Healing effect of acellular fish skin with plasma rich in growth factor on full-thickness skin defects

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Abstract
Acellular skin as a scaffold has a good potential to regenerate or repair damaged tissues. Growth factors such as Plasma Rich in Growth Factor (PRGF) as a rich source of active proteins can accelerate tissue regeneration. In this study, an acellular scaffold derived from fish skin with growth factors was used to repair full-thickness skin defects in a rat model. Cellular results demonstrated that epithelial cells adhere well to acellular scaffolds. The results of animal studies showed that the groups treated with acellular scaffold and growth factor have a high ability to close and heal wounds on the 28th day after surgery. Histological and staining results showed that in the treated groups with scaffold and growth factor, an epidermal layer was formed with some skin appendages similar to normal skin. Overall, such scaffolds with biological agents can cause an acceptable synergistic effect on skin regeneration and wound healing.

Keywords
acellular fish skin, animal study, full-thickness skin defects, growth factor, wound healing

Key Messages
- acellular skin derived from fish skin as a scaffold has a good potential to repair damaged skin tissue
- plasma rich in growth factor (PRGF) as a rich source of active proteins can accelerate the wound healing process
- groups treated with scaffold and growth factor showed a high ability to heal wounds on the twenty-eighth day after surgery
- treated groups showed an epidermal layer with some skin appendages similar to normal skin in rat animal models

1 | INTRODUCTION

The annual number of deaths due to burns is increasing in poor countries. On the other hand, the lack of a burn care system like effective wound dressing can increase the statistics. Use of the inexpensive dressings and available biomaterials in the field of regenerative medicine can help such communities. Tissue engineering is one of the novel fields in regenerative medicine with help of the scaffold, cells, and signals would be able to repair or
regenerate the damaged tissues and organs. The scaffold with or without cells and signals can play a key role in skin tissue regeneration. In skin tissue regeneration, the scaffolds act as a temporary extracellular matrix (ECM) for cells, and also a strict barrier against microbes to provoke and propel the process of wound healing. Various types of tissue-based therapies such as autograft, allograft, and xenograft have been used for this purpose, but none of the methods has been able to meet all the needs of an ideal skin replacement. Use of the biomaterials as natural scaffolds derived from animals such as bovine and porcine skins have been good alternatives for skin tissue regeneration. Unlike the mentioned mammals’ skins, the fish skin as a scaffold for wound healing does not contain prions or viruses that can be transmitted to humans. The fish skin is a multifunctional tissue with suitable physical and mechanical properties, and most importantly owning excellent antimicrobial properties against pathogens can be an ideal alternative for skin regeneration. Compared to fish collagen sponges or hydrogels, Acellular Fish Skin (AFS) retains its collagen and natural structure and has a significant advantage over mammalian skin. These properties maintain the mechanical strength of the structure and create a suitable microenvironment for tissue repair and regeneration. The fish skin does not need complex chemical processes to decellularize, and even keeps the structure and composition of biologically active compounds, including omega-3 unsaturated fatty acids during the decellularization process. In general, studies have shown that the fish skin can accelerate the wound healing process, even faster than mammalian-derived matrices. Although many studies focused on the skin of tilapia or wild Atlantic cod, we chose the skin of grass carp as a scaffold for wound healing. Grass carp is one of the domestic fishes in Iran that is widely cultivated and has a high annual production. Availability, high growth rate, and the large area of carp skin compared to tilapia as well as its low price are the advantages of this species of fish. Plasma Rich in Growth Factors (PRGF) as part of autologous plasma rich in proteins and effective growth factors can speed up the process of tissue regeneration. The Platelet Rich Plasma (PRP) derivatives successfully have been used in clinical applications such as wound healing, cosmetic surgery, nerve regeneration, orthopaedics, and chronic ulcers. PRGF like PRP plays an important role in the processes of tissue regeneration due to owning growth factors such as Transforming Growth Factor (TGFβ), Insulin-like Growth Factor (IGF-1), Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), and different cytokines & chemokines. Although the growth factors have a high potential for tissue regeneration, the half-life of most of them is defined in the range of minutes, so sustained delivery of the growth factors is a key factor in achieving great clinical results. The synergetic effects of a scaffold and a biological agent like the growth factors have been proven as an effective method in tissues regeneration. Extracellular matrices have been demonstrated as promising carriers for growth factors. For example, resorbable collagen sponges well were bonded with recombinant BMP-2 (Bone Morphogenetic Protein-2) and BMP-7 (Bone Morphogenetic Protein-7) and used as carriers for the growth factors in preclinical and clinical processes. The effective adsorption and sustained release of the growth factors linked to Hydrated Acellular Dermal Matrix (HADM) were studied on different days, and their kinetics were investigated for HADM, with an initial burst release and a stable amount of the growth factor release within a secondary phase, so it may be useful for the long-term tissue regeneration. In this paper, the synergistic effects of acellular fish skin as a scaffold with PRGF/PRF gel were studied on acute full-thickness wounds in a rat model, and then results were evaluated using histological and immunohistochemical analyses.

2 | MATERIALS AND METHODS

2.1 | Preparation of scaffold/PRGF

Acellular Fish Skin (AFS) as a scaffold was designed with different methods in the previous study. In brief, Fresh Grass Carp skin (Mazandaran Fisheries Department, Tonekabon, Iran) was prepared, cleaned, and then plunged in hypertonic solution for a significant time. The solution contains 0.5 M sodium chloride and 25–50 mM tris (hydroxyl methyl) aminomethane and 10 mM ethylene di-amine tetra-acetic acid (EDTA). The skin was exposed to Triton X100 (0.5% w/v) for 24 hours, then washed with PBS and cold distilled water after exposure to hypertonic saline, and finally freeze-dried. The resulting acellular fish skin was about 0.25 mm thick. To prepare PRGF, blood was collected from the rat jugular vein before induction of anaesthesia. For each rat, 5 mL of the blood samples were drawn into 12 different tubes containing buffered 3.8% CPDA-1 (Sigma-Aldrich, Germany C4431-50ML), and then the samples were centrifuged (850 g) for 10 minutes. This procedure separates the blood samples into different fractions such as platelets and growth factors like PRGF. Before using at the anastomosis site, 1 mL of the prepared suspension was transferred to a Petri dish with 50 μL of 10% calcium gluconate (Caltrex, Sina Daru Pharmaceutical Company, Tehran, Iran) to activate the sample. Finally, the AFS scaffolds
(15 × 15 mm) were incubated for 10 minutes in 2000 μL of PRGF solution under immersion in sterile conditions at 37°C. The prepared fibrin gel containing growth factors (GF gel) was also used to place at the bottom of the wound. All processes are shown in Figure 1.

### 2.2 Cell study

Epithelial cells (CHL-1: human melanoma cell line) were selected to assess cell activities such as cell adhesion and morphology in samples. The scaffolds were cut into 1 × 1 cm² and exposed with 10,000 cells in a culture medium containing RPMI1640 and 10% bovine fetal serum and penicillin/streptomycin and L-glutamine (Gibco, Germany) at 37°C with 5% CO₂. The samples were dehydrated with ethanol (sigma, Germany), then exposed to osmium tetroxide (Sigma, Germany) vapour at 4°C for 2 hours. The samples were coated with gold and examined by Scanning Electron Microscope (SEM) (Cambridge Stereo-scan, S-360).

![Decellularization process diagram](image)

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### 2.3 Surgical procedures

At the beginning of the study, forty male Wistar rats aged approximately 4–8 weeks and weighing 180–220 g were divided into four groups. The protocols were approved by the institutional animal care and use committee of Shahid Beheshti University of Medical Sciences (Iran) [Ethical code: IR.SBMU.REC.1398.059]. Animals were handled according to the guidelines established for animal care at the center. They were anaesthetised by 80 mg/kg ketamine; 10 mg/kg xylazine injection, and their back were shaved and swabbed with povidone-iodine followed by 70% ethanol. Full-thickness wounds of 15 × 15 mm were made by excising the skin within the confines of the square down to the level of subcutaneous panniculus carnosus. Acellular fish skins were disinfected in hydrogen peroxide solution, rinsed thoroughly with PBS buffer, and purified water. Finally, the samples were sterilised with gamma ray (60Co; dose: 15 kGy). The samples were grafted on the wounds using 10–0 nylon at 0.75 cm intervals. Among 40 skin defects, 10 defects were grafted with AFS without GF gel, 10 defects only with GF...
gel (2000 μL), 10 defects with AFS loaded with GF gel (2000 μL), and also 10 defects with gauze as a blank control (control group). The wounds were covered with a standard compress to prevent the scaffolds from detaching and drying out. Every day, the animals were examined for pain, distress, or postoperative complications.

2.4 Histological assays

After 28 days of transplantation, the wounds were removed and stained for histological evaluations. The tissues were cut, and fixed in 10% formalin for 5 days at 4°C, then dehydrated, and embedded with paraffin. Paraffin sections (2 μm) were prepared with a Senior Precision Rotary Microtome (Model-RMT-30, Radical Instruments, and Haryana, India) and stained with haematoxylin and eosin according to histological protocols. The presence of skin appendages such as hair follicles & sebaceous glands, epithelialization, inflammation, and angiogenesis were qualitatively and quantitatively evaluated by optical microscope (Nikon, Mellville, NY). Some of the histological parameters were scored from 1 to 3 according to previous reports (Table 1).

2.5 Immunohistochemical analysis

The presence of epidermal keratinocytes in the rat skin equivalents was investigated by cytokeratin 10 expression. The formalin-fixed skin sections were exposed with Anti-Cytokeratin 10 antibody DE-K10 (ab9026, Abcam), and then the sections were heated in a citrate buffer (pH: 6) for 20 minutes to improve antigen retrieval for K10. Finally, the samples were incubated with the monoclonal antibody to cytokeratin at 1:17 (v/v) in TBS-2% BSA for 16 hours at 4°C.

2.6 Statistical analysis

The values for all samples were evaluated as the mean ± standard deviation (SD). Statistically significant differences; \( P < .05 \) in a time group and \( P < .01 \) between groups were investigated using Student’s t-test and one-way analysis of variance (ANOVA) with Tukey’s post hoc multiple comparison test for all groups.

3 RESULTS AND DISCUSSION

Figure 2A,B show cross-sectional images of the decellularized samples. The porous AFS showed a variable pore size from 20 to 100 μm which can be a suitable environment for cell adhesion and proliferation. In general, the dense surface layer, as well as the porous middle layer of the scaffold, can promote the growth of cells in the skin regeneration process. Figure 2C,D show cell adhesion on the re-cellularized AFS scaffolds by SEM at different magnifications. The results demonstrated that epithelial cells have good adhesion on the scaffolds.

The results also showed that the wounds in all groups closed after 28 days. The closest difference was observed among the groups of GF gel, AFS scaffold, and AFS/GF with wound closure of almost 82%, 85%, and 100%, respectively. By contrast, the control groups demonstrated 76% wound closure. Although there was no statistically significant difference between the groups of GF gel and AFS scaffolds, a significant improvement in wound healing was observed in the groups treated with the combination of GF gel and AFS scaffolds (Figures 3 and 4).

On day 28, histological evaluation of wound healing according to the degree of inflammation, angiogenesis, granulation tissues, and re-epithelialization were performed (Figure 5 and Table 2). There were no signs of severe inflammation in all treated groups (Table 2). There was illustrated any inflammation in the treated samples with AFS. Several studies demonstrated that acellular fish skin (AFS) can decrease antimicrobial, anti-inflammatory and analgesic effects. The fish skin as an absorbable scaffold can prevent bacterial attacks and accelerate the regeneration process due to the presence of ECM materials such as collagen and omega-3 fatty acids. Unsaturated fatty acids in fish skin increase signalling activity in the inflammatory phase of wound healing as well as tissue remodelling. Figure 5 shows histological images of all treated groups and normal skin. Well-improved epidermal layers also were shown in histological images, especially

| Score | Re-epithelialization | Granulation tissue deposition | Collagen deposition | Inflammatory cell | Angiogenesis |
|-------|----------------------|-----------------------------|---------------------|------------------|-------------|
| 0     | No                   | No or immature              | No                  | No               | No          |
| 1     | Partial              | Small amount                | Small amount        | Small amount     | Less than 5 vessels |
| 2     | Completed immature   | Moderately mature           | Moderate            | Moderate         | 6–10 vessels |
| 3     | Completed mature     | Mature                      | Abundant            | Abundant         | More than 10 vessels |
in the AFS-treated groups. Skin appendages and a thick bundle of collagen deposition as well as granulation tissue were observed in the dermal layer for the AFS/GF grafts, while they were well not found in the treated groups with GF only. Most likely, this low effectiveness can be due to their short lifespan, which will not provide enough time to regenerate skin appendages. However, PRGF can accelerate the regeneration process by recalling fibroblasts, angiogenesis, as well as ECM secretion.  

Identification of keratinocytes was performed with cytokeratin 10, a specific marker for epithelial differentiation. The increase in epidermis formation and the presence of keratinocytes in the treated groups with AFS/GF was almost similar to the normal skin (Figure 6).

The presence of a rich source of growth factors and cytokines in platelets can accelerate wound healing. In the process of re-epithelialization, growth factors such as EGF, IGF-1, FGF-2, and TGFα, as key elements, play an important role in processes such as angiogenesis, cell migration, cell proliferation, and differentiation to ultimately enhance extracellular matrix production. Growth factor-rich plasma (PRGF) is considered as a rich source of proteins and circulating growth factors for tissue regeneration. Simple extraction, availability, cost-effectiveness, and safety are some of the advantages of PRGF in wound healing. Our study showed that wound

![FIGURE 2](image-url) SEM images of the Acellular Fish Skin (AFS) scaffold in cross-section (A and B), and re-cellularized scaffold at different magnifications (C and D). The yellow arrow indicates the cell attachment to the scaffold and the formation of an extracellular matrix and filamentous appendages, and the red arrow indicates the cells dividing on the surface of the scaffold substrate.

![FIGURE 3](image-url) Wound healing for control samples (Non-treated on the first day (A) and the 28th day (B)), and grafted samples: GF only (C), scaffold without GF (D), and scaffold with GF (E) on the 28th day.
healing using PRGF/PRF with AFS is faster than the others, which may be due to the protection of growth factors against enzymes, as well as control of their release in the damaged area by scaffolds. Several studies have shown that such factors may inhibit hyper-inflammation, and affect macrophages to improve wound healing. According to our results, some authors observed a small or moderate inflammatory process using PRGF/PRF (GF gel) on day 28, and others on day 7. López-Jornet et al. used PRGF as an effective factor for wound healing in rabbits. Their results showed that the use of PRGF accelerates epithelialization and reduces inflammation on day 28. Our study also showed that the epithelialization process occurs for all samples, especially the treated samples with AFS/GF. Some studies have shown that PRGF is able to accelerate the re-epithelialization process.

Histological studies using tilapia skin as a scaffold for wound healing revealed a stratified squamous epithelium.
with broad layers of collagen. Collagen is one of the most important components of ECM in mammalian skin. Studies have shown that type I collagen in fish skin stimulates Fibroblast Growth Factors (FGF), as well as Keratinocyte Growth Factor (KGF), as two important cytokines for wound healing. Moreover, the use of skin dressings keeps tissue moisture, which is a key factor in speeding up the epithelialization process. Our results showed that the scaffold allows the epithelial cells to grow, thereby reducing wound size and the epithelial distance between the wound margins. The AFS/GF scaffold increases the process of epithelialization and wound closure by the formation of dense collagen and some skin appendages. The results showed that the GF gel with AFS scaffold eventually improved the wound closure process and the wounds were completely closed on day 28 (P < .05) (Figures 3 and 4). Angiogenesis and the number of blood vessels are other important factors in the wound healing process because blood vessels provide the oxygen and nutrients of cells in the regeneration process. VEGF is one of the most potent angiogenic cytokines, is secreted by platelets, macrophages, fibroblasts, and induces angiogenesis. The results showed that AFS or GF gel and their combination increased the number of newly formed blood vessels, and this function can regulate and accelerate the wound healing process. The AFS scaffolds and PRGF also showed a high ability to form collagen at the wound site. Therefore, the Acellular Fish Skin (AFS) scaffold due to its rich source of collagen and amino acids such as proline and alanine can enhance the proliferation of fibroblasts, formation of the granulation tissue, and synthesis of collagen in the wound site.

| Sample                          | Control | GF  | AFS | AFS/GF |
|---------------------------------|---------|-----|-----|--------|
| Size of deep scar tissue (mm)   | 7.8     | 1.8 | 1.5 | 0.8    |
| Collagen deposition             | 2       | 2   | 3   | 3      |
| Re-epithelialization            | 1       | 3   | 3   | 3      |
| Inflammatory cells PMN / Monocyte| 1       | 1   | 0   | 0      |
| Angiogenesis                    | 2       | 3   | 3   | 3      |
| Granulation tissue deposition   | 2       | 3   | 3   | 3      |

Note: Inflammatory cells are composed of lymphocytes and mast cells.

**TABLE 2** Histological evaluation of the tissues in all groups on day 28 after grafting

**FIGURE 6** Expression of epidermal markers (cytokeratin-10) on day 28. (A) The normal skin, (B) GF only, (C) AFS without GF, (D) AFS with GF. Scale bar: 50 mm
CONCLUSION

The advantages of using biodegradable scaffolds based on fish collagen include high biocompatibility, no allergic and inflammatory reactions, and invoking wound margin cells to accelerate wound healing, as well as its easy and cost-effective preparation and use. One of the important points in this research is the use of autologous growth factor sources, which can be mentioned as a turning point in personalised medical goals in terms of safety and no worries about disease transmission. In this study, the Acellular Fish Skin (AFS) as a rich source of collagen and other factors with GF gel as an effective growth factor were used for healing the acute full-thickness skin wounds in a rat model. The acellular fish skin as a scaffold with GF gel showed an intact epithelium with the formation of some skin appendages almost similar to the normal skin. The synergistic potential of the AFS loaded with GF gel can be used as an efficient skin graft in wound healing.

CONFLICT OF INTEREST

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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