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Original Article

3D printed gelatin-alginate bioactive scaffolds combined with mice bone marrow mesenchymal stem cells: a biocompatibility study

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Abstract: Objective: This study was aimed to determine the biocompatibility of gelatin-alginate scaffolds with 3D bio-printer and the mice bone marrow mesenchymal stem cells (BMSCs), and the histocompatibility during skin wound healing. Methods: BMSCs was separated and cultured using pan-marrow attachment method and the third generation combined with gelatin-alginate was added to the 3D printer for the preparation of bioactive scaffolds that concluded the BMSCs. Then the following characteristics of BMSCs, including survival, adhesion, aggregation and growth, were observed under inverted microscope and electron microscope. We created the full-thickness skin wound model on the mouse back and observed the wound surface's fusion situation and the corresponding histopathological changes after putting the bioactive scaffolds on the surface. Results: It was found that BMSCs was dispersing on the scaffolds' surface evenly and singly, and gradually growing into the inside of the bracket, whose morphology was closed to the performance in culture medium. The scaffold was attached completely on the wound surface and integrated with issues in a week. And the inflammatory cells seeped into the scaffold and promoted the growth of granulation tissue. Conclusion: Overall, the results suggest that the 3D-printed bioactive scaffold carrying the BMSCs has good biocompatibility and is safe for skin tissue engineering without rejection and biotoxicity.

Keywords: Biocompatibility, 3D bioprinting, tissue engineering, gelatin-alginate scaffold, mesenchymal stem cells

Introduction

The skin as the largest organ in human body and the barriers to contact with the external environment has the function of the maintenance of homeostasis. Meanwhile, the skin, a region vulnerable to injury extremely, tends to repair the minor injury by itself except the extensive damage that comes hard to be healed relying on its own ability [1] and often is referred for the skin transplantation [2]. Autologous skin grafting not only can result in the second trauma for patients, but also is confined to the physical status and skin lesions of patients, especially when treating in the condition of the extensive burns with the fully finite availability of autologous skins, however allogenic skin grafting always accompany the problems like immune suppression or infection [3]. Therefore, with increasingly requirements of the substitutions of skin in clinical, the tissue engineering technology seems to appear as a turning point for management of trauma. Compared with the application of autologous and allogenic skins, the tissue engineering technology is superior in covering and closing wound without the limitation of skin damaged area, which is the most proper method to solve the extensive injuries [4]. With the development of tissue engineering technology, the increasing applications of substitutions of skin for extensive injuries has contributed a lot to reconstruct skin destructions.

Although the traditional artificial skin substitutes easy to industrial production has been difficult in satisfying personalized necessary and the growth of cells because of the absence of epidermis, prone to be infected, get low success rate of transplantation [5]. By comparison, the 3D printing active scaffolds consisted of cells and stents are more beneficial to the

growth and multiply [6], and this kind of technology has the possibility to prepare the personalized supporter suited for various patients rapidly and accurately, regulate the size, porosity and distribution of pores in materials. 3D printing is suitable for tissue engineering and regenerative medicine extraordinary as the transformative tool applied to biomedicine. The bioactive scaffolds planted into skins have the advantages in promoting skin wound healing, eliminating rejection and biological toxicity, and excellent biocompatibility referring to the scaffold that has no obviously adverse effect on cells [7]. The most of studies are focused on the application of the bioactive scaffold, such as the case that Tonsomnoon K [8] replaced cornea with the gelatin-alginate bioactive scaffolds while pressed for the investigations on biocompatibility of the bioactive scaffolds. Consequently, our study, taking the compound bioactive scaffolds with the bone marrow mesenchymal stem cells as an object by 3D printing and exploring the biocompatibility of the cells and scaffolds as a key point, is devoted to the confusion of scaffolds and wound surface reflected from histopathology in the period of wound healing after applying the scaffolds for the full-thickness destructions on the mouse.

Materials and methods

Culture of rat bone marrow mesenchymal stem cells

Five, 6-week-old healthy rat were used in this study. Under aseptic conditions, their tibia and femur were removed and flushed 3 times with phosphate-buffered saline (PBS) sterilized by high pressure. Then we cut the ends of those bones to exposing the bone marrow cavity and rush out of the marrow using a syringe to inject the nutrient solution into the tibia and femur that was repeated flushing 3-5 times, until bone marrow cavities gradually faded to white. Acquired marrows were made into single cell suspension and cultured in a 25 cm^2 culture bottle at 37°C in a CO_2 incubator. Then full amounts of the culture medium were replaced with fresh ones after first 48 h and next each 3 days. While covering the bottom of the bottle and fusing into a single layer, those cells were subcultured at 70%-80% confluence using 0.25% trypsin in a 1:2 ratio. Then the third generation was selected and reserved within an observation of the mouse BMSCs growth situations in scaffold under electron microscope.

Preparation of the bioactive scaffolds

25% and 5% solutions were prepared by dissolving a certain amount of sodium alginate and gelatin in PBS partly and stirred to clarify by magnetic force. Then the above two kinds of solutions were under intermittent sterilization and was mixed into a mixture in predetermined proportions. Cell suspension with a density of 4×10^8 cells/ml was made by digestion and centrifugation of the third generation of BMSCs induced 14 days. The mixture and cell suspension were placed into the cartridge of the 3D printer that had been programmed with parameters preset by a computer control system, then released through a 300 μm nozzle and deposited layer-by-layer into a culture dish, under aseptic conditions, to form a functional 3D bioactive scaffold after a soaking in 100 mM CaC_{l2} solution for 10 minutes to complete crosslinking of the two suspension.

Culture of the bioactive scaffolds

The bioactive scaffolds coupled with BMSCs were cultivated 3 days and observed to determine the growth and proliferation of BMSCs around the scaffolds under electron and light microscope.

Preparation and treatment of animal model

We selected twenty mice (scxkLu 20130001) aged 6-week-old and weighted 20~25 g. After anesthesia with an intraperitoneal injection of pentobarbital sodium and fixation in supine position, those mice, taking the spine as the center line, had a preparation of fur on the dorsal sized 3×3 cm^2 [9] using 8% Na_2S and were sterilized in a 1 cm circular area, marked with methylene blue, on the exposed dorsal skin with 1% Iodophors and 75% ethanol solution, and a full-thickness excision was made using in cession scissors. Then the wound was covered with the bioactive scaffolds and sterile gauze and dressed with elastic bandage. The wound was observed and imaged every day.

Histological examination

On day 1, 3, 7 and 14, the mouse wound tissue sample, including 0.5 cm of normal skin, was

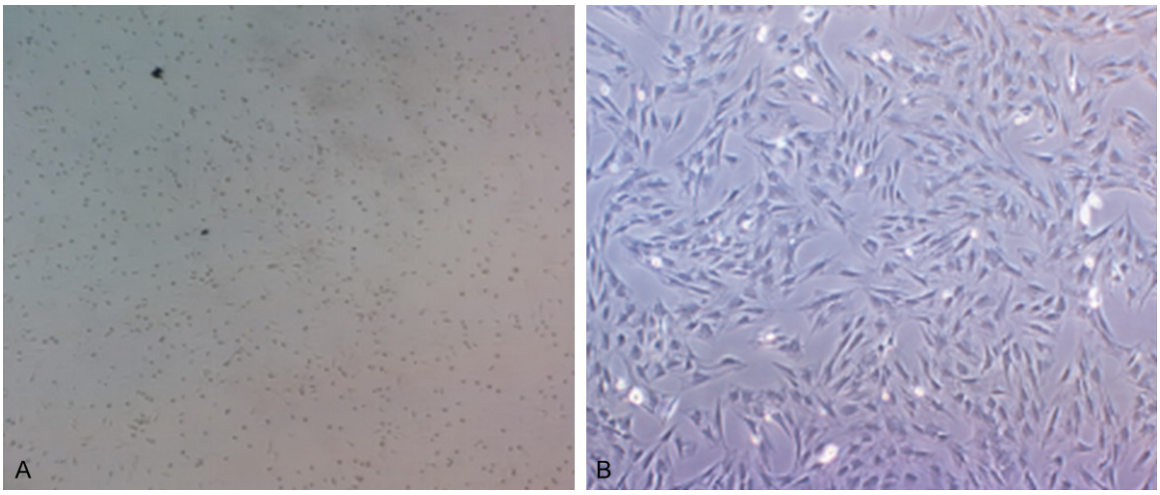

Figure 1. Mice mesenchymal stem cells' subculturing.

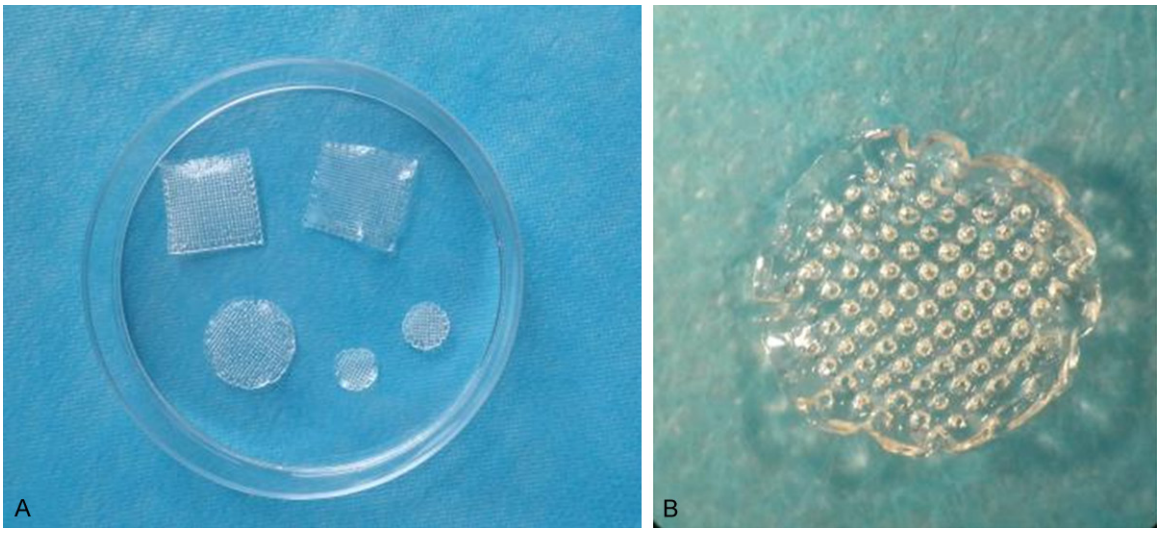

Figure 2. Morphology of 3D bioactive scaffolds carrying BMSCs.

harvested respectively. Those samples were fixed in neutral formaldehyde solution for 24 h, and dehydrated, cleared and embedded in paraffin. Then those samples were cut in sections, stained with hematoxylin-eosin (HE), that were observed under a light microscope.

Result

Mice mesenchymal stem cells' subculturing

Initially, many types of cells were found in the cell suspension including mice bone marrow mesenchymal stem cells, which were kept into a single cell state and nearly spherical (**Figure 1A**). After 6-10 days inoculation, BMSCs, mainly in polygonal cells, were proliferated and gradually formed colonies to reach the degree of fusion of 90% eventually and gathered in a whirl-like cluster way, which was accompanied with the decreasing of other types of cells. Subcultured BMSCs grew faster and were observed to adhere on the cell bottle after each vaccination immediately. Then the next subculture was began in 2~3 days. Being subcultured to the third generation, the same majority of BMSCs became polygonal with a few spindle-shaped ones and arranged in swirling or clustered conditions and reached to the degree of fusion of 70% (**Figure 1B**).

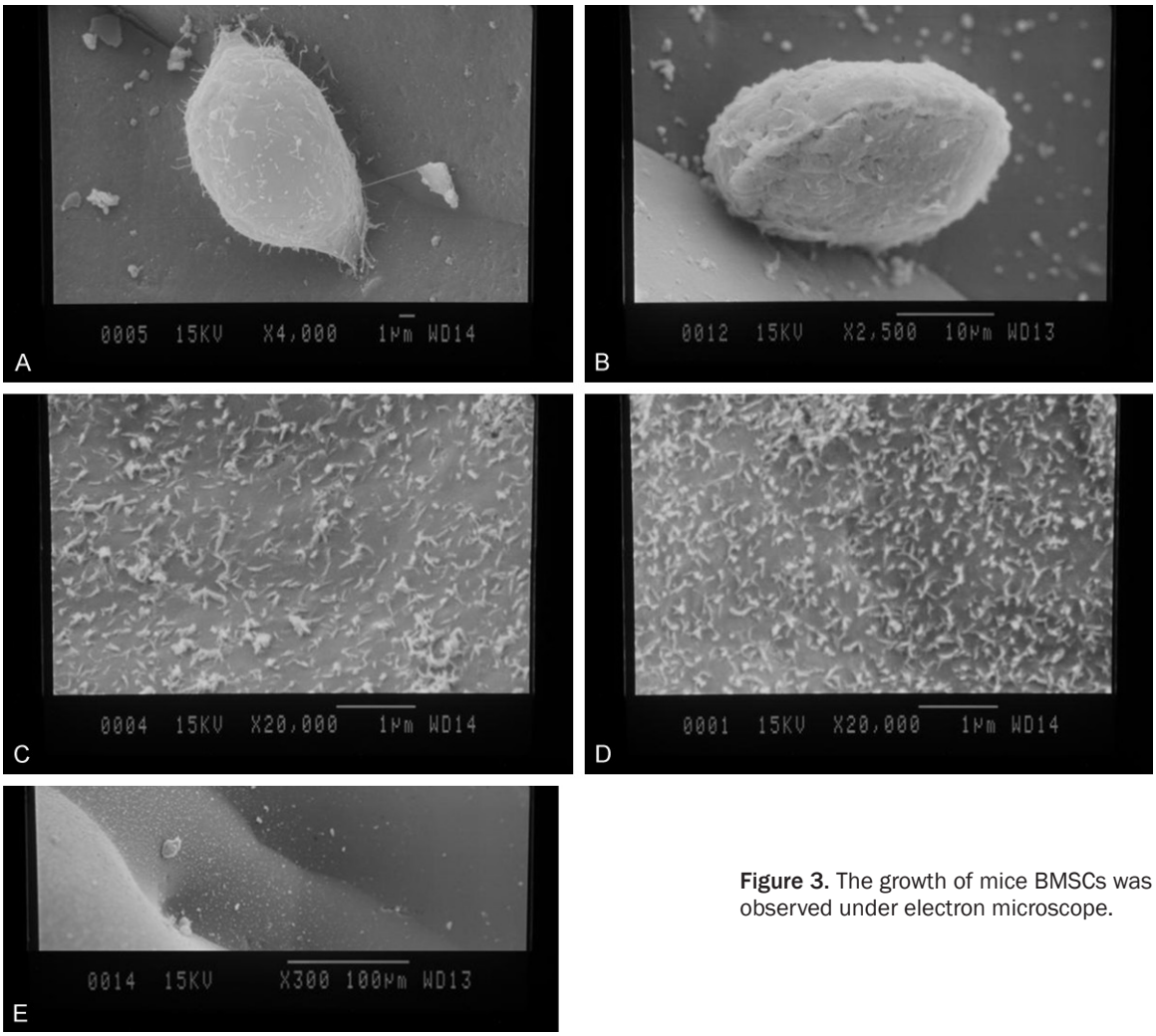

Figure 3. The growth of mice BMSCs was observed under electron microscope.

Morphology of 3D bioactive scaffolds carrying BMSCs

By using 3D bioprinter, BMSCs, gelatin and sodium alginate were used as the raw materials to produce the bioactive scaffolds with 300 μm and interval 600 μm arrangement (**Figure 2A**). The scaffold's structure was a colloidal mesh that have soft texture, ivory-white whole and even-distributed pores (**Figure 2B**).

The growth of mice BMSCs was observed under electron microscope

Under the electron microscope, all mice BMSCs scattering individually have a good attachment on the surface of scaffolds and are even-distributed. The mice BMSCs grown on the surface of gelatin-alginate scaffolds mostly exist in a distended-spindle shapes with short cell protuberances at both (**Figure 3A**) ends that is unlike the growth on rigid glass surfaces in culture flasks. Minority of the cells are elliptical (**Figure 3B**) rather than polygon or long spindle that grew adherently with obvious processes. Those BMSCs cells were plump and extended, which had the cell membrane that was covered with tiny and uneven-distributed villi that located with less villi on the intumescent middle part of (**Figure 3A** and **3C**) and two ends relatively dense (**Figure 3B** and **3D**). The stretched villi was observed on the surface of the cells with the enlargement of magnifier and minority areas' villi concentrated tuftedly (**Figure 3C**), in which of the scaffold appeared small sag with the implantation of cells (**Figure 3D** and **3E**).

The results of optical microscopy

In the sight of light microscope, BMSCs had an obvious attachment to the scaffolds in the

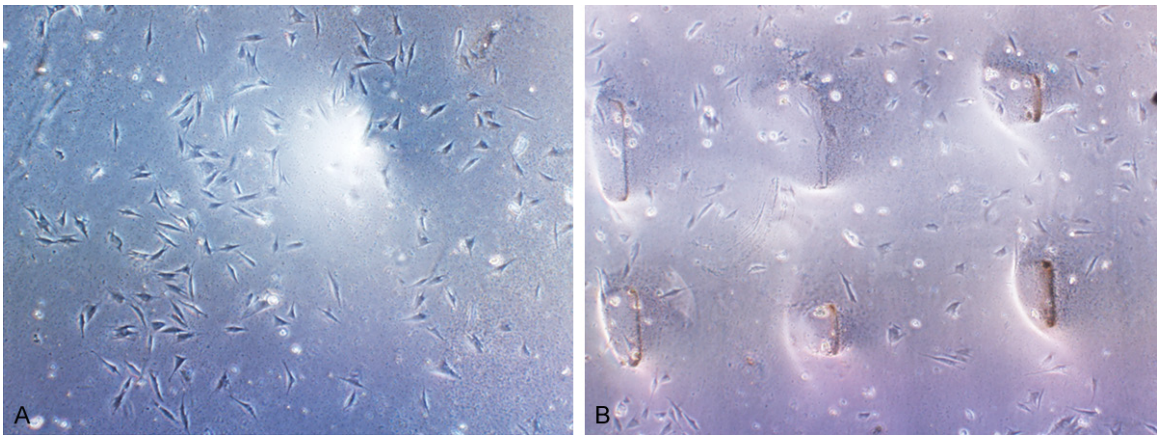

Figure 4. The results of optical microscopy.

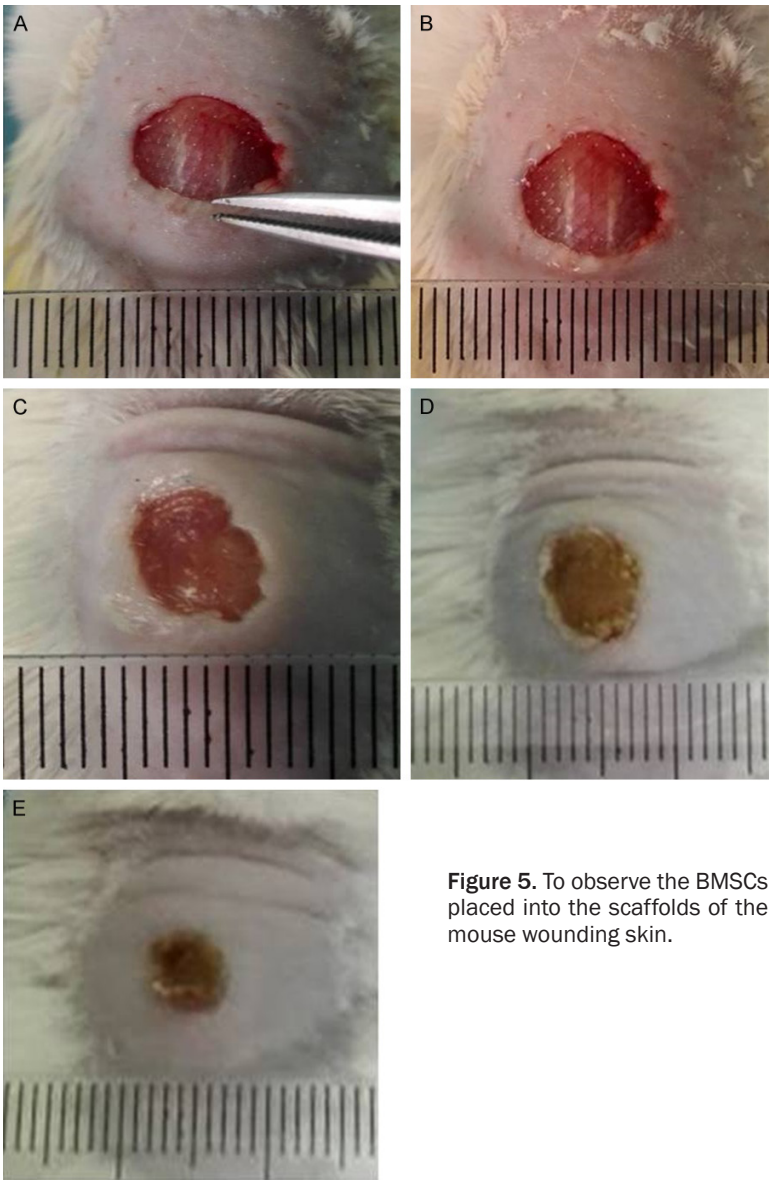

Figure 5. To observe the BMSCs placed into the scaffolds of the mouse wounding skin.

early stage of its formation and distributed quite uniformly as single, tiny cluster, polygon or long fusiformis, that was similar to the growth state in the culture flask (**Figure 4A**). With the extension of incubation time, the inside of the scaffolds was gradually grown into the cells distributed uniformly, While increasingly scarce polygonal cells outside the bracket led to the small lacuna on the surface (**Figure 4B**).

To observe the BMSCs placed into the scaffolds of the mouse wounding skin

In this experiment, the full-thickness tissue of the back of mice was prepared in 1 cm diameter. The bioactive scaffolds, touching softly and cut in the size based on the shape and size of the wound, achieved the complete attachment with the defect after covering the wound (**Figure 5A**, **5B**). After 24 hours, the scaffolds began to combine with the edge of the wound appearing to significant swelling that moved to the middle of the wound and enwrapped the scaffolds under the edge of

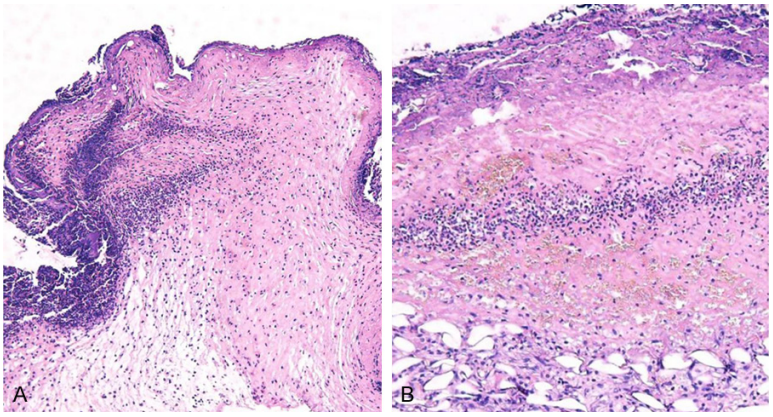

Figure 6. Histological study of the safflods coupled with BMSCs after placed into the wound of the mice.

wound, which narrowed to the average diameter of 0.8 cm and was not neat, jagged in some areas. That a small amount of gray-yellow exudation passed through the scaffolds pores deepened the color of the scaffolds surface from transparent to pale-yellow semitransparent (**Figure 5C**), while the activity of those mice was unaffected. The scaffolds had been saturated with the gray-yellow exudation and were firmly fixed to the wound in the third working day. The exposed wound of the mice was in dry state and the wound fringe reducing the swelling was in a position below the marginal mixture consisting of partial stent dissolution and exudation. The wound surface gradually covered with the grayish-yellow crust that the scaffold was seen indistinctly below and the wound's diameter was 0.6 cm to 0.7 cm (**Figure 5D**). In the 7th day, with the reduction of the marginal mixture to only a small amount of remain, the crust became darker and harder gradually causing the scaffold under it to be completely invisible. The diameter of the wound was 0.5 cm (**Figure 5E**). Subsequently, the wound of the mice gradually contracted until it had a primary healing for 2 weeks.

Histological study of the scaffolds coupled with BMSCs after placed into the wound of the mice

On the first day of experiment, we observed using the way of tissue slices under light microscope that the scaffolds and skin wound were separated and not fixed firmly. The skin had an breakage in combination with the subcutaneous tissue adhering to a large amount of loose exudation packed with neutrophils and cellulose mostly and apart from small amounts of necrotic material on the surface (**Figure 6A**). On day 3, the wound's surface covered with the crust composed of necrosis and exudation, in which the scaffold was found under it. A great quantity of fresh granulation tissue grew into the bottom and the edge of the wound, while the scaffolds were dissolving in the top granulation tissue, where bleeding and few amounts of inflammatory cells existed in. In addition, rich inflammatory cells gathered below the residual scaffolds, in which some small cracks appeared subtly, that produced the tendency of granulation tissue formation and some inflammatory cells penetrating into the scaffolds (**Figure 6B**). As the wound reached to the recovery, the scaffolds dissolved gradually until it disappeared in the visual field of microscope on the day 7. New granulation tissue had been gradually thickening all the time. On the day 14, some change had taken place that stratified squamous epithelium covered the wound surface entirely and granulation tissue began to aging, even form the scar rich in collagen, until the defect eventually achieved primary healing.

Discussion

Immense potentials exists in the modern and future medical field of tissue engineering, especially in the skin tissue trauma, providing some more stable alternative for the treatment of clinical skin defects [10]. While the study on tissue engineering skin has aroused broad attention of researchers, most of them focus so extremely on the source of seed cells, differentiation mechanisms, large-scale cultivation and proliferation technology, preparation technology and design proposals of scaffold materials, and bioactive scaffolds in clinical study that they has not paid enough attention to figure out the biocompatibility of active scaffolds yet [11]. In this study, through electron microscopy, not only the growth states of the bone marrow mesenchymal cells and scaffolds, but the fusion and other corresponding histopathological changes of active scaffolds and tissues in the

wound of mice were observed to analyze the biocompatibility of bioactive scaffolds.

Mesenchymal stem cells have a good performance in proliferation capacity, self-renewal and multiple differentiation potential in vitro [12-15]. In this study, we used the method of the bone marrow attachment to isolate mice bone marrow mesenchymal stem cells mixing with other types of cells. After 6-10 days of culture, that the insufficient proliferation capacity of other mixed cells reduced gradually raised the BMSCs in a mass proliferation state that had the typical form of homogeneous polygonal or long spindle shape when cultured to the third generation. This kind of performance may result from the effect of the telomerase inner BMSCs, which can regulate and maintain cell proliferation capacity that make the subcultured BMSCs excelling in fast proliferation [16, 17], culture stability, unchangeable quality and morphological agreement. Therefore, the BMSCs cultured in this way can be easily applied to tissue engineering research.

3D bioprinting relying on computer aided imaging uses gelatin/sodium alginate carrying bone marrow mesenchymal stem cells as the print carrier, which serve as the ideal biological materials containing no cytotoxicity and immunogenicity, conducing to cell adhesion, absorbing wound exudation, improving inflammatory response and expediting wound healing to be the scaffold material for cell culture [18-20]. The complex structures of defective skin tissue can be reproduced and reconstructed quickly and accurately applying the 3D bioprinting technology in vitro [21, 22]. However, as mentioned early, the research on the biocompatibility of cells and scaffolds has been insufficiency so far. Instead, our experiment finds that the scaffold cultured for three days was uniformly distributed with the mesenchymal cells on the surface by electron microscope, the cells remaining the characteristics its own, such as mostly fusiform, obvious process, cell filling, stretch nap, and growth in good condition. The BMSCs perform a near similar proliferation situation and morphology as growth in the culture medium, which proves that the biological scaffold can support the growth of the cells and distribute the cells in the tissues evenly with scarcely any effect on the growth state of the adherent cells. The adhesion and proliferation of the cells on the scaffolds is demonstrated by the excellent growth status of BMSCs on bioactive scaffolds. Apart from the well biological compatibility between BMSCs and scaffolds in vitro culture, the controllable pore size and functions of the 3D scaffolds prepared through this way arrange the cells in a more uniform distribution in the scaffolds to obtain nutrient substance to the utmost, which are beneficial to the development of BMSCs on the scaffold. The cells grew inner of the scaffolds producing the depression around in electron micrographs (**Figure 3E**). Such phenomenon was also found when using the light microscope (**Figure 4B**). Therefore, we suppose that the formation of depression is due to the nutrients essential to the cells, existing in the composition of scaffolds, engulfed by the cells. Such mechanism indicates that the scaffolds can be absorbed by the cells carried, feed the cells to grow up constantly while serving as the support simultaneously and promote the fusion of cells and scaffolds. All above build the foundations for protecting the cell growth in the scaffold from necrosis and exudation in tissue defect repair application subsequently.

For the study of skin tissue engineering, researchers have put most of the focus on the performance and application of the active stent itself rather than the biocompatibility of cells and stents. For example, Nathan J. Castro [23] created a series of new constructs to combine to stress the capability of key characteristic nanocomposite scaffold manufactured by 3D printing. The scaffold was easy to separate from the wound when put in the surface of mice wound in the first day in our study, hence such operation had no effect on the discharge of a large amount of inflammatory exudate and necrotic materials. That the stent dissolved within one week didn't influence the formation of granulation tissue in the bottom of the wound at all. What's more, extra new granulation tissue emerged below the undissolved stent, which seems to one of the mechanisms of the scaffolds containing cells to promote wound healing. The histological changes happened in wound after the dissolution of the stent are consistent with our previous studies. We observed that the wound tissue covered with the cell-contained scaffolds grew similarly to the normal tissue, which demonstrates the good biocompatibility of bioactive scaffold without

biotoxicity and rejection form the perspective of histopathology. The safe application of the bioactive scaffolds with Bone Marrow Mesenchymal Stem Cells prepared by 3D bioprinting for skin tissue engineering provides a theoretical basis for the practices of active scaffolds in clinical, which turns out that active scaffolds will achieve more research progress in skin tissue engineering in the future.

Disclosure of conflict of interest

None.

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