences (Franklin Lakes, NJ), except for the polypropylene 96-well plates which were sourced from Phenix Research Products (Hayward, CA). Nominally 10 ml sterile pyrogen-free borosilicate glass septum vials were obtained from Allergy Labs (Oklahoma City, OK, actual volume 12.8 ml). Syringes and needles were from Becton Dickinson.

Cells and cell culture:

Deidentified human bone marrow aspirates were obtained through our institutional Hematopoietic Stem Cell Core Facility from a single healthy adult volunteer donor (female, 23 y.o., Caucasian), using a procedure reviewed and approved by the Institutional Review Board and after informed consent was obtained. Briefly, 10 – 15 ml of bone marrow aspirate were combined with DMEM-LG with 10% FBS and centrifuged at 500 × g for 5 minutes. The cell pellet was resuspended in 5 ml of DMEM-LG with 10% FBS, and subjected to centrifugation over a preformed Percoll gradient. After centrifugation at 460 × g, the top 25% of the gradient was collected, washed with serum-containing medium, and re-centrifuged at 500 × g. Cells from the resulting pellet were seeded onto 10 cm diameter plates at 1.8×10^5 nucleated cells/cm^2, and

incubated for four days. At this point, non-adherent cells were removed by replacing the medium. At this and subsequent feedings, the medium was supplemented with 10 ng/ml FGF-2 (complete growth medium) [15]. Subsequently, the medium was replaced every third day for approximately two weeks, after which the MSCs were subcultured using 0.05% Trypsin EDTA [14] and re-plated in T-175 flasks at 5×10^3 cells/cm^2. At the end of first passage (7 days), the MSCs were again trypsinized, counted using a hemacytometer, and then resuspended at 2×10^6 or 4×10^6 cells per ml in complete growth medium.

For chondrogenic differentiation experiments, a defined chondrogenic medium was used, composed of DMEM-HG supplemented with 1% ITS$^+$ Premix™, 100 µM ascorbate-2-phosphate, 10^{-7} M dexamethasone, and 10 ng/ml TGF-β1 [28]. L-glutamine, antibiotic antimycotic, non-essential amino acids, and sodium pyruvate were added at 1%.

Injection protocol:

There were seven experimental groups. Two milliliter aliquots of the cell suspension were taken from the syringe using only the extension tubing, but no needle (no injection controls). Aliquots were then passed through either a 22 Ga or a 25 Ga needle either directly into an open collection vial at atmospheric pressure (no backpressure) or into a sterile septum vial against a ~ 207 kPa (30 PSI, low backpressure) or ~ 414 kPa (60 PSI, high backpressure) head. Injections were done by hand and took 26 ± 3 s (mean ± SD). Timing and pressures were monitored and recorded.

Live/dead assay:

Round glass coverslips were cleaned in a 68% nitric acid bath for 1 hour, repeated once, rinsed extensively in diH_2O and autoclaved. Following syringing, 4-6 replicate aliquots from each treatment condition were plated at 23,000 cells/well onto the coverslips in 24-well plates. 24 and 48 hours later, live dead assays were performed following the manufacturers protocols (L-3224, Molecular Probes/Invitrogen). Stock solutions (Calcein AM, 4 M in DMSO, ethidium homodimer, 2 mM in 25% DMSO in H_20) were diluted 1:4000 in sterile Tyrode's solution. These dilutions had previously been found to work well with human MSCs. The coverslips were covered with 500 µl of the staining mixture for 30 minutes, after which the cover slips were wet-mounted and photographed immediately at 5 × by fluorescent microscopy. Live (green cytoplasm) and dead (red nuclei) cells were counted on 3-5 random fields, using ImageJ. Results were expressed as percent of total cell number which stained positive with ethidium homodimer.

Chondrogenic medium and aggregate culture:

Microplate chondrogenic aggregate cultures were prepared using the remainder of the cells.[29] Briefly, the cells were resuspended in chondrogenic medium (see above) at 1.25×10^6 cells per ml, and 200 µl of cell suspension were then pipetted into each well of an autoclaved 96-well, V-bottom, 300 µl polypropylene microplate. The plates were centrifuged for 5 minutes at 500 × g and then placed in a cell culture incubator (37 °C, humidified atmosphere of 95% air and 5% CO_2). Twenty-four hours later, the newly formed aggregates were released from the bottom of the wells to ensure that they float freely by pipetting a small volume of the medium with an 8-channel pipette. The

chondrogenic medium was replaced every three days.

At 21 days, the aggregates were harvested, formalin-fixed, and paraffin-embedded as described previously. Seven micrometer sections were stained for proteoglycan content using Toluidine Blue-O. All sections (several from each aggregate) were imaged in a single session in bright-field using a Leica DM-LB2 microscope and a Spot-RT camera. Quantitative image analysis was performed on the resulting 8-bit per channel RGB tiff images. We used ImageJ with the “Threshold Color” plugin as a band pass filter in hue/saturation/brightness (HSB) mode, with a pass band of 165-255 set in the Hue channel. [30] This identified the cross-sectional area of the aggregates that stained metachromatically (**Fig. 1B, C, & D**), which was expressed relative to the total cross-sectional area (**Fig. 1E**) of the section.

Modeling:

We used computational modeling to determine peak shear forces on the cells at the transition of the syringe hub to the needle bore using a Comsol Multiphysics model. To this end, molds were made of the needle hub using poly(dimethyl siloxane) (**Fig. 2**). The molds were carefully measured and the dimensions used for the model.

We solved axi-symmetric Navier-Stokes equations in the transition region (**Fig. 3**). The transition region is where the cells are expected to encounter maximum shear forces in the entire system; it corresponds to the region where the conical body connects to the cylindrical needle (**Fig. 2 (arrow) and 3**). Viscosity correction for cell suspensions was modeled using the Einstein’s suspensions model; for the volume fraction corresponding to the cell density used in the experiments, the cells had very little impact (<0.1%) on the viscosity (1.001 mPa-s) of the fluid. The density of the fluid was set to 1000 kg/m^3.

Boundary conditions included: no slip conditions at the solid walls, inlet average velocity was specified based on the flow rate, and the outlet pressure value was specified based on the measurements. Meshing was carried out to obtain extrafine meshing near the walls and the transition region where the changes were expected to be dramatic. The model was solved under steady-state conditions to obtain velocity and shear rate profiles. Post-processing of the data was carried out to obtain shear stress profiles in the system.

Software packages

Software packages used for data acquisition and analysis include Excel (Microsoft, Redmond, WA), ImageJ [30], SPOT-RT software (Diagnostic Instruments, Sterling Heights, Michigan), Comsol Multiphysics (Comsol, Inc., Burlington, MA) and Sigmaplot / Sigmastat (Systat Software, San Jose, CA), and SAS Version 9.3 (Sas Institute, Inc., Cary, NC).

Results

Modeling:

Velocity profiles in the system indicate that the peak values occurred along the axis of the needle with values as high as 2.5 m/s (**Fig. 3A**). Despite these large values, the maximum Reynolds number was about 12 in the needled groups indicating laminar flow situations in the system (**Fig. 3**). Pressure profiles in the system indicate that the pressure drop across the model section was around 8000 Pa. Shear rate profiles showed that peak wall shear stress values occurred in the transition region (**Fig. 3B**) and, for the conditions used in the experiments, they ranged from 143-247 Pa. Dwell

time in the transition region was <<1 sec.

Cell-based experiments:

All manipulations were accomplished under sterile conditions; there were no experimental losses due to contamination.

Cell viability:

There were almost no (1-5 per 5× field) non-attached cells in all groups, either at 24 or 48 hours, suggesting that the live-dead assay on cover-slip attached cells would be representative of the total population. On average, 1500 cells were counted per group for each day. Results of the live-dead assays are shown in **Fig. 4** for all groups at 24 and 48 hours after the injection procedure. With a few exceptions, the percentage of ethidium-positive cells was under 10%, and in general (10 out of 14 conditions) the percentage of dead cells was higher at 48 hours than at 24. However, the time effect was not significant in the ANOVA analysis, and both are fairly typical of passaged MSC in our hands.

For statistical analysis, the weighted mean and standard error of the proportion of dead cells for each slide was calculated using multiple readings from the same slide using a random-effects model used for meta-analysis [31]. The data were analyzed using a weighted 3-way ANOVA, including main effects for condition (the seven combinations of gauge/pressure), time (24 vs. 48 hours), and cell number (2 or 4 M cells/ml), where weights were the inverses of the squared estimated standard errors of the estimated percentages for each slide. Use of a main-effects only ANOVA was justified since the 2-way interactions were all non-significant (all p-values ≥ 0.45). The main effect of time

was not significant (p = 0.66), whereas main effects for cell concentration (p = 0.018) and condition (p = 0.015) were significant. The estimated percentage of dead cells was higher when the concentration was 4 M/ml as compared to 2 M/ml (mean difference = 0.020, 95% CI (0.004, 0.035). Pairwise comparisons of the 7 conditions correcting for multiple testing using the Tukey-Kramer method found that the 22 gauge needle – low backpressure condition had higher cell death as compared to both the 25 gauge needle-Low and the 25 gauge needle no backpressure conditions. No other differences reached statistical significance, although confidence intervals indicated that some of the conditions could differ by as much as 0.10 in the proportion of dead cells. (**Fig. 4, Table 2**)

Aggregate culture:

Within 24 hours of seeding the microwell plates, cells from all groups formed aggregates normally. After three weeks in aggregate culture, the cells had undergone chondrogenic differentiation in all cases, with morphological changes consistent with previously published aggregate culture experiments, including the expression of a proteoglycan (PG)-rich ECM. This suggests that none of the injection regimes had a deleterious effect on the subsequent differentiation potential of the cells. Interestingly, the relative metachomatic area of Toluidine blue O stained sections, an indication of PG-content, was lower on the cells (57.3 ± 19% for the 2M cells/ml group and 73.6 ± 16% for the 4M cells/ml group) which had not been passed through the needle compared to cells which had been passed through the needle (100% in all pellets examined).

Discussion

Cell therapies may be enhanced through interventional radiology. Image guided needle or catheter procedures in this setting potentially offer the advantages of a minimally invasive approach and improved efficacy through directed delivery. [25, 32, 33] When cells are handled, though, they are subjected to various forces. Key types of flow-induced forces include normal (pressure) and shear forces. It has been shown that the cells can withstand large amounts of normal forces for long durations; however, there is evidence that even brief exposure to shear forces can be deleterious to cells. In this study, we investigated the effects of injecting MSCs through spinal needles against various levels of backpressure. Our findings suggest that under the conditions tested, there was a slight but statistically significant increase in cell death in the 22 gauge needle – low backpressure treatment group vs. only two other groups, 25 gauge needle-Low and the 25 gauge needle no backpressure groups. These differences in total number of dead cells is quite small, so that we feel confident that there would be a negligible biological effect due to MSC viability in an intradisk injection scenario. This is also consistent with the lack of negative effects of the injection protocols on the function of the viable cells as determined by their chondrogenic potential in aggregate culture (see below). These findings are complemented by the results of modeling of the fluid dynamic characteristics of the system.

Live cells are routinely delivered through needles, e.g., in blood transfusions. Typically, the backpressure in such procedures is the venous pressure that is in the range of 3-8 mm Hg central or 10-12 mm Hg for peripheral circulation above atmospheric pressure. In a previous study, Walker et al. showed that at common infusion rates (60 – 500

ml/hour), through short needles of between 20 and 30 gauge, but against atmospheric backpressure, rat and human MSCs were not affected in terms of viability and multilineage differentiation potential. [26] That study applies to IV infusion of the cells, and not necessarily to injection into solid tissue. When cells are delivered to solid tissues, they can face significantly higher backpressures due to resistance to flow in the tissue.

The feasibility of delivering MSC by high-pressure injection to the IVD using a percutaneous image-guided needle has previously been shown in porcine models of degenerative disc disease. [25] In that study, radiolabelled xenogenic MSC were injected and imaged over time (0 and 3 days) for confirmation of delivery and containment. The imaging studies were confirmed by histology, [25] however, it was not clear whether the injected cells survived the procedure. In this study we show that, under clinically relevant conditions, resistance to flow during injection was not deleterious to MSC.

It is well understood that excess shear can lead to cellular damage; e.g., RBC lysis after passing blood through a needle is a known problem. [34] RBC lysis depends on the level of shear stress and the exposure time. In general nucleated cells are considered more fragile, for example with leukocyte damage being observed at 30 Pa. Both cell types can withstand higher stresses if the duration of exposure is brief. Generally large-bore needles and low pressures can mitigate damage, [34, 35] but spinal needles are generally smaller in diameter. To understand the fluid dynamics and shear stress conditions in our system, we utilized computational fluid dynamic modeling. Peak stresses typically occur at flow transition conditions such as sudden diameter transitions

in the flow conduit; in our system this is at the junction between the hub and the needle (**Fig. 2 (arrow) & 3**). Although the stresses are relatively high, at the flow velocities we used, dwell time in this high-shear region would be predicted to be on the order of milliseconds (**Fig. 3**), and even then only occur at the wall of the conduit (**Fig.** 3). Our results suggest that for the conditions cells encounter in the spinal needles used in this study, and using RBC criteria for MSCs, lysis would not be expected to occur.

The intent of the aggregate culture experiments was to determine whether there was a longer term negative impact of the injection on the subsequent differentiation potential of the cells. We selected chondrogenic differentiation as a general marker for the differentiation potential of the cells, as this is generally the first lineage to be lost [36]. Rather unexpectedly, even the brief, half minute exposure to the injection procedure resulted in an apparent enhanced chondrogenic differentiation. It is currently unknown whether other MSC properties are similarly affected.

Other groups have studied the effects of shear stress on MSCs and MSC differentiation. For example, Yourek et al. exposed MSCs to 24 hours of shear at 1Pa and found an upregulation of osteogenic markers. Interestingly, undifferentiated MSCs were more responsive to shear than partially differentiated cells [37]. In those cases, the shear magnitude was lower but the duration of shear exposure was much greater, and the cells were substrate attached; in such systems the contribution of shear exposure vs. improved medium convection is unclear. In other studies, even brief exposure to shear stresses has been shown to cause activation of certain genes in MSCs. [38] It is, thus, possible that this effect could be exploited to enhance differentiation in tissue engineering applications.

As interventional radiology expands and integrates with cellular medicine, multiple facets of image guided delivery that may potentially impact therapy should be considered, including cellular tracking, sites of cell action, efficacy of local delivery, and feasibility of needle or catheter delivery. This study investigated the effects of flow induced forces introduced through needle delivery on mesenchymal stem cells in a simulated model of in vitro intervertebral disc injection, indicating that the associated backpressure and shear do impact the cells. Currently, injection pressure is not usually recorded even when injecting into solid tissue, e.g., [39] but given the small but detectable biological effects, it could be useful to document them going forward. Using pressure monitoring equipment clinically would have advantages, as it has previously been documented that “syringe feel” by the operator is a poor predictor of the actual injection pressures. [40, 41] Further, pressure feedback can likely provide some objective assessment of the quality of the tissue being injected, and can potentially provide a final alert to erroneous needle tip placement. Larger in vitro and in vivo studies that analyze potential effects of this impact on disc regeneration vs. other cell delivery methods (surgical +/- scaffolds) are warranted.

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Table Legends

Table 1: Injection conditions used. No backpressure signifies injection against atmospheric pressure. Low and high were against 30 and 60 PSI (207 & 414 kPa, respectively) above atmospheric

Table 2. Differences in proportion of dead cells, estimated from a weighted analysis of variance. Because 2-way interactions with time (or among any other two factors) were not significant, when comparing conditions we averaged over the four time/concentration groups in each condition. Confidence intervals are simultaneous 95% Tukey-Kramer confidence intervals. Asterisks denote differences significant at the 0.05 level using the Tukey-Kramer multiple comparisons adjustment.

Figure Legends:

Fig. 1: Schematic of the image analysis approach used to measure metachromatically-stained cross-sectional area fraction in chondrogenic aggregates. **A:** original image mosaic. **B:** segmentation. **C:** binary transformation of **B. D:** area measured. **E:** cross sectional area of the whole section. Metachromatic fraction was defined as the area in **D** divided by the area in **E**.

Fig. 2: Silicone rubber mold of the interior of the needle hub. Arrow points to the transition from the hub to the needle. This mold was then measured to generate the geometry of the Comsol model

Fig. 3: Comsol Model output. **A) Velocity Profile:** Velocity profile (m/sec) in the transition region between the hub and the needle. The insert (red rectangular box) shows the transition region which is expanded to show changes in velocity profiles. Except for the transition region, the velocity profile is constant in the axial direction. **B) Shear Rate:** Shear rate profile (1/sec) in the transition region between the hub and the needle. The insert (red rectangular box) shows the transition region which is expanded to show changes in shear rates. Only in the 'neck' surface of the transition region, the shear rates are high. Note that the shear stress is proportional to shear rate for Newtonian fluids.

Fig. 4: Percent dead cells at 24 and 48 hours for each condition. See **Table 1** for group designations, **Table 2** for statistical analysis results.

Figure 1

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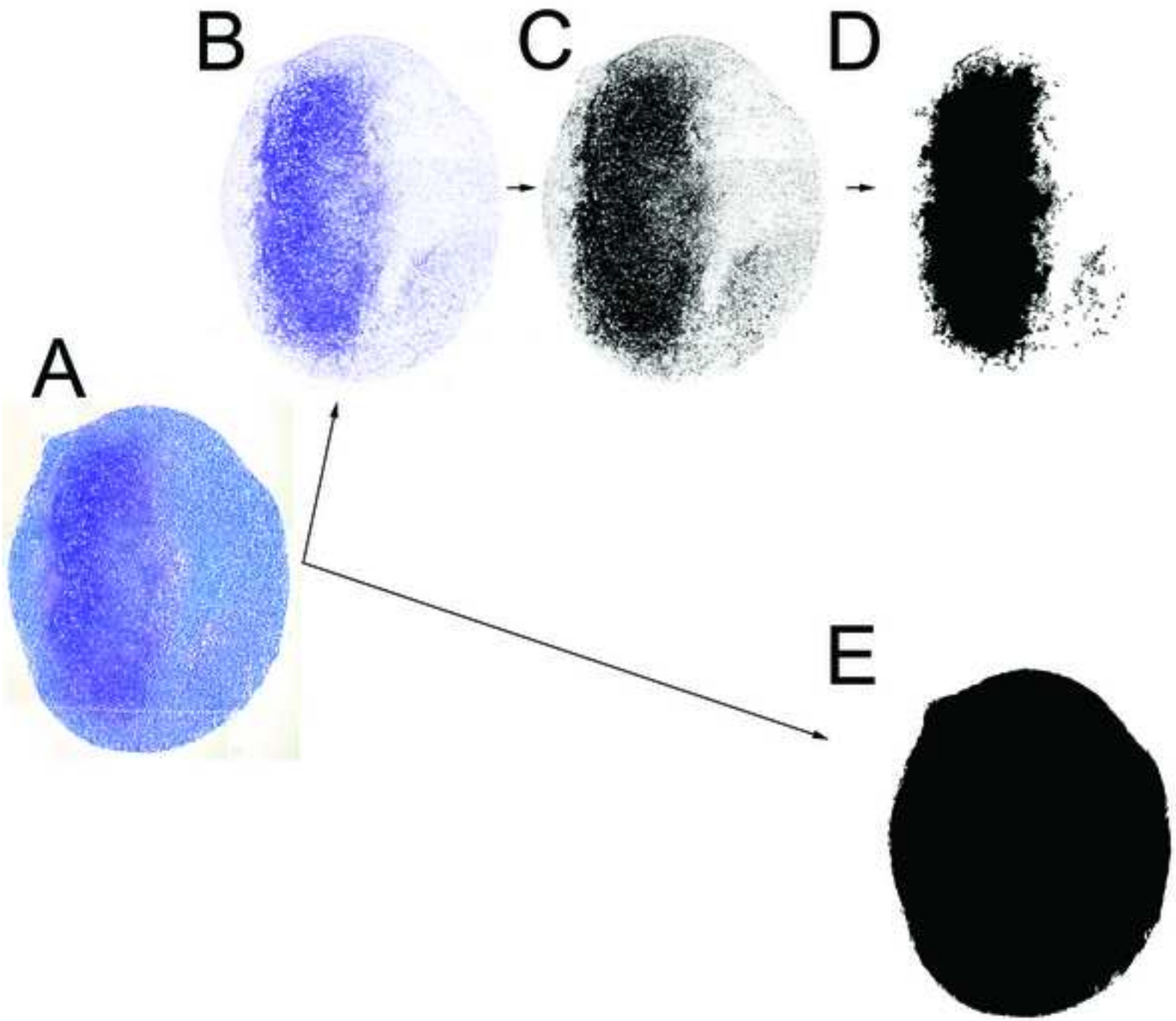

Figure 2

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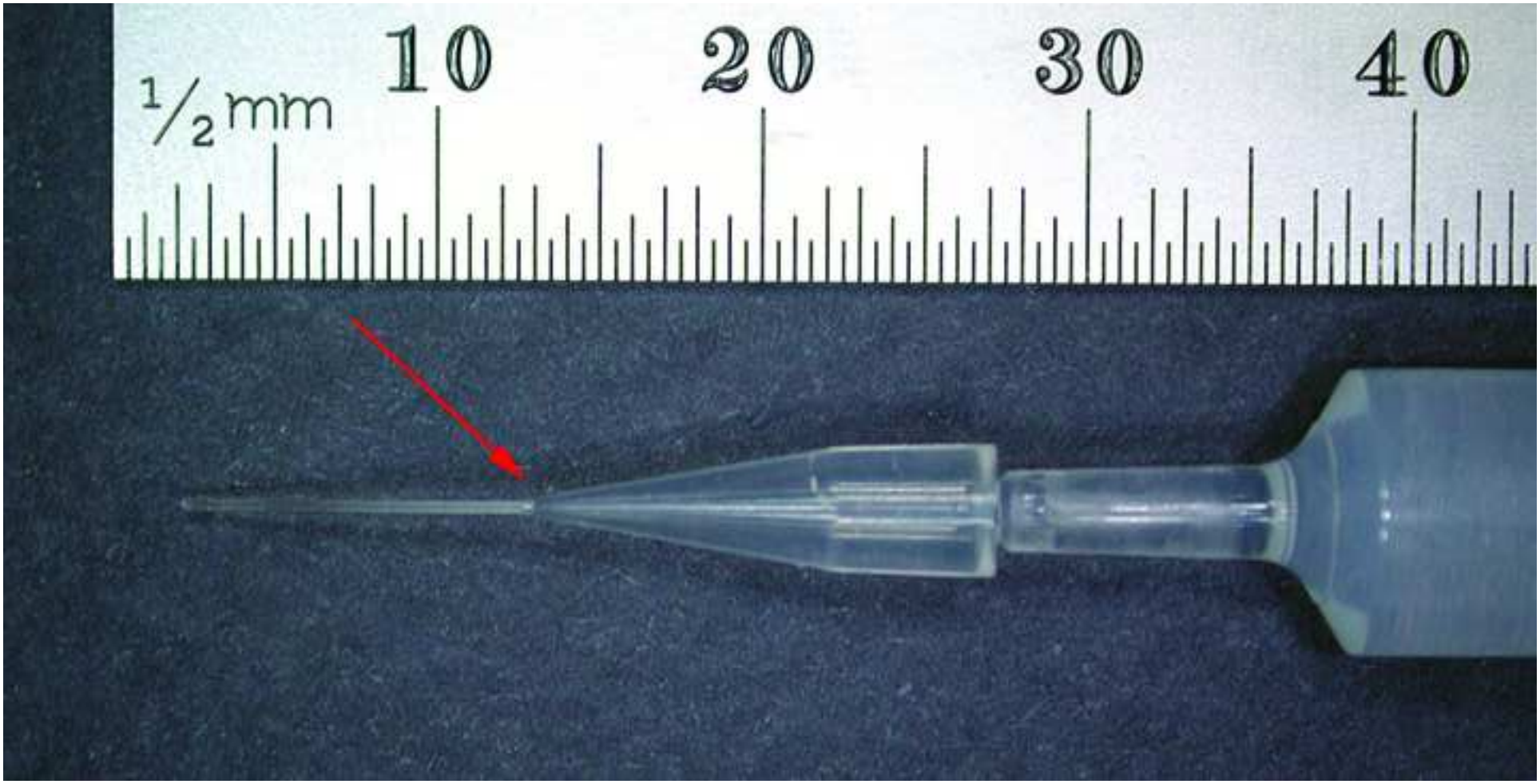

Figure 3

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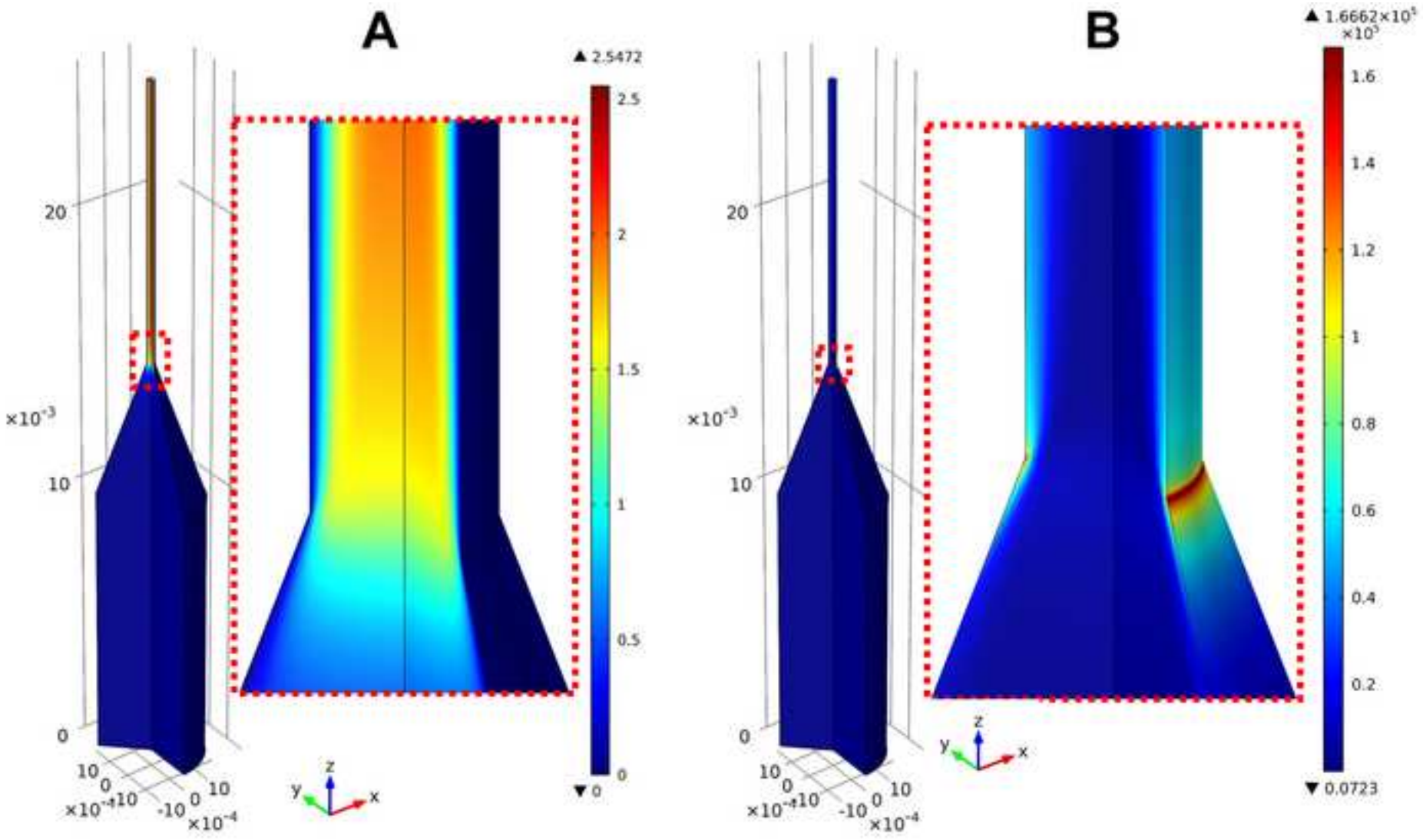

Figure 4

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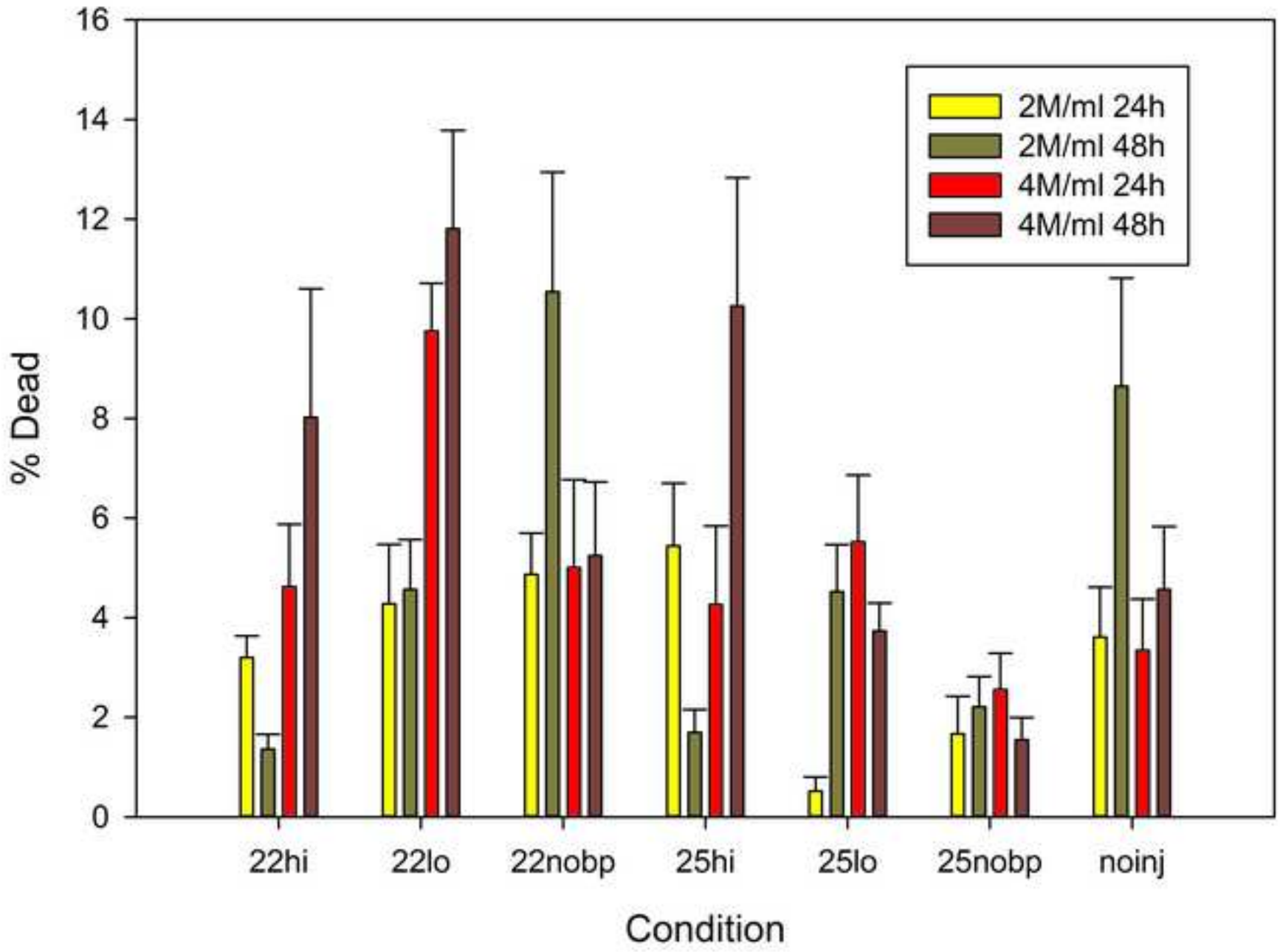

Table 1

	Needle	Backpressure	Designation
1	No	No	noinj
2	22g	No	22nobp
3	22g	Low	22lo
4	22g	High	22hi
5	25g	No	25nobp
6	25g	Low	25lo
7	25g	High	25hi

Table 1: Injection conditions used. No backpressure signifies injection against atmospheric pressure. Low and high were against 30 and 60 PSI (207 & 414 kPa, respectively) above atmospheric

Table 2

Comparison	Mean Difference	95% CI	
22hi - 22lo	-0.040	(-0.087, 0.006)	
22hi – 22nobp	-0.026	(-0.077, 0.025)	
22hi – 25hi	-0.003	(-0.038, 0.031)	
22hi – 25lo	0.010	(-0.016, 0.037	
22hi – 25nobp	0.013	(-0.018, 0.044)	
22hi – noinj	-0.009	(-0.058, 0.040)	
22lo – 22nobp	0.014	(-0.048, 0.077)	
22lo – 25hi	0.037	(-0.016, 0.090)	
22lo – 25lo	0.051	(0.005, 0.096)	*
22lo – 25nobp	0.053	(0.006, 0.100)	*
22lo – noinj	0.031	(-0.029, 0.091)	
22nobp - 25hi	0.023	(-0.034, 0.080)	
22nobp – 25lo	0.036	(-0.013, 0.086)	
22nobp - 25nobp	0.039	(-0.013, 0.091)	
22nobp - noinj	0.017	(-0.047, 0.081)	
25hi – 25lo	0.014	(-0.023, 0.051)	
25hi – 25nobp	0.016	(-0.023, 0.056)	
25hi – noinj	-0.006	(-0.061, 0.050)	
25lo – 25nobp	0.003	(-0.027, 0.033)	

25lo – noinj	-0.019	(-0.067, 0.028)
25nobp – noinj	-0.022	(-0.071, 0.027)

Table 2. Differences in proportion of dead cells, estimated from a weighted analysis of variance. Because 2-way interactions with time (or among any other two factors) were not significant, when comparing conditions we averaged over the four time/concentration groups in each condition. Confidence intervals are simultaneous 95% Tukey-Kramer confidence intervals. Asterisks denote differences significant at the 0.05 level using the Tukey-Kramer multiple comparisons adjustment.