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5-Fluorouracil Regulates Collagen Synthesis to Reduce Hypertrophic Scar Formation in Donkey Skin Wounds

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Abstract

Background: Excessive proliferation of fibroblasts, which produce collagen, leads to Hypertrophic Scar (HS) formation in the wound healing process. 5-Fluorouracil (5-FU) is an anti-metabolic drug that inhibits fibroblast proliferation. The aim of this study was to determine whether 5-FU could help in reducing the healing time of wounds and HS formation in donkey skin.

Materials and methods: Full-thickness excisional skin wounds were created laterally on the back of each donkey. The wounds were treated topically with 5-FU or saline. Wound healing was evaluated clinically, histologically, and through gene expression.

Results: Compared with the control group, the proliferation phase of the wound healing process was delayed in 5-FU treated group, and HS formation did not finally appear. Histological staining confirmed decreases in epidermal thickness, as well as fibroblast numbers and the collagen fibrous tissue accumulation in the 5-FU treated group. On day 17 after wounding, quantitative RT-PCR analysis showed that the expression of collagen synthesis-related genes (TGF-β1, Smad3, COL1A1, COL1A2 and COL3A1 genes) was significantly lower in the 5-FU treated group than that in the control group.

Conclusions: Our study suggests that 5-FU reduces collagen deposition and HS formation, which highlights this as a potential strategy to achieve scar-free wound healing.

Keywords: Donkey; Wound healing; Hypertrophic scar; Collagen.

Introduction

Wound healing is a complex process that occurs in three phases that can be categorized as inflammation, proliferation and re-modeling or scar formation [1,2]. Wound closure is mediated by the proliferation of fibroblasts, which secrete collagen types I and III [3]. However, excessive fibroblast proliferation results in overproduction of collagen, which is a possible cause of Hypertrophic Scar (HS) or keloid formation [4]. HS formation results from dysregulation of the wound healing process [5,6]. Factors such as inflammation, collagen accumulation, and reduced fibroblast apoptosis or skin mechanical tension as well as excessive contraction at the wound site have been implicated in the formation of HS, although the mechanism remains to be fully elucidated [7-9]. Donkey skin contains large amounts of collagen secreted by fibroblasts. In our preliminary experiment, we showed that HS formation occurs in donkey skin, while keloid production does not. The mechanism by which donkey skin suppresses excess collagen secretion in wound healing remains unclear.

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Transforming growth factor-β1 (TGF-β1) has been widely reported to play an essential role in wound healing [10,11]. Furthermore, enhanced TGF-β1 expression stimulates type I and type III collagen synthesis in fibroblasts by activating the Smad2/3 signaling pathway, resulting in HS formation [12]. Therefore, TGF-β1 is a key target for the development of novel therapeutic strategies for HS [13].

5-Fluorouracil (5-FU) is a derivative of uracil in which the hydrogen at position five is replaced by fluorine. This anti-metabolic drug inhibits the biochemical activity of rRNA and mRNA in the nucleus, as well as the synthesis of DNA and proteins, leading to cell damage and death. 5-FU exerts its cytotoxic effects at all stages of cell proliferation, and also inhibits collagen synthesis [14]. It can also inhibit the growth of tumours and normal tissues [15]. 5-FU is widely used as a chemotherapeutic agent because of its capacity to induce apoptosis in malignant cells [16]. Subconjunctival 5 -FU injections administered after glaucoma surgery led to apoptotic cell death in the conjunctival epithelium [17,18].

In this study, we investigated the ability of 5-FU to inhibit excessive collagen secretion and reduce HS formation in donkey skin wound to provide a theoretical basis for the development of strategies to achieve scar-free wound healing.

Materials and methods

Donkeys

In this study, we used six female donkeys (aged 2–4 years; average weight, 200–230 kg). Before the experiment, all donkeys underwent a health examination to confirm the absence of systemic disorders or lameness that could hinder wound healing. The donkeys were fed 0.8 kg concentrate and allowed free access to hay and water.

Wound generation and wound images

Each donkey was anesthetized by intraperitoneal injection with 10% chloral hydrate (4 mL/kg) before dorsum shaving and application of antiseptic. Six full-thickness skin wounds (diameter 1.5 cm) were created with a metal template on both sides of the back midline of each donkey (three wounds on each side) with a minimum 50 cm space between each wound. The donkeys were then allocated randomly to the control or the 5-FU (Choitec Pharmaceuticals Co., Ltd. Hainan, China) treatment groups (n = 3 per group). On days 4, 5, 6, 7, 9, 11, 13 and 15 after wound creation, 6 mL 5-FU liquid (25 mg/mL) was applied topically and injected in two separate sites around each wound; control wounds were treated with 10 mL saline. Wound images were captured using a camera on days 0, 7, 17, 30, 40 and 100, with a ruler included in the image for reference. The wound healing areas were analyzed using Image J software and the wound healing rate was calculated according to the following formula:Wound healing rate (%) = [(area on day 0−area on day n)/area on day 0] × 100%.

Skin biopsy collection and staining observation

On days 0, 7,17 and 100, a full-thickness skin sample (diameter 2 cm) was collected from the wound margins and including 0.25 cm of normal skin. Each sample was equally divided into two parts. One was immediately frozen in liquid nitrogen for qRT-PCR analysis, and the other was immediately placed in 10% formaldehyde and store for 2 weeks prior to histological staining. The histological samples were embedded in optimum cutting temperature compounds, and sectioned (5μm thickness) on a microtome. The sections were stained with Hematoxylin and Eosin (HE) to evaluate the morphological features of the tissues and skin thickness, and with Masson's trichrome (MS) to evaluate the fibrous connective tissues after wound healing. Histological examinations were conducted under a microscope. The epidermis thickness and collagen density were determined with Image J software.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from samples using TRIzol reagent (Invitrogen company, Shanghai, China). The RNA samples were quantified, and reverse transcribed into first-strand cDNA using transcriptor first strand cDNA synthesis Kit (Roche, Basel, Switzerland). Each reaction was carried out in volumes of 20 μL, which included 10 μL Tip Green qPCR Super Mix, 1 μL of each primer, and 1 μL cDNA template. The PCR amplification conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, target gene annealing temperature for 30 s, and 72°C for 30 s. The following primers were used in this study: TGF-β1-F: 5′-ATTCCTGGCGCTACCTCAGT-3′, and R: 5′-CGCACGACTCCAGTGACATC-3′; Smad3-F: 5′-AGAGACCAGCGACCACCAGATG-3′, and R: 5′-GCTGCGAGGCGTGGAATGTC-3′; Smad7-F: 5′-CTCGGAAGTCAAGAGGCTGTGTTG-3′, and R: 5′-TCTAGTTCGCAGAGTCGGCTAAGG-3′; COL1A1-F: 5′-GCAACGTGTTGTGCGATGAC-3′, and R: 5′-CGACTCCTGTGGTTTGGTCG-3′; COL1A2-F: 5′-GCTGGTAGTCGTGGTGCAACTG-3′, and R: 5′-TTGGTCCAGGTCTGCCGTCTATAC-3′; COL1A3-F: 5′-TGCTGCTGGTACTCCTGGTCTG-3′, and R: 5′-CACCTGCACTGCCTGGTTCAC-3′; β-actin-F: 5′-CGACATCCGTAAGGACCTGT-3′, and R: 5′-CAGGGCTGTGATCTCCTTCT-3′. Relative gene expression was normalized against internal controls and calculated using the $^{\Delta\Delta}$CT method.

Enzyme-linked immunosorbent assay (ELISA)

Protein levels of TGF-β, COL1A1, and COL1A2 in donkey skin wound samples were analyzed using specific ELISA kits (Shanghai Enzyme Union Biotechnology Co. Ltd, Shanghai, China). The absorbance at 450 nm (OD450) was measured using a microplate reader (Thermo Fisher Scientific).

Statistical analysis

All statistical analyses were performed using SPSS 19.0. Data were presented as the mean ± standard deviation and analyzed by one-way ANOVA. $P < 0.05$ was set as the threshold for statistical significance.

Results

Macroscopic Evaluation

Wound healing occurred gradually over time (Figure 1A). On day 7, the wounds started to shrink due to re-epithelialization at the wound edge. Before day 30 post-wounding, healing occurred more slowly in the 5-FU treatment group than that in the control. By day 100, there was no HS formation in the 5-FU injection group, while slight HS formation was observed in the control group.

Compared with the control group on days 17 and 30, wound healing was significantly slower in the 5-FU group (Figure 1B). However, after day 40, there were no significant differences in the

wound healing rate of the 5-FU and control groups.

The epidermal thickness

Skin samples collected on days 0 and 100 post-wound creation were stained with HE to determine the epidermal thickness (Figure 2). In the 5-FU group, there was no significant difference in the epidermal thickness of samples collected on day 0 and 100. In contrast, the epidermal thickness in the control group was significantly increased on day 100 compared with that on day 0.

Collagen deposition

MS staining showed the presence of coarse collagen bundles in the reticular dermis of the 5-FU and control groups (Figure 3). In all wounds, the collagen bundles were distributed more sparsely on day 100 compared with 0. Furthermore, on day 100, the collagen fibers were more ordered, with wider gaps, less deposition and lower density in the 5-FU group compared with the control group.

Collagen synthesis-related gene expression

Compared with the control group, gene expression of TGF-β1, Smad3, COL1A1, COL1A2 and COL3A1 was significantly reduced in the 5-FU group on days 7 or 17 ($P < 0.05$), while the opposite pattern of expression was observed for Smad7 (Figure 4).

Collagen synthesis-related protein expression

Compared with the control group, TGF-β1, COL1A1, and COL1A2 protein expression was significantly reduced in the 5-FU group in the proliferation stage of skin wound healing at days 7 and 17 ($P < 0.05$) (Figure 5).

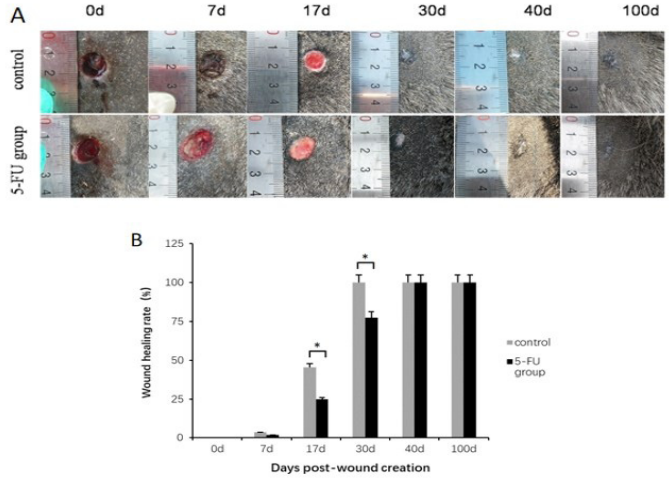

Figure 1: 5-FU slowed wound healing rate compared to that of control. **A** Images show the morphology of the healing wound. **B** Comparison of wound healing rates in the 5-FU and control groups. Data represent the mean ± SD, *$P < 0.05$.

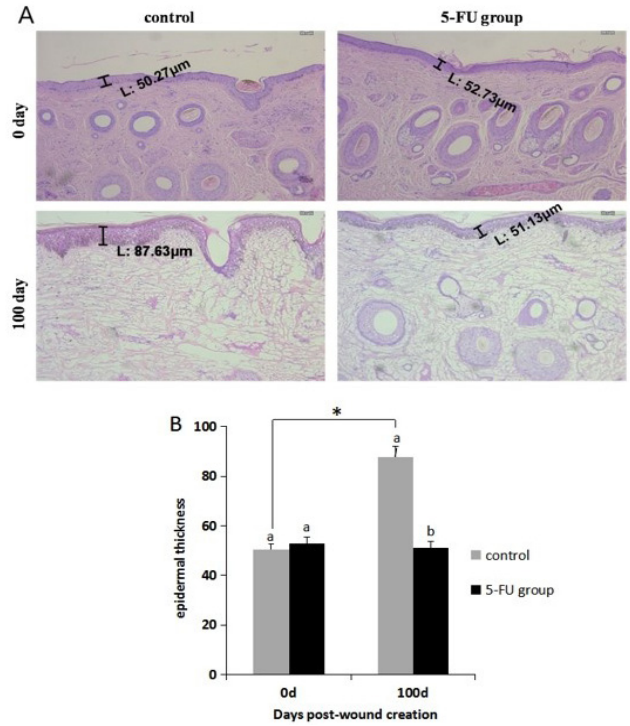

Figure 2: 5-FU had no change skin thickness in donkey skin wound healing. **A** HE staining showing skin thickness (100× magnification). **B** Epidermal thickness before and after wound healing. At the same time, values with different letter superscripts mean significant difference, and with the same letter superscripts mean no significant difference; Data represent the mean ± SD, *$P < 0.05$, $^{a,b}P < 0.05$.

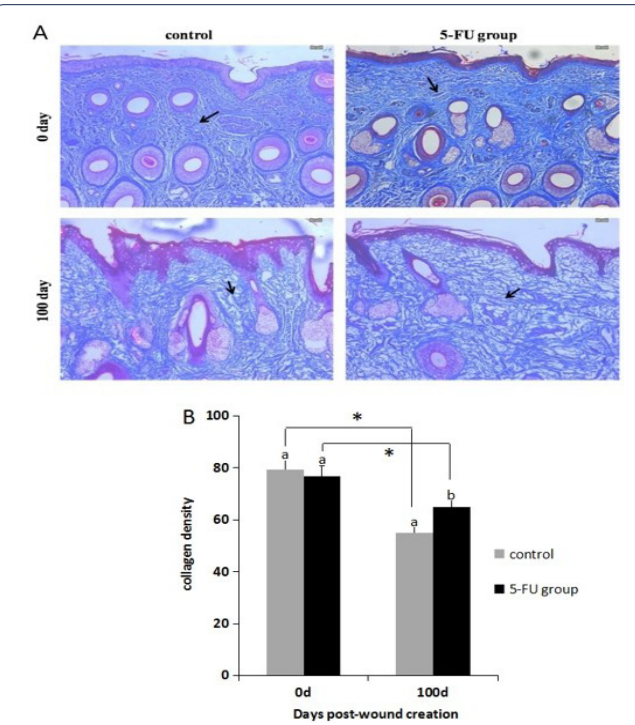

Figure 3: 5-FU reduced collagen density in donkey skin wound healing. **A** MS staining of skin dermis (100× magnification), arrow showed collagen fiber bundles. **B** Comparison of collagen density before and after wound healing. At the same time, values with different letter superscripts mean significant difference, and with the same letter superscripts mean no significant difference; Data represent the mean ± SD, *$P < 0.05$, $^{a,b}P < 0.05$.

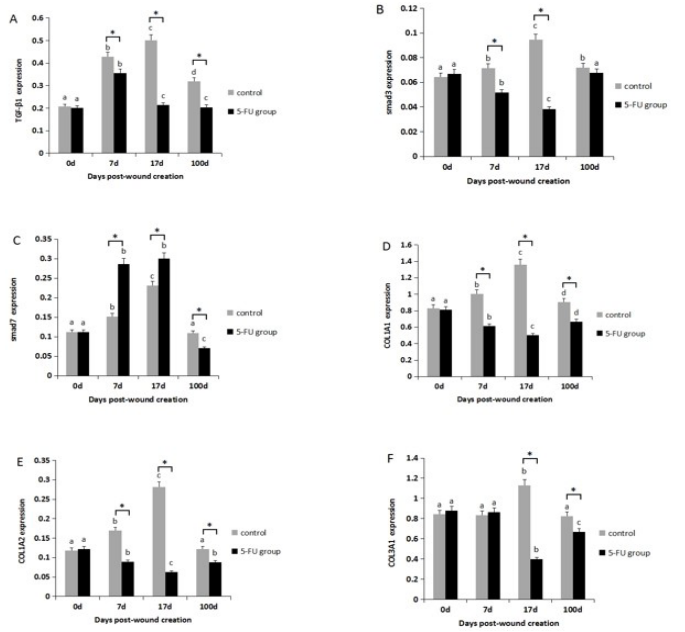

Figure 4: 5-FU reduced collagen density in donkey skin wound healing. **A** MS staining of skin dermis (100× magnification), arrow showed collagen fiber bundles. **B** Comparison of collagen density before and after wound healing. At the same time, values with different letter superscripts mean significant difference, and with the same letter superscripts mean no significant difference; Data represent the mean ± SD, $*P < 0.05$, $^{a,b}P < 0.05$.

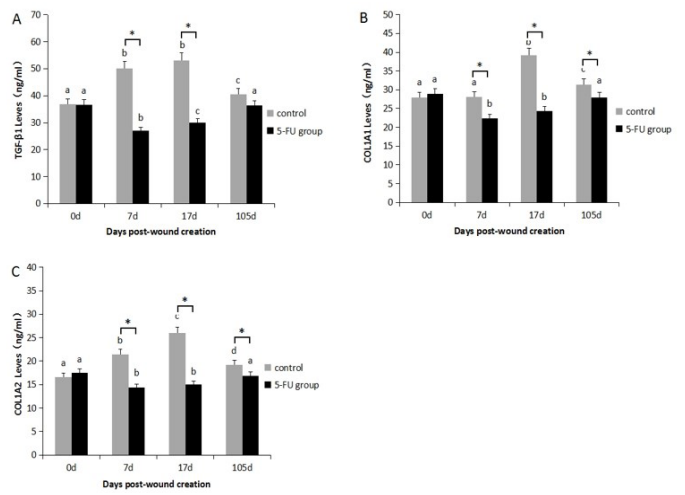

Figure 5: The effect of 5-FU treatment on proteins expression of collagen synthesis-related proteins in skin wound healing. **A-C** ELISA analysis of TGF-β1, COL1A1 and COL1A2 levels in skin samples collected at different time-points after wound creation. In the same group, values with different letter at different times superscripts mean significant difference, and with the same letter superscripts mean no significant difference; Data represent the mean ± SD, $*P < 0.05$, $^{a,b}P < 0.05$.

Discussion

In this study, we examined the effect of 5-FU on wound healing and HS formation in donkey skin model, and we specially investigated differences in proliferation stage. Our results showed that 5-FU could reduce collagen deposition and HS formation by inhibiting the proliferation of fibroblasts. It has been known that scar formation is due to the proliferation of collagen fibers, and disruption of the tissue structure [19-21]. 5-FU Could Inhibit 2 type I collagen gene (COL1A2) expression in human fibroblasts [13]. Although donkey skin can secrete a lot of collagens, our study suggests that 5-FU not only prolonged the period of proliferation during wound healing, but also reduced collagen deposition, allowing sufficient time for orderly arrangement of collagen and scar-free healing. In the pre experiment, observation of skin wound contraction combined with analysis of the expression of collagen synthesis-related genes indicated that the proliferation period of donkey skin wound healing occurred approximately 5-20 days after the trauma. Therefore, we started 5-FU treatment before the granulation phase.

The TGF-β/Smad pathway is considered to be one of the main mechanisms of fibroblast proliferation and is associated with regulation of the genes that cause scar fibrosis [22,23]. TGF-β1 promotes fibroblast growth, and increases the synthesis of collagen and other extracellular matrixes to heal wounds [24-26]. 5-FU has been implicated as an effective treatment for keloids formation by inhibiting the expression of TGF-β1 gene and blocking the signaling pathways involved in collagen synthesis [27,28]. Smad3 is activated by TGF-β1 to promote the expression of type I collagen genes [29]. Fibroblast proliferation and collagen deposition is induced in response to skin trauma, and can lead to HS formation. Inhibition of Smad3 or increased expression of Smad7 results in reduced collagen deposition [30]. To investigate the role of the TGF-β/Smad signaling pathway donkey skin wound healing and the effects of 5- FU treatment, we analyzed the expression of TGF-β, Smad3, and Smad7. We found that the expression of TGF-β1, COL1A1 and COL1A2 were significantly reduced in the 5-FU group at both the gene and protein levels compared with the control group, which may be mediated through TGF-β/Smad signaling pathway, indicating that 5-FU not only inhibited the growth of fibroblasts, but also downregulated the expression of collagen.

Conclusions

This study shows that 5-FU reduces collagen deposition and HS formation in donkey skin wounds, which highlights this as a potential strategy to achieve scar-free wound healing.

Declarations

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Ethics approval: All animal experiments in this study were approved by the Animal Ethics Committee at Shihezi University (Xinjiang, China).

Disclosure statement: No potential conflict of interest was reported by the author(s).

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