Effects of *Citrus sunki* Peel Extract on Matrix Metalloproteinase-1 Expression

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Flavonoids are one of the major components found in the peels of citrus fruits. Present evidence has suggested that polymethoxyflavonoids, including nobiletin and tangeretin isolated from *Citrus sunki*, have many biological properties, such as anti-inflammatory, anti-oxidant, and anti-obesity capabilities. Here, we investigated the effect of *Citrus sunki* peel extract and its possible mechanisms on oxidative stress-induced MMP-1 expression, a major marker of skin photoaging. H$_2$O$_2$ induced MMP-1 expression in a dose- and time-dependent manner. Extract of *Citrus sunki* peel (1-25 µg/ml) dose-dependently decreased MMP-1 mRNA levels. When H$_2$O$_2$ was combined with *Citrus sunki* peel extract, the phosphorylation of ERK was further decreased compared to a single treatment with H$_2$O$_2$ alone. Moreover, U0216, an MEK inhibitor, markedly prevented the production of MMP-1. These data suggest that *Citrus sunki* peel extract has demonstrated protective activity against oxidative damage on MMP-1 expression, and ERK MAP kinase may be involved.

**Key words**: *Citrus sunki*, matrix metalloproteinase-1, oxidative stress, skin aging

**Introduction**

Human skin is exposed to potentially harmful compounds and/or irradiation and becomes aged gradually. It is well known that ultraviolet radiation, one of potent factors for skin aging, induce oxidative stress through the generation of reactive oxygen species (ROS) such as superoxide anion radical (O$_2^-_*$) and hydrogen peroxide (H$_2$O$_2$) in human dermal cells. Oxidative damage induced by ROS is responsible for inflammatory disorders and skin aging [2, 8, 12]. Aged skin shows a decrease in collagen synthesis, which is related to matrix metalloproteinases (MMPs) regulation [6]. MMPs are a family of matrix degrading enzymes consisting of different members with substrate specificity that play important roles in various destructive processes by remodeling extracellular matrix (ECM) [10]. Among these, MMP-1, known as collagenase, is a key enzyme involved in collagen degradation due to oxidative stress, which is mediated by mitogen activating protein kinase (MAPK) - extracellular signal-regulated kinase (ERK)/p38/JUN-N-terminal kinase (JNK) activation [3, 8]. Especially, ERK and JNK are known to be involved in MMP-1 expression via activation of the MMP-1 promoter by the transcription factor AP-1 and NF-kB [2, 4, 8].

The peel of *Citrus* fruit contains a wide range of flavonoids constituents has been used as a traditional medicine in Korea. Flavonoids isolated from *Citrus* peel are categorized into two types. One is flavones glycosides including naringin, the other is polymethoxylated flavones (PMF) including nobiletin and tangeretin [11]. Following a report analyzing the composition of *Citrus* peel, the peel of *Citrus sunki* Hort. *ex* Tanaka is a rich source of polymethoxylated flavones [9]. A number of biological activities of polymethoxylated flavonoids isolated from *Citrus sunki* Hort. *ex* Tanaka such as anti-inflammation and anti-obesity activities have been suggested [5, 9]. In this study, we investigated the effect of *Citrus sunki* Hort. *ex* Tanaka peel extract including polymethoxylated flavones on the expression of MMP-1 against oxidative stress in human skin fibroblast. *Citrus sunki* peel extract was found to prevent oxidative stress-induced MMP-1 expression by inhibiting ERK MAPK signaling. These results indicate that *Citrus sunki* peel extract may be a useful product for skin anti-aging.

**Materials and Methods**

Preparation of *Citrus sunki* peel extract

*Citrus sunki* Hort. *ex* Tanaka peel extract was isolated as described previously [9].
Cell culture

HS-68 cells obtained from Korea Cell Bank (KCLB) were cultured in DMEM containing 10% FBS (fetal bovine serum) and 1% antibiotics at 37°C in humidified atmosphere of 5% CO2.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using easy blue kit and RT-PCR reaction was carried out by Maxime RT-PCR PreMix kit. The conditions for MMP-1 and GAPDH were as follows: 30 cycles of 94°C for 1 min, 68°C for 1 min, 72°C for 1 min. Forward and reverse primers were 5'-ATTCTACTGATA-TGGGGCTTTGA-3' and 5'-AGTCCTTGCTGGGATCCGTGTAG-3', respectively, for MMP-1 and 5' - ACCACAGTCC- ATGCCATCAC-3' and 5'- TCCACCAC CCGTGGCCTGA-3', for GAPDH. Amplified product was separated through 1.5% agarose gel and visualized on an UV transilluminator.

Western blot

Cells were lysed in buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1mM β-Glycerolphosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF) and centrifuged at 14,000 rpm for 10 min at 4°C. Protein (30 μg) was separated in 10% SDS-PAGE and transferred to nitrocellulose membrane. The blots were incubated with anti-ERK, anti-pERK and anti-actin antibody, followed by incubation with peroxidase-conjugated secondary antibodies and then detected by enhanced chemiluminescence kit.

Results and Discussion

Protective effect of Citrus sunki peel extract on MMP-1 expression

Oxidative stress due to the ROS production is related to the skin aging. Oxidative stress-induced skin damage results from MMP's up-regulation, which causes collagen degradation [1, 2, 6]. Since MMPs-induced collagenous matrix damage is a main phenomenon of aged skin, MMPs inhibition is thought to be a useful method for skin anti-aging. It has been reported that natural product plays a role on MMP's down-regulation in UV-irradiated cells [2, 3, 13, 15]. Polymethoxyflavonoids have been shown to exhibit a broad spectrum of pharmacological actions including anti-cancer, anti-inflammation, anti-oxidant and anti-obesity [5, 7, 14].

Recently, Kang et al. reported that extract isolated from Citrus sunki Hort. ex Tanaka peel was a rich resource of polymethoxyflavonoid such as nobiletin and tangeretin [9]. In this study, we examined that effect of Citrus sunki Hort. ex Tanaka peel extract on MMP-1 inhibition in oxidative stress-induced skin aging model. To check up-regulation of MMP-1 by oxidative stress, H2O2 was treated to the HS-68 cells and RT-PCR analysis was performed. As shown in Fig. 1A and 1B, H2O2 induced MMP-1 expression in a dose and time dependent manner. For further studies, 500 μM of H2O2 were chosen as an optimal dose. Next, we investigated whether Citrus sunki peel extract might affect oxidative stress-induced MMP-1 expression. H2O2 induced increase in MMP-1 level was significantly reduced by treatment with 25 μg/ml Citrus sunki peel extract (Fig. 1C). These results indicate that Citrus sunki peel extract inhibits the H2O2-induced production of MMP-1 in human skin fibroblasts.

Involvement of ERK on MMP-1 expression

The activation of mitogen activating protein kinase (MAPK) is well known to be associated with MMP-1

![Fig. 1. Protective effect of Citrus sunki peel extract (CPE) on MMP-1 expression. Cells were exposed to H2O2 with indicated dose (A) or time (B), respectively. (C) Cells were treated with each indicated dose of CPE for 30 min prior to H2O2 treatment and harvested 24 hr later. MMP-1 mRNA expression was analyzed by RT-PCR. GAPDH was used as an internal control.](image-url)
Fig. 2. Involvement of ERK on MMP-1 regulation. (A) Cells were incubated with 500 μM H2O2, 25 μg/ml CPE and H2O2 plus CPE for 30 min. Protein levels of ERK were analyzed by Western blot. (B) Cells were pretreated with 20 μM U0216 (U) or 25 μg/ml CPE for 30 min, and then exposed to 500 μM H2O2 for 24 hr. MMP-1 expression was detected by RT-PCR. GAPDH was used as an internal control.

regulation. Up-regulation of MMP-1 by UV irradiation as a potent factor of skin aging, which causes collagen degradation, is mediated by MAPK signaling via activation of transcription factor AP-1 and/or NF-kB [2, 3, 4, 8]. To investigate involvement of MAPK signaling cascades on Citrus sunki peel extract-induced MMP-1 down-regulation, total and the phosphorylation levels of ERK was measured by Western blot analysis. Incubation of cells with H2O2 induced ERK phosphorylation. Citrus sunki peel extract significantly inhibited the phosphorylation of ERK compared with single treatment of H2O2 (Fig. 2A). However, Citrus sunki peel extract did not affect the phosphorylation and expression of p38 and JNK (data not shown). Moreover, pretreatment of U0216, a MEK inhibitor, blocked the H2O2 induced increase MMP-1 expression markedly (Fig. 2B). These results suggest that protective effect of Citrus sunki peel extract on H2O2-induced MMP-1 expression is regulated by ERK inhibition.

In conclusion, MMP-1 inhibition is an important strategy for skin anti-aging. Our results demonstrate that Citrus sunki peel extract inhibits oxidative stress-induced MMP-1 expression via suppression of ERK signaling, suggesting that Citrus sunki peel extract could potentially be used in the prevention of skin aging through MMP-1 inhibition.

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References

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초록: 진귤 과피 추출물의 MMP-1 발현조절 효과

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본 연구는 제재감귤종의 하나인 진귤 과피 추출물이 산화적 스트레스에 의한 MMP-1의 발현 조절에 미치는 효과를 확인하기 위해 수행되었다. \(\text{H}_2\text{O}_2\)를 피부세포에 처리하여 산화적 스트레스를 유도한 결과 노화 유발에 중 요한 역할을 하는 것으로 알려진 MMP-1의 발현량이 증가하였고 진귤과피 추출물의 처리는 산화적 스트레스에 의해 증가된 MMP-1의 활성을 현저히 감소시켰다. 이러한 활성 조절이 ERK signaling을 통해 조절되는지 확인한 결과 산화적 스트레스에 의해 증가된 ERK의 인산화는 진귤과피 추출물의 처리로 억제되었고 MEK 억제제인 U0216을 처리하였을 경우 MMP-1의 활성도 또한 저해시키는 것을 확인하였다. 이상의 결과로 보아 \(\text{H}_2\text{O}_2\)에 의해 유도된 산화적 스트레스는 MMP-1의 발현을 촉진시켰고 진귤과피 추출물은 ERK 신호전달 경로를 통해 MMP-1의 발현을 조절하는 것으로 보여진다.

