ORIGINAL ARTICLE

The antifibrotic effects of naringin in a hypochlorous acid (HOCl)-induced mouse model of skin fibrosis

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ABSTRACT

**Objectives:** Fibrosis is a chronic inflammation caused by the loss of innate compensational mechanisms. Naringin (NR) is a flavonoid with antineoplastic and anti-inflammatory effects. Here, we aimed to investigate the antifibrotic effects of NR and underlying mechanisms in a Hypochlorous acid (HOCl)-induced mouse model of skin fibrosis.

**Materials and methods:** A total of 24 six-week-old female BALB/c mice were randomly allocated into five groups: HOCl, Sham, PBS, HOCl + NR and DMSO and selected skin regions were treated for 6 weeks, until sacrifice. The histopathologic and collagenesis of skin resections were analyzed using H&E and PR staining. The mRNA levels of COL1, COL3 and \(\alpha\)SMA genes were quantified. Serum samples were also used to evaluate TGF-\(\beta\) levels and LDH activity.

**Results:** HOCl could increase the relative collagen content, while NR administration on HOCl-treated biopsies decreased collagenesis. COL1, COL3 and \(\alpha\)SMA mRNA levels were significantly increased among HOCl-treated skin samples, while NR treatment could decrease these mRNA levels of genes to the extent equal to the levels in the Sham group. Similarly, Naringin-treated samples could decrease TGF-\(\beta\) levels.

**Conclusions:** We demonstrated that Naringin could exert protective effects against fibrotic complications of HOCl in skin tissue in vivo, by reducing the collagenesis and decreasing the levels of fibrosis-associated genes.

Introduction

Fibrosis is demonstrated by restricted wound healing, majorly caused by the loss of innate compensational mechanisms and abnormal replication of embryological developmental codes \cite{1,2}. The inflammatory condition of fibrosis is soared through several reinforcement chains which are initiated as a result of tissue injury, incremented rigidity of the extracellular matrix (ECM), oxidative stress, hypoxia, and congestion of damage-associated molecular patterns (DAMPs), which foster the activation and differentiation of fibroblasts concerning the innate immune signaling \cite{3}. Several diseases including rheumatic diseases, representing the chronic, inflamed, and/or protracted tissue injury are commonly associated with fibrosis \cite{1}. Fibrosis is thought to be a possibly reversible process \cite{4}, while the general immunosuppressive approaches are not effective in the management of fibrosis \cite{3,5}.

Most of the fibrosis-associated disorders including systemic sclerosis engage skin and develop Scleroderma-like cutaneous fibrosis \cite{6}. More than 70% of the skin weight, as the largest human organ, is comprised of ECM \cite{7}. The majority of ECM is found within the dermis and is primarily composed of collagen-1 and protein components such as fibronectin and proteoglycans \cite{8}. Fibroblasts are the principal mesenchymal cell type and the central effectors of the ECM homeostasis, controlling its constant transition \cite{9}. The fibrogenesis, actually known as an unbecoming consequence of improper management of tissue repair, is a polyphase process in which the production of enormous signaling molecules including growth factors and cytokines are deregulated, and tissue homeostasis is inclined to inordinate congestion of extracellular matrix (ECM) and interstitial hyperplasia \cite{1,10–12}. Since current therapeutic approaches against cutaneous fibrosis are limited to the management of complications, it is crucial to better study the underlying mechanisms of fibroblast activation to introduce novel therapeutic targets or redesign previously approved drugs against countless fibrosis-associated diseases.

Hypochlorous acid (HOCl), in addition to other important oxidative molecules within the ROS family such as hydroxyl radical (OH\(^{-}\)), peroxynitrite (ONOO\(^{-}\)), hydrogen peroxide (H\(_2\)O\(_2\)), and superoxide radical (O\(^{-}\)) and selected drugs such as bleomycin are considered to possess key roles in the
initiation and propagation of fibrosis-associated diseases including systemic sclerosis (SSc) [13]. These chemicals are effective in developing fibrosis conditions by increasing the expression of related proteins and genes including collagen type 1α, α-SMA and TGF-β, and currently used in expanding fibrosis-associated animal models, such as scleroderma [14]. Moreover, the ligand-dependent activation of immune response signaling molecules including cytokines could result in the elevation of ROS [15]. In this regard, the pro-fibrotic response signaling molecules including cytokines could result in the elevation of ROS [15]. In this regard, the pro-fibrotic response signaling molecules including cytokines could result in the elevation of ROS [15].

Flavonoids are known as natural polyphenolic compounds that are thought to possess antifibrotic effects in addition to the prevention of numerous inflammatory diseases [19]. Naringin (NR; PubChem CID: 442428; Molecular Formula: C_{22}H_{22}O_{14}\_; molecular weight: 580.5 g/mol) is the principal active flavonoid in citrus-related species and grapefruit [20]. Several antineoplastic and an anti-inflammatory effect of NR have been reported in previous studies with minimal adverse effects [21–26]. To the best of our knowledge, no previous study has evaluated the effectiveness of NR on skin fibrosis.

Hereupon, we aimed to investigate the antifibrotic effects of NR and elucidate the underlying mechanisms in a Hypochlorous acid (HOCl)-induced mouse model of skin fibrosis.

Materials and methods
The maintenance of animals, and chemicals
Specific pathogen-free, 6-week-old female BALB/c mice were purchased from Chakavak Laboratory Institute (Sari, Mazandaran, Iran) and maintained with adequate and sterile food and water in autoclaved cages. They were given humane care following the ethical guidelines of handling laboratory animals in our institution (12 h light/dark cycles, 24 ± 2 °C, and 35–60% humidity). Naringin (CAS Number: 10236-47-2) was purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Picro-Sirius red (0.1%) (CAS Number: 2610-10-8) was also purchased from Sigma-Aldrich. Human TGF Beta 1 and human/Mouse TGF beta ELISA kit (CAT Number: 2610-10-8) and vehicle (DMSO 20%) alone (n = 8) for 6 weeks. Prior to the induction of fibrosis and/or administration of treatment compounds, the mice backs were shaved. The initial thickness of skin was measured using a digital caliper and repeated every week during the whole experiment by a trained and unaligned investigator. Moreover, the body weights of mice were scaled and recorded every week using a digital balance.

In order to induce fibrosis in mice, 300 μl of HOCl was injected subcutaneously in two sites of the shaved backs in predetermined mice with a 29 G needle, 5 days a week for 6 weeks [d0 to d42]. The control mice were given PBS under the same settings. Naringin (50 mg/kg/BW) was dissolved in 20% DMSO and was administrated into mice’s back once every three days for 6 weeks via subcutaneous injection.

After 6 weeks and a 2-day recovery time without HOCl injections, at the end of the experimental period (7th week equal to d45) all groups were sacrificed by cervical dislocation, following the ethical guidelines. Blood samples were collected and serum was separated after centrifugation (1,500 g, 10 min) and stored at −20 °C for ELISA and LDH assay. Skin biopsies (6 mm punches) were performed on the backs of the mice and washed in PBS. Samples were stored at −80 °C for RT qPCR and collagen content determination or fixed in 4% formaldehyde for histopathological analysis [27].

Lactate dehydrogenase assay
The lactate dehydrogenase (LDH) activity in the sera of the mice in all treatment groups was assessed by measuring the NADH oxidation rate using an enzyme colorimeter kit (Pars Azmoun Co., Tehran, Iran) (CAT Number: 97203232). Absorption changes were detected at 340 nm and the results were expressed as unit protein/mg [28].

Histopathological analysis
All skin sections were obtained from the injection sights in all treated mice. Fixed skin samples were then embedded in paraffin. A 5-μm-thick tissue section was prepared from the midportion of paraffin-embedded tissue and stained either with hematoxylin eosin and Picrosirius red. Slides were prepared and evaluated using standard brightfield microscopy (Olympus BX60, Tokyo, Japan) by a pathologist who was blinded to the placement of the groups [29].
Histomorphometric analysis of skin collagenesis by picrosirius red (PR) staining and image analysis

The Picrosirius red (PR) stained slides were evaluated by an irrespective assessor and six micrographs per each slide were generated at the magnification of 100× using light microscopy (Olympus BX60, Tokyo, Japan).

As previously described by Caetano et al. [30], the Threshold function within ImageJ software was used to examine the intensity and percentages of pixels for PR in each captured image, while the red dye specified the existence of collagen fibers in each sample. Data were recorded as the mean of pixels in ten specimens per day.

Quantitative RT-PCR analysis

TRizol Reagent (Invitrogen, USA) (CAT Number: 15596026) was used to extract total RNA from duplicate skin biopsy samples, according to the suggested protocols by the manufacturer. A total amount of 1μg DNase I (Yekta Tajhiz Azma, Iran) (CAT Number: YT8054) treated RNA was used for cDNA synthesis (Yekta Tajhiz Azma, Iran) (CAT Number: YT4500) using the manufacturer’s instructions. The gene expression levels were evaluated using qRT-PCR method by SYBR green master mix (Ampliqon Inc., Denmark) (CAT Number: A325402), and exon-specific primers (Table 1) for the collagen type I alpha 1 (COL1), collagen alpha-1(III) chain (COL3) and smooth muscle alpha-actin (αSMA) genes. The amplification of target genes was performed in the following conditions: incubation at 95°C for 10 min, 40 cycles of the 95°C, 10 s; annealing at 60°C for 30 s and the extension at 72°C for 40 s. The GAPDH gene expression was used as a housekeeping gene and the 2^-ΔΔCt method was used for the gene expression normalization. The qRT-PCR experiments were carried out by using the LightCycler system (Roche Diagnostics Corporation, Indianapolis, USA) [20].

Measurement of serum TGF-β levels

Serum samples were isolated and kept at −20°C at the time of sacrifice, and all samples were analyzed at the same time. TGF-β levels were evaluated by a commercially available ELISA kit (eBioscience, USA) for the detection of mouse TGF-β, with the minimum detection limit of 8 pg/ml. Assays were performed in triplicates and according to the manufacturer’s guidelines.

Statistical analysis

All quantitative data were represented as means ± SEM. All combinations and treatment counterparts were compared using the Mann-Whitney test for nonparametric measures, Student’s t-test for parametric values, and one-way ANOVA with Tukey post hoc test for more than 2 groups. All statistical analyses were performed using GraphPad Prism 8.3 software. P-values less than 0.05 were considered significant.

Results

Naringin showed no adverse effects on body weight or LDH activity in vivo

In order to better monitor and possibly normalize the physiological effects of treatments on each experiment, the alterations of body weights (gr) were recorded for each treatment group during the experimental procedures and at the beginning of each week. As illustrated in Figure 1(a), the average body weight of all groups was not significantly different with each other at a specific time point, while the growing rate of body weights for each treatment group was increasing.

We also evaluated the toxic effects of each treatment on vital organs especially liver by evaluating the LDH enzyme activity at the end of treatment procedure (day 43). As shown in Figure 1(b), two-way Annona statistical results revealed that LDH enzyme activity was not significantly different between treatment groups.

The dermal thickness was effectively decreased in naringin-treated skin biopsies

In order to evaluate the dermal thickness of HOCl-treated samples macroscopically and microscopically, as a major consequence of fibrosis, and the possible effects of Naringin on altering the thickness of skin biopsies, two major methods were performed. At the end of the experimental procedure and after sacrificing the tested animals, the skin biopsies from each group were obtained, fixed, paraffin-embedded and the prepared slides were stained using hematoxylin and eosin (H&E) (Figure 2(a)). A curated semi-quantitative method using Image J software (Supplementary results; Figure 1) was used to calculate the epithelial height of the cellular part of the dermis. Our results revealed that the microscopic dermal thickness was significantly higher in skin biopsies treated with HOCl compared to all other treatment groups, while the dermal thickness in Naringin-treated skin resections were

| Table 1. The specific primers for the mRNA expression of fibrosis-associated genes. |
|-----------------------------------------------|-----------------|-----------------|
| Gene          | Primers 5′-3′ | bond size (bp) | GenBank Accession Number |
|---------------|--------------|----------------|--------------------------|
| COL1          | Forward      | CAGGGTACAGTTTGAA | 87 | NM_007742.4 |
|               | Reverse      | CCTGAACTCCAGTACA |              |               |
| COL3          | Forward      | GGACAGAGGGCTTTGAT | 92 | NM_009930.2 |
|               | Reverse      | CGGTGTCTTCTGGAGAA |              |               |
| αSMA          | Forward      | TGAAGAGCCTCCGACACT | 168 | NM_007392.3 |
|               | Reverse      | CCCACGCACTACACGTTG |              |               |
| GAPDH         | Forward      | GTGCCGGCGCTGAGTACGTG | 587 | NM_001289726.1 |
|               | Reverse      | GAGGAGCCACCTGGTGTCAG |              |               |
markedly decreased, almost equal to the Sham and PBS group. Although the dermal thickness of DMSO-treated samples was less than the HOCl group, no significant difference was observed (Figure 2(b)). A digital caliper was used to calculate the macroscopic skin thickness during the experimental procedure. Our results showed that the skin thickness in HOCl-treated mice was significantly higher than all treatment groups, while the thickness of Naringin-treated skin samples was markedly decreased compared to the HOCl-treated counterparts.
samples were markedly decreased compared to the HOCl-treated counterparts. In consistent with the microscopic evaluations, the skin thickness of DMSO-treated samples was less than the HOCl group and higher than all other treatment groups (Figure 2(c)).

The collagen content was significantly decreased in the skin biopsies of naringin-treated mice

In order to evaluate and compare the expression levels of collagen in all experimental groups, a picrosirius red staining (PRS) method followed by an ImageJ quantification was performed. Our results showed that HOCl could significantly increase the relative collagen content (to the Sham group) compared to PBS and DMSO treatments, regarding both area and integrated density of the collagen. Moreover, the administration of Naringin on HOCl-treated skin biopsies was associated with lower levels of collagen in both area and IntDen categories (Figure 3).

Naringin treatment could successfully decrease the levels of fibrosis-associated genes and cytokines

We evaluated the mRNA expression levels of specific fibrosis-associated genes using RT qPCR. As demonstrated in Figure 4(a–c), COL1, COL3 and αSMA mRNA levels were significantly increased among HOCl-treated skin samples. Although DMSO-treated samples expressed lower levels of all three genes, the mean differences were not significant. Interestingly, Naringin treatment could effectively decrease these mRNA levels of tested genes to the extent equal to or even lower than the levels in the Sham group. We also demonstrated that the serum levels of TGF-β were significantly increased in HOCl-treated groups (Figure 4(d)). Similar to the mRNA expression levels, Naringin-treated samples could markedly decrease TGF-β levels to the extent equal to the Sham group.

Discussion

Naringin is a dihydroflavonoid that can be found in grapefruits and the citrus of Rutaceae [22,31]. Naringin exhibits several biologically active effects, including anti-inflammation, hepato-protection, anti-apoptosis, antioxidant in addition to inhibiting genetic toxicity [25]. The aim of the current study was to investigate the anti-fibrotic effects of Naringin on HOCl-induced skin fibrosis in mice. The data obtained from the study demonstrated the protective effects of Naringin treatment against fibrotic complications of HOCl in skin tissue in vivo.

The anti-fibrotic role of Naringin was previously reported in other fibrotic models including liver [4], pulmonary [24], cardiac [21] and renal [32] fibrosis. It is worth mentioning that various methods for fibrosis induction have been applied in different studies depending to the target tissue which can rely on different molecular mechanism of fibrotic

![Figure 3](image-url)

Figure 3. Evaluating the collagen content using picrosirius red staining (PRS); PRS staining was used to evaluate the content levels of produced collagen on the paraffin-embedded, skin biopsies of all experimental groups (20X magnification) (a). The ratios of area (b) and integrated density (IntDen) (c) of stained collagen in all microscopic samples to the Sham groups were quantified using ImageJ software. Skin biopsies from HOCl-treated mice showed the highest relative levels of collagen content, while Naringin-treated samples expressed significant lower levels of collagen which were almost similar to the contents in the PBS control group. Two-way Annona and Tukey’s post hoc statistical measures were used to compare the mean differences between the treatment groups. *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant. H: HOCl; H + N: HOCl + Naringin; H + D: HOCl + DMSO.
response. In the current study, we used HOCL injection (subcutaneous, 6 weeks) for induction of skin fibrosis in mice. It has been reported that HOCL injection results in a primary inflammatory step at day 21 characterized by infiltration of proliferative myofibroblast and immune cells followed by second step of matrix fibrosis establishment at day 42 featured by less inflammation but high collagen deposition [27]. Meng et al. (2019) also observed significant fibrosis following 6 weeks of treatment with HOCL, obvious in skin and lung tissues in a mechanism related to elevation of TGF-β, NF-κB, Smad3, p-Smad3, STAT3, and p-STAT3 [13]. In accordance with the previously mentioned study, we also found statistically significant increased skin thickness, only after 6 weeks of treatment by HOCL at day 43 but not earlier. The rate of body weight was increasing through time in all treatment groups and no significant change was detected in LDH enzyme activity, delineating no toxic effect from Naringin. The fibrosis was confirmed by elevation of collagen content (stained with picrosirius red) and increased expression of COL1, COL3, and αSMA genes in skin tissue of the HOCL treated mice which was ameliorated in Naringin (50 mg/kg weight) treated animals.

Administration of HOCL either by subcutaneous [33] or intradermal injection [34] can efficiently induce fibrosis in both skin and lung tissues of mice which resembles the diffused systemic sclerosis. In current study we used subcutaneous injection method to generate HOCL induced fibrosis model. Since we were aimed to investigate the anti-fibrotic application of Naringin in skin, fibrosis of the other tissues like lung were not explored; however, we found remarkable

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**Figure 4.** The mRNA expression of fibrosis-associated genes and serum levels of TGF-β; we quantified and compared the mRNA expression levels of the fibrosis-associated genes on all experiments. 2−ΔΔCt method using GAPDH was used to normalize the gene expression levels in all experiments. Our results demonstrated that COL1 (a) COL3 (b) and αSMA (c) mRNA levels were significantly increased among HOCl-treated skin samples. Naringin treatment could effectively decrease these mRNA levels to the extent equal to or even lower than the levels in the Sham group. Similarly, we demonstrated that the serum levels of TGF-β were significantly increased in HOCl-treated groups, while Naringin-treated samples could markedly decrease these levels to the levels equal to the Sham group (d). Two-way Annona and Tukey’s post hoc statistical measures were used to compare the mean differences between the treatment groups. (All unspecified comparisons are performed between the experiments and the Sham group). *p < 0.05, **p < 0.01, ns: not significant. H: HOCl; H + N: HOCl + Naringin; H + D: HOCl + DMSO.
elevation of TGF-β in circulation of HOCL-treated mice which indicates systemic effect of HOCL induced fibrotic response which was reduced in Naringin treated group.

There are various mechanisms suggested for the anti-fibrotic effect of Naringin in different studies which are dependent to PI3K/Akt pathway regulation [4,23], anti-oxidant activity [24], PKC-β modulation [21] or Rock inhibition [32]. Previously, Khouloud Alaa El-Mihia et al. (2017) showed that simultaneous administration of Naringin (40 mg/kg/day, oral) alleviates the fibrosis in hepatic tissue of thioacetamide induced liver fibrosis, restoring the normal histology through modulation of the PI3K/Akt pathway. They showed oral administration of the Naringin has anti-oxidant and anti-inflammatory function and can ameliorate liver fibrosis by decreasing collagen and fibronectin deposition [4]. Moreover, Naringin can attenuate the proliferation and motility of the fibroblasts in a mechanism dependent to Akt pathway; thus it can restrain the development of hypertrophic scar through suppression of Akt phosphorylation at Ser473/Thr308 in hypertrophic scar fibroblasts [23].

Oral delivery of Naringin also can ameliorate the pulmonary fibrosis induced by Bleomycin (5 mg/kg; via the tracheal cannula) in rats. Nergiz H. Turgut et al. (2016) reported that rats treated with Naringin (80 mg/kg body weight, for 14 days) had significantly decreased level of hydroxyproline content in lung tissue and TNF-α and IL-1β in bronchoalveolar lavage fluid in bleomycin-induced pulmonary fibrosis model. Moreover, Naringin treatment increased the anti-oxidant activity through elevation of the Glutathione peroxidase and superoxide dismutase suggesting it as a protective compound against toxic effects of bleomycin in pulmonary fibrosis induction [24]. Similar anti-fibrosis effect of Naringin has been reported in other tissue fibrosis models. For example oral delivery of Naringin (50 mg/kg body weight) can reduce the fibrosis in cardiac tissue of the hyperglycemic diabetic rats through antioxidants and modulation of the p38 and PKC-β protein expression [21].

Another mechanism of HOCL-induced fibrosis is enhancement of RhoA/ROCK activity. It has been shown that ROCK inhibition by Fasudil (Rho kinase inhibitor and vasodilator) can reduce the expression of αSMA and skin fibrosis through inhibition of phosphorylation of Smad2/3 and ERK1/2 [34]. In a diabetic nephropathy mouse model, treatment with Naringin by gavage or injection of Fasudil ameliorated renal fibrosis with decreased level of RhoA, ROCK1, ROCK2, Collagen types 1 and 3, p-MYPT1 Thr853, laminin and fibronectin; suggesting anti-fibrotic function of Naringin in a mechanism related to RhoA/ROCK inhibition in diabetic nephropathy model [32]. Similarly, in the current study we found anti-fibrotic effect of Naringin when administrated subcutaneously in skin fibrosis model which was associated with down-regulation of fibrosis-associated genes (COL1 & 3, αSMA), decreased collagen deposition and serum TGF-β level. Although it is strongly suggested to confirm the mRNA expression results by protein expression methods such as western blot analysis, or ELISA, we were not capable of implementing these confirmatory studies due to lack of financial supports, which is listed as a major limitation to our study. However, these studies could be used to increase the quality of future research works.

Conclusion

Our findings demonstrated that Naringin could exert protective effects against fibrotic complications of HOCL-induced model of skin fibrosis in vivo, by reducing the collagenesis and decreasing the levels of fibrosis-associated genes. However, the underlying mechanism remains to be investigated more clearly.

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Ethical approval

Animal subjects

This research project was approved by the committee of ethics at Golestan University of Medical Sciences (Code of ethics: IR.GOU.MS.REC.1398.157) and conducted following all applicable international, national, and/or institutional guidelines for the care and use of animals. The ethics certificate could be found using the following link: https://ethics.research.ac.ir/ProposalCertificateEn.php?id=71865&Print=true&NoPrintHeader=true&NoPrintFooter=true&NoPrintPageBorder=true&LetterPrint=true.

Disclosure statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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