The effects of the metformin on inhibition of UVA-induced expression of MMPs and COL-I in human skin fibroblasts

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Abstract
This study was to investigate the effects of metformin (MF) on ultraviolet A (UVA)-induced expression of matrix metalloproteinases (MMPs) and type I collagen (COL-I) in human skin fibroblasts (HSFs). HSFs were cultured in vitro and divided into control group, UVA group, and UVA + MF group. Cell proliferation was detected by CCK-8 method, and intracellular reactive oxygen species (ROS) level was detected by flow cytometry with fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA) staining. Meanwhile, real-time polymerase chain reaction (PCR) was used to examine the relative messenger RNA (mRNA) expression of aging-related genes, including MMP1, MMP3, and COL-I. Moreover, the expression of MMP1, MMP3, and COL-I proteins was further detected by western blot. Compared with the control group, the ROS content in the UVA group was increased significantly ($P < 0.05$), while the ROS content in the UVA + MF group was evidently lower than that in the UVA group ($P < 0.05$). In addition, the MMP1 and MMP3 mRNA levels were significantly elevated, while the COL-I mRNA was significantly decreased in UVA-induced HSF cells compared with the control cells. However, MF could significantly inhibit the improved MMP1 and MMP3 mRNA level and increase the COL-I mRNA level. Moreover, MF could significantly reverse the increasing MMP1 and MMP3 protein level and decreasing COL-I protein level induced by UVA. In conclusion, MF can increase the antioxidant capacity of cells and increase the synthesis of COL-I by inhibiting the level of intracellular ROS and the expression of related MMPs, thereby inhibiting the UVA-induced photoaging effect of HSF.

Keywords
COL-I, human skin fibroblasts, metformin, MMP1, MMP3, ultraviolet A

Date received: 19 November 2018; accepted: 18 June 2019

Introduction
It was demonstrated in the related data that the extracellular matrix (ECM) of the skin is mainly composed of type I collagen (COL-I) secreted by human skin fibroblasts (HSFs), which plays an important role in maintaining skin tension and tolerance. Sunlight is an important factor in inducing photoaging of the skin. A large amount of ultraviolet A (UVA) can penetrate the dermis layer of the skin, causing photoaging phenomenon of skin such as skin damage, cutis laxa, and wrinkles. The photoaging of the skin induced by ultraviolet radiation is mainly manifested in the increase of skin wrinkles, decreased elasticity, and pigmentation, which is associated with increased secretion of matrix metalloproteinases (MMPs) and decreased synthesis of COL-I caused...
by ultraviolet B (UVB) irradiation. Metformin (MF) is a widely used anti-diabetic drug, and it is further verified in the recent studies that MF possessed the effects of reducing cancer incidence, anti-aging, and prolonging life. MF can inhibit the activation of I kappa B kinase (IKK) kinase and reduce the occurrence of chronic inflammatory reactions by reducing the production of mitochondrial reactive oxygen species (ROS), thereby inhibiting aging.

This study was to observe whether MF can protect against UVA-induced photoaging of HSF, so as to provide a basis for the application of MF in photoaging prevention.

**Methods**

**CCK-8 assay for cell proliferation**

HSF was provided by Shanghai Aolu Biological Technology Co., Ltd. and cultured in a high-glucose DMEM (Dulbecco’s Modified Eagle Medium; Beijing Qingda Tianyi Technology Co., Ltd) containing 10% fetal bovine serum (ScienCell Co., Ltd., Carlsbad, CA, USA). This study was approved by the Ethics Committee of Shunde Hospital Affiliated to Guangzhou University of Traditional Chinese Medicine. The cells were plated at a density of 2000 cells/well in a 96-well plate and cultured at 37°C under 5% CO2 condition. After adherence, a concentration of 0.01 mmol/L of MF (Taize Ruida Technology Co., Ltd., Beijing, China) was added, and after 72 h of culture, CCK-8 reagent was added and operated according to the kit instructions (Aviva Systems Biology, Beijing, China). The absorbance (A) value was measured with a microplate reader at an excitation wavelength of 450 nm. The experiment was repeated three times separately.

**Detection of ROS level by flow cytometry**

The cell culture conditions are the same as “CCK-8 assay.” And the cells were seeded at a density of 10^5 cells/well in a six-well plate and divided into control group, UVA group, and UVA + MF group. The UVA + MF group was treated with 0.01 mmol/L MF, while the control group and UVA group were treated with DMEM medium instead. After 24 h of drug treatment, the culture solution was removed, and then the cells were washed three times with phosphate-buffered saline (PBS). Finally, the UVA group and UVA + MF group were added with 500 μL of PBS and irradiated at a dose of 9 J/cm2. The control group did not receive any dose of irradiation. After the irradiation, the UVA + MF group was further cultured by adding 0.01 mmol/L of MF. After 72 h of culture, the cells were collected by trypsinization, then ROS-sensitive fluorescent probe, 2’,7’-dichlorofluorescein diacetate (DCF-DA; Sigma Aldrich, Germany), was added, followed by incubation at 37°C for 30 min. It was washed three times with PBS. A FACScan flow cytometer and FACSCanto II version 4.1 (BD Biosciences, San Jose, CA, USA) were used to perform the detection. The experiment was repeated three times separately.

**Real-time polymerase chain reaction method for detecting relative expression of MMP1, MMP3, and COL-I mRNA**

Cell culture and treatment are the same as “flow cytometry assay.” After 72 h of culture, the cells were collected by trypsinization, and total RNA was extracted, purified, and quantified. The RNA was reverse transcribed into complementary DNA (cDNA) according to the instructions of the reverse transcription kit, pre-denatured at 95°C for 5 min, denatured at 95°C for 15 s, annealed at 60°C for 30 s, extended at 72°C for 30 s, 40 cycles, and maintained at 72°C for 10 min. β-actin was served as the internal control, and the expression levels of MMP1, MMP3, and COL-I genes were calculated based on the cycle threshold (Ct). The primer sequences are shown in Table 1 (GenePharma Co., Ltd, Shanghai, China). The experiment was repeated three times separately.

Table 1. Primer sequences of each gene.

| Primer        | Sequences               |
|--------------|-------------------------|
| MMP1-forward | 5’-TTGGAGGGGATGCTATT-3’ |
| MMP1-reverse | 5’-ACACGCTTTTGGGTGTT-3’ |
| MMP3-forward | 5’-GCGGTTTGGCTACGCTATC-3’ |
| MMP3-reverse | 5’-TCCAGAGCTGGCTGAGTCC-3’ |
| COL-I-forward| 5’-CCA GTG GCG GTT ATG ACT-3’ |
| COL-I-reverse| 5’-GCT GCG GAT GTT CTC AAT-3’ |
| β-action-forward | 5’-AGCGGACGATTCCTAAAGTT-3’ |
| β-action-reverse | 5’-GGGCACGAGGGCTCATCATT-3’ |

MMP: matrix metalloproteinase; COL-I: type I collagen.
Western blot analysis of MMP1, MMP3, and COL-I protein expression

Cell culture and treatment are the same as “flow cytometry assay.” After 72 h of culture, the culture medium was discarded. And the radioimmunoprecipitation assay (RIPA) lysis buffer was added after washing with PBS. Then, the cells were placed on the ice for 30 min, were centrifuged at 4°C, 12,000 r/min for 15 min, and the total protein was extracted and quantified. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the protein in the gel was transferred to the nitrocellulose (NC) membrane using a transfer electrophoresis chamber at a constant current of 280 mA for 90 min, and then blocked in 5% skim milk for 2 h, followed by incubation with a MMP1 antibody, MMP3 antibody (1:1000; Proteintech Group, Inc.) or COL-I antibody (1:1000; SinoMab BioScience Limited) at 4°C overnight. After washing three times with TBST, the secondary antibody was added and incubated at 37°C for 1 h. After washing 3 times with TBST (Tris-buffered saline with Tween 20), ECL (chemiluminescent agent) was added. The bands were exposed under a chemiluminescence detection kit (Nanjing KeyGen Biotech Co. Ltd., Jiangsu, China) and analyzed by Image J version 1.8.0 (National Institutes of Health, Bethesda, MD, USA) and normalized to β-actin. The experiment was repeated three times separately.

Statistical analysis

All data were analyzed by SPSS 21.0 analysis software. The data were expressed as mean ± standard deviation (x ± SD). Comparison between the groups was analyzed using one-way analysis of variance (ANOVA) analysis. The P value of <0.05 indicated that the difference was statistically significant.

Results

Effect of MF on proliferation of HSF cells

To choose the suitable concentration of MF in HSF cells, CCK-8 assay was adopted to detect the effect of different concentrations of MF on cell proliferation. As shown in Figure 1(a), the results showed that concentration of MF at 0.01 mmol/L exerted no significant cytotoxic effect after 72 h of treatment. However, concentrations of MF at 0.1 and 1 mmol/L resulted in a marked decrease in cell proliferation. Therefore, we finally chose 0.01 mmol/L as the concentration of MF in this study.

MF can reduce ROS level in HSF cells irradiated by UVA

Flow cytometry was used to determine whether MF can reduce UVA-induced ROS level. Compared with the control group, the ROS content in the UVA group was increased significantly. However, the upregulation of ROS level was significantly attenuated by MF, as shown in Figure 1(b).

Effects of MF on mRNA and proteins expression of MMP1, MMP3, and COL-I in HSF cells irradiated by UVA

To investigate the role of MF on the expression of MMP1, MMP3, and COL-I mRNA in UVA-treated HSF cells, real-time polymerase chain reaction (RT-PCR) method was performed in this study. The MMP1 and MMP3 mRNA levels were significantly elevated, while the COL-I mRNA was significantly decreased in UVA-induced HSF cells compared with the control cells. However, MF could significantly inhibit the improved MMP1 and MMP3 mRNA level and increase the COL-I mRNA level, as shown in Figure 1(c). Moreover, western blot analysis was carried out to further explore the function of MF on the expression of MMP1, MMP3, and COL-I proteins against UVA. Interestingly, MF could significantly reverse the increasing MMP1 and MMP3 protein level and decreasing COL-I protein level induced by UVA, as shown in Figure 1(d) and (e).

Discussion

MF, as a first-line oral drug for T2DM, has a safe and effective hypoglycemic effect. In addition, MF can regulate the mannose transport activity of dermal fibroblasts through AMPK (5′ AMP–activated protein kinase) and inhibit the carcinogenic effect of benzopyrene on mouse skin. Moreover, it also inhibits the carcinoma cell proliferation of squamous cell by inhibiting nuclear factor kappa B (NF-κB) and mammalian target of rapamycin (mTOR) signaling pathways. Long-term chronic repeated exposure to ultraviolet light can cause
skin photoaging, which was manifested as slack, dryness, roughness, and wrinkle increase. The phenomenon of photoaging is related to the decrease of human dermal fibroblast (HDF) and the decline or abnormality of secretory and synthetic functions. Repeated UV A irradiation can accumulate ROS in HDF cells, which exceeds their clearance capacity and breaks the balance between oxidation and anti-oxidation. Consequently, it causes oxidative stress to occur and regulates a series of programmed cellular responses, even regulating aging-related signaling pathways, thereby promoting cellular senescence. The intracellular accumulation of ROS is an important part of the development of skin photoaging. In this study, MF can eliminate intracellular ROS.

Collagen is the main component of skin protein and one of the most important parameters for evaluating the aging state of HDF. COL-I is the most important collagen component in the dermis. In the pathological state, the synthetic decomposition loses the balance and leads to collagen abnormality. MMPs are one of the most important factors involved in the decomposition mechanism. In the process of photoaging, MMPs can degrade COL-I, thereby attributing to the reconstruction of skin connective tissue structure and aggravating skin aging. Ultraviolet radiation will increase the secretion of MMPs in the skin. UVB irradiation can cause the cells to produce a large amount of ROS, which can increase the phosphorylation of the membrane surface of the cell, thereby activating the mitogen-activated protein kinase (MAPK) pathway and activator protein-1 (AP-1) signal pathway, consequently leading to increased secretion of MMP-1, MMP-3, and elevated degradation of collagen. In this experiment, MF could significantly reverse the increasing MMP1 and MMP3 mRNA and protein level and decreasing COL-I mRNA and protein level induced by UV A.

In conclusion, MF can improve the antioxidant capacity of cells and increase the synthesis of COL-I by inhibiting the expression of intracellular ROS and related MMPs, thereby inhibiting the photoaging effects of UVA-induced HDF cells. This study provides a certain experimental basis for the anti-aging effect of MF in delaying the photoaging of skin.
Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) received no financial support for the research, authorship, and/or publication of this article.

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