Chlorpheniramine maleate exerts an anti-keloid activity in vivo and in vitro

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Abstract

Background: Keloid can cause numerous adverse consequences, severely affecting patients' physical and mental health. The mechanisms of keloid scarring remain unclear, as revealed by the inability of patients to satisfactorily manage this benign neoplastic disease. Studies suggest that H1 blockers, such as Tranilasts, inhibit keloid overgrowth and hyperplasia. Chlorpheniramine maleate (CPM) has been extensively employed as an H1 receptor blocker, whereas its treatment effects on keloid formation are unknown. The present study aimed to evaluate the inhibiting effect of CPM on keloid, which might provide a novel strategy for keloid treatment.

Methods: *In vitro*, Cell Counting Kit-8 (CCK-8), apoptosis, cell cycle and migration assays of keloid fibroblasts (KFs) were performed to evaluate the effects of CPM on inhibiting the proliferation and apoptosis of dermal fibroblasts (DFs) and keloid fibroblasts (KFs), and the potential mechanism of action was analyzed by qPCR. *In vivo*, nude mice keloid scar model was built to assess the therapeutic effect of CPM and explore its possible mechanism.

Results: CCK-8 and apoptosis experiments revealed that the concentration of 0.15 mM CPM could inhibit keloid fibroblasts proliferation and promote apoptosis with minimal impact on DFs. Cell cycle and migration experiments confirmed that a 0.15 mM CPM could inhibit the proliferation and migration of keloid fibroblasts. Moreover, qPCR revealed that CPM down-regulated the expressions of TGF- β , STAT3, JAK1, and Ki-67 genes in KFs. Together with *in vitro* experiments, CPM could inhibit keloid scar formation and down-regulate the expressions of TGF- β , STAT3, JAK1, and Ki-67 genes in the tissue.

Conclusion: Chlorpheniramine maleate could be an ideal localized therapeutic strategy in keloid treatment.

Background

Keloid, a fibrotic disease, is characterized by hyper-proliferation of responsive fibroblasts, with continuous extracellular matrix (ECM) components synthesis. The progressive uncontrolled proliferation of keloids is usually accompanied by chronic inflammation, extremely high morbidity, and malignant tendencies (1). Keloid commonly leads to adverse outcomes, such as functional impairment, limited mobility, impaired cosmetic effects, and adverse psychological effects (2, 3). Reports have proposed that several factors attributing to keloid formation, skin tension, infection, ECM differences, and immune dysfunction take the lead (4, 5). Most of the factors mentioned above involve inflammatory responses and aberrant ECM production (6–8). Intra-lesioned injection with steroids is currently used as the first-line therapy because of its advantages (i.e., localized treatment with less injury, high remission efficiency, and high tolerance) (9). However, numerous side effects, tissue atrophy, de-pigmentation, telangiectasis, drug deposition, localized paper-thin skin, possible Cushing's syndrome, and systemic adrenal insufficiency, are associated with steroid treatment (10–12). Although various novel drugs have been applied as treatment options over the past few years, including immune-suppressants, calcium channel blockers, and

interferon, their efficacy is not ideal in terms of safety and disease recurrence (13, 14). Therefore, effective and safe drugs are urgently required to bridge the gap in keloid treatment.

Microenvironment immune-mediated inflammatory response stimulates collagen synthesis in wound repair, and moderate inflammatory reaction facilitates wound healing and repair (15, 16). Keloid can be associated with early inflammatory infiltration of various inflammatory cells (e.g., neutrophils, macrophages, and mast cells). These cells are activated and release an excess of inflammatory cytokines (e.g., IL-1 β , IL-6 and TNF- α) (1, 17). In keloids, considerable mast cells are closely associated with fibroblasts, and mast cell phagocytosis of collagen fibres is considered a common ultra-structural feature (18). Abnormal collagen synthesis and subsequent accumulation of collagen fibres in keloids can induce mast cell recruitment and increase collagen phagocytosis (19, 20). Products of mast cells (e.g., histamine, heparin, and various cytokines) can stimulate fibroblasts and initiate keloid formation (21). Antihistamine drugs can stabilize mast cells' cell membrane and inhibit histamine release from mast cells. Additionally, the histamine-induced inflammation, fibrosis and keloid scarring can be antagonized by preventing the binding of histamine to effector cells H1 receptor (22–24).

Chlorpheniramine maleate (CPM) has been extensively applied in propylamine H1-receptor antagonists as an antihistamine. The clinical evaluation suggests that over-healing states in keloids could be suppressed by H1 blocker agents (25, 26). In addition, antihistamine usage can inhibit the growth of fibroblasts derived from human skin, scar, and keloid (25, 27). Tranilast is an H1 histamine receptor antagonist. In a rabbit ear keloid model, tranilast can inhibit keloid hyperplasia by blocking the TGF β -I/Smad3 signal pathway to reduce collagen synthesis and type I/ III collagen ratio (11). In a clinical study, oral use of tranilast has been effective in keloid treatment. It can relieve itching, pain, scar control with high safety (28). In addition, in vivo experiments revealed that tranilast can be administered effectively through ion electro-osmosis (29) or acid penetration enhancer (30) in localized keloid treatment, which has been more beneficial than oral drugs, especially in the form of oral drugs in relieving pain and itching symptoms. However, the effect of CPM for treating keloid has not yet been studied, and its underlying mechanism remains unknown.

The present study aimed to clarify the effect of CPM on KFs viability in vitro and keloid hyperplasia in vivo to present a novel therapeutic attempt of CPM on the keloid.

Methods

Reagents

Chlorpheniramine maleate (National Pharmaceutical Group Rongsheng Pharmaceutical Co., Ltd., China) was dissolved in dimethyl sulfoxide (DMSO) and then stored at -20°C. For all samples, the final DMSO concentration was kept at 0.1%.

Cell culture

Human adult dermal fibroblasts (DFs) and primary keloid fibroblasts (KFs) received the culture as described. The use of human samples (keloid tissue and normal tissue) was conducted by complying with a protocol reviewed and approved by the Human Research Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine. In brief, keloid fibroblasts originated from earlobe keloid tissue resulting from ear piercing in three patients aged from 20 to 30 (two females and one male). Normal dermal fibroblasts were acquired from the back skin of the three patients in their thirties (two females and one male) without systematic diseases when they were undergoing other unrelated operations. DFs and KFs were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, CA, USA) containing 10% Fetal Bovine Serum (FBS, Gibco, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Furthermore, cells were grown in a humidified atmosphere of 5% CO₂ at 37 °C. The culture medium was changed per 3 days. Moreover, all experiments were performed after 3 to 4 cell passages.

Cell proliferation assay

The primary KFs and human adult DFs were seeded in 96-well plates with 5000 cells per well along with 100 µl of medium, respectively. KFs and DFs, after being starved in serum-free medium for 24h to allow for cell synchronization, were replaced with fresh culture medium in the absence or presence of different doses of CPM (0.01, 0.05, 0.10, 0.15, 0.30mM), and then tested by employing a cell counting kit-8 (CCK-8; Lianke Biological, China) at 24h and 48h. In brief, at each testing time point, 10 µl of sterile CCK-8 solution was introduced to each well and then incubated for 1h at 37 °C, and then the medium was harvested to measure the optical density (OD) values at 450 nm by using a micro-plate reader (Thermo Scientific). To be specific, cell proliferation rate (%) = OD value of the experimental group/OD value of the control group 100%. On the whole, all the assays were performed in quintuplicate and then repeated by applying three cell samples.

Apoptosis assay

The experimental concentration was further taken by complying with the results of IC₅₀. The two cells were cultured according to the growing conditions and the groups described above. Subsequently, the cells were detached with 0.25% trypsin/0.01% EDTA (Gibco) and then washed with cold PBS for three times. Next, the cells were re-suspended in 1x annexin-binding buffer to 1x10⁶ cells/ml and then stained with 100 µl binding buffer containing 5 µl Annexin V (Beyotime Biotechnology) for 15 min and 1 µl of 100 mg/ml PI for additional 5 min at ambient temperature. Cells were analyzed using FACS Caliber (BD biosciences). The results were analyzed with Flowjo software (BD biosciences).

Cell cycle analysis

As indicated from the result of CCK-8 and Apoptosis assay, 0.15 mM CPM could inhibit proliferation of KFs and promote KFs apoptosis, while 0.15 mM CPM did not induce mass death of KFs. Thus, 0.15mM

CPM was employed for the following experiment.

Cell cycle analysis was performed to evaluate the effect of CPM on KFs. In brief, KFs and DFs were cultured for 48h without or with CPM at a concentration of 0.15 mM, respectively. Subsequently, the cells were trypsinized, centrifuged at 1500 rpm for 5 min, washed with ice-cold PBS and then suspended in PBS containing 1 ml DNA Staining solution and permeabilization solution. The samples were incubated at ambient temperature for 30 min, and flow cytometric analyses were conducted with a flow cytometer (Beckman Coulter) equipped with Flowjo software (TreeStar). The mentioned analyses were repeated in four cell samples.

Cell migration assay

An *in vitro* scratch wound assay was performed to evaluate cell migration. For the *in vitro* wound assay, KFs and DFs, when reaching 90 % confluence in 6-well plates, were scratched with a sterile 200-μl pipette tip and then incubated in the culture medium with or without 0.15 mM CPM. The cell status was recorded for 0 h after the scratching process. The serum free medium was replaced and then photographed after 48h. Besides, photographs were taken before and after the scratching and at the indicated time point. The photographed area was quantified with computer-assisted image analysis with IPP 6.0 software.

Quantitative real time qPCR analysis

To analyze the effect of CPM to the mRNA levels of TGF-β1, JAK1, STAT3 and Ki-67 in DFs and KFs, RT-PCR were performed (Table 1). In brief, after 48h of incubation with or without CPM (0.15 mM) in serum-free culture medium, total mRNA was extracted by using RNA Rapid Extraction Kit (Generay) and then reverse transcribed to cDNA with PrimeScript™ RT reagent Kit (Takara, Japan). In addition, RT-PCR was performed by using ChamQ SYBR Color qPCR Master Mix (Vazyme) based on CFX connect Real-Time PCR System (Bio-Rad). With the standard $2^{-\Delta\Delta Ct}$ method, the mRNA expressions of Target genes were normalized to GAPDH and calculated.

Table 1

Primer sequences for RT-qPCR

Gene	Primer	Sequence (5' - 3')
TGF- β 1	Forward	CAGTACAGCAAGGTCCTTGC
	Reverse	ACGTAGTAGACGATGGGCAG
JAK1	Forward	GCATCGAGCGCACAAAGTTA
	Reverse	GTGATGGTGCGATTTGGAGC
STAT3	Forward	CAAGGGCTTCTCCTTCTGGG
	Reverse	CCTGGGTCAGCTTCAGGATG
Ki-67	Forward	TTACCGGGCGGAGGTATGAA
	Reverse	ACGTCCAGCATGTTCTGAGG
GAPDH	Forward	GCTCTCTGCTCCTCCTGTTC
	Reverse	GACTCCGACCTTCACCTTCC

Western blot assay

DFs and KFs were seeded on six well plated, cultured overnight, and treated with CPM for 48h. Then Cells were lysed in RIPA containing 1% protease inhibitors (Solarbio, Beijing). Different groups of protein concentration were measured by BCA Protein Assay Kit (Beyotime, China). The protein of each sample was separated on 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore, IPVH00010) at 300 mA V for 90 min. After blocking in 5% nonfat milk for 1 h at room temperature, the membranes were incubated with primary antibodies against TGF- β (1:1000; Abcam, ab179695), JAK1 (1:1500; Abcam, ab133666), STAT3 (1:2000; Abcam, ab68153) and Ki67 (1:1000; Abcam, ab243878) overnight, and GAPDH (1:5000; Abcam, ab8245) was used as a control. Next, after washing with TBST for three times, the membranes were incubated with Goat anti-Rabbit IgG $\times$ H+L $\times$ Secondary antibody (1:5000; Thermo Pierce, 31210) for 1 h at room temperature. Finally, according to instruction of Super Signal® West Dura Extended Duration Substrate, the membranes were detected using enhanced chemiluminescent (ECL). Band Scan 5.0 software was used to quantify the density of protein bands.

Animal model study design

The experimental procedures were approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (Approval number: 2020-948, Date: 2020/12/18, Hangzhou, China). A total of 18 animals were treated by complying with the facility's guidelines for laboratory animal treatment and care. Human keloid fragments were acquired from three surgically treated patients. Next, each human keloid fragment was split into multiple small specimens (0.5*0.5cm with full

thickness), soaked in ice PBS and then processed in 2 h. After the general anesthesia of the nude mice (BALB/nu-nu; SLAC Laboratory Animal Company, Shanghai, China) was achieved, a 0.5*0.5cm full-thickness skin was excised on backs of the nude mice, specimens were subcutaneously sutured and then fixed on the backs of nude athymic mice, with two specimens in the respective mouse. One week later, 18 mice with all the keloid scar tissues on the back were selected to perform the next experiment. From day 8, drugs were injected once a week. The specimens in the respective mouse fell to two study groups, i.e., control group, 0.9% normal saline (100 μ l/site) and experimental group, CPM (Volume ratio 6%, 0.6mg/ml, 100ul/site). All mice were euthanized on day 56, anesthetized with chloroform and separated by guillotine. The volume ratio change of the back keloid was measured, and scar samples were collected for histology and immunohistochemistry. All of the animal experiments in this study were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the laboratory animal ethical committee of Zhejiang University.

Keloid Tissue Weight

Five weeks after the implantation, the keloids were harvested from the backs of the mice. The weight variation of the explanted keloids was compared between the treatment and control groups.

Histology and immunofluorescence

Furthermore, the samples were fixed in 4% paraformaldehyde for 48 h, dehydrated through an alcohol gradient and then embedded in paraffin blocks. Five-micron-thick histological sections were cut at the center of the embedded specimens and then stained with Hematoxylin and Eosin (H&E) and Masson. The sections were observed under an optical microscope, and the images were captured with an inverted microscope (Leica, German). For immunohistochemistry, skin sections were dewaxed and then pretreated with antigen retrieval solution. Next, the sections were stained with TGF β 1 antibody (Sc-146), Ki-67 antibody (27309-1-AP), Stat3 antibody (4904S), JAK1 antibody (ET1705-84) and further incubated with Enzyme-labeled goat anti-mice/rabbit IgG polymer covered tissue in the histochemistry kit (Fuzhou Maixin Bio). Afterwards, the color was rendered with DAB (Shanghai Gene), and counterstained nucleus with Hematoxylin for about 4min before was observed under an inverted fluorescence microscope (Leica, German). VS2000 scanning system was used to collect images, and Image J was employed for the analysis.

Statistical analysis

Triplicate experiments were performed. All quantitative data with normal distribution were expressed as mean $\pm$ standard deviation. Comparisons among multiple groups were drawn by conducting one-way analysis of variance (ANOVA) and then Tukey post hoc multiple comparisons test. For the comparisons

between two groups, the analyses were conducted by Student's t test. $P < 0.05$ was considered with statistical significance. All statistical analyses were conducted with GraphPad Prism 6.0 software.

Results

The isolation and identification of keloid fibroblasts and normal skin fibroblasts

In this study, keloid tissue abiding by the clinical diagnostic criteria was selected (See Supplementary Figure 1a, Additional File 1), and after surgical resection, pathological diagnosis was conducted (See Supplementary Figure 1b, Additional File 1). Moreover, keloid fibroblasts and normal skin fibroblasts were successfully separated. The purity of keloid fibroblasts was identified based on immunofluorescence staining for Vimentin (See Supplementary Figure 1e, Additional File 1). To maintain primary characteristics, all primary cells were preserved for 3-7 generations.

Chlorpheniramine maleate inhibited cell proliferation and facilitated apoptosis in DFs and KFs

As impacted by the aberrant proliferation of dermal fibroblasts having been suggested to contribute significantly to keloid overgrowth and expansion, the ability of CPM to modulate the proliferation of DFs and KFs was initially investigated.

As indicated from the result of CCK-8 assay, low dosage of CPM (0.01 mM and 0.05 mM) did not noticeably impacted DFs as compared with the control, whereas when the dose was elevated to 0.1 mM, it could inhibit DFs from being proliferated. A consistent trend was identified at 48h (Fig.1a). For KFs, however, the inhibiting effect of CPM was lower than that of DFs. The inhibition of cell proliferation was still observed at 24h under high doses (0.3mM and 0.15mM), and after 48h of stimulation, 0.01 mM dose also inhibited cell proliferation. On the whole, CPM impaired the growth of DFs and KFs.

Moreover, IC50 values were adopted to indicate the effect of CPM on cell viability. As demonstrated from the results, both DFs and KFs growth were inhibited, with CPM IC50 values ranging from 0.05 mM to 0.15 mM at both 24 h and 48 h (Fig.1b). Furthermore, the inhibition effect of CPMs was slightly stronger at 48h compared with that at 24h after the administration.

Next, this study aimed to determine whether CPM inhibits cell proliferation by facilitating the interference with fibroblast apoptosis. As suggested from the results of CCK-8, the concentration of 0.05-0.15 mM had significant impact on cell viability. Thus, the concentration gradient of 0.05 mM, 0.10 mM and 0.15 mM was employed for the study in the apoptosis experiment. When the two types of cells were administrated with different concentrations of CPM, the results (Fig. 2) indicated that CPM could significantly induce cell apoptosis and display dose-dependent and time-dependence characteristics.

According to the result of CCK-8, CPM in 0.1 mM above the sensitive concentration had a significant inhibition of cell proliferation and apoptosis. As shown by the experiments, CPM was sensitive to the inhibition of cell proliferation effect by promoting apoptosis, and 0.1 mM of CPM was the minimal concentration that showed a significant effect on promoting apoptosis. Moreover, the result of the combination with IC50 inhibited the concentration of 50% cell vitality between 0.05 mM and 0.15 mM, so the concentration of CPM was taken as the experimental time point in the following *in vitro* experiments, and 24h was selected as the experimental time point, which could inhibit the cell vitality, promote cell apoptosis, and not cause considerable cell death.

Chlorpheniramine maleate inhibited cell cycle in DFs and KFs

Cell cycle refers to one of the critical processes in cell proliferation. Flow cytometry was adopted to detect the inhibiting effects of CPM on two types of cells. As indicated from the results (Fig. 3), the cycle changes of DFs and KFs were consistent after the addition of CPM stimulation. In comparison with the CK group, the proportion of G0/G1 phase cells in the CPM group was significantly up-regulated, the proportion of S phase cells remarkably decreased, and the G2/M phase was basically unchanged. The cell cycle of fibroblasts was inhibited by CPM, thereby indicating G0/G1 phase arrest.

Chlorpheniramine maleate inhibited cell migration of DFs and KFs

Since the enhanced migration and invasion properties of DFs act as the critical parameters in the development of keloid disease (31), whether CPM affects the migration of DFs and KFs *in vitro* was investigated. According to Fig. 4a, in the control, DFs and KFs efficiently migrated into the scratched area. In particular, KFs migrated to such an extent that the wound boundary was insignificant after 24 h of the culture. Fig. 4b shows that the migration ability of 24h KFs was greater than that of DFs, and the wound healing rate of DFs was nearly $51.19 \pm 4.18\%$, while that of KFs was approximately $69.26 \pm 3.02\%$. After the treatment with CPM, the migration ability of both DFs and KFs was reduced. The migration capacity of DFs was evidently reduced, as only $30.16 \pm 3.19\%$ of the scratched area was filled after treatment with CPM. For KFs, however, a similar phenomenon was observed, with the wound healing percentage of only $48.65 \pm 10.08\%$, significantly lower than that of the control. Accordingly, CPM was suggested to counteract the elevated capacities of migration in both DFs and KFs.

mRNA and protein expression of DFs and KFs after Chlorpheniramine maleate treatment

DFs and KFs were cultured in medium with and without CPM. Fig. 4c showed that the expressions of TGF- β 1, JAK1, STAT3 and Ki-67 in KFs were significantly higher than those in DFs. After CPM treatment for 24 h, the expressions of TGF- β 1, JAK1, STAT3 and Ki-67 in DFs and KFs were down-regulated compared with those in the control. Specific to KFs, the expressions of TGF- β 1, JAK1, STAT3 and Ki-67 were significantly down-regulated after CPM treatment for 24h. Consistent with mRNA expression, the WB results (See Blot Image 1, Additional File 2) showed that CPM treatment decreased the protein expression of TGF- β 1, JAK1, STAT3 and Ki-67 in both DFs and KFs (Fig.4d, e).

Chlorpheniramine maleate reduces keloids in vivo

In vivo experiments further elucidated the potential therapeutic effect of CPM in keloids. In this study, the effect of CPM on keloid was determined by using nude mice keloid scar model. In Fig. 5, CPM and normal saline were injected to the established nude mice keloids. An obvious volume reduction was observed after the administration of CPM for 8 injections (Fig. 5b). The keloid in the control was obviously raised, which was firm and red in color. As opposed to the mentioned, palpation and observation on the CPM-treated side showed a flatter, softer tissue displaying a significantly reduced area. As compared with normal saline, the injection of CPM efficiently reduced the volume of keloids (Fig. 5c). The weight of the excised keloids was measured as a percentage of the original weight calculated. In normal saline group, there was a natural degradation of the keloids, and the weight was down-regulated to 70 percent of the original. The highest decrease in keloid weight was noted in CPM group, reduced to 30 percent of the original weight (Fig. 5h). As further confirmed by histological examinations, CPM significantly decreased scar size, demonstrating that CPM hindered the growth of keloids (Fig. D). Thus, CPM inhibited the development of keloids.

HE staining was used to observe the scar tissue section under light microscope. The tissue treated with CPM injection (Fig. 5f, g) was flatter than that of the control (Fig. 5d, e), with complete dermal tissue and raised fibrotic tissue, consistent with the appearance of the naked eye. Besides in the CPM group, keloid tissue was loose, with cavities and homogeneous red-stained necrotic matter, suggesting that CPM could facilitate the apoptosis of keloid scar tissue, complying with the results of *in vitro* experiments. The degree of scar reduction was quantified according to the thickness of the keloid scar indicated by HE staining. As indicated from the results, the mean weight and thickness of scar tissue in the CPM group were significantly lower than those in the control, proving that CPM could significantly reduce keloid.

Masson trichrome staining was conducted to further observe the effect of CPM on collagen deposition in scar. In the control, the collagen fibers were densely packed and then arranged in parallel. As opposed to the control, the CPM groups had less collagenous fibers arranged.

According to immunohistochemistry results, the expressions of TGF- β 1, JAK1, STAT3, and Ki-67 protein were significantly different between the control and the CPM group, and the histogram directly showed this difference. After 56 days of CPM treatment, the expressions of TGF- β 1, JAK1, STAT3, and Ki-67 were significantly down-regulated, consistent with the results of qPCR analysis *in vitro*.

Discussion

Keloid is guided by continual inflammatory stimulation of the dermal reticular layer during the healing process after various wounds (32). A keloid scar, accompanied by pain, itching, burning, and other symptoms, deteriorates the patient's mental and physical conditions and life quality (2). Its formation consists of inflammation, proliferation, and remodelling. The construction of keloid involves numerous factors, and the process is complicated (33). The abnormal activation of KFs may be regulated by the inflammatory factors secreted by themselves and local inflammatory cells (34).

Various critical chemical mediators regulate chemotaxis inhibition, proliferation, and collagen synthesis in fibroblasts (35). Studies have revealed that hyperplastic scars and keloid tissues possess a large proportion of histamine released from mast cells, and the histamine can assist the proliferation and growth of fibroblasts (25, 36–38). Thus, antihistamine drugs decreasing histamine secretion is considered a promising treatment. Antihistamines (e.g., avil, diphenhydramine, and emedastine difumarate) can inhibit scar growth (25, 39, 40). In rabbit ear hyperplastic scar, long-acting intra-lesioned injection of histamine drug can inhibit the TGF beta I/Smad3 signalling pathway to reduce the collagen synthesis and I/collagen type III proportion, thereby inhibiting scar hyperplasia (41). The regulatory effect of histamine on wound fibroblast function (activity/metabolic activity or TGFβ1 secretion) is determined by H1 receptor stimulation (42, 43). CPM acts as an H1 receptor antagonist with a more substantial antihistamine effect than diphenhydramine and others, and it has been applied clinically as an antihistamine with less dosage and side effects (44, 45). H1 receptor blocker reduces histamine-induced collagen synthesis by fibroblast-like cells and can reduce scar formation (37).

Excessive abnormal proliferation and insufficient relative apoptosis rate of human KFs can cause excessive extracellular matrix deposition and continual proliferation of keloid tissue. Accordingly, inhibiting proliferation and inducing apoptosis of human KFs are critical in treating keloid. CCK-8 detection experiments revealed that CPM could effectively inhibit the proliferation of human KFs, and it increased with the increase in the concentration. Flow cytometry suggested that the apoptosis rate of KFs treated with CPM was significantly higher than that of the control, with significant differences between the groups. The cell cycle is one of the vital basic processes of cell life activities. Inhibition of the cell cycle can inhibit cell division and proliferation. Flow cytometry was conducted to detect the effect of CPM on the cell cycle of human KFs. Results indicated that CPM had an inhibiting effect on the cell cycle of human KFs, both of which resulted in the accumulation of G0/G1 phase cells and hindered the progress of G1 phase cells into the S phase. Furthermore, CPM significantly down-regulated the wound healing rates in normal and scar fibroblasts according to wound healing experiments.

Of note, qPCR and WB assay indicated that the mRNA and protein expressions of JAK1 and STAT3 in the cells were significantly down-regulated after CPM treatment, suggesting that CPM might inhibit scar cells via the JAK1/STAT3 pathway. TGF-β1 has long been recognized as the vital mediator for tissue repair and fibrosis after injury. Overexpression of TGF-β1 is capable of promoting angiogenesis, synthesising collagen, reducing the degradation of the extracellular matrix, breaking the homeostasis between

collagen generation and decomposition, and causing keloid formation and fibrosis disease (13, 46). TGF- β 1 expression was significantly down-regulated after CPM treatment. Ki-67 protein was involved in cell dysplasia and other processes, which could assist the infiltration of cells (47, 48), and its expression varied with the phase of the cell cycle. Its expression began in the G1 phase, gradually increased in the S phase, and reached its peak in the M phase. The expression of Ki-67 was down-regulated after the treatment with CPM, complying with the cell cycle results.

To prove the inhibiting effect of CPM on keloid in detail, an animal model was constructed for *in vivo* experiment. The results revealed that CPM could significantly reduce the volume and weight of keloid, and according to HE results, CPM might reduce the scar by facilitating the apoptosis of scar tissue. The results of immunohistochemistry complied with those of the *in vitro* experiment. Subsequent experiments will conduct an in-depth investigation of the mechanism by which CPM reduces scar hyperplasia.

Keloid refers to a complex network of cellular, molecular, and mechanical factors, and the disease can act as a model system for fibrotic diseases (1, 34). By elucidating the key pathogenic steps causing the accumulation of extracellular matrix, it is expected to determine the target of drug intervention, which is suitable for keloid treatment. Additionally, it is suitable for other hereditary and acquired fibrous diseases, such as scleroderma. Moreover, from the perspective of local drug use in the immune microenvironment, the current study could explore the treatment of localized inflammatory diseases, hypertrophic eczema for instance, providing novel treatment ideas for similar diseases, which can directly assist the clinical application.

Conclusion

In the present study, primary normal fibroblasts and keloid fibroblasts were employed to evaluate the effect of CPM *in vitro*. It was significantly demonstrated that CPM could inhibit KFs from proliferating and migrating, promote KFs apoptosis, and exert G0/G1 phase blocking effect on KFs. As also revealed from *in vivo* experiments, CPM could significantly reduce keloid. As suggested from the results of qPCR and immunohistochemistry, CPM might affect the growth of keloid scar cells via the JAK1/STAT3 pathway, which also provided ideas for us to study the mechanism of CPM for treating keloid more specifically. In brief, as indicated from *in vivo* and *in vitro* results, CPM acted as a promising agent to treat keloid, and our findings provide new possibilities for therapeutic intervention or prevention of keloid.

Abbreviations

CPM: Chlorpheniramine maleate

CCK-8: Cell Counting Kit-8

KFs: keloid fibroblasts

DFs: dermal fibroblasts

ECM: extracellular matrix

DMSO: dimethyl sulfoxide

OD: optical density

H&E: Hematoxylin and Eosin

ANOVA: analysis of variance

Declarations

Ethical Approval and Consent to participate

Written informed consent was obtained from all participants. The study was approved in writing by the Medical Ethic Committee of the Second Affiliated Hospital, College of Medicine, Zhejiang University.

Consent for publication

Informed written consent was obtained from the patient for publication of this study, including all individual details. The written consent form is available for review upon request.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MZ and HQ planned and designed the study. HQ conducted the database search, screened studies for inclusion. FJS extracted data. CW, MWZ, and ZFW assessed in animal study. HQ planned and performed

the statistical analysis. HQ wrote the first draft of the manuscript. MZ revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Figures

Figure 1

Cell proliferation in DFs and KFs. The histogram of keloid fibroblast cells and normal dermal fibroblast cells proliferation in various groups at 24h and 48h by CCK-8 analysis (a) and IC50 analysis (b). $n = 3$, $*p < 0.05$, $**p < 0.01$.

Figure 2

Cell apoptosis in KFs. (a) Apoptosis assay of keloid fibroblast cells after 24 and 48 hours of treatment with CPM in different concentration. (b) Bar diagram shows percentage of cells undergoing apoptosis. $n = 3$, $*p < 0.05$, $*p < 0.01$.

Figure 3

Cell cycle in DFs and KFs. (a) Examination of the cell cycle by flow cytometry analysis in keloid fibroblast cells and normal dermal fibroblast cells after 24 hours with or without Chlorpheniramine treatment. (b) Bar diagram shows the difference of cell cycle in each group. $n = 3$, $*p < 0.05$, $*p < 0.01$.

Figure 4

Chlorpheniramine significantly reduced migration and the expression of some genes. (a, b) *In vitro* wound healing assays showed that keloid fibroblast cells and normal dermal fibroblast cells migrated at 0 and 24 hours with/without Chlorpheniramine treatment. (c) Expression of TGF-β1, JAK1, STAT3 and Ki-67 mRNA in the two cells with/without Chlorpheniramine treatment. (d) The protein expression of TGF-β1, JAK1, STAT3 and Ki-67 in the two cells with/without Chlorpheniramine treatment, and (e) the

quantification of the Western blot was determined by densitometry using Image J software. (*: compared with DFs, $*p < 0.05$, $*p < 0.01$. #: compared with KFs, $\#p < 0.05$, $\#\#p < 0.01$.). Scale bars: 400 μ m (a).

Figure 5

Establishment of animal model of keloid and histopathological evaluation of keloid scar tissue after treatment.(a) Nude mice were implanted with keloid for 7 days.(b) 56 days after injection of normal saline (left, 100ul/site) and Chlorpheniramine (right, 0.6mg/ml, 100ul/site).(c) After 56 days of treatment, scar was removed in the experimental group and control group.(d, e) Morphological images of HE stained scar specimens in the control group after 56 consecutive days of treatment.(f, g) Morphological images of HE stained scar specimens in the experimental group after 56 consecutive days of treatment. (h) Percentage of residual keloid weight of the control group and the experimental group after 56 days of treatment. (i) Thickness of keloid in the two group. Scale bars: 1cm (c), 500 μ m (d, f), 200 μ m (e, g).

Figure 6

(a) Histopathological evaluation of keloid scar tissue after treatment. Masson trichrome staining was used to further observe the effect of Chlorpheniramine on collagen deposition in scar. TGF-β1, JAK1, STAT3 and Ki-67 expression in keloid by treatment with normal saline and Chlorpheniramine at 56 days after animal model establishment. (b) Bar diagram shows percentage of positive rate. Scale bars: 200μm

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