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Enhancement of skin wound healing with decellularized scaffolds loaded with hyaluronic acid and epidermal growth factor

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ABSTRACT

Current therapy for skin wound healing still relies on skin transplantation. Many studies were done to try to find out ways to replace skin transplantation, but there is still no effective alternative therapy. In this study, decellularized scaffolds were prepared from pig peritoneum by a series of physical and chemical treatments, and scaffolds loaded with hyaluronic acid (HA) and epidermal growth factor (EGF) were tested for their effect on wound healing. MTT assay showed that EGF increased NIH3T3 cell viability and confirmed that EGF used in this study was biologically active in vitro. Scanning electron microscope (SEM) showed that HA stably attached to scaffolds even after soaking in PBS for 48 h. ELISA assay showed that HA increased the adsorption of EGF to scaffolds and sustained the release of EGF from scaffolds. Animal study showed that the wounds covered with scaffolds containing HA and EGF recovered best among all 4 groups and had wound healing rates of 49.86%, 70.94% and 87.41% respectively for days 10, 15 and 20 post-surgery compared to scaffolds alone with wound healing rates of 29.26%, 42.80% and 70.14%. In addition, the wounds covered with scaffolds containing EGF alone were smaller than no EGF scaffolds on days 10, 15 and 20 post-surgery. Hematoxylin–Eosin (HE) staining confirmed these results by showing that on days 10, 15 and 20 post-surgery, the thicker epidermis and dermis layers were observed in the wounds covered with scaffolds containing HA and EGF than scaffolds alone. In addition, the thicker epidermis and dermis layers were also observed in the wounds covered with scaffolds containing EGF than scaffolds alone. Skin appendages were observed on day 20 only in the wound covered with scaffolds containing HA and EGF. These results demonstrate that the scaffolds containing HA and EGF can enhance wound healing.

1. Introduction

Skin is the largest organ system in animals and provides the front defense barrier against different damages to animal bodies [1,2]. Injuries to skins caused by various physical factors or chemical agents can induce wound healing and skin regeneration [3,4]. Wound healing is the process in which cells in the body regenerate and repair to reduce size of damaged or necrotic area [5]. Wound healing involves a set of events that include inflammation surrounding a region of injury, wound cell migration and mitosis, angiogenesis and the development of granulation tissue, repair of the connective tissue, regeneration of extracellular matrix and remodeling that leads to a healed wound [6,7]. The effects of growth factors on wound healing have been extensively studied and are found to be involved in these processes [8,9].

Almost all growth factors are peptides that bind to the target cell surface receptors. Commonly, the receptor kinase activity is essential for inducing the biological activities of various growth factors including epidermal growth factor (EGF), transforming growth factor β (TGF-β) and vascular endothelial growth factor (VEGF), and different growth factors can affect the normal and pathological wound healing process [10]. EGF is known to be a potent stimulator of cellular proliferation. It has been tested for its effects on wound healing [11,12]. EGF is beneficial to wound healing because of its effects on keratinocytes, fibroblasts and vascular endothelial cells and promotes the formation of granulation tissue and re-epithelialization. The action of EGF on the cells including keratinocytes, fibroblasts and endothelial cells in wound area can be regulated by local production of EGF through autocrine and paracrine mechanisms [10]. EGF can stimulate the growth and

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differentiation of keratinocyte as well as the proliferation and migration of fibroblast and vascular endothelial cells [10,13].

Besides growth factors, other molecules also play important roles in wound healing. Hyaluronic acid (HA) is a major extracellular matrix component. It is important for wound healing because of its various biological activities [14]. Due to its unique physical properties, HA creates an excellent wound healing environment and has multifaceted roles in wound healing and scarring [15]. HA can be induced to be synthesized in wounds and plays an important role in scarless wound healing of fetal skin [16]. Wound dressing composed of HA and collagen sponge containing EGF has been investigated [17,18].

Decellularized tissues as biological scaffolds are tested in pre-clinical animal studies and clinical applications [19]. Decellularized scaffolds possess many good properties compared with artificial scaffolds [20]. Their exceptional cellular affinity and the ability to provide extracellular matrix resembling in vivo environment enable them to provide a proper environment for cellular growth, migration and differentiation and for protein expression, tissue deposition and angiogenesis [21]. Decellularized scaffolds are used for the skin engraftments and cosmetic surgery and are an ideal tissue source for tissue transplantation [22]. They are able to accelerate skin regeneration after they are applied to full-thickness skin defects [23]. They can promote re-epithelialization, formation of granulation tissue and other skin appendages as well as neovascularization in the early phase of implantation [24]. Decellularized scaffolds represent a promising biomaterial in future clinical applications.

The effective wound healing strategies have been extensively studied for decades. Due to high complexity and poorly-known mechanisms of wound healing processes, many unavoidable obstacles and shortcomings exist with the current methods. These obstacles are divided into 3 groups: (1) Low secretion of growth factor in chronic wound. Previous study found that fluid from acute healing wounds contained approximately ten-fold higher level of EGF than from chronic wounds [25]. (2) Excessive degradation of growth factor in the wound because of high concentration of digestive enzymes. Previous study reported that an ointment containing EGF and a protease inhibitor could accelerate wound healing through stabilizing the topical EGF [26]. (3) The change of physiological response of skin cells to growth factor. For example, many studies indicate that aberrant transforming growth factor β (TGF-β) signaling plays a key role in the etiology of the pathophysiological mechanisms involved in hypertrophic scarring which often occurs after deep burn injury or trauma [27]. Many previous studies applying EGF in wound area failed to provide positive effects [28]. To address the under-secretion of the growth factors in the wound, artificial administration of growth factors through wound is explored, but the complexity has caused not satisfactory wound healing outcome in various wounds, including burns, diabetic, trauma and other chronic ulcers [29]. Thus, studies have been performed to find strategies to overcome those obstacles and increase EGF effect on wound healing, and for example, use of highly positive charged LMWP conjugated to the N-terminal of EGF significantly can increase the EGF permeability and decrease effective dose of EGF [12]. Studies of prolonged retention and sustained release of EGF are urgently required.

In this study, decellularized scaffolds were prepared from pig peritoneum by a series of physical and chemical treatments. Decellularized scaffolds containing HA and EGF were compared with EGF alone for effect on the recovery of skin wounds. This study may provide effective method for the use of decellularized scaffolds in combination of EGF and HA for the promotion of wound healing.

2. Materials and methods

2.1. Materials

DMEM cell culture medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Penicillin/streptomycin and dimethyl sulphoxide (DMSO) were purchased from Sigma (St Louis, MO, USA). Ethanol, phosphate buffered saline (PBS), SDS and sodium chloride (NaCl) were purchased from Chemical Regent (Guangzhou, China). Benzalkonium bromide solution was purchased from Baiyun Pharmaceutical (Nanchang, China). Polypropylene oxide was purchased from Hainan Petrochemical Works (Jiangsu, China). HA was purchased from Freda Biotechnology Company (Shandong, China). EGF was purchased from PeproTech (Rocky Hill, NJ, USA). Atropine sulfate was purchased from Jixing Pharmaceutical Company (Shandong, China). Pelltobarbitalum natricum was purchased from Chemical Plant (Beijing, China).

2.2. MTT assay

To understand the optimum concentration of EGF for the best interaction with the cells so that it can be used in further stages of the research and confirm that EGF used in this study is biologically active, MTT assay was performed. NIH3T3 cells (mouse embryonic fibroblasts) were obtained from American Type Culture Collection (ATCC) and seeded at passage 3 to 5 in 24-well plate (Corning, Acton, MA, USA) at a density of 8×10^3 cells/well. The culture medium was DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. After cells were cultured for 24 h, the culture medium was replaced with DMEM containing 0.5% FBS, and cells were cultured for 12 h. Cells were cultured for 72 h in DMEM containing 0.5% FBS and different concentrations of EGF. Then, 100 μl MTT solution was added to each well and incubated for 4 h. The culture medium was replaced by 300 μl DMSO. Then, 100 μl liquid was transferred from each well to a 96-well plate (Corning, Acton, MA, USA) and measured at 570 nm by a microplate reader (Bio-Rad 680, Hercules, CA, USA). The group containing 0 ng/ml EGF was used as a negative control.

2.3. Preparation of decellularized scaffolds

Decellularized scaffolds were prepared from fresh and healthy pig peritoneum. The peritoneum was soaked overnight at 4 °C in 0.1% benzalkonium bromide solution as an antiseptic to inhibit the microbial growth in the scaffolds. It was soaked for 1 h in 70% aqueous solution and overnight in 30% aqueous solution. It was repeatedly rinsed in water to remove residual ethanol and soaked in water for at least 1 h at 4 °C to make cells in the tissue expand. Cells in the tissue were removed by ultrasonic treatment as previously described [30]. Briefly, the decellularization system using sonication treatment consisted of an ultrasonic horn (Sonifier 250A, Branson Ultrasonics Co., Shanghai, China), a constant temperature water bath (Shanghai Anfamayaxi Bio Co., Shanghai, China) and a custom-made reactor. A solution (pH 5.6) containing 2% SDS and 0.3% NaCl was used as detergent solution to improve the efficiency of cell removal. Ultrasonic power was set to 30 W of continuous oscillation, and the frequency of ultrasound was 20 kHz. The ultrasonic treatment was carried out for 24 h. Then, 0.1 M polypropylene oxide was prepared and adjusted to pH 8.5–10.5 with NaOH. The peritoneum, following sonication treatment, was soaked in 0.1 M polypropylene oxide for one 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 PBS to remove residual polypropylene oxide. It was cut into different sizes, and the surface was coated with 1% sodium hyaluronate. It was sterilized in a plastic bag with ^{60}Co irradiation.

2.4. Examination of the attachment of HA to scaffold by scanning electron microscope (SEM) and HA release experiment

Decellularized scaffolds were placed at room temperature to dry before SEM, and cut into pieces of 0.5 × 0.5 cm. A layer of conductive adhesive was pasted to the dedicated SEM object stage, and samples were adhered to the stage, keeping observation surface up. Sample was sputtered with gold (Au) for 60 s using a Fine Coater