Summary. The aim of this study was to investigate the effects of resveratrol (Res) on hydrogen peroxide (H$_2$O$_2$)-treated fibroblast-like synoviocytes (FLSs) in vitro. We studied the phosphoinositide 3-kinase (PI3K)-Akt pathway inhibition-mediated effects of Res on forkhead box O (FoxO) mRNA expression levels. FLS viability was determined by Cell Counting Kit-8 (CCK-8) assay, and FLS apoptosis was measured by terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and flow cytometry. FoxO1, FoxO3 and FoxO4 mRNA expression levels in FLSs were determined by RT-PCR, and p-Akt, Akt, p-FoxO1, FoxO1, p-FoxO3, FoxO3, Bcl-2 and Bax protein expression levels were determined by western blotting. Our results showed that low H$_2$O$_2$ concentrations (20 μM) can promote FLS growth and that Res significantly inhibited FLS activity. Moreover, Res significantly increased the number of apoptotic cells and the ratio of Bax/Bcl-2 protein expression in the group treated with Res compared with the group treated with H$_2$O$_2$ and LY294002, a PI3K inhibitor. Res also decreased FoxO1, FoxO3 and FoxO4 mRNA expression levels and p-Akt/Akt, p-FoxO1/FoxO1, p-FoxO3/FoxO3 protein expression levels. Taken together, these findings indicate that Res can induce apoptosis in H$_2$O$_2$-treated FLSs in part by inhibiting the PI3K-Akt signaling pathway.

Key words: Fibroblast-like synoviocytes, Oxidative stress, PI3K-Akt pathways, Resveratrol, FoxO gene

Introduction

A chronic systemic autoimmune inflammatory disease, rheumatoid arthritis (RA) develops as a result of the influence of many factors, such as heredity and environmental exposures. The main pathological changes of RA include synovial lining thickening, inflammatory cell infiltration, pannus formation, and cartilage and bone tissue destruction -which eventually leads to joint deformity and loss of function- as well as abnormal cell proliferation, differentiation and apoptosis (Marrelli et al., 2011; Bugatti et al., 2012). The current evidence indicates that fibroblast-like synoviocytes (FLSs), which constitute the synovial lining in the rheumatoid synovium and are one of the principal cells involved in joint pannus formation, play key roles in cartilage and bone destruction in RA (Bartok and Firestein, 2010). FLSs have been shown to lack contact inhibition, proliferate continuously, and produce cytokines and oncogenic proteins continuously (Lafyatis

Abbreviations. Res, resveratrol; H$_2$O$_2$, hydrogen peroxide; FLSs, fibroblast-like synoviocytes; PI3K, phosphoinositide 3-kinase; FoxO, forkhead box O; TUNEL, terminal deoxyribo nucleotidyl transferase-mediated dUTP nick-end labeling; RA, rheumatoid arthritis; ROS, reactive oxygen species; PKB, protein kinase B; NF-κB, nuclear factor kappa B; COX-2, cyclooxygenase 2.
et al., 1989). These findings indicate that FLSs are a distinct well-differentiated cell type dependent on the local articular environment (Ritchlin et al., 1994). Therefore, FLS may display specific phenotypic characteristics and become activated during the process of RA development, thereby having profound effects on cellular interactions, matrix destruction, and tissue remodeling.

Previous studies have shown that oxidative stress inducers, such as reactive oxygen species (ROS), serve as inflammatory mediators in the inflamed joints of RA patients. Inflammation is induced by activated multinucleated granulocytes and macrophages that have infiltrated arthritic joints (Bae et al., 2009). ROS include hydroxyl radicals (OH·), superoxide anions (O2·-), and hydrogen peroxide (H2O2). The forkhead box O (FoxO) family, which comprises FoxO1, FoxO3 and FoxO4, belongs to the forkhead box class of transcription factors. These proteins are downstream effectors of the phosphoinositide 3-kinase (PI3K)-Akt pathway and play general roles in the stress response. Specifically, FoxO proteins play an important role in maintaining intracellular redox balance and biological survival under different environmental stresses (Wang et al., 2007; Nakamura and Sakamoto, 2008). FoxO proteins can be activated by manganese superoxide dismutase (MnSOD) and catalase, which increases cell antioxidant ability and prevents peroxidase-induced mitochondrial oxidative damage. Thus, by protecting cells from oxidative stress-induced damage, FoxO has anti-apoptotic effects (Arden, 2006; Jagani et al., 2008). Similarly, FoxO proteins have been shown to play critical roles in RA synovial tissue proliferation and survival (Ludikhuite et al., 2007). PI3K-dependent protein kinase B (PKB) activation has been observed in RA synovial tissue, and mechanisms interfering with this process have been shown to have protective effects in animal models of arthritis (Kok et al., 2013).

Resveratrol (Res) is a natural phenolic compound found in various fruits. Res is particularly abundant in the skin of red grapes. Mechanistic studies have demonstrated that Res possesses anti-inflammatory, antioxidant, anti-cancer and anti-aging properties (Udenigwe et al., 2008). It has been reported that Res can inhibit autoimmune diseases, such as RA (Chen et al., 2013). However, the mechanisms underlying the effects of Res are not fully understood. We propose that Res may modulate the functions of key cellular receptors, inflammatory genes and signaling-related transcription factors, including nuclear factor kappa B (NF-κB) and cyclooxygenase 2 (COX-2), which play critical roles in the pathogenesis of RA (Chen et al., 2014). In this study, we used an in vitro model of H2O2-induced oxidative stress to observe the effects of Res on FLS growth. Our preliminary results indicate that Res inhibited FoxO gene expression in oxidative stress mediator-treated FLSs in vitro by inhibiting the PI3K-Akt pathway.

**Materials and methods**

**Reagents**

Resveratrol (purity, 99%), H2O2, LY294002 and collagenase I were obtained from Sigma (St. Louis, MO, USA), and Res was dissolved in dimethylsulfoxide (DMSO). High-glucose Dulbecco’s modified Eagle medium (DMEM) was obtained from HyClone Laboratories, Inc. (Logan, UT, USA). Fetal calf serum (FCS) was obtained from Gibco BRL (Carlsbad, CA, USA). An annexin V-FITC apoptosis detection kit containing propidium iodide (PI) was obtained from Solarbio Biosciences (Beijing, China). Rabbit polyclonal anti-vimentin (Abcam, Cambridge, MA, USA), rabbit polyclonal anti-phospho-Akt (Thr208), anti-phospho-FoxO1 (Ser253), and anti-phospho-FoxO3 (Ser253), as well as antibodies to Akt, FOXO1, FOXO3, Bcl-2, Bax, and GAPDH, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies were purchased from Sigma (St. Louis, MO, USA). An In Situ Cell Death Detection Kit was purchased from Roche Applied Science (Mannheim, Germany). TRizol reagent was obtained from Gibco BRL, and a Revert Aid™ First-strand cDNA Synthesis Kit was purchased from Fermentas Life Sciences (Vilnius, Lithuania). A real-time qPCR Master Mix Kit was purchased from Shine Gene Molecular Biotech Co. Ltd. (Shanghai City, China). Sodium dodecyl sulfate (SDS) and polyacrylamide gel electrophoresis (PAGE) reagents were obtained from Bio-Rad Company (Hercules, CA). A stock solution of DMSO was prepared, and the final concentration of the DMSO solution, which contained diluted phosphate-buffered saline (PBS), was 0.05% (w/v). All other reagents were of analytical purity.

**Fibroblast-like synoviocyte culture**

Synovial biopsy samples were obtained from the knee joints of patients with clinically active RA disease who were undergoing needle arthroscopy. The RA patients fulfilled the revised criteria of the American Rheumatology Association for the diagnosis of RA (Takayanagi et al., 1997). The study was approved by the Medical Ethics Committee of the Academic Committee of Anhui Medical University. FLSs were isolated as described previously (Itano et al., 2002) and incubated using the adherence method. Briefly, synovial tissue samples were cut into small pieces and digested with collagenase I for 2 hours at 37°C, after which the non-adherent cells were removed. After centrifugation, the cells were washed extensively and cultured in complete high-glucose DMEM containing 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 ml of glutamine in a humidified atmosphere of 5% CO2. The cells were trypsinized and passaged when they
reached confluence. FLSs from the third to eighth passages were used for the experiments in our study. The cells were stained with anti-vimentin (Abcam, Cambridge, MA, USA). Approximately 99% were anti-vimentin-positive and considered FLSs. The FLSs were maintained at 37°C in complete medium (DMEM supplemented with 10% FCS) in a humidified atmosphere of 5% CO₂. After approximately 1 week, the size of the cell population had increased to the extent that the cells gradually coalesced. The cells were subsequently stimulated with TNF-α (10 ng/ml) for 24 hours.

Effects of different concentrations of H₂O₂ or Res on FLS viability

FLS viability was determined using Cell Counting Kit-8 (CCK-8, Kumamoto, Japan) assay. Briefly, FLSs were seeded in 96-well microtiter plates (approximately 1×10⁴ cells/well) and cultured in DMEM with 10% FCS for 24 hours prior to stimulation. The next day, the cells were treated with different concentrations of H₂O₂ (0 μM, 5 μM, 10 μM, 50 μM, 100 μM, 200 μM, and 500 μM) for 24 hours or different concentrations of Res for the same time period (0 μM, 20 μM, 50 μM, 100 μM, 200 μM, 300 μM, 400 μM, and 500 μM). After the FLSs had been washed with PBS three times for ten minutes each, 100 μl of fresh medium and 10 μl of CCK-8 were added to each well, after which the cells were incubated for 1 hour at 37°C. Cell viability in the group treated with different concentrations of H₂O₂, as determined by CCK-8 assay, was expressed as a percentage of the absorbance (A) of the vehicle control-treated group. The absorbance in the group of cells treated with the above Res concentrations was measured on Microplate Reader Model 550 (Bio-Rad; Hercules, CA) at 492 nm. The experiment was performed in triplicate (Wang et al., 2016).

Terminal deoxyribo nucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis for apoptosis

Synovial fibroblasts were maintained in complete medium (DMEM supplemented with 10% FCS) at 37°C in a humidified 5% CO₂ atmosphere. The FLSs were washed and harvested with 0.01% trypsin at 37°C. Then, the cells were seeded in six-well plates containing cover slips at a density of 2×10⁵ cells/well and incubated for 24 hours, after which they were divided into the following 5 groups for subsequent experiments: a control group, in which the FLSs were cultured in DMEM with 10% FBS and incubated at 37°C with 5% CO₂ for 24 hours; an H₂O₂ group, which the FLSs were treated with 20 μM H₂O₂ in DMEM with 10% FBS for 24 hours; an H₂O₂ + Res group, in which the FLSs were pre-incubated with 20 μM H₂O₂ for 24 hours before being incubated with 300 μM Res in complete medium for 24 hours; an H₂O₂ + LY294002 group, in which the FLSs were pre-incubated with 20 μM H₂O₂ for 24 hours before being incubated with the PI3K-Akt inhibitor LY294002 (20 μM) in complete medium for 6 hours; and an H₂O₂ + LY294002 + Res group, in which the FLSs were pre-incubated with 20 μM H₂O₂ in complete medium for 24 hours before being exposed to LY294002 for 6 hours and then incubated with 300 μM Res in complete medium for 24 hours. Apoptotic cell numbers were detected with an in situ cell death detection kit, according to the manufacturer’s instructions. Briefly, the cells were fixed with 4% paraformaldehyde for 1 hour at room temperature before being permeabilized with 0.1% Triton X-100. After a TUNEL reaction mixture was added to the cells, they were incubated in a humidified atmosphere for 1 hour at 37°C in the dark. All the samples were analyzed using fluorescence microscopy. The cell populations were analyzed at ×200 magnification. Approximately 500 to 800 cells per slide were examined, and the proportions of cells displaying nuclear staining were determined.

Annexin V-FITC/PI detection for apoptosis

FLS apoptosis was detected by flow cytometry. The cells were trypsinized and collected for apoptosis detection, which was performed with an Annexin V-FITC Apoptosis Detection Kit, according to the manufacturer’s protocol. Briefly, FLSs (approximately 1×10⁵ ~ 2×10⁵ cells/well) were cultured in six-well plates in DMEM with 10% FCS and divided into a normal control group, an H₂O₂ group, an H₂O₂ + Res group, an H₂O₂ + LY294002 group, and an H₂O₂ + LY294002 + Res group and treated with the above agents at the indicated dosages and for the indicated times. The FLSs were subsequently washed with PBS and harvested with 0.01% trypsin at 37°C, resuspended in 400 μl of Annexin V binding buffer at a density of 1×10⁶ cells/ml and incubated with 5 μl of Annexin V-FITC and 10 μl of PI for 15 min at 4°C in the dark. Finally, the cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences).

RNA isolation and cDNA synthesis

The FLSs were collected after receiving the above treatments. Total RNA was isolated, and its quality and purity was determined using an ND-1000 UV/Vis spectrophotometer (NanoDrop®, Wilmington, DE, USA). PCR was performed using 1 μl of cDNA from each sample. The total RNA (0.5 μg) was pretreated with RNase-free DNase I (TaKaRa, Otsu, Japan) before being reverse-transcribed into cDNA with an M-MLV Reverse Transcriptase Kit and oligo (dT) primers, according to the manufacturer’s instructions. The abovementioned cDNAs were diluted 20-fold for qRT-PCR analysis. The FoXO1, FoXO3 and FoXO4 primer sequences have been reported previously (Mei et al., 2012). See Table 1 for additional details.

Western blotting analysis

The FLSs were collected after receiving the
abovementioned treatments. Protein expression levels were quantified using the Bradford method (Bio-Rad, Hercules, CA). The four groups of harvested RA-FLS cells were lysed with radio immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors on ice for 10 min and then centrifuged. After the protein concentrations were determined using a BCA protein assay kit, the cell lysates were loaded onto a 10% acrylamide gel at a concentration of 30 µg/lane and separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred onto polyvinylidenedifluoride (PVDF) membranes, which were blocked for 1 hour at room temperature in Tris buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl)/0.05% Tween 20 (TBST) containing 2% milk protein. The membranes were subsequently incubated with the indicated primary antibodies overnight at 4°C (1:1000-diluted rabbit polyclonal anti-Akt, anti-FoXO1, and anti-FoXO3; 1:1500-diluted rabbit polyclonal anti-phospho-Akt (Thr308), anti-phospho-FoXO1 (Ser256), and anti-phospho-FoXO3 (Ser253); 1:2000-diluted rabbit polyclonal anti-Bcl-2 and anti-Bax; and 1:3000-diluted rabbit polyclonal anti-GAPDH) in TBST. The blots were then washed extensively before being incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1:3000) for 1 hour at room temperature, washed again, and developed using an Enhanced Chemiluminescence (ECL) Detection System (GE Life Sciences, Piscataway, NJ, USA). Akt, p-Akt, Bcl-2 and Bax levels were quantified using densitometric analysis software (Lab Image, Hercules, CA, USA) and were normalized to GAPDH levels in the same sample. To quantify the chemiluminescent signals, we scanned the PVDF membranes with a Fuji Film LAS-1000 system and analyzed them using Multi Gauge software (version 3.0). The results were expressed as p-Akt/Akt, p-FoXO1/FoXO1, p-FoXO3/FoXO3, Bcl-2/GAPDH and Bax/GAPDH ratios in arbitrary units.

Statistical analysis

The data are expressed as the mean±standard deviation. Between-group comparisons were performed using one way analysis of variance (ANOVA) followed by Tukey’s post hoc test. All analysis were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered statistically significant.

Results

Morphology of the cultured synovial cells

FLSs are long, thin and fibroblast-like, as shown in Fig. 1A. We conducted morphologic observations of FLSs from the knee joints of RA patients and subjected the cells to immunohistochemical staining against vimentin. The FLSs stained dark purple (Fig. 1B).

Table 1. Sequences of the oligonucleotide primers used for RT-PCR.

| Gene   | Sequence (5’ 3’) Size (bp)      |
|--------|---------------------------------|-------------------|
| FoXO1  | GCTCGGCAGGATGGAAGA                 | 155               |
|        | GGCTGAGGCTGGCATTTC                  |                   |
| FoXO3  | TCTGGGCACCGGAGGAAA                 | 208               |
|        | TGTGCGGATGGGAGTCTT                  |                   |
| FoXO4  | TGGCCGCTCTGTGCTGAT                  | 434               |
|        | TGCGGTGCAATGAAAT                    |                   |
| β-actin| CTCCATCTGGGCTCTCGT                   | 268               |
|        | GCTGTCACCTTCCAGGTTCC                |                   |

Fig. 1. In vitro culture of FLSs collected from RA patients. A. Three passages of FLSs from RA patients. B. Vimentin expression in cultured FLSs from RA patients. Scale bars: A, 100 µm; B, 50 µm.
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Fig. 2. A. Effects of different H$_2$O$_2$ concentrations on FLS viability. B. Effects of different Res concentrations on synovial fibroblast viability. Data are presented as the mean±SD (n=6). **P<0.01, ***P<0.001 versus control, P<0.05, P<0.01 versus DMSO group.

Fig. 3. Res increases apoptosis in FLSs treated with H$_2$O$_2$, as determined by TUNEL assay. A. Representative images of TUNEL staining, which was analyzed using microscopy (200×magnification). B. Analysis of TUNEL-positive cell numbers and signal densities. a: P<0.01 versus control, b: P<0.05 versus control, c: P<0.05 versus LY294002 + H$_2$O$_2$ group.
Res inhibited FLS viability

We investigated the effects of different H$_2$O$_2$ concentrations on FLS viability in this study. FLS viability increased after the cells were incubated with a low concentration of H$_2$O$_2$ (≤20 μM) for 24 hours but decreased in concentration-dependent manner after the cells were incubated with a high concentration of H$_2$O$_2$ (>20 μM) for the same time period (Fig. 2A). We also investigated the effects of different Res concentrations on FLS viability. FLS viability gradually decreased in concentration-dependent manner after the cells were incubated with Res at the following concentrations for 24 hours: 0 (control), 20, 50, 100, and 200 μM (Fig. 2B).

TUNEL assay of the effects of Res on FLS apoptosis

No TUNEL-positive cells were noted in the normal-control FLS or H$_2$O$_2$-treated FLS groups (P>0.05), while increased numbers of TUNEL-positive FLSs were noted in the H$_2$O$_2$ (20 μM) + Res (300 μM) group (P<0.01). TUNEL-positive cell nuclei stained significantly brown in the cells of the H$_2$O$_2$ (20 μM) + Res (300 μM) group, findings indicative of an increased signal density and an increased number of apoptotic cells (Fig. 3A). The number of TUNEL-positive FLSs was decreased in the H$_2$O$_2$ (20 μM) + LY294002 (20 μM) + Res (300 μM) group compared with the H$_2$O$_2$ (20 μM) + Res (300 μM) group (P<0.01) (Fig. 3B).

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**Fig. 4.** Res increases apoptosis in FLSs treated with H$_2$O$_2$, as determined using flow cytometry. A. The bottom right quadrant represents Annexin V-FITC-stained cells (early phase apoptotic cells), and the top right quadrant represents PI- and FITC-dual-stained cells (late-phase apoptotic or necrotic cells). B, C. The apoptosis rates are represented as the percentage of Annexin V-FITC single-positive or Annexin V-PI double-positive cells. Data are presented as the mean±SD (n=4). a: P<0.01 versus control, b: P<0.05 versus control, c: P<0.05 versus LY294002+H$_2$O$_2$ group.
Flow cytometric analysis of the effects of Res on FLS apoptosis

To confirm that Res induced apoptosis in FLSs, we treated the cells with Res and determined the apoptosis rate of each group of FLSs using an Annexin V-FITC Apoptosis Detection Kit. Double-staining for Annexin V and PI revealed the presence of necrotic cells or cells in late apoptosis, while single-staining for Annexin V revealed the presence of cells in early apoptosis. The results of the analysis showed that the FLS apoptosis rate was low in cells treated only with low concentrations of H$_2$O$_2$. However, the FLS apoptosis rate increased after the addition of Res to the cells. The FLS apoptosis rate was significantly decreased in the H$_2$O$_2$ + Res + LY294002 (the PI3-Akt pathway inhibitor) group compared with the H$_2$O$_2$ + Res group (Fig. 4A). The numbers of cells in early and late apoptosis, as well as the number of cells undergoing necrosis, were significantly increased in the H$_2$O$_2$ (20 μM) + Res (300 μM) group (P<0.01) and decreased in the H$_2$O$_2$ (20 μM) + LY294002 (20 μM) + Res (300 μM) group (P<0.01) compared with the control group (Fig. 4B,C).

Effect of Res on FoxO gene expression levels in FLS treated with low concentrations of H$_2$O$_2$

The results of the RT-PCR analysis show that FoxO gene expression levels were not different between FLSs treated with low concentrations of H$_2$O$_2$ and FLS treated with Res (P>0.05). However, FoxO1, FoxO3, and FoxO4 gene expression levels were decreased in FLSs pretreated with Res compared with FLSs treated with H$_2$O$_2$ alone (P<0.05), while FoxO1, FoxO3 and FoxO4 gene expression levels were increased in FLSs treated with Res + LY294002 + H$_2$O$_2$ compared with cells treated with H$_2$O$_2$ alone (P<0.05) (Fig. 5A,B). Taken together, these results suggest that Res decreased FoxO1, FoxO3 and FoxO4 gene expression levels.

Res decreased p-Akt, p-FoxO1, FoxO1, p-FoxO3, FoxO3 expression levels in FLSs treated with H$_2$O$_2$

p-FoxO1, FoxO1, p-FoxO3, and FoxO3 expression levels were not different between FLSs treated with H$_2$O$_2$ and FLSs treated with the vehicle control (P>0.05). p-FoxO1, FoxO1, p-FoxO3, FoxO3, p-FoxO1/FoxO1 and p-FoxO3/FoxO3 expression levels were decreased in FLSs pretreated with LY294002 or Res (P<0.05). However, p-FoxO1, FoxO1, p-FoxO3, FoxO3, p-FoxO1/FoxO1 and p-FoxO3/FoxO3 expression levels were increased in the Res + LY294002 + H$_2$O$_2$ group compared with the LY294002 + H$_2$O$_2$ group (P<0.05) (Fig. 6A-C). p-Akt/Akt and Bax/Bcl-2 expression levels were not different between the H$_2$O$_2$- and control-treated group (P>0.05). The ratio of p-Akt/Akt expression was decreased after the cells were treated with LY294002 or Res. In contrast, the ratio of Bax/Bcl-2 expression was increased after the cells were treated with the above agents (P<0.05). However, the ratio of Bax/Bcl-2 expression was decreased, and the ratio of p-Akt/Akt expression was increased in the Res +

![Fig. 5. Res decreased FoxO gene expression levels. FoxO1, FoxO3, and FoxO4 levels in each group of FLSs were determined by RT-PCR. A. Changes in the mRNA expression levels of the above proteins in each group, as demonstrated by electrophoresis. B. Changes in the optical density of the mRNA expression levels of the above proteins in each group relative to the expression levels of β-actin in each group. Data are presented as the mean±SD (n=4). a: P<0.01 versus control, b: P<0.05 versus control, c: P<0.05 versus LY294002+H$_2$O$_2$ group.](image-url)
Discussion

Although the pathogenesis of RA remains unknown, researchers have determined that synovial membrane inflammation is a key feature of the disease. Synovitis occurs secondary to cartilage degradation and is characterized by the invasion and activation of granulocytes and macrophages, which release large amounts of pro-inflammatory mediators into the joint cavity and increase the vascularity of the synovial membrane (Connor et al., 2012). FLSs can be isolated from the synovial tissues of RA patients and cultured for prolonged periods of time. These cells can proliferate in an anchorage-independent manner, lack contact inhibition and express several oncogenes characteristic of cells that have escaped normal growth regulation mechanisms. Furthermore, RA FLSs cultured long term (but not normal FLSs or osteoarthritis FLSs) invade the cartilage matrix after being co-implanted with cartilage explants into severe combined immunodeficiency (SCID) mice (Müller-Ladner et al., 1996). FLSs were stimulated with TNF-α (10 ng/ml) for 24 hours in the experiments to simulate the internal environment characteristic of RA in vivo. After 3~8 passages, proliferating FLSs had become the predominant cell type, resulting in a relatively homogenous population. These cells, which have a doubling time of 5-7 days, can be maintained for several months in vitro. We also found that FLSs display many of the characteristics of fibroblasts, including vimentin expression.

ROS are a key mediator of many processes in mammalian cells and are associated with many diseases, such as diabetes (Datta et al., 2014), as well as processes such as aging and inflammation (including RA). In RA patients, large numbers of polymorphonuclear leukocytes and macrophages infiltrate into the joint space. The activation of phagocytic cells is accompanied by the generation of ROS, including O₂•−, OH•, hydroperoxyl (HO₂•), H₂O₂ and other species. ROS liberation stimulates phagocytes in the synovial cavity to generate immune complexes, such as inflammatory agents or pyrophosphate crystals, which are considered responsible for RA (Mirshafiey and...
Resveratrol decreases FoXO protein expression in synoviocytes

Resveratrol (Res) is a natural polyphenolic compound possessing anti-inflammatory and antioxidant properties. However, only very limited data regarding the effects of Res on RA have been obtained, and the mechanisms underlying the effects of Res on RA remain largely unknown. In this study, we found that Res can significantly inhibit FLS growth in a concentration-dependent manner, indicating that Res can be used for the treatment of RA patients displaying excessive synovial cell growth. Res can inhibit FLS growth and promote FLS apoptosis under oxidative stress. Previous reports have shown that these cells can produce low concentrations of antioxidants, such as superoxide, under oxidative stress conditions. These antioxidants play a normal messenger role in signal transduction pathways (Shaykhalishahi et al., 2009; Kim et al., 2010). The PI3K-Akt signaling pathway plays an important role in the cell cycle, apoptosis, and development and is one of the most important anti-apoptotic pathways enabling cells to resist stress-induced apoptosis (Stambolic and Woodgett, 2006). In this study, we found that Res could inhibit FLS growth in a concentration-dependent manner. When Res was administered at a concentration of 300 μM, FLS viability was approximately 50% of that of cells treated with H₂O₂. Previous studies have reported that Res inhibits skin tumor genesis by interacting with PI3K and Akt, proteins involved in cancer development and progression (Roy et al., 2009). We found that 300 μM Res can induce apoptosis in 20 μM H₂O₂-treated FLSs; however, these effects can be partially inhibited by the PI3K-Akt signaling pathway inhibitor LY294002. These findings suggest that Res may be able to inhibit H₂O₂-stimulated FLS growth and induce apoptosis at least in part by down-regulating Akt signaling.

Resveratrol decreases FoXO protein expression in synoviocytes

The mammalian FoXO family consists of several gene expression regulatory and cellular processes, such as cell proliferation, cell survival and cell oxidation defenses (Paik, 2006). In this study, FLSs were stimulated with TNF-α (10 ng/ml) for 24 hours and then exposed to a low concentration of H₂O₂, FoXO1, FoXO3 and FoXO4 mRNA expression levels were not significantly increased in these cells, results consistent with the findings of the indicated study (Li et al., 2012). We also found that Res can inhibit Akt phosphorylation, and decrease the mRNA expression levels of FoXO family members; however, these changes can be partially blocked by LY294002, indicating that the PI3K-Akt-FoXO signaling pathway may play an important protective role in Res-induced apoptosis in FLSs treated with low concentrations of H₂O₂. However, a previous paper showed that FoXO1 downregulation is required for the promotion of synovial cell survival (Grabiec et al., 2015). In addition, another article reported that FoXO1 protein expression levels were also markedly increased in the fasted state, a change associated with a decrease in phospho-Akt expression (Wondisford et al., 2014). These results are not consistent with ours. The discrepancy is likely a result of the effects of a molecular protein or receptor induced by Res.

Imbalances in the expression levels of the members of the Bcl-2 family can result in cell apoptosis. Previous studies have shown that Bcl-2 is an anti-apoptotic protein on the mitochondrial membrane and that Bcl-2/Bax are downstream proteins of the PI3K-Akt signaling pathway, which plays a role in regulating apoptosis (Gross et al., 1999). Some studies have shown that the PI3K-Akt signaling pathway participates in antioxidant processes to protect cells from the damage and that Res can increase Bcl-2 protein expression and protect synovial cells from the effects of oxidative stress through the PI3K-Akt signaling pathway (Chogle et al., 1996). In contrast, in this experiment, we found that Res decreased the expression of the anti-apoptotic protein Bcl-2 and increased the expression of the pro-apoptotic protein Bax in H₂O₂-treated RA FLS; however, these effects could be partially blocked by LY294002.

Conclusions

In summary, Res increase the apoptosis of FLS which were obtained from RA patients and inhibited FoXO1, FoXO3 and FoXO4 mRNA levels and proteins expression by decreasing Akt phosphorylation, increasing the Bax/Bcl-2 expression under low concentration of H₂O₂. Therefore, we think that Res is a promising agent, which can inhibit abnormal proliferation of synovial cells.
Resveratrol decreases FoxO protein expression in synoviocytes

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Author Contributions. Bin Xu and Gaoyuan Wang performed the data acquisition. Zhicheng Wang and Wei Cao analysed the data. Junqiang Zhang and Gaoyuan Wang performed the cell culture and relevant cell treatment. Xiaoyu Chen edited the paper. All authors reviewed the manuscript.

Ethics statement. The experiments were conducted in strict accordance with the Guide of the National Institutes of Health for the Care and Use of Laboratory Animals and were approved by the Laboratory Animal Committee of Anhui Medical University, China.

Competing interests. The authors declare that no competing interests exist.

References


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