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Original Article

Evaluation of the proliferation and viability rates of nucleus pulposus cells of human intervertebral disk in fabricated chitosan-gelatin scaffolds by freeze drying and freeze gelation methods

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Abstract

Background: Low back pain is one of the most significant musculoskeletal diseases of our time. Intervertebral disk herniation and central degeneration of the disk are two major reasons for low back pain, which occur because of structural impairment of the disk. The reduction of cell count and extracellular matrix, especially in the nucleus pulposus, causes disk degeneration. Different scaffolds have been used for tissue repairing and regeneration of the intervertebral disk in tissue engineering. Various methods are used for fabrication of the porosity scaffolds in tissue engineering. The freeze drying method has disadvantages such as: It is time consuming, needs high energy, and so on. The freeze-gelation method can save a great deal of time and energy, and large-sized porous scaffolds can be fabricated by this method. In this study, proliferation of the nucleus pulposus (NP) cells of the human intervertebral disk are compromised in the fabricated Chitosan-gelatin scaffolds by freeze drying and freeze gelation methods.

Materials and Methods: The cells were obtained from the nucleus pulposus by collagenase enzymatic hydrolysis. They were obtained from patients who were undergoing open surgery for discectomy in the Isfahan Alzahra Hospital. Chitosan was blended with gelatin. Chitosan polymer, solution after freezing at -80°C, was immersed in sodium hydroxide (NaOH) solution. The cellular suspension was transferred to each scaffold and cultured in plate for 14 days. Cell viability and proliferation were investigated by Trypan blue and MTT assays.

Results: The MTT and Trypan blue assays demonstrated that cell viability and the mean of the cell number showed a significant difference between three and fourteen days, in both scaffolds. Accordingly, there was a significantly decrease in the fabricated chitosan-gelatin scaffold by the freeze-drying method.

Conclusion: The fabricated chitosan-gelatin scaffold by the freeze-gelation method prepared a better condition for proliferation of NP cells when compared with the fabricated chitosan–gelatin scaffold by the freeze drying method.

Key Words: Chitosan, freeze drying, freeze gelation, gelatin, intervertebral disk

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INTRODUCTION

Degeneration of intervertebral disks is associated with back pain and elevated levels of inflammatory cells.[1] It is now well-established that the nucleus pulposus (NP) is prematurely affected by degenerative events.[2]

The IVDs Intervertebral disks are located between spines, which contain three parts. The outer part is the annulus fibrosis (AF), the middle part is the transitional zone (TZ), and the inner part is the NP, which produces the nucleus of the disk.[3-5] The IVD cells comprise of only 1% of the volume of the IVD. Water, proteoglycans, and collagen in the extracellular matrix (ECM) of the NP tissue provide fluidity and viscoelasticity to the structure, acting as a shock absorber, and maintaining loads in the IVDs.[6]

The main pathological changes occur in the cells and the extracellular matrix (ECM), which lead to changes in the biomechanical behavior.[7] Tissue-engineering scaffolds need to be built with functions that help to interact with cells at different spatial and temporal scales to invoke complex, tissue-like patterns.[8] Newly developed biodegradable polymers and modifications of previously developed biodegradable polymers have enhanced the tools available for creating clinically important tissue-engineering applications.[9]

It is important for the tissue-engineering product developers to have many biomaterial options:

Support for new tissue growth, Prevention of cellular activity (where tissue growth, Guided tissue response, Enhancement of cell attachment and cell migration cellular, Inhibition of cellular attachment and/or activation and so on.[9-11]

Chitosan is a biosynthetic polysaccharide that is the deacylated derivative of chitin.[12,13] Chitosan gels, powders, films, and fibers have been formed and tested for such applications as encapsulation, membrane barriers, contact lens materials, cell culture, and inhibitors of blood coagulation,[14-16] for example, in the repair of bone, cartilage, and different organs in tissue engineering.[14-16]

Gelatin biopolymer added to chitosan can improve its mechanical and biological virtues and increase the biological activity of the scaffold because of its specific sequence that increases cell adhesion and migration.[17]

Various methods are used to produce porosity in the scaffolds of tissue engineering, for example, progen leaching, saturation, release of Co2, freeze drying, freeze gelation, and so on.

In the freeze drying method, the sample is dried after freezing by vacuum and is synthesed for strength and porosity scaffold. This method has disadvantages, such as: It is time consuming, needs high energy, fabricates surface skin because of uncontrolled temperature during drying, limits selection of solvent, and so on.[18]

Thus, the freeze-gelation method can save much time and energy, and large-sized porous scaffolds can be fabricated by this method.[18]

Although freeze gelation had no disadvantages of the freeze drying method, a scaffold created by this method has not strength and is not suitable for implantation in hard tissues such as bones and the like.[18]

Hesieh *et al*., fabricated the chitosan scaffolds by the freeze drying and freeze gelation methods after incubation by the recombinant human protein (rhBMP2) and reported that the fabricated chitosan scaffold by the freeze gelation method released more rhBMP2 in the environment and resulted in this method being more suitable as a carrier system and for fabricating small-sized scaffolds.[19]

Ming *et al*. cultured ROS cells in poly (lactic-co-glycolic acid) (PLGA) and poly (l-lactic acid) (PLLA), and fabricated them by the freeze drying and freeze gelation methods. They found that the fabricated scaffolds by the freeze gelation method were more suitable for proliferation and attachment of cells. It has been reported that this method is more suitable for chitosan and alginate scaffolds.[18]

Bahramian *et al*. fabricated the chitosan-gelatin scaffold by the freeze drying method and after seeding the NP cells on this and the alginate scaffolds, they reported that the alginate scaffold was more suitable for the growth and proliferation of NP cells. The cause for the decrease in growth and proliferation was expressed as a change in the freezing and drying temperatures and glutaraldehyde was a cross-linker for chitosan and gelatin.[20] It should be noted that the measure of the scaffold pores depended on the freeze temperature before drying. Small size pores increased the strength of the biomechanical structure of the scaffold.[21]

Miranda *et al*., seeded adipose-derived stem cells in the fabricated chitosan gelatin scaffold by the freeze drying method and observed that the growth of cells decreased after day three.[22]

In the present study, we fabricated chitosan-gelatin scaffolds with the freeze drying and freeze gelation

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methods and compared the morphology, proliferation, and viability of the NP cells in the scaffolds.

MATERIALS AND METHODS

Synthesis of chitosan–gelatin by the freeze drying method:

Acetic acid and chitosan were purchased from Aldrich Chemicals, USA.

To make the chitosan–gelatin scaffold, chitosan was dissolved in 1 wt% aqueous acetic acid at room temperature. The gelatin was dissolved in water at 42°C. These solutions were mixed in equal parts to obtain a final concentration of 1.5% chitosan and 0.5% gelatin each. The mixed solutions were poured into 10 cm tissue culture dishes to a depth of approximately 4 mm.

The scaffold was re-cross-linked for using with glutaraldehyde solution and lyophilized for 24 hours.

The solution was placed in −27°C freezer for 24 hours. The frozen solution was then dried for 36 hours. Grade ethanol series was used to eliminate the remains of acetic acid and washed thrice and dried again.

Synthesis of chitosan–gelatin by the freeze gelation method

Briefly, chitosan was dissolved in acetic acid aqueous solution to form a 3.5 wt% chitosan polymer solution. Following this, amino acid and acetic acid solutions were added to form a viscous polymer solution, which was continually stirred at 48°C for six 6 hours. The polymer solution was centrifuged for 15 minutes at 3000 g to remove the insoluble impurities, and then the polymer solution was poured into a square stainless steel plate with a specially made mold and frozen at −80°C for six hours. The frozen chitosan solution was immersed in a pre-cooled NaOH aqueous solution for 24 hours, and gelation occurred when the temeperature of the polymer solution reached below the freezing point. Subsequently the scaffolds were washed with 95% ethanol and phosphate-buffered saline (PBS) solution. Finally the scaffolds were kept in a moist condition at 48°C until further experiments were carried out.

Isolation and culture of human nucleus pulposus cells

Human nucleus pulposus (hNP) cells were collected from IVD donors at the 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. The NP cells were then isolated from these slices in an enzymatic solution (0.2% collagenase and 0.04% pronease, purchased from Sigma) for four hours 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 minutes and 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% CO2 before the subsequent experiments. The culture medium was changed thrice a week.

Flow cytometry

After proliferation of the NP cells in a monolayer culture, the expression of Cyotokeratin 18 (CK-18) markers was demonstrated with a flow cytometry technique. After being trypsinized, the 10^5 passage-3 cells were washed with phosphate-buffered saline, and fixed with 4% paraformaldehyde for 15 minutes.

The cytokeratin 18 marker is cytoplasmic, thus we penetrated the membrane of the NP cells by adding Fixation/Permeablization (BD Cytifix/Cytoperm) kit. Two hundred microliters of the BD kit solution were added to the samples and they were kept in a dark place for 45 minutes.

After membrane permeability, the cells were washed and suspended in 500 μL PBS containing 10 μL cytokeratin 18 antibody conjugated with FITC (Fluorescein isothiocyanate) against cytoplasmic markers and kept in a dark place, at 4°C temperature, for 45 minutes. Cell fluorescence was measured by flow cytometry using a FACSCalibur instrument (Becton Dickinson).

Culture of NP cells in chitosan–gelatin scaffolds

A prepared chitosan–gelatin scaffold was cut into pieces of 5 mm diameter and 4 mm width, and was sterilized by ultraviolet (UV) radiation for 30 minutes and distributed in 24 wells. The human NP cell monolayer culture was trypsinized with trypsine/EDTA and centrifuged. One hundred milliliters of cellular suspension, which contained 4×10^5 cells, was transferred to the chitosan–gelatin scaffold via a pipette. The chitosan–gelatin gel that was fabricated by the freeze-gelation method was added to the cellular precipitate, with 4×10^5 cells and 3 cc gel, and the cells were injected into each of the 24 wells, which contained the F12 medium (FBS 10% and Penicillin-Streptomycin (pen/strep)) and then transferred to the incubator and cultured for 14 days. The culture medium was changed thrice a week.

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Trypan blue

The cell number and viability were evaluated via Trypan blue exclusion in three, seven, and fourteen days. In the chitosan–gelatin scaffolds, isolation of the NP cells was done by immersion of the scaffold in a solution containing trypsin/EDTA. Trypan blue of 10 ml was added to almost 10 ml of cellular suspension of each scaffold after the suspension was centrifuged. Next, 10 ml of this solution was put on a neobar slide to calculate the number of dead cells through the inverted microscope.

MTT assay

Scaffolds with cells in three, seven, and fourteen days were cultured in 12 wells for 24 hours, and then excluded from the medium and washed with PBS. After that, the medium was added with MTT to each well for 4 hours and incubated at 37°C and 5% CO2. The next step was discharging the medium, adding dimethyl sulfoxide (DMSO), and pipetting. The aftermath residue was transferred to the 96 wells and read with an ELISA reader on 540 nm.

Statistical analysis

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

RESULTS

Flow cytometery

The flow cytometery technique was used for recognition and confirmation of NP cells. This method was used to confirm the cytokeratin 18 (CK 18) that existed in the cytoplasm of NP cells.[23]

Curve 1: Isotope control group of NP cells without the added cytokeratin 18 antibody

Curve 2: Unstained group, NP cells without the added cytokeratin 18 antibody that exposed the CD45 negative antibody of the mouse

Curve 3: NP cells group with added CaK18 antibody: Peak of curve showed that most NP cells expressed CK18.

Nucleus Pulposus cell culture

Cultured NP cells in a monolayer condition had a small size and a tape shape [Figure 1a]. However, 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; hence, the first passage cells were used to reduce the morphological changes.

Trypan blue

The results of the cell count showed that the mean of the cell numbers for both scaffolds had a significant reduction and this was more significant in the fabricated chitosan–gelatin scaffold by the freeze gelation method in three and seven days [Curve 4].

Curve 4: The comparison percent of the live NP cells in fabricated chitosan–gelatin scaffolds by the freeze gelation and freeze drying methods. (*: Significant difference, $P < 0.05$).

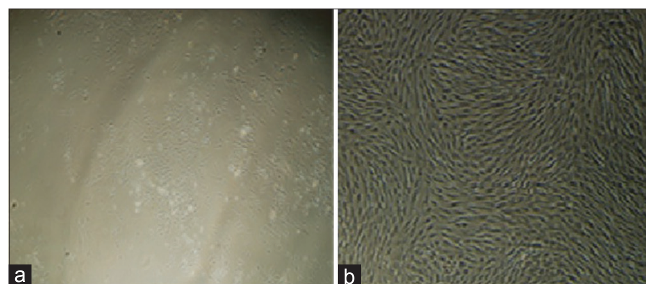
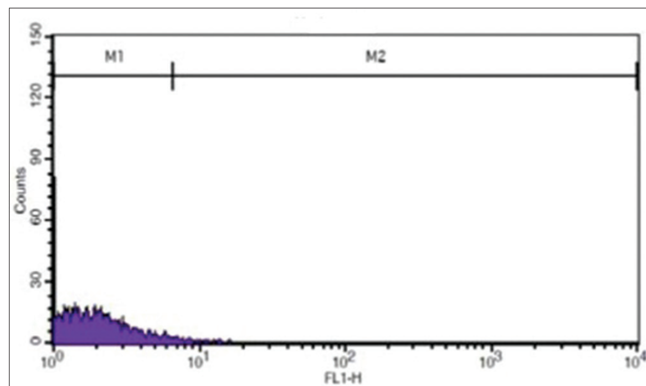
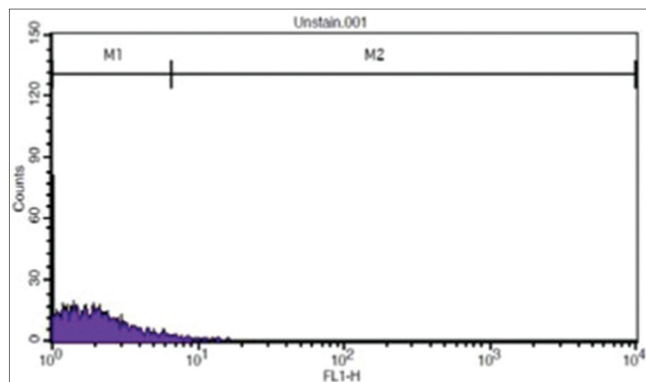


Figure 1: Light microscopic images of NP cells cultured on tissue culture dish. NP cells have polygonal (a) and fibroblastic morphology (b) (×60)



Curve 1: Isotope control group of NP cells without added cytokeratin 18 antibody



Curve 2: Unstain group, NP cells without added cytokeratin 18 antibody that exposed with CD45 negative antibody of mouse

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MTT

The results of the MTT assay demonstrated that the cell viability in both scaffolds decreased between the third day and fourteenth day and this reduction was more significant in the fabricated chitosan–gelatin scaffold by the freeze drying method [Curve 5].

Curve 5: Comparison of the viability and proliferation of NP cells in the fabricated chitosan–gelatin scaffolds by freeze gelation and freeze drying methods (*: Significant difference between the third day and fourteenth day, $P < 0.05$).

DISCUSSION

In present study, the rate of proliferation and viability of NP cells of the human intervertebral disk were surveyed in fabricated chitosan–gelatin scaffolds by the freeze drying and freeze gelation methods.

A novel freeze gelation method saves time and energy, and is suitable for fabricating large-sized scaffolds. In other studies using the freeze drying method, small-sized porous scaffolds were usually prepared.

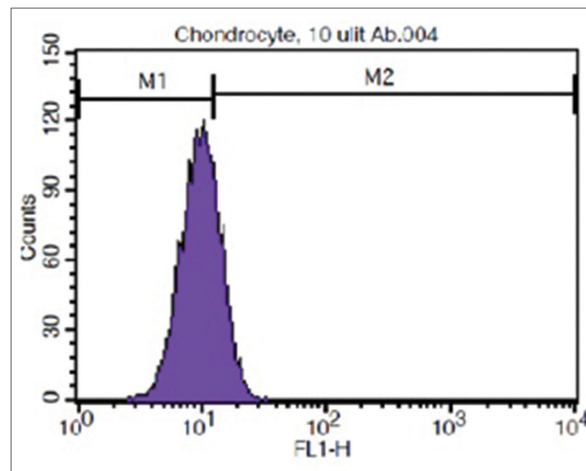
In large-sized studies, freeze drying and freeze gelation were used for the synthesis of scaffolds, especially for chitosan-gelatin scaffolds.

According to studies, the effects of chitosan and its composites, evaluated in the proliferation of NP cells and rate of the produced extracellular matrix (ECM), and their results, showed that this scaffold increased the proliferation of NP cells and production of ECM by these cells.

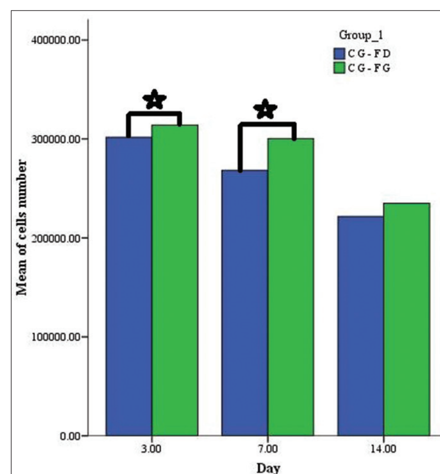
Gelatin is an element of the ECM that improves cell attachment.[17] Biocompatibility, biodegradability, and non-stimulation of the immune system are excellent properties of gelatin that can be used in the structure composition of scaffolds and synthesis and are ideal composites for tissue engineering.[24-26]

Results of trypan blue showed that the mean of the cell numbers for both scaffolds had a significant reduction and this was more significant in a fabricated chitosan–gelatin scaffold obtained by the freeze gelation method, in three and seven days.

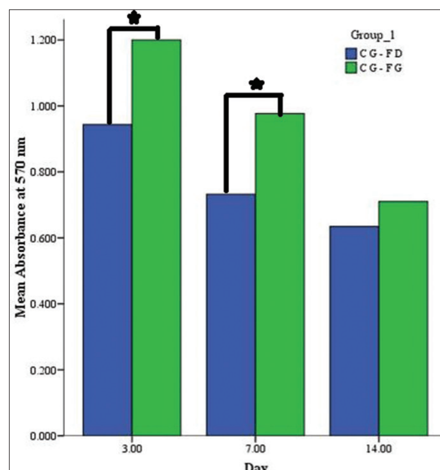
Ghorbani *et al.,* in a study, compared the rate of proliferation of NP cells and production of ECM in a fabricated chitosan-gelatin scaffold and an alginate scaffold by the freeze drying method and showed that the percent of live NP cells increased between the third and fourteenth days. The reported cause of this difference between the proliferation and production of ECM was



Curve 3: NP cells group with added CaK18 antibody: peak of curve showed that most of NP cells expressed CK18



Curve 4: Comparison percent of alive NP cells in fabricated chitosan-gelatin Scaffolds by freeze gelation and freeze drying methods. (*:significant difference ,$P<0.05$)



Curve 5: Comparison of viability and proliferation of NP cells in fabricated chitosan-gelatin Scaffolds by freeze gelation and freeze drying methods (*: Significant difference between 3 and 14 days. $P<0.05$)

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the toxic effect of glutaraldehyde, which was used as a cross-linker in the chitosan–gelatin scaffold that could release from scaffold structure to the medium. Hence the reported cause of decrease in the nutrient transport and oxygen was obstruction of the scaffold pores by production of ECM between days three and fourteen.[27]

Paradoxically, reports exist on the effects of using glutaraldehyde as a cross-linker. It seems that glutaraldehyde can be excreted from the fabricated chitosan–gelatin scaffold by the freeze drying method in a timely manner, which leads to degradation and destruction of the scaffold (change of the color of medium is proof of scaffold destruction). On the other side, this toxic substance causes cell death and decreases cellular proliferation. Of course, in some studies that have cultured bone marrow–derived stem cells on this scaffold, the glutaraldehyde (0.1%) has not affected cellular viability.[28]

Roughley *et al*., cultured NP cells on chitosan–genipin gel and illustrated that chitosan hydrogels could keep NP cell secretion of the ECM and lead to obturation of transport of the medium. Chitosan hydrogel also increased cellular proliferation.[23]

In hydrogels, the cells communicate more and are suitable for nutrition and oxygen transportation.[29]

Studies reported that the freeze gelation method saves time and energy, and is suitable for fabricating large-sized scaffolds.[28] The size of the pore scaffold is also more, and the nutrition, proliferation of cells, and secretion of ECM is better.[30-32]

Nitar *et al*. cultured fibroblast cells on the fabricated chitosan-collagen scaffold by freeze gelation and reported that this scaffold increased the growth and proliferation of cells, and also, the fabricated scaffold had a suitable mechanical strength. They expressed that freeze gelation was a novel method that could synthesize the composite scaffold with various-sized pores and excellent specifics.[28]

We concluded that the freeze-gelation process is a promising method for fabricating various chitosan-based composite biomaterials and the freeze gelation method for hydrogel scaffold fabricated scaffolds is a better method for the growth, proliferation, and viability of NP cells. Otherwise the freeze drying method can be used for hard and non-hydrogel scaffolds.

CONCLUSION

We fabricated porous chitosan–gelatin composite scaffolds by common methods freeze drying and freeze gelation. We concluded that the freeze-gelation process is a promising method for fabricating various chitosan-based composite biomaterials, especially for hydrogel structures such as NP tissues of the intervertebral disk.

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