

Use of Decellularized Scaffolds Combined with Hyaluronic Acid and Basic Fibroblast Growth Factor for Skin Tissue Engineering

Zhengzheng Wu, MS,^{1,*} Lina Fan, MS,^{2,*} Bin Xu, MS,³ Yongliang Lin, MS,³
Peng Zhang, MS,³ and Xing Wei, PhD¹

Skin damage is one of the common clinical skin diseases, and the main cure is the use of skin graft, especially for large area of skin injury or deep-skin damage. However, skin graft demand is far greater than that currently available. In this study, xenogeneic decellularized scaffold was prepared with pig peritoneum by a series of biochemical treatments to retain normal three-dimensional tissue scaffold and remove cells and antigenic components from the tissue. Scaffold was combined with hyaluronic acid (HA) plus two different concentrations of basic fibroblast growth factor (bFGF) and tested for its use for the repair of skin wounds. HA enhanced bFGF to adsorb to the decellularized scaffolds and slowed the release of bFGF from the scaffolds *in vitro*. A total of 20 rabbits were sacrificed on day 3, 6, 11, or 14 postsurgery. The wound healing rate and the thickness of dermis layer of each wound were determined for analyzing the wound repair. Statistical analysis was performed by the two-tailed Student's *t*-test. Wounds covered with scaffolds containing 1 $\mu\text{g}/\text{mL}$ bFGF had higher wound healing rates of 47.24%, 74.69%, and 87.54%, respectively, for days 6, 11, and 14 postsurgery than scaffolds alone with wound healing rates of 28.17%, 50.31%, and 61.36% and vaseline oil gauze with wound healing rates of 24.84%, 42.75%, and 57.62%. Wounds covered with scaffolds containing 1 $\mu\text{g}/\text{mL}$ bFGF showed more dermis regeneration than the other wounds and had dermis layer of 210.60, 374.40, and 774.20 μm , respectively, for days 6, 11, and 14 postsurgery compared with scaffolds alone with dermis layer of 116.60, 200.00, and 455.40 μm and vaseline oil gauze with dermis layer of 82.60, 186.20, and 384.40 μm . There was no significant difference in wound healing rates and thickness of dermis layer between wounds covered with scaffolds containing 1 and 3 $\mu\text{g}/\text{mL}$ bFGF on days 3, 6, 11, and 14 postsurgery. The decellularized scaffolds combined with HA and bFGF can be further tested for skin tissue engineering.

Introduction

SKIN IS THE LARGEST ORGAN in the human body and consists mainly of the outermost epidermis, the underlying dermis, a subcutaneous adipose-storing hypodermis layer, and various appendages, such as hair follicles, sweat glands, and sebaceous glands.¹ Skin defect is one of the common clinical diseases.^{2–6} In the developed countries, it has been estimated that 1% to 2% of the population will experience chronic wounds during their lifetime.⁷ In the United States alone, chronic wounds affect about 6.5 million patients and over \$25 billion is spent each year on wound-related complications.⁸ Wound healing is a complex biological process and involves four continuous and overlapping phases:

hemostasis, inflammation, proliferation, and remodeling.^{9–11} Because the epidermis layer is very thin, the common skin damages often hurt the dermis layer and even the subcutaneous tissue. So far, the main method of skin defect treatment is by skin transplantation.⁴ But, lack of available skin from autografts and allografts is a major limiting factor for wound healing. It demands the development of various artificial skin replacements. However, artificial skin replacements are relatively expensive, difficult to use, and prone to infection.¹² Some studies reported the successful use of decellularized tissue scaffolds in the repair of injured skin, liver, trachea, cartilage, blood vessels, and other tissues.^{13–18} Xenogenous decellularized dermis was used in clinic for skin burns and accelerated skin tissue repair.^{19,20}

¹Institute of Biomedicine, College of Life Science and Technology, National Engineering Research Center of Genetic Medicine, Jinan University, Guangzhou, China.

²Multidisciplinary Research Center, College of Science, Shantou University, Shantou, China.

³Grandhope Biotech Co. Ltd, Guangzhou International Business Incubator, Guangzhou Science Park, Guangzhou, China.

*The first two authors contributed equally to this article.

Studies showed that basic fibroblast growth factor (bFGF) promoted wound repair.²¹ It can significantly accelerate the proliferation of epidermal cells, fibroblasts, vascular endothelial cells, and keratinocytes *in vitro*.²² The bFGF effect on wound healing is closely related to its dose and action duration.²³ The main disadvantage of bFGF is its short half-life *in vivo*. So, it is necessary to provide sustained release of bFGF in the wound healing process.²⁴ Hyaluronic acid (HA) is a natural polysaccharide composed of N-acetylglucosamine and glucuronic acid sugar units.²⁵ HA is one of the composition materials present in skin and lacks immunogenicity as an ideal product for human use.²⁶ It is easily degraded and is used in different forms for wound repair.^{25–29}

The immune response of allogeneic skin grafts results mainly from the cellular components of skin. Dermis contains fibroblasts, microvascular endothelial cells, and cells for forming appendages. Decellularized dermal matrix for repairing skin defects can overcome the problem of immune rejection. Skin regeneration process is slow and easily leads to excessive scar formation and poor wound healing. Decellularized scaffold combined with growth factors, such as bFGF and epidermal growth factor, may provide an effective solution for promoting wound healing process.^{21,22,30–33} The objective of this study is to test decellularized scaffold combined with HA and bFGF for wound healing.

Materials and Methods

Experimental animals

The animals used in this study were from Medical Laboratory Animal Center of Guangdong (Guangzhou, China). Peritoneum was removed from healthy pigs for the preparation of decellularized scaffold. Healthy New Zealand white rabbits were used for skin experiment. All procedures involving experimental animals were conducted in accordance with the institutional guideline and approved by the Animal Care Committee of the Jinan University.

Preparation of decellularized scaffold

Pigs of the same species and age were raised in a clean house. The peritoneum was processed soon after it was removed from healthy pigs. Each batch of scaffolds was processed as follows in a sterile hood by the same restrict procedure to insure the same good quality. Decellularized scaffolds were kept at 4°C before the transplantation into rabbits. Peritoneum was soaked overnight at 4°C in 1‰ benzalkonium bromide solution (Baiyun Pharmaceutical, Nanchang, China). It was soaked for 1 h in 70% ethanol (Chemical Reagent, Guangzhou, China) and overnight in 30% ethanol. It was repeatedly rinsed with pure water to remove residual ethanol and soaked in pure water at 4°C for at least 1 h to make cells in the tissue expand. Cells in the tissue were removed by ultrasonic treatment. Then, 0.1 M polypropylene oxide (Haian Petrochemical Works, Jiangsu, China) was prepared and adjusted to pH 8.5 to 10.5 with NaOH. The peritoneum was soaked in 0.1 M polypropylene oxide for 1 week at room temperature for fully crosslinking the tissue and the solution was changed every 2 to 3 days. The peritoneum was thoroughly rinsed with phosphate buffered saline (PBS; Chemical Reagent) to remove residual polypropylene oxide. It was

cut into different sizes, and surface was coated with 1% sodium hyaluronate. It was sterilized with Co⁶⁰ irradiation after packaging to get decellularized scaffold.

Scanning electron microscopy (SEM)

Decellularized scaffold was placed at room temperature to dry before SEM (Philips XL-30, Amsterdam, Netherlands), and was cut into pieces of 0.5×0.5 cm². A layer of conductive adhesive was pasted to the dedicated SEM object stage, and samples were adhered to the stage, keeping observation surface up. The samples were examined by SEM.

Cytotoxicity assay

The decellularized scaffolds were soaked for 24 h in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco, Grand Island, NY). The culture medium was collected and used for the culture of NIH3T3 cells (American Type Culture Collection, ATCC, Manassas, VA). The original culture medium alone or containing 5% DMSO (Sigma, St Louis, MO) was used as controls. After incubation for 24, 48, and 72 h, cell viability was analyzed by MTT assay (Sigma). Optical densities were measured at 570 and 650 nm as reference wavelengths using a spectrophotometer (Bio-Rad 680, Hercules, CA).

Examination of the attachment of HA to scaffold

Decellularized scaffold was cut into pieces of 2.5×2.5 cm² in sterile procedure. It was placed in a sterile six-well plate (Corning, Corning, NY), and 2.5 mL of 1% HA (Freda Biotechnology Company, Shangdong, China) solution was added to each well. After scaffold was incubated for 24 h at room temperature, it was washed with PBS with gentle shaking for 5 min each time for a total of three times. Then, scaffold containing HA was soaked in 2.5 mL PBS at 37°C for 12, 24, and 72 h to release adsorbed HA from scaffold. Scaffold containing HA was examined by SEM. Scaffold containing no HA was soaked in PBS as a control. Soaking solution was replaced with fresh PBS after soaking for 12 and 24 h. Each wash solution and soaking solution were analyzed for the release of HA by enzyme-linked immunosorbent assay (ELISA) test (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. Absorbance was read at 450 and 630 nm as reference wavelengths by a spectrophotometer (Bio-Rad 680).

Incubation of decellularized scaffold with bFGF solution

Decellularized scaffold containing HA was prepared according to the method described previously and soaked for 12 h at room temperature with 2.5 mL bFGF (PeproTech, Rocky Hill, NJ) solution at concentration of 1 or 3 µg/mL in a sterile six-well plate. Fifty microliters of soak solution was taken from each well after 3, 6, 9, or 12 h of incubation, and stored at –20°C for ELISA test (R&D Systems). Decellularized scaffold was washed with PBS with gentle shaking for 5 min each time for a total of three times. Wash solutions were stored at –20°C for ELISA test. The concentrations of bFGF were detected by ELISA test, and absorbance was read at 450 and 630 nm as reference wavelengths by a spectrophotometer (Bio-Rad 680).

Release of bFGF from decellularized scaffold

Decellularized scaffold containing HA and bFGF was soaked with 2.5 mL PBS in a sterile six-well plate and placed in a 37°C incubator to release adsorbed bFGF. Soak solution was replaced with 2.5 mL PBS after 12, 24, 72, or 144 h of incubation and was stored in a -20°C freezer for ELISA test, and absorbance was read at 450 and 630 nm as a reference wavelength by a spectrophotometer (Bio-Rad 680).

Animal skin experiment

Decellularized scaffold was cut into 4×5 cm² in the sterile condition. It was soaked for 24 h in 8 mL of 1% HA solution and washed for three times with PBS. Then, it was soaked for 12 h in 8 mL of 1 or 3 µg/mL bFGF solution and used for the following animal experiment.

Four full-thickness rectangular wounds were created at sizes of 2.5×3 cm² on each animal's skin. Rabbits are less expensive and easy to be taken care, have a relatively large skin surface, and are commonly used for research. So, rabbits were chosen for this study. A total of 20 rabbits were treated, and 80 defects were created. Rabbits were anesthetized by the injection of 0.5 mL atropine sulfate (Jixing Pharmaceutical Company, Shandong, China) and 0.15 mL of 3% pelltobarbitalum natricum (Chemical Plant, Beijing, China) per kg. The hair in four skin areas on the left and right sides of each rabbit spine was removed and the four skin areas were disinfected. The wounds were created by removing skin epidermis and dermis layers (about 1.2-mm depth). The wounds were covered by the vaseline oil gauzes or the scaffolds, which were sewed to the wound edge by nonabsorbable 0 suture (Jinhuan Medical Devices Factory, Yangzhou, China). The excised skin was kept for hematoxylin and eosin (HE) staining (Beyotime Biotechnology Co., Shanghai, China) as a normal control. One wound was covered with vaseline oil gauze, the second wound with

decellularized scaffold alone, the third with decellularized scaffold containing HA and 1 µg/mL bFGF, and the fourth with decellularized scaffold containing HA and 3 µg/mL bFGF. Animals were sacrificed on day 3, 6, 11, or 14 postsurgery, and vaseline oil gauzes and decellularized scaffolds were gently peeled off the wounds. A piece of skin was taken from each wound on different days postsurgery and processed for HE staining. The thickness of dermis layer of each wound was determined and blood was taken from each animal on day 3, 6, 11, or 14 postsurgery for the detection of bFGF in serum by ELISA test.

Calculation of wound healing rate

The lengths of upside, downside, left side, and right side of each wound were measured after the excision of skin areas as original wound areas and on day 3, 6, 11, or 14 postsurgery. The sizes of wound areas were calculated as follows: area of wound = (upside + downside)/2 × (left side + right side)/2. The wound healing rate was calculated as follows: wound healing rate = (original wound area - wound area on different days postsurgery)/original wound area.

Statistical analysis

All experiments in this study were independently repeated at least three times with similar results. The relative percentage and values are presented as the mean and standard deviation (SD) of the mean. Statistical analysis was performed by the two-tailed Student's *t*-test, and *p*-value < 0.05 was considered as statistically significant. In this study, there are a small number of samples. The data have a normal distribution, and the SD is unknown. So, Student's *t*-test was used. In this study, the average values (e.g., wound healing rates or the thickness of dermis layer) of experimental groups were compared with the control groups (e.g., vaseline oil gauze group). Only one test of statistics was made on the data so that Bonferroni correction was not used.

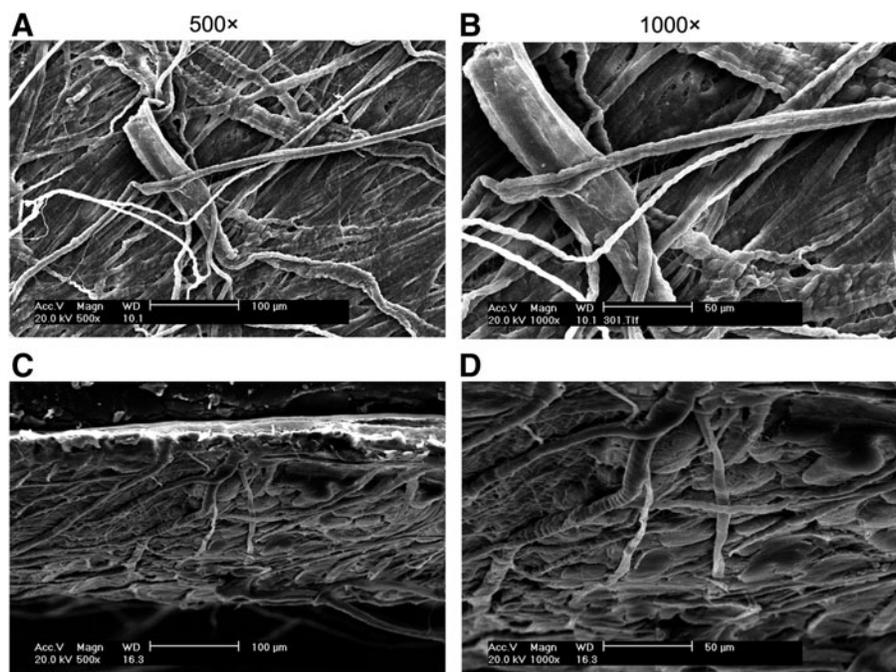


FIG. 1. Analysis of decellularized scaffolds by scanning electron microscopy (SEM). Both surface (A, B) and longitudinal sections (C, D) of decellularized scaffold were examined by SEM. Results showed that the scaffold was composed of the compact and long fibers and contained no cells.

Results

Analysis of scaffolds by scanning electron microscopy (SEM)

Decellularized scaffold was examined by SEM. The examination of both surface and longitudinal sections of decellularized scaffold showed that it was composed of the compact and long fibers and contained no cells (Fig. 1).

Cytotoxicity assay

After NIH3T3 cells were incubated for 24, 48, and 72 h, cell viability was analyzed by MTT assay. There is no significant difference in the viability of cells cultured with

the original culture medium or the culture medium collected after soaking with scaffolds, suggesting that the scaffolds are not toxic to NIH3T3 cells (data not shown).

Examination of the attachment of HA to decellularized scaffold

Longitudinal sections of scaffold containing HA were examined by SEM. Scaffold containing no HA was used as a control. Results showed that scaffold was covered by HA before and after soaking in PBS (Fig. 2A). Each wash solution and soaking solution were analyzed for the release of HA by ELISA test. Results showed that HA concentrations in wash solution decreased significantly after each wash, and there was no

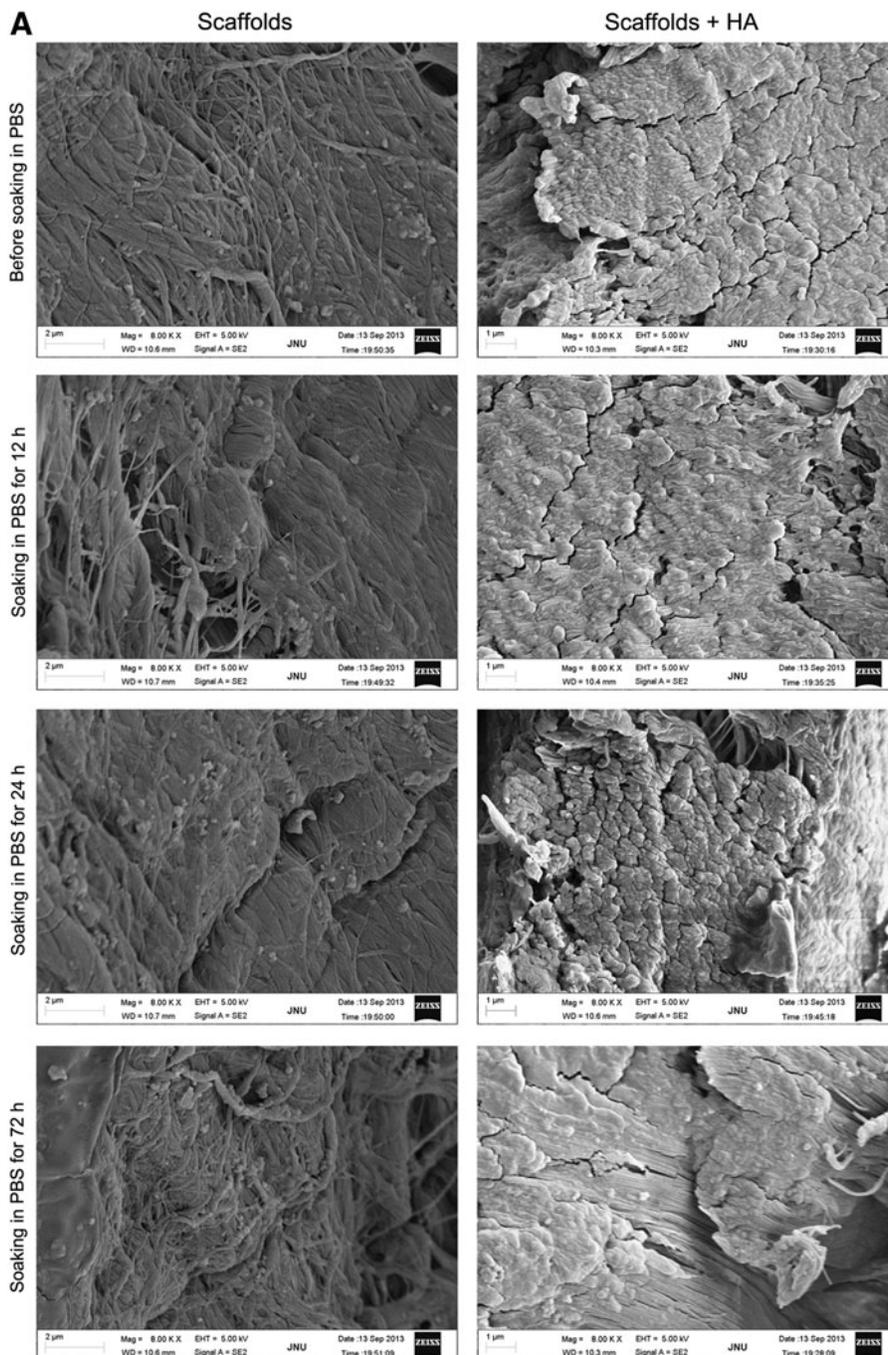
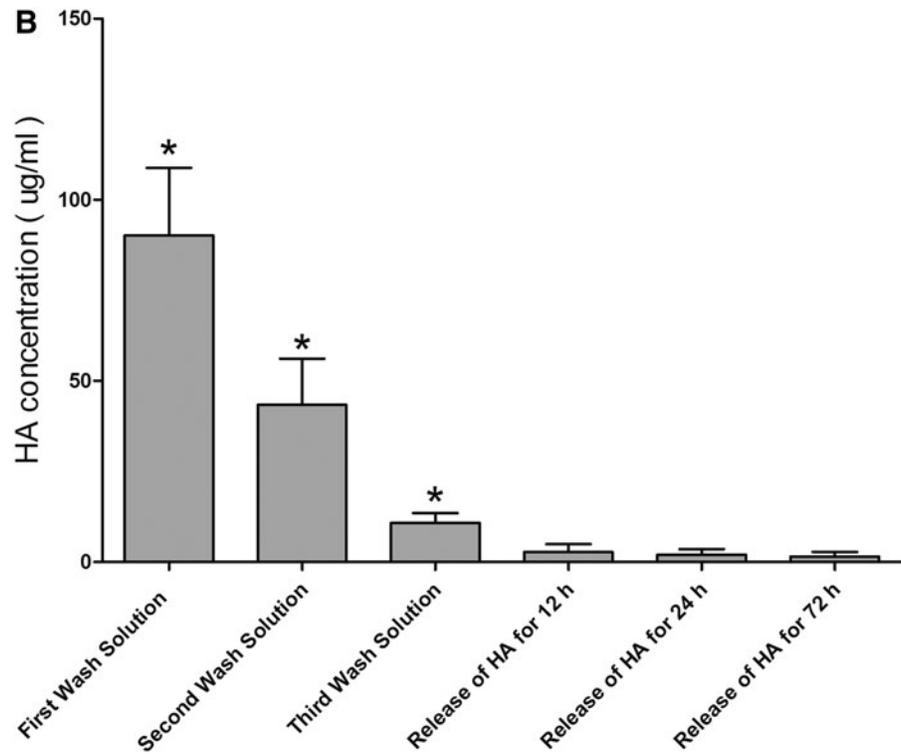


FIG. 2. Examination of the attachment of HA to decellularized scaffold. (A) After scaffold was soaked in 1% HA solution for 24 h, it was washed with PBS for three times. Then, scaffold containing HA was soaked in PBS for 12, 24, and 72 h to release adsorbed HA from scaffold. Longitudinal sections of scaffold containing HA were examined by SEM. Scaffold soaking in PBS containing no HA as a control. Results showed that scaffold was covered by HA before and after soaking in PBS. (B) Soaking solution was replaced with fresh PBS after soaking for 12 and 24 h. Each wash solution and soaking solution were analyzed for the release of HA by ELISA test. Results showed that HA concentrations decreased significantly after each wash and there was no significant difference in HA concentrations after soaking for different periods of time. * $p < 0.01$ compared with the release of HA for 72 h ($n = 5$). ELISA, enzyme-linked immunosorbent assay; HA, hyaluronic acid; PBS, phosphate-buffered saline.

FIG. 2. (Continued).



significant difference in HA concentrations in soaking solution after soaking for different periods of time (Fig. 2B). These results suggest that the attachment of HA to scaffold is stable.

Detection of bFGF in soak solution by ELISA test

The bFGF concentrations in soak solution decreased gradually along with the increase of immersion time when the initial concentration of bFGF was either 1 or 3 $\mu\text{g/mL}$, suggesting that amount of bFGF adsorbed into scaffold increased gradually along with the increase of immersion time (Fig. 3A, B). The higher initial concentration of bFGF (3 $\mu\text{g/mL}$) increased the adsorption of bFGF into the scaffold, compared with the lower initial concentration (1 $\mu\text{g/mL}$). In addition, the bFGF concentrations in soak solution decreased faster with scaffold previously soaked in HA solution than no-HA controls, suggesting that HA increased the adsorption of bFGF into scaffold.

Detection of bFGF released from decellularized scaffold by ELISA test

Concentration of bFGF decreased significantly in the second- and third-wash solutions compared with the first-wash solution, indicating that free bFGF was almost completely removed during the third wash (Fig. 3C, D).

Scaffold following three washes was soaked in PBS to release bFGF from scaffold. The concentrations of bFGF released from scaffold were detected by ELISA test and decreased along with the increase of incubation time (Fig. 3E, F). In addition, more bFGF was released from scaffold previously soaked in HA-containing solution than non-HA-containing solution, suggesting that HA increased the binding of bFGF to scaffold and prolonged the release of bFGF.

Examination of wound healing on different days postsurgery

On day 3 postsurgery, vaseline oil gauzes or decellularized scaffolds were gently peeled off the wounds. No empyema and no inflammatory reaction were observed in all four wounds, and wounds remained moist and contained vascular growth (Fig. 4A, B). There was no significant difference in wound healing rates on day 3 postsurgery (Fig. 4A, B and Table 1).

On day 6 postsurgery, decellularized scaffold stuck tightly to the wounds so that it was difficult to peel the scaffold off the wounds. Wounds covered with scaffold containing either 1 or 3 $\mu\text{g/mL}$ bFGF were significantly smaller than with vaseline oil gauzes or with scaffold alone (Fig. 4A, B and Table 1). Wounds covered with scaffolds containing 1 $\mu\text{g/mL}$ bFGF had higher wound healing rate of 47.24% on day 6 postsurgery than scaffolds alone with wound healing rate of 28.17% and vaseline oil gauze with wound healing rate of 24.84% (Fig. 4A, B and Table 1). In addition, wounds covered with scaffold containing either 1 or 3 $\mu\text{g/mL}$ bFGF had a thicker layer of white translucent granulation tissue that plays a key role in skin wound healing process, compared with the wounds covered vaseline oil gauzes.

On day 11 postsurgery, wounds covered with scaffold containing either 1 or 3 $\mu\text{g/mL}$ bFGF were also significantly smaller than with vaseline oil gauzes or with scaffold alone (Fig. 4A, B and Table 1). Wounds covered with scaffolds containing 1 $\mu\text{g/mL}$ bFGF looked close to normal skin and had higher wound healing rate of 74.69% on day 11 postsurgery than scaffolds alone with wound healing rate of 50.31% and vaseline oil gauze with wound healing rate of 42.75% (Fig. 4A, B and Table 1).

On day 14 postsurgery, the wound covered with scaffold containing 1 $\mu\text{g/mL}$ bFGF looked close to normal skin, and

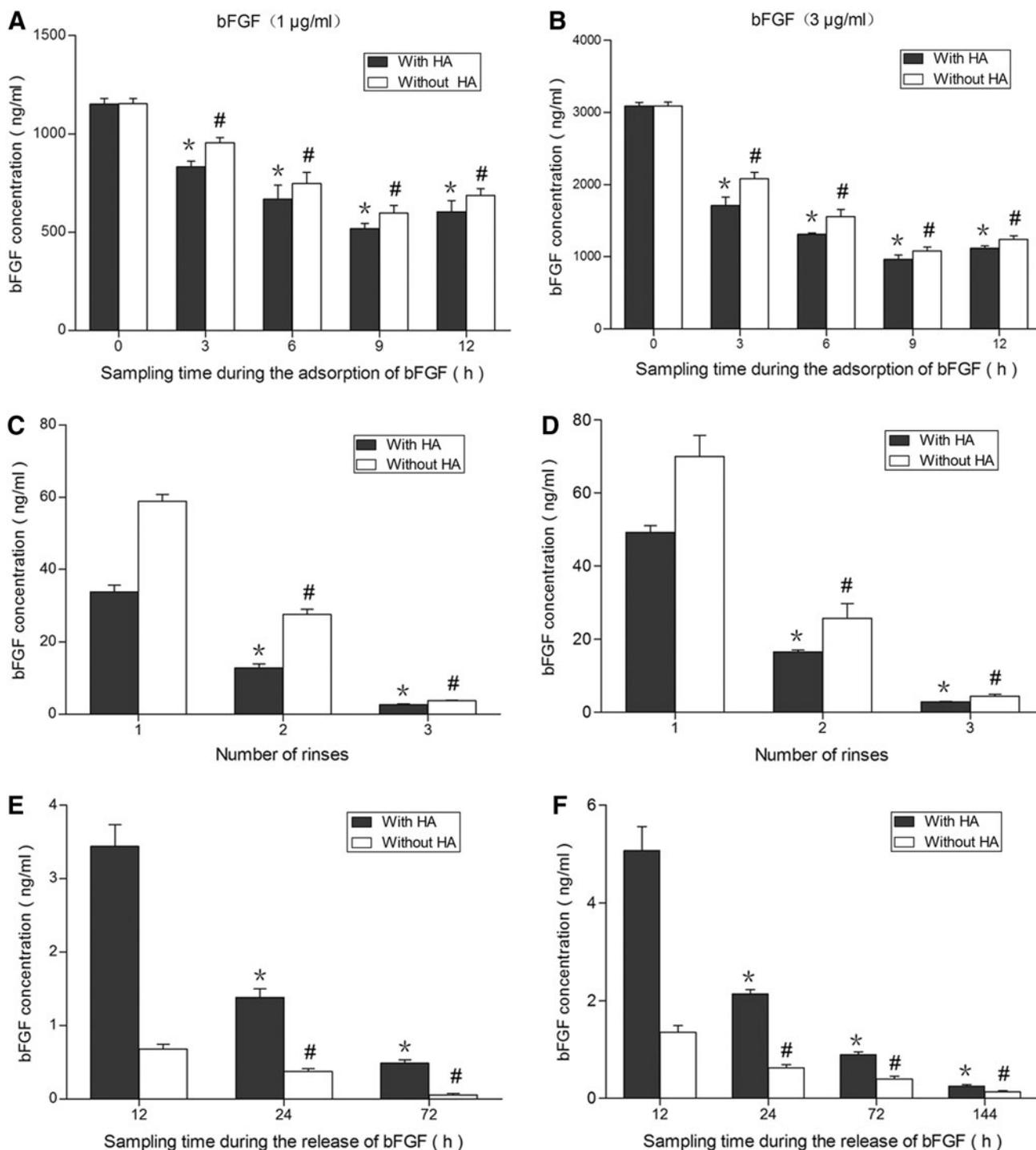
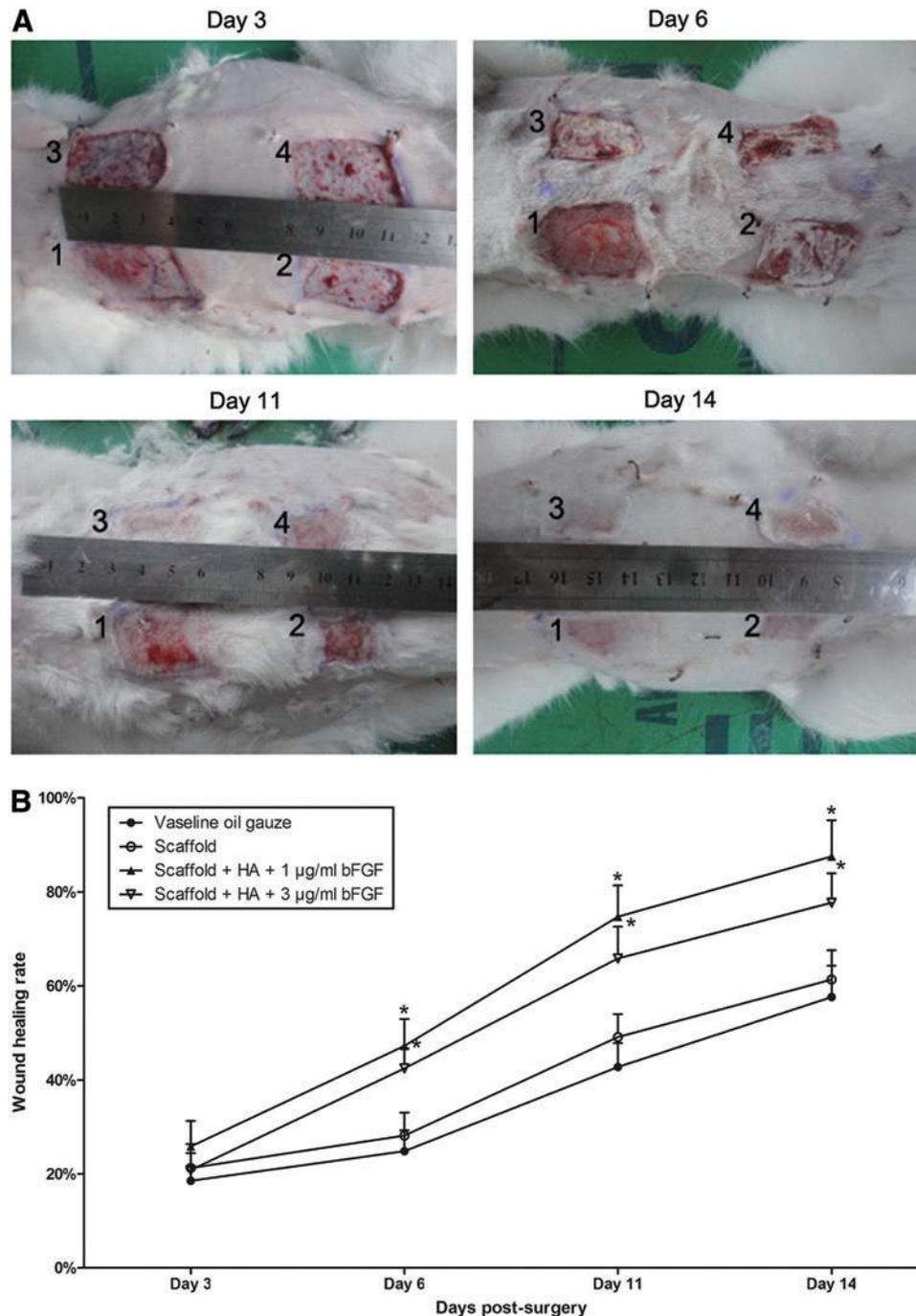


FIG. 3. Detection of bFGF by ELISA test. Decellularized scaffold was soaked in a solution containing either 1 µg/mL (A) or 3 µg/mL bFGF (B). bFGF remaining in the soak solution was detected by ELISA to estimate bFGF adsorbed into scaffold. The amount of bFGF in soak solution decreased gradually along with the increase of immersion time, suggesting that bFGF adsorbed into scaffold increased gradually along with the increase of immersion time. (C, D) Decellularized scaffold containing bFGF was washed three times with PBS to release the free bFGF. Concentration of bFGF decreased significantly in the second- and third-wash solutions compared with the first-wash solution. (E, F) Scaffold following three washes was soaked in PBS to release bFGF from scaffold. The concentrations of bFGF released from scaffold decreased along with the increase of incubation time. Effect of HA on bFGF adsorption into scaffold was also compared with no-HA control. * or # $p < 0.01$ compared with respective first sample ($n = 5$). bFGF, basic fibroblast growth factor.

FIG. 4. Examination of wound healing on different days postsurgery. (A) Four skin wounds were generated in each rabbit's back-skin area. One wound was covered with vaseline oil gauze (1). The second was covered with decellularized scaffold alone (2). The third was covered with decellularized scaffold containing HA and 1 $\mu\text{g}/\text{mL}$ bFGF (3). The fourth was covered with decellularized scaffold containing HA and 3 $\mu\text{g}/\text{mL}$ bFGF (4). Vaseline oil gauzes or decellularized scaffold was gently peeled off the wounds on day 3, 6, 11, or 14 postsurgery. (B) Calculation of wound healing rate. The lengths of upside, downside, left side, and right side of each wound were measured after the excision of skin area as original wound area and on day 3, 6, 11, or 14 postsurgery. Size of wound area and wound healing rate were calculated for each wound. Results showed that wounds covered with scaffold containing either 1 or 3 $\mu\text{g}/\text{mL}$ bFGF were significantly smaller than with vaseline oil gauzes or with scaffold alone, and the wound covered with scaffold containing 1 $\mu\text{g}/\text{mL}$ bFGF recovered best among all four wounds. * $p < 0.01$ compared with the respective vaseline oil gauzes ($n = 5$). Color images available online at www.liebertpub.com/tea



had higher wound healing rate of 87.54% than scaffolds alone with wound healing rate of 61.36% and vaseline oil gauze with wound healing rate of 57.62% (Fig. 4A, B and Table 1). It contained no scar tissue and no scab formation. There was no significant difference in wound healing rates between wounds covered with scaffolds containing 1 and 3 $\mu\text{g}/\text{mL}$ bFGF on days 3, 6, 11, and 14 postsurgery.

Examination of skin tissue after HE staining

On day 3 postsurgery, HE staining showed that hypodermis layer was the main tissue observed in each wound (Fig. 5A, E). Epidermis and dermis layers were removed by

surgery and were not yet regenerated in all four wounds. There was no significant difference in the regeneration of four wounds. On day 6 postsurgery, new epidermis and dermis layers were regenerated from outside toward inside of each wound. Thicker dermis layers were observed in the two wounds covered by decellularized scaffold containing HA and either 1 or 3 $\mu\text{g}/\text{mL}$ bFGF than the other two wounds (Fig. 5B, E and Table 2). Wounds covered with scaffolds containing 1 $\mu\text{g}/\text{mL}$ bFGF had dermis layer of 210.60 μm on day 6 postsurgery compared with scaffolds alone with dermis layer of 116.60 μm and vaseline oil gauze with dermis layer of 82.60 μm . On day 11 postsurgery, thicker epidermis and dermis layers were observed in the

TABLE 1. WOUND HEALING RATES ON DIFFERENT DAYS POSTSURGERY IN THE ANIMAL EXPERIMENT

Days postsurgery	n	Wound healing rate (%)			
		Vaseline oil gauze	Scaffold	Scaffold+ HA + 1 µg/mL bFGF	Scaffold+ HA + 3 µg/mL bFGF
Day 3	1	17.18	19.63	20.19	20.96
	2	20.21	23.50	31.15	23.61
	3	14.18	29.03	24.19	15.56
	4	19.34	17.67	24.96	19.42
	5	21.76	16.53	31.74	24.54
	Average	18.53 ± 2.94	21.27 ± 5.08	26.45 ± 4.91	20.82 ± 3.58
Day 6	1	25.12	29.95	40.84	49.37
	2	29.60	32.90	49.53	39.82
	3	25.81	31.95	41.84	39.37
	4	26.13	21.91	54.23	43.75
	5	17.56	24.15	49.78	39.87
	Average	24.84 ± 4.42	28.17 ± 4.88	47.24 ± 5.72	42.44 ± 4.26
Day 11	1	37.48	41.46	81.09	68.34
	2	50.80	54.01	65.01	67.91
	3	43.48	56.46	71.09	58.34
	4	39.65	51.96	79.87	73.43
	5	42.34	47.65	76.43	64.13
	Average	42.75 ± 5.07	50.31 ± 5.91	74.69 ± 6.66	66.43 ± 5.60
Day 14	1	59.23	67.46	81.60	84.05
	2	67.90	65.71	93.31	75.81
	3	57.21	58.46	77.60	68.05
	4	50.76	52.03	95.54	79.06
	5	52.98	63.14	89.65	81.56
	Average	57.62 ± 6.65	61.36 ± 6.22	87.54 ± 7.68	77.71 ± 6.20

bFGF, basic fibroblast growth factor; HA, hyaluronic acid.

wound covered by scaffold containing HA and either 1 or 3 µg/mL bFGF than the other two wounds (Fig. 5C, E and Table 2). Wounds covered with scaffolds containing 1 µg/mL bFGF had dermis layer of 374.40 µm on day 11 postsurgery compared with scaffolds alone with dermis layer of 200.00 µm and vaseline oil gauze with dermis layer of 186.20 µm. On day 14 postsurgery, thicker epidermis layers were observed and stratum corneum was more visible in the two wounds covered by decellularized scaffold containing HA and either 1 or 3 µg/mL bFGF than the other two wounds (Fig. 5D, E and Table 2). Wounds covered with scaffolds containing 1 µg/mL bFGF had dermis layer of 774.20 µm on day 14 postsurgery compared with scaffolds alone with dermis layer of 455.40 µm and vaseline oil gauze with dermis layer of 384.40 µm. Skin appendages were observed only in the wound covered by decellularized scaffold containing HA and 1 µg/mL bFGF. There was no significant difference in the thickness of dermis layer between wounds covered with scaffolds containing 1 and 3 µg/mL bFGF on days 3, 6, 11, and 14 postsurgery.

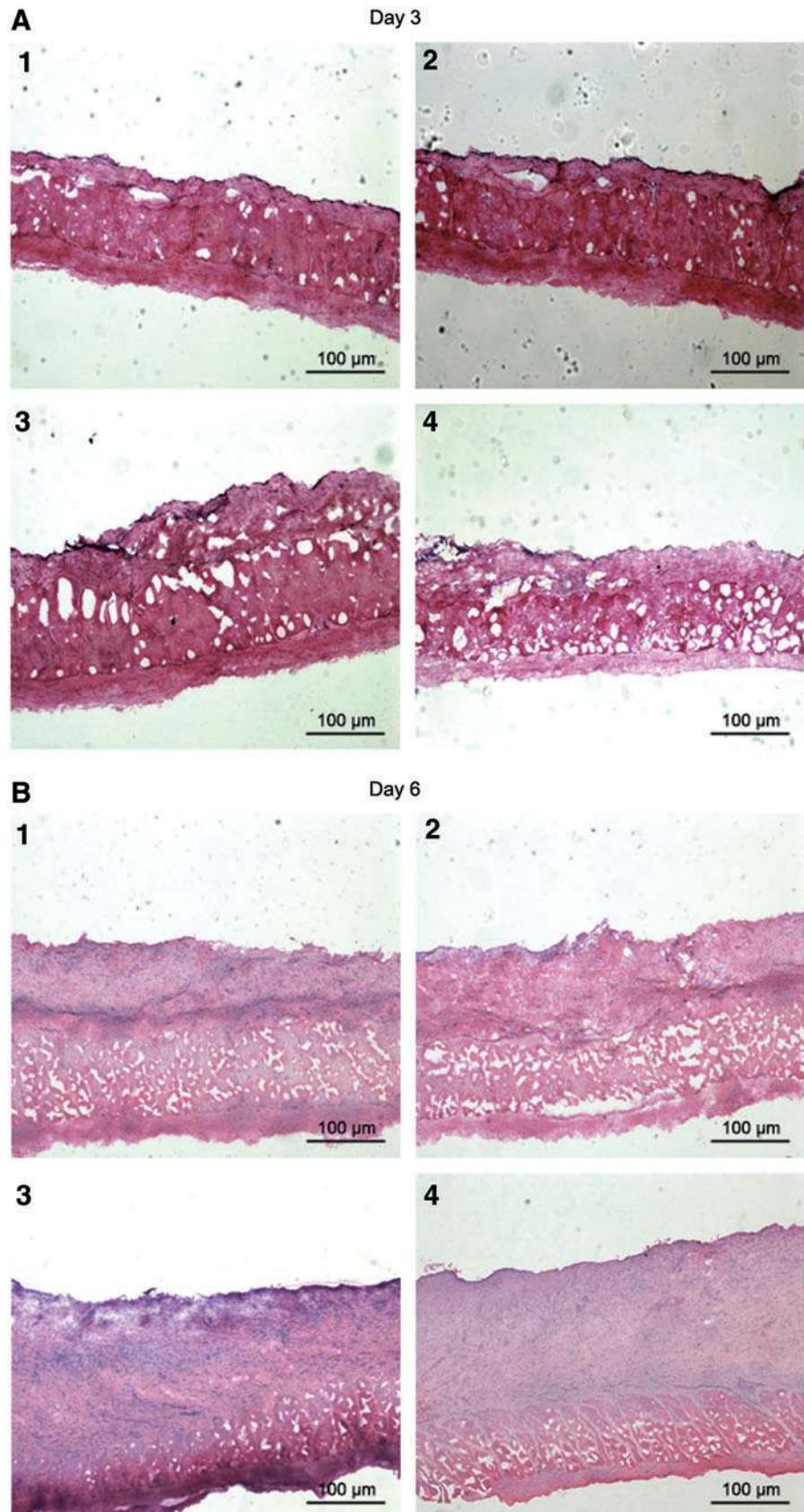
Discussion

Primary function of skin is to provide a protective barrier against the environmental damage. The loss of integrity of a skin portion due to burn, trauma, or illness may result in significant disability or even death.⁵ Skin grafting is the most common method for the treatment of skin defects, but there are a lot of limitations in traditional autologous skin grafts, far from being able to meet different requirements.³⁴

Therefore, it is urgent to find applicable skin substitutes for wound healing. The major focus of current research is to find a safe and effective scaffold, which can not only protect the wound, but can also promote wound healing and effectively repair skin defects. In recent years, a variety of skin substitutes was used in clinic and increased the cure rate of burn of a large and deep skin area.^{12,18,35,36} Currently, xenogeneic skin and xenogeneic decellularized dermal matrix were used in the clinic. Although xenogeneic skin has a high degree of similarity with human skin, it is immunogenic to cause immune rejection after implantation and may spread infectious agents, such as viruses and bacteria. Xenogeneic decellularized dermal matrix has a poor adhesion with a wound because of its texture.³⁷

The ideal skin substitutes should have following characteristics: good bionics, no immunogenicity and immune rejection after transplantation, good biocompatibility, good degradability, degradability after a period of wound healing, good elasticity, suitability for irregular wound surface, protective effect to prevent bacterial invasion, sufficient supply, and promotion of wound healing, which is the most important.³⁸ In this study, xenogeneic decellularized scaffold was used for wound healing. A series of biochemical techniques was used to remove cells and antigenic substances from tissues and retained normal three-dimensional tissue scaffold. This scaffold has excellent biocompatibility, good biomechanic compliance, and proper degradation. The methods used for the preparation of decellularized scaffold and addition of HA and bFGF to the scaffolds are relatively mild, do not cause physical or chemical damage to

FIG. 5. Examination of skin tissue after HE staining. Rabbits were sacrificed on day 3, 6, 11, or 14 postsurgery. A piece of skin was taken from each wound and processed for HE staining. The wound was covered with vaseline oil gauze (1), decellularized scaffold alone (2), decellularized scaffold containing HA and 1 $\mu\text{g}/\text{mL}$ bFGF (3), or decellularized scaffold containing HA and 3 $\mu\text{g}/\text{mL}$ bFGF (4). (A) Rabbits were sacrificed on day 3 postsurgery. Results showed that hypodermis layer was the main tissue observed in each wound. Epidermis and dermis layers were removed by surgery and were not yet regenerated in all four wounds. There was no significant difference in the regeneration of four wounds. (B) Rabbits were sacrificed on day 6 postsurgery. Results showed that thicker dermis layers were observed in the two wounds covered by decellularized scaffold containing HA and 1 and 3 $\mu\text{g}/\text{mL}$ bFGF than the other two wounds. (C) Rabbits were sacrificed on day 11 postsurgery. Results showed that thicker epidermis and dermis layers were observed in the wound covered by decellularized scaffold containing HA and 1 $\mu\text{g}/\text{mL}$ bFGF than the other three wounds. (D) Rabbits were sacrificed on day 14 postsurgery. Results showed that thicker epidermis layers were observed and stratum corneum was more visible in the two wounds covered by decellularized scaffold containing HA and 1 and 3 $\mu\text{g}/\text{mL}$ bFGF than the other two wounds. Skin appendages were observed only in the wound covered by decellularized scaffold containing HA and 1 $\mu\text{g}/\text{mL}$ bFGF. (E) The thickness of dermis layer of each wound was determined. Results showed that the thickness of dermis layer of the wound covered with scaffold containing 1 or 3 $\mu\text{g}/\text{mL}$ bFGF was significantly thicker than with vaseline oil gauzes or with scaffold alone on day 6, 11, or 14 postsurgery. $*p < 0.01$ compared with the respective vaseline oil gauzes ($n = 5$). HE, hematoxylin and eosin. Color images available online at www.liebertpub.com/tea



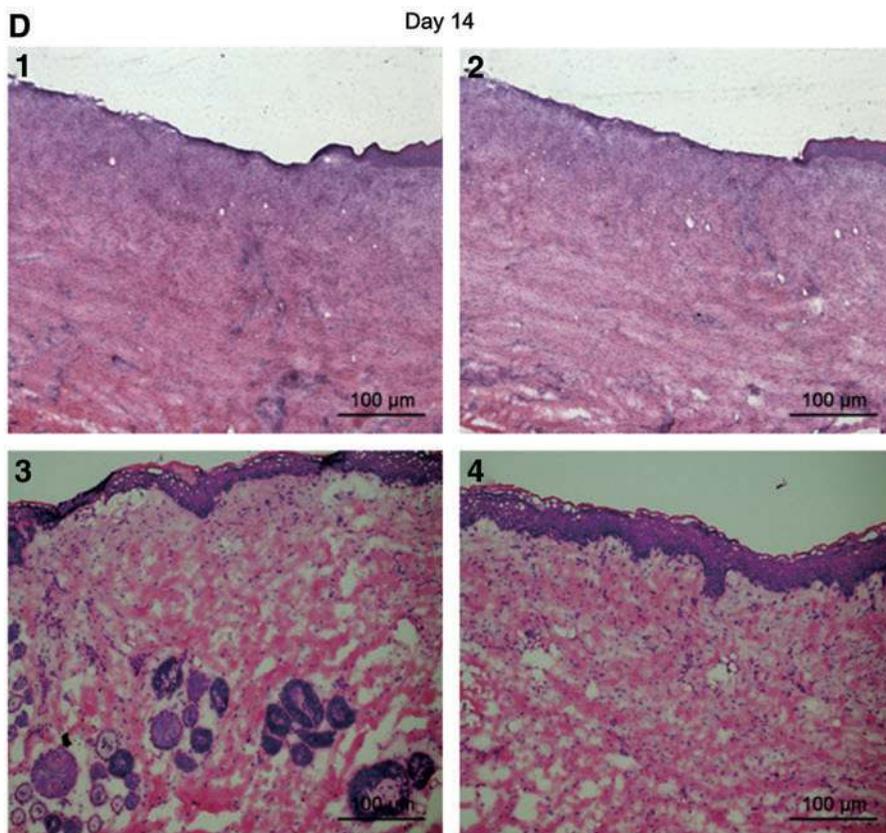
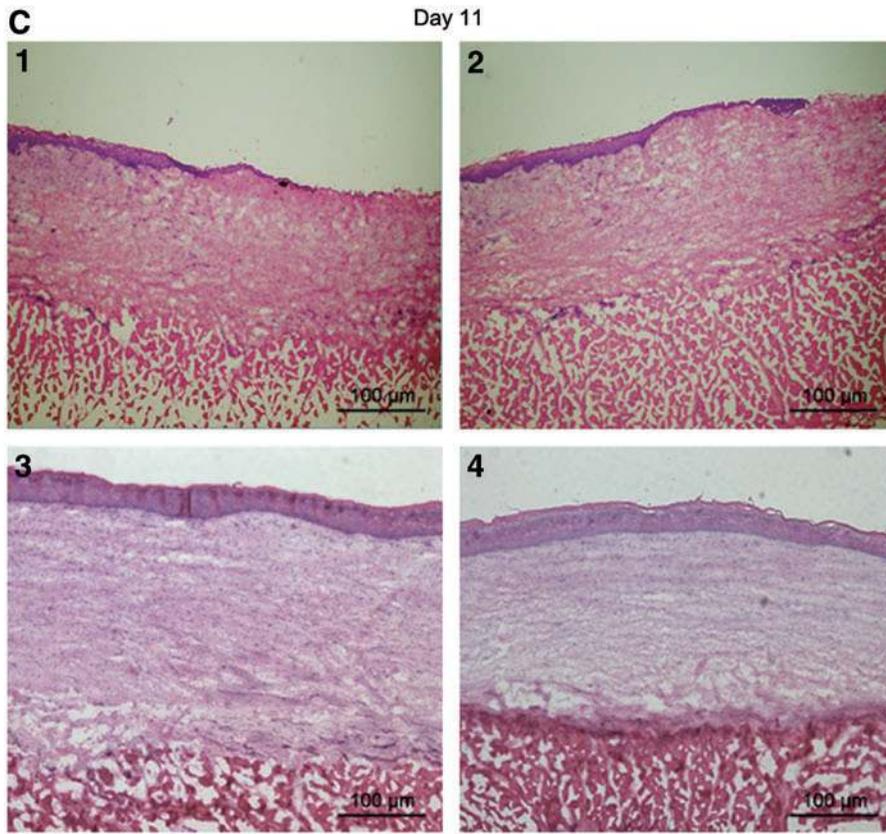


FIG. 5. (Continued).

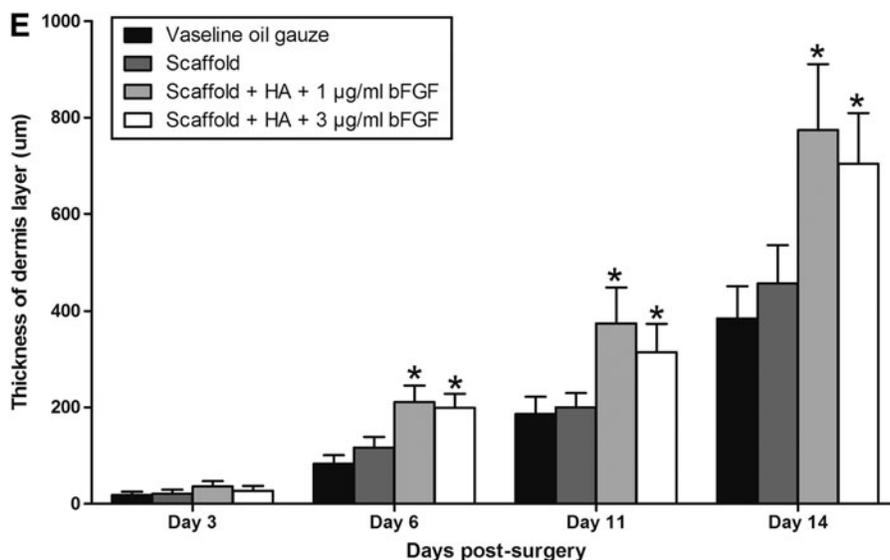


FIG. 5. (Continued).

decellularized scaffold, maintain its natural features, and increase the adsorption of HA and bFGF into the scaffold.

HA is a component of human skin tissue and an acidic mucopolysaccharide present in the form of polyanion. It has good moisture-retention ability and is an active ingredient for the skin healing without scar formation.²⁷ bFGF can promote wound healing and exists as a cationic polypeptide in a neutral environment. But excessive bFGF can cause inflammation and increase perivascular inflammatory re-

sponse and scar formation. In this study, HA was added to decellularized scaffold and bFGF was added later. HA enhanced bFGF to adsorb and bind tightly to the scaffold *in vitro* (Fig. 3). In addition, HA slowed the release of bFGF from the scaffold *in vitro*. After its transplantation into animals, HA may also slow the release of bFGF into the wounds and extend its effective time. Two different concentrations of bFGF were separately added into decellularized scaffold, and results showed that 1 µg/mL of bFGF

TABLE 2. THICKNESS OF THE DERMIS LAYER ON DIFFERENT DAYS POSTSURGERY IN THE ANIMAL EXPERIMENT

Days postsurgery	n	Thickness of the dermis layer (µm)			
		Vaseline oil gauze	Scaffold	Scaffold + HA + 1 µg/mL bFGF	Scaffold + HA + 3 µg/mL bFGF
Day 3	1	27	32	49	41
	2	11	14	22	15
	3	23	27	37	25
	4	13	13	29	19
	5	16	17	43	33
	Average		18.00 ± 6.78	20.60 ± 8.44	36.00 ± 10.77
Day 6	1	107	148	263	204
	2	83	127	188	236
	3	92	114	207	201
	4	63	91	174	153
	5	68	103	221	199
	Average		82.60 ± 17.90	116.60 ± 22.03	210.60 ± 34.34
Day 11	1	194	166	451	356
	2	135	217	340	296
	3	167	176	266	223
	4	221	238	421	374
	5	214	203	394	323
	Average		186.20 ± 35.48	200.00 ± 29.47	374.40 ± 73.07
Day 14	1	496	589	983	843
	2	324	381	611	549
	3	354	414	714	693
	4	381	437	759	703
	5	367	456	804	732
	Average		384.40 ± 65.84	455.40 ± 79.75	774.20 ± 136.91

promoted wound healing more efficiently and faster and caused less scar formation than 3 µg/mL (Figs. 4 and 5). It is unknown why 1 µg/mL of bFGF is more effective, and it may be due to the fact that only suitable amount of bFGF can most effectively promote the wound healing. Decellularized scaffold combined with HA and bFGF can be tested in human for its effect on the wound healing.

Previous study showed that among patients with endometrial cancer, the serum level of bFGF was higher in women with metastatic disease than in those with localized disease.³⁹ The serum level of bFGF is elevated in patients with lung cancer, renal cell cancer, non-small-cell lung cancer, and metastatic colorectal cancer.^{40,41} bFGF can promote cancer stem cell proliferation in many solid tumors⁴² and promote the development of tumor and the metastasis of tumor cells.⁴³ Previous study also showed that bFGF has a short half-life and can be quickly degraded *in vivo*. The amount of bFGF in serum is microscale and easily decomposed by proteinase. The tissue half-life of bFGF is only about 3–10 min.^{44–46} In this study, bFGF was undetected in the serum of the rabbits on day 3, 6, 11, or 14 postsurgery (data not shown). Although bFGF in serum can have adverse effect on tumor patients, bFGF on scaffolds may not be harmful to tumor patients due to the undetectable bFGF in rabbit serum and a short half-life *in vivo*.

Conclusions

Our results demonstrated that the decellularized scaffold combined with HA and bFGF significantly promoted wound healing and clearly increased the regeneration of epidermis and dermis layers in rabbits. It may be further studied for skin tissue engineering.

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant No. 30870650 and Grant No. 31171304 to Xing Wei), Research Foundation for Doctoral Discipline of Higher Education (Grant No. 20114401110007 to Xing Wei), and the Fundamental Research Funds for the Central Universities (Grant No. 21612107 to Xing Wei).

Disclosure Statement

No competing financial interests exist.

References

1. Brohem, C.A., da Silva Cardeal, L.B., Tiago, M., Soengas, M.S., de Moraes Barros, S.B., and Maria-Engler, S.S. Artificial skin in perspective: concepts and applications. *Pigment Cell Melanoma Res* **24**, 35, 2011.
2. Kondo, T., and Ishida, Y. Molecular pathology of wound healing. *Forensic Sci Int* **203**, 93, 2010.
3. Larson, B.J., Longaker, M.T., and Lorenz, H.P. Scarless fetal wound healing: a basic science review. *Plast Reconstr Surg* **126**, 1172, 2010.
4. van der Veen, V.C., van der Wal, M.B.A., van Leeuwen, M.C.E., Ulrich, M.M.W., and Middelkoop, E. Biological background of dermal substitutes. *Burns* **36**, 305, 2010.
5. Huang, S., and Fu, X. Naturally derived materials-based cell and drug delivery systems in skin regeneration. *J Control Release* **142**, 149, 2010.

6. Rodero, M.P., and Khosrotehrani, K. Skin wound healing modulation by macrophages. *Int J Clin Exp Pathol* **3**, 643, 2010.
7. Sen, C.K., Gordillo, G.M., Roy, S., Kirsner, R., Lambert, L., Hunt, T.K., *et al.* Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair Regen* **17**, 763, 2009.
8. Wong, V.W., and Gurtner, G.C. Tissue engineering for the management of chronic wounds: current concepts and future perspectives. *Exp Dermatol* **21**, 729, 2012.
9. Akhundov, K., Pietramaggiore, G., Waselle, L., Darwiche, S., Guerid, S., Scaletta, C., *et al.* Development of a cost-effective method for platelet-rich plasma (PRP) preparation for topical wound healing. *Ann Burns Fire Disasters* **207**, 25, 2012.
10. Li, J., Chen, J., and Kirsner, R. Pathophysiology of acute wound healing. *Clin Dermatol* **9**, 25, 2007.
11. Young, A., and McNaught, C.E. The physiology of wound healing. *Surgery (Oxford)* **475**, 10, 2011.
12. Nguyen, D.Q.A., Potokar, T.S., and Price, P. An objective long-term evaluation of Integra (a dermal skin substitute) and split thickness skin grafts, in acute burns and reconstructive surgery. *Burns* **36**, 23, 2010.
13. Shirakigawa, N., Ijima, H., and Takei, T. Decellularized liver as a practical scaffold with a vascular network template for liver tissue engineering. *J Biosci Bioeng* **114**, 546, 2012.
14. Zang, M., Zhang, Q., Chang, E.I., Mathur, A.B., and Yu, P. Decellularized tracheal matrix scaffold for tissue engineering. *Plast Reconstr Surg* **130**, 532, 2012.
15. Ting, T.S., Hashimoto, H.Y., Kishida, K.A., Ushida, U.T., and Furukawa, F.K. High-hydrostatic pressurization decellularized bovine articular cartilage scaffolds for cartilage tissue engineering. *J Tissue Eng Regen Med* **6**, 74, 2012.
16. Ye, Q., Wang, J., Deng, V., Kaplunovsky, A., Bhatia, M., Hofgartner, W., *et al.* Decellularized human placental vascular scaffold provides a platform for tissue and organoid engineering. *J Tissue Eng Regen Med* **6**, 169, 2012.
17. Mangold, S., Schrammel, S., Huber, G., Schmid, C., Liebold, A., and Hoenicka, M. Comparison of denuded and decellularized human umbilical vein as scaffolds for vascular tissue engineering. *J Tissue Eng Regen Med* **6**, 174, 2012.
18. Sun, T., Han, Y., Chai, J., and Yang, H. Transplantation of microskin autografts with overlaid selectively decellularized split-thickness porcine skin in the repair of deep burn wounds. *J Burn Care Res* **32**, e67, 2011.
19. Wainwright, D.J., and Bury, S.B. Acellular dermal matrix in the management of the burn patient. *Aesthet Surg J* **31**, 13S, 2011.
20. Patel, K.M., Nahabedian, M.Y., Gatti, M., and Bhanot, P. Indications and outcomes following complex abdominal reconstruction with component separation combined with porcine acellular dermal matrix reinforcement. *Ann Plast Surg* **69**, 394, 2012.
21. Heybeli, T., Kulacoglu, H., Genc, V., Ergul, Z., Ensari, C., Kiziltay, A., *et al.* Basic fibroblast growth factor loaded polypropylene meshes in repair of abdominal wall defects in rats. *Chirurgia (Bucur)* **105**, 809, 2010.
22. Yang, Y., Xia, T., Zhi, W., Wei, L., Weng, J., Zhang, C., *et al.* Promotion of skin regeneration in diabetic rats by electrospun core-sheath fibers loaded with basic fibroblast growth factor. *Biomaterials* **32**, 4243, 2011.
23. Yang, Y., Xia, T., Chen, F., Wei, W., Liu, C., He, S., *et al.* Electrospun fibers with plasmid bFGF polyplex loadings

- promote skin wound healing in diabetic rats. *Mol Pharm* **9**, 48, 2012.
24. Ayvazyan, A., Morimoto, N., Kanda, N., Takemoto, S., Kawai, K., Sakamoto, Y., *et al.* Collagen-gelatin scaffold impregnated with bFGF accelerates palatal wound healing of palatal mucosa in dogs. *J Surg Res* **171**, e247, 2011.
 25. Ferguson, E.L., Alshame, A.M.J., and Thomas, D.W. Evaluation of hyaluronic acid-protein conjugates for polymer masked-unmasked protein therapy. *Int J Pharm* **402**, 95, 2010.
 26. Slavkovsky, R., Kohlerova, R., Jiroutova, A., Hajzlerova, M., Sobotka, L., Cermakova, E., *et al.* Effects of hyaluronan and iodine on wound contraction and granulation tissue formation in rat skin wounds. *Clin Exp Dermatol* **35**, 373, 2010.
 27. Gao, F., Liu, Y., He, Y., Yang, C., Wang, Y., Shi, X., *et al.* Hyaluronan oligosaccharides promote excisional wound healing through enhanced angiogenesis. *Matrix Biol* **29**, 107, 2010.
 28. Galeano, M., Polito, F., Bitto, A., Irrera, N., Campo, G.M., Avenoso, A., *et al.* Systemic administration of high-molecular weight hyaluronan stimulates wound healing in genetically diabetic mice. *Biochim Biophys Acta* **1812**, 752, 2011.
 29. Anilkumar, T.V., Muhamed, J., Jose, A., Jyothi, A., Mohanan, P.V., and Krishnan, L.K. Advantages of hyaluronic acid as a component of fibrin sheet for care of acute wound. *Biologicals* **39**, 81, 2011.
 30. Hu, D.H., Zhang, Z.F., Zhang, Y.G., Zhang, W.F., Wang, H.T., Cai, W.X., *et al.* A potential skin substitute constructed with hEGF gene modified HaCaT cells for treatment of burn wounds in a rat model. *Burns* **38**, 702, 2012.
 31. Choi, J.K., Jang, J.H., Jang, W.H., Kim, J., Bae, I.H., Bae, J., *et al.* The effect of epidermal growth factor (EGF) conjugated with low-molecular-weight protamine (LMWP) on wound healing of the skin. *Biomaterials* **33**, 8579, 2012.
 32. Song, R., Bian, H.N., Lai, W., Chen, H.D., and Zhao, K.S. Normal skin and hypertrophic scar fibroblasts differentially regulate collagen and fibronectin expression as well as mitochondrial membrane potential in response to basic fibroblast growth factor. *Braz J Med Biol Res* **44**, 402, 2011.
 33. Ackermann, M., Wolloscheck, T., Wellmann, A., Li, V.W., Li, W.W., and Konerding, M.A. Priming with a combination of proangiogenic growth factors improves wound healing in normoglycemic mice. *Int J Mol Med* **27**, 647, 2011.
 34. Iyyam Pillai, S., Palsamy, P., Subramanian, S., and Kandaswamy, M. Wound healing properties of Indian propolis studied on excision wound-induced rats. *Pharm Biol* **48**, 1198, 2010.
 35. Leclerc, T., Thepenier, C., Jault, P., Bey, E., Peltzer, J., Trouillas, M., *et al.* Cell therapy of burns. *Cell Prolif* **44**, 48, 2011.
 36. Lataillade, J.J., Bey, E., Thepenier, C., Prat, M., Leclerc, T., and Bargaes, L. Skin engineering for burns treatment. *Bull Acad Natl Med* **194**, 1339, 2010.
 37. Malkowski, A., Sobolewski, K., Jaworski, S., and Bankowski, E. FGF binding by extracellular matrix components of Wharton's jelly. *Acta Biochim Pol* **54**, 357, 2007.
 38. Horch, R.E., Kopp, J., Kneser, U., Beier, J., and Bach, A.D. Tissue engineering of cultured skin substitutes. *J Cell Mol Med* **9**, 592, 2005.
 39. Dobrzycka, B., Mackowiak-Matejczyk, B., Kinalski, M., and Terlikowski, S.J. Pretreatment serum levels of bFGF and VEGF and its clinical significance in endometrial carcinoma. *Gynecol Oncol* **128**, 454, 2013.
 40. Szarvas, T., Jäger, T., Laszlo, V., Kramer, G., Klingler, H.C., vom Dorp, F., *et al.* Circulating angiostatin, bFGF, and Tie2/TEK levels and their prognostic impact in bladder cancer. *Urology* **80**, 737.e13, 2012.
 41. Bremnes, R.M., Camps, C., and Sirera, R. Angiogenesis in non-small cell lung cancer: the prognostic impact of neoangiogenesis and the cytokines VEGF and bFGF in tumours and blood. *Lung Cancer* **51**, 143, 2006.
 42. Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N.M., *et al.* Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell* **9**, 391, 2006.
 43. Sun, B., Xu, H., Zhang, G., Zhu, Y., Sun, H., and Hou, G. Basic fibroblast growth factor upregulates survivin expression in hepatocellular carcinoma cells via a protein kinase B-dependent pathway. *Oncol Rep* **30**, 385, 2013.
 44. Li, S.H., Cai, S.X., Liu, B., Ma, K.W., Wang, Z.P., and Li, X.K. *In vitro* characteristics of poly (lactic-co-glycolic acid) microspheres incorporating gelatin particles loading basic fibroblast growth factor. *Acta Pharmacol Sin* **27**, 754, 2006.
 45. Skaat, H., Ziv-Polat, O., Shahar, A., and Margel, S. Enhancement of the growth and differentiation of nasal olfactory mucosa cells by the conjugation of growth factors to functional nanoparticles. *Bioconjug Chem* **22**, 2600, 2011.
 46. Nie, S.P., Wang, X., Qiao, S.B., Zeng, Q.T., Jiang, J.Q., Liu, X.Q., *et al.* Improved myocardial perfusion and cardiac function by controlled-release basic fibroblast growth factor using fibrin glue in a canine infarct model. *J Zhejiang Univ Sci B* **11**, 895, 2010.

Address correspondence to:

Xing Wei, PhD

Institute of Biomedicine

College of Life Science and Technology

National Engineering Research Center of Genetic Medicine

Key Lab for Genetic Medicine of Guangdong Province

Jinan University

Guangzhou

Guangdong 510632

China

E-mail: wei70@hotmail.com

Received: April 29, 2013

Accepted: August 1, 2014

Online Publication Date: October 9, 2014