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Design, Fabrication and *In-Vitro* Characterization of Injectable Chitosan/Polyvinyl Alcohol Hydrogel Scaffold for Heart Tissue Engineering

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Abstract

Background and objectives: The principal problem in the treating heart damages is the restricted ability of the heart to restore. Injectable hydrogels offer considerable potential for repairing cardiac tissue. According to the features of chitosan and polyvinyl alcohol (PVA) in the cardiac repair, preparing a hydrogel that imitates the extracellular matrix (ECM) of the heart and is a new treatment for heart patients, especially heart infarction, the purpose of the current study is to design, fabrication and *in-vitro* specification of injectable chitosan/PVA scaffold for heart tissue engineering.

Materials and methods: The prepared hydrogel consisted of 2% natural chitosan and 5% synthetic PVA. The characteristics of the prepared hydrogel were assessed by experiments of gelation time, pH determination, microbial culture, biodegradability, swelling ratio, toxicity and microscopic evaluation of three-dimensional culture.

Results: The evaluation of chitosan/PVA hydrogel experiments displayed that the gelation time was 7.66 ± 1.69 minutes and the pH measurement was 7.03 ± 0.12. The bacteria did not grow in the microbial culture. The degradability of hydrogel occurred after one week of immersion in PBS, which indicated its biocompatibility. The highest swelling was observed at 3 hours after immersion in PBS and then, the hydrogel started to lose water and shrank. The hydrogel was not toxic to adipose-derived mesenchymal stem cells (ADMSCs) and created a suitable three-dimensional structure for the growth and proliferation of ADMSCs and the cells maintained their attached and morphology.

Conclusion: We demonstrated the development of biocompatible and injectable chitosan/PVA hydrogel scaffold with high cellular compatibility, which along with other evaluations can use as a biomaterial in cardiac tissue engineering.

Keywords: Chitosan; Polyvinyl alcohol; Hydrogel scaffold; Injectable; Heart tissue engineering.

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1. Introduction

Cardiovascular diseases, especially myocardial infarction (MI), are the most common reasons of human death in different countries. Therefore, it is very important to

study its pathological process and its treatment methods (1). During the past decades, major improvements have been achieved in management of patients with MI (2). Existing treatments face many limitations. A part of the heart muscle that has suffered ischemia cannot be replaced with common treatment methods such as opening the vessels through angiography or coronary artery bypass and using Anticoagulant (3). The principle problem in the treating heart damages is the restricted ability of the heart to restore (4). Therefore, more attention should be paid to new therapies to regenerate or substitute the damaged tissue of the heart (5).

Injectable hydrogels possess strong potential for cardiac repair and regeneration after MI. They can be used for cells, biomolecules or drugs delivery to induce a special reaction, or for the formation of functional heart tissue structures in the damaged tissue of the heart (6,7). Recently, several hydrogels have been assessed in the regeneration and restoration of the heart (8). Hydrogels are three-dimensional materials enriched with water (about 99% water) that are connected by cross-linked and hydrophilic polymer chains and form a network. They are used as scaffolds that mimic the ECM and are used in various tissue engineering applications. Hydrogels are usually made of natural or synthetic polymer chains (9). The production of a suitable scaffold with biocompatibility and good mechanical properties that can simulate the natural microenvironment for cells is one of the key principles in tissue engineering (10).

Chitosan hydrogel, a temperature-sensitive hydrogel, is based on a natural polymer prepared through N-deacetylation of chitin and due to its structural resemblance to natural glycosaminoglycans is determined with low toxicity and high biocompatibility. Moreover, due to high crystallinity, it causes its solubility in acidic medium, which makes the use of chitosan very efficient (11).

Today, research on natural and synthetic scaffolds for use in cardiac injuries has expanded greatly. One of the drawbacks of these scaffolds is their lack of electrical conductivity, which causes cell-cell communication and heart coordination to decrease (12). To solve this problem, synthetic polyvinyl alcohol (PVA) can be used to strengthen mechanical properties and especially electrical conductivity.

PVA is manufactured via the polyvinyl acetate hydrolysis. Polyvinyl alcohol can bind to several biological molecules. Considering the elastic property of PVA and its role in improvement of mechanical signal transmission, it can be used as a matrix. The adhesive features of PVA are low because it is a neutral hydrogel. PVA hydrogel has low wear coefficient and robust mechanical features. These great features of PVA have hopeful applications in tissue engineering and regenerative medicine, particularly the repair of heart tissue damage (13,14).

Therefore, based on the characteristics and role of chitosan and PVA in the repair of heart tissue damage, obtaining a hydrogel with optimal properties that can imitate the ECM of the heart and in the near future can be a potential and new treatment for heart patients, especially heart infarction, the purpose of the current research was to design, fabrication and *in-vitro* characterization of injectable chitosan/polyvinyl alcohol hydrogel scaffold for heart tissue engineering.

2. Materials and methods

The used materials in this study are: chitosan powder (Sigma-Aldrich (CAS No. 9012-76-4)), polyvinyl alcohol lyophilized powder (MP Biomedicals (CAS No. 9002-89-5)), and beta-glycerol phosphate (βGP) (Sigma-Aldrich (CAS No. 154804-51-0)).

The prepared hydrogel scaffold in this study was a hydrogel based on 2% natural chitosan polymer, to which 5% synthetic PVA was added to strengthen the mechanical properties and especially electrical conductivity. First, all the materials needed to make hydrogel were sterilized by UV chamber and then chitosan, PVA, and finally the desired hydrogel were prepared as follows.

2.1. Chitosan synthesis

To make 2% chitosan, 0.2 gram of chitosan powder was mixed with 0.1 M acetic acid, and sterile distilled water and the resulting mixture was placed on the magnetic stirrer at 50°C till the chitosan powder was fully dissolved.

2.2. PVA synthesis

To prepare PVA 5%, 0.5 gram of polyvinyl alcohol lyophilized powder was combined with sterile distilled water and in order to dissolve this compound, it was placed on the magnetic stirrer at 100°C. The container opening was covered by parafilm to prevent vaporization of solution and the powder was permitted to completely dissolve in sterile distilled water.

2.3. Injectable hydrogel synthesis

To prepare hydrogel, chitosan 2% and PVA 5% were mixed together with a volume ratio of 80/20 on a magnetic stirrer for a few minutes. Then, for chemical crosslinking (for the preparation of thermosensitive hydrogel) to next to ice and at a temperature of 4°C, βGP 50% (100 µl to maintain the injectability of the hydrogel) was added drop by drop to the solution. Finally, the characteristics of the prepared hydrogel were assessed.

2.4. *In-vitro* assessments of injectable chitosan/PVA hydrogel scaffold

2.4.1. Gelation time

A tube test was used to check the gelation time. The prepared hydrogel was placed in a tube inside the incubator at 37°C and removed from the incubator every two minutes and its gelation time was checked. The evaluation was done by turning the tube upside down and observing the movement of the gel. This test was achieved in three replicates.

2.4.2. pH determination

The pH value was measured by pH meter for three replicates.

2.4.3. Microbial culture

After preparing the hydrogel, it was cultured in blood agar, MacConkey agar, and Sabouraud dextrose agar media and the results were checked after 72 hours.

2.4.4. Biodegradability test

First, the weight of the dry hydrogel (W0) was measured. Then, to evaluate the degradability of the hydrogel, amount of the hydrogel was immersed in PBS (pH = 7.4) and was placed in an incubator with 37°C temperature. The hydrogel was then dried and weighed (Wt) at days 1, 3, 5, 7, 10, 14, and 21 and the changes were recorded and their amount were compared with the amount at day zero (W0). This test was achieved in three replicates and the percentage of degradation was determined according to the following formula:

$$\text{Degradation (\%)} = (W0 - Wt) / W0 \times 100$$

W0: the dry hydrogel weight (before immersion in PBS)
Wt: the wet hydrogel weight (after immersion in PBS)

2.4.5. Swelling ratio

This test was measured by immersing the hydrogel in PBS (pH = 7.4) and incubating at 37°C. For this purpose, the weight of the dry hydrogel (W_{dry}) was first measured. After immersing the hydrogel in PBS, the changes in hydrogel weight (W_{wet}) were recorded during one week and at times of 3, 6, 24, 72, 120 and 168 hours. This test was achieved in three replicates and the swelling ratio was determined according to the following formula:

$$\text{Swelling ratio (\%)} = (W_{wet} - W_{dry}) / W_{dry} \times 100$$

W_{dry}: the dry hydrogel weight before immersion in PBS
W_{wet}: the wet hydrogel weight after immersion in PBS

2.4.6. Fourier Transform Infrared Spectroscopy (FTIR)

The Chitosan, βGP, PVA and hydrogel were analyzed using a Nicolet Nexus 470 Fourier Transform Infrared Spectrophotometer. The FTIR spectrum was collected in the 4000-400 cm^{-1} range with 4 cm^2 resolution averaging 120 scans.

2.4.7. Toxicity test

Alamar blue test was used to evaluate the toxicity of hydrogel for cells (cell viability). The adipose-derived mesenchymal stem cells (ADMSCs) in the third passage were used to perform the test. At first, a suspension containing ADMSCs and DMEM culture medium with 10% FBS was prepared. 10^4 cells per well were seeded in the 96-well plate with a complete culture medium and the plate was located in the incubator at 37°C for 24 hours. After the cells sticked to the plate bottom (the confluence of about 80%), the hydrogel was poured into each test well. After 24, 72, 120 and 168 hours, the culture medium was removed and Alamar blue dye was poured into each test well and located in the incubator at 37°C for 4 hours. Then, the rate of fluorescent absorption was measured with a spectrophotometer at a wavelength of 530 to 590 nm. Each time was experimented in triplicate and finally the cell viability was calculated at different times according to the following formula:

$$\text{Cell viability (\%)} = (\text{Control group} / \text{Hydrogel group}) \times 100$$

2.4.8. Microscopic evaluation of three-dimensional culture

In this method, three-dimensional cultivation of ADMSCs was done in hydrogel and the morphology of ADMSCs in the hydrogel was evaluated. For this purpose, the cells were first seeded in the 96-well plate with a complete culture medium. After 24 hours' incubation when the cells sticked to the plate bottom, the hydrogel was poured into each test well. The morphology of cells was checked

by inverted microscope in the control and hydrogel groups after 24, 72, 120 and 168 hours.

2.4.9. Statistical analysis

The software of SPSS version 26 (IBM) was utilized for the statistical evaluation of the results. After checking the normality of the data with the Kolmogorov-Smirnov test, independent-samples T-Test and repeated measurement were utilized for compare the data. The data with $p \leq 0.05$ were estimated significant.

3. Results

3.1. Gelation time and pH

The gelation time chitosan/PVA hydrogel was check via the tube test for a few minutes. The produced hydrogel was injectable in terms of the degree of gelation. The gelation time was 7.66 ± 1.69 minutes (Figure 1). Also, the pH measurement was in the neutral range and 7.03 ± 0.12, which showed a suitable feature for injection.

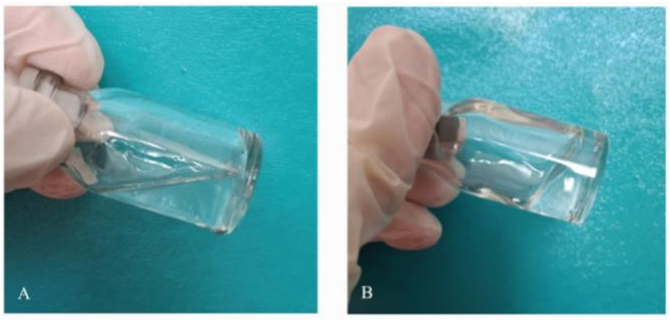

Figure 1. A tube test to check the gelation time of chitosan/PVA hydrogel at temperatures of 25°C (A) and 37°C (B).

3.2. Microbial culture

The results of microbial culture indicated the bacteria did not grow in any of the media after 72 hours. This confirmed the observance of sterile conditions and proper sterilization of materials during the production of hydrogel, which removed the concern of contamination for direct injection into the tissue.

3.3. Biodegradability test

The degradability degree of chitosan/PVA hydrogel was assessed through immersion in PBS for duration of three weeks. The data showed that there was no noticeable change in the weight of the hydrogel at different times, statistically. But after a week, the hydrogel started to degrade, which the highest degradation of the hydrogel occurred on day 21 (Figure 2). This test displayed that the hydrogel is biocompatible.

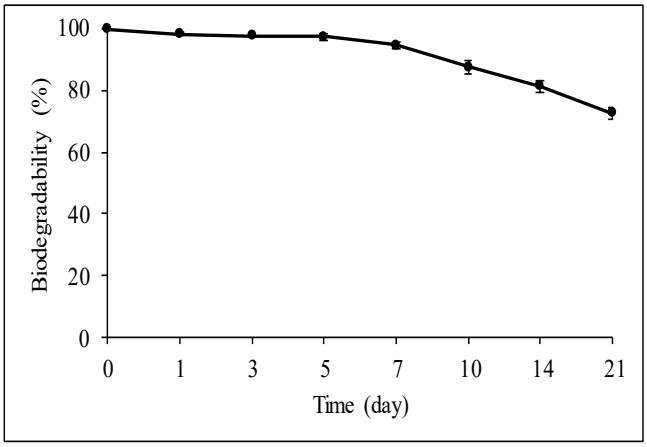

Figure 2. The biodegradability of chitosan/PVA hydrogel through immersion in PBS and an incubation with 95% O2, 5% CO2 and 37°C temperature for a duration of three weeks.

3.4. Swelling ratio

The swelling ratio of chitosan/PVA hydrogel was measured through immersion in PBS for duration of one week. According to the results (Figure 3), the highest swelling was observed in 3 hours and then, the hydrogel started to lose water and shrank. But statistical analysis did not reveal any significant difference between different times of hydrogel measurement. Therefore, through this test, the amount of hydrogel that does not cause pressure necrosis in the heart tissue was determined for injection.

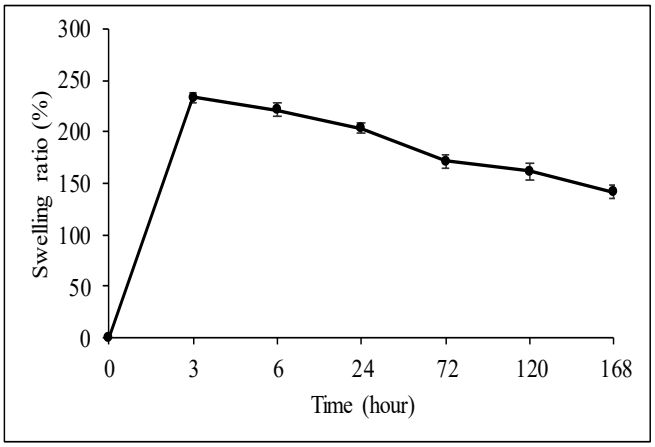

Figure 3. The swelling ratio of chitosan/PVA hydrogel through immersion in PBS and an incubation with 95% O2, 5% CO2 and 37°C temperature for a duration of one week.

3.5. FTIR analysis

As shown in Figure 4, the chemical structure of the samples was analyzed by FTIR characteristic peaks for chitosan, βGP, PVA, and Hydrogel, and the presence of chemical interactions was confirmed in the hydrogel. The hydrogel spectra showed the peak at 3374 cm^{-1} assigned to the combined vibration of OH and NH. OH bending is observed at 1641 cm^{-1}, CH3 bending at 1417 cm^{-1}, and CH bending band at 753 cm^{-1}. All of the peaks are present in chitosan and PVA spectra. The absorption band at 2089 cm^{-1} signifies, which shows that the ammonium groups are crosslinked with βGP.

The characteristic peaks in FTIR spectra of chitosan are given as follows:

The peak at 3432 cm^{-1} is due to the stretching of OH and NH, and the peak at 2974 cm^{-1} is attributed to the stretching of CH. The stretching of CO2, caused by absorption during tableting, is observed in 2361. The stretching of C=O is observed in 1634 cm^{-1} and the bending of CH3 is observed in 1385 cm^{-1}. C-O stretching is observed at 1085 $cm^{-1}$and CH bending vibration at 668 cm^{-1}.

The spectrum of βGP showed characteristic peaks at 3260 cm^{-1} of the hydroxyl stretching, 1670 cm^{-1} of the OH bending, and 782 cm^{-1} of the CH bending. In the PVA spectrum, the characteristic peaks were observed for hydroxyl stretching at 3430 cm^{-1}, CH stretching at 2936 cm^{-1}, Carbonyl band at 1735 cm^{-1}, a peak at 1634 cm^{-1} associated with OH bending, a peak at 1383 cm^{-1} related to CH3, a peak at 1090 cm^{-1} attributed to a C-O group, and CH band bending at 607 peaks.

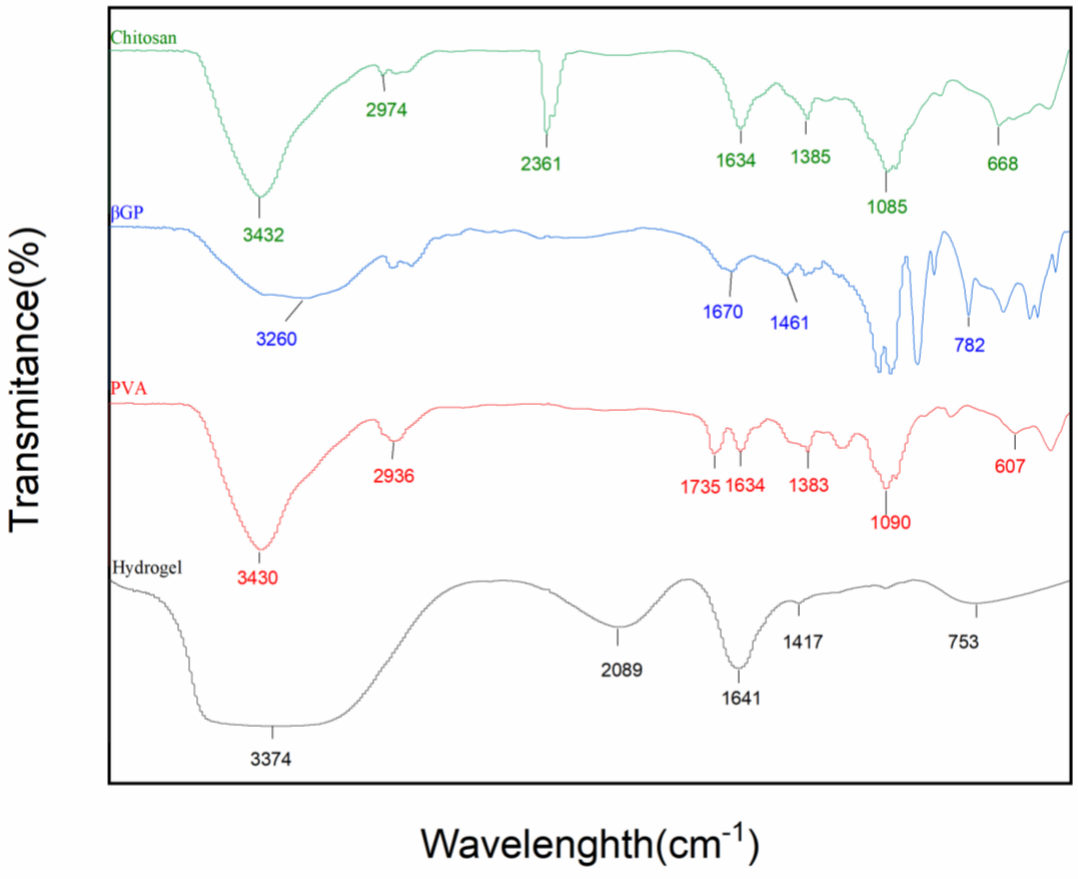

Figure 4. FTIR spectra of chitosan, βGP, PVA and hydrogel in the 4000-400 cm^{-1} range.

3.6. Toxicity test

The toxicity of chitosan/PVA hydrogel for ADMSCs(cell viability) was evaluated by Alamar blue test for a duration of one week. The data revealed that the population of ADMSCs in the group of hydrogel compared to the group of control decreased at time of 24 hours. But the population of ADMSCs increased at times of 72, 120 and 168 hours (Figure 5) and no significant statistical difference was observed between the two groups ($p > 0.05$), which demonstrates the chitosan/PVA hydrogel created a suitable three-dimensional structure for the

growth and proliferation of ADMSCs and the chitosan/PVA hydrogel was not toxic to ADMSCs.

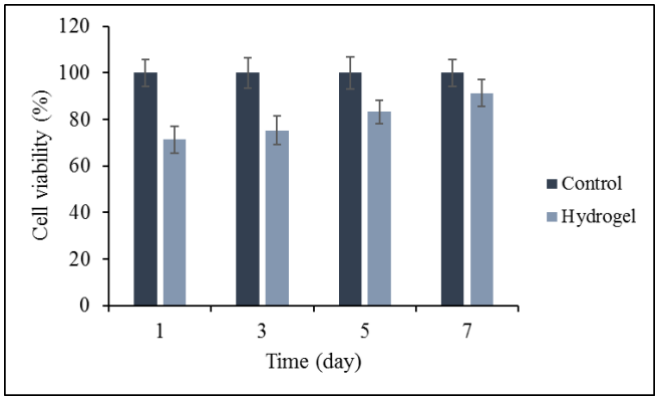

Figure 5. Evaluation of chitosan/PVA hydrogel toxicity for ADMSCs (cell viability) by Alamar blue test for one week.

3.7. Microscopic evaluation of three-dimensional culture

The morphology of three-dimensional cultivation of ADMSCs on chitosan/PVA hydrogel was evaluated by inverted microscope for a period of one week (Figure 6). The observations displayed that in all the investigated times in the group of hydrogel, the cells did not change significantly in shape and at all times and at the end of one week, the cells had a normal shape similar to the group of control, and in other words, the cells had maintained their attached and spindle shape. This shows that chitosan/PVA hydrogel is suitable as a scaffold for keeping cells.

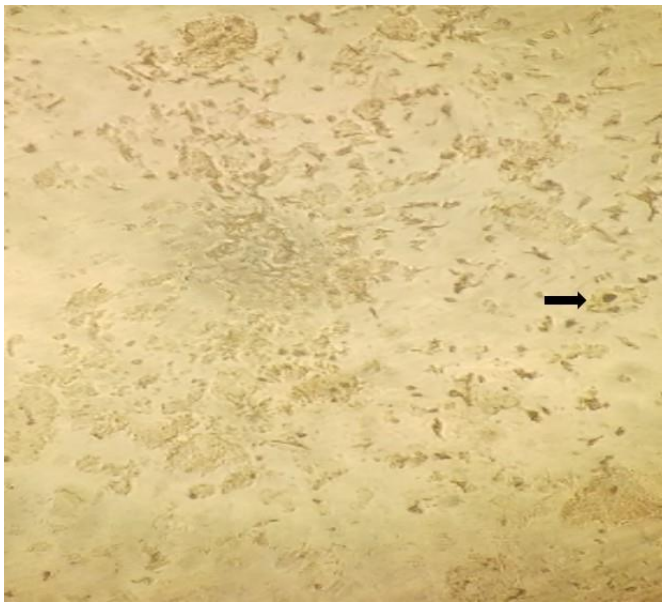

Figure 6. Morphology evaluation of three-dimensional cultivation of ADMSCs on chitosan/PVA hydrogel by inverted microscope. Flash point: ADMSC encapsulated cells.

4. Discussion

We demonstrated the design, fabrication and *in-vitro* characterization of injectable chitosan/PVA hydrogel scaffold for heart tissue engineering. The results displayed the gelation time and the pH measurement were 7.66 ± 1.69 minutes and 7.03 ± 0.12, respectively. The degradability of hydrogel occurred after one week, which indicated its biocompatibility. The highest swelling was observed at 3 hours and then, the hydrogel started to lose water and shrank. The hydrogel was not toxic to ADMSCs and the cells maintained their attached and morphology.

Today, research in the field of tissue engineering is expanding on a large scale, so that tissue engineering has the potential to build organs and tissues (15). The process of progress in the field of using engineered scaffolds is on the rise quickly, and materials alike the goal tissue are used to promotion their performance. Scaffolds are constructions based on materials in the ECM. At first, these substances were only used to transfer drugs and hormones to the body; but later the study indicated, which these substances have the capability to preserve, store and transfer cells to the body (16).

MI is one of the most important cardiovascular diseases that causes death worldwide, and the cases number of this disease is expected to increase continuously in the coming years (17). The treatment choices available after a heart attack are concentrated on decreasing the reappearance risk and do not repair the impaired tissue. New study has been directed towards the regeneration of impaired tissue with stem cells or adult cardiac cells. Nevertheless, cell transplantation has shown limited success due to cell death at the transplant site (18,19). Therefore, for the complete transplantation of stem cells, materials that can protect cell survival and persistence throughout regeneration are necessary (20). Additionally, these scaffolds should act similar to heart when exposed to the heart milieu (19).

Constructs based on chitosan, a cheap biopolymer, which is available in large quantities, offer a hopeful method. Nevertheless, the use of chitosan only has displayed inadequate protect for development of heart tissue. Therefore, efforts should be made to find alternative approaches (21). Hydrogels formed using chitosan alone or together with mesenchymal, umbilical cord and embryonic stem cells have been investigated in cardiac

tissue engineering. It was shown that the delivery of a hydrogel consisting of only chitosan improved cardiac action. Nonetheless it was stated, which similar to the delivery of stem cells alone, most of the transplanted stem cells with chitosan hydrogel were destroyed within a week. However, even with the death of most cells, the improvement in heart action was observed (22). Chitosan hydrogels slightly promoted the microenvironment for survival of stem cell, but by adding binding domains, they can increase cell endurance and promote transplantation.

Challenges arising from the use of natural hydrogels are low cell survival in a nutrient-poor milieu, rapid degradation time, and poor mechanical features (23). To overcome the restrictions of degradation and mechanical features of chitosan, one method is to combine it with natural biomaterials such as collagen and gelatin or with synthetic biomaterials such as PVA and polycaprolactone (PCL). These additives promote hydrogel efficiency via adjusting degradation rates, introducing cell attachment sites, or enhancement mechanical stability. One of the most plentiful proteins in the body is collagen that it is a great addition for chitosan hydrogel. In addition to attachment sites, this protein is necessary for stimulating angiogenesis and cell migration in damaged tissue. Chiu and colleagues applied a collagen-chitosan hydrogel to transport thymosin-β4 to the infarct location in rat (24). They revealed, which there is no noteworthy difference in degradability and swelling ratio of hydrogel on different days of the study, which the findings of the current research are consistent with their study.

One of the drawbacks of these scaffolds is their lack of electrical conductivity, which causes cell-cell communication and heart coordination to decrease. Dvir and colleagues have applied gold nanowires in the construction of alginate scaffolds to increase the conductivity of these types of scaffolds. The results showed that gold nanowire promoted the expression of connexin 43 (a protein involved in relationship among cells and mechanical-electrical coupling) and caused electrical stimulation of cells (25).

In this study, PVA was used to strengthen the mechanical properties and eliminating the lack of electrical conductivity of the hydrogel scaffold. PVA due to its excellent properties such as use as a matrix with regards to elasticity and promoting transmission of mechanical signals, binding to several biological molecules, low wear coefficient and robust mechanical features has led to promising applications in tissue engineering and regenerative medicine, particularly the repair of heart tissue damage (13,14). The *in-vitro* finding of the current study demonstrated, which the hydrogel scaffold based on chitosan/PVA created a suitable three-dimensional structure for maintenance and proliferation of cells with injectability.

The findings of our research were consistent with the studies of Dattula et al. (26) and Huang et al. (27). Dattula et al. developed a three-dimensional scaffold composed of PVA for use in cardiac tissue engineering. Their scaffold was a biocompatible porous matrix with suitable mechanical characteristics that had an elastic behavior similar to the muscles ECM. Also, the scaffold showed the ability to support cell growth and proliferation similar to the present study (26). Huang et al. designed a flexible film prepared of PVA with chitosan based thermosensitive hydrogel for periodontal regeneration. Their hydrogel was biocompatible and was not toxic to MG63 cells and meaningfully improving cell proliferation (27). Kook et al. presented a multi-scale scaffold containing PCL fibers with bilayer hydrogels including fibrin and alginate for cardiovascular tissue regeneration. Their findings indicated that the scaffold was mechanically stable and supported cell attachment and proliferation during culture periods (28).

The various studies have stated which three-dimensional scaffolds with the appropriate architecture improve the cell growth and proliferation. In the present study, we revealed that the chitosan/PVA hydrogel scaffold with suitable biocompatibility properties caused the growth and proliferation of ADMSCs *in-vitro* and the cells maintained their attached and morphology during the study.

5. Limitations

A limitation of this study could be the non-uniform distribution of cells on the hydrogel. Therefore, it is more desirable to load the cells on the hydrogel using a 3D printer. It is suggested that the chitosan/PVA hydrogel scaffold, along with other evaluations, can be used as a suitable choice for heart tissue damage.

6. Conclusion

The study presents a biocompatible and injectable chitosan/PVA hydrogel scaffold suitable for cardiac tissue engineering. The hydrogel showed high cellular compatibility, was non-toxic to ADMSCs, and provided a conducive environment for cell growth and proliferation. Its degradation started after one week, and the appropriate swelling ratio was established to prevent pressure necrosis

in heart tissue.

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Ethics

Not applicabe.

Author contributions

Abolfazl Najd Ghahremani: Investigation, Writing – Original draft

Sirous Sadeghian Chaleshtori: Project administration, Conceptualization, Writing – Review & editing

Mohammad Reza Mokhber Dezfouli: Project administration, Conceptualization, Writing – Review & editing

Mohammad Mehdi Dehghan: Formal analysis, Software

Fatemeh Barzegar: Investigation, Resources

Neda Sabetzadeh: Investigation, Resources

Massoumeh Jabbari Fakhr: Investigation, Valdation, Writing – Original draft

Conflict of interest

The authors declare no conflicts of interest.

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