Designing robust chitosan-based hydrogels for stem cell nesting under oxidative stress

Zahra Olfat Noubari*1, Asal Golchin1, Marziyeh Fathi1*2, Ailar Nakhlband1*2

1Research Center for Pharmaceutical Nanotechnology, Biomedicine Institute, Tabriz University of Medical Sciences, Tabriz, Iran
2Research Center of Psychiatry and Behavioral Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

Abstract

Introduction: Hydrogels are unique candidates for a wide range of biomedical applications including drug delivery and tissue engineering. The present investigation was designed to consider the impact of chitosan-based hydrogels as a scaffold on the proliferation of human bone marrow mesenchymal stem cells (hBM-MSCs) besides neutralization of oxidative stress in hBM-MSCs.

Methods: Chitosan (CS) and CS-gelatin hydrogels were fabricated through ionic crosslinking using β-glycerophosphate. The hBM-MSCs were cultured on the prepared matrices and their proliferation was evaluated using DAPI staining and MTT assay. Furthermore, the effect of hydrogels on oxidative stress was assessed by measuring the expression of NQO1, Nrf2, and HO-1 genes using real-time PCR.

Results: The developed hydrogels indicated a porous structure with high water content. The toxicity studies showed that the prepared hydrogels have a high biocompatibility/cytocompatibility. The expression of intracellular antioxidant genes was studied to ensure that stress is not imposed by the scaffold on the nested cells. The results showed that Nrf2 as a super transcription factor of antioxidant genes and its downstream antioxidant gene, NQO1 were downregulated. Unexpectedly, the upregulation of HO-1 was detected in the current study.

Conclusion: The prepared CS-based hydrogels with desired properties including porous structure, high swelling ability, and cytocompatibility did not show oxidative stress for the nesting of stem cells. Therefore, they could be attractive scaffolds to support stem cells for successful tissue engineering purposes.

Introduction

Mesenchymal stem cells (MSCs) have gained growing attention as an efficient therapeutic approach for various chronic diseases. These cells can be isolated from various sources such as bone marrow, skeletal muscle, adipose tissue, amniotic fluid, fetal blood, synovium, dental pulp, and umbilical cord.1 It is well documented that extracellular matrix (ECM) is vitally required to surround and support the cells structurally. Moreover, ECM plays an important role in regulating the cell function by providing biochemical and biomechanical signals.2 Evidence supports that the interaction with microenvironment affects the function of the injected stem cells in tissue engineering approaches.3 Engineered biomaterials provide a suitable platform to best mimic the microenvironment of the MSCs and to improve cell function and viability.3,4

Hydrogels are considered as one of the best candidates for supporting MSC growth and differentiation.3,5 It is well established that hydrogels are water-swollen networks that facilitate cell attachment, proliferation, and biomolecule transportation.6,9 Moreover, injectable hydrogels demonstrated unique properties for drug delivery and tissue engineering applications. They can encapsulate cells, or therapeutic substances including drugs, proteins, and even genes under a controlled manner. The most unique properties of hydrogels include hydrophilic and environment-friendly structure, ability to form a solid gel, and sensitivity to temperature and pH. Hydrogels are biocompatible biomaterials and have a similar structure to ECM with low protein absorption due to low surface tension. These properties make hydrogels efficient biomaterials for various medical applications including...
tissue engineering, controlled release of therapeutic agents (proteins, drugs, genes), contact lens fabrications, and wound healing.²⁰⁻²²

Chitosan (CS) as the most abundant biocompatible, biodegradable, and mucoadhesive biopolymer can be utilized for the preparation of thermosensitive injectable hydrogels using β-glycerophosphate (β-GP) as an ionic crosslinking agent in physiological conditions.¹⁶⁻¹⁸ The low mechanical strength of CS-based hydrogels has restricted their biomedical applications. The combination of CS with gelatin not only result in the improvement of mechanical properties of CS but also provides a suitable scaffold for cell survival in tissue engineering due to the similar structure to the collagen and mimicking the ECM.¹⁹

Regarding regenerative medicine, the damage of individual cells and host cells due to reactive oxygen species (ROS)-induced oxidative stress is a major obstacle to successful tissue engineering and regeneration. Decreased oxidative stress leads to MSCs’ promoted proliferation besides DNA stability.²⁰⁻²¹ Despite high contents of glutathione and an active antioxidant system in MSCs, they endure ROS production as they replicate. Elevated levels of ROS lead to a decreased cell differentiation though in adipose tissue, it is the opposite.²² Therefore, we aimed to design a CS/gelatin hydrogel system for promoting the therapeutic potential of stem cells with a focus on overcoming oxidative stress.

Materials and Methods

Materials
CS (medium molecular weight with a deacetylation degree of (75%–85%), gelatin (porcine skin), β-glycerophosphate (GP) disodium salt hydrate, and methyl thiazolyl tetrazolium (MTT) reagent were provided from Sigma-Aldrich Corp. (St. Louis, MO, United States). MSCs were obtained from the National Institute of Genetic Resources (Tehran, Iran). Cell culture flasks and plates and all disposable equipments were bought from IWAKI (CITY, Japan). Low Glucose DMEM medium, fetal bovine serum (FBS), and trypsin-EDTA (0.02–0.05%) were bought from Gibco (Paisley, UK). Ethanol, HCL, chloroform, and isopropanol were purchased from Merck (Kenilworth, United States). Complementary DNA synthesis kit was bought from Fermentas (Waltham, Canada). All the primers were provided by MWG Biotech (Ebersberg, Germany).

Preparation of temperature-sensitive CS and CS/gelatin hydrogels
To fabricate hydrogels, 0.2 g of CS powder with a medium molecular weight was autoclaved at 121°C for 20 minutes. Afterward, 0.1M hydrochloric acid was filtered by a 0.22 μm syringe filter and added to the sterile CS powder to dissolve it. Then 1 g of GP was dissolved in distilled water. The GP solution was filtered using a 0.22 μm syringe filter. For the preparation of CS hydrogel (A), the GP solution was added dropwise to the CS solution and mixed in an ice bath to prevent the gelation. After mixing for 15 minutes, the pH of each mixture was evaluated and set to be 7.4. The obtained solutions were placed in an incubator of 5% CO₂ and 37°C for hydrogel formation. Moreover, for the preparation of CS-gelatin hydrogels (B, C), the sterile gelatin solution (4% w/v) was prepared using autoclaved gelatin powder with sterile distilled water by mixing 0.4 g gelatin in 10 mL distilled water.²² Hydrogels B and C were prepared in the same way by adding gelatin to the CS solution. Table 1 indicates the composition of the prepared hydrogels.

Structural analysis by FT-IR and morphology study of hydrogels using scanning electron microscopy
The chemical structure of the hydrogels was studied through Fourier transform infrared (FT-IR) spectroscopy. KBr tablets were used for FT-IR analysis. Scanning electron microscopy (SEM, Zeiss DSM-960A, Germany) was used to investigate the morphology and structural features of the hydrogels.

Investigation of swelling behavior of hydrogels
To calculate the swelling ratio of hydrogels, the hydrogel samples were freeze-dried and incubated at 37°C in phosphate-buffered saline (PBS) solution and their weight was measured at different time intervals. The swelling percentage was calculated by the following formula (1):

\[
\text{Swelling ratio} \% = \left( \frac{W_t - W_0}{W_0} \right) \times 100
\]

Where \(W_t\) is the weight of dried gel and \(W_i\) is the weight of the swollen hydrogel.

Ex Vivo red blood cell hemolysis assay
Ex vivo red blood cell hemolysis assay was performed to consider the hemolytic potential of the hydrogel in human whole blood. To evaluate the effect of hydrogels on hemolysis, 0.5 mL of hydrogels were added to the tubes. Afterward, 0.5 mL of blood was poured into each falcon plus 3 mL of PBS and incubated at 37°C for one hour with shaking. Afterward, the tubes were subjected to centrifugation at 160 g for 12 minutes. After the separation of the supernatant, its light absorption percentage was measured by a spectrophotometer at 545 nm. PBS was employed as the negative control (0% lysis) and distilled
water was used as the positive control (100% lysis). The percentage of hemolysis was calculated by the following formula (2):

\[
\text{Hemolysis (\%)} = \frac{(OD_{\text{sample}} - OD_{\text{negative}})}{(OD_{\text{positive}} - OD_{\text{negative}})} \times 100
\]

**Biocompatibility and cell toxicity assay**

To assess the cytotoxicity of hydrogels, an MTT assay was implemented according to the literature. hBM-MSCs were cultured and incubated in a humidified incubator with 95% air and 5% CO₂ at 37°C. The cell culture medium was composed of low glucose DMEM supplemented with 10% (v/v) fetal bovine serum and antibiotics (penicillin G (100 U/mL) and streptomycin (100 µg/mL)). Cytotoxicity of CS-based hydrogels on MSCs was evaluated using the MTT assay via extraction based on ISO 10993-5 guidelines. To this end, hydrogels' solutions were developed as mentioned above and added to 6-well culture plates (1 mL/well) and incubated at 37°C to form the gels. Then the complete medium (5 mL/well) was added to the formed hydrogels and incubated at 37°C for 24 hours. The obtained leachates were stirred through filtering with a 0.22 µm syringe filter. To perform the MTT test, MSCs were cultured in 96-well plates at their exponential phase. After 24 hours of cultivation, the cells were exposed to the whole extracted solution of the hydrogel. A normal complete culture medium was added to provide positive control. To prepare the leachates, 5 mL complete medium was added to 1 mL of the formed hydrogels for 24 hours at 37°C. They were filtrated through a 0.22 µm syringe filter.

After the incubation period for 24 and 48 hours, the culture medium was replaced with a solution containing 50 µL MTT solution (2 mg/mL in PBS) and 150 µL of fresh medium. After 4 hours of incubation at 37°C, the MTT solution was removed and substituted with 200 µL of DMSO and 25 µL of Sorenson buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). Through this procedure, metabolically active cells produced formazan crystals which were dissolved in DMSO and the absorbance was measured at 570 nm via a spectrophotometric plate reader, ELx 800 (Biotek, CA, USA).

**DAPI staining for detection of apoptotic cells**

The DAPI staining was conducted according to a previously published report to detect the possible incidence of nucleus condensation in cells injected into hydrogels. Briefly, 48 hours after injection of treated cells into the hydrogels, the cells were fixed with the freshly prepared ice-cold paraformaldehyde (4%) and exposed to 0.1% Triton X-100 in PBS for 5 minutes and permeabilized. Subsequently, cells were stained with DAPI (1 µg/mL in PBS) for 5 minutes in the dark. After removal of surplus stain, the cells were washed (3×) using 0.1% Triton X-100 in PBS. The image acquisition was achieved by Olympus IX81 inverted fluorescence microscope equipped with an Olympus DP70 camera.

**Real-time PCR studies**

Hydrogels' impact on oxidative stress in hBM-MSCs was studied via a quantitative iQ5 real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, USA). To this end, the expression levels of oxidative stress-related genes including nuclear factor erythroid 2–related factor 2 (Nrf2), heme oxygenase 1 (HO-1), NAD(P)H (Nicotinamide Adenine Dinucleotide plus Hydrogen), and quinone oxidoreductase 1 (NQO1) were investigated. Total RNAs from the control and hBM-MCSs cultured on the hydrogel were isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) based on the company’s protocol. The integrity of the isolated RNA was assessed by agarose gel electrophoresis while the purity and concentration of RNAs were tested by optical density measurement (A260/A280 ratio) with a nanodrop device (ND 1000, USA). Subsequently, 1 µg of the isolated RNA sample was used to develop complementary DNA (cDNA). Primers were designed using published gene bank sequences via Oligo 7.56 (Molecular Biology Insights, Inc., USA) as shown in Table 2. Each reaction composition included 1 µL cDNA, 1 µL primer (100 nM each primer), 12.5 µL 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA), and 10.5 µL RNase/DNase free water. Thermal cycling settings were as follows: 1 cycle at 94°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60–63°C for 30 seconds, and 72°C for 25 seconds. Results were interpreted by the Pfaff method and the CT values were normalized to the expression proportion of β-actin as a housekeeping gene. All reactions were implemented in triplicate and each assay included negative control.

**Table 2. The sequences of the used primers**

| Gene     | Sequence                        | Size (bp) | Tm (°C) | Ref. |
|----------|---------------------------------|-----------|---------|------|
| Nrf2     | Forward GAGACAGGTGAAATCTCCCAAT  | 120       | 54      | 20   |
|          | Reverse TTTGGGAAATGGGGCAAC       |           |         |      |
| HO-1     | Forward ACGGCTTCAGGCTGGTGATG    | 118       | 59      | 20   |
|          | Reverse TGAGCCTTCTTGGGGAAGTAG    |           |         |      |
| NQO1     | Forward ATGTATGACAAGGACCTTCC    | 124       | 60      | 20   |
|          | Reverse TCCCTTCGAGAGACATAGG     |           |         |      |

**Statistical analysis**

One-way analysis of variance (ANOVA) followed by a
Tukey post hoc test was performed to study the average differences between data (SPSS 13.0 software; SPSS Inc., Chicago, IL, USA). All results were presented as mean ± SD. A P value less than 0.05 was defined as statistically significant.

**Results**

**Evaluation of hydrogel structure**

In the FT-IR spectra of hydrogels (Fig. 1), CS-related functional groups exist at different wavelengths. The broad peak in the spectrum of hydrogel (A) exists at 1070 cm⁻¹ that is related to the tensile vibrations of C-O. The peaks in 1642 cm⁻¹ and 1529 cm⁻¹ are related to the tensile vibration of the CS amide group and bending vibration of the CS amine group, respectively. The N-H CS bending vibration of the amide II has appeared at 1461 cm⁻¹. The broad peaks of the hydroxyl and CS amine groups can be seen at 3000-3500 cm⁻¹. The peak in 972 cm⁻¹ belongs to the GP group. There was no obvious difference in FT-IR spectra between hydrogel sols and gels (Fig. 1). The spectra of hydrogels B and C, which contain gelatin are similar to the spectrum of hydrogel A due to the overlapping of their functional group peaks and they only indicated some wider peaks compared to hydrogel A.

The microstructure of hydrogels A, B, C was evaluated by SEM (Fig. 2). According to the SEM images, hydrogels indicated interconnected porous structures with different sizes. The porous structure of the hydrogels allows the high permeability of the hydrogels, resulting in the loading of drugs and cells into the hydrogel matrix, making them suitable for drug release and tissue engineering. Gelatin affected the microstructure of hydrogels and hydrogel with the looser structure was obtained with gelatin incorporation.

The swelling property of hydrogel has an important effect on their biomedical applications. The swelling ratio of hydrogels was evaluated by soaking them in PBS solution (Fig. 3). Hydrogels B and C showed a more swelling ratio compared to hydrogel A which indicated that gelatin causes more water uptake in the hydrogel matrix.

A hemolysis test was performed after three hours of incubation. The supernatants were clear compared to the negative control. In comparison to the control group, the calculation of adsorption percentage did not show any significant difference in hydrogel samples (Fig. 4).

**Cell biocompatibility of the scaffold**

Toxicity of hydrogels and hBM-MSCs viability were assessed via MTT colorimetric assay (Fig. 5). No significant toxicity was observed on mesenchymal cells injected in type A, B, and C hydrogels after 24 hours. It seems that the eluted materials from hydrogel are interfering with absorption, hence the chart had an ascending trend. Based on the cytotoxicity evaluation, the results presented that no significant in vitro toxicity was observed compared with untreated cells (P<0.001). However, it seems that gelatin in hydrogel formulation may result in some toxicity which can be attributed to more release of free β-GP molecules.

**Fig. 2.** SEM images of the fabricated hydrogels (A, B, and C). (A) Hydrogel A: 1 mL Chitosan 2% + 200 μL Glycerophosphate 40%; (B) hydrogel B: 600 μL Chitosan 2% + 200 μL Glycerophosphate 40% + 400 μL Gelatin 4%; (C) Hydrogel C: 800 μL Chitosan 2% + 200 μL Glycerophosphate 40% + 200 μL Gelatin 4%.

**Fig. 3.** Swelling behavior of hydrogels (A, B, and C) at different time intervals.
due to the less crosslinked network. Therefore hydrogels A would be selected to be the best.

**DAPI staining**

The viability of injected cells in the hydrogel was assessed with DAPI staining. The results demonstrated that cells injected to hydrogels did not show any morphological changes compared to the control cells (Fig. 6). Moreover, no apoptosis and nuclear fragmentation were observed in the cells cultured on the scaffold compared to the control samples. However, not much growth was observed in the cells injected in the hydrogel, but the viability of the cells in hydrogel A was observed and confirmed.

**Intracellular antioxidants gene expression**

The effect of hydrogel on intracellular oxidative stress was determined by examining the mRNA expression of some key intracellular antioxidants enzymes. Nrf2 is a transcription activator that attaches to the antioxidant response elements in the promoter regions of target antioxidant genes and is critical for the harmonized up-regulation of genes in response to oxidative stress. As shown in Fig. 7, a decreased profile level of Nrf2 was detected in the cells nested at the CS scaffold ($P=0.003$). This finding indicates that the designed hydrogel does not induce oxidative stress on the established cells. It was expected that Nrf2 downstream signaling genes including HO-1 and NQO1 be down-regulated, but surprisingly HO-1 upregulation was observed in nested cells.

**Discussion**

Applying hydrogels for therapeutic applications began in the 1960s when Lim and Wichterle synthesized the poly(hydroxyethyl methacrylate) bridge. As water is the largest component of the human body and a hydrogel can have a high water absorption, the use of hydrogels has been potentially considered in biomedicine in areas such as tissue engineering, drug delivery, cell therapy, and anesthetic delivery. Hydrogels, compared to other types of biological materials, have the advantages of increasing biological compatibility, biodegradability, adjustability, good mechanical strength, and porous structure. However, due to the low mechanical strength and brittle nature of hydrogels, the possibility of using hydrogels is still limited; thus, the studies which are concerned with their effective types including CS-based hydrogels are still in progress only due to the high biocompatibility of such gels. Stem cells such as MSCs are regarded as useful cells
Owing to their potential immunomodulatory properties in alleviating diseases caused by inflammatory responses of immune cells in some diseases such as type 1 diabetes, rheumatoid arthritis, and multiple sclerosis.\(^{13-36}\) 

CS and CS/gelatin hydrogels were synthesized and characterized in the current study.

In the FT-IR spectra (Fig. 1), the characteristic peaks of CS, gelatin, and β-GP confirmed the fabrication of hydrogel. According to the SEM images (Fig. 2), all hydrogels have a porous structure, however, the addition of gelatin caused the increase of pores size. It is assumed that the presence of gelatin (Figs. 2B and C) caused the decrease of electrostatic reaction of CS functional groups in the presence of β-GP as an ionic crosslinking agent that resulted in the larger pores. This was confirmed via swelling data (Fig. 3), as the hydrogels B and C have a relatively higher swelling ratio compared to hydrogel A.

The cytotoxicity assays via hemolysis (Fig. 4) and MTT (Fig. 5) demonstrated the compatibility of the developed hydrogels. CS/gelatin and GP as non-toxic materials are accepted for biomedical applications that resulted in nontoxic hydrogels.

In the present study, the effect of the CS-based hydrogels on the proliferation and redox state of hBM-MSCs was evaluated. Nrf2 gene was studied to identify the pathway of resistance to oxidative stress caused by the hydrogel. The results showed that there was no significant increase in Nrf2 gene expression. To recognize the exact pathway of resistance to oxidative stress by stem cells, another gene called NQO-1, antioxidant genes involved in the cellular oxidative stress pathway, was studied. It can be interpreted that culturing cells in hydrogels reduces oxidative stress-induced gene expression. Since the NQO-1 gene possesses a key role in the pathway of cellular oxidative stress, therefore we can demand that hydrogels have a protective effect on stem cells against oxidative stress. Another gene that was examined in this study was HO-1. Culturing cells in hydrogels reduces the HO-1 gene expression level. Because of the importance of the HO-1 gene in the pathway of cellular oxidative stress,\(^{29,30}\) it can be concluded that hydrogel has a protective effect on stem cells against oxidative stress.

In a study carried out by Tang et al., it was concluded that one of the causes of cell death was oxidative stress that was induced by apoptosis and caspases. By inhibiting caspase, catalase degradation continued and ROS increased which led to cell death. They found that this cell death was caused by autophagy.\(^{23}\)

The results of the recent study showed that Nrf2 has been developed as a major regulator of oxidant resistance and has been used in a range of toxicities and chronic diseases associated with oxidative stress. Nrf2 is activated by the evolutionarily conserved Keap1-dependent mechanism of depression signaling.\(^{14}\) While Nrf2 is inhibited under basal conditions by protease cleavage and keap1-controlled ubiquitination, it is activated by oxidants and ultrafilters by modulating the cysteine thiols of Keap1 and Nrf2. Activated Nrf2 mediates the expression of the induction of each of the signaling enzymes and proteins for metabolic regulation and antioxidant defense and oxidant signaling; therefore, it affects the physiology and pathobiology of the oxidant.\(^{14}\) Nrf2 participates in the control of several programming roles, such as inhibitory signaling autophagy, apoptosis, mitochondrial biogenesis, and stem cell regulation. This is mainly done by regulating oxidant levels and oxidant signaling.\(^{22}\)

The present study showed that culturing cells in hydrogels increases the expression of the HO-1 gene. According to the literature, upregulation of HO-1 expression levels and elevated activity of HO-1 are needed for MSCs growing and differentiation.\(^{27}\) In addition, MSCs increase the expression of HO-1 due to their anti-inflammatory activity.\(^{28}\) Therefore, MSCs may always need high levels of HO-1 naturally for normal activity, and the upregulated profile is not related to the stressed condition.

**Conclusion**

The CS and CS/gelatin hydrogels were fabricated and characterized as a scaffold for the proliferation of hBM-MSCs. The prepared hydrogels indicated the porous structure with the high swelling ability and cytocompatibility properties. The results of gene expression evaluation in MSCs nested on prepared hydrogel indicated that the Nrf2 and its downstream antioxidant gene, NQO1 were downregulated which confirms no induction caused by oxidative stress on the established cells. On the other hand, the upregulation of HO-1 was detected as well which may be due to the natural need of MSCs for HO-1 activity to growth and differentiation.

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Research Highlights

What is the current knowledge?
✓ The CS and CS/gelatin hydrogels have been fabricated and evaluated as a scaffold for the proliferation of hBM-MSCs.
✓ Oxidative stress is a major issue in successful tissue engineering.

What is new here?
✓ The effect of prepared hydrogels on oxidative stress was assessed by investigating the expression of NQO1, Nrf2, and HO-1 genes using real-time PCR.
✓ The cellular and molecular evaluation demonstrated no oxidative stress induced by the prepared CS-based hydrogels.
✓ The CS-based hydrogel could be considered as an attractive candidate for supporting stem cells for successful cell therapies.

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Ethical statement
There is none to be disclosed.

Competing interests
The authors declare that there is no conflict of interests.

Authors’ contribution
This work was carried out in collaboration with all authors. MF and AN designed the study. ZON did the experiments, collected the data, analyzed the data, and wrote the manuscript. AG collaborated in cellular experiments. MF and AN edited the manuscript. All authors read and approved the final manuscript.

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