Inhibition effects of a negative electret 5-FU patch on the growth of a hypertrophic scar

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Abstract
In this study, the hypertrophic scar (HS) model in rats was established. 5-fluorouracil (5-FU) patch, −1000 V and −2000 V polypropylene (PP) electret 5-FU patches were prepared and applied onto the wound. The in vitro permeation experiment was performed using the Franz diffusion cell system to determine the permeation cumulative amount and retention amount of 5-FU through/scar skin. The inhibition effect of negative electret on growth of HS was studied by hematoxylin-eosin (HE) staining, Masson staining and the immunohistological methods. The permeation study indicated that a negative electret could enhance the permeation and retention of 5-FU through and in scar skin respectively. HE staining and Masson staining indicated a better effect for −1000 V and −2000 V electret 5-FU patches on HS inhibition after 28 d post-wounding compared with 5-FU patch. The immunohistological study showed much more reduced expressions of collagen type I, collagen type III, TGF-β1 and HSP47 in scar tissue after application of negative electret 5-FU patches than those of 5-FU patch. A negative electret 5-FU patch may be advantageous for HS treatment.

Keywords: electret, hypertrophic scar, 5-fluorouracil, patch, collagen, TGF-β, HSP47

(Some figures may appear in colour only in the online journal)

1. Introduction
Although wounds are common in our daily life, it is always accompanied with a hypertrophic scar (HS) if the healing process biases the normal physiologic response. It is reported that the incidence of HSs varied from 40% to 94% after surgery and 30% to 90% after burns [1, 2]. The formation of a HS is usually considered to be related with over proliferation of inflammatory cells and fibroblasts, excessive collagen deposition, disorder of growth factors and cytokines [1, 3, 4]. Transforming growth factor β (TGF-β) plays a key role in HSs because it is a potent regulator of multiple cellular functions [5, 6]. TGF-β-1 and β-2 conduct signal through the Smads and Wnt signaling pathways, upregulate the synthesis of collagen type I and type III, and promote the formation of HS [7]. Heat shock protein 47 (HSP47) is also called a collagen binding protein serving as a collagen-specific molecular chaperone [8]. Its upregulation is closely related to excessive deposition of collagen type I and type III in scar tissue. Studies also revealed that expression of HSP47 is related to TGF-β1 [9, 10]. Thus, it is important to find the strategies to regulate the expression of TGF-β1, HSP47, collagen type I and type III in clinical treatment of HSs.

5-fluorouracil (5-FU) is a fluorinated pyrimidine typically used as an antineoplastic agent. It is also effective in treatment of established hypertrophic scar through a intralosomal injection by inhibiting fibroblast proliferation and TGF-β induced expression of type I collagen. However, it also have been reported that 5-FU can lead to pain at injection site, wound ulceration and hyperpigmentation [7, 11]. Besides, systemic 5-FU may cause anaemia, leukopenia and thrombocytopenia [12]. Electret 5-FU transdermal system would
contribute all advantages of transdermal drug delivery system to 5-FU current administration status in scar treatment. It is an non-invasive and improved safty profiles to provide systemic or local drug therapy with a reduced total drug dosage because of its avoidance of first-pass metabolism in the liver [13]. The drug concentrations are reached gradually and can be controlled with higher concentration in scar tissue but reduced concentration in plasma by using the external electrostatic field as permeation driving force, which further improves the safety of medication.

The present study aims to investigate the efficacy of electret 5-FU transdermal patches in HS treatment by techniques of hematoxylin and eosin (HE) staining and Masson staining, and its mechanism of actions by the quantitative measurement of TGF-β1, HSP47, collagen type I and type III using immunohistological methods. The skin permeability and retention of 5-FU through/in the HS were also studied to further elucidate the mechanism.

2. Materials and methods

2.1. Preparation of electret

Non-metalized polypropylene (PP) film (Toray Industry Co., Japan) with thickness of 13 μm was corona charged for 15 min with the corona voltage being kept at −1000 V and −2000 V respectively. A grid voltage of 15 kV was employed. The surface potential of the negative electrets was measured using a surface potentiometer (ESR102A, Beijing Huajinghui Technology Co. Ltd, China).

2.2. Preparation of transdermal patches

A 5-FU patch and electret 5-FU patch using Eudragit 100 as pressure sensitive adhesive, tributyl citrate as the plasticizer and 3% azone as the chemical permeation enhancer were prepared respectively.

The 5-FU patch was prepared by dissolving Eudragit E100 and tributyl citrate in pure ethanol under ultrasonication for 20 min. Then 10 mg of 5-FU and a certain amount of 3% azone were added and dissolved. The resultant drug-in-adhesive mixture was cooled to room temperature and then cast onto the PP film as backing layer in size of 5 cm × 5 cm. It was allowed to air dry at room temperature for 12 h. The prepared 5-FU patch was covered with a release liner.

The electret 5-FU patches were fabricated by covering the adhesive-free backing layer of 5-FU patch onto the charged surface of electret. The prepared electret 5-FU patches were evaluated for surface potential.

2.3. Hypertrophic scar induction and treatment

Male Sprague-Dawaly (SD) rats weighing 200 ± 10 g were anesthetized by intraperitoneal injection of chloral hydrate (4 ml kg⁻¹). Two full-thickness skin wounds with 16 mm diameter were created on each side of the spine under sterile condition. The epidermis and dermis in each wound were removed. The wounds were covered by sterile gauze for one day. On the second day, animals were grouped into four categories at random. Wounds in group A were not treated by anything and would be used as control in staining and immunohistological studies. Wounds in group B to D were covered by a 5-FU patch, −1000 V electret 5-FU patch, and −2000 V electret 5-FU patch respectively. By day 28 after wound creation, HS began to appear in group A. Animals in all groups were sacrificed on day 28 by over dose intraperitoneal injection of 10% chloral hydrate. Skin samples containing whole scar area were excised and the subcutaneous tissues were removed carefully. The scars harvested from groups A to D were fixed in 4% paraformaldehyde for 48 h. After fixation, samples were dehydrated in a graded series of ethanol, embedded in paraffin and sectioned from the same direction for use. The animal care and treatment in this study were with the approval of the Animal Experimentation Ethics Committee of Second Military Medical University.

2.4. Skin permeation and retention of 5-FU

The scar skin was constructed in the same manner as group A described in section 2.3. After the rat being sacrificed at day 28 post-wounding, skin samples containing whole scar area were excised and the subcutaneous tissues were removed carefully from the undersurface. The in vitro permeation studies were performed using Franz diffusion cells. The scar skin was sandwiched between donor and receptor compartments with the stratum corneum (SC) facing the donor cell. The patch was pressed on the skin with the adhesive side facing the SC. The receptor cell with the volume of 7 ml and effective permeation area of 3.14 cm² was filled with pH 7.4 phosphate buffer solution (PBS). The receptor solution was maintained at 32°C ± 0.5°C and stirred continuously at 500 rpm. At 2, 4, 8, 12, 24, 36, 48, 72, 84 and 96 h, the diffusion cells were taken out. The contents of 5-FU in receptor cell and the whole skin were analyzed by high performance liquid chromatography (HPLC).

The permeation contents of 5-FU was expressed by cumulative permeation amounts which was calculated from the amount of 5-FU in receptor cell divided by effective permeation area at each time.

For detereroration of the 5-FU in whole skin, the patch was removed, rat skin was taken out from the diffusion cell and swung in distilled water for three times. Then the skin was cut into small pieces and homogenized in 10 ml of ethanol containing 10% hydrochloric acid for five times. Each time the homogenate was centrifuged and the supernatant was collected together for 5-FU analysis. The retention amount of 5-FU in skin was calculated from the amount of drug divided by effective permeation area.

The HPLC system consisted of a Shimadzu instrument (LC-2010) and Diamonsil C18 column (250 mm × 4.6 mm 5 μm, Dikma Company). The mobile phase was methanol and water containing 0.15% acetic acid and 0.15% ethanolamine (5:95, V/V). The flow rate was 1 ml min⁻¹. The analysis
wavelength was 265 nm. 10 μl of each sample was injected for analysis.

2.5. HE staining and Masson staining

The scar sections were deparaffinized, stained with hematoxylin and eosin (HE) for histological analysis, and with Masson’s trichrome for collagen fiber observation, respectively [13]. All samples were examined under light microscope.

2.6. Expressions of collagen Type I, collagen type III, TGF-β1 and HSP47

Immunostaining techniques were performed for quantitative determination of collagen type I and type III, TGF-β1 and HSP47. The scar sections were deparaffinized, and incubated with primary antibodies of collagen type I and type III, TGF-β1 and HSP47, respectively. Then tissue sections were prepared after treatment by secondary antibody, color reaction using 3, 3’-diaminobenzidine tetrahydrochloride (DAB), hematoxylin staining and dehydration [14].

Five representative fields (up, middle, down, left and right) of each section were photographed under a microscope and quantified using Image Pro Plus 6.0 analysis software by mean optical density. The background was removed by optical density calibration. The areas of interest in each image were set through hue: 0–30, saturation: 0–255, and intensity: 0–230. It covered the majority of the DAB-positive areas. Then the images were converted into grey scale frames and values were measured. The intensity of collagen type I and type III, TGF-β1 or HSP47 expression was measured as the mean optical density = integrated optical densities (sum)/areas (sum).

2.7. Statistical analysis

Data were expressed as mean ± standard deviation. The ANOVA test was employed for statistical analysis using SPSS21.0 software. The significance level P was set at 0.05.

3. Results

3.1. Skin permeation and retention of 5-FU

The scar skin permeation results are shown in figure 1. It can be observed that the permeation profiles were similar for all patches. However, a higher cumulative permeation amount was obtained for negative electret 5-FU patches compared with that of the 5-FU patch at each sampling time. The cumulative amounts at 72 h for −1000 V and −2000 V electret 5-FU patches were 1.28 times and 1.61 times that of the 5-FU patch (P < 0.05), demonstrating that the permeation enhancement of negative electret in 5-FU through rat scar skin was positively controlled by the magnitude of surface potential of electrets.

The retention profiles for 5-FU in whole scar skin were different after the skin being treated by the 5-FU patch, −1000 V electret 5-FU patch and −2000 V electret 5-FU patch, respectively (figure 2). Higher drug retention amounts were clearly observed when negative electret 5-FU patches were applied. The retention amounts of 5-FU in skin after treatment of −1000 V electret 5-FU patch and −2000 V electret 5-FU patch were 1.40 times and 1.73 times at 36 h, 1.24 times and 1.44 times at 72 h, respectively, compared with those of the 5-FU patch (P < 0.05), respectively. Electrets with higher magnitude of surface potential could lead to a higher retention amount of 5-FU in skin tissue, which could result in the sustained release of drugs locally.

3.2. HE staining and Masson staining

The histological profiles of the study groups after HE staining are shown in figure 3. In comparison with the unwounded skin, control group showed obvious dermis and epidermis hyperplasia, higher epithelial thickness. Collagen fibers were dense, thick and arranged irregularly. Fibroblasts densely accumulated around collagen fibers. In contrast, 5-FU patch could decrease epidermal thickness and fibroblasts density in dermis. The collagen deposition was reduced to some extent. However, fibroblasts density and collagen deposition were further reduced when wounds were treated by electret 5-FU patches. There was no obvious epidermis hyperplasia as well.

Figure 1. 5-FU permeation profiles through rat scar skin (n = 6).

Figure 2. 5-FU retention in whole HS rat skin (n = 6).
The results suggested that $-2000$ V electret 5-FU patch had more favorable effect on HS than 5-FU patch. The influence of 5-FU patch and electret 5-FU patch in collagen synthesis was further examined by Masson’s tri-chrome staining with stronger staining indicating more disorganized collagen (figure 4). In contrast with control group, the skin tissue showed lighter staining, more regular arrangement of collagen fibers, and reduced collagen density in 5-FU patch group and $-2000$ V electret 5-FU patch group. These results were consistent with findings from HE staining. The electret 5-FU patch had better effect in inhibition of collagen synthesis.

3.3. Expressions of collagen type I, collagen type III, TGF-$\beta$1 and HSP47

The expression levels of collagen type I, collagen type III, TGF-$\beta$1 and HSP47 in untreated and treated scar tissues were examined using immunohistological techniques to investigate the mechanism of the inhibition of HS proliferation by electret 5-FU patches. Results were summarized in figure 5. It indicated that HS in control group yielded significant increases of 1.66 fold for collagen type I, 2.63 fold for collagen type III, 3.63 fold for TGF-$\beta$1 and 1.47 fold for HSP47 in scar tissue compared with those of normal skin tissue ($P < 0.05$). The higher level of those expressions in the control group also indicated the successful establishment of the HS model.

However, collagen type I, collagen type III, TGF-$\beta$1 and HSP47 revealed significantly reduced expressions in scar tissues treated by 5-FU patch. The decreases of 26.4%, 37.3%, 34.6% and 26.8% for expressions of collagen type I, collagen type III, TGF-$\beta$1 and HSP47, respectively, were obtained compared with those of in the HS control group ($P < 0.05$). What is more, the expression levels of collagen type I, collagen type III, TGF-$\beta$1 and HSP47 were further reduced to 51.3%, 36.0%, 30.7% and 60.7% those of the HS control group when $-2000$ V electret 5-FU patches were applied onto the wounds ($P < 0.05$). Electrets with higher absolute value of surface potential showed better inhibition effect on HS growth.

4. Discussion

Wound healing is divided into three stages: inflammation, proliferation and remodelling. The formation of hypertrophic scar is closely related to excessive proliferation of inflammatory cells and fibroblasts, abnormal metabolism of collagen, and over expression of cytokine and growth factors [2]. And high expression of inflammatory cells, macrophage and neutrophil will appear after deep dermis injury. The inflammatory cells secrete large amount of cytokine as TGF-$\beta$1 etc and growth factor to induce the proliferation of fibroblasts, which lead to obvious increase of fibroblasts and
aggregation at the location of the wound. Besides, fibroblasts are activated and differentiated into myofibroblasts, both of which are responsible for wound contraction and closure during HS formation. The proliferative fibroblasts produce massive collagens, resulting in significant increase of collagen type I and type III in HS [14, 15]. Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone for collagen folding and secretion. Highly elevated HSP47 was detected during HS formation [8]. Therefore, the significant high expressions of collagen type I, collagen type III, TGF-β1 and HSP47 can be used to confirm the formation of HS.

In this study, we observed obvious dermis and epidermis hyperplasia, thick, densely aggregated and irregularly arranged collagen fibers in control group on day 28 post-wounding. And significantly higher expressions of collagen type I and type III, TGF-β1 and HSP47 in scar tissue were detected, which satisfied the biological characteristics of HS. Thus, we successfully established the HS model.

5-FU is a pyrimidine analog with antimetabolite activity. Studies have shown that 5-FU inhibits the TGF-β-induced expression of collagen type I in human fibroblasts and fibroblast proliferation [16, 17]. Since intralosional injection of 5-FU may cause several side effects, the 5-FU patch was used to investigate its effect on HS in this study. After application of the 5-FU patch on wounds for 28 days, more organized collagen fibers, reduced density and enlarged space among collagen fibers were observed. Besides, the quantitative immunohistological studies indicated that 5-FU patches decreased the levels of collagen type I and type III, TGF-β1 and HSP47 in wound tissues by 26.4%, 37.3%, 34.6% and 26.8% respectively in comparison to scar tissue. Therefore, 5-FU could inhibit HS formation to some extent by decreasing TGF-β1 level to further reduce the expression of HSP 47 and inhibit synthesis of collagen type I and type III.

For HS treatment, it is expected that more 5-FU could be delivered to the scar tissue. However, the excessive aggregation of collagen in HS may result in difficult penetration of compounds through the skin. In our in vitro scar permeation study, we observed that electret 5-FU patch could result in higher permeation and retention of 5-FU through and in scar skin as compared with 5-FU patch, which is favorable for HS inhibition. Electret is a kind of functional material that can produce a stable electrostatic field and microcurrent for long periods. It can not only control the release rate and amount of 5-FU from the patch, but safely regulate the permeation rate and retention of the drug in skin tissue by changing the distribution of drug molecules in the patch and reducing the intermolecular force between the matrix molecules, both of which being beneficial for drugs to migrate in matrix. And an electret with higher surface potential could produce a stronger electrostatic field, thus the surface potential-dependent enhancing effects on permeation amount and retention amount were observed. Besides, electrets can also increase the injury current of the wound and accelerate directional cell
migration towards the wound, which promotes wound healing. The external electrostatic field of electret has the effect on improvement of tissue microcirculation and decrease of inflammatory response, thereby reducing the synthesis of collagen and inhibiting the formation of HS in later stage. Therefore, results in this study showed much reduced levels of collagen type I, collagen type III, TGF-β1 and HSP47 when electret 5-FU patches were applied onto the wound. And electret 5-FU patch had a better effect on the inhibition of HS formation.

5. Conclusions

5-FU could inhibit HS formation to some extent by decreasing TGF-β1 levels to further reduce the expression of HSP 47 and inhibit synthesis of collagen type I and type III. More of the 5-FU amount was observed in scar skin tissue when the electret 5-FU patch was applied because the external electrostatic field and microcurrent that the electret produced could change the distribution of drug molecules in the patch and reduce the intermolecular force between the matrix molecules. The increased 5-FU retention amount in the scar skin resulted in a much more reduced level of collagen type I, collagen type III, TGF-β1 and HSP47. Therefore, the electret 5-FU patch in HS treatment is worth studying.

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