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The Effects of Fibrobalastic Stem Cells with Collagen Hydrogel on Burn Wound Healing in Animal Model

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Abstract: Although burn wounds are complicated wounds that are difficult to heal, stem cells may have positive role in burn wound healing. The purpose of this study was to investigate whether fibroblast cells combined with collagen hydrogel accelerate burn wound healing in an animal model. Burn wounds were created in male Wistar rats. The rats were divided into groups treated with fibroblast cells, collagen hydrogel, "collagen + fibroblast cells", and control untreated group. Macroscopic and microscopic examination were applied to evaluate wound healing. The data were analyzed using ANOVA. Treatment with collagen + fibroblast cells led to higher accelerated wound healing rate than other groups. It can be concluded that collagen hydrogel facilitates the formation of a suitable substrate to increase the fibroblast activity, which is followed by increased skin epithelial cell proliferation resulting in accelerated wound healing rate.

Keywords: Fibroblast, Collagen hydrogel, Burn wound, Rat

1 Introduction

Burns are wounds that cause superficial or deep skin damage with protein breakdown in cells and biochemical abnormalities, and increase the risk of functional and cosmetic consequences (Franck et al., 2019). Autologous stem cell (SC) injection was observed to

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significantly accelerate wound healing. In a mouse cytokine array experiment, transplantation of SCs showed a significant increase in angiogenesis and accelerated wound healing by increasing cell proliferation in wound area (Zhou et al., 2019) and increasing regeneration (Feng et al., 2019) and also SCs increased type III collagen deposition (Franck et al., 2019). Transplantation of stem cells into the burn wound bed increases re-epithelialization and wound closure (Yan et al., 2020). The use of hydrogels can be effective in transporting SCs to the wound site, with fewer side effects and anti-scar activity (Franck et al., 2019).

SCs suppress apoptosis with immunomodulatory and antioxidant angiogenic activities (Abbas et al., 2018). They can reduce pathological wounds in the healing process (Wang et al., 2021). Levels of anti-inflammatory cytokines such as TGF-β1 levels in SCs-treated wounds were also significantly elevated (Xiao et al., 2020; Oryan et al., 2019). The findings show that stem cells stimulate the migration of fibroblasts *in vitro* and *in vivo* (Kobayashi et al., 2018). The *in vivo* results showed the effectiveness of using SCs in reducing the time required for complete recovery. In addition, skin wound perfusion was significantly improved in SC-treated mice (Rodriguez et al., 2015). SCs affect paracrine activity on skin cells (Wiśniewska et al., 2021). Injury causes significant diversity among fibroblasts (Guerrero-Juarez et al., 2019) and it has been shown that exosomes derived from stem cells are involved in the healing of skin wounds (Ma et al., 2019). Promising results were also observed in complete skin regeneration using fibroblast growth factor (Matsumine et al., 2019). It has been shown that the neonatal stem cells effectively healed severe burn wounds (Mahmood et al., 2019). The use of fibroblasts in wound healing eliminates the need for a ready-to-use fibroblast reservoir (Nilforoushzadeh et al., 2017). Basic fibroblast growth factor (BFGF) containing hydrogels have been shown to dramatically improve cell proliferation, wound epithelialization, collagen deposition, and contraction without causing toxicity or inflammation (Zhang et al., 2018; Wang et al., 2019). Fibroblast growth factor mediates a variety of biological and pathological processes, including angiogenesis, wound healing, embryonic growth, and metabolic control via paracrine or endocrine signals (Hui et al., 2018). Autologous fibroblast grafts improve grade 3 burn wounds in diabetic surgery (Nilforoushzadeh et al., 2019). Mice treated with hydrogel cells have relatively more collagen deposition (Mohamad et al., 2019; Hoyle et al., 2017). Fibroblasts have been reported to play an important role in the deposition of extracellular matrix (ECM) components, wound contraction, and regeneration of new ECM (Desjardins-Park et al., 2018). Wound closure, granulation tissue growth, angiogenesis, and re-epithelialization were all greater in fibroblast growth factor 2 (FGF2) treated mice (Tran-Nguyen et al., 2021). FGF2-treated wounds may induce epithelial keratinocyte-mesenchymal transmission (EMT) and accelerate wound healing (Koike et al., 2020; Monsuur et al., 2017). However, the distinction between mouse and human skin regeneration, such as contraction of the panniculus carnosus and the importance of specific niches of skin stem cells, makes it difficult to bridge the gap between preclinical and clinical research (Zomer and Trentin, 2018). Although some clinical trials have shown that chronic wound healing is enhanced after SC transplantation, these clinical trials have drawbacks, including the small number of cases and the incomplete method of larger-scale controlled multicenter trials required. During recent years the safety and effectiveness of treating SC-based wounds with standard techniques are enhanced, especially for the production and quality control of mesenchymal stem cells, as well as for establishing a

strong body of clinical evidence (Huang et al., 2020). Adult-derived stem cells can be used in a variety of products, however *in vivo* stem cell therapy is limited due to the need for harvesting techniques, immunogenicity, and cell viability.

Considering the contradictory results on the effects of fibroblast cells on burn wound healing, the present study was carried out to investigate the effects of fibroblast cells combined with collagen hydrogel on burn wound healing in animal model.

2 Materials and Methods

2. 1. Fibroblast cells isolation

Newborn human foreskin tissue obtained following routine circumcision, was collected in 0.15 N NaCl containing 400 units/ml penicillin and 200 /lg/ml streptomycin (GIBCO (USA)) and stored at 4°C for up to 24 hr before use. The foreskin was cut into small sized pieces using a scalpel and the pieces of skin were placed in a 15 mL centrifuge tube (containing 10 mL of 1x dispase solution and incubated overnight (~ 16 h) at 4°C. Phosphate buffered saline was used to wash away excess dispase. The dermis was then separated from the epidermis and cut into very small pieces. The pieces were transferred into centrifugation tube containing 5 mL of collagenase and incubated at 37°C for 4-8 hours. 5 mL Dulbecco's Modified Eagle Medium (DMEM) containing fetal bovine serum10% (FBS) was added to dilute the collagenase and the solution, and then passed through 70 um cell strainer to obtain a single-cell suspension. The suspension was centrifuged and the supernatant was discarded and the cell pellet was cultured in DMEM culture medium. The cultured cells were used for *in vivo* experiments after 3-4 passages (Monsuur et al., 2017).

2. 2. Evaluation of cell viability

Cell viability is assessed by flow cytometry using a fluorescent dye called PI (propidium iodide).

2. 3. Characterization of fibroblast cells

Immunocytochemistry (ICC) staining of obtained cells was performed to confirm the identification of the isolated fibroblast cells. The cells were grown on coverslips for 24h and then fixed in acetone for 30 min at -20°C. After blocking with 3% H2O2 in 1% Sodium Azide PBS for 30 minute and several washes with PBS and again blocking with 1.5% goat serum for 60min at room temperature, cells were stained with primary mouse monoclonal anti-vimentin antibodies. The coverslips were dehydrated and mounted onto microscopic slides. Obtained slides were observed using fluorescence microscope.

2. 4. Preparation of hydrogel and seeding the fibroblast into hydrogel

To prepare the collagen hydrogel, 41 mg of bovine collagen was dissolved in 25 mL of sterile water and a mixture of PBS buffer (16 mL) and HEPES buffer (8 mL) was added to it. After reaching the desired confluency in culture medium, fibroblast cells were trypsinized and transferred onto the hydrogel. Cell-containing hydrogel was placed in the incubator and

examined under microscope. Propidium iodide-acridine fluorescent staining was used to detemine the viability of seeded fibroblast cells in the hydrogel.

2. 5. Animal studies

2. 5. 1. Establishment of skin burns

3-months male Wistar rats weighing between 200-220g were anesthetized by intraperitoneal injection of 2 mg ketamine and 0.2 mg xylazine and their backs were moistened and completely shaved. The shaved area was disinfected with chlorhexidine gluconate. A heated sterile biopsy punch was used to create a full thickness burn wound with the diameter of 8 mm at the dorsal supracostal region on both sides of the midline. The wound area was sterilized by gauze and washed by normal saline. The dressings were fixed on the wound using Vaseline gauze and transparent adhesive tape. Images of burn wound area were captured on days 0, 7, 14 and 21 after wound establishment using a digital camera.

2. 5. 2. Histological examination

The rats were sacrificed by spinal cord destruction method on post-treatment days 7, 14 and 21. The wound bed and the surrounding intact skin containing dermis and hypodermis were excised. Harvested specimens were fixed with 10% formalin and were stained with H&E staining for histological analysis. Fixed and stained tissue slides were photographed using a digital camera.

2. 6. Statistical studies

The data were analyzed by one-way ANOVA. P value less than 0.05 was considered statistically significant in all analyses

3 Results and Discussions

3. 1. Cell viability

The results showed that a high percentage (89.9%) of fibroblast cells were alive on the day of isolation (Fig. 1).

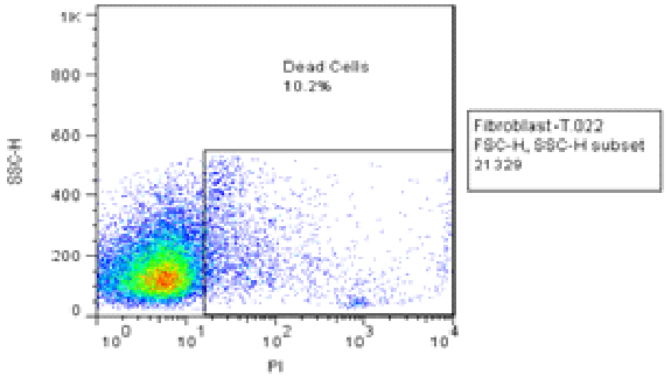

Figure 1. Viability of fibroblast cells on the first day after isolation. According to the figure above, 89.8% of the cells were alive.

3. 2. Fibroblast cell staining

On microscopic examination of fibroblast cells isolated from tissue and stained with ICC for Vimentin marker, all cells were uniformly brown. The results showed that the isolated cells expressed the Vimentin marker at a high level confirming the identity of fibroblast cells (Fig. 2).

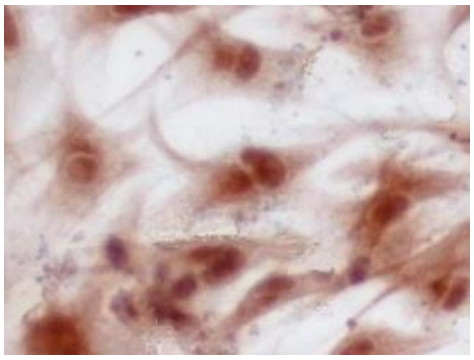

Figure 2. Fibroblast cells were observed by ICC staining (1000X). Due to the presence of Vimentin, the cells were colored brown indicating the fibroblast identity.

3. 3. Fibroblast cells viability on collagen hydrogel

Fluorescent investigation of cells loaded on hydrogel with propidium-acridine staining showed that 96% of the cells were alive.

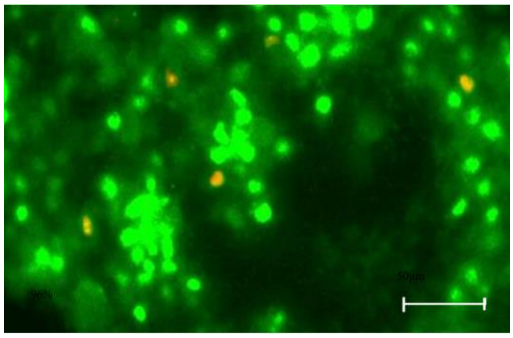

Figure 3. The green color in the image above indicates live cells cultured on the hydrogel, and the red color shows dead cells.

3. 4. Macroscopic and microscopic observation of wound area

Macroscopic observation showed accelerated wound healing in treatment groups compared to control group on days 14 and 21 post-treatments. Animals treated with collagen + fibroblast cells had significantly lower wound area than other groups on day 21 post-treatment, indicating that (Fig. 4). Microscopic examination of skin tissue samples showed that the number of neutrophils infiltrated to the wound site increased significantly in all groups and was almost the same in all 3 intervals of the study, indicating that the wound has stimulated immune system, which was followed by inflammatory reaction leading to increased neutrophils count at the wound site. Epithelial tissue started to form during first week post-treatment and continued to expand during weeks 2 and 3 after operation. The epithelial formation was higher in group

treated with collagen + fibroblast than other groups on day 21 post-treatments (Fig. 5).

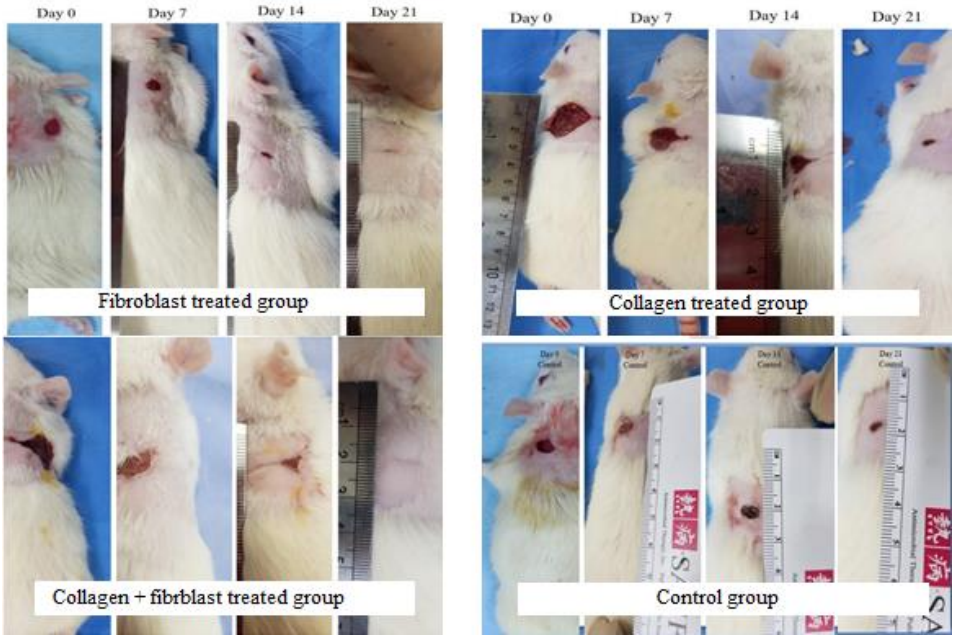

Figure 4. Wound closure on days 0, 7, 14 and 21 in control and experimental rats.

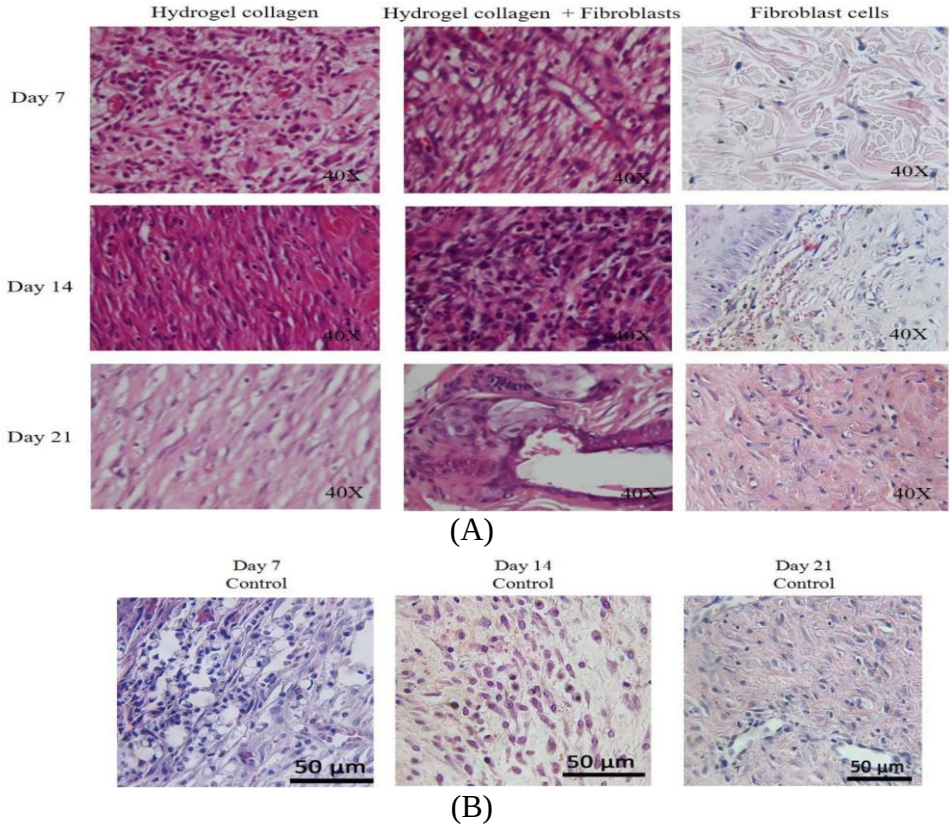

Figure 5. Histological sections of harvested wound area specimens on days 7, 14 and 21 in control and experimental groups.

In the present study animals treated with collagen + fibroblast showed the highest accelerated wound healing rate and completed formation of epidermis in the wound site 21 days after wound-establishment. In consistent with our findings the reasearch has shown that fibroblasts can induce angiogenesis to increase burn wound healing in conditions where skin wounds are difficult to repair and close (Van den Broek et al., 2014). Fibroblasts also have been reported to increase the number of cells, the synthesis of collagen, and the thickness of the skin layers at the wound site, which ultimately accelerate the wound healing process (Yates et al., 2017). The use of fibroblast growth factor in the treatment of skin ulcers has been shown to increase the quality of skin ulcers (Gorecka et al., 2019).

Collagen scaffolds are used as a biological support substrate for wound healing because they are well adapted and have good strength (Chattopadhyay and Raines, 2014) and hydrogels are suitable for wound healing because they have a cooling function and protect damaged tissue. Their adhesion preserves wound tissue (Ruszczak, 2003). Collagen also causes blood to coagulate, which plays a key role in wound healing, creating a large surface area for cells to connect and speeding up the angiogenesis process. Collagen is well able to bind to surrounding tissues and has little antigenicity and supports the biological activity of cells (Shen et al., 2015). Fibroblast cells can be used to repair burn wounds because theses cells, along with keratinocytes, produce a variety of cytokine growth factors that differentiate cells in the wound area. However, the exact mechanism of this effect is not still completely clear, but it has been shown that these cells stimulate the initial growth of the skin in the bed of healing wounds. Therefore, using fibroblasts and scaffolds at the same time is very helpful.

4 Conclusion

The results of the present study showed that applying fibroblast cells with collagen hydrogel can significantly accelerate burn wound in animal model. Fibroblast cells seeded into collagen hydrogel can cause more proliferation of epithelial cells causing accelerated wound closure and repair.

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Conflict of interests

The authors state that there are no conflicts of interest regarding the publication of this article.

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