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Triamcinolone acetonide suppressed scar formation in mice and human hypertrophic scar fibroblasts in a dose-dependent manner

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ABSTRACT

A hypertrophic scar is a complex medical problem. The study of triamcinolone acetonide for the treatment of scars is necessary. The 7mm full-thickness skin wounds were created on the back of BALA/c mice to construct the animal scar model. The different doses of triamcinolone acetonide injection or normal saline were injected into the wound on the 15th, 30th and 45th day after the operation. The skin histopathological changes of mice were observed by Hematoxylin-Eosin (H&E) staining. The proteins and mRNA expression level of scar-biomarkers (COL1, COL3, α-SMA) in mice scar tissue were detected by western blot and qRT-PCR. Besides, the effect of triamcinolone acetonide on the proliferation, invasion, and migration of human hypertrophic scar fibroblast (hHSFs) in vitro was also explored by cck-8, transwell and wound healing assays. After triamcinolone acetonide was injected into the wound, the proportion of scar was significantly reduced, and the treatment effect was concentration-dependently. H&E staining showed that the skin histopathological of mice was improved dose-dependently after injecting the low/middle/high-dosage of triamcinolone acetonide. The proteins and mRNA expression levels of COL1, COL3, and α-SMA were reduced dose-dependently in mice scar tissue. Furthermore, triamcinolone acetonide dose-dependently suppressed the proliferation, invasion, and migration of hHSFs in vitro. Together, triamcinolone acetonide suppressed scar formation in mice and human hypertrophic scar fibroblasts in a dose-dependent manner, phenotypically and mechanistically. The research and further exploration of triamcinolone acetonide in treating scar formation may find new effective treatment methods for the scar.

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Introduction

Scar tissue is a natural product in the process of human wound repair (1). During normal wound healing, the synthesis and degradation of collagen maintain a balance, while in pathological scars, this balance is broken, leading to excessive hyperplasia of scar tissue (2, 3). Pathological scar mainly includes hypertrophic scar and keloid (4). A hypertrophic scar is a complex medical problem. It is estimated that 100 million people suffer from the global disease burden caused by scar (5).

The common pathogenic factors of pathological scarring include trauma, burn, surgery, vaccination, skin puncture, acne, and herpes zoster (6). The formation of pathological scars is related to the mechanism of growth factor regulation, collagen abnormality, heredity, immune abnormality, and sebum reaction (7). Pathological scarring seriously affects the physiological function and mental health of patients, so active treatment measures should be taken. Hypertrophic scar and keloid have similarities in clinical manifestation and response to treatment, and their treatment methods also have similarities (8).

At present, surgical resection, radiotherapy, local injection of drugs, laser therapy, pressure therapy and combination therapy are used to treat scars clinically (9, 10). In drug therapy, intrascar injection of drugs has played an important role in the treatment of scars (11). Triamcinolone acetonide is the most used corticosteroid hormone in the clinical treatment of scar (12), but the relationship between the concentration of triamcinolone acetonide and the proliferation of scar fibroblasts is still unclear. In the clinical application of triamcinolone acetonide in the treatment of scars, different regions and different doctors have their drug concentration habits, and there is no unified clinical standard. Too high a concentration of triamcinolone acetonide is likely to cause long-term residues of local drugs, resulting in skin pigmentation or deepening, skin atrophy, capillary necrosis, skin ulcer formation, and cortisol increase (13). Therefore, it is very necessary to explore the appropriate concentration of triamcinolone acetonide for the treatment of scars.

In this study, 7mm full-thickness skin wounds were created on the back of BALA/c mice to construct the animal scar model for investigation. The different doses of triamcinolone acetonide (high/middle/low-dose) or normal saline were injected into the wound on the 15th, 30th and 45th day after the operation. The skin histopathological changes of mice were recorded and observed by Hematoxylin-Eosin (H&E) staining. The proteins and mRNA expression level of scar-biomarkers in mice scar tissue were detected by western blot and qRT-PCR, including COL1, COL3, and α-SMA. Besides, the effect of triamcinolone acetonide on the proliferation, invasion, and migration of Human hypertrophic scar fibroblast (hHSFs) in vitro was also explored by cellular experiments. This research highlights the important effect of triamcinolone

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acetonide on scar formation in mice and human hypertrophic scar fibroblasts.

Materials and Methods

Animals and groups

A total of 24 SPF female BALA/c mice (20-25 g) (License No. of Shanghai Slake Laboratory Animal Co., Ltd.: SCXK (Shanghai) 2017-0005) were raised in separate cages, the drinking water and food were disinfected, the indoor air was clean and ventilated, and the room temperature was kept at 22°C ~ 27°C. The animal experiments were approved by the Medical Ethics Committee of Nanping First Hospital (Animal Ethics Approval No.: NPSY202002041). Triamcinolone Acetonide Injection (TAI, 40 mg/mL, Kunming Jida Pharmaceutical Co., Ltd, Kunming, China) was prepared for treatment. The mice were divided into 4 groups, including (a) Normal saline group (0.3 mL); (b) Low-dose triamcinolone acetonide group (0.075 mL TAI); (c) Middle-dose triamcinolone acetonide group (0.15 mL TAI); (d) High-dose Triamcinolone acetonide group (0.3 mL TAI). The mice were injected with 5% chloral hydrate (anesthetized to create 7mm full-thickness skin wounds on the back, respectively (14). On the 15th, 30th and 45th days after the operation, each wound in the experimental group and the control group was treated with injections (normal saline group, low-dose/middle /high-dose triamcinolone acetonide). After 8 weeks, the mice were euthanized, and scar tissue was photographed and taken to store and observe the changes of the wound.

Hematoxylin-Eosin staining

After obtaining scar tissue samples, each group takes one sample, which is fixed with 10% polymethanol, dehydrated, embedded, and made into pathological sections for H&E staining. After absorbing scar tissue samples, immerse the tissue in hematoxylin dye solution for 5 min, and wash with water until no dye solution flows out. Immerse the tissue in PBS and turn blue for 5 min. Immerse the tissue in eosin dye for 15 s. Immerse the tissue in 95% ethanol for fast decolorization for 3 s. Immerse the tissue in absolute ethanol for fast decolorization for 2 s. Wash with water until no dye flows out. The tissue was immersed in absolute ethanol and dehydrated for 10 minutes. Immerse the tissue in xylene for 10 minutes. Use neutral gum for sealing.

Western blot

Using the whole protein extraction kit (KeyGEN Bio TECH, Nanjing, China) to take 100 mg of samples for protein extraction, add 1 ml lysis buffer, 10 μL phosphatase inhibitor and 10 μL PMSF+1 μL protease inhibitor until they are ground into homogenate (operation on ice), pour them into EP tube, put them into a centrifuge for centrifugation (12000 g / 5 min), and take the supernatant. Next, configure SDS-PAGE gel, electrophoresis time is 1.5 h, run for~30 min with 80 V, and run for~1h after the marker starts to separate, adjust the voltage to 120 V. Put the PVDF membrane (Immobilon-P; Millipore, Billerica, Massachusetts) and blocked with 5% milk or bovine serum albumin, followed by incubation with primary and secondary antibodies (Goat Anti-Rabbit IgG H&L (1:5000, Abcam, Cambridge, MA, USA, ab205718). Primary antibodies were COL1 (1:2000, Abcam, Cambridge, MA, USA, ab255601), COL3 (1:1000, Abcam, Cambridge, MA, USA, ab23445), α-SMA (1:1000, Abcam, Cambridge, MA, USA, ab5831), and GAPDH (1:10000, Abcam, Cambridge, MA, USA, ab181602) antibodies.

RNA extraction and qRT-PCR

TRIzol was used to extract total RNA from the tissues, and then RNA concentration and purity were determined by RNA quantizer. Then, the genome DNA was removed and incubated at 42°C for 5min. After the reaction, it was placed on ice. A reverse transcription reaction was carried out, incubate at 50°C for 15 min, and 75°C for 5 min, terminate the reaction, and store the transcripts at -20°C. PCR reaction, reaction system (2 μL cDNA, 0.4 μL upstream primer, 0.4 μL downstream primer, 10 μL 2 × NovoStart ® SYBR qPCR SuperMix Plus, 6.8 μL ddH2O, 0.4 μL Dye I). Reaction conditions step 1: 95°C for 1 min; step 2: 95°C for 20 s, 56°C for 20 s, 72°C for 30 s, 40 cycles were conducted; step 3: 95°C for15 s, 60°C for 60 s, 95°C for 15s, 60°C for 15s. The primer sequence is as follows: COL1-Forward: 5'-CAGTCGATTCACCTACAGCACG-3'; COL1-Reverse: 5'-GGGATGGAGGGAGTTTACACG-3'. COL3-Forward: 5'-TTGAAGGAGGATGTTCCCATCT-3'; COL3-Reverse: 5'-ACAGACACATATTTGGCATGGTT-3'. α-SMA-Forward: 5'-TGGCTGATGGAGTACTTC-3'; α-SMA-Reverse: 5'-GATAGAGAAGCCAGGATG-3'. GAPDH-Forward: 5'-ACAGCAACAGGGTGGTGGAC-3'; GAPDH-Reverse: 5'-TTTGAGGGTGCAGCGAACTT -3'.

Cell culture

Human hypertrophic scar fibroblast (hHSFs) was bought from Shanghai Guandao Biological Engineering Co., Ltd. (Shanghai, China). The cells were cultured in DMEM medium (containing 5% fetal bovine serum) and grew in an environmental incubator at 37°C and 5% CO_2.

CCK-8 assay for proliferation

Taking 1.5×10^4/ml cells of hHSFs in a logarithmic growth period and inoculate them on 96 well culture plates. Each well is inoculated with 100 μL of triamcinolone acetonide culture solution with different concentrations (3/6/12 mg) for intervention, respectively. Cell proliferation is measured every 24 hours for 3 consecutive days. According to the operating instructions of the kit, after replacing the common culture solution, added 10 μL of CCK8 reagent into each well, and incubate at 37°C for 2 hours in the incubator with 5% CO_2. Then measured the light absorption value (OD value) at 450 nm wavelength with an enzyme-linked immunosorbent detector. The light absorption value indirectly reflects the number of living cells.

Transwell assay for invasion and migration

Inoculated 8×10^4 cells per well in 24 well plates and cultured for 24 hours under conventional conditions. Discard the culture medium, then resuspend the cells with serum-free culture solution, count, and adjust the cell density to 300000/ml. Place the transwell culture chamber into a 24-well plate and divide it into 4 groups (n=3). Each well-added 600 μL triamcinolone acetonide was diluted with serum-free medium to the lower layer of the chamber (3/6/12 mg). Then added 100 μL cell suspension contai-

ning 3 × 10^4 cell into the chamber, routinely cultured for 12 hours. Take out the transwell cell, fix it with methanol for 15 mins, dye it with 0.1% crystal violet for 20 minutes, carefully wipe the upper cells of the microporous membrane with a cotton swab, and clean it twice with PBS. Taking pictures of the cells in the lower layer of the microporous membrane under the fluorescence microscope, take 3 visual fields for each hole, and counted the cell number.

Wound healing

Draw a parallel line at the bottom of the six-well plate with a marker pen. The distance between the parallel lines is 0.5cm. Inoculated 2 × 10^6 cells per well in 6 well plate for 24 h. Thereafter, a ruler was used to make scratches with a 10 μL gun head. The scratch direction is perpendicular to the marking line. PBS was rinsed 3 times to wash the floating cells and add serum-free medium. Then the cell was cultured for 24 hours under conventional and triamcinolone acetonide-treated conditions (specific concentration). Take out cells at 0 h and 24 h time points and take pictures under the microscope.

Statistical analysis

The statistical analysis data were analyzed by Statistic Package for Social Science (SPSS) 22.0 software (IBM, Armonk, NY, USA). The data between groups were compared by one-way ANOVA test. The data between the two groups were compared by Tukey in Post Hot Multiple Comparisons test. The difference was statistically significant if $P<0.05$.

Results

Triamcinolone acetonide dose-dependently reduces hypertrophic scar in mice

First, a 7 mm full-thickness skin wound was created on the back of mice, then normal saline (control group) and low-/middle-/high- doses of triamcinolone acetonide (3/6/12 mg) were injected into the wound of mice at 15 d, 30 d, 45 d. The mice in each group survived normally after injection, and the back skin of the mice was thin, ruddy, and the veins and blood vessels on the back could be observed. The texture was soft, and the skin tissue was small, without adhesion. In the triamcinolone acetonide treatment group, the scar area of mice can be observed on the 15th, 30th, 45th and 60th days to decrease significantly with time. In the control group, obvious scar reduction occurred 45 days later. After triamcinolone acetonide was used, the proportion of scar was significantly reduced, and the treatment effect was concentration-dependent (Figure 1). The results of Hematoxylin-Eosin (H&E) staining showed that in the control group, the dermis of mice was significantly thickened, the collagen fibers were significantly proliferated and expanded, the subdermal vascular wall became thicker, the lumen became narrower, the subcutaneous hair follicles were significantly reduced, and the fat layer became thinner with more inflammatory cell infiltration. Compared with the control group, the above skin histopathological changes of mice in the low/middle/high dosage groups of triamcinolone acetonide were improved with dose dependence, and the thickness of skin tissue was lower than that of the control group (Figure 2). These results suggest that triamcinolone acetonide dose-dependently reduces hypertrophic scar in mice.

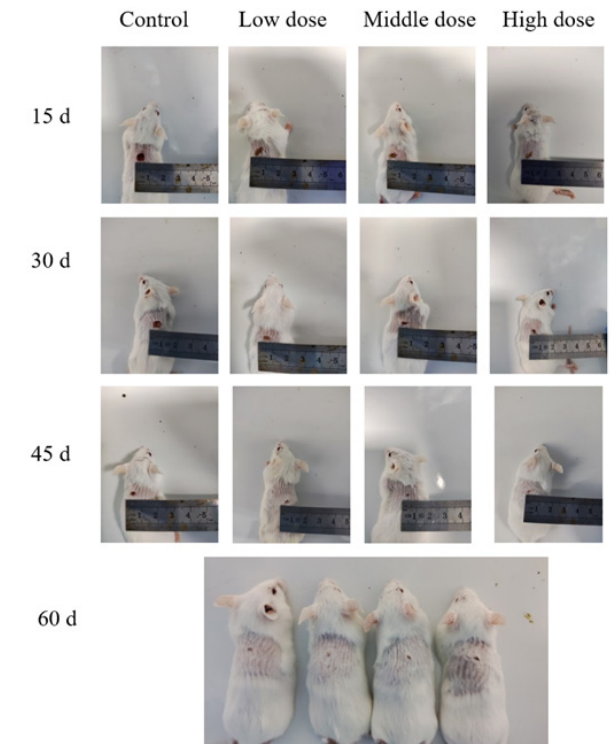

Figure 1. Triamcinolone acetonide dose-dependently reduced scar formation in mice. The scar of mice became smaller with time (n=6). After using triamcinolone acetonide, the proportion of scar decreased with the increase of drug dosage. Control group: 0.3 mL normal saline; Triamcinolone acetonide injection (40 mg / mL) group: high dose: 0.3 mL; middle dose: 0.15 mL; low dose: 0.075 mL).

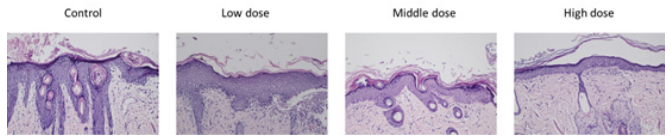

Figure 2. H&E staining of mice scar tissue. H&E staining showed histopathological changes in scar tissue when treated with different concentrations of triamcinolone acetonide. With the increase of triamcinolone acetonide concentration, the skin histopathological changes of scar gradually decreased.

Triamcinolone acetonide dose-dependently reduces scar biomarkers in mice scar tissue

In the next step, the scar-biomarkers in mice scar tissue were assessed by WB and qRT-PCR at proteins and mRNA levels. As presented in Figure 3A, triamcinolone acetonide could inhibit the protein expression level of collagen COL1, COL3, and α-SMA, while the normal saline has no significant effect. The suppression effect of triamcinolone acetonide was concentration-dependently and remarkably (Figure 3B). In addition, the mRNA expression level of COL1, COL3, and α-SMA were also suppressed by triamcinolone acetonide concentration-dependently, and that was consistent with the trend of protein level (Figure 3C). These results revealed that triamcinolone acetonide can improve the scar condition in mouse skin tissue at the molecular level.

Triamcinolone acetonide dose-dependently suppressed the proliferation, invasion, and migration of hHSFs in vitro.

Next, human hypertrophic scar fibroblast was cultu-

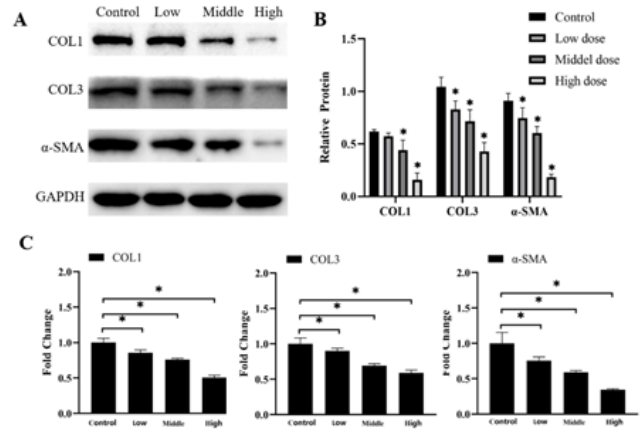

Figure 3. Triamcinolone acetonide dose-dependently reduces the expression level of COL1, COL3, and α-SMA in mice scar tissue. (A) The western blot presented the protein expression level of COL1, COL3, and α-SMA in groups treated with different concentrations of acetonide. (B) The histogram shows the statistics of protein expression of COL1, COL3, and α-SMA. With the increase of triamcinolone acetonide concentration, the protein expression of COL1, COL3, and α-SMA protein decreased significantly. (C)The histogram shows mRNA expression levels of COL1, COL3, and α-SMA under different concentrations of triamcinolone acetonide. With the increase of triamcinolone acetonide concentration, the protein expression of COL1, COL3, and α-SMA protein decreased significantly. *, P<0.05, n=3.

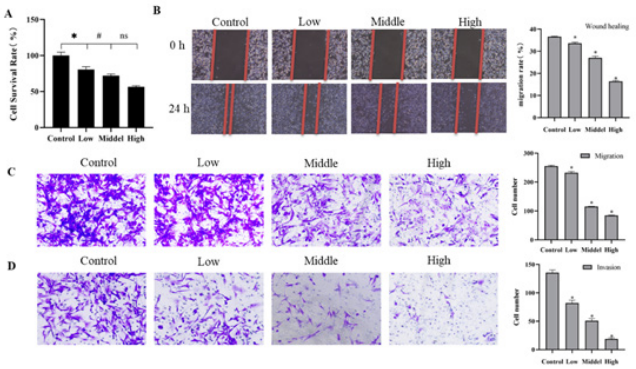

Figure 4. Triamcinolone acetonide suppressed the proliferation, invasion, and migration of human hypertrophic scar fibroblast in vitro. (A) The CCK-8 assay was performed to detect the proliferation ability of hHSFs treated with different concentrations of triamcinolone acetonide. (B) The wound healing assay was carried out to detect the migration ability of hHSFs treated with different concentrations of triamcinolone acetonide. (C and D) The transwell assay was conducted to detect migration (C) and invasion. (D) ability of human hypertrophic scar fibroblast (hHSFs) treated with different concentrations of triamcinolone acetonide. *, control vs. low-dose group, P<0.05; #, low- vs. middle-dose group, P<0.05; ns, not significant. n=3.

red to explore the effect of triamcinolone acetonide at the human cell level. As shown in Figure 4A, the results of CCK-8 indicated that triamcinolone acetonide concentration-dependently suppressed the proliferation of hHSFs in vitro. In addition, the result of the wound healing assay also confirmed the suppression of triamcinolone acetonide on the hHSFs (Figure 4B). What's more, the representative images of the transwell assay presented that the cell migration and invasion were suppressed gradually with the increased concentration of triamcinolone acetonide. (Figure 4C and D). This evidence proved the effective inhibition function of triamcinolone acetonide on human hypertrophic scar fibroblast.

Discussion

In this study, we used the BALA/c mice to create 7mm full-thickness skin wounds on the back to construct the animal scar model for investigation. After triamcinolone acetonide was injected into the wound, the proportion of scar was significantly reduced, and the treatment effect was concentration dependent. H&E staining showed that the skin histopathological of mice was improved dose-dependently after injecting the low/middle/high dosage of triamcinolone acetonide. The proteins and mRNA expression level of scar biomarkers were reduced dose-dependently in mice scar tissue, including COL1, COL3, and α-SMA. Furthermore, triamcinolone acetonide dose-dependently suppressed the proliferation, invasion, and migration of hHSFs in vitro. These results revealed that triamcinolone acetonide suppressed scar formation in mice and human hypertrophic scar fibroblasts in a dose-dependent manner.

The scar is different from the normal skin, it is mainly composed of abnormal structure and excessive dense accumulation of collagen fibers, accompanied by the loss of hair follicles and glands (15). The pathological scar can be divided into hypertrophic scar and keloid (16). A hypertrophic scar is when too much collagen tissue is produced at the site where the skin is physically damaged, and these tissues remain within the boundary of the original lesion (17). The hypertrophic scar can be divided into the linear hypertrophic scar and extensive hypertrophic scar (18). Unlike hypertrophic scar, keloid involves hypertrophic scar tissue, which is characterized by the expansion of the scar beyond the scope of the initial lesion (19). In view of the lack of animal models of keloid, the basic research on pathological scar mainly focuses on hypertrophic scar. In this research, we used the BALA/c mice to create 7 mm full-thickness skin wounds on the back to construct the hypertrophic scar model refer to the study of Vorstandlechner et al. (14). The scar model was successfully established for the following experiments.

Triamcinolone acetonide is a synthetic long-term corticosteroid chemical drug (20). Triamcinolone acetonide can inhibit cellular immunity, reduce inflammatory reaction and early telangiectasia, maintain capillary permeability, and induce changes in the function of vascular endothelial cells by inhibiting the conversion of the extravascular matrix (21, 22). Triamcinolone acetonide has long-lasting anti-inflammatory and anti-allergic effects on various skin diseases such as neurodermatitis, eczema, psoriasis, keloid, hypertrophic scar, etc. (23-25). Grabowski et al. showed that triamcinolone acetonide has a good therapeutic effect on hypertrophic scar (26). However, there is no agreed standard for the drug concentration of triamcinolone acetonide. Therefore, this study explored the appropriate concentration of triamcinolone acetonide for the treatment of hypertrophic scar in mice. Triamcinolone acetonide dose-dependently reduces scar on mice, H&E staining showed that skin histopathology of mice was improved dose-dependently.

The study of hypertrophic scars mainly focuses on fibroblasts (27). Previous studies have confirmed that these

kinds of cells are the main effector cells in the pathological process of hypertrophic scar (28). Changes in its biological behavior, such as changes in its ability to proliferate, migrate, and contract, can have an important impact on the formation and remodeling of the extracellular matrix during wound healing, leading to the formation of hypertrophic scar (1, 29, 30). Phenotypically, triamcinolone acetonide concentration dependently suppressed the proliferation, invasion, and migration of hHSFs in vitro. As for the molecular mechanism, triamcinolone acetonide dose-dependently reduces scar biomarkers in mice scar tissue, including COL1, COL3, and α-SMA. In a previous study, COL1, COL3, and α-SMA are served as biomarkers for hypertrophic scar fibroblasts (31). These results indicated that triamcinolone acetonide has a remarkable treatment effect on the hypertrophic scar fibroblasts.

Together, triamcinolone acetonide suppressed scar formation in mice and human hypertrophic scar fibroblasts in a dose-dependent manner, phenotypically and mechanistically. The research and further exploration of triamcinolone acetonide in preventing scar formation may find new effective treatment methods for the scar.

Funding

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Conflict of interest

There is no conflict of interest associated with this work.

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