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In vitro and in vivo biological performance of collagen-chitosan/silicone membrane bilayer dermal equivalent

Lie Ma · Yanchao Shi · Yixin Chen · Haiguang Zhao · Changyou Gao · Chunmao Han

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Abstract Skin loss or damage affects severely the life quality of human being and can even cause death in many cases. We report here a bilayer dermal equivalent (BDE) composed of collagen-chitosan porous scaffold and silicone membrane, which can effectively induce the regeneration of dermis in an animal model of full thickness skin loss. The in vitro biosecurity test showed that the BDE had no cytotoxicity, and no remarkable sensitization and irritability. In vitro cell culture proved that the BDE had good biocompatibility to support the proliferation of fibroblasts. Animal test was performed on Bama miniature pig skin. Gross view and histological sections found plenty of fibroblasts and extracellular matrix in the regenerative scaffold after transplantation of the BDE for 4 weeks. Immunohistochemistry results proved that the BDE has the ability to support the angiogenesis of the regenerated dermis. All these results indicate that the BDE might be a promising equivalent in treating dermal loss.

L. Ma · Y. Shi · H. Zhao · C. Gao (✉)
Key Laboratory of Macromolecule Synthesis and Functionalization, Ministry of Education and Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China
e-mail: cygao@mail.hz.zj.cn

Y. Chen · C. Han
Faculty of Burn, Second Affiliated Hospital of Zhejiang University, Hangzhou 310027, China

Introduction

Skin, the biggest organ of body, is frequently damaged by burn, hurt and trauma [1]. There are millions of patients suffered from skin lose annually. The cost for skin recovery is about $36,000–117,000 each patient [2–5]. Full-thickness skin defects in large scale can not be repaired spontaneously. Therefore, a proper dermal equivalent plays a crucial role in the skin regeneration [6, 7].

Although applied in clinic widely, traditional autograft and allograft have the limitations of time, space, donors and antigenicity. Most importantly, supplying of enough fresh skin is often limited [8–10]. Tissue engineering skin provides a prospective source of advanced therapy for treatment of skin defects because it has comparable results with traditional methods while can avoid those limitations [1].

By far, many dermal equivalents have been developed for the full-thickness skin defects in hospital, such as Integra®, Dermagraft® and so on [8]. The bilayer dermal equivalent (BDE) was first developed by Yannas and coworkers. Its commercial product, Integra®, has been approved by the Food and Drug Administration (FDA) in 1996 and has been applied to dermal regeneration widely [11–13]. In this bilayer dermal equivalent, the collagen based three-dimensional scaffold provides an extra-cellular matrix analog which functions as a necessary template for host infiltration and a physical support to guide the differentiation and proliferation of cells into the targeted functional dermis. Meanwhile, the silicone membrane functions as a temporary epidermis, which can play a role in controlling water loss and inhibiting bacterial entry until an ultrathin epidermal autologous grafts applied [14, 15].

We have developed a BDE which is composed of collagen-chitosan scaffold and silicone membrane [16].

Chitosan is able to accelerate tissue regeneration and collagen synthesis from fibroblasts. Moreover, it has functions of hemostasis and sterilization, and has been widely investigated for applications in wound healing [17–21]. Therefore, a porous dermal equivalent composed of collagen and chitosan possesses the functionality of fibroblast infiltration, proliferation and differentiation, and can accelerate vascularization in vivo. Meanwhile, the cost of this BDE is comparatively lower. We shall show here that this BDE can effectively induce dermal regeneration of full skin loss in an animal test. Moreover, the biosecurity, biocompatibility shall also be evaluated.

Experiment

Materials

Chitosan (Mη: $1.0 \times 10^5 - 1.7 \times 10^5$, 75–85% decetylation degree) was purchased from Sigma. Gelatin and glutaraldehyde (GA, 25% water solution) was purchased from Shanghai Pharm Co. (China). Silicone membrane is a medical grade product from Shanghai Xincheng Co. (China). Collagen type I was isolated from fresh bovine tendon by trypsin digestion and acetic acid dissolution method [22]. All other reagents and solvents were of analytical grade and used as received.

Preparation of collagen-chitosan/silicone membrane bilayer dermal equivalent

Collagen and chitosan were dissolved in 0.5 M acetic acid solution to form a 0.5% (w/v) solution in a mass ratio of 9:1. The collagen and chitosan composite was injected into a round mould and freeze-dried to form collagen-chitosan porous scaffold. Then the collagen-chitosan porous scaffold was further treated by 0.25% GA.

The collagen-chitosan scaffold and silicone membrane were combined with gelatin. A 10% (w/v) gelatin solution was homogeneously spread on the silicone membrane with an amount of 10 μl/cm^2. After cooled to room temperature, the gelatin solution on the surface was transferred into a gel layer. Then the GA treated scaffold was put on the surface slightly to obtain the bilayer dermal equivalent (BDE).

Macroscopic shape and microstructure observation

Macroscopic images of the collagen-chitosan/silicone membrane BDE were taken by a digital camera. The microstructure was observed by scanning electron microscopy (SEM, Cambridge stereoscan).

Biosecurity evaluation

Cytotoxicity evaluation

The cytotoxicity of the BDE was evaluated by fibroblast culture in vitro. The BDE was immersed in 4.5 mL Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with penicillin (100 U/mL), streptomycin (100 U/mL) and 10% FBS (complete medium) for 24 h. Then the lixivium was used for cell culture. Meanwhile, the normal culture medium was used as a control.

The human fibroblasts were isolated from foreskins and were routinely cultured [23]. 20 μl human dermal fibroblast suspension at a density of 3×10^5 cells/mL was seeded in a 96-well polystyrene culture plate, to which 180 μl sample extractant or normal culture medium was added. The culture medium was changed with the same medium every 2 days. Then the cell viability was measured by 3-(4,5-dimethythiazol-2-yl) -2,5-diphenyltetrazoliumbromide (MTT) assay [24] as a function of time.

Sensitization evaluation

The lixivium for sensitization test was obtained by immersing the BDE in 10 mL 0.9% NaCl solution at 37 °C for 72 h. The negative control is the cellulose acetate membrane (with a diameter of 13 mm) and the positive control was the same membrane treated by immersing in 5% phenol solution for 24 h.

20 guinea pigs weighting 300–400 g were separated into three groups. Five guinea pigs were used for negative and positive control groups, respectively. 10 guinea pigs were used for sample group. A depilatory cream was applied to remove the hairs. Then 0.1 mL lixivium of the sample, negative and positive control were injected into the subcutaneousness. After induced for 14 days, the skin situation was evaluated.

Irritability evaluation

The lixivium for irritability test was obtained by the same process of the sensitization evaluation. The pledgets (2.5 cm × 2.5 cm) immersed in each kind of lixivium were attached on the rat's back and enswathed by elastic bandages. Then the skin situation was evaluated after 1, 24, 48 and 72 h.

Cell proliferation in vitro

The sterilized BDE was placed on a 24-well polystyrene plate and seeded with 200 μl human dermal fibroblast suspension at a density of 3×10^6 cells/mL. The fibroblasts

were cultured in a 5% CO_2 incubator at 37 °C and the culture medium was changed every 3 days. The cell proliferation as a function of culture time was evaluated by MTT assay according to the methods of Mosmann with minor modification [24]. 21 days past seeding, the scaffolds seeded with fibroblasts were observed under scanning electron microscopy (SIRION-100, Philips).

In vivo animal test

5 Bama miniature pigs weighing about 15 kg were obtained from the animal laboratory. The BDE was sterilized by immersion in 75% (v/v) ethanol solution for 30 min and washing with PBS (pH 7.4) (5 times × 5 mins). Before implantation, the dorsal surface were shaved to remove hairs and sterilized with 5% PVP-I. All pigs were anesthetized by celiac injection of 0.5 mL/kg pentobarbital sodium. Then 4 full-thickness skin defects, 3 cm diameter each, were made symmetrically on the back of each animal. In this experiment, a polypropylene wound isolation chamber was used to resist the skin shrinkage. Then the sterilized BDE was applied on the skin defect as shown in Fig. 1. Harvests were performed at 1, 2, 4 weeks after transplantation. At each harvest, the transplantation sites were cut in a full thickness manner. Paraffin sections were stained with hematoxylin-eosin (HE) reagent for histological observations.

Immunohistochemistry

Immunohistochemistry was preformed to identify the new-formed blood vessels by Factor VIII related antigen. Briefly, samples transplanted for different time were fabricated into paraffin section. Paraffin-embedded samples were pretreated with 3% H_2O_2 and 50% methanol diluted in distilled water for 5 min. To block non-specific binding sites the tissue sections were incubated in 3% (w/v) bovine serum albumin/PBS (pH 7.4) (BSA/PBS) for 60 min. Due to the high homology of pig with human, rabbit anti-human Factor VIII antibody was adpopted as the primary antibody. The sections were incubated in the primary antibody at a 1:500 dilution for 24 h at 4 °C and washed with PBS (pH 7.4) (3 times, each for 5 min). Subsequently, the sections were incubated for 30 min at 37 °C with biotinylated goat anti-rabbit IgG (diluted 1:300) and washed with PBS (pH 7.4). The slides were then reacted with avidin-conjugated peroxidase (diluted 1:30) at 37 °C for 30 min. Finally, the sections were displayed with DAB and embedded by paraffin to yield a positive stain. The number of new-formed blood vessels was determined by averaging the number of 3 un-overlapped vision fields at a magnification of 100.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using two-population Student's *t*-test. The significant level was set as $p < 0.05$.

Results and discussion

Macroscopic shape and microstructure of the BDE

The shape and the microstructure of the BDE are displayed in Fig. 2. The collagen-chitosan scaffold has a foam-like shape, and is well combined with the silicone membrane. SEM observation shows clearly the bilayer structure

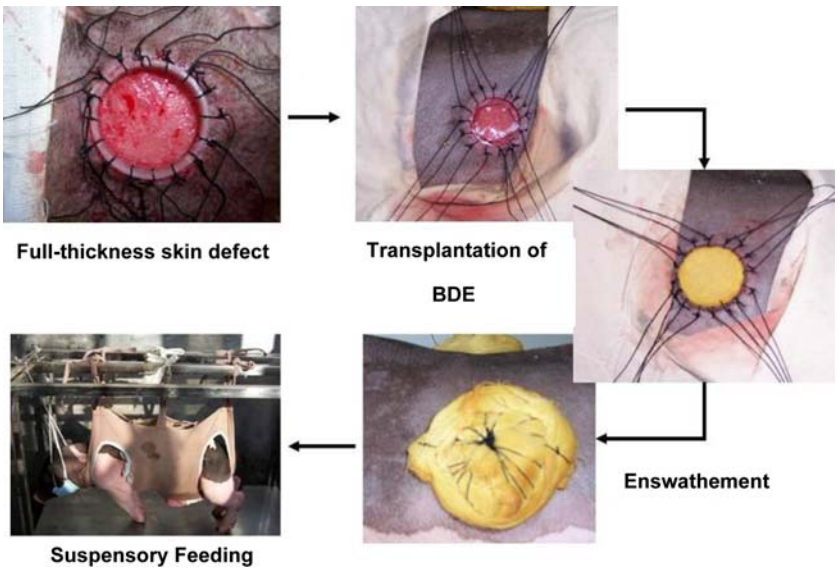

Fig 1 Transplantation process of the bilayer dermal equivalent (BDE) on the pig's back

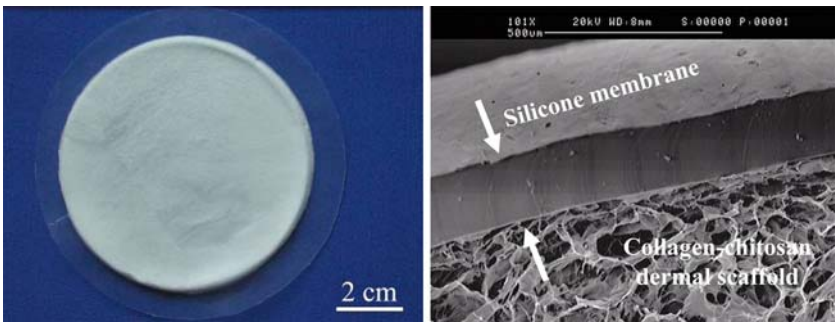

Fig 2 Macroscopic appearance (**a**) and microstructure (**b**) of the BDE

(Fig. 2b). The pore size of the crosslinked collagen-chitosan scaffold is within the range of 80–200 μm, which is suitable for fibroblast to infiltrate.

Biosecurity

Cytotoxicity

The cytotoxicity of the collagen-chitosan dermal scaffold and the silicone membrane was evaluated by in vitro fibroblast culture. The cell viability in the BDE lixivium and normal culture medium were compared in Fig. 3. The cell viability increased linearly as a function of culture time. No significant difference of the cell viability was found in this whole culture period. The results indicate that the BDE has not shown any cytotoxicity.

Sensitization

For the BDE group, no obvious edema and erythema reaction was observed during the sensitization test, corresponding to a skin reaction score of 0 (the best one). By contrast, the erythema could be observed in the positive group, corresponding to a skin reaction score of ≥3. These comparative results prove that the BDE has no potential ability to induce sensitization.

Irritability

The contact area of the sample group and the negative control were observed after taking off the skin pitches after 1, 24, 48 and 72 h. There were no obvious edema and erythema reaction on the skin and the skin reaction score was 0, suggesting that the BDE has no remarkable irritative reaction.

Cell proliferation behavior

Figure 4 shows the cell proliferation behavior measured by MTT assay. Culturing in the BDE, the fibroblast viability increases with the culture time in the first 14 days, demonstrating that the BDE has good cytocompatibility to support the fibroblast proliferation. Then the cell viability decreases at 21 days, which may most possibly caused by

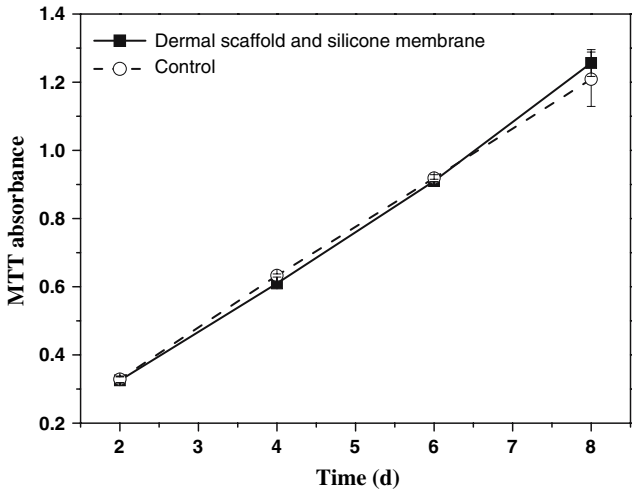

Fig 3 Cytotoxicity of the BDE evaluated by in vitro fibroblast culture and MTT assay. Control was the normal DMEM medium. Values are mean ± SD (n=3).

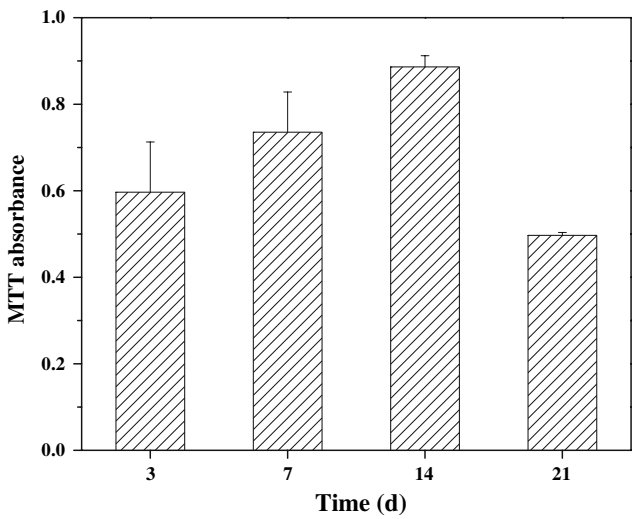

Fig 4 Viability of fibroblasts seeded in the BDE as a function of culture time. Values are mean ± SD (n = 3)

cell apoptosis after cultured for long time. The SEM images of the cell-seeded BDE are displayed in Fig. 5. Many fibroblasts and their abundant extracellular matrix were observed on both the surface (Fig. 5a) and the inner of the scaffold (Fig. 5b).

In vivo transplantation test

Macroscopic appearance and histological examination

The macroscopic appearance and the corresponding histological sections of the wounds treated with BDE for different time are displayed in Fig. 6. The macroscopic appearance shows no obvious difference between the samples transplanted for 1 week and 2 weeks (Fig. 6a, b). Both present a white and relative smooth surface, indicating that few of new formed capillary vessels have infiltrated into the inner of the BDE at this stage. After transplantation of the BDE for 4 weeks, the surface of the wound shows more blood-red (Fig. 6c).

The histological images show that few of fibroblasts were found in the BDE in the first week except for plenty of inflammatory cells (Fig. 6d). After transplantation of the BDE for 2 weeks (Fig. 6e), more fibroblasts were observed in the BDE while the inflammatory cells began to decline. The porous structure of the BDE was still preserved and little of new-secreted matrix had filled into the scaffold. The histological image of 4 weeks shows a notable difference (Fig. 6f). Plenty of fibroblasts and new-formed extracellular matrix (ECM) were observed within the collagen-chitosan scaffold of the BDE. Meanwhile, few of inflammatory cells were observed in the scaffold at this stage. Therefore, one can safely draw the conclusion that the BDE has effectively induced the regeneration of the dermis after 4 weeks.

Analysis of the regenerative dermis

In order to analyze the regenerative effect of the BDE, the histological images of the normal pig dermis, acestoma and the regenerative dermis are compared in Fig. 7. In the normal pig skin, high density and regularly arrayed ECM can be observed, which is related to the normal structure and property of skin (Fig. 7a). In contrast, the acestoma presents a disordered and low-dense ECM (Fig. 7b). However, the density of the new-formed ECM in the regenerative dermis is close to the normal skin (Fig. 7c). The arrangement of the ECM is more regular too. This comparison proves that the BDE indeed has the ability to regenerate a damaged dermis with a similar structure of the normal skin.

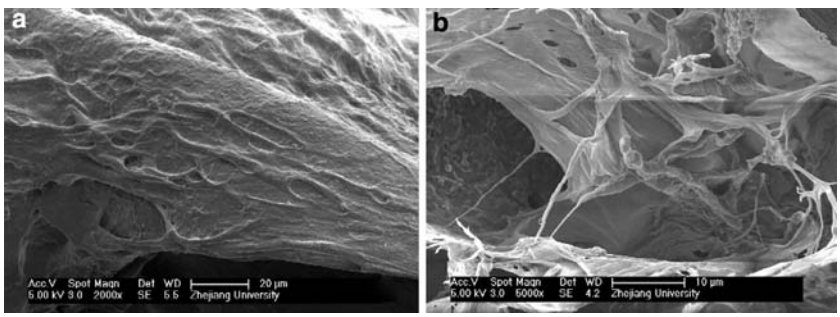

Fig 5 SEM images of the BDE after seeded with fibroblasts for 21 days. (**a**) Surface and (**b**) cross-section view

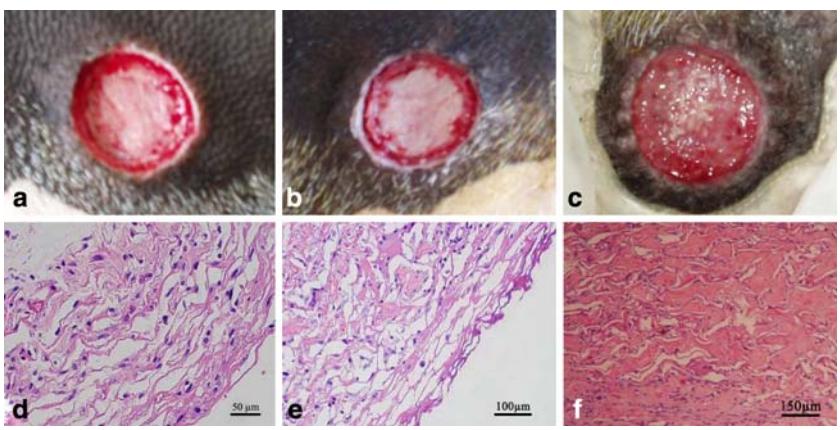

Fig 6 Macroscopic appearance of the wounds after BDE transplanted for 1 week (**a**), 2 weeks (**b**), and 4 weeks (**c**) and their corresponding histological images (**d–f**) . All the images were obtained after the silicone membranes were removed

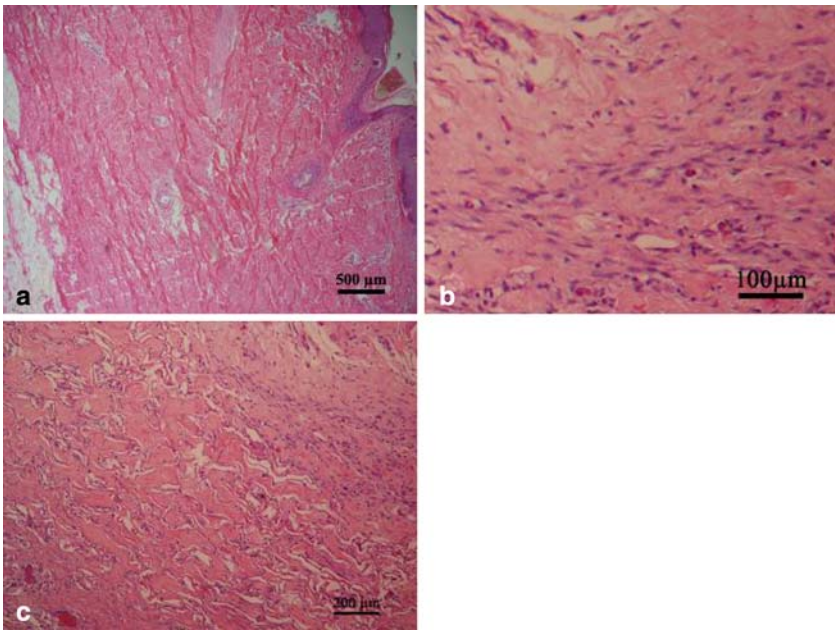

Fig 7 Histological comparison between the normal pig skin (**a**), acestoma for 4 weeks (**b**), and the wound after BDE transplanted for 4 weeks (**c**). All the images were obtained after the silicone membrane were removed

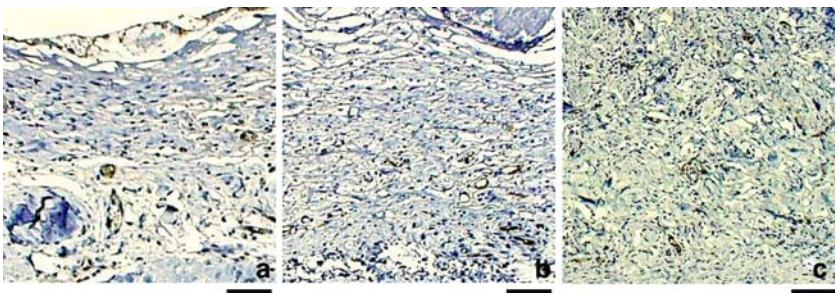

Fig 8 Immunohistochemistry images of the wounds after BDE transplanted for 1 week (**a**), 2 weeks (**b**) and 4 weeks(**c**). Bars indicate 100 μm

New-formed blood vessel analysis

The quick angiogenesis plays a key role in the dermal regeneration process. Here, an immunohistochemistry test was performed to evaluate the BDE's property with respect to new blood vessel formation (Fig. 8). In the first week, only a few of blood vessels (brown-yellow points) can be detected (Fig. 8a). More blood vessels can be found after 2-week transplantation (Fig. 8b). It is worth mentioning that at this time most of the new-formed blood vessels are in the interface between the tissue and the scaffold. After 4-week transplantation, a plenty of new-formed blood vessels was shown in the immunohistochemistry image (Fig. 8c). Figure 9 quantitatively compares the number of new-formed blood vessels, indicating that the number of blood vessels increased along with the transplantation time. Significant increase was found during the 2 week transplantation. All these results indicate that the BDE takes a very positive performance in supporting the angiogenesis of the regenerated dermis.

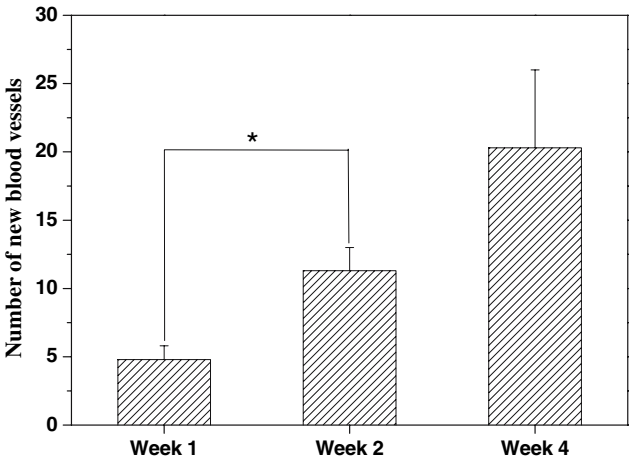

Fig 9 The number of the new-formed blood vessels as a function of the BDE transplantation time. Asterisk indicates a significant difference

Conclusion

A collagen-chitosan/silicone membrane bilayer dermal equivalent (BDE) is fabricated with a suitable microstructure for the fibroblast infiltration. No cytotoxicity, remarkable potential sensitization and irritability are detected in the biosecurity test. Cell culture in vitro proves that the BDE has good biocompatibility that can support the proliferation of fibroblasts. In vivo transplantation indicates that the BDE has the ability to regenerate a damaged dermis with a similar structure of the normal skin, and to support the angiogenesis of the regenerated dermis. Therefore, one can conclude that the BDE is a promising dermal equivalent for skin regeneration.

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