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Research Article
Carvacrol Inhibits the Proliferation and Extracellular Matrix Deposition of Keloid Fibroblasts Through Nrf2/GPX4 and TGF-β1/Smad Signaling Pathways

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Abstract

Background and Objective: Keloid (KD) is a kind of fiber proliferative disease, usually caused by abnormal wound healing after a burn, trauma or infection and characterized by proliferation of cells and excessive deposition of extracellular matrix (ECM) in keloid fibroblasts (KFs). This study investigated the effect of carvacrol (CV) on the proliferation and ECM deposition of KFs. **Materials and Methods:** The proliferation of KFs was determined by scratch assay and cloning assay. The contents of iron, MDA and GSH were measured in KFs. The expressions of Nrf2/GPX4 and TGF-β1/Smad proteins were detected using Western blotting. All data were analyzed by GraphPad Prism 9.0 software. **Results:** The CV showed the ability to inhibit cell proliferation and migration, it promoted the expressions of iron and MDA and inhibited the expression of GSH in KFs. The CV promoted ferroptosis of KFs by inhibiting the protein expressions of Nrf2, HO-1, GPX4 and xCT. It also inhibited collagen deposition of KFs by inhibiting protein levels of collagen I, collagen III, fibronectin, α-SMA, p-Smad2 and p-Smad3. **Conclusion:** The CV promoted ferroptosis of KFs by regulating Nrf2/GPX4 signaling pathway to inhibit cell proliferation and regulated TGF-β1/Smad signaling pathway to inhibit ECM deposition.

Key words: Carvacrol, keloid fibroblasts, ferroptosis, Nrf2/GPX4, extracellular matrix deposition, TGF-β1/Smad

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The biological characteristics of KFs are similar to those of tumors and abnormal proliferation and persistent tissue fibrosis caused by overexpression of collagen are common manifestations of skin scars[1]. At present, the commonly used clinical treatment methods for keloids include surgery, oral drug therapy, pressure therapy, radiotherapy, laser therapy, corticosteroid injection, stem cell therapy, autologous fat transplantation and mental health counseling[2,3]. However, there is still no clear gold standard treatment for KD. Therefore, it is of great significance to develop new therapeutic methods or find effective drugs to treat keloids, while some natural products showed promising effects[4], which may be a viable option.

Ferroptosis is involved in the occurrence and development of KD[5,6]. The ferroptosis-related genes Glutathione Peroxidase 4 (GPX4), cystine transporter SLC7A11 (xCT) and Nuclear Factor E2 Related Factor 2 (Nrf2) are lowly expressed, while iron content is highly expressed in KD[5]. The Nrf2 is a key regulator of anti-oxidant responses and a major ferroptosis signaling molecule in the nucleus[7]. Many ferroptosis-related proteins are Nrf2 target genes, such as ferritin, ferroportin, Heme Oxygenase 1 (HO-1), NADPH oxidase, xCT and GPX4[8,9]. Therefore, KD may be improved by interfering with proteins related to Nrf2/GPX4 signaling pathway.

Transforming Growth Factor-β1 (TGF-β1) has been identified as an important regulator of wound healing and it was found to induce KD formation[10]. Increased TGF-β1 levels are accompanied by collagen I overexpression in KD[11]. It has also been suggested that the mechanism of KD formation is related to fibroblasts secreting high levels of TGF-β1[12]. In addition, Smad2 and Smad3 are highly phosphorylated in KFs compared to normal fibroblasts[13,14] and inhibition of Smad2 and Smad3 by RNA interference resulted in a significant reduction in collagen I and collagen III expression in KD[14,15]. Therefore, TGF-β1/Smad signaling pathway may play a key role in KD and it may serve as an important target for the treatment of KD.

The CV is a liquid phenolic monoterpene found in the essential oils of oregano, thyme, pepper, wild bergamot and other plants[16]. Mbese and Aderibigbe[17] and Silva *et al.*[18] shows that the CV had anti-oxidant activity, anti-tumor activity and the gram positive and negative bacteria produced inhibition. At present, there are no relevant reports on the effect of CV on KD. Therefore, the present study aimed to investigate the inhibitory effect and mechanism of CV on KFs, providing a basis for the application in the treatment of KD.

MATERIALS AND METHODS

Study area: The experiments were carried out from August, 2022 to December, 2023 at Yanbian University Hospital, Yanji, Jilin Province, China.

Materials and reagents: The CV (HY-N0711) was purchased from MedChemExpress (New Jersey, USA). The KFs were purchased from American Type Culture Collection Cell Bank (Maryland, USA). The KFs were cultured in DMEM medium containing 10% fetal bovine serum at 37°C with 5% CO_2 (Sheldon Manufacturing, Cornelius, Indiana, USA). Antibodies against Nrf2 (12721), HO-1 (70081), GPX4 (52455), xCT (12691), p-Smad2 (3104), Smad2 (5339), p-Smad3 (9520), Smad3 (9523) and β-actin (4967) were purchased from Cell Signaling Technology (Boston, USA). Antibodies against collagen I (AF7001), collagen III (AF5457), fibronectin (AF5335) and α-Smooth Muscle Actin (α-SMA) (AF1032) were purchased from Affinity Biosciences (Melbourne, Australia).

CCK8 assay: The viability of KFs was measured using the CCK8 cell proliferation Assay kit (Solarbio, Beijing, China) according to the manufacturer's instructions. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well and treated with different concentrations of CV (0, 12.5, 25, 50, 100, 200 and 400 μmol/L) for 24 and 48 hrs, respectively, followed by incubation with CCK8 solution for an additional 4 hrs. The OD values of each well were determined at 450 nm using a multifunctional microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Scratch assay: The KFs were seeded in 6-well plates at a density of 5×10^5 cells/well and treated with different concentrations (0, 200 μmol/L) of CV. A 200 μL pipette tip was used to draw a straight line evenly in the 6-well plate, then the cells were cultured for another 24 or 48 hrs. The scratch width was observed and recorded at 0, 24 and 48 hrs, respectively[19]:

$$\text{Mobility}\ (\%) = \frac{\text{Scratch width at 0 hr} - \text{Scratch width at 24 or 48 hrs}}{\text{Scratch width at 0 hr}} \times 100\%$$

Cloning assay: The KFs were seeded in 6-well plates with 500 cells per well, treated with different concentrations (0, 200 μmol/L) of CV and cultured in an incubator for 14 days. Fresh medium was replaced every 3 days, fixed with 4% paraformaldehyde and stained with crystal violet. Excess staining solution was removed by washing with tap water and allowed to dry before photographs were taken.

Iron detection: The cells were divided into control group, TGF-β1 group, CV group and TGF-β1+CV group. A density of 5×10^5 cells/well was cultured in 6-well plates. The CV and TGF-β1+CV groups were treated with 200 μmol/L CV for 23 hrs. Except for control and CV groups, KFs were added with 10 ng/mL TGF-β1 in other groups. After treatment with TGF-β1 for 1 hr, the cells of each group were collected. The absorbance was detected at 510 nm by a multifunctional microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the instructions of the iron assay kit (Solarbio, Beijing, China) and the intracellular iron content was calculated.

Malondialdehyde (MDA) and Glutathione (GSH) detection: The KFs were cultured in 6-well plates and after treatment with CV, the cells were digested and collected. The MDA and GSH detection kits (Nangjing Jiancheng Bioengineerina Institute, Nanjing, China) were used. The absorbance was detected at 530 and 405 nm (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the contents of MDA and GSH in each group were calculated.

Western blotting: The KFs were cultured in 6-well plates, digested and harvested after treatment and cellular proteins were extracted. Sample sizes of 15 to 20 μL and 15 to 30 μg were selected for loading. The SDS-PAGE gel electrophoresis (Bio-Rad, Hercules, California, USA) was performed for 1.5-2 hrs, transferred onto PVDF membrane (Millipore, Sigma-Aldrich, California, USA) with a transmembrane apparatus (Bio-Rad, Hercules, California, USA) and placed in 5% nonfat milk. Membrane resistance was incubated with primary antibodies overnight at 4°C. Subsequently, the secondary antibodies were added and incubated at 37°C for 2 hrs. Subsequently, the membranes were imaged and analyzed.

Statistical analysis: All data were analyzed by GraphPad Prism 9.0 software. All data were presented as the Mean±Standard Deviation (SD). The differences among the experimental groups were calculated by One-way Analysis of Variance (ANOVA). Student's t-test was used to detect significant differences between the two groups and $p<0.05$ was considered statistically significant.

RESULTS

Effect of CV on the proliferation of KFs: As shown in Fig. 1a, CCK8 results showed that 25, 50, 100, 200 and 400 μmol/L CV had significant inhibitory effects on KFs at 24 and 48 hrs ($p<0.05$). Based on the above results, 200 μmol/L CV was selected for subsequent experiments. As shown in Fig. 1(b-c), compared with control group, the scratch distance of cells in CV group was significantly wider and the migration ability was significantly decreased ($p<0.05$). As shown in Fig. 1(d-e), compared with control group, the number of clonal formations was reduced by 200 μmol/L CV treatment ($p<0.01$). Therefore, CV inhibited the proliferation of KFs *in vitro*.

Effect of CV on ferroptosis in KFs after TGF-β1 treatment: As shown in Fig. 2, compared with control group, the expressions of iron and MDA were significantly increased (Fig. 1a-b, $p<0.01$) and the expression of GSH was significantly decreased (Fig. 1c, $p<0.05$) in TGF-β1 and CV groups. Compared with TGF-β1 group, the expressions of iron and MDA were significantly increased ($p<0.01$) and the expression of GSH was significantly decreased in TGF-β1+CV group ($p<0.01$).

Effect of CV on the expressions of Nrf2/GPX4 signaling pathway-related proteins in KFs after TGF-β1 treatment: As shown in Fig. 3, compared with control group, the expression levels of Nrf2, HO-1, GPX4 and xCT in TGF-β1 and CV groups were significantly decreased (Fig. 3a-e, $p<0.01$). Compared with TGF-β1 group, the expressions of these proteins were further suppressed after 200 μmol/L CV treatment ($p<0.01$).

Effect of CV on the protein expressions of collagen I, collagen III, fibronectin and α-SMA in KFs after TGF-β1 treatment: As shown in Fig. 4, compared with control group, the expressions of collagen I, collagen III, fibronectin and α-SMA in TGF-β1 group were significantly increased (Fig. 4a-e, $p<0.05$). Compared with control group the expression levels of the above proteins were significantly decreased in CV group ($p<0.01$). Relative to the TGF-β1 group, these proteins were significantly decreased in TGF-β1+CV group ($p<0.01$).

Effect of CV on the protein expressions of p-Smad2 and p-Smad3 in KFs after TGF-β1 treatment: As shown in Fig. 5, compared with control group, the expression levels of p-Smad2 and p-Smad3 were significantly increased in TGF-β1 group (Fig. 5a-c, $p<0.01$) and the expressions of p-Smad2 and p-Smad3 were significantly decreased in CV group ($p<0.01$). Compared with TGF-β1 group, 200 μmol/L CV inhibited the expressions of p-Smad2 and p-Smad3 ($p<0.01$).

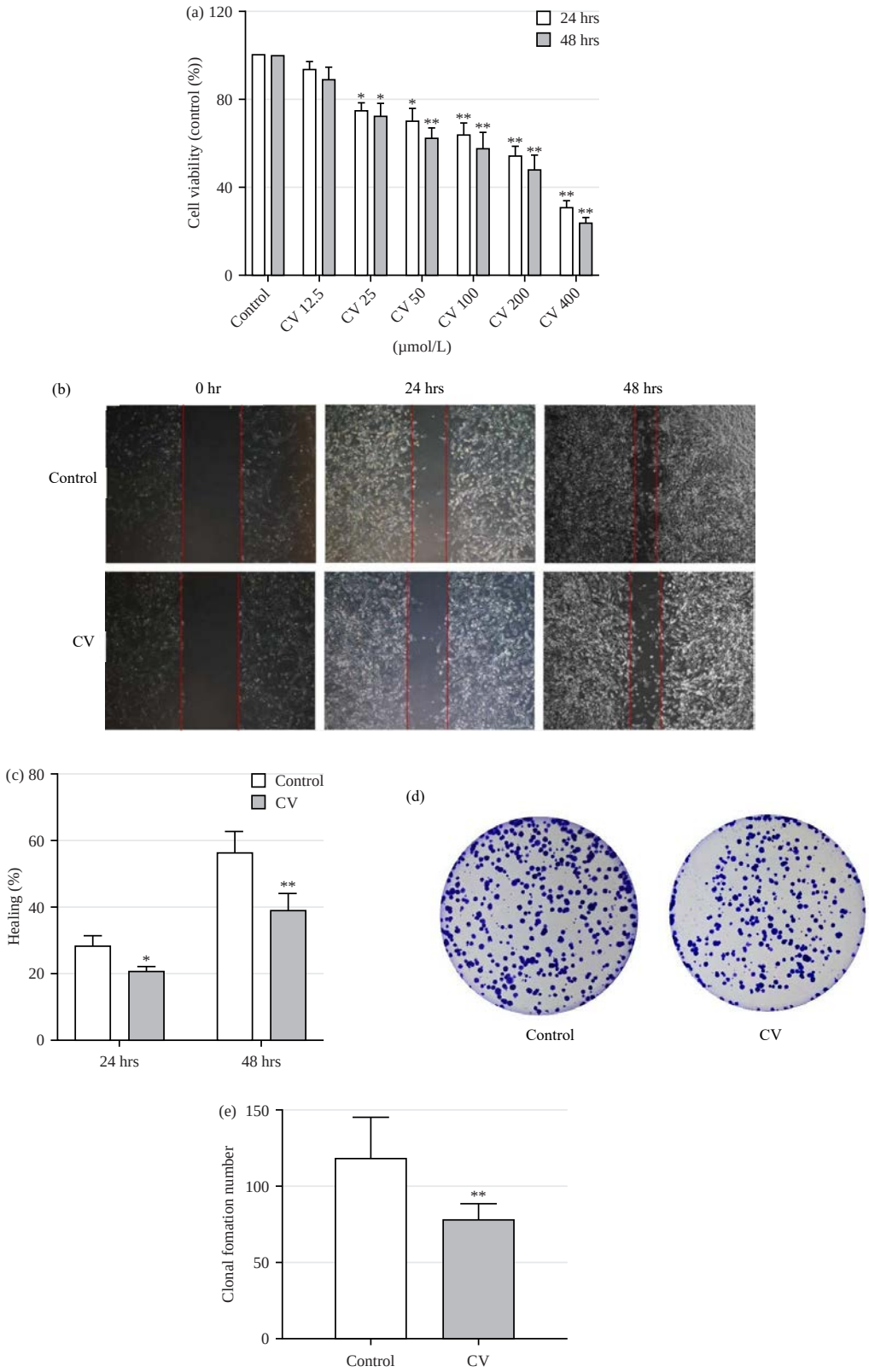

Fig. 1(a-e): Effect of CV on the proliferation of KFs, (a) Cell viability was detected by the CCK-8 assay, (b-c) Scratch assay and healing and (d-e) Cloning assay and clonal formation number

Data are expressed as Mean±SD, *p<0.05 vs control group and **p<0.01vs control group

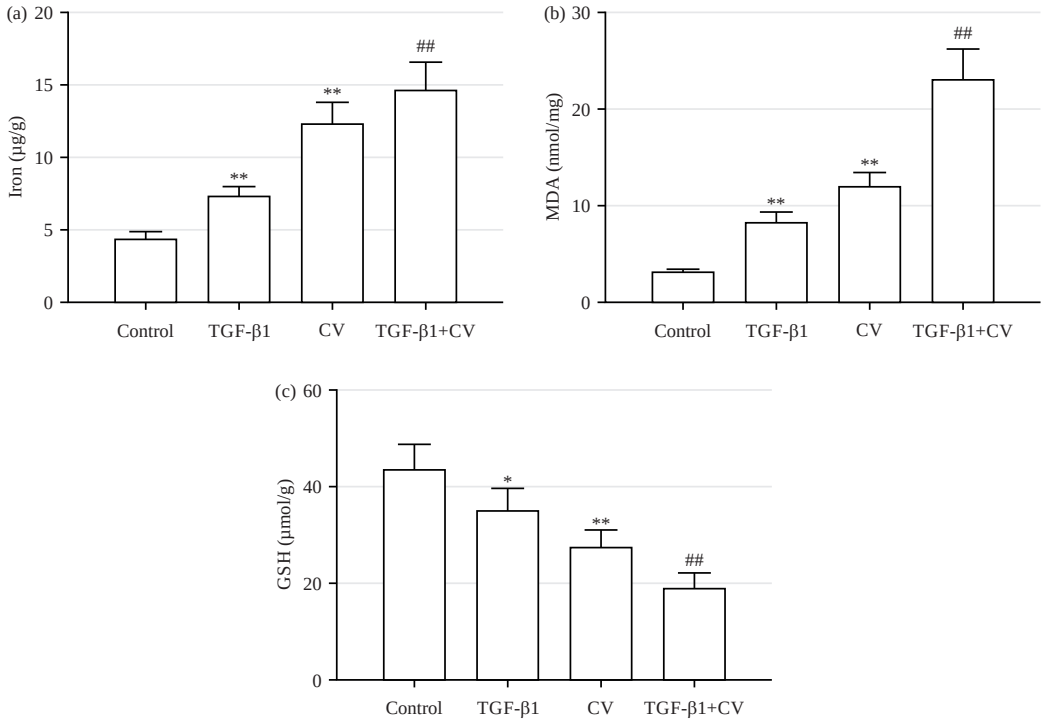

Fig. 2(a-c): Effect of CV on the ferroptosis of KFs, these indicators were detected according to the kit instructions, (a) Iron, (b) MDA and (c) GSH

Data are expressed as Mean±SD, *p<0.05 vs control group, **p<0.01 vs control group and ##p<0.01 vs TGF-β1 group

DISCUSSION

In the present study, KFs were treated with 12.5, 25, 50, 100, 200 and 400 μmol/L CV to determine the effect of CV on the proliferation of KFs for 24 and 48 hrs. The CCK8 results showed that 25, 50, 100, 200 and 400 μmol/L CV had a significant inhibitory effect on KFs at 24 and 48 hrs. Scratch assay and Cloning assay also showed that 200 μmol/L CV inhibited the proliferation of KFs. These findings indicated CV may be a promising drug for KFs, which also offers the possibility of further treating KDs that cause itching and pain in humans[20]. In addition, CV has been tried for treatments of other diseases, given that it has broad-spectrum antibacterial, anti-inflammatory, anti-tumor, anti-oxidation and bone protection, while the advantages of being widely available, low price, safety and reliability contribute to its subsequent widespread use[21,22]. The current study is a good addition to its use as a potential drug option for the treatment of diseases.

Ferroptosis is a type of cell death caused by programmed oxidative damage, which plays an important regulatory role in the development of a variety of diseases and has become the focus of the treatment and improvement of many diseases[23-25]. In recent years, a number of studies have reported that ferroptosis plays an important role in KD. Bioinformatics analysis showed that in keloid several diagnostic markers associated with ferroptosis in KD, indicating that ferroptosis is involved in the occurrence and development of KD[5]. In addition, 5-Aminolevulinic Acid-Based Photodynamic Therapy (5-ALA-PDT) may inhibit KFs by increasing reactive oxygen species (ROS) and inducing ferroptosis[6]. Notably, iron overload is one of the characteristics of ferroptosis[23]. The current study results illustrated that the iron content substantially increased after TGF-β1 treatment in KFs. However, the iron content can be further increased after CV treatment, indicating that CV may induce ferroptosis in KFs. The contents of MDA and GSH in KFs were also examined and the results showed that CV could promote KFs exhibiting high MDA and low GSH expression after TGF-β1 stimulation. The Nrf2 is an important nuclear transcription factor, cell oxidation can be activated[26]. Under the condition of oxidative stress, Nrf2 translocation to the nucleus, activates the downstream of HO-1 and GPX4, affected by the generation of lipid peroxidation and regulating ferroptosis[27]. The activity of GSH is GPX4 cofactors, so consumption of GSH or direct inhibit the activity of GPX4 can improve ferroptosis happened[28]. Therefore, the Nrf2/GPX4 pathway plays a critical role in ferroptosis regulation. The

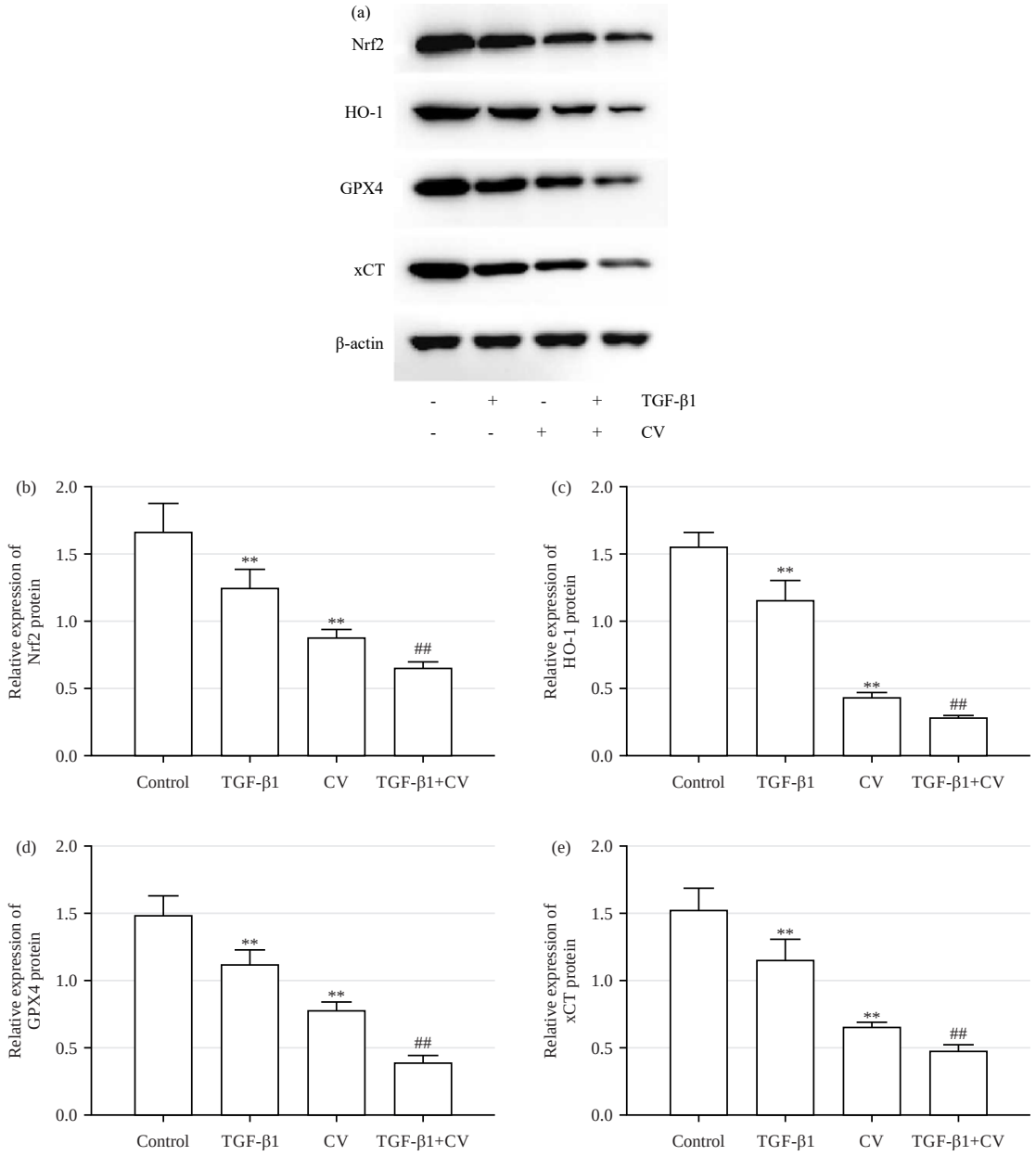

Fig. 3(a-e): Effect of CV on the protein expressions of Nrf2, HO-1, GPX4 and xCT in KFs. Western blotting was used to detect the protein expressions of Nrf2, HO-1, GPX4 and xCT in KFs, (a) Western blot images, (b-e) Densitometric analysis of (b) Nrf2/β-actin, (c) HO-1/β-actin, (d) GPX4/β-actin and (e) xCT/β-actin

Data are expressed as Mean±SD, **p<0.01 vs control group and ##p<0.01 vs TGF-β1 group

present study results implicated that Nrf2/GPX4 pathway was remarkably activated by CV after TGF-β1 treatment in KFs. These results suggest that CV may inhibit the proliferation of KFs by mediating the Nrf2/GPX4 signaling pathway.

The KD is characterized by activation of skin fibroblasts and ECM deposition after abnormal repair of trauma or surgical injury. In the process of skin wound healing, fibroblasts are activated to highly express α-SMA and differentiate into myofibroblasts, which synthesize a large amount of collagen[29]. Excessive accumulation of ECM is an important feature of KD[30]. The expressions of collagen I, collagen III, fibronectin and α-SMA were significantly enhanced in KFs compared to normal fibroblasts[31,32]. Inhibition of fibroblast activation and ECM deposition has been proposed as an effective strategy for the treatment and prevention of KD. In this study, Western blotting results

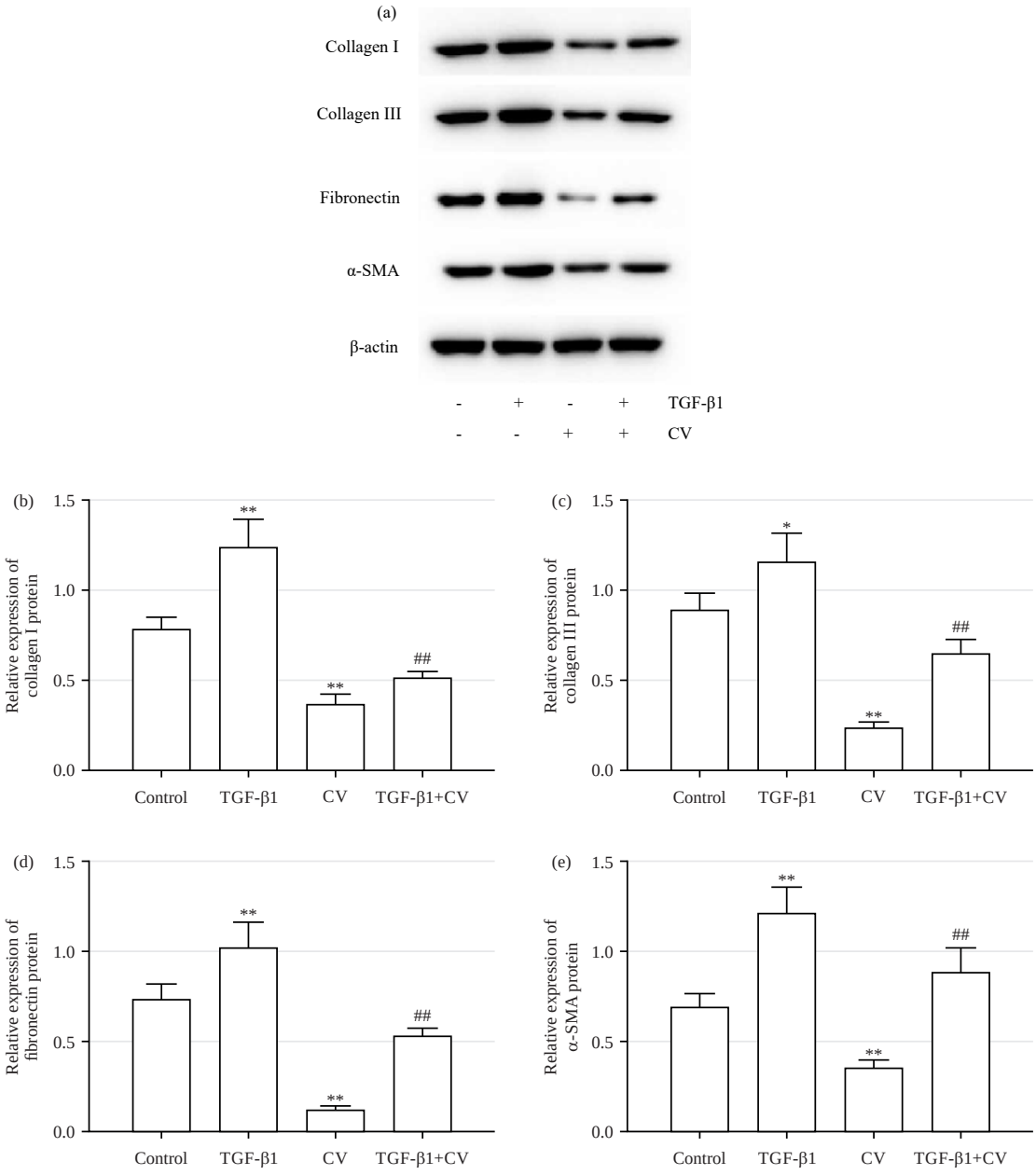

Fig. 4(a-e): Effect of CV on the protein expressions of collagen I, collagen III, fibronectin and α-SMA in KFs. Western blotting was used to detect the protein expressions of collagen I, collagen III, fibronectin and a-SMA in KFs, (a) Western blot images, (b-e) Densitometric analysis of (b) Collagen I/β-actin, (c) Collagen III/β-actin, (d) Fibronectin/β-actin and (e) a-SMA/β-actin

Data are expressed as Mean±SD, **p<0.05 vs control group and ##p<0.01 vs TGF-β1 group

showed that CV significantly inhibited the protein expressions of collagen I, collagen III, fibronectin and α-SMA after TGF-β1 treatment in KFs. Therefore, CV could significantly improve the deposition of ECM in KFs.

The TGF-β plays diverse biological regulatory roles in the control of cell proliferation and differentiation, wound healing and immune system[33]. As a mediator of extracellular matrix production, TGF-β1 can promote tissue repair, inhibit the synthesis of collagenase, lead to excessive synthesis of collagen, make fibroblasts differentiate into myofibroblasts and eventually lead to scar fibrosis[32]. The TGF-β1 can stimulate the proliferation and differentiation of KFs and up-regulate the expression of α-SMA and collagen, which leads to increased stiffness of KD tissue. The possibility that the Smad family is

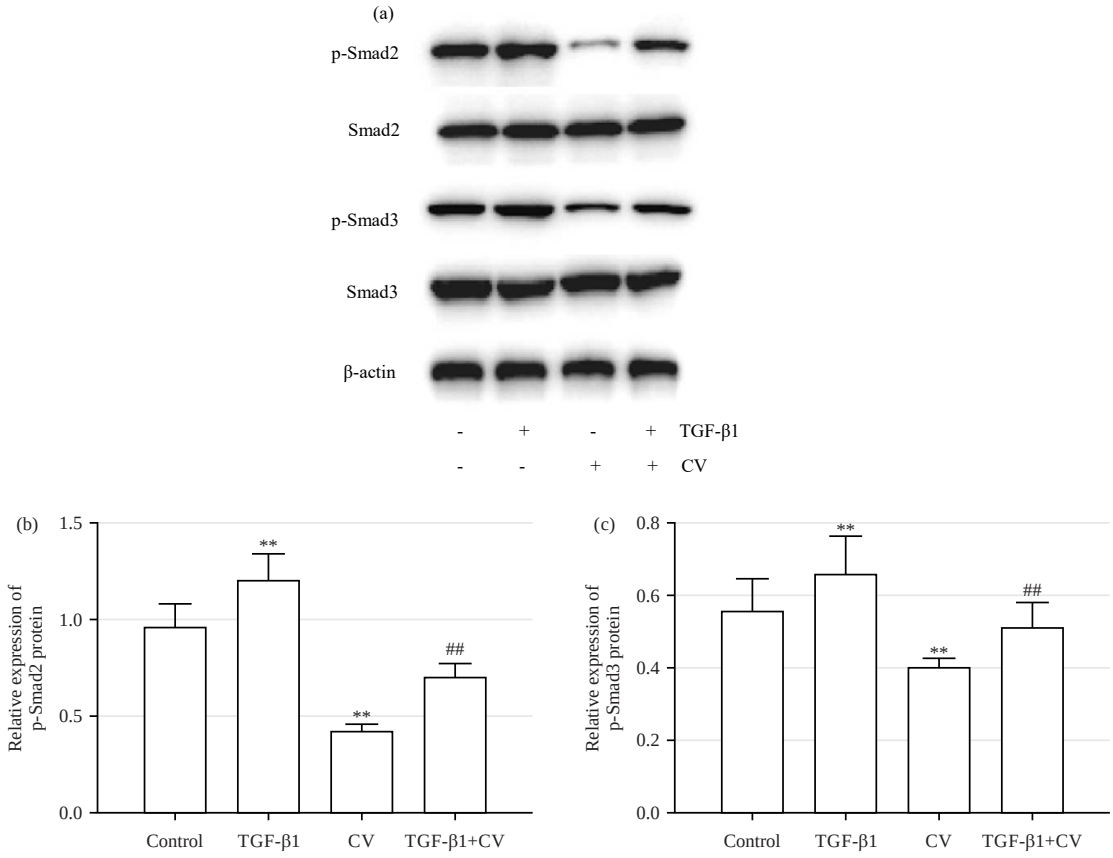

Fig. 5(a-c): Effect of CV on the protein expressions of p-Smad2 and p-Smad3 in KFs. Western blotting was used to detect the protein expressions of p-Smad2 and p-Smad3 in KFs, (a) Western blot images, (b-c) Densitometric analysis of (b) p-Smad2/Smad2 and (c) p-Smad3/Smad3

Data are expressed as Mean±SD, **p<0.01 vs control group and ##p<0.01 vs TGF-β1 group

involved in the pathogenesis of KD disease. It has been found that Smad2 and Smad3 are overexpressed and highly phosphorylated in KFs compared to normal fibroblasts[13-15]. Inhibition of TGF-β1/Smad signaling pathway can reduce the proliferation and collagen. Therefore, the roles of downstream pathways of TGF-β1 in KFs were explored by Western blotting and it was found that CV could improve KFs by inhibiting the expressions of p-Smad2 and p-Smad3 proteins after TGF-β1 treatment. Therefore, CV may mediate TGF-β1/Smad signaling to inhibit ECM deposition in KFs.

Taken together, CV inhibited the proliferation and collagen deposition of KFs probably by inducing ferroptosis by activating Nrf2/GPX4 and reducing collagen deposition by inhibiting TGF-β1/Smad signaling. This study provides some experimental support for CV treatment of KD, which offers a theoretical basis for the rational use of CV in clinical practice. However, the present study mainly focused on *in vitro* experiments, while *in vivo* studies and prospective clinical trials are still needed in order to further evaluate the therapeutic effects of inflammatory CV.

CONCLUSION

This study provided evidence that CV treatment could dramatically reduce KFs proliferation and ECM deposition by regulating Nrf2/GPX4 and TGF-β1/Smad signaling pathways. These findings suggested that the effect of CV on KD warrants further in-depth study and that it has the potential to be developed into an alternative drug for the treatment of KD.

SIGNIFICANCE STATEMENT

Keloid is a kind of fiber proliferative disease that is characterized by proliferation of cells and excessive deposition of extracellular matrix (ECM) in keloid fibroblasts (KFs). The study was designed to investigate the inhibitory effect of carvacrol (CV) on the proliferation and ECM deposition of KFs. Results showed that CV could inhibit cell proliferation and ECM deposition in KFs by regulating Nrf2/GPX4 signaling pathway-mediated ferroptosis and TGF-β1/Smad signaling pathology-mediated ECM production. These findings initially

reveal that CV can counteract KD through multiple targets and pathways, which provides some experimental bases for the subsequent development and clinical application of CV in treating KD. On the basis of this *in vitro* experiment, more *in vivo* experiments can be carried out in the future to evaluate the efficacy and safety of CVs for the treatment of KDs in living subjects, which will provide a better basis for its clinical application.

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