Oxymatrine promotes hypertrophic scar repair through reduced human scar fibroblast viability, collagen and induced apoptosis via autophagy inhibition

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Abstract
Scars are common complications of burns and trauma, resulting in mental trauma, physical pain, and a heavy financial burden for patients. Specific and effective anti-scarring drugs are lacking in clinical practice. Phytochemicals are easily accessible, low in toxicity, and have various biological and pharmacological properties. Oxymatrine is a phytochemical that regulates autophagy networks. Autophagy is closely related to the maintenance, activity, differentiation, and life-death of skin fibroblasts during wound repair, which results in pathological scars. We hypothesised that oxymatrine may promote hypertrophic scar repair by inhibiting fibroblast autophagy. In vitro studies showed that inhibition of autophagy by oxymatrine decreased viability and collagen metabolism, and increased apoptosis of human scar fibroblasts (HSFs). In vivo studies showed that inhibition of autophagy by oxymatrine promoted scar repair, resulting in a significantly improved final outcome of the hypertrophic scars, a smaller scar area, decreased epidermal and dermal thickness, and a significant downregulation of CK10, P63, collagen I, α-SMA, and TGF-β1. In summary, oxymatrine promoted hypertrophic scar repair by decreasing HSF viability and collagen, and inducing apoptosis via autophagy inhibition. This study provides a new perspective on the mechanism of hypertrophic burn scar formation, as well as key scientific data for the application of the phytochemical oxymatrine as a new method for the prevention and treatment of hypertrophic scars.

Keywords: autophagy inhibition, hypertrophic scar remediation, oxymatrine

Abbreviations: CK10, cytokeratin 10; FBS, fetal bovine serum; GHNMU, General Hospital of Ningxia Medical University; H&E, haematoxylin and eosin; HSF, human scar fibroblasts; HUVECs, human umbilical vein endothelial cells; LC3, light chain 3; NC, 0.9% NaCl; OMT, oxymatrine; SFM, serum-free media; SG, silicone gel; TGF-β1, transforming growth factor-beta 1; α-SMA, alpha-smooth muscle actin.

Xingwang Deng, Yongzhao Zhu, and Yan Xie are co-first authors.

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### Key Messages

- Autophagy is closely related to the maintenance, activity, differentiation, and life-death of skin fibroblasts during wound healing, which leads to the formation of pathological scars.
- Phytochemicals are easily accessible, low in toxicity, and have a wide range of pharmacological and biological effects. Oxymatrine is a phytochemical that regulates autophagy networks.
- Oxymatrine promoted hypertrophic scar repair by decreasing HSF viability and collagen, and inducing apoptosis via autophagy inhibition.
- This study provides a new perspective on the mechanism of hypertrophic burn scar formation, as well as key scientific data for the application of the phytochemical oxymatrine as a new method for the prevention and treatment of hypertrophic scars.

## 1 INTRODUCTION

Scars are common complications of burns and trauma, resulting in mental trauma, physical pain, and a heavy financial burden for patients.\(^1\,^2\) There are approximately 10 million new burn patients worldwide each year, and the incidence of scars after burns is 91.4%. Scars are associated with a high medical burden for patients and society, and the annual cost of scar treatment is expected to be at least 4 billion US dollars. In addition, there are many complications of scars, such as itching, pain, insomnia, anxiety, loss of function, and limited tissue movement.\(^3\) Scar tissue can undergo malignant transformation into scar cancer.\(^4\,^5\) At present, specific and effective anti-scarring drugs for use in clinical practice are lacking.\(^6\)

Phytochemicals are easily accessible, low in poisonousness, and have various biological and pharmacological properties. They are an important source for drug preparation and play critical role in the discovery of innovative medicines.\(^7\) Phytochemicals are natural plant-based compounds present in grains, fruits, and vegetables. They are regarded as an important discovery for humans in recent years, and their significance is comparable to the discovery of antibiotics and vitamins.\(^7\) Phytochemicals are easy to obtain, have less poisonous effects than synthetic molecules, and have various pharmacological and biological properties, including antimicrobial, antitumor, antimitogenic, and antioxidant effects.\(^8\,^9\) Several phytochemicals recently passed clinical trials and are being used in clinical practice, such as in the treatment of cancer.\(^8\,^10\) Phytochemicals such as oxymatrine (OMT) also regulated autophagy networks.\(^11\)

Autophagy, which has recently become a research hotspot, performs a vital role in maintaining the steady state of tissue structure and function, and has a close relationship with various human diseases.\(^12\) Autophagy is an evolutionarily conservative catabolic pathway in eukaryotic cells. It mediates the degradation of organelles and cytoplasm through lysosomes, thereby maintaining cell metabolism and homeostasis and providing raw materials for cell proliferation. However, excessively activated autophagy can result in a non-apoptotic mechanism of death named autophagic cell death.\(^13\) Autophagy is closely related to the maintenance, activity, differentiation, and life-death of skin fibroblasts during wound repair, which results in pathological scars.\(^14\,^16\)

OMT, the active ingredient of *Sophora flavescens*, has various biological activities.\(^17\) A recent study showed that OMT inhibits cell autophagy in human umbilical vein endothelial cells (HUVECs).\(^11\) However, OMT has not been reported to inhibit scar fibroblast autophagy to date. Given the link between OMT and autophagy in cancer, as well as the involvement of autophagy in scar formation, we hypothesised that OMT may promote hypertrophic scar repair by inhibiting fibroblast autophagy.

## 2 MATERIALS AND METHODS

### 2.1 Reagents

OMT (6 mg/mL, Jiangsu Zhengda Tianqing Pharmaceutical Co. Ltd.) was provided by the Department of Dermatology at General Hospital of Ningxia Medical University (GHNMU) and stored at 4°C. The 0.9% NaCl (NC) and silicone gel [SG] (15 g, Unitrump Bio, Jiangsu, China) were obtained from the Burn and Plastic Surgery Department of the GHNMU.

### 2.2 Ethics

This research described has been undertaken in accordance with the Declaration of Helsinki. Ethical approval...
was obtained from the GHNMU Review Board (Approval number: 2017-205). This research, which involved animals, strictly followed the Ningxia Medical University ethical review of laboratory animal welfare.

### 2.3 | Cellular studies

Human scar fibroblasts (HSFs) were isolated and cultured as previously described. HSFs were utilised for autophagy, proliferation, viability, collagen, apoptosis, and western blot assays.

### 2.4 | Autophagy assay

An autophagy assay kit (Sigma-Aldrich, St. Louis, MO, USA) was utilised to measure autophagy in HSFs exposed to OMT. HSFs were seeded and treated with OMT for 4, 8, or 12 hours. The number of autophagosomes was quantified using microplate readers and images were developed with a microscope system (Echo Laboratories, San Diego, CA, USA).

### 2.5 | Alamar blue assay

The Alamar blue assay was used to inspect the changes in HSF viability and proliferation caused by OMT, as previously described. HSFs were seeded and treated as follows: OMT (3 mg/mL); OMT + rapamycin (RAPA [an autophagy inducer]; 0.01 mg/mL); and RAPA (0.01 mg/mL) for 24 hours (viability) or 48 hours (proliferation). The changes in HSF viability and proliferation were then quantified.

### 2.6 | Sirius red assay

The Sirius red staining was used to inspect the changes of collagen present in HSF caused by OMT. HSFs were treated and tested after 48 hours of treatment as described above. Briefly, Sirius red (0.1% v/v) was added to wells and incubated for 90 minutes. Wells were washed and dried overnight. Images were acquired. Finally, cells stained with Sirius red were dissolved in 0.1 M NaOH, and the absorbance was read.

### 2.7 | Apoptosis assay

The ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore, USA) was used to measure apoptosis. In brief, HSFs (2 x 10⁴ cells/300 μL) were added in cell imaging slide-8 chambers (Eppendorf, USA) and treated with OMT (4.5 mg/mL), OMT + RAPA, and RAPA (0.01 mg/mL) for 48 hours. Fluorescence images were captured and cells with apoptosis were quantified.

### 2.8 | Western blot assay

HSFs were seeded and treated with RAPA, OMT (4.5 mg/mL), or OMT + RAPA for 12 hours, lyed, and collected. Proteins were separated, transferred, and blocked for 1 hour. The primary antibodies against light chain 3 (LC3) (Santa Cruz Biotechnology, Texas, USA) and p-ULK (Cell Signalling Technology, Danvers, USA) were added and incubated. The secondary antibody was then added and incubated. The expression of the labelled proteins was identified by a chemiluminescence reagent on an Amersham Imager 600 chemical imaging system (GE healthcare Sciences AB, Sweden). The intensity of the band was quantified by densitometry and expressed relative to GAPDH loading controls.

### 2.9 | The burn hypertrophic scar model

Porcine full-thickness burn hypertrophic scar models were produced following previously described protocols reported by our group (Figure 2C,D). Scar models were topically treated with SG (0.3 mL [positive control], 0.9% NC (1 mL [negative control]), or OMT (1 mL [4.5 mg/mL]) every 2 days. The effect of OMT on scar repair was measured through clinical scar assessment, the epidermal and dermal thickness, and protein expression.

### 2.10 | Clinical scar assessments

Clinical scar characteristics were assessed following previously described protocols reported by our group. The scar colour, scar texture, and the final outcome were graded from 0 to 5. The scar height was graded from −1 to 5. The scar areas were photographed and quantified from images acquired.

### 2.11 | Histology

Specimens were acquired from hypertrophic scars at 0, 1, 4, and 8 weeks. Samples were fixed and stained with haematoxylin and eosin (H&E). H&E-stained images were captured, and the thickness of the epidermis and dermis was measured. The reduction rate of epidermal and dermal thickness was calculated with the formula previously described.
2.12 | Immunohistochemistry

Immunohistochemical analysis was performed to detect protein expression as reported previously. Paraffin sections of scar tissue were incubated with antibodies against P63 (1:200; Biocare Medical, Concord, CA, USA), cytokeratin 10 (CK10) (1:200) (Abcam, Cambridge, UK), collagen I (1:100; Epitomics, Burlingame, Concord, CA, USA), alpha-smooth muscle actin (α-SMA) (1:150) (Biocare Medical, Concord, CA, USA), transforming growth factor-beta 1 (TGF-β1, 1:150; Abcam, Cambridge, UK), and MAP LC3 alpha/beta (G-4) (1:500) (Santa Cruz Biotechnology, Texas, USA). Immunohistochemistry-stained images were captured, and the protein expression was quantified as previously described.

2.13 | Statistical analysis

SPSS 18.0 software (IBM Co., Chicago, IL, USA) was utilised for statistical data analysis. All experiments were independently executed three times, with each different treatment examined independently in triplicate on three distinct female mini-pigs or on cells from three different patients. The data were expressed as mean ± standard error and analysed with one-way ANOVA and Tukey’s post hoc test. P values <.05 were regarded as statistical significance.

3 | RESULTS

3.1 | OMT inhibits autophagy in HSFs

Autophagy was assessed in HSFs exposed to OMT using an autophagy assay. As shown in Figure 1A, autophagosomes (blue emission) were evident in HSFs treated with SFM, but not in those treated with OMT. The number of autophagosomes assessed using a fluorescence microplate reader was significantly smaller in HSFs treated with OMT than in HSFs treated with SFM at 4 and 8 hours (25.4% ± 1.7% and 15.2% ± 3% lower than the SFM control, respectively) (P < .01) (Figure 1B). OMT inhibited autophagy in HSFs at 4 and 8 hours.

3.2 | OMT decreases HSF viability by autophagy inhibition

Inhibition of autophagy by OMT (3 mg/mL) had a significant effect on HSF viability (Figure 1C), which decreased to 20% ± 0.2% of the SFM control (P < .01) (Figure 1D). Treatment with RAPA, an inducer of autophagy, suppressed the effect of autophagy inhibition on HSF viability in cells exposed to OMT, with values 9% ± 0.4% above those in cells treated with OMT alone (P < .05) (Figure 1D). OMT decreased HSF viability by autophagy inhibition.

3.3 | OMT decreases HSF collagen metabolism by autophagy inhibition

Inhibition of autophagy by OMT (4.5 mg/mL) decreased HSF collagen metabolism (Figure 1E) to 45% ± 7.7% of the SFM control (P < .01) (Figure 1F). Treatment with RAPA suppressed the effect of OMT-induced autophagy inhibition on HSF collagen metabolism, with values 9.1% ± 2.6% above those of cells treated with OMT alone (P < .05) (Figure 1F). OMT decreased HSF collagen metabolism by autophagy inhibition.

3.4 | OMT induces HSF apoptosis by autophagy inhibition

Apoptotic cells (green) were apparent in HSFs exposed to OMT (Figure 1G). Inhibition of autophagy by OMT (4.5 mg/mL) decreased HSF apoptosis to 34.7% ± 2% of the SFM control (P < .01) (Figure 1H). Treatment with RAPA suppressed the effect of OMT-induced autophagy inhibition on HSF apoptosis, with values 27.7% ± 2% above those of cells treated with OMT alone (P < .05) (Figure 1H). OMT induced HSF apoptosis by autophagy inhibition.

3.5 | Effects of OMT on autophagy-related protein expression

OMT (4.5 mg/mL) downregulated the expression of LC3 II and p-ULK (Figure 1I) in HSFs by 34.3% ± 5.9% and 62.4% ± 2.7%, respectively, compared with the controls (P < .01; Figure 1J,K). RAPA blocked the effects of OMT on down-regulating LC3 II expression by 28.99% ± 2.3% (P < .01). The effect of OMT on decreasing LC3 II and p-ULK expression suggested that OMT inhibited cell autophagy.

3.5.1 | Clinical scar assessment of hypertrophic scars

The porcine full-thickness burn hypertrophic scar model was used to evaluate the potential of OMT on scar repair. A total of 27 hypertrophic scars were created and analysed for this study. Hypertrophic scars give the impression of dark purple, firm, raised, and without hair (Figure 2A,D). The area of hypertrophic scars was measured, and the
The original scar area was 6.97 ± 0.20 cm². The mean thickness of the original epidermis was 213.17 ± 4.49 μm, while the thickness of the dermis was 5189.89 ± 36.58 μm.

Scars were topically applied with OMT, NC, or SG. At 28 days, scars treated with SG or OMT displayed red colour, were less contracted, had reduced height, and had less hardness, compared with the scars in the NC group. Fifty-six days later, scars in SG or OMT group were pink in colour, were less pigmented, flat, softer, and less contracted compared with the NC. Clinical scar assessment showed that scars treated with SG or OMT were significantly better than the NC group at 28 and 56 days (P < .01, Figure 2E).

3.5.2 | Reduced area of the hypertrophic scars

The areas of the scars topically applied with OMT, SG, or NC were measured (Figure 2F). The scar area in SG group decreased to 4.34 ± 0.13 cm² at 28 days and 3.69 ± 0.08 cm² at 56 days. The scar area in OMT group decreased to 4.45 ± 0.24 cm² at 28 days and 3.83 ± 0.16 cm² at 56 days. The scar area in NC group decreased to 6.42 ± 0.13 cm² at 28 days and 4.85 ± 0.18 cm² at 56 days. The scar area of the SG-treated group or OMT-treated group at 28 or 56 days was clearly smaller than that of the NC-treated group (P < .01, Figure 2F).

3.5.3 | Histological characteristics of hypertrophic scars

The epidermal and dermal morphology of scars was evaluated via histology (Figure 2B). The initial hypertrophic scars showed a thick epidermis and many keratinocytes. The scars topically applied with OMT or SG had a thinner epidermis and decreased numbers of keratinocytes compared with the NC group at 28 and
The epidermal thickness of the SG-treated group decreased to 95.82 ± 6.36 μm at 56 days. The epidermal thickness of the OMT-treated group decreased to 100.83 ± 4.92 μm at 56 days. The thickness of scar epidermis applied with SG or OMT at 28 and 56 days was clearly thinner than the NC group (P < .05, Figure 2G,H).

The initial hypertrophic scars showed large numbers of fibroblasts, and dense and disordered collagen fibres in the dermis. At 56 days, scars in OMT or SG group displayed reduced numbers of fibroblasts in the dermis and loose bundles of collagen fibres compared with the NC group (Figure 2B). The dermal thickness of the SG-treated group decreased to 196.98 ± 14.30 μm at 28 days and 171.56 ± 12.10 μm at 56 days. The thickness of scar epidermis applied with SG or OMT at 28 and 56 days was clearly thinner than the NC group (P < .05, Figure 2G,H).
NC-treated group decreased to 4655.31 ± 75.00 μm at 28 days and 3712.55 ± 102.86 μm at 56 days. The thickness of scar dermis in OMT or SG group at 28 and 56 days was clearly less than the NC group (P < .01, Figure 2I,J). Taken together, these data showed that OMT enhanced the ultimate outcome of the
hypertrophic scars, reduced the epidermal and dermal thickness, and decreased the scar area.

### 3.5.4 Protein expression in hypertrophic scars

At day 0, strong positive staining of CK10 (a differentiation marker) and P63 (a proliferation marker) was identified in the epidermis of scar tissue (Figure 3A). At 28 and 56 days, positive staining for CK10 and P63 in each group was weaker than that at day 0 (Figure 3B,C). The positive staining for CK10 and P63 in the SG-treated group or OMT-treated group was considerably weaker than NC group at days 28 and 56 ($P < .01$, Figure 3D-E, 3I-J). OMT resulted in a steady decrease in the expression of collagen I, α-SMA, and TGF-β1 in hypertrophic scars.

In the dermis of hypertrophic scars, strong positive staining for collagen I, α-SMA, and TGF-β1 in the dermal layer of the scar. At 28 and 56 days, positive staining for collagen I, α-SMA, and TGF-β1 progressively decreased in each group compared with that at day 0 (Figure 3B,C). The positive staining for collagen I, α-SMA, and TGF-β1 in the SG group or OMT group was considerably weaker than the NC group at days 28 and 56 ($P < .05$, Figure 3F-H, 3K-M). OMT resulted in a steady decrease in the expression of collagen I, α-SMA, and TGF-β1 in hypertrophic scars.

LC3, which is a fundamental element of autophagosomal membranes, is branded as a marker for mammalian autophagy. At day 0, strong positive staining for LC3 was identified in the dermal layer of the scar. At 7, 14, and 56 days, positive staining for LC3 progressively decreased in each group compared with that at day 0 (Figure 4A). At 7 days, LC3 expression reached a maximum of 64.46% in the OMT-treated group, then gradually decreased, reached 11.96% at 14 days, and then gradually decreased to 36.44% at 56 days. At 7 and 56 days, the expression of LC3 in scar tissues treated with OMT decreased by 82.05% ($P < .05$) and 43.96% ($P < .05$), respectively (Figure 4B,C). In general, positive staining for LC3 in scar tissues treated with OMT at 7 and 56 days was less than that of the scars applied with NC.
4 | DISCUSSION

Skin wound repair is an extremely synchronised and multifaceted process encompassing many factors. During normal wound healing, fibroblasts nearby the wound site are activated and transformed into myofibroblasts and migrate to the wound site to increase collagen synthesis, thereby promoting wound repair. After the wound has healed, myofibroblasts undergo apoptosis. Conversely, under pathological circumstances, such as long-standing infection and inflammation, the persistence of myofibroblasts deposits excessive collagen, which leads to “over repair”, namely, scar formation. Abnormal proliferation, apoptosis, and excessive deposition of collagen of fibroblasts can result in hypertrophic scars. \(^{26-28}\) Currently, there is no effective method to treat hypertrophic scars, mainly because the pathogenesis is unknown. \(^1\)

Phytochemicals, which are natural plant-based compounds present in vegetables, fruits, and grains, have a wide variety of biological and pharmacological effects. They are an important source for drug preparation, and they are valuable for the development of innovative drugs. \(^7\) Recently, several phytochemicals have passed clinical trials and are being used in clinical practice for managing various diseases such as cancer. \(^6,10\) In this study, we showed that the phytochemical OMT promoted scar tissue remediation. OMT, the active ingredient extracted from Sophora flavescens, has various biological activities. \(^17\) Recent studies show that OMT inhibits cell autophagy in HUVECs. \(^11\) We showed that OMT inhibited autophagy in HSFs. Autophagy, a research hotspot, plays a significant role in maintaining tissue structure and function and is related to various human diseases. \(^12\) Autophagy is involved in the maintenance, activity, and life-death of skin fibroblasts during wound healing, which leads to pathological scars. \(^14-16\)

Autophagy has a significant impact on cellular survival. Karamanova et al, reported that amyloidogenic

![Diagram showing the effect of OMT on scar repair](image)

**Figure 5** OMT promotes hypertrophic scar repair by decreasing HSF viability and collagen deposition, and by inducing apoptosis via autophagy inhibition.
medin impairs endothelial cell autophagy and viability.29 Another study showed that reduced viability of T cells via increased apoptosis was caused by autophagy inhibition.30 We found that inhibition of autophagy by OMT decreased HSF viability, which is consistent with the Kapuy study, indicating that cell viability was enhanced by autophagy induction during endoplasmic reticulum stress.

Apoptosis, also acknowledged as type I programmed cell death, is manifested by cell shrinkage, chromatin condensation, DNA degradation and fragmentation, and cell division into apoptotic bodies, which are engulfed and degraded by phagocytes.31 Autophagy has a dual effect on cellular apoptosis. It can impede the induction of apoptosis, but also promote apoptosis.32 Cao et al33 reported that ursolic acid-induced apoptosis in HSF was stimulated by the inactivation of Beclin-1-dependent autophagy, which is consistent with our study, indicating that inhibition of autophagy by OMT decreased HSF apoptosis.

Collagen is a rich and unique extracellular matrix protein secreted by fibroblasts in different tissues. Autophagy performs a significant role in regulating collagen metabolism in cells. Increasing evidence from studies investigating the occurrence and development of fibrotic diseases shows that autophagy is closely related to collagen synthesis or degradation.34,35 Xu et al showed that puerarin reduces collagen synthesis by fibroblasts through inhibiting autophagy, which is consistent with our study, indicating that inhibition of autophagy by OMT decreased HSF collagen synthesis.36

Inhibition of autophagy by OMT promoted scar repair. OMT enhanced the ultimate outlook of the hypertrophic scars, reduced the scar area, lessened epidermal and dermal thickness, and significantly downregulated the expression of CK10, P63, collagen I, TGF-β1, and α-SMA. Although we have reported OMT promoted hypertrophic scar repair by decreasing HSF viability and collagen deposition, and by inducing apoptosis via autophagy inhibition in vitro and in vivo, this is a preliminary and superficial study. Further studies are required to verify these findings and investigate the mechanism underneath these phenomena, such as the function-blocking assay, double or triple labelling experiments, the gene study, etc.

5 CONCLUSIONS

In summary, OMT promoted hypertrophic scar repair by decreasing HSF viability and collagen deposition, and by inducing apoptosis via autophagy inhibition (Figure 5). This study provides a new perspective on the mechanism underlying hypertrophic burn scar formation, as well as key scientific data for the application of OMT as a new method for the management of burn hypertrophic scars.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the finding of this study are available in the article.

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