Research Article

Effect of Saikokeishito, a Kampo medicine, on hydrogen peroxide-induced premature senescence of normal human dermal fibroblasts

Takanobu Takata¹, Yoshiharu Motoo¹,², Naohisa Tomosugi¹,³
¹. Medical Research Institute of Kanazawa Medical University, Ishikawa, Japan
². Department of Medical Oncology, Kanazawa Medical University, Ishikawa, Japan
³. Department of Nephrology, Kanazawa Medical University, Ishikawa, Japan

OBJECTIVE: Saikokeishito (TJ-10) is a Kampo (traditional Japanese herbal) medicine, clinically used for hundreds of years in East Asia. Among its various mechanisms elucidated so far, TJ-10 inhibits the production of transforming growth factor-β1 (TGF-β1) and development of pancreatic fibrosis in vivo. Oxidative damage of normal human dermal fibroblasts (NHDFs) in the corium is a cause of human dermal senescence. Our aim was to determine whether TJ-10 protects NHDFs from premature senescence by hydrogen peroxide (H₂O₂).

METHODS: Premature senescence was induced in NHDFs by 200 μmol/L H₂O₂ for 4 h. Cell viability and the expressions of p53, AMP-activated protein kinase α1 (AMPKα1), AMPKα2, and 14-3-3 protein sigma (14-3-3 σ) were measured in NHDFs treated with TJ-10 for 48 h before exposure to H₂O₂ for 4 h.

RESULTS: Cell viability after treatment with 200 μmol/L H₂O₂ for 4 h was similar (about 80%) to after pre-treatment with TJ-10. Ascorbic acid as a control did not protect NHDFs from damage by 200 μmol/L H₂O₂. Treatment with 200 μmol/L H₂O₂ tended to up-regulate p53 and to down-regulate SIRT1 and AMPKα1, but had no effect on AMPKα2 and 14-3-3 σ expression. Pretreatment with TJ-10 inhibited H₂O₂-induced up-regulation of p53 and enhanced AMPKα1 expression.

CONCLUSION: It is suggested that Saikokeishito has a protective effect on oxidative stress-induced senescence of NHDFs.

KEYWORDS: Saikokeishito; medicine, Kampo; premature senescence; p53; fibroblasts

1 Introduction

Saikokeishito (TJ-10; ref: KCONSORT http://kconsort.umin.jp) is one of the most popular Kampo medicines. TJ-10 has been used for hundreds of years and is mainly against acute infectious diseases. It contains Radix Bupleuri (Saiko), Tuber Pinelieae (Hange), Radix Glycyrihizeae (Kanzo), Radix Ginseng (Ninjin), Radix Scutellaiae (Ogon), Corex Clinnamomi (Keihi), Radix Paenoniae (Shakuyaku), Fructus Zizphi (Taiso), and Rhizoma Zingiberis (Syokyo) and is also used for chronic pancreatitis, covered by health insurance in Japan. Previously, we reported that TJ-10 suppressed the expression of transforming growth factor-β1 (TGF-β1) and other mediators, elucidating its anti-inflammatory, anti-fibrotic, anti-apoptotic, and

http://dx.doi.org/10.1016/S2095-4964(14)60052-2

Received March 27, 2014; accepted July 18, 2014.
Correspondence: Yoshiharu Motoo, MD, PhD, Professor; Tel: +81-76-8284; E-mail: motoo@kanazawa-med.ac.jp
antioxidative properties in pancreatic tissues of chronic pancreatitis model rats (WBN/Kob rats)\cite{3}, TJ-10 delayed the onset of chronic pancreatitis, and also suppressed the expression of pancreatitis-associated protein (PAP)\cite{27}.

Although there are various mechanisms in dermal senescence, the main cause is oxidative stress\cite{21–23}. In vitro, reactive oxygen species induce the expression of senescence-associated proteins (including p53) in cytoplasm. There is no obvious morphological change. This phenomenon is called ‘premature senescence’\cite{6,7}.

In these studies, premature senescence of normal human dermal fibroblasts (NHDFs) was induced by hydrogen peroxide (H$_2$O$_2$). Substances that control the expressions of senescence-associated proteins in NHDFs have been previously assessed\cite{8}. The expressions of senescence-associated proteins are mutually regulated. A decreased expression of AMP-activated protein kinase (AMPK) has been shown to increase the expression and phosphorylation of p53\cite{9}. Moreover, the expression of silent information regulator T1 (SIRT1) is required for activation of AMPK\cite{10}. SIRT1 inhibits TGF-induced renal fibrosis in rats via deacetylation of decapentaplegic homolog 3 (Smad3)\cite{11}. SIRT1 also inhibits TGF-induced apoptosis via degradation of Smad7 in glomerular mesangial cells\cite{12}. The tumor suppressor protein p53 is deacetylated (inactivated) by SIRT1\cite{13,14}. The 14-3-3 protein sigma (14-3-3 $\sigma$) is a well-known downstream target of p53. Activation of p53 leads to the up-regulation of 14-3-3 $\sigma$\cite{15}, while 14-3-3 $\sigma$ stabilizes p53\cite{16}.

TGF-$\beta$1 is associated with fibrosis, senescence and apoptosis\cite{17,18}. Crosstalk between TGF-$\beta$1 and p53 affects apoptosis\cite{19}. Oxidative stress induces changes in the expressions of AMPK, SIRT1, p53, 14-3-3 $\sigma$ and TGF-$\beta$1.

In this study, we investigated the effects of TJ-10 on premature senescence in NHDFs by analyzing changes in the expressions of above mentioned proteins. Ascorbic acid was used as a control.

2 Materials and methods

2.1 Reagents and samples

NHDFs (CC-2511) were obtained from Takara Bio Inc. (Otsu, Japan). The TACS MTT Cell Proliferation Assay Kit (catalog# 4890-25-K; Cell Biolabs Inc., San Diego, CA, USA) was used to measure cell growth. This assay kit contained “Reagent A (catalog: 4890-25-01)” and “Reagent B (catalog: 4890-25-02)”. The FGM-2 Bullet Kit (CC-3132: Takara Bio Inc.) was used for incubation of NHDFs, and Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin was used during the ‘pretreatment period’.

H$_2$O$_2$ solution (30% w/v) was obtained from Waco Pure Chemical Industries, Ltd. (Osaka, Japan). TJ-10 was obtained from Tsumura & Co. (Tokyo, Japan). Primary antibodies including anti-p53 (ab26), anti-14-3-3 $\sigma$ (ab87209), anti-SIRT1 (ab110304), anti-AMPKa1 (ab110036), and anti-AMPKa2 (ab3760) were purchased from Abcam Plc (Cambridge, UK). Secondary antibodies were obtained from Thermo Scientific (Yokohama, Japan), including goat anti-mouse IgG (H+L), min x HnBVs Sr Prot, peroxidase conjugated (product number: 31432) and donkey anti-rabbit IgG H+L, min x v ChGtGuHaHs-HnMsRsSh Sr Prot, peroxidase conjugated (product number: 31458).

2.2 Cell culture

NHDFs were maintained in FGM-2 medium with 2.0% FCS, 0.05% human fibroblast growth factor (hFGF), and 0.05% insulin (FGM-2 Bullet Kit [CC-3132]). For the present experiments, the cell passage number was not more than 5, and the doubling time was within 10 d.

2.3 Saikokeishito and ascorbic acid samples for assay

TJ-10 (10 g) was dissolved in serum free DMEM (35.2 mL) and sterilized\cite{20}. After sterilization, FCS (4.0 mL, final 10%), 200 mmol/L glutamine (400 $\mu$L, final 2.0 mmol/L), penicillin and streptomycin solution (400 $\mu$L, final 100 U/mL and 100 mg/mL) were added to make Pre-TJ-10-DMEM (250 mg/mL of TJ-10). Separately, sterilized DMEM (88 mL) was supplemented with FCS (10 mL, final 10%), 200 mmol/L glutamine (1.0 mL, final 2.0 mmol/L), penicillin, and streptomycin solution (1.0 mL, final 100 U/mL and 100 mg/mL). Each concentration of pre-TJ-10-DMEM (125, 60, 30, 15, 5, 2.5, 1.0, 0.5, and 0.1 mg/mL) were prepared by dilution of pre-TJ-10-DMEM (250 mg/mL of TJ-10) with the sterilized DMEM. Last, all samples of pre-TJ-10-DMEM were centrifuged (7 000 x $\mu$ for 10 min), and the supernatants were named as TJ-10-DMEM\cite{21}.

Ascorbic acid was dissolved in sterilized DMEM to final concentrations of 100 and 200 $\mu$mol/L. Then, the solution was filtered (pore size: 0.22 $\mu$m)\cite{22–24}.

2.4 MTT assay

NHDFs (4.2 x 10$^3$ cells/well) were cultured in 96-well microplates. This cell number was calculated based on the standard number of cells seeded in a 75-cm$^2$ culture flask (1.0 x 10$^5$ NHDFs)$^{[8]}$. The area at the bottom of one well of a 96-well plate is 0.31 cm$^2$. These cells were maintained in FGM-2 medium.

After 72 h, the medium was removed and replaced after washing the cells with phosphate-buffer saline (PBS) three times. Reagent A (10 $\mu$L) was added to the medium and the cells were incubated at 37 $^\circ$C for 4 h; reagent B (100 $\mu$L) was added to the medium and the cells were incubated for 16 h at room temperature (in the dark). The absorbance (ABS) at 570 nm minus the ABS at 650 nm...
(the absorbance of impurities) was calculated and used to determine the cell number from a standard curve. The percent viability of cells exposed to H₂O₂ was measured relative to a control (100%).

### 2.5 H₂O₂ exposure and preparation of cell lysate

NHDFs (2.9 × 10⁵) were cultured in 60-mm dishes (area of the base: 21.5 cm²; medium volume: 5.0 mL; 2.9 × 10⁵ NHDFs, calculated from the number of cells per unit area on the bottom of the dish). After culture of the cells in FGM-2 medium for 72 h, the medium was changed to serum free DMEM, and 14.2 μL of H₂O₂ solution diluted in PBS (final concentration: 0, 100, 150, 200, and 250 μmol/L) was added. After H₂O₂ exposure for 4 h, cells were washed three times in PBS and solubilized in 25 mmol/L-Tris HCl containing EDTA-free protease inhibitor cocktail, 150 mmol/L NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% sodium lauryl sulfate (SLS). The solution was sonicated for 20 min and centrifuged (10 000×g, 10 min) to collect the supernatant (cell lysate). Protein concentration was measured using the bicinchoninic acid (BCA) method.

### 2.6 Analysis of the effects of TJ-10 and ascorbic acid

NHDFs (4.2 × 10⁵ cells/well) were seeded, cultured in 96-well microplates (area of each well, 0.31 cm²; medium volume, 100 μL), and maintained in autocalved DMEM supplemented with 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin for 72 h before MTT assay. For the assay, NHDFs were incubated in sterilized DMEM, washed with PBS (3 times), and incubated with DMEM containing ascorbic acid or TJ-10-DMEM (125, 60, 30, 15, 5, 2.5, 1.0, 0.5, and 0.1 mg/mL) for 48 h. The medium was changed, cells were washed, and the medium was replaced with serum-free DMEM. MTT assay was performed according to the same protocol above (2.4).

Effect of pretreatment with TJ-10 or ascorbic acid: NHDFs (4.2×10⁵ cells/well) were seeded and cultured before the MTT assay (as described above). NHDFs were incubated with autocalved DMEM for 24 h, washed with PBS, pretreated with autocalved DMEM containing ascorbic acid or TJ-10-DMEM (125, 60, 30, 15, 5, 2.5, 1.0, 0.5, and 0.1 mg/mL) for 48 h, and washed with PBS (3 times). Serum-free DMEM was added followed by H₂O₂ (in 2.8 μL of PBS; final concentration, 200 μmol/L). After 4 h of incubation, the medium was changed, and the cells were washed with PBS (3 times), and the MTT assay was performed.

### 2.7 Preparation of cell lysate after pretreatment with TJ-10 or ascorbic acid

NHDFs (2.9 × 10⁵) were seeded, cultured in 60-mm dishes (area of the base: 21.5 cm²; medium volume: 5.0 mL), and maintained in autocalved DMEM supplemented with 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin for 48 h. Then, the medium was removed, and the cells were washed with PBS three times and incubated with various amounts of TJ-10-DMEM or ascorbic acid (200 μmol/L) in autoclaved DMEM for 48 h. Cell lysates were prepared as described above and protein concentrations were measured as described above.

### 2.8 Western blot analysis

Protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard (Protein Assay kit; Bio-Rad, Hercules, CA, USA). Lysates (12.5 μg protein/lysat) were mixed with SDS sample buffer (6.25 mmol/L Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% mercaptoethanol, 0.005% bromophenol blue) and resolved by SDS-PAGE on 10%–20% gradient polyacrylamide gels. Proteins were detected immunologically following semidy transfer (Trans-Blot SD semidy electrod transfer system, Bio-Rad) onto PVDF membranes (Millipore). The membranes were treated with 5% non-fat dry milk in Tris-buffered saline with Tween 100 for 20 min at room temperature to block nonspecific immunoglobulin binding, incubated with primary antibodies including anti-p53 (1:500, mouse monoclonal antibody), anti-14-3-3 σ (1:1 000, rabbit polyclonal antibody), anti-SIRT1 (1:1 000, mouse monoclonal antibody), anti-AMPKα1 (1:1 000, mouse monoclonal antibody), anti-AMPKα2 (1:1 000, rabbit polyclonal antibody), and anti-actin (1:1 000, mouse monoclonal antibody) for 2 h at room temperature, washed three times with 0.5% non-fat dry milk in Tris-buffered saline with Tween 100, incubated with horseradish peroxidase-conjugated secondary antibody to mouse immunoglobulin (diluted 1:1 000) and to rabbit immunoglobulin (diluted 1:5 000) for 2 h at room temperature, and incubated using the HRP-DAB detection kit (Wako) to visualize the bands. Band densities were measured using the LAS-4000 fluorescence imager (Fujifilm, Tokyo, Japan) and expressed in arbitrary units.

### 2.9 Statistical analysis

Stat Flex (ver. 6) software was used for statistical analysis of the cytotoxicity and protein expression. Data are expressed as mean ± standard deviation. The significance of difference in mean cell viability between groups was determined by one-way analysis of variance. Then we used the Dunnett’s t-test for analysis of variance. P < 0.05 was considered to be statistically significant.

### Results

#### 3.1 Premature senescence induced by H₂O₂ and analysis of cell viability

We investigated the effects of H₂O₂ on NHDF viability (used here as an index of premature senescence). H₂O₂ (final concentration: 100, 150, 200, and 250 μmol/L)
was added to the media of NHDFs in 96-well plates (4.2×10³ cells/well) and the cells were incubated for 4 h. Cytotoxicity was detected throughout the concentration range, but viability remained above 80% (Figure 1).

Baseline level, SIRT1 and AMPKα1 expression decreased to 60%–70% of the baseline level, but 14-3-3 σ and AMPKα2 expression remained unchanged (Figure 3).

3.3 Cell viability of NHDFs treated with ascorbic acid and TJ-10

Then, we investigated whether pretreatment with TJ-10-DMEM or ascorbic acid DMEM protected NHDFs from 200 μmol/L H₂O₂ exposure (TJ-10-DMEM was labeled as “TJ-10”, and the sterilized DMEM containing ascorbic acid was labeled “ascorbic acid”). Before testing the anti-senescence effect of TJ-10 and ascorbic acid, the MTT method was used to determine the viability of TJ-10-treated and ascorbic acid-treated NHDFs. The treated NHDFs were incubated 72 h before MTT assay. At 100 and 200 μmol/L, ascorbic acid was not cytotoxic. On the other hand, at more than 2.5 mg/mL, TJ-10 was very cytotoxic. Therefore, viability of NHDFs treated with 0.1, 0.5, and 1.0 mg/mL of TJ-10 was indicated (Figure 4). Viability at 0.1, 0.5, and 1.0 mg/mL of TJ-10 was 90% to 95% without significant difference between concentrations.

3.4 Cell viability of NHDFs treated with ascorbic acid and TJ-10 before H₂O₂ exposure

We investigated the viability of NHDFs pretreated with ascorbic acid (200 μmol/L) and TJ-10 (0.1, 0.5, and 1.0 mg/mL) for 48 h. The medium was removed and the cells were washed with PBS and treated with serum-free medium containing 200 μmol/L H₂O₂ (final concentration). The MTT assay confirmed that ascorbic acid (200 μmol/L) and TJ-10-DMEM (0.1, 0.5, and 1.0 mg/mL) had no toxicity on cell viability, which remained at the control level (Figure 4), and pretreatment with TJ-10 at only 0.1 mg/mL protected NHDFs against H₂O₂ stress (Figure 5).

3.5 Effects of ascorbic acid and TJ-10 on H₂O₂ stress-induced changes in the expressions of senescence-associated proteins

We investigated the effects of TJ-10 and ascorbic acid on premature senescence markers such as p53, SIRT1 and AMPKα1. NHDFs were pretreated with TJ-10 and ascorbic acid for 48 h, incubated with serum-free DMEM containing H₂O₂ (final concentration 200 μmol/L) for 4 h, and lysed as above. The markers were assayed by Western blotting (Figure 6). Though ascorbic acid could not inhibit the expression of p53, TJ-10 (0.5 and 1.0 μg/mL) indicated the inhibition of p53 (Figure 7).

Pretreatment with ascorbic acid and TJ-10 (0.1, 0.5, and 1.0 mg/mL) up-regulated AMPKα1 expression that was inhibited by H₂O₂ exposure (Figure 7). TJ-10 when compared with the control (H₂O₂ exposure alone) was especially effective. It significantly increased AMPKα1 expression of H₂O₂-treated cells by 60%–70%. On the other hand, neither ascorbic acid nor TJ-10 pre-treatment affected SIRT1 expression (Figure 7).
4 Discussion

First, we confirmed that H₂O₂ induces premature senescence of NHDFs. More than 80% of NHDFs survived after exposure to 100–250 μmol/L H₂O₂. In the previous study on premature senescence in keratinocytes, the selected concentration of H₂O₂ was 50 μmol/L⁹⁷. This difference may be due to a difference in the resistance of cell types to H₂O₂ damage.

We cannot confirm that there is a difference in morphology between normal mature cells and senescent cells after treatment with H₂O₂ for 4 h. Although a previous study determined the p53 expression in NHDFs treated with 150 μmol/L H₂O₂ for 4 h⁹⁸ and cell viability of NHDFs treated with 250 μmol/L H₂O₂ for 24 h⁹⁹, the authors did not address changes in morphology therein. An observation of cells after 24 or 48 h of H₂O₂ exposure might reveal a

---

**Figure 3** Quantitative analysis on the effects of H₂O₂ on the expressions of senescence-associated proteins
A: p53; B: SIRT1; C: AMPKα1; D: 14-3-3 σ; E: AMPKα2. Data are presented as mean ± standard deviation, n=3; *P<0.05, **P<0.01, vs control.

**Figure 4** Cell viability of NHDFs treated with ascorbic acid and TJ-10
Cells were incubated for 24 h and the medium was changed to new medium containing ascorbic acid or TJ-10. Then cells were incubated for 48 h. The MTT assay was done in 8 wells. Data are presented as mean ± standard deviation, n=8; **P<0.01, vs control.
Figure 5  Cell viability of NHDFs treated with ascorbic acid and TJ-10 before H$_2$O$_2$ exposure

Cells were incubated for 24 h and the medium was changed to new medium containing ascorbic acid or TJ-10. Then, cells were incubated for 48 h, the medium was changed to serum-free medium, and the cells were exposed to 200 μmol/L H$_2$O$_2$ stress. ‘A’ indicates ascorbic acid. The MTT assay was done in 8 wells. Data are presented as mean ± standard deviation, $n$=8; $^{*}$P<0.05, vs blank control; $^{△}$P<0.05, vs H$_2$O$_2$ control.

Figure 6  Effects of ascorbic acid and TJ-10 on H$_2$O$_2$ stress-induced changes in the expressions of senescence-associated proteins

Figure 7  Effects of ascorbic acid and TJ-10 on H$_2$O$_2$-induced changes in the expression of p53, SIRT1, and AMPKα1

Cells were incubated for 48 h and the medium was changed. Then cells were exposed to 200 μmol/L H$_2$O$_2$ for 4 h. ‘A’ indicates ascorbic acid at 200 μmol/L. The data are presented as mean ± standard deviation, $n$=3; $^{**}$P<0.01, vs blank control; $^{△}$P<0.05, $^{△△}$P<0.01, vs H$_2$O$_2$ control.

In this study, we researched the expression of the proteins that associated with the premature senescence under 100–250 μmol/L H$_2$O$_2$ exposure. The up-regulation or down-regulation of proteins did not indicate the dose dependence of H$_2$O$_2$. The expression of p53 was the maximum at a dose of 200 μmol/L of H$_2$O$_2$ exposure, and morphological difference.
The up-regulation was 38% (Figure 3A). Levels of p53 are elevated in premature senescent cells. It has been reported that p53 induces 14-3-3σ expression and 14-3-3σ activates p53. Therefore, we predicted that up-regulation of p53 would be associated with 14-3-3σ. However, the exposure to H2O2 did not up-regulate 14-3-3σ expression. In H2O2-induced senescence, p53 might be induced by some other pathways. Also, 14-3-3σ induction might need more than 4 h exposure to induce p53.

Crosstalk between p53 and TGF-β1 has been reported. Wild-type p53 and TGF-β1 are key tumor suppressors which regulate an array of cellular responses. TGF-β1 acts in part via the Smad3 signal transduction pathway. Wild-type p53 and Smads interact and coordinately induce transcription of a number of key tumor suppressive genes. So p53 might support the role of TGF-β1.

Treatment with H2O2 (200 μmol/L) decreased the expression of SIRT1 and AMPKα1. The down-regulation of AMPKα1 may induce the suppression of SIRT1. TGF-β1 induces renal fibrosis in rats via deacetylation of Smad3. SIRT1 inhibits TGF-β1-induced apoptosis via degradation of Smad7 in glomerular mesangial cells. Down-regulation of SIRT1 cannot protect NHDFs from TGF-β1-induced fibrosis. Also, SIRT1 binds and deacylates p53. In this study, up-regulation of p53 and down-regulation of SIRT1 indicated that SIRT1 protects p53. As a result, SIRT1 acts as an anti-senescence protein. We thought that one of the reasons for premature senescence was reduction of SIRT1. On the other hand, AMPK is upstream of the SIRT pathway. SIRT1 is required for AMPK activation. The down-regulation of AMPK may induce down-regulation of SIRT1 and up-regulation of p53. AMPK inhibits TGF-β1-induced fibrosis. However, there is no evidence that AMPK directly inhibits TGF-β expression or activation of TGF-β. AMPKα1 and AMPKα2 belong to the AMPK family of kinases. H2O2 stress has different effects on AMPKα1 and AMPKα2. In keratinocytes, AMPKα2 is resistant to 50 μmol/L H2O2 and AMPKα1 is down-regulated. The function of AMPKα2 may be to respond to low oxidative stress. AMPKα1 knockdown with shAMPK RNA induces the up-regulation of p53 and increases SA-β-Gal activity. However, the role of AMPKα2 in anti-senescence has not been investigated.

TGF-β1 is known to induce fibrosis in the pancreas and other organs. TGF-β1 inhibits fibrosis and TGF-β1 expression in the pancreas in vivo. We hypothesized that TJ-10 inhibits premature senescence, and one of its abilities is to inhibit TGF-β1-induced fibrosis.

H2O2 induces expression of TGF-β1 and TGF-β2 in retinal pigment epithelial cells and secretion into conditioned medium where the secreted TGF-β1 and TGF-β2 might induce cellular senescence. Moreover, TGF-β1 is increased in the human diploid fibroblasts and induces cellular senescence. However, in both investigations, the interval between TGF-β1 or TGF-β2 measurement and H2O2 exposure was long. The protein and mRNA expressions of TGF-β1 or TGF-β2 were measured 12, 24, 48, or 72 h from H2O2 exposure. Moreover, other researchers have measured ‘secreted TGF-β1 and TGF-β2’ because of the possibility that the simultaneous attachment of both to the TGF-β receptor on the cell surface is necessary for activity. In this study, TGF-β1 might not induce the premature senescence directly because the time of H2O2 exposure was too short.

If TJ-10 is a candidate of anti-senescence herbal medicine, its safety should be confirmed. Indeed, pretreatment with TJ-10 inhibited up-regulation of p53 and down-regulation of AMPKα1 in NHDFs exposed to H2O2. Direct suppression of TGF-β1 and p53 by AMPKα1 has not been reported, but these three proteins may interact in the cellular senescence pathway. Though the AMPKα1 gene is upstream of the SIRT1 gene, TJ-10 did not alter the effect of H2O2 exposure on AMPKα1 expression. Though AMPKα1 was up-regulated by TJ-10 pretreatment, premature senescence reduced the expression of SIRT1. Because the expression of SIRT1 requires phosphorylation of AMPKα1, the level of phosphorylated AMPKα1 induced by TJ-10 may be low. Other proteins may also be needed for SIRT1 expression.

AMPKα2 was not down-regulated or up-regulated by H2O2 exposure before TJ-10 treatment. So no relationship between AMPKα2 and premature senescence was shown. TJ-10 down-regulated p53 and up-regulated AMPKα1 but had no effect on SIRT1 and AMPKα2. Also, ascorbic acid pretreatment (the positive control for anti-oxidative stress and anti-premature senescence) did not protect against damage by 200 μmol/L H2O2, and NHDF viability decreased. However, pretreatment with 0.1 mg/mL of TJ-10 (but not 0.5 and 1.0 mg/mL of TJ-10) did protect NHDFs against stress induced by exposure to 200 μmol/L H2O2 (Figure 5). Possible stress induced by 0.5 and 1.0 mg/mL of TJ-10 might account for our inability to detect NHDF protection at these higher TJ-10 concentrations under the conditions of the experiment. We used the MTT method to measure cell survival. The MTT method measures mitochondrial enzyme activity. The activation and expression of AMPKα1 depend on the mitochondria. Up-regulation of AMPKα1 might account for TJ-10 treatment-induced protection of cells against premature senescence (H2O2 exposure for 4 h).

These results suggest that Saikokeishito has a protective effect on oxidative stress-induced senescence of NHDFs.

5 Acknowledgements

This work was supported by the Grant for Research Promotion from Kanazawa Medical University (S2011-11).
6 Competing interests

The authors declare that they have no competing interests.

REFERENCES


27 Kim KH, Park GT, Lim YB, Rue SW, Jung JC, Sonn JK, Bae YS, Park JW, Lee YS. Expression of connective tissue...


Submission Guide

Journal of Integrative Medicine (JIM) is an international, peer-reviewed, PubMed-indexed journal, publishing papers on all aspects of integrative medicine, such as acupuncture and traditional Chinese medicine, Ayurvedic medicine, herbal medicine, homeopathy, nutrition, chiropractic, mind-body medicine, Taichi, Qigong, meditation, and any other modalities of complementary and alternative medicine (CAM). Article types include reviews, systematic reviews and meta-analyses, randomized controlled and pragmatic trials, translational and patient-centered effectiveness outcome studies, case series and reports, clinical trial protocols, preclinical and basic science studies, papers on methodology and CAM history or education, editorials, global views, commentaries, short communications, book reviews, conference proceedings, and letters to the editor.

● No submission and page charges
● Quick decision and online first publication

For information on manuscript preparation and submission, please visit JIM website. Send your postal address by e-mail to jcim@163.com, we will send you a complimentary print issue upon receipt.