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Effect of Pre-Incubation of Electrospun Silk Fibroin Scaffold in Complete and Serum-Free Media on Survival and Proliferation of Rat Bone Marrow Mesenchymal Stem Cells

Maryam Janitermi
(PhD Candidate) Department of Biology, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran

Esmail Fattahi
(PhD) Department of Biology, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran

Seyed Gholam Ali Jorsarai
(PhD) Fatemeh Zahra Infertility and Reproductive Health Research Centre, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

Corresponding author: Esmail Fattahi

Email: esmail_fattahy@yahoo.com

Tel: +989113255311

Address: Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran

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ABSTRACT

Background and objectives: Developing scaffolds is important for tissue engineering and repairing damaged tissues. The present study aimed to investigate effects of pre-incubation of an electrospun silk fibroin scaffold in complete and serum-free media on proliferation and survival of cells seeded on the scaffold.

Methods: After removing sericin from the silk cocoon and preparing the fibroin solution (3% w/v), the electrospun silk fibroin scaffold was fabricated and its morphology was evaluated by scanning electron microscopy. The scaffolds were pre-incubated in complete and serum-free Dulbecco's Modified Eagle media for one hour (short-term) and 10 days (long-term), and the hydrophilicity of scaffolds was evaluated by measuring the water contact angle. Rat bone marrow mesenchymal stem cells were seeded onto the scaffolds, and cell survival and genomic DNA concentration were evaluated after 21 days.

Results: The short-time pre-incubation of electrospun silk fibroin scaffolds in the complete medium increased the proliferation of seeded cells because of serum protein adsorption. In addition, long-term pre-incubation of the scaffolds in the complete and serum-free media increased cell proliferation due to the increased hydrophilicity of the scaffold ($p<0.05$). However, only long-term pre-incubation of the scaffolds in the complete medium had a significant effect on cell survival.

Conclusion: The results demonstrated that long-term pre-incubation of the scaffolds in the complete medium have more profound positive effects on cell survival and proliferation compared to short-term pre-incubation.

Keywords: Tissue Culture Techniques, Blood Proteins, Culture Media Serum-Free.

INTRODUCTION

As a promising technology, tissue engineering has an important role in repairing damaged tissues and organs using cells and scaffolds (1, 2). Over the past decades, various methods have been proposed to fabricate scaffolds that could be utilized for tissue engineering, one of which is electrospinning. This simple and easy method uses electricity to produce nano-to-micrometer-thick fiber structures from their polymeric solutions. This conversion increases the surface-to-volume ratio of the structure, thereby increasing its flexibility, strength, and hardness (3). These new properties improve the applicability of the structures for tissue engineering as scaffolds, just similar to an extracellular matrix. Electrospun scaffolds are a suitable option for bone, cartilage, and nerve tissue engineering (4). Nowadays, electrospun scaffolds are considered to be compatible with cells' attachment and proliferation, and their interactions with cells have been evaluated in cartilage and bone tissue engineering (5, 6). According to literature, cells cannot directly attach to these substances. In other words, these biological structures must first adsorb serum or blood proteins to be biocompatible for the attachment (7, 8). Similar to in vivo conditions in which cells bind to proteins in the extracellular matrix, cell attachment to the culture surface also occurs through adhesive proteins in a complete culture medium (9, 10). Serum proteins that have different binding properties compete with each other to bind to the polymer (11). Over time, the layer containing the adsorbed proteins (e.g. albumin) is rapidly replaced by the layer containing the protein with a higher molecular weight (12, 13). After binding to the scaffold, the protein affects the properties of the three-dimensional scaffold. Previous studies have shown that immersing three dimensional polymer scaffolds in fibronectin solution significantly increases cell adhesion (14, 15). On the other hand, exposure of synthetic scaffolds to fetal bovine serum (FBS) significantly reduced apoptosis (16) and some properties of the polymer, e.g. hydrophilicity, as a result of pre-incubation in the culture medium (17). All of these changes may be due to the rearrangement on the scaffold surface, which may be time-dependent and intensified with increasing incubation time of the polymer in the culture medium (18). Previous studies also showed that pre-incubation of polymer in culture medium can increase cell adhesion (19), and pre-incubation of synthetic three dimensional scaffold for more than seven days in culture medium increases cell proliferation but not cell attachment (20).

One of the events that occur in the pre-treatment of biomaterials in a complete culture medium is the change in the hydrophilicity of these materials and the adsorption of serum protein over time, which may affect cell attachment and proliferation. The present study aimed to evaluate this hypothesis by constructing electrospun silk fibroin scaffolds and then pre-incubating them in complete and serum-free medium for an hour (short-term) and 10 days (long-term). The water contact angle was used to evaluate the hydrophilic properties of pre-incubated scaffolds. The effect of this pre-treatment on the survival and proliferation of rat bone marrow mesenchymal cells was evaluated by MTT assay and genomic DNA concentration assay.

MATERIALS AND METHODS

Bombyx mori silk cocoons were obtained from a silk farm located in the Babol Kenar District, Babol, Iran. After transferring the silk cocoons to the laboratory and removing the larvae, mincing was carried out. To remove sericin from the cocoons, the fibers were boiled in sodium bicarbonate solution (0.02 M) for 30 minutes. The samples were then washed with distilled water and dried at room temperature for 24 hours. Next, 9.3 M lithium bromide solution was prepared to dissolve the dried fibroin at 60 °C for 5 hours. The resulting solution was dialyzed with distilled water for 72 hours to remove residual lithium bromide salts. The dialyzed fibroin solution was centrifuged at 4 °C for 20 minutes, frozen at -80 °C for 24 hours, and dried (freeze-dried) (21).

Silk fibroin was dissolved in formic acid to obtain a concentration of 3% (w/v). Then, the electrospinning process was performed using a lab-scale electrospinning machine (ES1000, Fnm, Iran). The process was carried out with an 18 cm distance of needle from the collector, the flow rate of 0.9 ml/hour, and the voltage of 35 kV. Finally, an electrospun silk fibroin scaffold was obtained in the collector (1, 22).

The shape and morphology of the fabricated electrospun silk fibroin scaffolds were evaluated using a scanning electron

microscope (SEM, Philips, Netherlands) after coating the fibers with gold.

The effect of pre-incubation of scaffolds (with the constant elasticity) in culture medium on the proliferation and survival of cells seeded on the scaffolds was evaluated. The electrospun silk fibroin scaffolds were placed in 24-well plates containing 70% ethanol for 1 hour. The alcohol was then discarded and the scaffolds were washed with phosphate buffer saline (PBS) and subjected to UV radiation. After sterilizing the scaffolds, they were transferred to 24-cell plates containing complete Dulbecco's Modified Eagle Medium (DMEM, containing 10% FBS) and serum-free DMEM. The plates were incubated for 1 h and 10 days under standard conditions. Moreover, a control group was not pre-incubated.

The hydrophilicity of the pre-incubated and control scaffolds in the culture media was evaluated by measuring the water contact angle. After the pre-incubation, the scaffolds were washed with water and dried. Then, the water contact angle was measured in triplicates for each electrospun scaffold using a contact angle measurement system (CA-500, Sharifsolar, Iran).

For isolation and culture of rat bone marrow mesenchymal cells, rats (28-45 days old and 130 to 140 g) were anesthetized with isoflurane under sterile conditions. The rats were then dissected, the distal end of the femur and tibia were opened, and bone marrow cells were collected by rinsing the end with complete medium. The bone marrow mesenchymal stem cells were isolated by Ficoll density gradient centrifugation (1.073 g/ml Ficoll at 1,500 rpm for 30 minutes). In this method, the tip of the pipet containing Ficoll medium was carefully placed to the bottom of a conical tube containing single-cell suspensions, and the medium was slowly released to isolate cells. The cells were washed and then cultured (5×10^6 cells) in a flask containing DMEM with FBS (10 %), penicillin-streptomycin (100 U/ ml), amphotericin B (0.25/g/ml), and fibroblast growth factor (1 ng/ml). After 4 days of incubation at 37 °C with 5% carbon dioxide, non-adherent cells were removed by washing. To passage the cells, they were incubated with trypsin-EDTA solution (500 µl) at 37 °C for 5 minutes and then neutralized with FBS. After centrifugation at 1,800-2,000 g for 5 minutes, the cell suspension was transferred to two or more culture flasks. After passaging and replacing the culture medium, the cells were counted using a hemocytometer and trypan blue (23).

Culture medium of the pre-incubated electrospun scaffolds was removed and the cells were added to the scaffold and incubated for at 37 °C for 3 hours. Then, DMEM medium containing 10% FBS, 100 nM dexamethasone, 1,000 U/ml streptomycin solution, 100 U/ml penicillin, 0.25 mg/ml amphotericin B, 1 mM sodium pyruvate, 10 ng/ml TGF b1, 50 mg/ml ascorbic acid, 1X ITS3, 2 mM L-glutamine, and 40 mg/ml L-proline was prepared to incubate the seeded cells for subsequent days. The culture medium was replaced every 2 days and incubation continued for 21 days (23).

Then, survival of the cells seeded onto the electrospun scaffolds was assessed by MTT assay. For this purpose, MTT powder (5 mg) was dissolved in 1 ml of PBS buffer containing 10% FBS, and the resulting solution was sterilized with a 0.2 µm filter. Mesenchymal stem cells (100,000 cells/ml) were seeded onto the scaffolds for 21 days, and then the culture medium was replaced with the MTT solution (0.5 mg/ml). After 4 hours of incubation at room temperature and in the dark, the MTT solution was removed and dimethyl sulfoxide was added. Finally, the adsorption rate of the prepared solution was read at 570 nm (24). Moreover, genomic DNA extraction was carried out using a DNA extraction kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's protocol. The scaffolds were first washed with PBS, transferred to a microtube, mixed with TG1 buffer (200 µl), and vortexed with proteinase K solution (10 mg/ml). Several vortexing steps were carried out following incubation at 60 °C. Then, TG2 buffer and ethanol (96%) were added to the sample. The DNA solution was collected after several steps of centrifugation and washing. Finally, DNA concentration was measured by spectrophotometry (NanoDrop 2000, Thermo Scientific, USA) (25).

Statistical analysis was performed with SPSS software (version 24).

Data were expressed as mean±standard deviation. Given that the data had one variable in several groups, one-way analysis of variance (ANOVA) with the Tukey's test was used. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

According to SEM images, the electrospun fibroin scaffolds with a concentration of 3% (w/v) had a diameter of 38 ± 12 nm (Figure 1).

Hydrophilicity of the scaffolds

As shown in figure 2, the highest water contact angle was obtained in control scaffolds (91±14 degrees). On the other hand, incubation of the scaffolds in complete medium for 10 days reduced the contact angle (10±4 degrees). While pre-incubation of the electrospun scaffolds in serum-free medium for 1 hour had no significant effect on water contact angle (p>0.05), pre-incubation in serum-free medium for 10 days led to a significant reduction in water contact angle (58±5 degrees).

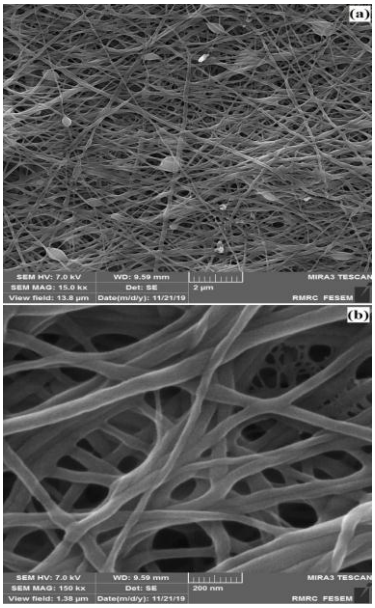

Figure 1- SEM images of an electrospun fibroin scaffold structure taken under 15 kx (a) and 150 kx (b) magnifications.

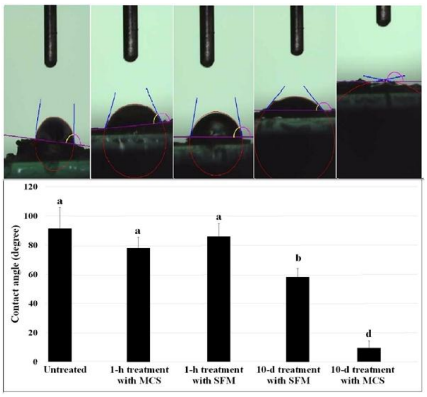

Figure 2- Comparison of water contact angle of untreated scaffolds as well as those treated with serum-containing medium (MCS) and serum-free medium (SFM) for an hour and 10 days. The letters a, b and d indicate significant differences between groups (p<0.05).

Cell viability

A significant difference in cell viability percentage was observed after 10 days of pre-incubation of the scaffolds in complete medium (Figure 3).

Genomic DNA concentration

According to figure 4, short-term and long-term pre-incubation of electrospun scaffolds in the complete and serum-free media had a significant effect on the genomic DNA concentration of cells. Genomic DNA concentration of cells seeded onto pre-incubated scaffolds in complete medium for 1 hour differed significantly with that of cells seeded onto control scaffolds (p<0.05). However, pre-incubation of the scaffolds for 1 hour in the serum-free medium had no significant effect on DNA concentration (p>0.05). On the other hand, pre-incubation of

the scaffolds in the complete and serum-free media for 10 days significantly increased DNA concentration compared to the control group (p<0.05).

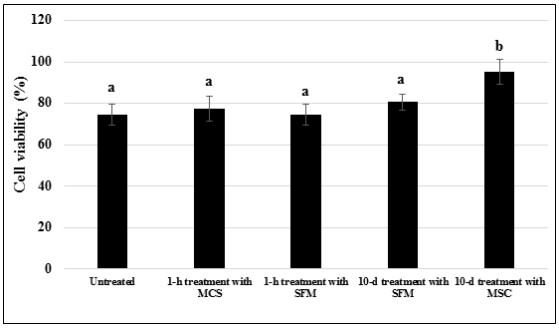

Figure 3-The percentage of viable cells seeded onto pre-incubated and control scaffolds in the medium containing serum (MCS) and serum-free medium (SFM) after 1 hour and 10 days. The same letters indicate no significant difference between groups (p> 0.05).

Genomic DNA concentration

According to figure 4, short-term and long-term pre-incubation of electrospun scaffolds in the complete and serum-free media had a significant effect on the genomic DNA concentration of cells. Genomic DNA concentration of cells seeded onto pre-incubated scaffolds in complete medium for 1 hour differed significantly with that of cells seeded onto control scaffolds (p<0.05). However, pre-incubation of the scaffolds for 1 hour in the serum-free medium had no significant effect on DNA concentration (p>0.05). On the other hand, pre-incubation of the scaffolds in the complete and serum-free media for 10 days significantly increased DNA concentration compared to the control group (p<0.05).

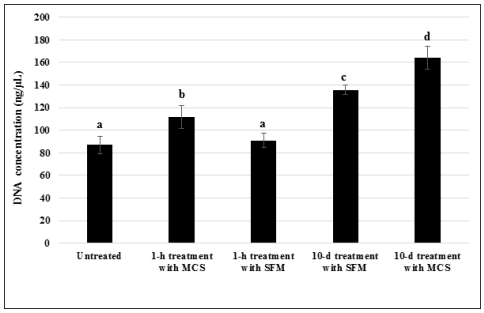

Figure 4- The concentration of genomic DNA after pre-incubation of the scaffolds in complete culture medium (MCS) and serum-free medium (SFM) for 1 hour and 10 days. The same letters indicate no significant difference between the groups (p> 0.05).

DISCUSSION

The present study showed that short-term pre-incubation of the electrospun silk fibroin scaffold in complete or serum-free media did not have a significant effect on water contact angle, while long-term pre-incubation in both culture media significantly reduced the water contact angle. Moreover, short-term pre-incubation in complete medium and long-term pre-incubation in both complete and serum-free media significantly increased genomic DNA concentration. This study aimed to investigate impact of long-term and short-term pre-incubation of an electrospun silk fibroin scaffold with complete and serum-free media.

Various synthetic and natural polymers have been used to produce scaffolds in tissue engineering, among which natural silk is a favorable option in the field of cartilage formation, due to its high mechanical strength, flexibility, biocompatibility, and potential in repairing damaged cartilage (26). Moreover, fibroin materials have a slow and controllable biodegradability as well as low immunogenicity, making them suitable for in vivo studies (23). In the present study, 3% (w/v) fibroin concentration was used to fabricate the scaffold. Our previous study showed that electrospun fibroin scaffolds fabricated from 3% (w/v) fibrin solution had the best effect on growth and differentiation of rat bone marrow mesenchymal stem cells to chondrocytes (27). In this study, bone marrow

was used as a source of mesenchymal stem cells because this organ is the best source for supplying stem cells to differentiate into chondrocytes (28). In the presence of dexamethasone and TGF-beta, bone marrow-derived stem cells easily differentiate into chondrocytes, and our recent study confirmed the differentiation of these cells on electrospun fibroin scaffolds (27).

In the present study, it was hypothesized that exposure of electrospun fibrin scaffolds to complete medium for various periods can affect protein adsorption by the scaffolds, thereby influencing cell proliferation and survival. The adsorption of protein by biomaterials is key to a biological response to these substances. In tissue engineering, serum proteins adsorption by scaffolds play an important role in determining the response of cells seeded on the scaffold (20).

When biodegradable materials (e.g. silk fibroin) are exposed to a culture medium, several phenomena can alter the properties of the biomaterial. Adsorption of water by the scaffold and its swelling and surface adsorption of protein can affect the behaviors of seeded cells (29). The present study showed that the hydrophilicity of fibroin scaffold increases (decreasing water contact angle) with increasing pre-incubation time. Although there was no significant difference between the hydrophilicity of scaffolds treated with complete and serum-free medium for an hour, this difference was significant after long-term pre-incubation. DNA concentration was used for quantitative assessment of cell attachment to the scaffold because the amount of DNA is directly proportional to the number of cells (20). Although pre-incubation of the scaffolds in serum-free medium for an hour did not notably change DNA concentration compared to the control scaffold, pre-incubation for 1 hour in the complete medium significantly increased DNA concentration. On the other hand, long-term pre-incubation of the scaffolds in complete and serum-free media significantly increased DNA concentration compared to the control scaffolds.

A previous study showed that pre-incubation of sponge polycaprolactone scaffolds in culture medium for various periods could significantly alter DNA concentration. In addition, pre-incubation of scaffolds for 5 minutes and 1 day had a significant effect on the amount of protein adsorption by the scaffold, but this difference between pre-incubated scaffolds for 1 and 7 days was not significant (20). In other words, when the scaffolds are incubated in the culture medium for more than one day, the amount of protein adsorption does not change. Chen et al. (2008) showed that pre-incubation of a glass surface (non-degradable and non-absorbable) in a culture medium for 7 days did not increase cell attachment (19). Based on the results obtained from the present study, it seems that protein adsorption in short-term pre-incubation (1 hour) led to an increase in DNA concentration, while long-term pre-incubation of the electrospun scaffolds in culture medium increased hydrophilicity in terms of water contact angle, which affects cell proliferation and adhesion. These results are consistent with findings of previous studies (29, 30). In a similar study, Amirikia et al. (2017) investigated the effect of long-term pre-incubation of three dimensional silk fibroin scaffolds in the complete medium on cell attachment and proliferation (17). In the present study, the results showed that only pre-treatment of scaffolds in complete medium for 10 days could significant change cell survival.

CONCLUSION

The findings indicate that short-term and long-term pre-incubation of electrospun fibroin scaffolds with constant elasticity in the complete and serum-free media can increase protein adsorption and scaffolds' hydrophilicity. Furthermore, long-term pre-incubation in the complete medium had the most positive effect on cell survival and proliferation. These findings have important implications for seeding cells onto scaffolds for tissue engineering.

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DECLARATIONS

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Ethics approvals and consent to participate

This study was approved by the Ethics Committee of Babol University of Medical Sciences, Iran (ethical code: IR.IAU.SARI.REC.1399.104)

Conflict of interest

The authors declare that there is no conflict of interest regarding publication of this article.

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