Stem Cell Delivery With Polymer Hydrogel for Treatment of Intervertebral Disc Degeneration: From 3D Culture to Design of the Delivery Device for Minimally Invasive Therapy

Deepak Kumar,*[1] Alex Lyness,* Irini Gerges,†[2] Christina Lenardi,†[3] Nicholas R. Forsyth,‡ and Yang Liu*

*Centre of Biological Engineering, Wolfson School of Mechanical and Manufacturing Engineering, Loughborough University, Loughborough, UK
†Fondazione Filarete, Viale Ortles, Milan, Italy
‡Guy Hilton Research Centre, Keele University, Staffordshire, UK

Nucleus pulposus (NP) tissue damage can induce detrimental mechanical strain on the biomechanical performance of intervertebral discs (IVDs), causing subsequent disc degeneration. A novel, photocurable, injectable, synthetic polymer hydrogel (pHEMA-co-APMA grafted with PAA) has already demonstrated success in encapsulating and differentiating human mesenchymal stem cells (hMSCs) toward an NP phenotype during hypoxic conditions. After demonstration of promising results in our previous work, in this study we have further investigated the inclusion of mechanical stimulation and its impact on hMSC differentiation toward an NP phenotype through the characterization of matrix markers such as SOX-9, aggrecan, and collagen II. Furthermore, investigations were undertaken in order to approximate delivery parameters for an injection delivery device, which could be used to transport hMSCs suspended in hydrogel into the IVD. hMSC-laden hydrogel solutions were injected through various needle gauge sizes in order to determine its impact on postinjection cell viability and IVD tissue penetration. Interpretation of these data informed the design of a potential minimally invasive injection device, which could successfully inject hMSCs encapsulated in a UV-curable polymer into NP, prior to photo-cross-linking in situ.

Key words: Regenerative medicine; Cell encapsulation; Tissue engineering; Cell delivery; Device design

INTRODUCTION

Biomechanical and pathohistological structure changes occur in the degenerated intervertebral disc (IVD). The difficulty in IVD tissue to repair and regenerate itself is attributed to its relatively acellular (5,000 cells/mm^3), acidic, and avascular environment. Subsequently, when considering an acellular or tissue engineering therapy for IVD, several factors must be considered, including extracellular matrix architecture, hydrated tissue matrix, hypoxic nature, and the mechanical stresses and strains imposed upon the nucleus pulposus (NP) tissue. For this reason, hydrogels are an ideal and popular choice, as they are able to offer a hydrated environment, they have the ability to mimic native fibrillar structure, and are biocompatible[1,2]. Furthermore,

photocurable hydrogels display additional benefits, including fine-tuning mechanical properties and swelling behavior, short reaction times, minimal heating, and minimally invasive delivery. To date, only a single study exists that has displayed the successful use of a photocurable polyethyelene glycol diacrylate hydrogel to encapsulate and differentiate mesenchymal stem cells (MSCs) toward NP-like cells, proven by the positive expression of typical NP matrix proteins (aggrecan and collagen II)[3].

Various parameters have been demonstrated to influence the differentiation of human MSCs (hMSCs) toward an NP cell-like phenotype including a 3D environment supported by hydrogels[4], hypoxia and chemical stimulation, and disc microenvironment[5]. A study by Stoyanov et al.
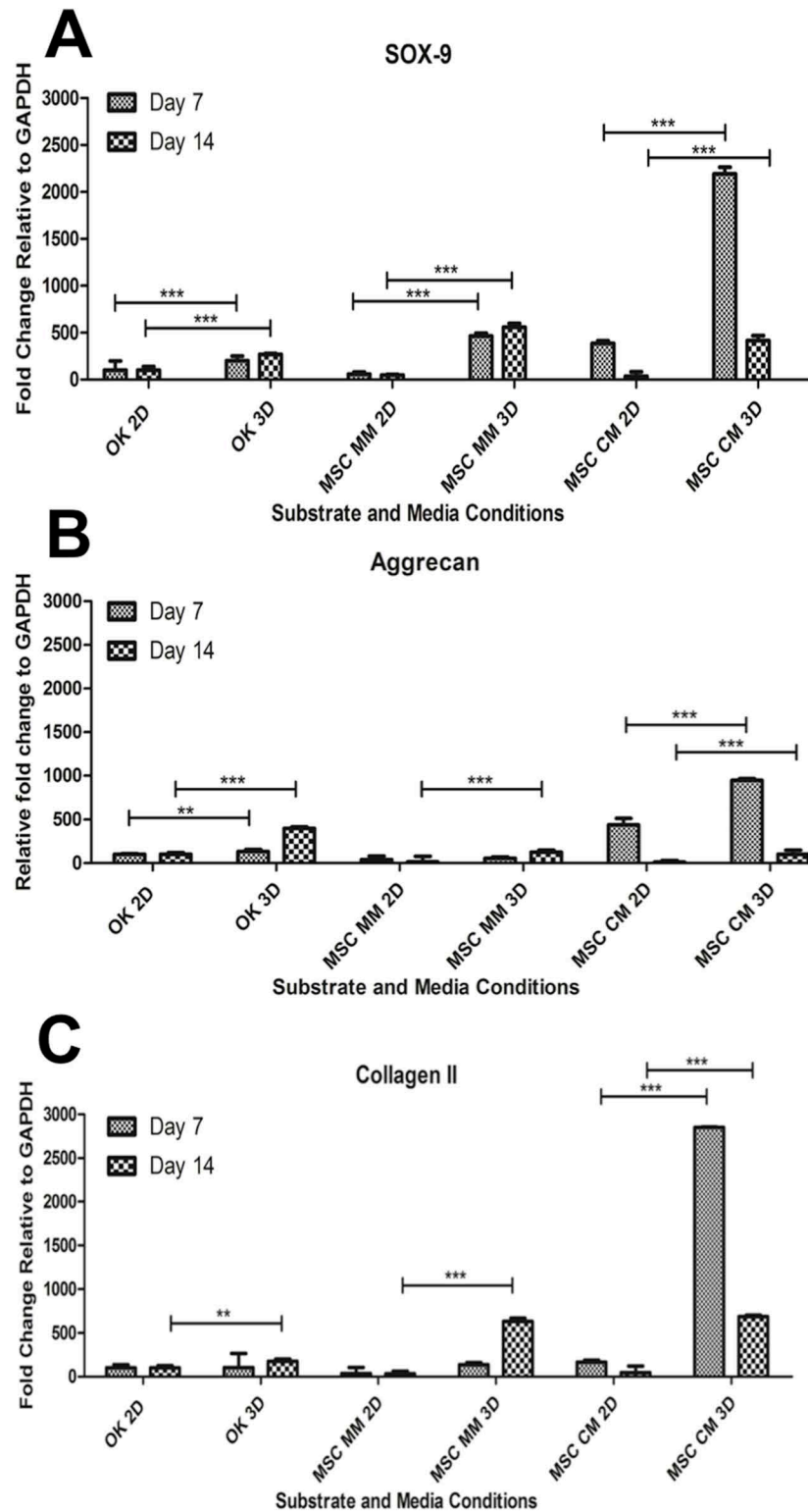
Figure 1. Gene expression. Real-time polymerase chain reaction (RT-PCR) demonstrating the expression of typical intervertebral disc (IVD) markers: (A) SOX-9, (B) aggrecan, (C) collagen II in primary chondrocytes (OK cells), and human mesenchymal stem cells (hMSCs) cultured in 2D and 3D (hydrogels) environments, in hMSC or chondrogenic media, under static or stimulated conditions in hypoxia up to 14 days. Annotations: human chondrocytes (OK), MSCs cultured in MSC expansion media (MSC MM), MSCs cultured in chondrogenic media (MSC CM), on tissue culture plastic (2D) and within hydrogels (3D), glyceraldehyde-3-phosphate dehydrogenase glyceraldehyde 3-phosphate dehydrogenase (GAPDH). ***$p < 0.001$, statistically significant.

demonstrated the successful differentiation of hMSCs toward NP cells under the combined influence of 3D encapsulation using alginate beads and chemical media[6]. Recently, our group demonstrated the successful use of a novel, synthetic, ultraviolet (UV) photocurable hydrogel polyhydroxyl ethyl methacrylate-co-*N*-3-aminopropylme thacrylamide grafted with polyamidoamine [p(HEMA-co-APMA)g PAA] for the encapsulation of hMSCs and their differentiation toward an NP-cell like phenotype[7]. In this case, differentiation was achieved through the multiple combinatorial effects of 3D encapsulation, chondrogenic media, and hypoxia. In this study, we have further investigated the additional influence of mechanical stimulation to hMSC differentiation and also demonstrated the feasibility of cell delivery using injection devices.

MATERIALS AND METHODS

Preparation of Photocurable Hydrogel Solution

pHEMA-APMA-PAA polymer (Fondazione Filarete, Milan, Italy) was prepared as described previously[7]. Briefly, hydrogels were prepared at 13.5% (w/v) concentration by dissolving the required amount of polymer in phosphate-buffered saline (PBS; Sigma-Aldrich, Dorset, UK). Irgacure 2959 [2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone; 50% (w/v)] was prepared using dimethyl sulfoxide (DMSO; Sigma-Aldrich) solution, which was added to the polymer solution at 1% volume of the total polymer solution. Polymer solution was briefly vortexed and sterile filtered before use.

hMSC Expansion, Encapsulation in Hydrogels, and Differentiation Toward NP-Like Cells

hMSCs from fresh human bone marrow aspirate supplied by Lonza (Catalog No. 1M-125; IRB approved; Walkersville, MD, USA) were recovered, isolated, and expanded as described previously by Pittenger et al.[8]. hMSCs were expanded using hMSC expansion media, which consisted of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS; Lonza), 1% L-glutamine (L-glut; Lonza), 1% nonessential amino acids (NEAA; Lonza), and 1% penicillin and streptomycin and incubated in a hypoxic chamber at 2% O_2 SCI-Tive workstation (RUSKINN Technology; The Baker Company, Bridgend, UK). Seeding solutions consisted of 200,000 cells/50 μl of hMSC media per hydrogel. Cell seeding solution and cross-linking were performed as mentioned in our previous work[7]. Chondrogenic medium [consisting of DMEM, 1% FBS, 1% NEAA, 1% L-glut, 0.1 μM dexamethasone, 50 μM ascorbic acid, 40 μg/ml L-proline, 1% sodium pyruvate, and 10 ng/ml transforming growth factor-β3 (TGF-β3)] was conditioned in hypoxia at 2% O_2 using the SCI-Tive workstation for 24 h prior to use. Mechanical stimulation to biological samples was

applied using the BOSE bioreactor (Minneapolis, MN, USA). Stimulation parameters of cyclic compression (5%) at 1 Hz for 15 min a day, up to day 7 or 14, were applied. hMSCs encapsulated in hydrogels were cultured in hMSC media under normoxic conditions or in chondrogenic media under hypoxic conditions and then were loaded into the biodynamic chamber using sterile forceps. Mechanical stimulation was performed for 15 min, after which point samples were placed back into culture within the SCI-Tive workstation until the following day.

PCR Analysis of Chondrogenic Markers (Static vs. Mechanical Stimulation)

One-step quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the SuperScript III Platinum SYBR Green enzyme (Part No. 11736051; Invitrogen, Paisley, UK) according to manufacturer's instructions. Primers (F indicates forward primer, R indicates reverse primer, and annealing temperature used is indicated for each primer pair) were purchased from Qiagen (Manchester, UK) [aggrecan (ACAN), Part No. QT00001365; collagen II, SLCO2A1, Part No. QT0009 5375; SOX-9, Part No. QT00001498; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Part No. QT011 92646]. All qRT-PCRs comprised a single cycle of 50°C for 3 min, a single cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, annealing temperature of 45°C for 30 s and 72°C for 1 min, finally followed by a single cycle of 40°C for 1 min using the thermocycler machine. Raw cycle threshold (Ct) values of interested genes were normalized to raw Ct values of the housekeeping gene GAPDH to attain ΔCt values per gene. These values were then converted to ΔΔCt values, which were the final values used in Figure 1.

Evaluation of Cell Viability After Injection Through Different Needle Gauges

Trypsinized hMSCs were resuspended homogenously into the polymer hydrogel solution (prephotocuring) at a cell density of 100,000 cells/50 μl polymer solution. hMSC/polymer solutions were injected using different surgical gauge size needles (19, 22, 27, and 30 gauge; Terumo; Fisher Scientific, Loughborough, UK) into a six-well plate and incubated at 2% O_2 for 1 and 24 h. Cell viability was evaluated using trypan blue and live/dead staining solutions (Sigma-Aldrich), as used previously in our study[7].

IVD Penetration Force Study

Frozen oxtail IVDs obtained from a local butcher shop were thawed to room temperature prior to use. Various size needles (22, 25, 27, and 30 gauge) were fixed to a compressive testing machine (Model 5848; Instron Ltd., High Wycombe, UK). This was achieved

using a stainless steel adaptor (Fig. 4A) that could receive a 2-ml syringe barrel with reduced length and a tip for needle attachment (Terumo; Fisher Scientific). The bespoke adaptor attached to the mobile upper jaw of the testing machine.

The machine was set in compression mode with a 5-N load cell installed, and the mobile upper jaw of the tester was programmed to travel at a rate of 1 mm/s. The oxtail was affixed to a polystyrene sheet using spare needles to prevent the sample from moving during penetration (Fig. 4B). For each experiment, the needle was pushed into the IVD by approximately 5 mm. This penetration depth ensured the needle would push past the annulus fibrosis (AF) and enter into the NP.

RESULTS AND DISCUSSION

Effects of 3D Culture and Cyclic Compression

In this study, further insights were examined in terms of specifying the power and impact of each of the stimulating parameters (hydrogel, hypoxia, chemical environment, and mechanical stimulation) individually on the differentiation process of hMSCs toward an NP cell-like phenotype. PCR analysis revealed that the 3D culture of hMSCs within the hydrogel under hypoxic conditions triggered the differentiation of hMSCs toward an NP cell-like phenotype (Fig. 1). This was shown through elevated gene expression of SOX-9 (Fig. 1A), aggrecan (Fig. 1B), and collagen II (Fig. 1C) at day 14 when compared to the 2D culture in hMSC media. When combining cell–gel constructs under hypoxia and in chondrogenic media, the gene expression of SOX-9 (Fig. 1A), aggrecan (Fig. 1B), and collagen II (Fig. 1C) was further elevated at day 14. However, the significant decrease in gene expression from day 7 to day 14 in hypoxia and chondrogenic media may have been attributed to the complete transcriptional switch from a stem cell to a terminally differentiated NP cell-like phenotype and that translational expression of matrix proteins could take place after day 7 during in vitro culture.

We next integrated mechanical stimulation to the study and investigated the effects of 5% cyclic compression, 1 Hz, 15 min a day, and up to day 14, on hMSCs encapsulated within hydrogels, under hypoxia, in hMSC or chondrogenic media. Immunocytochemistry was used to reveal the translational expression of key NP matrix proteins aggrecan and collagen II. Minimal differences in the expression of aggrecan and collagen II were witnessed when culturing hMSCs [2D, on tissue culture plastic (TCP); or 3D, cells encapsulated within hydrogels] in hMSC media under hypoxia (Fig. 2A and C). However, it was apparent that after the addition of chondrogenic media, further elevated levels of aggrecan and collagen II were witnessed when cultured under hypoxic conditions

for 14 days (Fig. 2B and D). Interestingly, the greatest level of translational expression of both aggrecan and collagen II was witnessed in hydrogels cultured in hypoxia and chondrogenic media and exposed to mechanical stimulation up to day 14 (Fig. 2). This in vitro 3D culture with mechanical stimulation demonstrated the ability to support hMSC viability and differentiation toward an NP cell-like phenotype. However, further improvements to the model can be made in order to mimic the mechanical stimulation parameters close to the biomechanics of IVD by applying a more long-term mechanical stimulation regime.

Postinjection Viability of Cells

Coupled together with the ability of the hydrogels to provide protection to the encapsulated cells and mechanical support to the surrounding tissue in vivo, the ability to deliver cells in a minimally invasive procedure to the target site as well as the ability of the hydrogel to photocure in situ after delivery prompted the consideration in the delivery parameters for a device that would be used for local delivery of cell-laden hydrogel solution in vivo. To minimize the damage to the IVD from the needle penetration, needles with smaller diameter are ideal. However, narrower needle gauges could cause damage to the cells because of shear stresses imposed within the needle bore. Hence the logical step in determining the most suitable needle gauge (Fig. 3A) to deliver hMSC-laden hydrogels was to establish the effect of the inner diameter of the needle used on cell viability after delivery.

Heng et al.[9] have reported that passing hMSCs through a narrower catheter (26-gauge nitinol needle lumen) at flow rates, which are normally encountered, resulted in no changes in hMSC multipotency or viability. This was an intramyocardial injection of hMSCs directly into the heart muscle. In another study by Garvican et al.[10], no changes in stem cell differentiation were observed when injected through 19-, 21-, or 23-gauge needles. However, there was a compromise in viability injecting through 21- and 23-gauge needles. The general consensus therefore appears to be the preference by clinicians for smaller-bore needles for hMSC delivery[11].

As the proposed route of delivery is to 1) inject cell hydrogel solution in situ followed by 2) cross-linking using a UV source, it was imperative to investigate various needle gauges (22, 25, 27, and 30 gauge) and the impact on cell viability. Observations revealed that after injection hMSCs retained good cell viability on TCP with the ability to fully recover their typical fibroblastic morphology in 24 h (Fig. 3B). Insignificant differences in viability were observed between 22, 25, and 27 gauge, although significant differences in cell viability were witnessed with 30 gauge compared to all other sizes (Fig. 3B and C). These data are in agreement with literature suggesting the use of
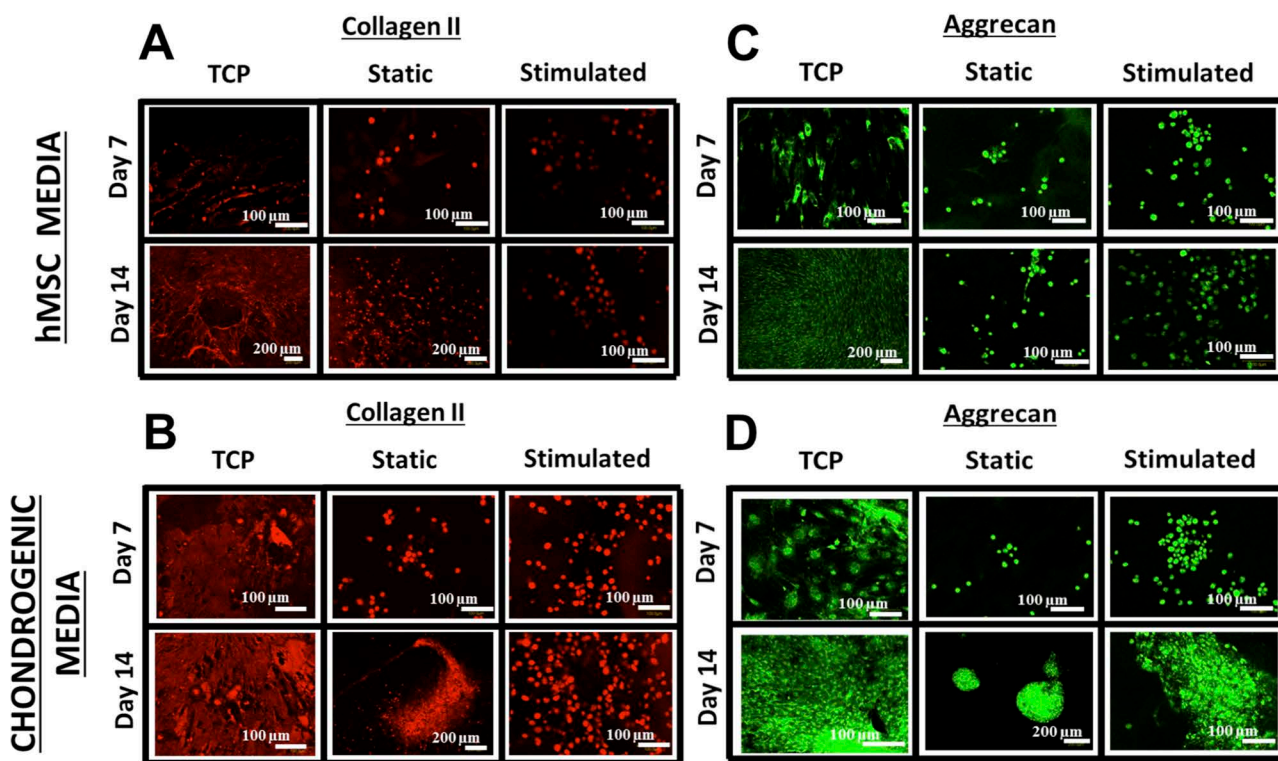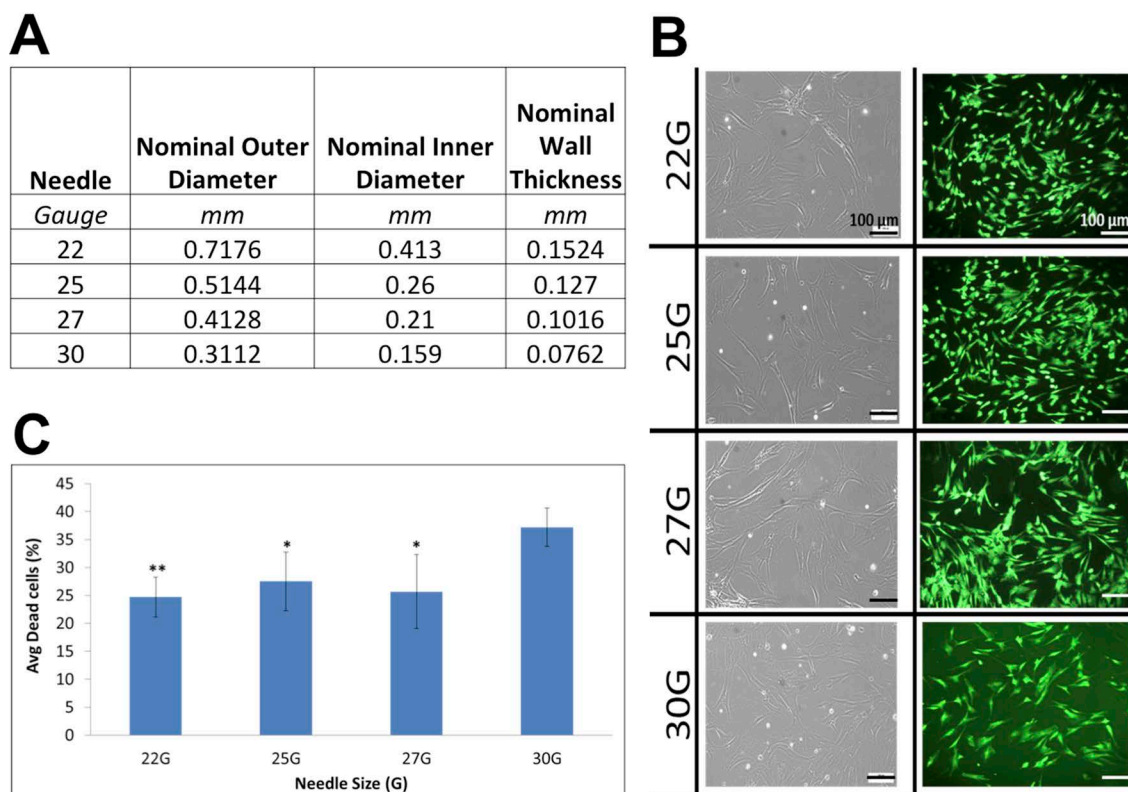
Figure 2. Intervertebral disc (IVD) matrix protein expression. Immunocytochemistry demonstrating the expression of typical IVD matrix proteins; collagen II expression in human mesenchymal stem cells (hMSCs) cultured on tissue culture-treated plastic (TCP) or within hydrogels under static and stimulated conditions at day 7 and day 14 of culture in (A) hMSC differentiation media and (B) chondrogenic media. Aggrecan expression in hMSCs cultured within hydrogels under static and stimulated conditions at day 7 and day 14 of culture in (C) hMSC differentiation medium and (D) chondrogenic medium.

22- to 27-gauge needles. The minimal cell death may be attributed to the masking/protection of cells through the viscosity of the polymer from the impact of shear forces applied by these needles[12,13]. However, a limitation of this experiment includes the inability to precisely control the flow rates, which could contribute to the large standard deviation on the average number of dead cells observed in Figure 3C. Future studies could include delivery using various flow rates controlled by an electrical motor, but we feel manual injection would reflect the manner of stem cell delivery more closely to the medical procedures deployed in a clinical setting[14,15].

After delivery, photo-cross-linking of cell-loaded hydrogel solution would take place in situ, where our previous study and others have confirmed insignificant compromise on viability using the photoinitiator and the UV parameters[7]. In addition, no secondary surgical intervention would be required after therapy, as the hydrogel is designed/tailored to break down with degradation products less than 20 kDa (limit for renal filtration), allowing efficient smooth excretion of the polymer by-products through the metabolic pathway, preventing subsequent localized accumulation of hydrogel by-products[16].

Quantification of Tissue Penetration Force

Needle penetration forces into IVD were investigated using an ex vivo tissue model. Explant IVD tissue from oxtail (with similar dimensions and mechanical properties to human IVD) was penetrated using different needle gauges (Fig. 4A–C). Greater variation in penetration force was observed using larger needle gauge and is probably attributed to the larger outer diameter of the needle, resulting in greater and controlled tearing of the tissue as it penetrates through the AF (Fig. 4D). The 22-gauge needle was shown to require the greatest mean force to penetrate 2.17 N (1.39% SD), the 30-gauge needle was shown to have the lowest force to penetrate with the penetration point being the most consistent with a mean of 0.51 N (0.04% SD) (Fig. 4D). Smaller penetration forces were encountered using smaller needles, but it is also important to ensure that cell viability is not compromised through direct shear forces, although this does not appear to be the case here. Hence the selection of delivery device parameters would be a negotiation between the internal diameter to ensure cell viability and the request of a small needle gauge to ensure negligible effect on IVD integrity[17,18]. It is anticipated that a suitable minimally

A

Needle Gauge	Nominal Outer Diameter mm	Nominal Inner Diameter mm	Nominal Wall Thickness mm
22	0.7176	0.413	0.1524
25	0.5144	0.26	0.127
27	0.4128	0.21	0.1016
30	0.3112	0.159	0.0762

B

C

Figure 3. Injection delivery route evaluation using different needle gauge sizes. (A) The table defines the dimensions of the different size gauge needles in millimeters (mm) used to inject human mesenchymal stem cells (hMSCs) into a well plate. (B) hMSC viability (live/dead staining) 24 h after injection through various size needle gauges (scale bars: 100 μm). (C) Quantification of the percentage of hMSC dead cells (trypan blue) 1 h after injection through various size needle gauges. $*p < 0.05$, $**p < 0.01$.

invasive delivery device must be able to exert approximately a force of 2.5 N, which is required to successfully penetrate through the AF of an IVD. The needle gauge of the device could be in the range of 19–30 gauge, although using a smaller-bore needle can minimize penetration forces, but also it will increase the pressure required on the back of a syringe, most likely when a viscous hydrogel solution is delivered.

Delivery Device Design

Having taken all parameters and outcomes into consideration, the formulated computer-aided design (CAD) model of the chosen delivery design is displayed in Figure 4E. Major design components include a consumable cell reservoir of hMSCs and hydrogel solution, battery-powered stepper motor with controlled injection, and corresponding control and feedback components. This allows accurate and precise volumetric delivery via an electromechanical device facilitating intelligent control of the delivery force. The possibility of a battery-powered device improves its ergonomic performance, as no wires would hinder the final positional control by

the surgeon. Briefly, injection of hMSC-loaded hydrogel solution is controlled through a rocker trigger activated with the user's forefinger. However, further investigations would be required to perform proof-of-concept studies using the device with an in vivo preclinical model (bovine or ovine).

Preliminary assessment of the injectable parameters has provided insights into the design of an injection device capable of delivering a UV-curable hydrogel loaded with hMSCs that could be used to inject through the AF and into the NP. It is anticipated that the delivery method would be minimally invasive. This is attributed to the fact that the device would have a narrow enough needle to penetrate through the AF and into the NP. The cannula would then be changed to allow injection/delivery of the hMSC-loaded polymer solution with the photoinitiator to the delivery site. Following delivery, a probe would then follow through the catheter to the target site allowing the exposure of UV light with optimized parameters to permit photocuring in situ. Once cured, the properties of the hydrogel would help to restore mechanical function of IVD for the time being[2,19] until the developmental
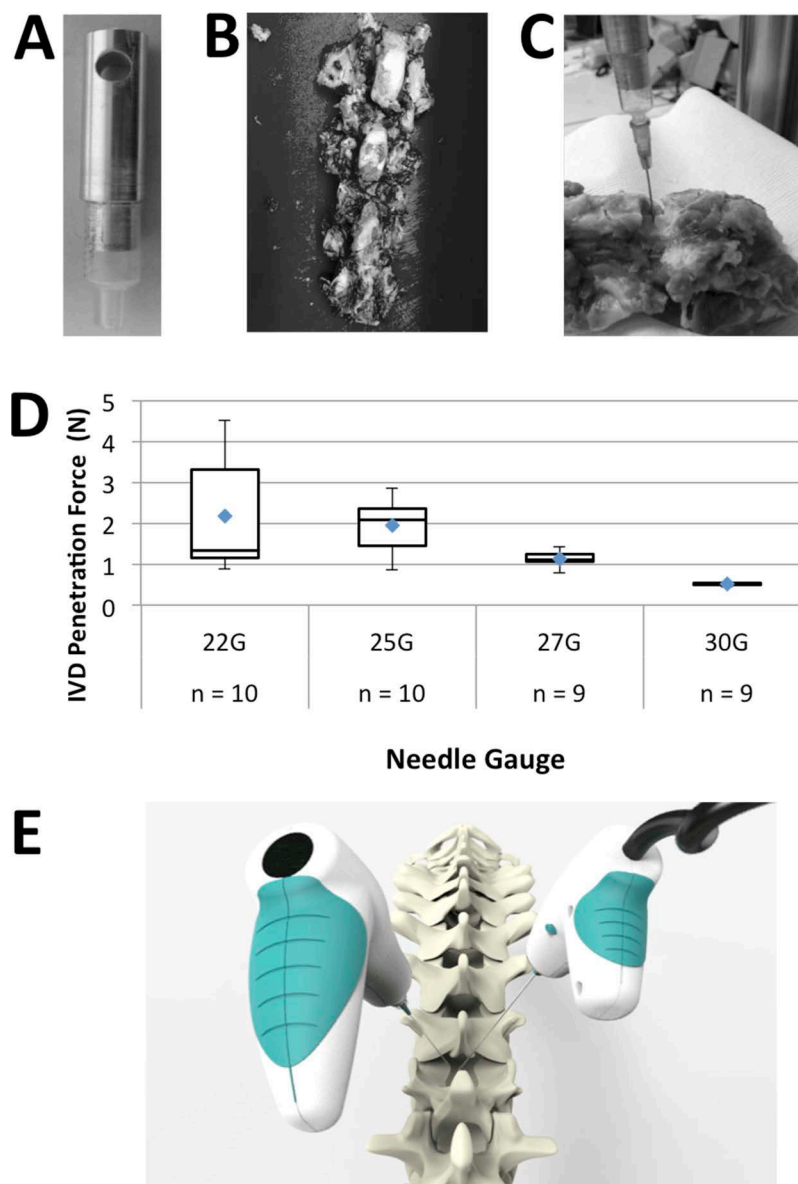
Figure 4. Intervertebral disc (IVD) penetration force in an ex vivo bovine model. (A) Bespoke adaptor assembled with the syringe component to connect the various size needle gauges used in the study. (B) Sectioned oxtail vertebrae. (C) Ex vivo tissue being injected using a needle mounted on the materials tester. (D) Box plot revealing IVD penetration forces required by the various needle gauge sizes used. (E) Computer-aided design (CAD) visualization of a potential injection device to deliver UV-curable hydrogel (left) and a UV light source (right) for the treatment of IVD in vivo.

state of hMSCs and cell/scaffold interactions are proper for effective extracellular matrix production, hence contributing to the repair of the NP tissue[5,20,21].

CONCLUSIONS

This study has demonstrated the successful use of a UV photocurable, synthetic hydrogel in encapsulating hMSCs and differentiating them toward an NP cell-like phenotype when cultured in a combined environment of hypoxia, chemical stimulation, and mechanical stimulation. In addition, we have considered the delivery route of the cell-loaded hydrogel solution in situ, have demonstrated adequate cell viability and morphology after injection using 22- and 27-gauge needles, and quantified the force required for successful penetration through the AF.

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