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Effect of DMSO Concentration, Cell Density and Needle Gauge on the Viability of Cryopreserved Cells in Three Dimensional Hyaluronan Hydrogel *

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Abstract— **For cells seeded in scaffolds, transplanted cell survival rate plays an important role for cell transplantation efficiency, and is essential for successful cell transplantation. Fibroblast viability in HyStem-C was examined by a double staining Live/Dead Viability/Cytotoxicity assay, and cell images were analyzed using MetaMorph software for calculating live cell percentage for fresh and cryopreserved cells at different incubation time points, delivery methods, differing DMSO and cell concentrations. The results of this research demonstrated that in HyStem-C, the viability of cryopreserved cells (85%) was significantly lower than fresh collected cells (96.7%). In addition, the physical force from a 27 gauge needle significantly decreased frozen cell survival rates to 83-85% compared to pipette delivered cells. Higher DMSO concentration (1.0%) and higher cell density (2 x 10^7 per milliliter) also significantly decreased cell survival to 73%. Cryopreserved cell viability in three dimensional scaffolding can be maintained over 80% with cell density of 1 x 10^7 per milliliter, total DMSO concentration of 0.5%, and passed through a 27-gauge needle. These results demonstrate the viability of cells seeded in hyaluronan hydrogel with commonly used storage and delivery methods can bring rather satisfactory cell transplantation efficiency.**

I. Introduction

Transplantation of stem cells into injured tissue can improve wound healing, tissue regeneration and functional recovery. Implanted cells rapidly lose their viability or fail to integrate into host tissue [1]. New strategies are needed to enhance transplanted cell survival *in vivo*. Biomaterials can mimic or include naturally occurring extracellular matrices and can instruct cell function in different ways [2-5]. However, the effects of biomaterials without cells disappear when the biomaterials degrade [6]. Therefore, different biomaterials have been used to deliver cells to local tissue for tissue regeneration [7-9]. Hyaluronan hydrogel (HyStem-C) is a synthetic biomaterial [10] that mimics the natural extracellular matrix component, hyaluronic acid [11], and can provide a biocompatible environment for cell attachment, survival, migration, growth and proliferation [12-14]. A previous study demonstrated that HyStem-C can protect encapsulated cells from inflammation and surrounding macrophages [6]. In addition, as a support vehicle HyStem-C also can control and retain implanted cells, allowing localization at the target site facilitating tissue repair [14, 15], and its functional recovery [16, 17]. Therefore, treatment with HyStem-C seeded with cells may accelerate the formation of new tissue and improve the quality of the newly generated tissue, serving as a potential engineering tool for clinical tissue regeneration applications.

Currently, there is paucity in the literature of the factors that affect biomaterial/cell viability that may increase transplantation efficiency for tissue regeneration. In this study, we selected mouse embryonic fibroblast cells (NIH 3T3 cells) to analyze cell viability of fresh and cryopreserved frozen cells with different cell-delivery methods (pipette or needle), dimethylsulfoxide (DMSO) concentration and cell density in three-dimensional (3-D) HyStem-C. The purpose of this study is to clarify which factors will be important for enhancing biomaterial-induced cell transplantation efficiency and provide much needed guidance for clinical trials.

II. Materials and Methods

A. A. Hyaluronan Hydrogel (HyStem-C) 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 cell culture hood to ensure sterility. At room temperature, HyStem-C casts in about 5 min. .

B. Maintaining Three Dimensional Cell Culture

NIH 3T3 cells come from a cell line isolated and initiated in 1962 at the New York University School of Medicine Department of Pathology; the cell line has since become a standard fibroblast cell line. In this study, NIH 3T3 cells were used for testing cell viability in 3-D HyStem-C. Cells were plated in cell culture dishes and incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf bovine serum (CBS), 100U/ml penicillin, 0.01 mg/ml streptomycin sulfate, and 1x none essential amino acid (all from Sigma, St. Louis, MO). For long-term storage, NIH 3T3 cells are suspended in freezing medium containing 5% DMSO, transferred into cryovials and then frozen by steps with slowly decreasing temperature to final -196ºC for cryopreservation. Before use, frozen cryopreserved cells were thawed into liquid in 37ºC, and then mixed with hydrogel.

For 3-D culture, cell suspension was mixed with hydrogel solution at final concentrations of 2 x 10^6, 1 x 10^7 and 2 x 10^7 per milliliter (ml). 0.5ml of this cell-gel mixture

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was placed into each well of 6-well plate with transwell permeable inserts (0.4µm membrane pore size, Millipore Inc. Billerica, MA) by pipette and 27-gauge needle (27G needle). After gelation (gel thickness was approximately 0.5mm), cell culture medium (DMEM-10%CBS) was added above and below the gel. Cell plates were kept in an incubator at 37ºC and 5% CO_2.

C. *Cell Viability Assay*

NIH 3T3 cell survival rates in 3-D HyStem-C were analyzed by a double staining procedure that uses calcein AM and ethidium homodimer-1 (EthD-1) (Live/Dead Viability/Cytotoxicity kit, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Calcein AM is non-fluorescent, cell-permeate molecule that is cleaved inside the cell by intracellular esterase to yield green fluorescence. EthD-1 is a nucleic acid stain permeate to viable cells and can diffuse through the membrane of dead cells where binds to the DNA and gives a red fluorescence. After 2 and 48 hours culture, inserts with cells and gel were washed three time with 1x PBS, pH 7.4, then incubated with the staining solution (2µM calcein AM and 4mµM EthD-1 in 1x PBS, (pH 7.4) for 30 minutes at room temperature. After incubation, samples were washed again three times in 1x PBS, and then covered in 1x PBS. Cells imaged with Nikon E600 florescence microscopy (Nikon Instruments Inc., Melville, NY) equipped Olympus DP71 CCD (Olympus America Inc., San Jose, CA) at x10 magnification using green and red filters. Percentage of live and dead cells was determined with MetaMorph software for each condition in quadruplicate.

D. *Statistical analysis*

Percentages of cell survival rate were expressed as mean ± standard deviation. Analysis of variance (ANOVA) with Fisher's protected least significant difference tests was performed to examine (1) effect of cell source, (2) effect of cell delivery methods, (3) effect of incubation time, (4) effect of final DMSO concentration, and (5) effect of cell density on cell survival rate. Prior to all analyses, data were rank-transformed. P-values less than or equal to 0.05 were considered significant. All analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC).

III. Results and Discussion

A. *Effect of physical force on fresh and cryopreserved cell viability in HyStem-C*

Viability of NIH 3T3 cells was determined by a double staining procedure that stains live cells green and dead cells red (Fig. 1). Three-dimensional cultured cells were examined 2 and 48 hours after seeding by pipette or 27G needle. The viability of fresh cells was maintained over 90% after 2 and 48h incubating in HyStem-C in both delivery methods (Fig. 2). At 2 hours, pipette-delivered cryopreserved cells also showed similar survival as fresh cells, however, viability of needle-delivered cryopreserved cells was 83±2.6%, which was significantly lower compared to pipette delivered cells (p<0.0001). After 48h incubation, viability of cryopreserved cells was significant lower (85-90%) than fresh cells (97%) (p<0.0001). Needle-delivered cryopreserved cell/biomaterial showed significant lower survival than pipette-delivered cells (p<0.05). Such differences may represent a higher sensitivity

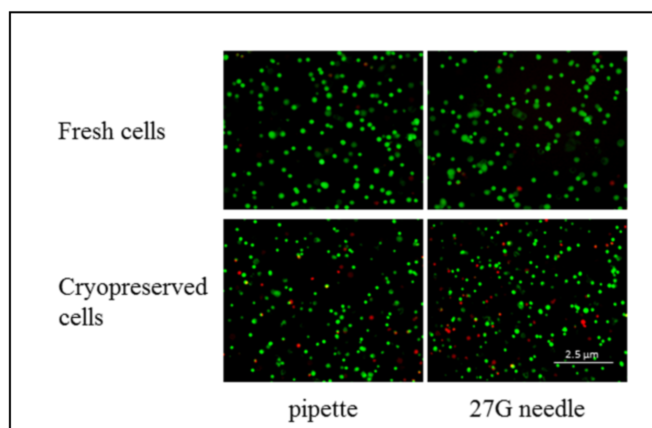

Figure 1. Fluorescent images of viable (green, calcein AM) and membrane damaged (red, ethidium homodimer-1) NIH 3T3 cells encapsulated in HyStem-C for 48 hours in pipette and 27G needle delivering methods. Cell images with x10 magnification were captured under florescence microscope. Scale bars, 2.5µm.

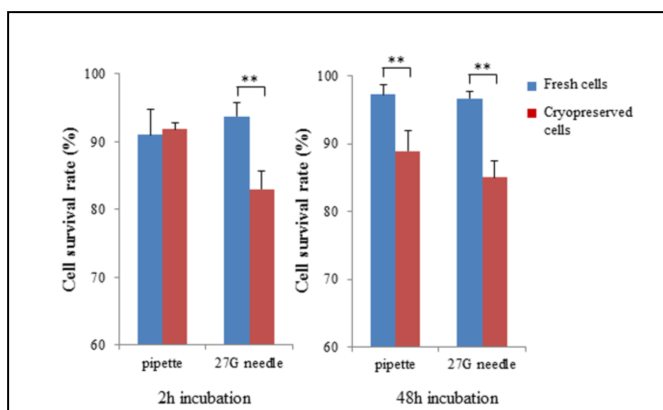

Figure 2. Percent cell viability in HyStem-C. After delivery by pipette and 27G needle, fresh and cryopreserved NIH 3T3 cells were incubated in 3-D HyStem-C. Cell survival rates were then evaluated at 2 and 48 hours after incubation. **p<0.0001.

of HyStem-C delivered cells to mechanical damage as suggested by previous experiments [18, 19].

For cell condition, we compared survival of fresh cultured to cryopreserved frozen cells. Slow-rate cooling cryopreservation using DMSO is a common and effective technique for cell preservation [20]. Cryopreserved cells can be stored, easily shipped worldwide in small tanks of liquid nitrogen, and recovered with satisfactory survival rates. This cell protectant method also can easily supply numerous cells for various research and clinical purposes. In addition to satisfactory viability, the application of cryopreserved cells in cell therapy also requires preservation of their function and proliferation ability. Previous research has demonstrated this freezing process does not significantly affect the proliferation, differentiation, differentiation capacity and immune-phenotype characteristics of cryopreserved cells [21], especially born marrow-derived mesenchymal stem cells [22] and embryonic stem cells [23]. In our experiment, we observed that at 48 hours although survival rate of needle-delivered cryopreserved cells was lower, it was still above 80%. This result suggested that both factors -- mechanical force (caused by cell delivery methods) and cell conditions

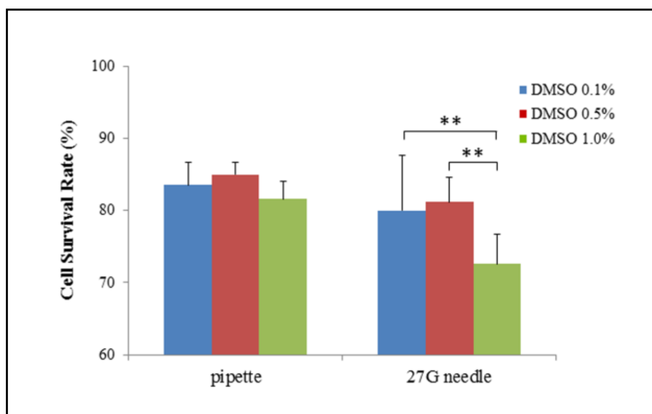

Figure 3. Effect of DMSO concentration on cell viability in HyStem-C. After delivered by pipette or 27G needle, cryopreserved NIH 3T3 cells were incubated in final 0.1, 0.5, and 1% DMSO in Hystem-C. Cryopreserved cell survival rates were then evaluated 48 hours after incubation. ** p<0.0001.

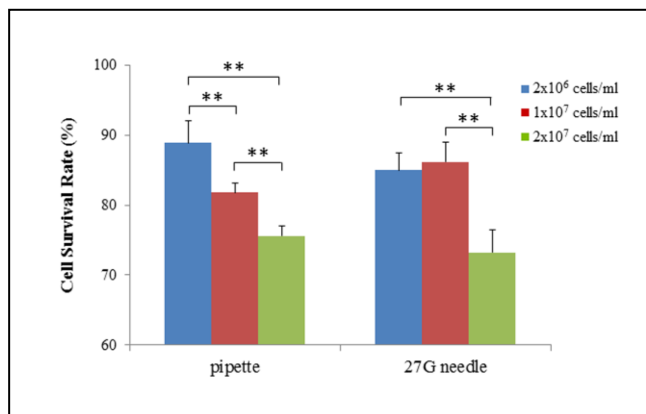

Figure 4. Effect of cell density on cell viability in HyStem-C. After delivery by pipette or 27G needle, cryopreserved NIH 3T3 cells were incubated in 2x106, 1x107, and 2x107/ml cell concentration in Hystem-C. Cryopreserved cell survival rates were then evaluated 48 hours after incubation. ** p<0.0001.

(fresh or frozen) are important for biomaterial-induced cell viability. In future investigations, it will be necessary to examine the morphologic features, function, proliferation and differentiation of biomaterial embedded cryopreserved cells prior to use in clinical application.

B. Effect of DMSO on frozen cell viability in HyStem-C

As a cryoprotective agent, DMSO is added to cell freezing medium to prevent the formation of ice crystals during the freezing process, otherwise cells would be destroyed and dead. It is commonly used in cell banking. DMSO has a low toxicity to cells. In order to test the effect of DMSO on survival of HyStem-C seeded cells, we designed to test a range of DMSO concentrations (0.1, 0.5, and 1.0% in final gel-cell solution) with two different cell delivery methods. As showed in Fig. 3, cells exhibited similar survival rates at 0.1 and 0.5% final DMSO concentrations for both pipette and needle delivery methods. When DMSO concentration was increased to 1%, needle-delivered cells displayed a very low survival rate of 72.6±4.1%, which was significant lower than other conditions (p<0.0001). Although it has been reported that 1.4% DMSO in cell culture medium does not affect cell growth [24], most studies have demonstrated that DMSO treatment substantially altered the morphology and attachment of cells in concurrence with a significant reduction in cell viability in a dose-dependent manner, and for cell culture, maximum DMSO concentration should be 0.5% [25]. Our results revealed that 1% DMSO had some cytoxicity for our HyStem-C seeded cells. This result is similar to previous reports [26], where cells lost their viability at DMSO concentrations higher than a critical value between 0.1% and 0.5%, because cells were permeabilzed by DMSO.

C. Effect of cell density on frozen cell viability in HyStem-C

We also examined the effect of cryopreserved cell density in HyStem-C on viability. Cryopreserved NIH 3T3 cells were subjected to HyStem-C over a wide range of cell concentrations, from $2x10^6$ to $2x10^7$ cells/ml. Cell survival rates decreased with increasing cell density in HyStem-C (Fig. 4). When cell density was higher than 1×10^7 cells/ml, the survival rates of pipette-delivered and needle-delivered cells dropped to 72-75%, which was significantly lower than the other cell densities -- $2x10^6$ and $1x10^7$ cells/ml, (p<0.0001 for both). These observations may reflect that the levels of survival/growth factors (such as CBS) are locally limited. At low cell seeding density, the level of survival factors is adequate, whilst at high cell density it is insufficient to prevent cell apoptosis and death. Similar results have also been reported in 3-D cultured bovine nucleus pulposus cells in alginate beads at a range of cell densities ($1.25 \ x10^5$-10^6 cells/ml) [27], where cell proliferation is inversely related to cell seeding density, and the number of apoptotic cells is positively correlated to cell seeding density. Our results suggest that at maximum cell density of $1x10^7$ cells per ml, needle-delivered cryopreserved cells can keep rather higher viability (about 81-89%). Hence, for enhancing cell transplantation efficiency, not only should total cell number be considered but cell density may also very important.

IV. Conclusion

Three dimensional cell survival rate in HyStem-C is very important for hydrogel induced cell therapy. Although frozen NIH 3T3 cells had lower viability than fresh cells, after 48 hours in HyStem, cells survival rate can still be maintained at 85 to 88%. 0.1-0.5% DMSO concentration or 27G needle-induced physical force did not affect frozen 3T3 cell viability significantly, whereas cell concentration in HyStem-C significantly affected cell viability. For hydrogel induced cell therapy, cryopreserved cells at particular cell densities ($1x10^7$ cells/ml, and DMSO concentrations (less than 0.5%) may provide better cell therapeutic approaches for regenerative medicine. In this study, we have selected mouse embryonic fibroblast cells for testing cell viability because this cell line is widely used. Further testing is necessary for other cell types, ie. mesenchymal stromal cells or pluripotent stem cells.

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REFERENCES

[1] M. Molcanyi, P. Riess, K. Bentz, M. Maegele, J. Hescheler, B. Schafke, et al. "Trauma-associated inflammatory response impairs embryonic stem cell survival and integration after implantation into injured rat brain," J Neurotrauma, vol. 24, pp. 625-637, 2007.

[2] L. R. Madden, D. J. Mortisen, E. M. Sussman, S. K. Dupras, J. A. Fugate, J. L. Cuy, et al. "Proangiogenic scaffolds as functional templates for cardiac tissue engineering," Proc Natl Acad Sci U S A, vol. 107, pp. 15211-15216, 2010.

[3] F. Guilak, D. M. Cohen, B. T. Estes, J. M. Gimble, W. Liedtke, C. S. Chen. "Control of stem cell fate by physical interactions with the extracellular matrix," Cell Stem Cell, vol. 5, pp. 17-26, 2009.

[4] M. E. Davis, J. P. Motion, D. A. Narmoneva, T. Takahashi, D. Hakuno, R. D. Kamm, et al. "Injectable self-assembling peptide nanofibers create intramyocardial microenvironments for endothelial cells," Circulation, vol. 111, pp. 442-450, 2005.

[5] S. Rodin, A. Domogatskaya, S. Strom, E. M. Hansson, K. R. Chien, J. Inzunza, et al. "Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511," Nat Biotechnol, vol. 28, pp. 611-615, 2010.

[6] S. Dobner, D. Bezuidenhout, P. Govender, P. Zilla, N. Davies, "A synthetic non-degradable polyethylene glycol hydrogel retards adverse post-infarct left ventricular remodeling," J Card Fail, vol. 15, pp. 629-636, 2009.

[7] T. Kofidis, D. R. Lebl, E. C. Martinez, G. Hoyt, M. Tanaka, R. C. Robbins, "Novel injectable bioartificial tissue facilitates targeted, less invasive, large-scale tissue restoration on the beating heart after myocardial injury," Circulation, vol. 112, pp. I173-I177, 2005.

[8] W. Dai, S. L. Hale, G. L. Kay, A. J. Jyrala, R. A. Kloner, "Delivering stem cells to the heart in a collagen matrix reduces relocation of cells to other organs as assessed by nanoparticle technology," Regen Med., vol. 4, pp. 387-395, 2009.

[9] J.M. Lu, X. Wang, C. Marin-Muller, H. Wang, P. H. Lin, Q. Yao, et al. "Current advances in research and clinical applications of PLGA-based nanotechnology," Expert Rev Mol Diagn, vol. 9, pp. 325-341, 2009.

[10] X. Z. Shu, Y. Liu, Y. Luo, M. C. Roberts, G. D. Prestwich, "Disulfide cross-linked hyaluronan hydrogels," Biomacromolecules, vol. 3, pp. 1304-1311, 2002.

[11] X. Z. Shu, Y. Liu, F. Palumbo, G. D. Prestwich, "Disulfide-crosslinked hyaluronan-gelatin hydrogel films: a covalent mimic of the extracellular matrix for in vitro cell growth," Biomaterials, vol. 24, pp. 3825-3834, 2003.

[12] L. Qian, W. Shim, Y. Gu, M. Shirhan, K. P. Lim, L. P. Tan, et al. "Hemodynamic contribution of stem cell scaffolding in acute injured myocardium," Tissue Eng Part A, vol. 18, pp. 1652-1663, 2012.

[13] X. Chen, S. L. Thibeault, "Biocompatibility of a synthetic extracellular matrix on immortalized vocal fold fibroblasts in 3-D culture," Acta Biomater, vol. 6, pp. 2940-2948, 2010.

[14] B. Q. Johnson, R. Fox, X. Chen, S. L. Thibeault, "Tissue regeneration of the vocal fold using bone marrow mesenchymal stem cells and synthetic extracellular matrix injections in rats," Laryngoscope, vol. 120, pp. 537-545, 2010.

[15] Y. Liu, X. Z. Shu, G. D. Prestwich, "Osteochondral defect repair with autologous bone marrow-derived mesenchymal stem cells in an injectable, in situ, cross-linked synthetic extracellular matrix." Tissue Eng., vol. 12, pp. 3405-3416, 2006.

[16] S. L. Thibeault, S. A. Klemuk, X. Chen, B. Q. Johnson, "In Vivo Engineering of the Vocal Fold ECM With Injectable HA Hydrogels-Late Effects on Tissue Repair and Biomechanics in a Rabbit Model," J Voice, vol. 25, pp. 249-253, 2010.

[17] S. Duflo, S. L. Thibeault, W. Li, X. Z. Shu, G. D. Prestwich, "Effect of a synthetic extracellular matrix on vocal fold lamina propria gene expression in early wound healing," Tissue Eng., vol. 12, pp. 3201-3207, 2006.

[18] M. Furuya, K. Yasuchika, K. Mizutani, Y. Yoshimura, N. Nakatsuji, H. Suemori, "Electroporation of cynomolgus monkey embryonic stem cells," Genesis, vol. 37, pp. 180-187, 2003.

[19] B. A. Aguado, W. Mulyasasmita, J. Su, K. J. Lampe, S. C. Heilshorn, "Improving viability of stem cells during syringe needle flow through the design of hydrogel cell carriers," Tissue Eng Part A, vol. 18, pp. 806-815, 2012.

[20] [20] G. N. Stacey, S. Dowall, "Cryopreservation of primary animal cell cultures." Methods Mol Biol., vol. 368, pp. 271-281, 2007.

[21] H. Yokomuro, N. Shiono, T. Ozawa, T. Fujii, Y. Watanabe, N. Koyama, et al. "Effect of cryopreservation on cell proliferation and immunogenicity of transplanted human heart cells," Ann Thorac Cardiovasc Surg., vol. 16, pp. 105-112, 2010.

[22] C. C. Doan, N. H. Truong, N. B. Vu, T. T. Nguyen, H. M. Nguyen, K. G. Nguyen, S. M. Do, N. K. Phan, P. V. Pham, "Isolation, culture and cryopreservation of human bone marrow-derived mesencnymal stem cells," Int. J. of Plant, Animal and Environmental Sciences, vol. 2, pp. 83-90, 2012.

[23] T. Fujioka, K. Yasuchika, Y. Nakamura, N. Nakatsuji, H. Suemori, "A simple and efficient cryopreservation method for primate embryonic stem cells," Int J Dev Biol., vol. 48, pp. 1149-1154, 2004.

[24] A. J. Schmidt, J. M. Lee, G. An, "Media and environmental effects on phenolics production from tobacco cell cultures," Biotechnol Bioeng., vol. 33, pp. 1437-1444, 1989.

[25] R. Pal, M. K. Mamidi, A. K. Das, R. Bhonde, "Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells," Arch Toxicol., vol. 86, pp. 651-661, 2012.

[26] C. H. Park, B. C. Martinez, "Enhanced release of rosmarinic acid from Coleus blumei permeabilized by dimethyl sulfoxide (DMSO) while preserving cell viability and growth," Biotechnol Bioeng., vol. 40, pp. 459-464, 1992.

[27] S. Stephan, W. E. Johnson, S. Roberts, "The influence of nutrient supply and cell density on the growth and survival of intervertebral disc cells in 3D culture," Eur Cell Mater., vol. 22, pp. 97-108, 2011.