Original Article

Determination and comparison of specifics of nucleus pulposus cells of human intervertebral disc in alginate and chitosan–gelatin scaffolds

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Abstract

Introduction: Low back pain is a major economical and social problem nowadays. Intervertebral disc herniation and central degeneration of disc are two major reasons of low back pain that occur because of structural impairment of disc. The intervertebral disc contains three parts as follows: Annulus fibrosus, transitional region, and nucleus pulposus, which forms the central nucleus of the disc. The reduction of cell count and extracellular matrix, especially in nucleus pulposus, causes disc degeneration. Different scaffolds (natural and synthetic) have been used for tissue repairing and regeneration of the intervertebral disc in tissue engineering. Most scaffolds have biodegradable and biocompatible characteristics and also prepare a fine condition for proliferation and migration of cells. In this study, proliferation of NP cells of human intervertebral disc compromised in Chitosan-gelatin scaffold with alginate scaffold was studied.

Materials and Methods: NP cells derived from nucleus pulposus by collagenase enzymatic hydrolysis. They were derived from patients who undergoing open surgery for discectomy in the Isfahan Alzahra hospital. Chitosan was blended with gelatin and glutaraldehyde was used for cross linking the two polymers. Then, alginate scaffold was prepared. Cellular suspension with 1×10^5 transferred to each scaffold and cultured for 21 days. Cell viability and proliferation investigated by trypan blue and (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Scanning electron microscope (SEM) was used to assert the porosity and to survey structure of scaffold.

Results: MTT assay dem1onstrated that cell viability of third day had significant difference in contrast by first day in both scaffolds. Accordingly, there was a significant decreased in cellular viability from day 3 to 21. Results of the cell count showed a punctual elevation cell numbers for alginate scaffold but there was no similar result for chitosan–gelatin scaffold.

Conclusion: Alginate scaffold prepared a better condition for proliferation of NP cells in comparison with chitosan–gelatin scaffold. Results of this study suggest that alginate scaffold could be useful in *in vivo* studies and treatment.

Key Words: Alginate scaffold, chitosan-gelatin scaffold, intervertebral disc, NP cells, tissue enginiaring

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INTRODUCTION

Low back pain is one of the most important musculoskeletal diseases nowadays. Sixty to eighty percent of people in United States of America have experienced low back pain. Then, it is a real challenge for economy and society.[1] About 11 billion pounds have been paid for low back pain each year in England.[2] Studies demonstrated a relation between degeneration of intervertebral disc and low back pain.[3] Herniation of intervertebral disc (IVD) and its degeneration are the major reasons of low back pain, which occur because of structural damage of the disc.[4] IVDs are located between spines, which contain three parts. The outer part is annulus fibrosis (AF), the middle part is transitional zone (TZ) and the inner part is nucleus pulposus (NP), which produce the nucleus of the disc.[5,6] AF and NP formation are mainly from extracellular matrix. IVDs cells comprise only 1% of the volume of the IVD.[7] Water, proteoglycans, and collagen in the extracellular matrix (ECM) of NP tissue provide fluidity and viscoelasticity to the structure, acting as a shock absorber, and maintaining loads in IVDs.[8] ECMs degradation is also increased in NP of aged individuals and makes difference in conformation, structure, and function of the disc.[9] Therapeutic strategies for disc degeneration treatment are cell therapy and gene transferring, which have done in laboratory animals.[9] A mesenchymal cell differentiated to NP-like cells by co-culturing with mature NP cells is one way to increase proliferation of NP cells and a treatment for degeneration.[10] Use of appropriate scaffold is an important point in tissue engineering and especially for cartilage restoration. Scaffolds prepare a three-dimensional condition for proliferation, production, and secretion of extracellular matrix and formation of normal tissue.[11-14] The purpose in tissue engineering is to find the proper substances with significant traits for restoration of tissue. These traits are biodegradable[13-15] and biocompatible, which mean do not induce inflammatory reactions and toxic production.[13-15] Having proper pores and controlled porosity, scaffold surface must be appropriate for adherence, proliferation, and migration of cells.[16] Polymers are subtypes of biomaterials, which have susceptibility for porosity and destruction. They include two major groups, natural and synthetic. [14,15] Alginate, Collagen, Chitosan, hyaluronic acid, agarose are among the natural polymers. Alginate is a natural biopolymer and is usually extracted from brown alga and minor from bacteria.[17] Lot of studies have been done on alginate scaffold. Guo-plated chondrocyte on alginate then investigated cellular morphology and observed maintenance of round shape of the cells. Meanwhile the alginate gel supported the chondrogenesis of the periosteum-derived cells and induced chondrogenesis in bone marrow stem cell and fatty tissue and had a role in mesenchymal stem cell differentiation.[17,18] Some studies showed also the elevation of NP cells proliferation and excretion of extracellular matrix like type II collagen, aggrecan, and glycosaminoglycan (GAG) on alginate scaffold.[19] These substances are discharged by NP cells. In some studies, NP cells isolated from human and rabbit IVDs, secreted type II collagen, aggrecan and GAG.[20,21] Chitosan is a glycosamine and N-acetyl glycosamine polymer, which is obtained from depolarization and deacetylation of chitin[22,23] Cation property of chitosan makes it a useful scaffold to induce proliferation and secretion of chondrocytes.[24,25] Biocompatibility and dissolubility (degradable) are two properties of a good scaffold. Chitosan have both characteristics.[26] This scaffold has been used in regeneration of bone and cartilage and also in tissue engineering.[27,28] Results of studies on efficacy of chitosan scaffold on proliferation of NP cells and the mass of extracellular matrix secretion, demonstrated an increase in both proliferation and secretion abilities.[28-30] Some polymers could help chitosan to improve its mechanical and biological virtues. Gelatin is one of them, which improves the biological activity of scaffold because of its specific sequence that increases cell adhesion and migration.[31] Gelatin is a natural biopolymer and is produced by collagen hydrolysis. Biocompatibility, biodegradability and not stimulating the immune system are perfect sights of gelatin.[32] Adding gelatin to chitosan scaffold increases hydrophilicity of chitosan and makes it more proper for keratinocyte culture and skin regeneration.[33] Gelatin binds to chitosan scaffold by cross linkers like glutaraldehyde or enzymes, which exist in different tissues like skin, cartilage and bone.[34-38] Chitosan–gelatin scaffold has been effective on proliferation of SHED cells, of course with weak attachment. There is a mass of studies on separation of NP cells from IVDs and proliferation of them on different scaffolds *in vitro.* Efficacy of alginate scaffold in NP cells proliferation, secretion of extracellular matrix, and expression of chondrocyte gene markers have been investigated by numerous studies. There are lots of studies on efficacy of chitosan–gelatin scaffold for proliferation of NP cells. Recently, it was reported that chitosan–gelatin scaffold has a proper structure for cellular proliferation compared with pure chitosan scaffold.

The goal of this study was to compare NP cells proliferation and viability in alginate scaffold with chitosan–gelatin scaffold to reach a proper scaffold for NP cells, which could be used for restoration of degenerative damages to IVDs in future studies *in vivo.*

MATERIALS AND METHODS

Scaffold synthesis andcharacterization

Synthesis of chitosan–gelatin scaffold

All reagents were prepared from Sigma Chemical Co (USA). Degree of deacetylation of chitosan was 85%. Mw range was 150 000.

Aqueous solutions of gelatin 0.5% and chitosan 1.5% were prepared. Each solution was mixed to have a weight ratio of 1:1gelatin to chitosan and stirred with a magnetic bar at 50°C for 12 h. A glutaraldehyde solution was then added for cross linking. The mixed solutions were poured into 10-cm tissue culture dishes to a depth of approximately 4 mm. The solution was placed in –27°C freezer for 24 h. The frozen solution was then lyophilized for 36 h. Grade ethanol series was used to eliminate the remains of acetic acid and washed by PBS for three times and lyophilized again.

Synthesis of alginate scaffold

Alginate powder was diluted in NaCl to produce 1.2% alginate solution; then, the solution was filtered.

Isolation and culture of human nucleus pulposus cells

Human nucleus pulposus (hNP) cells were collected from IVD donors of Alzahra hospital of Iran. These volunteers provided informed consent for the use of their nucleus pulposus cells, as required by the Ethics Committee of Isfahan University of Medical Science. Normal NP tissue harvested aseptically from donors was minced into pieces in Hanks balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY) along with antibiotics. NP cells were then isolated from these slices in an enzymatic solution (0.2% collagenase and 0.04% pronase, purchased from Sigma) for 4 h at 37°C. The cell suspension in the enzyme solution was filtered through a 40-μm nylon mesh (Falcon, NY), and then, centrifuged at 1800 rpm for 10 min, re-suspended in Dulbeccos modified Eagles medium (DMEM/F12) (Gibco BRL) with 10% fetal bovine serum (FBS). After isolation, it was incubated at 37°C in 5% CO_2 before subsequent experiments. The culture medium was changed three times a week.

Culture of NP cells in chitosan–gelatin scaffolds

Prepared chitosan–gelatin scaffold was cut in pieces of 5 mm diameter and 4 mm width, was sterilized by UV radiation for 30 min and distributed in 24 wells. Human NP cells monolayer culture was trypsinized by trypsine/EDTA and centrifuged. 100 mL of cellular suspension that contained 1×10^5 cells, transferred to the chitosan–gelatin scaffold by pipette. The alginate solution was added to cellular precipitate with 1×10^5 cells and 2–3 drops was injected into each well of 24 wells, which contained 102 m molar CaCl2 by 22 gage syringe. After 15 min, bubbles of cellular alginate became hydrogel and washed by NaCl for 10 minutes. Located beads in 24 wells were washed by medium. F12 medium (FBS 10% and pen/strep) was added to each well and aftermath 24 wells were transferred to incubator and cultured for 21 days. The culture medium was changed three times a week.

Scanning electron microscopy (SEM)

In order to determine the morphology and structure of chitosan–gelatin scaffold and distribution of cultured NP cells, the SEM test was performed. Samples exposed to 2.5% glutaraldehyde for 1 h and then went through ethanol series. Samples covered by gold and scanned by SEM.

Trypan blue

Cell number and viability were evaluated via trypan blue exclusion. In alginate scaffold, NP cells isolated by adding sodium citrate to alginate beads contained falcon. After 20 min, alginate scaffold was hydrolyzed and NP cells were exempted from scaffold. In chitosan–gelatin scaffolds, isolation of NP cells was done by immersion of scaffold in a soluble containing trypsin/EDTA. 10 mL trypan blue was added to almost 10 mL cellular suspension of each scaffold after the suspension was centrifuged. Then, 10 mL of this solution was put on neobar slide to calculate death cells by the inverted microscope.

MTT assay

Both kinds of scaffolds with cells were cultured in 12 wells for 24 h, then discharged from the medium and washed by PBS. After that, medium was added with MTT to each well for 4 h and incubated in 37°C and 5% CO_2. Next step was discharging the medium, adding DMSO and pipetting. Aftermath was transferred to the 96 wells and read by ELISA reader on 540 nm.

Statistical analysis

To compare the proliferation and cellular viability in alginate scaffold with those of chitosan–gelatin scaffold, we used SPSS-17 and Mann-Whitney U test. For all tests, $P<0.005$ was considered significant.

RESULTS

NP cells culture

Cultured NP cells in monolayer condition had small size and taped shape [Figure 1a]. But, in further passages they were changed to fibrocyte-like cells with long processes [Figure 1b]. In the first culture, cellular proliferation was almost high but decreased in the next passages and the morphology was changed;

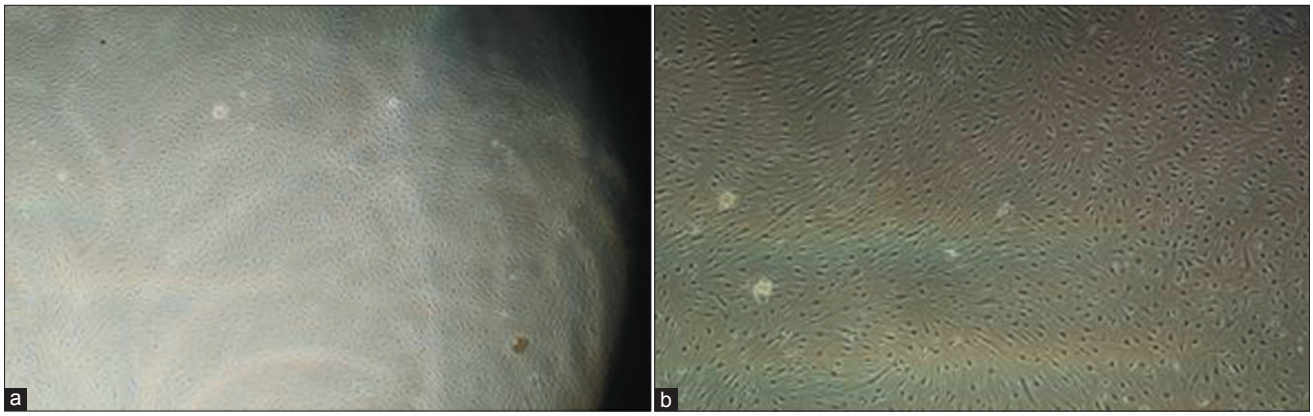

Figure 1: Light microscopic images of NP cells cultured on tissue culture dish. NP cells have polygonal (a) and fibroblastic morphology (b)(x60)

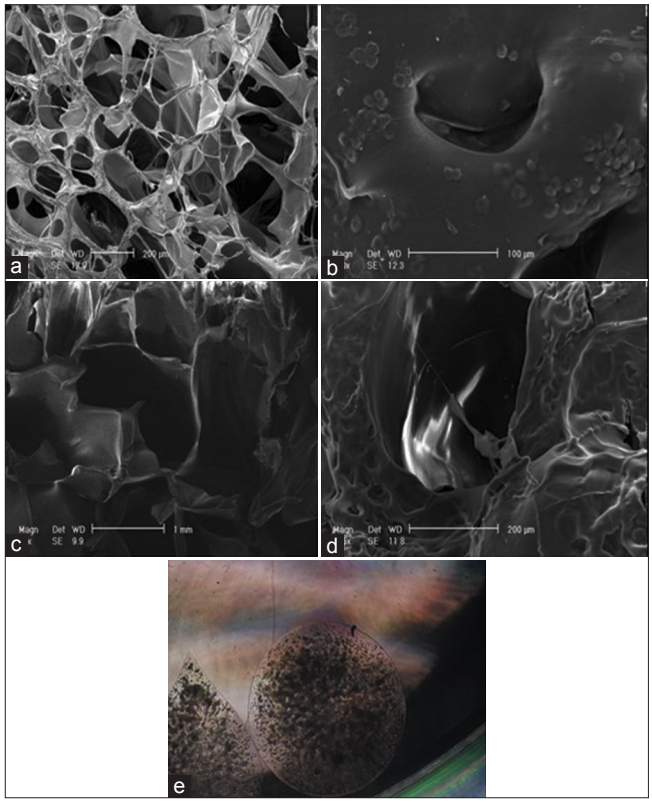

Figure 2: SEM Micrographs of porous chitosan-gelatin scaffold (a) surface (b) NP Cells grown on chitosan-gelatin scaffold for 1 days;note that the cells have spherical Morphology for 1 days in chitosan-gelatin scaffold (b) and alginate gel (e) and fobrobastic and elangated morphology of one NP cell for 3 days (d) cross-section of inner part (c)

hence, the first passage cells were used to reduce morphological changes.

Scanning electron microscope

Chitosan–gelatin scaffold SEM photos showed high porosity structure with mean 125-μm diameter (50–200 μm) [Figure 2a].

SEM of cell-scaffold hybrid demonstrated distribution NP cells on the surface of scaffold and their processes were tightly attached to the scaffold surface [Figure 2b].

Transverse sectional analysis of samples showed that depth porous could reach to 1 mm diameter [Figure 2c].

SEM evaluated NP cells morphology in chitosan–gelatin scaffold. It was figured out that NP cells morphology in the first day was spherical with short processes but after 3 days; they became fibroblastic like with long process and tight junctions to the scaffold [Figure 2d and b]. NP cells in alginate gel were trapped in pores of gel with spherical morphology [Figure 2e]

MTT assay

Results demonstrated that the cell viability after the third day had significant difference with that of the first day in both scaffolds [Figure 3]. Accordingly, there was a significant decrease in cellular viability from day 3 to 21.

Day

Trypan blue

Results of cell count showed a punctual elevation of cell numbers for alginate scaffold but there was no similar results for chitosan–gelatin scaffold [Figure 4].

Day

DISCUSSION

The goal of this study was comparison of the efficacy of chitosan–gelatin scaffold with alginate scaffold in proliferation, viability and morphology of human NP cells.

This study showed that proliferation and viability percentage were significantly higher in day 3 in contrast to day 1, in both kinds of used scaffolds. Also, in both kinds of used scaffolds we saw a punctual reduction in proliferation and viability in day 3 to day 21.

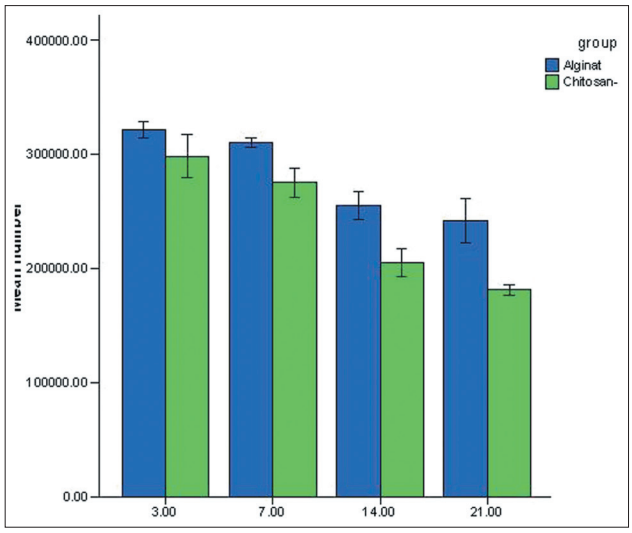

Figure 3: Comparison of viability and proliferation of alginate and chitosan-gelatin scaffolds (*: Significant difference between 3 and 14 days)

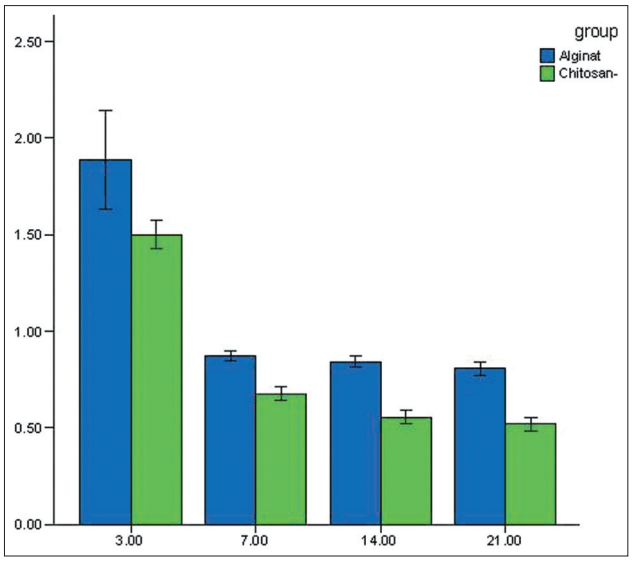

Figure 4: Comparison percent of alive NP cells in alginate and chitosan-gelatin Scaffolds. $P<0.05$

There is a vast majority of published reports about the effects of different scaffolds on proliferation of human or animal NP cells and secretion of extracellular matrix (ECM). Each scaffold has some benefits and of course some defects. Overall, alginate seems to be a perfect scaffold for intervertebral disc regeneration and ECM secretion and also has a roll in chondrogenesis and differentiation.[17-19]

Rabie *et al.* reported the effects of alginate scaffold on proliferation of Calvaria-derived osteoblasts.[39]

Studies showed that adipose-derived stem cells could be differentiated to chondrocytes in alginate scaffold by adding BMP-6, which differentiated chondrocytes secreted ECM.[40]

Counting of cultured NP cells demonstrated that viability percentage in day 3 significantly increased in comparison with that in day 1, also meaningfully decreased from day 3 to day 21. It was almost similar to Bertolo *et al.* study.[41] Bertolo *et al.* differentiated MSc to NP cells on alginate scaffold. Their results showed that cellular proliferation reached the maximum size in the first few days but had a punctual reduction in continue while secretion of ECM from NP cells began and achieved maximum range on day 35.

Chitosan scaffold has been used in tissue engineering and it is fine for NP cells proliferation and ECM secretion by these cells.[27-30] In tissue engineering, additional substances such as collagen and gelatin have been used to upgrade physiological and mechanical properties of scaffolds and also to increase cell attachment.[32,44,42]

Thein *et al.* reported that adding gelatin to chitosan scaffold increased its porosity, softness, flexibility, and elasticity.[43] So, we used gelatin in our study to promote mentioned criteria and also used glutaraldehyde for cross-linking of chitosan with gelatin.

A routine method to produce the spongy structure with large pores is the freeze-drying technique.[34] We used this technique to make the porous structure. In our study, SEM results showed porous and sponge-like structure. The porous had interconnections in chitosan–gelatin scaffold. Fine porosity has an impressive role in proliferation and diffusion of nutrition.[34] It should be noticed that size of porous depends on freezing temperature before freeze-drying. The less freezing the temperature, the smaller the porous. This is because of numerous ice crystals.[44] Small-size porous elevated authority of the scaffold biomechanical structure.[45] Arger porous improved diffusion of nutrients so increased cellular proliferation and ECM secretion.[46] Hsieh *et al.* reported that the most appropriate temperature to create a stable and porous scaffold is –20°C.[57] So, we used this temperature in our study.

SEM showed that the diameters of porous on the surface of chitosan–gelatin scaffold were 50–200 μm (mean of 125 μm) and they could reach to 1 mm in depth of chitosan–gelatin scaffold. According to the transverse sectional analysis after implantation of NP cells on chitosan–gelatin scaffold, cells were distributed on the surface of the scaffold. They were rounded and had long processes and tightly adhered to the scaffold.

MTT and trypan blue results demonstrated that the proliferation and viability percentage were

significantly higher in day 3 in contrast to day 1, in both kinds of used scaffolds. Also, in both kinds of used scaffolds, we saw a punctual reduction in proliferation and viability in day 3 to day 21.

These results are similar to Mao study, which reported scale down of cultured fibroblasts on chitosan–gelatin scaffold after day 7. This was because of restriction to accessibility of medium that results in decline in cellular proliferation.[48] Bertolo *et al.* differentiated MSc to NP cells on both alginate scaffold and chitosan one. They reported the elevation of ECM secretion and reduction in cell counts. Cells proliferated in the first few days and after that the secretion of ECM began.[41] Miranda study showed detraction of cultured bone-marrow-derived stem cells on chitosan–gelatin scaffold after day 3. They reported that day 3 is a convenient time for cellular transplantation *in vivo*.[51]

MTT and trypan blue results illustrated that cellular proliferation and viability on alginate scaffold are significantly higher than those in chitosan–gelatin scaffold. Difference of proliferation and viability on these scaffolds maybe because of first, glutaraldehyde, a toxic substance used in chitosan–gelatin scaffolds for cross-linking.[49] It seems that glutaraldehyde could be excreted from scaffold in a timely manner and result in degradation and destruction of scaffold (change of the color of medium is a proof for scaffold destruction). On the other site, this toxic substance caused cell's death and decreased cellular proliferation.[49] Of course, in some studies that cultured bone marrow derived stem cells on this scaffold, the glutaraldehyde (0.1%) did not affect the cellular viability.[47] Second, surface porous in some regions of chitosan–gelatin scaffold have micro-diameter. After few days of culturing NP cells on this scaffold, this micro-diameter porous was blocked because of cellular proliferation and aggrecan secretion. Hereby, potency of scaffold for more NP cells proliferation decreased and led to abatement maintenance of produced aggrecan by NP cells. Blocking the surface porous also caused a decline in exchanged nutrition to the depth cells and eventuated cell death and decreased cellular proliferation. Griffen *et al.* cultured chondrocytes on chitosan scaffold and reported as the surface porous become tighter, feeding and distribution to the depth cell decrease because of secretion of ECM by attached chondrocytes. This process results in cellular death and degeneration.[48] Third, hydrogel property of alginate caused better cell connection and more nutrition and oxygen transport.[40] Li *et al.* reported that chitosan-alginate scaffold increased cellular proliferation of chondrocytes and also increased ECM secretion from day 1 to day 21 compared with chitosan scaffold. These results showed that alginate is more appropriate than chitosan. So, alginate could be used instead of gelatin in mixture with chitosan.[32,50] Roughley *et al.* cultured NP cells on chitosan–genipin gel and illustrated that chitosan hydrogels could keep the NP cells secretion of ECM and prevent it from the medium. Chitosan hydrogel also increased cellular proliferation.[50,51] Nevertheless, it could claim that hydrogels are more proper than nonhydrogel scaffolds for proliferation and even secretion of ECM. Fourth, some studies demonstrated that incorporation of gelatin into chitosan improved the hydrophilicity of chitosan–gelatin scaffold[49] and caused wetting and hydrolyze of scaffold. So, it seems it is better to add less gelatin to the scaffold. Thus, alginate scaffold has better conditions for NP cells proliferation and viability than chitosan.

CONCLUSION

According to our study, it was figured out that alginate scaffold is more appropriate than chitosan–gelatin scaffold for human NP cells culture in *in vitro*. We suggest using this scaffold in tissue engineering and treatment of human IVDs degeneration for *in vivo* studies.

REFERENCES

1. Waddell G. Low back pain: A twentieth century health care enigma. Spine 1966;21:2820-5.
2. Le Maitre CL, Christine L Le Maitre, Judith A Hoyland and Anthony J Freemont. Interleukin-1 receptor antagonis derived directly and by Gene therapy inhibits matrix degradation in the intact degenerate human intervertebral disc: An *in situ* zymographic and gene therapy study. Arthritis Res Ther 2007;9:83.
3. Mauth C, Bono E, Haas S, Paesold G, Wiese H, Maier G, *et al.* Cell-seeded polyurethane-fibrin structures-a possible system. for intervertebral disc regeneration. Eur Cell Mater 2009;18:27-39.
4. Boss N, Rieder R, Schade V, Spartt KF, Semmer N, Aebim. The diagnostic accuracy of magnetic resonance imaging, work perception,and psychosocial factors in identifying symptomatic descherniations. Spine 1995;20:2613-25.
5. Oegema TR Jr. The role of disc cell heterogeneity in determining disc biochemistry: A speculation. Biochem Soc Trans 2002;30:839-44.
6. Hunter CJ, Matyas JR, Duncan NA. The notochordal cell in the nucleus pulposus: A review in the context of tissue engineering. Tissue Eng 2003;9:667-77.
7. Roberts S, Evans H, Trivedi J, Menage J. Histology and pathology of the human intervertevral disc. J Bone Joint Surg Am 2006;88:10-4.
8. Goupille P, Jayson MI, Valat JP, Freemont AJ. Matrix metalloproteinases: The clue to intervertebral disc degeneration? Spine 1998;23:1612-26.
9. Sobajima S, Vadala G, Shimer A, Kim JS, Gilbertson LG, Kang JD. Feasibility of a stem cell therapy for intervertebral disc degeneration. Spine J 2008;8:888-96.
10. Nishida K, Suzuki T, Kakutani K, Yurube T, Maeno K, Kurosaka M, *et al.* Gene therapy approach for disc degeneration and associated spinal disorders. Eur Spine J 2008;17 Suppl 4:459-66.
11. Watanabe T, Sakai D, Yamamoto Y, Iwashina T, Serigano K, Tamura F, *et al.* Human nucleus pulposus cells significantly enhanced biological properties in a coculture system with direct cell-to-cell contact with autologous mesenchymal stem cells. Orthop Res 2010;28:623-30.
12. TabatoY. Recent progress in tissue engineering. Drug Discov Today 2001;6:483-7.
13. Tsuchiya K, Chen G, Ushida T, Matsuno T, Tateishi T. The effect of coculture of chondrocytes with mesenchymal stem cells on their cartilaginous phenotype *in vitro*. Mat Sci Eng 2004;24:391-6.

14. Bronzino JD. Tissue engineering and artificial organs. 3rd ed. The Biomedical Engineering Handbook. Vol. 37. UK: Tylor and Francis; 2007. p. 1-8.
15. Vunjak-Novakovic G, Freshney RI. Culture of cells for tissue engineering. United States: Wiley-Liss; 2006. p. 131-155
16. Haringham T, Tew S, Murdoch A. Tissue engineering: Chondrocyes and cartilage. Arthritis Res 2002;4:63-8.
17. Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. Biomaterials 2000;21:2529-43.
18. Morris VJ. GElation of polysaccharides,IN Functional properties of food macromolecules. Norwich.Mitchell and DA Ledward; 1986. p. 121-8.
19. Guo JF, Jourdian GW, Macallum DK. Culture and growth characteristics of chondrocytes encapsulated I alginate beads. Connect Tissue Res 1989;19:277-84.
20. Stevens MM, Qanadilo HF, Langer R, Shastri PV. A rapid-curing alginate gel system: Utility in periosteum-drived cartilage tissue engineering. Biomaterials 2004;25:887-94.
21. Leone G, Torricelli P, Chiumiento A, Facchini A, Barbucci R. Amidic alginate hydrogel for nucleus pulposus replacement. J Biomed Mater 2008;84:391-401.
22. Chelberg MK, Banks GM, Geiger DF, Oegema TR Jr. Identification of heterogeneous cell populations in normal human intervertebral disc. J Anat 1995;186:43-53.
23. Sive JI, Baird P, Jeziorsk M, Watkins A, Hoyland JA, Freemont AJ. Expression of chondrocyte markers by cells of normal and degenerate intervertebral discs. Mol Pathol 2002;55:91-7.
24. Suh JK, Matthew HW. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: A review. Biomaterials 2000;21:2589-98.
25. Khor E, Lim LY. Implantable applications of chitin and chitosan. Biomaterials 2003;24:2339-49.
26. Chenite A, Chaput C, Wang D, Combes C, Buschmann MD, Hoemann CD, *et al.* Novel injectable neutral solutions of chitosan form biodegradable gels *in situ*. Biomaterials 2000;21:2155-61.
27. Elder SH, Nettles DL, Bumgardner JD. Synthesis and characterization of chitosan schffolds for cartilage-tissue engineering. Methods Mol Biol 2004;238:41-8.
28. Laiji A, Sohrabi A, Hungerford DS, Frondoza CG. Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. J Biomed Mater Res 2000:51:586.
29. Lee JY, Nam SH, Im SY, Park YJ, Lee YM, Seol YJ, *et al.* Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials. J Control Rel 2002;78:187-97.
30. Roughley P, Hoemann C, Desrosiers E, Mwale F, Ntoniou J, Alini M. The potential of chitosan-based gels containing intervertebral disc cells for nucleus pulposus supplenetation. Biomaterials 2007;27:388-96.
31. Dang JM, Sun DD, Shin-Ya Y, Sieber AN, Kostuik JP, Leong KW. Temperature-responsive hydroxybutyl chitosan for the culture of mesenchymal stem cells and intervertebral disc cells. Biomaterials 2006;27:406-18.
32. Huang Y, Onyeri S, Siewe M, Moshfeghian A, Madihally SV. *In vitro* characterization of chitosan-gelatin scaffolds for tissue engineering. Biomaterials 2005;26:7616-27.
33. Hong SR, Lee SJ, Shim JW, Choi YS, Lee YM, Song KW, *et al.* Study on gelatin containing artificial skin iv: A comparative study on the elect of antibiotic and EGF on cell proliferation during epidermal healing. Biomaterials 2001;22;2777-83.
34. Mao J, Zhao L, De Yao K, Shang Q, Yang G, Cao Y. Study of novel chitosan-gelatin artificial skin *in vitro*. J Biomed Mater Res A 2003;64: 301-8.
35. Mao JS, Zhao LG, Yin YJ, Yao KD. Structure and properties of bilayer chitosan-gelatin scaffolds. Biomaterials 2003;24:1067-74.
36. Chen T, Embree HD, Brown EM, Taylor MM, Payne GF. Enzyme-catalyzed gel formation of gelatin and chitosan: Potential for *in situ* applications. Biomaterials 2003;24:2831-41.
37. Xia W, Liu W, Cui L, Liu Y, Zhong W, Liu D, *et al.* Tissue engineering of cartilage with the use of chitosan–gelatin complex scaffolds. J Biomed Mater Res 2004;71:373-80.
38. Yin Y, Ye F, Cui J, Zhang F, Li X, Yao K. Preparation and characterization of macroporous chitosan–gelatin/beta-tricalcium phosphate composite scaffolds for bone tissue engineering. J Biomed Mater Res A 2003;67:844-55.
39. Rabie A, Esfandiari E, Fesharaki M, Sanaie M, Aminmansur B, Hashemibeni B. Access to a three dimentional osteoblasts culture in Iran. J Isfahan Med School 2010;27:778-85.
40. Hashemibeni B, Razavi SH, Esfandiary E, Karbasi S, Mardani M, Sadeghi F, *et al.* Effect of transforming Growth factor-B3 and bone morphogenetic protein-6growth factors on chondrogenic differentiation of adipose-drived stem cells in alginate scaffold. J Isfahan Med School 2010;28:608-17.
41. Bertolo A, Mehr M, Aebli N, Baur M, Ferguson SJ, Stoyanov JV. Influence of different commercial scaffolds on the *in vitro* differentiation of human esenchymal stem cells to nucleus pulposus-like cells. Eur Spine J 2011; [Epub ahead of print]
42. Risbud M, Ringe J, Bhonde R, Sittinger M. *In vitro* expression of cartilage-specific markers by chondrocytes on a biocompatible hydrogel: Implications for engineering cartilage tissue. Cell Transplant 2001;10:755-63.
43. Thein-Han WW, Saikhun J, Pholpramoo C, Misra RD, Kitiyanant Y. Chitosan-gelatin scaffolds for tissue engineering: Physico-chemical properties and biological response of buffalo embryonic stem cells and transfectant of GFP-buffalo embryonic stem cells. Acta Biomater 2009;5:3453-66.
44. Sun LP, Wang S, Zhang ZW, Wang XY, Zhang QQ. Biological evaluation of collagen-chitosan scaffolds for dermis tissue engineering. Biomed Mater 2009;4:1-6.
45. Mao J, Zhao L, De Yao K, Shang Q, Yang G, Cao Y. Study of novel chitosan-gelatin artificial skin *in vitro*. J Biomed Mater Res A 2003;64:301-8.
46. Kon M, de Visser AC. A poly (HEMA) sponge for restoration of articular cartilage defects. Plast Reconstr Surg 1981;67:288-94.
47. Hsieh CY, Tsai SP, Ho MH, Wang DM, Liu CE, Hsieh CH, *et al.* Analysis of freeze-gelation and cross-linking processes for preparing porous chitosan scaffolds. CarbohyDr. Polym 2007;67:124-32.
48. Griffon DJ, Sedighi MR, Schaeffer DV, Eurell JA, Johnson AL. Chitosan scaffolds: Interconnective pore size and cartilage engineering. Acta Biomater 2006;2:313-20.
49. Suzana CC. Miranda A, Gerluza AB, Silva A, Rafaela CR, Hell B, *et al.* Goes; Three-dimensional culture of rat BMMSCs in a porous chitosan-gelatin scaffold: A promising association for bone tissue engineering in oral reconstruction. Arch Oral Biol 2011;56:1-15.
50. Li Z, Zhang M. Chitosan-alginate as scaffolding material for cartilage tissue engineering. J Biomed Mater Res A 2005;75:485-93.
51. Roughley P, Hoemann C, DesRosiers E, Mwale F, Antoniou J, Alini M. The potential of chitosan-based gels containing intervertebral disc cells for nucleus pulposus supplementation. Biomaterials 2006;27:388-96.

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