European Review for Medical and Pharmacological Sciences 2022; 26: 787-798

Muscle-derived stem cells in silk fibroin hydrogels promotes muscle regeneration and angiogenesis in sheep models: an experimental study

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Abstract. – OBJECTIVE: Silk fibroin (SF) hydrogels are of high interest in tissue engineering. However, angiogenesis is one of the major challenges in tissue regeneration and repair. In this study, we present a simple and effective method to develop a 1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol sodium salt (DMPG)-SF hydrogel. The SF hydrogels had no immunogenicity and approached natural tissues.

MATERIALS AND METHODS: The SF scaffolds were first prepared from Bombyx mori silkworms and DMPG. The SF scaffold was seeded with muscle-derived stem cells derived from sheep embryo and implanted in the tibialis anterior muscle of mature sheep. Gelation time, H&E staining, and histochemistry were conducted and observed. The suitability of the hydrogels for 3D cell culture was assessed by living cell stain CM-Dil.

RESULTS: The results showed that the SF hydrogels resembled the mechanical properties of natural soft tissues better. The results of H&E staining and histochemistry revealed that the degradation rate showed an S-type change, and muscle regeneration and angiogenesis were clearly visible. Adverse effects were not observed in the sheep models.

CONCLUSIONS: DMPG-induced SF hydrogels can be successfully used for in situ cell encapsulation. It provides promising opportunities in biomedical applications, such as in tissue engineering and regeneration.

Key Words:
Tissue engineering, Silk fibroin, Hydrogel, DMPG, Muscle-derived stem cells, Angiogenesis.

Introduction

Current tissue regeneration strategies primarily rely on the tissue repair of artificial or natural implants[1]. However, the limitations of existing strategies increase the need for organizational engineering methods[2]. The right cell source, effective cell modification, and suitable supporting substrings are the three foundations of tissue engineering[3]. Choosing suitable cell sources and method for cell stimulation, stent synthesis, and tissue transplantation plays a decisive role in the success of tissue engineering[4]. Recent research in this area has focused on the development and maintenance of functional tissues, in which vascular regeneration plays a significant role. Several types of cells have been used in tissue engineering, but the most promising are stem cells[5]. Stem cells, because of their ability to renew themselves, can be amplified *in vivo* and then transplanted to where they are needed. Most importantly, after induction, stem cells have the ability to differentiate into most cell lines[6]. The first type that was applied to tissue engineering was bone satellite cells[7-9]. By simulating the extracellular substation of bone cells, repairing bone defects and bone loss, and using the characteristics of low blood vessel density of bone tissue to easily form new blood vessels, defect repair was successfully carried out on bone tissue sites[10]. Subsequent *in vivo* tests have been performed, including the successful proliferation and differentiation of human fibroblasts on silk fibroin (SF) hydrogels[11]. Furthermore, muscle-derived stem cells have also been explored. These types of stem cells are distinct because they have the ability to differentiate into other additional lineages, and may not be limited to just mesenchymal tissues or the myogenic lineage[12]. Myod1 and Desmin genes are determinants of muscle development. They jointly guide

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the transformation of progenitor cells into skeletal muscle cells. Muscle stem cells are the main seed cells for muscle regeneration and repair. Research has shown that transplantation of muscle-derived stem cells into injured skeletal muscle resulted in an improvement in muscle repair following injury by promoting angiogenesis[13].

As previous researches[14,15] indicated, scaffolds of suitable material have a vital part in neovascularization through their ability to maintain cell function and viability and providing angiogenic factors locally. One of the most researched and promising materials is silk, which is a well-known natural fiber produced by silkworm (*Bombyx mori*) cocoons. SF is a natural polymer with excellent biocompatibility, adjustable properties, low inflammatory response, and is easy to process[16-18]. SF can be processed into different structures, such as films, fibers, meshes, sponges, and hydrogels, which have been shown to induce stem cell adhesion, proliferation, and differentiation[5].

Hydrogels have a certain morphological stability, in which water molecules are locked inside the mesh structure. Meanwhile, its hydrophobic group molecular chains are interlinked with chemical or physical bonds. This results in water expansion to form a mesh hole wall, leading to hydrophobic residues and water binding[7,19]. The abundance of water in hydrogels gives it the basic properties of fluidity, which can make gas, liquid, and small molecular substances penetrate and spread from it. Since the human environment contains a large amount of water-like liquid, hydrogel has become an excellent material for studying the growth of cells, tissues, organs, as well as metabolic mechanisms[5,20-22].

In solution, SF goes through structural changes that turn its unordered structure gradually into β sheets, resulting in the formation of gel. This whole process is known as gelation, and takes place between several days to weeks, depending on the protein temperature and concentration[5,17]. Therefore, different methods have been applied to accelerate the natural gelation process. These methods can be physical, such as pH reduction and sonication, or chemical by means of polyethylene oxide or phosphoryl glycerols[23]. A variety of factors have been studied that can improve the SF stent to better adapt to the needs of different environments, such as the silk egg stent[24]. In these studies, the silk stent was implanted into the animal and sliced for observation and analysis[25]. SF stents have the advantage of man-made regulation, both *in vivo* and *in vitro*, to meet the needs of modern medicine. However, most experiments in recent years have been conducted in mice, rabbits and other small animals, leading to limited similarities to humans[26]. In addition, fetal sheep have many developmental and physiological characteristics in common with humans. Therefore, they are deemed as very valuable and useful in research[27].

Based on the above findings, this study investigated whether SF hydrogels seeded with muscle-derived stem cells improved muscle regeneration and vascularization of injured muscle tissue *in vivo*. Innovative methods by injection were used that can minimize the impact on animal experiments[28,29]. The results demonstrated the regenerative and angiogenic potential of muscle-derived stem cell-seeded SF hydrogel when injected intramuscular in a sheep.

Materials and Methods

Silk Fibroin Solution Preparation

The SF solution was prepared from the cocoons of *Bombyx mori* silkworms (Hangzhou Lin Ran Biotech, China). Methods have been described in previous research[17,30]. Briefly, the silk was cut with scissors into lengths of 2 cm and degummed by boiling in a 0.5% sodium bicarbonate solution (1:200, Ryan, Shanghai) for 30 min, while stirring with a glass rod every 15 min. The resulting silk was obtained from the solution and washed three times with deionized water. The degumming process was repeated again for 45 min. After this process, the silk was washed 6 times with deionized water and transferred to an oven at 37°C to allow drying for 12h. To dissolve the degummed silk, it was added to a 9.3 mol/l lithium bromide solution (Aladdin, Shanghai, China) with a ratio of 1:4, and then, transferred to an oven at 60°C for 2h. It was taken out every 15 min and stirred until the degummed silk was totally dissolved. After three days of dialysis with deionized water in a dialysis tube (MWCO 12,000-16,000; Viskase Company Inc., Tokyo, Japan), the SF solution was subjected to gauze filtration. Then, it was centrifuged for 20 min in a low-temperature high-speed centrifuge (Biobase, Shanghai, China) at 7000 r/min. Subsequently, SF was filtered with a 0.22 μm bacterial filter and stored at 4°C for preparation of the scaffold. Figure 1 represents a simplified flow diagram of the process.

Preparation of Silk Scaffolds

1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol sodium salt (DMPG, TCI, Tokyo, Japan) was

used to induce SF from disordered compositions into thermodynamically stable β-sheet structures. Muscle-derived stem cells of *Hu sheep* were cultured in F12 medium. After 30 min of UV irradiation (Panasonic, Osaka, Japan), DMPG was dissolved in deionized water. Then, it was mixed with the sterile SF solution in three different concentrations; 5, 10, and 15 mM. The pH of the solutions was managed with 10 mM of HEPES buffer (pH 7.4). Pure 3% SF solution without stem cells was used as control.

Preparation of Freeze-Dried Scaffolds

The SF solution was first frozen in liquid nitrogen and then transferred to a lyophilizer for freeze-drying at -20°C for 24 h. The freeze-dried scaffolds were measured with a digital micrometer (Mitutoyo, Takatsu-ku, Japan) and their surface morphologies were observed under a scanning electron microscope (SEM, KYKY, Shanghai, China).

Gelation Time Determination

The gelation time was derived from the turbidity change, which was measured by optical density with a microplate reader (Synergy 2, BioTek, Shanghai, China) at 550 nm. The gelation time was established from the point in time at which a half-maximum value of optical density was reached.

Muscle-Derived Stem Cells Seeding in SF Scaffolds

The SF scaffolds were first pre-seeded in a 24-well plate containing DMEM, supplemented by 10% FBS and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Then, 1×10^5 muscle-derived stem cells were seeded on them. The stem cell-seeded SF scaffolds were incubated (Seymogen) for 3 days at 37°C with 5% CO_2 and 95% humidity.

Experimental Animals

The experimental procedures were approved by the Ethics Committee of the College of Animal Science and Technology of Zhejiang A & F University. All experiments and procedures involving animals were performed in compliance with guidelines established by the China Animal Welfare Association. All the 8 experimental *Hu sheep* were obtained from the Lin'an *Hu sheep* farm. They weighted 50±5 kg and were approximately 5 months old. The standards of normal feeding and enclosure feeding were followed by daily providing total mixed ration and free access to water. The experimental animals implanted with the SF hydrogel were equally divided into two groups of SF hydrogel+stem cells and pure SF as control (N=4 per group).

Hu Sheep Muscle-Derived Stem Cell Extraction and Identification

After mating of the 12 months old male and female sheep with a weight of 80.7 kg, pregnancies were conceived. These were monitored by animal B-ultrasound (Ysenmed, China). The *Hu sheep* embryos with placenta and membranes were taken out by artificial caesarean section under anesthesia at 60 days and transported to the laboratory in ice bags within 4 h. Medical alcohol of 75% was used to wash the embryos three times. Then, the membranes were gently cut open with surgical scissors and the embryo was taken out, and washed again three times with 75% medical alcohol. Subsequently, the leg muscle was taken out and skin, fat, blood vessels, and connective tissue were removed, and totally immersed in PBS buffer (CellMax, Shanghai, China) containing 1% rabbit antibodies and anti-rabbit secondary antibodies (Flow Laboratories, McLean, VA, USA). To harvest viable muscle-derived stem cells the two-step trypsinization procedure was followed. One, ophthalmic scissors were used to cut the muscle tissue into pieces, while adding a suitable amount of type IV collagenase for digestion in the incubator at 37°C with 5% CO_2 for 15 min. Two, DMEM/F12 (10% FBS and 1% penicillin/streptomycin, HyClone, Foster City, MA, USA) was used as culture medium to stop the digestion. The cell suspension was transferred to a 100-mesh cell sieve (Thomas Scientific, Swedesboro, NJ, USA) to remove debris. Then, it was collected into a centrifuge tube for low-speed centrifuging at 1200r/min for 15min, after which the supernatant was discarded. DMEM/F12 supplemented with 1% rabbit antibodies and anti-rabbit secondary antibodies was added to resuspend the remaining cells. Subsequently, the cells were inoculated onto 100 mm cell culture dishes. After observation with an inverted phase contrast microscope (Nikon, Tokyo, Japan), the cells were cultured in an incubator at 37°C with 5% CO_2.

Immunofluorescence Staining

The myogenic cell surface marker proteins, Desmin and Myod1, were identified using cellular immunofluorescence technology. The cells were first seeded into a 6-well plate. After the cells adhered to the wall, 4% polyformaldehyde was used

to fix the cells for 15 minutes. Then, they were washed with PBS 3 times and permeabilized with 0.25 Triton X-100 for 15 minutes. After which, they were washed again with PBS 3 times, sealed for 1h with 10% goat serum, and washed 3 times with PBS. Desmin and Myod1 of 1:100 mouse primary antibody were incubated at 4°C overnight and washed 3 times with PBS. The FITC-labeled goat anti-mouse secondary antibody 1:50 was incubated with the cells at room temperature for 1h, and washed 3 times with PBS. The cells were counterstained with 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) for 15 minutes at room temperature and observed under a fluorescent microscope (Okang, China).

The muscle-derived stem cells were labeled with CM-Dil (Invitrogen Life Technologies, Waltham, MA, USA) fluorescent staining to track them and to analyze their cell viability. Digested stem cells were first rinsed with PBS and then placed with CM-Dil in an incubator at 37°C for 5 minutes, followed by another 15 minutes at 4°C. Fluorescence microscopy was used to visualize and locate the CM-DiI-labeled stem cells.

In Vivo Experiment

Cardiotoxin (CTX; Sigma Chemical, St. Louis, MO, USA) mixed with 100 µl of 10 µM PBS was injected into the tibialis anterior muscle of the sheep. Then, under aseptic conditions, the cell suspension containing either SF seeded with muscle-derived stem cells, or pure SF were aspirated into a medical syringe. Subsequently, it was injected into the tibialis anterior muscle in a hydrogel state, after which the cell suspension coagulated in the *Hu sheep*'s leg. After disinfecting the injection points with dressing, the sheep were returned to normal feeding. Potential changes (including abnormal behavior, not eating or drinking, and signs of inflammation) in the *Hu sheep* were recorded daily.

Histological Assessment

The tissues were removed surgically on day 1, 3, 6, 9, and 11 after implantation. The removed tissues were immediately put into liquid nitrogen and transported back to the laboratory for hematoxylin and eosin (H&E) staining and immunohistochemical analysis. The tissues were fixed

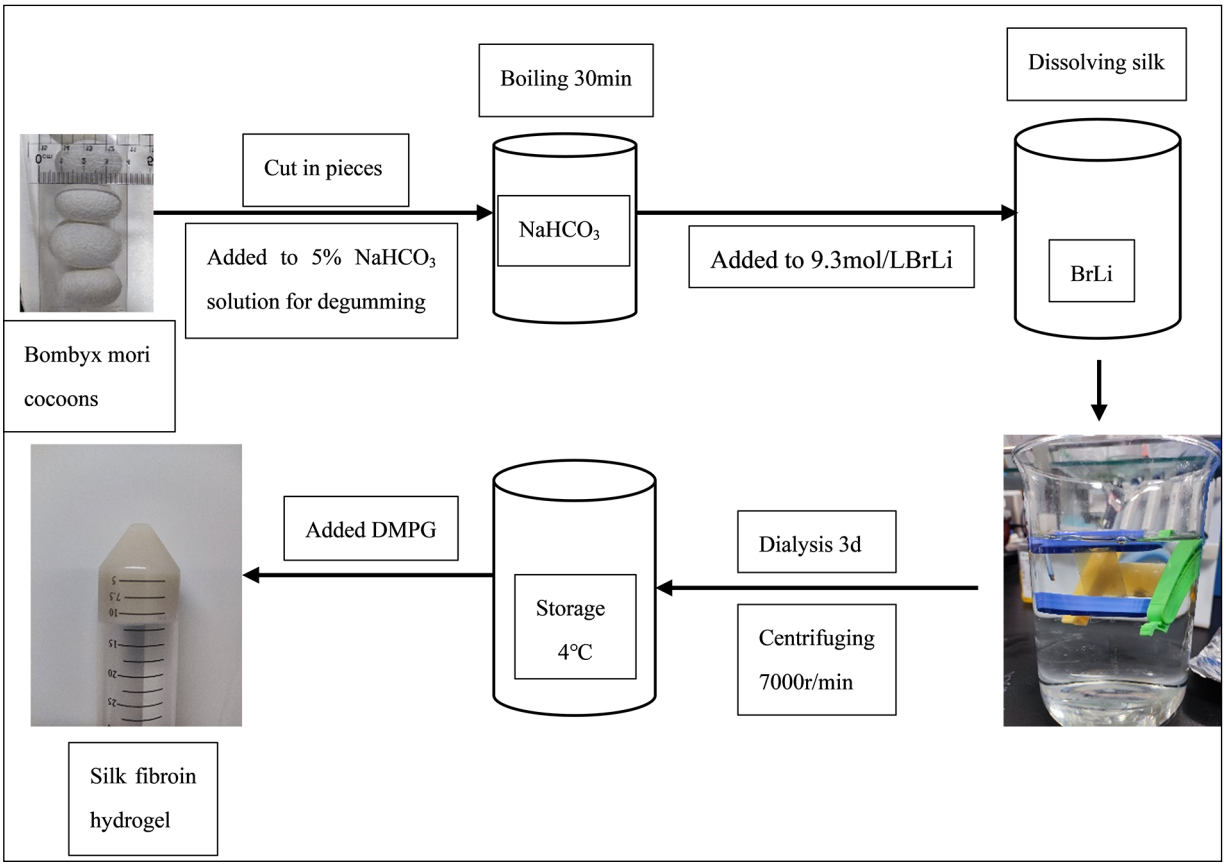

Figure 1. Flow diagram showing the process of silk fibroin hydrogel preparation.

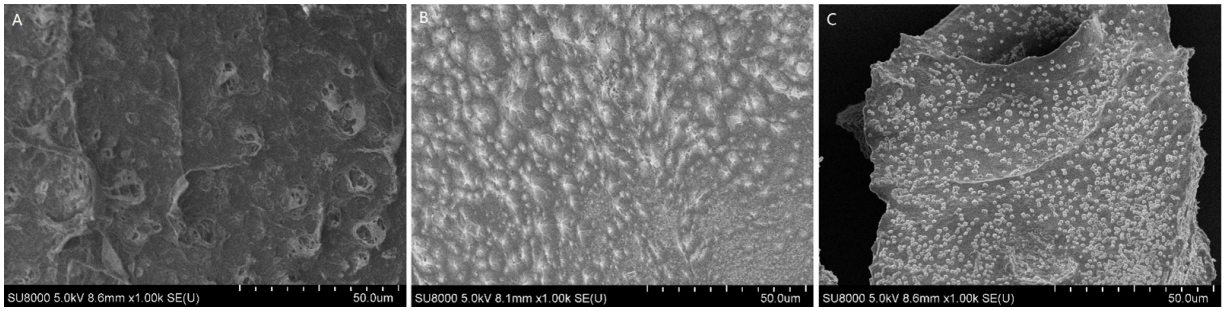

Figure 2. SEM images of silk fibroin hydrogels after mixing with DMPG in the concentrations, 5 (**A**), 10 (**B**), and 15 (**C**) mM. Scale bar 50 μm.

in a 10% formalin solution for 48h, embedded in paraffin, and sliced into 8 μm sections. The sections were stained with H&E to observe the nucleus and cytoplasm under a microscope. After the occlusion of rabbit serum, anti-CD34 (1:100) and CD31 (1:100) were added by drip at 4°C overnight, and then washed three times with PBS for histochemical analysis.

Statistical Analysis

Data were expressed as mean ± SD unless otherwise indicated. Statistical analysis was performed with SPSS 13.0. To analyze the differences between different concentrations of DMPG, One-way ANOVA and Bonferroni post-hoc test was performed. A value of $p < 0.05$ was considered statistically significant.

Results

SF Hydrogel Morphology

The inner structures of SF hydrogels prior to seeding were evaluated using a SEM after freeze-drying (Figure 2). We compared the structures of SF hydrogels after mixing it with DMPG in the concentrations 5, 10, and 15 mM. SEM showed that the pores in the SF scaffolds were highly porous and interconnected, with an average pore diameter of 481.34 ± 6.21 μm.

Primary Cell Immunofluorescence

Muscle-derived stem cells of *Hu sheep* were identified two weeks after induction. The cell surface marker proteins used for identification of muscle-derived stem cells were Myod1 and Desmin. Myod1 has a high expression capacity in muscle cells and can regulate the cell cycle of muscle cells. Myod1 is the main regulatory gene of muscle development in embryos, and its main function is to promote the formation and differentiation of skeletal muscles. A lack of the Myod1 gene can lead to the failure of normal proliferation and differentiation of muscle cells. The transformation of muscle-derived stem cells into muscle cells during rest requires the involvement of muscle genes. The images in Figure 3 show that the positive rate of Myod1 and Desmin was 99%, demonstrating the ability of primary cells to differentiate into muscle cells.

Gelation Time and Viability

Living cells were stained with CM-Dil to show the growth of cells in the scaffold (Figure 4A). The co-existence of the scaffold and the cells was the decisive factor determining whether the scaffold could be applied in practice. DAPI and CM-DiI staining confirmed that the labeled muscle-derived stem cells could be observed in the SF scaffold before implantation. These results show that as a water-based scaffold, it could perfectly coexist with the stem cells.

The optical density at 550nm was similar between the SF hydrogels supplemented with 10 and 15 mM of DMPG. Whereas that of 5 mM was statistically significantly less, as analyzed by ANOVA ($p<0.01$). This could indicate that there was more cellular activity in the SF hydrogels with higher concentrations of DMPG. Figure 4B shows the gelation time of the solutions, which was determined from the time-point at which the optical density reached the half-maximum value. The setting time of scaffolds depended on protein concentration and temperature. For water-based scaffolds, the gelation time induced by DMPG would not exceed 24 hours, provided the protein concentration was determined. We tested the gelation time of 5, 10, and 15mM DMPG, in addition to 3% SF solution as control at 37°C. The continuous measurement results of od550 (Figure

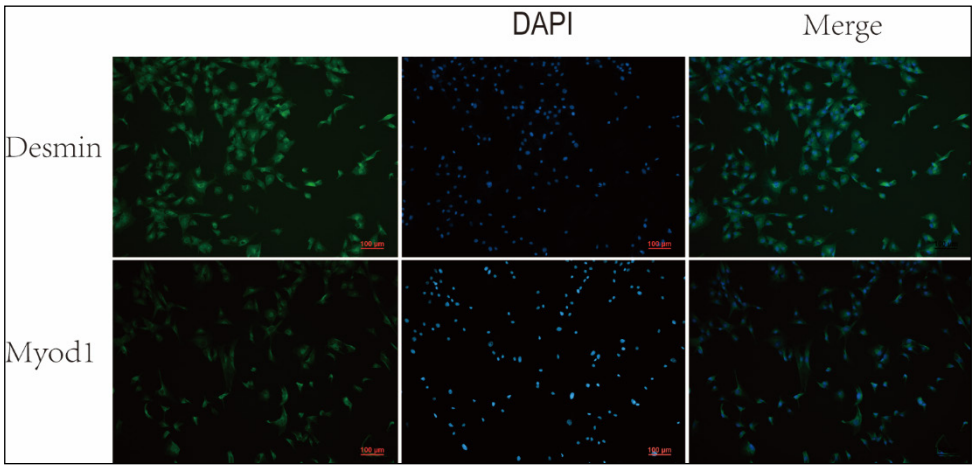

Figure 3. Immunofluorescence images of muscle-derived stem cell induction. Nucleus (blue) was stained with DAPI. Desmin and Myod1 are markers on the surface of muscle cells and Merge represent the mixture of Desmin and Myod1; scale bar: 100 μm.

4B) showed that the optical density continued to increase under the concentration of 5mM until 120 min. This was much faster than the 3% SF solution, which took 7 days to reach the half-maximum value and could thus not be measured. The gelation times after adding different concentrations of DMPG to SF showed statistically significant differences [$F(1.16) = 5.59$, p=0.03]. An interesting phenomenon was observed herein: the optical density value of the hydrogel at the concentration of 10 and 15 mM changed instantaneously when the gel was ready. However, thereafter the optical density value dropped to the same level as before, which we speculate may be related to the similar structure of DMPG and cell membrane. After that, the optical density continued to rise because the moisture in the scaffold was gradually lost under the condition of 37°C. For further experiments we chose to use the SF hydrogel mixed with 10 mM DMPG, because it showed more cellular activity and the gelation time seemed to be the most appropriate, whereas that of 5mM was too fast and 15 mM too slow.

Tissue Analysis

Degradation is the key to understand three-dimensional porous scaffolds. Judging whether the scaffold degradation can meet the specific needs of tissue regeneration can be done by studying the scaffold degradation. Water-based scaffolds should maintain a certain structural integrity at the initial stage of implantation, especially in the center of the scaffolds. The degradation process determines the growth of the tissues[22,23]. The degradation rate of the scaffold is also limited by the size of the pore diameter, which determines the difficulty of macrophage invasion. The large pore diameter formed by a low concentration of SF can accelerate the degradation of the scaffold to meet the requirements of the rapidly growing muscle tissue[18,24]. The results of H&E (Figure 5F) staining showed that the injected new tissue resembled normal skeletal muscle fiber tissues within two weeks. Compared with the first day of injection (Figure 4A), the scaffold (blue area) was degraded, and the new muscle fiber tissue was completely exposed. The scaffold degradation rate (Figure 5H) was the fastest at 6 days after injection. At the same time, no evident structural integrity of the scaffold was destroyed 6 days before (Figure 5A-C).

Different from the degradation of proteins, scaffold degradation is not only accomplished by surface enzymatic hydrolysis but also assisted by internal macrophages. When the number of macrophages in the scaffold reaches a certain level, the degradation rate of the scaffold will accelerate.

During the study, the *Hu sheep* tolerated the scaffolds well and no abnormality in behavior and food intake, and signs of inflammation occurred.

The SF hydrogels seeded with stem cells could form new muscle tissues as shown in Figure 6. The length of muscle regeneration showed statistically significant differences between the SF hydrogel with stem cells compared to the pure SF hydrogel (p<0.05). The growth rate of muscle tissues appeared to be faster until day 9 of implantation and then slowed down. In addition, the

subcutaneous degradation rate of the control hydrogel was much faster as it could not form new tissues and quickly degraded.

Immunohistochemistry Analysis

The degradation rate showed a general trend of first fast and then slow. Angiogenesis is related to the rate of scaffold degradation and the site of implantation. The number of blood vessels is also a key factor that reflects the role of scaffolds in animals. The histochemical analysis results of CD31 and CD34 as shown in Figure 7 indicates that no obvious angiogenesis was detected on day 1 and 3 due to the structural integrity of scaffolds in the early stage of implantation. With the speed of scaffold degradation, the number of blood vessels increased gradually as indicated by the red arrows in Figure 7. Immunohistochemical staining of the CD34 proliferation marker suggested that there was widespread endothelial cell proliferation, whereas staining with CD31 visualized the neo-vascularization as shown by the red arrows in Figure 7A, B.

Immunohistochemical staining of the specimens taken from sheep implanted with pure SF hydrogel (control) showed the process of degradation over the two-week period (Figure 8). The hydrogels without cells showed the forming of tissue masses, which were non-muscle tissues. However, it quickly degraded, which is related to the ability of the hydrogel to recruit cells in the animal's body. Clearly less endothelial cell proliferation and angiogenesis was observed in the sheep implanted with pure SF hydrogel.

Discussion

Regenerating functional tissue through tissue engineering entails a stable structure and an appropriate microenvironment that mimics the host site closely. SF is an excellent option due to its great mechanical properties, biodegradability, and biocompatibility. In this study, a hydrogel was prepared from SF and showed a porous structure. Then DMPG was added in three different concentrations to analyze the gelation time. A concentration of 10 mM seemed the most feasible and was applied in further *in vivo* experiments. Muscle-derived stem cells were derived from sheep embryos and seeded on SF scaffolds, after which the SF hydrogel was implanted in the tibialis anterior muscle of mature sheep. After implantation, muscle regeneration and angiogenesis were observed, while no reverse reactions occurred.

SF solutions usually become gel after a self-assembly process, in which hydrogen bonding and chain rearrangement takes place. This leads to the

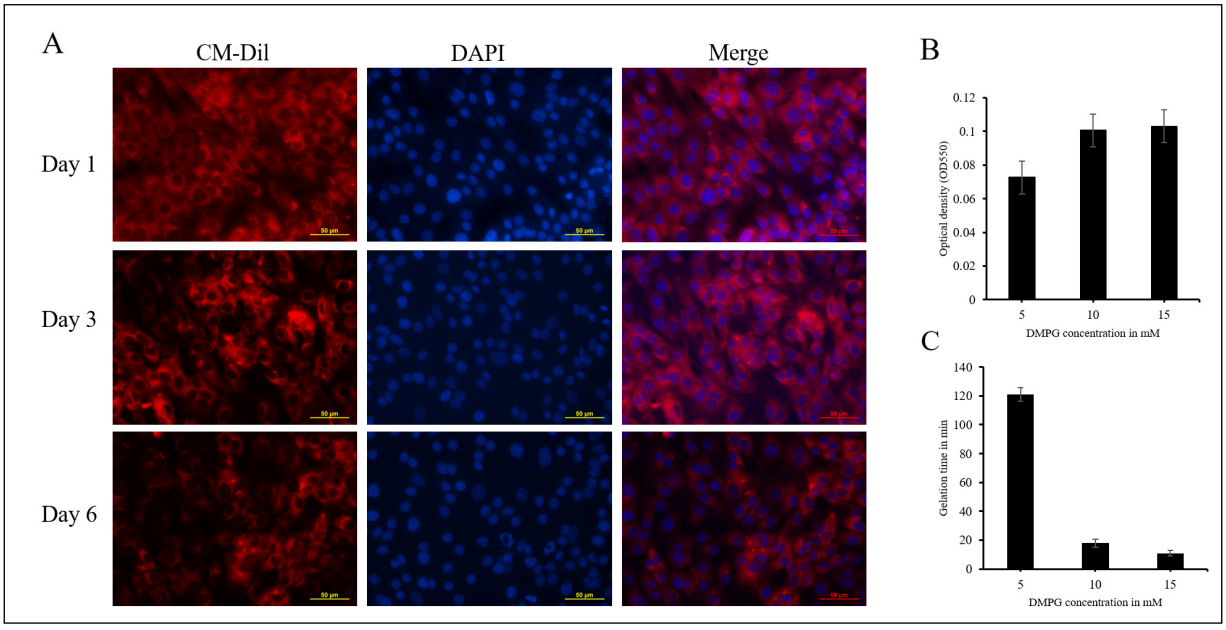

Figure 4. A, Cytoplasm of living cells stained with CM-Dil, DAPI staining for nucleus, Merge was mixture of Desmin and Myod1; scale bar: 100 µm. **B,** Absorbance at 550 nm of the muscle-derived stem cells seeded in the SF scaffolds over 24h. **C,** The average gelation time after induction by DPMG in varying concentrations.

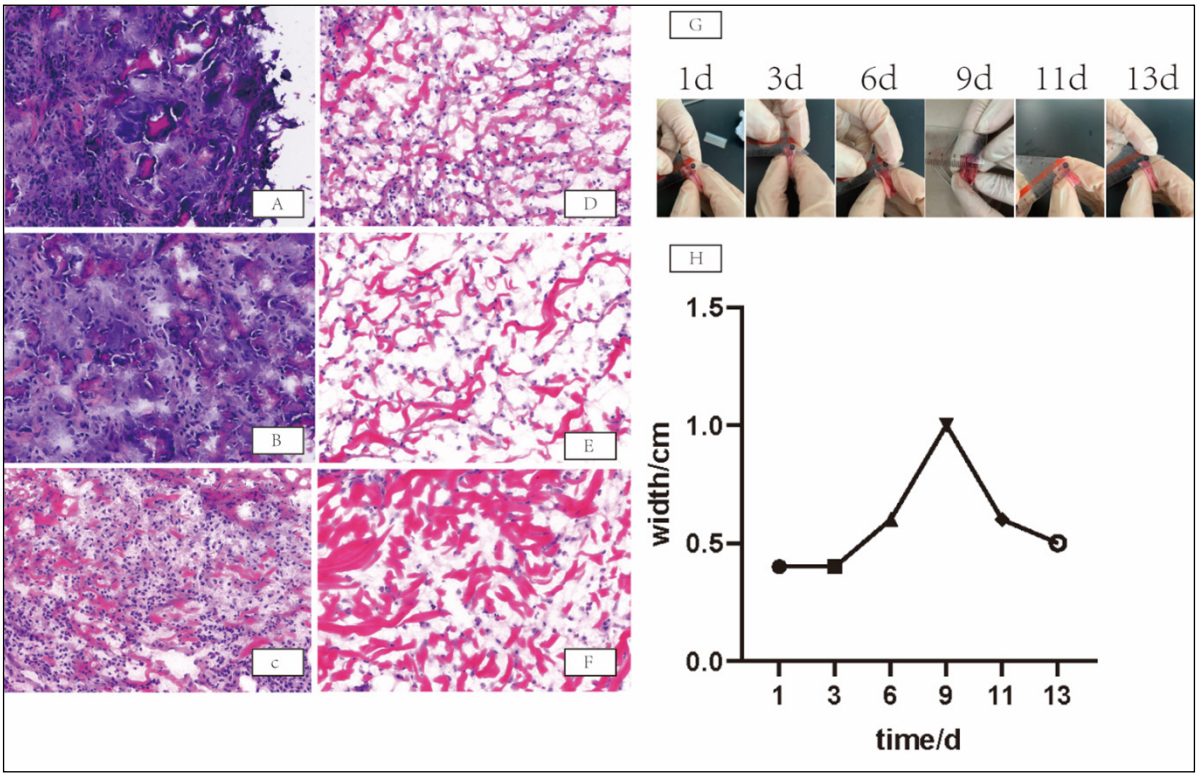

Figure 5. A-F, Representative photomicrographs of the scaffolds with H&E staining, A: day 1, B: day 3, C: day 6, D: day 9, E: day 11, F: day 13. The blue areas are hydrogels, and the red areas indicate muscle fibers. **G,** Represents the tissue mass taken from the *Hu sheep* on different days, and the diameter was measured with a 10 cm ruler. **H,** Variation in the diameter of tissues over time.

process of random coils transforming into highly stable β sheets. Since this process takes a very long time, they are not feasible as a 3D cell carrier.

Immunohistochemical methods were used to identify the muscle stem surface markers Desmin and Myod1 to distinguish muscle-differentiated cells. The proliferation and differentiation of cells in hydrogels were observed by using CM-Dil staining combined with fluorescence technique. CM-Dil, a derivative of Dil, was used to label cells, which is suitable for cell movement monitoring and cell localization analysis. Because of its good dye maintainability, it can be used to track the movement characteristics of cells passing through multiple generations. The results showed that the high-water retention of the hydrogel could meet the basic needs of cell proliferation. The H&E dyeing test were used to observe the dynamic changes of the hydrogels in the sheep and revealed the mechanisms of the hydrogel as a scaffold. Since hydrogels in animals need certain time and space for their own β folding, we used a simulation test to control the setting time of water gel *in vitro*. The results indicated that the simulation results of in vitro experiments could also condense in the body and have the effect of an extracellular matrix. One of the biggest challenges in tissue engineering is the lack of a vascular network that can transfer nutrients and oxygen to the transplanted scaffold, which is vital for its survival and the full recovery of tissue structure and functionality[31-33]. The gel acts as an extracellular matrix, which can deliver oxygen and nutrients to the cells. At the same time, it discharges metabolic waste products, thereby supporting the needs of the cell for early growth. To examine the degradation, its tolerance, and growth, we implanted the SF hydrogel into the tibialis anterior muscle of sheep. In the process of degradation, hydrogel is bound to enhance the immune capacity of the body. Our results showed that the 3% SF hydrogel could not form new tissues and was degraded and absorbed in 24h, which resulted in it being undetectable in our specimens. A study by Wang et al[25], in which they compared water-based SF scaffolds to scaffolds with an organic solvent *in vivo*, suggested that scaffolds prepared from lower concentrations of SF solution were completely degraded by the

host animals. Although, they used a 6% SF solution, the degradation in our study resulting from implanting a 3% SF hydrogel solution is in line of expectation and similar to their results. However, the 10% DMPG SF solution was well tolerated, did not generate adverse reactions, and promoted cell growth. CD34 staining was applied to detect the newly formed vessels in this study. Immunohistochemical staining of CD34 visualized a large number of endothelial cells, indicating that SF degradation could induce proliferation of endothelial cells. In addition, CD31 staining indicated that the degradation products of the SF hydrogel could promote neo-vascularization.

Although the precise origin of muscle-derived stem cells has not been elucidated yet, it has been reported that these cells express the endothelial cell marker, von Willebrand factor, and seem to play a part in angiogenesis following injection into skeletal muscle[34]. Multiple studies[34-36] have shown that stem cells could release angiogenic growth factors, such as vascular endothelial growth factor (VEGF). Endothelial proliferation could result in the formation of new capillaries. VEGF and other nutrients can be directly exposed to muscle fiber tissue, accelerating the formation and change of blood vessels. SF hydrogel incorporated with VEGF and bone morphogenic protein-2 (BMPR2) has been reported to interact with each other resulting in an enhancement of angiogenesis and bone development[16,37, 8]. These results indicate a potential relationship between muscle-derived stem cells and endothelial cells[39]. Simultaneously, because the muscle-derived stem cells were derived from the sheep themselves, an immune rejection was avoided to the greatest extent which increased the survival rate of cells[12,40]. Hydrogels can begin to degrade over time, and the degradation products are digested and absorbed by *Hu sheep*. In short, hydrogels can be used as extracellular matrix in animals. Scaffolds can deliver nutrients needed by cells, as well as metabolites of cells to the culture medium. This reticular interconnection system is a perfect substitute for the extracellular matrix.

There are several limitations to this study. Firstly, we used a small sample size in our in vivo experiments, which could influence the statistical power of the research findings. Secondly, although we observed that the SF hydrogels seeded with muscle-derived stem cells promoted muscle regeneration and angiogenesis *in vivo*, we only did this for two weeks. Observation for an extended period of time is necessary to determine its long-time ability of regeneration and angiogenesis. Thirdly, the long-term safety of SF is still unknown. Experiments conducted with a longer timespan could also evaluate its safety by measuring the immunogenicity and adverse effects. Further studies with larger sample sizes and extended duration are necessary to comprehensively evaluate its effectiveness.

Conclusions

The findings in this study have shown that SF hydrogels seeded with muscle-derived stem cells can promote muscle regeneration and an-

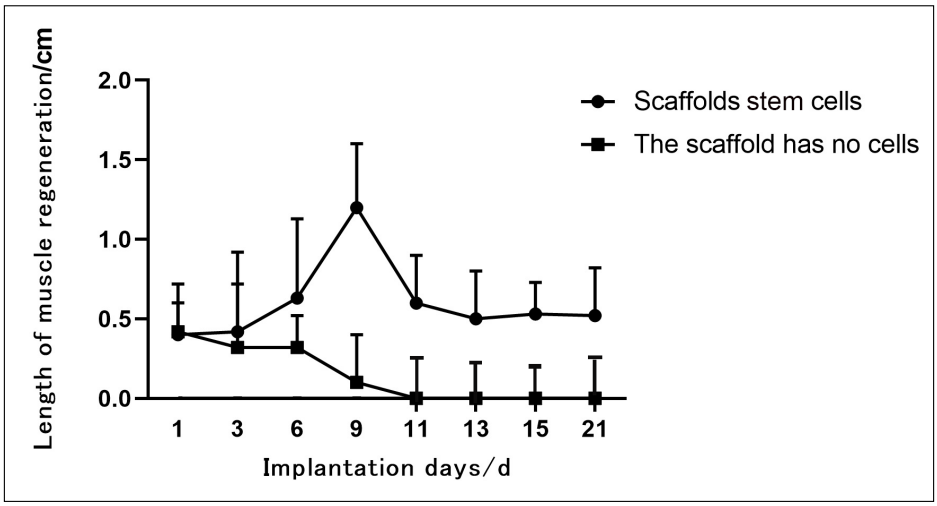

Figure 6. Length of muscle regeneration in the sheeps following implantation of SF hydrogel with muscle-derived stem cells, or control pure SF.

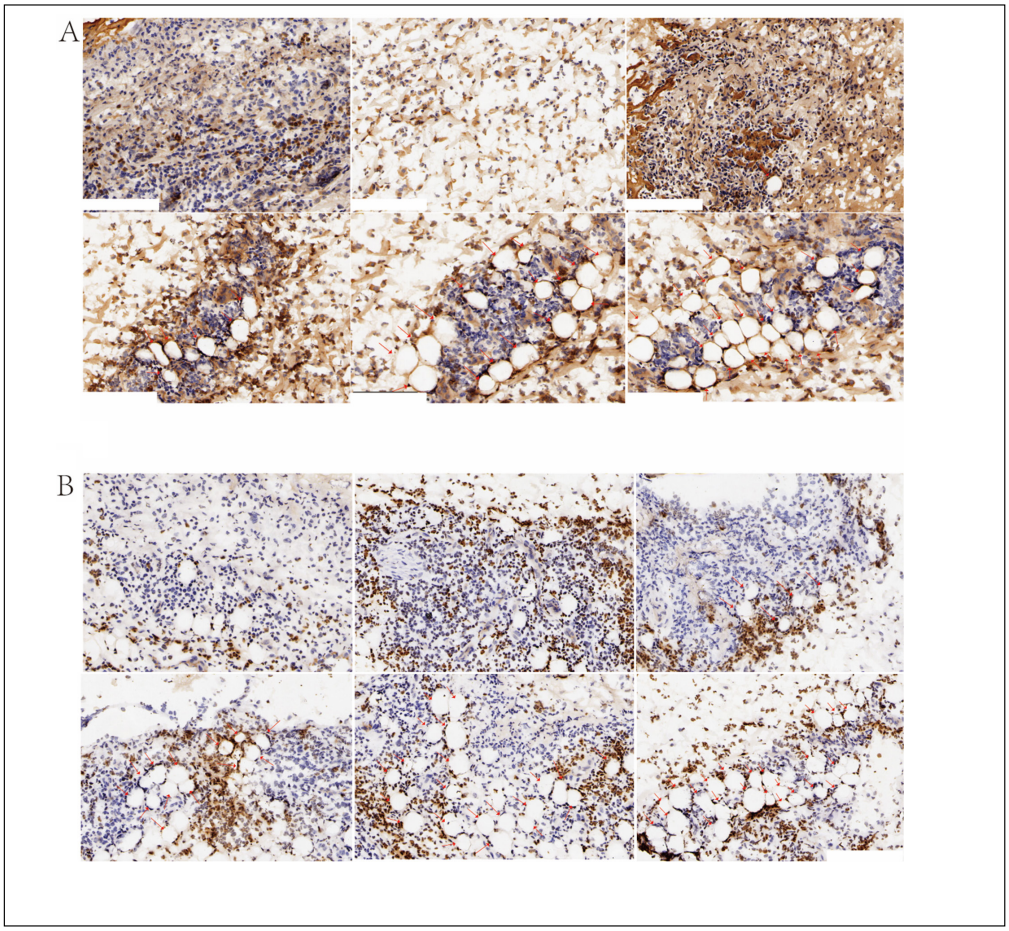

Figure 7. Immunohistochemistry analysis. (**A**) Photomicrographs of CD34 stained tissues (**B**) Photomicrographs of CD31 stained tissues indicating day 1 to 13 of vascular changes, the red arrows indicate blood vessels; scale bar: 100 μm.

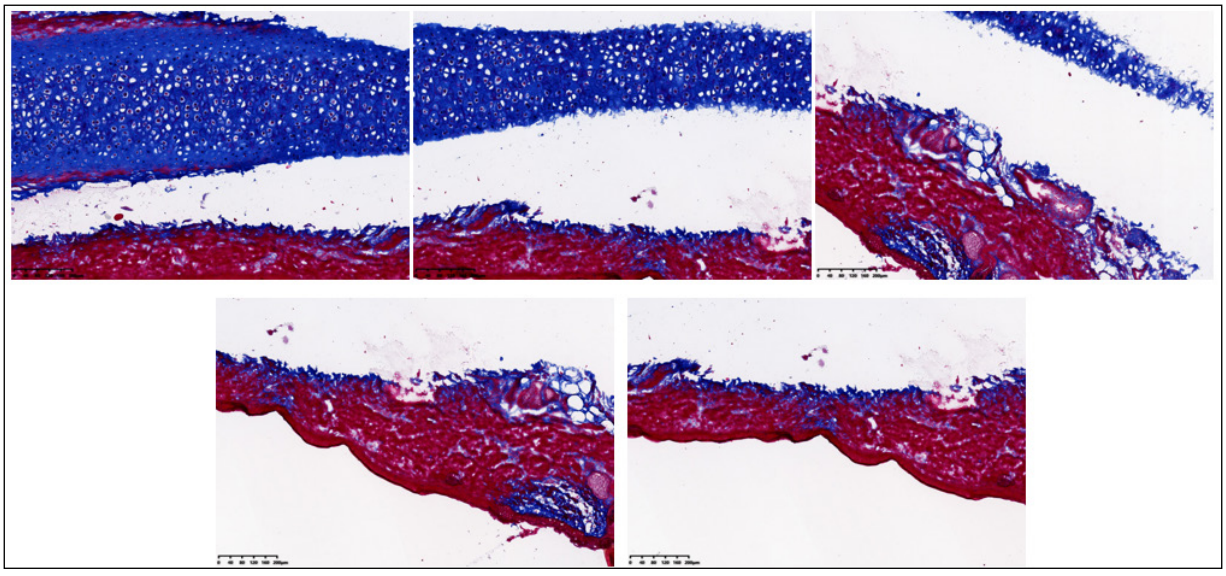

Figure 8. Immunohistochemistry analysis. Photomicrographs indicating day 1 to 13 of changes following implantation of pure SF hydrogel; scale bar: 200 μm.

giogenesis in a sheep model without leading to any adverse reactions or immunogenicity. Furthermore, intramuscular injection has proven to be an innovative and relatively simple method which minimizes the impact on animals in experiments.

With more future studies conducted in this field, we expect that SF scaffolds will increase their potential in tissue engineering and regenerative medicine.

Acknowledgements

This study was supported by the Zhejiang Basic Public Welfare Research Project; Qingshan Lake Science and Technology City Joint Fund, grant number LQY19F010002.

Conflicts of Interest

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

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