Original Research

Thymoquinone-induced reactive oxygen species causes apoptosis of chondrocytes via PI3K/Akt and p38kinase pathway

Seon-Mi Yu and Song-Ja Kim

Department of Biological Science, College of Natural Sciences, Kongju National University, Gongju, Chungnam 182, Republic of Korea
Corresponding author: Song-Ja Kim, Department of Biological Science, Kongju National University, Shinkwan-Dong, Gongju, Chungnam 182, Republic of Korea.
Email: ksj85@kongju.ac.kr

Abstract

Thymoquinone (TQ), a bioactive ingredient of the volatile oil of black seed (*Nigella sativa*), has been shown to possess anti-neoplastic and anti-inflammatory effects on a variety of tumours. However, the precise mechanism of action is not clear in normal cells such as primary chondrocytes. So, we have investigated the effects of TQ on the apoptosis of chondrocytes with a focus on reactive oxygen species (ROS) production. In *in vitro* experiments, chondrocytes were cultured with increasing concentrations of TQ for 24 h or with 20 μmol/L TQ for the indicated time periods, and various experiments were performed to detect the apoptotic effects caused by TQ. The results showed that TQ significantly increases apoptosis. Apoptosis was dose- and time-dependently expressed, and the generation of ROS also dramatically increased in a dose-dependent manner. Pretreatment of *N*-acetyl-L-cysteine (NAC), an inhibitor of ROS, inhibited both TQ-induced apoptosis and ROS generation. Also, TQ up-regulated phosphorylation of phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinases ([MAPKs] p38kinase, ERK-1/-2, and JNKinase), and these effects were prevented by pretreatment of NAC. However, pretreatment with inhibitors of PI3K/Akt and MAPKs did not inhibit TQ-caused ROS generation. Among the inhibitors of PI3K/Akt, p38kinase, ERK-1/-2, and JNKinase, pretreatment with LY294002 and SB203580 abolished TQ-induced apoptosis, but PD98059 and SP600125 did not have any effect on TQ-caused apoptosis. These findings suggest that TQ-induced ROS generation regulates apoptosis by modulating PI3K/Akt and p38kinase pathways.

Keywords: Thymoquinone (TQ), Reactive oxygen species (ROS), Chondrocytes

***Experimental Biology and Medicine* 2013; 238: 811–820. DOI: 10.1177/1535370213492685**

Introduction

Osteoarthritis (OA) is a painful bone and joint disease that develops as a result of insufficient and aberrant repair of damaged synovial joint tissue.[1] It is characterized by a loss of cartilage, alteration of subchondral bone, and low-grade synovitis, leading to a gradual loss of articular cartilage.[2] Although symptoms involved in OA are well understood, the trigger onset for cartilage matrix destruction and changes remain largely unknown. Chondrocytes in OA cartilage is accompanied by morphological changes that are characteristic features of apoptosis.[3] During the OA progression, to maintain the chondrocytes viability and their ability to resist apoptosis play an important role.[4]

Apoptosis of chondrocytes has been reported to be involved with the strictness of OA and is correlated with the progression of the disease.[3] It plays a critical role in development, maintenance of homeostasis, and a wide range of diseases.[5,6] Although apoptosis of chondrocytes is crucial event in the pathogenesis of cartilage degradation, the detailed apoptosis pathways in pathology of arthritis have not yet clearly elucidated.[7,8] To understanding the causes of arthritis, we need studies of signalling pathway related with apoptosis modulated by a number of stimuli.

Apoptosis is characterized by a number of well-defined features, including the presence of apoptotic bodies, chromatin condensation, nuclear fragmentation, and the activation of caspases.[9] Also, p53 and $p21^{WAF1}$ have been shown to function as an apoptosis-promoting protein, and the mechanisms by which p53 and $p21^{WAF1}$ promote apoptosis may be associated with their interaction with the DNA repair machinery.[10]

Reactive oxygen species (ROS) including oxygen ions, free radicals and peroxides usually have highly reactive properties and are produced as by-products of cellular metabolism. During physiological homeostasis, oxidative balance is maintained by the removal of ROS through

diverse antioxidants to match the production of ROS from a variety of stimuli.[11] When cellular generation of ROS exceeds its antioxidant facility, it damages lipids, protein, and DNA.[6,12] ROS also promotes oxidative stress and chondrocytes apoptosis by altering mitochondrial function.[13] ROS has been hypothesized to contribute to the development of OA. Overproduction of ROS can lead to activation of the receptor for advanced glycation end products, which modulate chondrocytes and synovial responses in OA.[14]

Thymoquinone (TQ), a drug derived from *Nigella sativa*, has a variety of biological effects that include antimicrobial, antiviral, anti-inflammatory, and immunomodulatory activities. TQ belongs to a family of quinones, which are known to regulate as redox couples within the mitochondrial respiratory chain.[15]

Many findings indicate that the activation of receptor tyrosine kinases, which are transmembrane receptors with an intrinsic ability to phosphorylate tyrosine residues in their cytoplasmic domains, such as phosphatidylinositol (PI) 3-kinase/Akt (PI3K/Akt), results in the activation of nuclear transcription factors that regulate apoptosis.[16,17] Mitogen-activated protein kinases (MAPKs) are also involved in relaying extracellular stimulations to intracellular responses and coordinately regulate cell proliferation, differentiation, motility, and survival.[18,19] It has been shown that a number of events including PI3K/Akt and MAPKs signalling pathways could play a role in mediating apoptosis.[20,21]

However, no reports exist on the effect of TQ on the apoptosis of chondrocytes. In this study, we have investigated the effect of TQ on apoptosis with a focus on the generation of ROS and its related mechanistic pathways to identify an association between ROS and joint diseases, such as OA. Here, we demonstrated that TQ strongly produces ROS and this oxidative stress causes apoptosis through the activation of PI3K/Akt and p38kinase signalling in rabbit articular chondrocytes.

These studies evidenced that PI3K/Akt and p38kinase signalling play a critical role in TQ-induced apoptosis. Also, these findings can be provided a scientific data basis for understanding the process of cartilage damage.

Materials and methods

Culture and treatment of rabbit articular chondrocytes

Articular chondrocytes were isolated from cartilage slices of two-week-old New Zealand White rabbits by enzymatic digestion, as previously described.[22] Briefly, cartilage slices were enzymatically dissociated in 0.2% collagenase type II (381 units/mg solid, Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Burlington, ON, Canada). Individual cells were suspended in DMEM supplemented with 10% (v/v) bovine calf serum (Invitrogen), 50 μg/mL streptomycin, and 50 units/mL penicillin. The cells were seeded on culture dishes at a density of 5×10^4 cells/cm^2. The medium was replaced every two days, and cells reached confluence after approximately five days. For these experiments, 3.5-day cell cultures were treated with TQ (Sigma-Aldrich). To explore the signalling pathway in TQ-induced apoptosis, 10 μmol/L p38kinase inhibitor SB203580 (SB; Tocris Bioscience, Ellisville, MO, USA), 10 μmol/L MEK-1/-2 inhibitor PD98059 (Tocris Bioscience), 10 μmol/L PI3kinase inhibitor LY294002 (San Diego, CA, USA), 10 μmol/L JNK inhibitor SP600125 (Tocris Bioscience), 5 mmol/L ROS inhibitor *N*-acetyl-L-cysteine (NAC; Sigma-Aldrich), and 200 μmol/L mitochondrial anion channel inhibitor (4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid [DIDS], Sigma-Aldrich) were used. Chondrocytes were pre-incubated with each inhibitor for 1 h, followed by co-incubation with TQ. The study was approved by the ethics committee of Kongju National University, Gongju, Republic of Korea.

Western blot analysis

After the indicated TQ treatment, the medium was removed, and the cells were rinsed with phosphate-buffered saline (PBS) twice. After the addition of cold radio immuno precipitation buffer (10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulphate [SDS], and 1% Triton X-100), supplemented with protease inhibitors (10 g/mL leupeptin, 10 g/mL pepstatin A, 10 g/mL aprotinin, and 1 mmol/L of 4-[2-aminoethyl] benzenesulphonyl fluoride) and phosphatase inhibitors (1 mmol/L NaF and 1 mmol/L Na_3VO_4). Cells were scraped at 4°C. Cell lysate was then subjected to a centrifugation of at 13,000 × *g* for 10 min at 4°C. Resultant protein samples were separated by sodium dodecyl sulphate SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose (NC) membrane (Millipore, Billerica, MA, USA). The membrane was stained by ponceau S solution to confirm the uniform transfer of all samples and was then incubated in blocking solution (Tris-buffered saline [TBS] with 0.05% Tween 20 and 5% nonfat dry-milk) for 1 h at room temperature. The antibodies used in this study, pro-caspase-3, p53, p21, and actin, were obtained from Santa Cruz Biotechnology Inc (CA, USA), and phospho-p38, phopho-ERK-1/-2, phospho-JNK, and phospho-Akt were purchased from Cell Signaling (Beverly, MA, USA). The membrane was reacted first with the desired primary antibodies for 24 h at 4°C. The membrane was then incubated with appropriate horseradish peroxidase conjugated secondary antibody (Sigma-Aldrich) for 2 h, washed with TBST, and developed using the ECL kit.

Measurement of ROS

ROS production was determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich). The acetoxymethyl group on DCFH-DA is cleaved by non-specific esterases within the cell, resulting in a nonfluorescent-charged molecule that did not cross the cell membrane. Intracellular ROS irreversibly oxidized DCFH-DA to dichlorofluorescein (DCF), which is a fluorescent product. After the cells were treated with various concentrations of TQ for 30 min to 2 h, the cells were washed with PBS twice and then loaded for 30 min with DCFH-DA (10 μmol/L) in DMEM without phenol red. After the media were removed,

the chondrocytes were centrifuged to collect cells, and the fluorescence was assessed using an Flx 8000 fluorometer (excitation 485 nm/emission 525 nm; Bio-tek Instruments Inc., Winooski, VT, USA) and flow cytometry (Partec, Munster, Germany, excitation at 495 nm and emission at 529 nm). For visualization of ROS by fluorescent microscopy, cells were labelled and placed in darkness for 30 min at 37°C with a DCFH-DA (10 μmol/L) probe. The chondrocytes were washed with PBS twice. Fluorescence was then observed using an inverted Olympus BX50 microscope (Tokyo, Japan).

Cell cycle analysis

The cells were serum starved for 24 h to synchronize them in the G_0 phase of cell cycle. Synchronous populations of cells were subsequently treated with TQ for 24 h. The cells were washed twice with cold PBS and then centrifuged. The pellet was fixed in 70% (vol/vol) ethanol for 1 h at 4°C. The cells were washed once with PBS and resuspended in cold PI solution (50 μg/mL) containing RNase A (0.1 mg/mL) in PBS (pH 7.4) for 30 min in the dark. Flow cytometry analyses were performed using flow cytometer. Forward light scatter characteristics were used to exclude the cell debris from the analysis. The sub-G_1 population was calculated to estimate the apoptotic cell population.

Determination of proliferation

Cell proliferation was monitored using Cell Proliferation Reagent Kit I (MTT) (Roche Applied Science, Indianapolis, IN, USA). Chondrocytes (2×10^4 cells/well) were allowed to grow in 96-well plates and treated with reagents. After the exposure period, media were removed. Thereafter, the medium was changed and incubated with MTT (0.1 mg/mL) for 4 h. The viable cell number per well was directly proportional to the production of formazan, which was solubilized in dimethyl sulphoxide, and measured spectrophotometrically at 595 nm. All experiments were performed in triplicate.

Determination of apoptosis

Apoptotic death of chondrocytes was determined by examining DNA fragmentation using standard procedures. Briefly, cell pellets were resuspended in 750 μL of ice-cold lysis buffer (100 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 200 mmol/L NaCl, and 0.2% SDS) for 45 min with occasional shaking. DNA was extracted with phenol and precipitated with alcohol. The pellet was dried and resuspended in 100 μL of 20 mmol/L Tris-HCl, pH 8.0. After digesting RNA with RNase (0.1 mg/mL) at 37°C for 1 h, the samples (5 μg) were electrophoresed through a 1.5% agarose gel in 450 nmol/L Tris-acetate-EDTA buffer, pH 8.0. DNA was photographed under ultraviolet light.

4,6-Diamidino-2-phenylindole staining

Cells were plated onto 12 mm^2 coverslips in dishes and cultured with complete medium. After treating with 20 μmol/L TQ in the absence or presence of 5 mmol/L NAC, the cells were fixed with 3.5% paraformaldehyde for 15 min at room temperature and were then washed with PBS. The membrane-permeable fluorescent dye 4,6-diamidino-2-phenylindole (DAPI), 2 μg/mL, which binds to the chromatin of cells, was added to the fixed cells, and the cells were examined by an Olympus BX50 microscope. Apoptotic cells were identified by condensation and fragmentation of nuclei. For each experiment, nuclei from 10 random fields of each coverslip were examined at × 1000 magnification.

Data analysis and statistics

The results are expressed as mean values with standard deviation. The values were calculated from the specified number of determinations. An analysis of variance test was used to compare individual treatments with their respective control values. Significance was defined at the $P < 0.01$ level.

Results

TQ induces intracellular ROS generation

To test the hypothesis that TQ is an inducer of oxidant stress, we first examined TQ-treated cells for the accumulation of ROS. Chondrocytes were treated with various concentrations of TQ (5–20 μmol/L) for 2 h and the level of intracellular ROS was monitored using a DCF-DA probe, which emits a green fluorescence on oxidation (Figure 1). TQ caused the accumulation of ROS, resulting in a substantial increase in the number of fluorescent cells as detected by fluorescence microscopy (Figure 1a, left panel). Quantification of the fluorescent signal by fluorescence microscopy showed a 1.37-fold increase in the ROS content of TQ-treated cells relative to that observed in control cells (Figure 1a, right panel). A fluorometer and flow cytometry analysis also indicated that the concentration of intracellular ROS increased transiently in a dose-dependent manner in TQ-treated chondrocytes (Figure 1b and c). These observations indicated that TQ induces the generation of ROS in rabbit articular chondrocytes (Figure 1).

TQ inhibits proliferation and causes apoptosis

We investigated whether TQ could regulate the proliferation and apoptosis of articular chondrocytes. When cells were treated with various concentrations of TQ for 24 h, the cell viability was significantly reduced (Figure 2a). To assess the cellular apoptosis induced by TQ, cells were treated with TQ at different concentrations for 24 h, then stained with DAPI dye, and examined by fluorescence microscopy (Figure 2b). The staining of cell nuclei with DAPI revealed characteristics typical of apoptosis, including chromatin condensation and nuclear fragmentation (Figure 2c). Consistent with the morphological changes seen by DAPI staining, a ladder pattern of fragmentation was distinctly seen for TQ-treated cells. The fragmentation pattern of a DNA ladder also significantly increased at 20 μmol/L TQ, while cells did not show distinct fragmented DNA at concentrations of less than 20 μmol/L (Figure 2c). Flow cytometry was performed to analyse the cell cycle perturbation and apoptosis (Figure 2d). As expected, TQ-induced cell

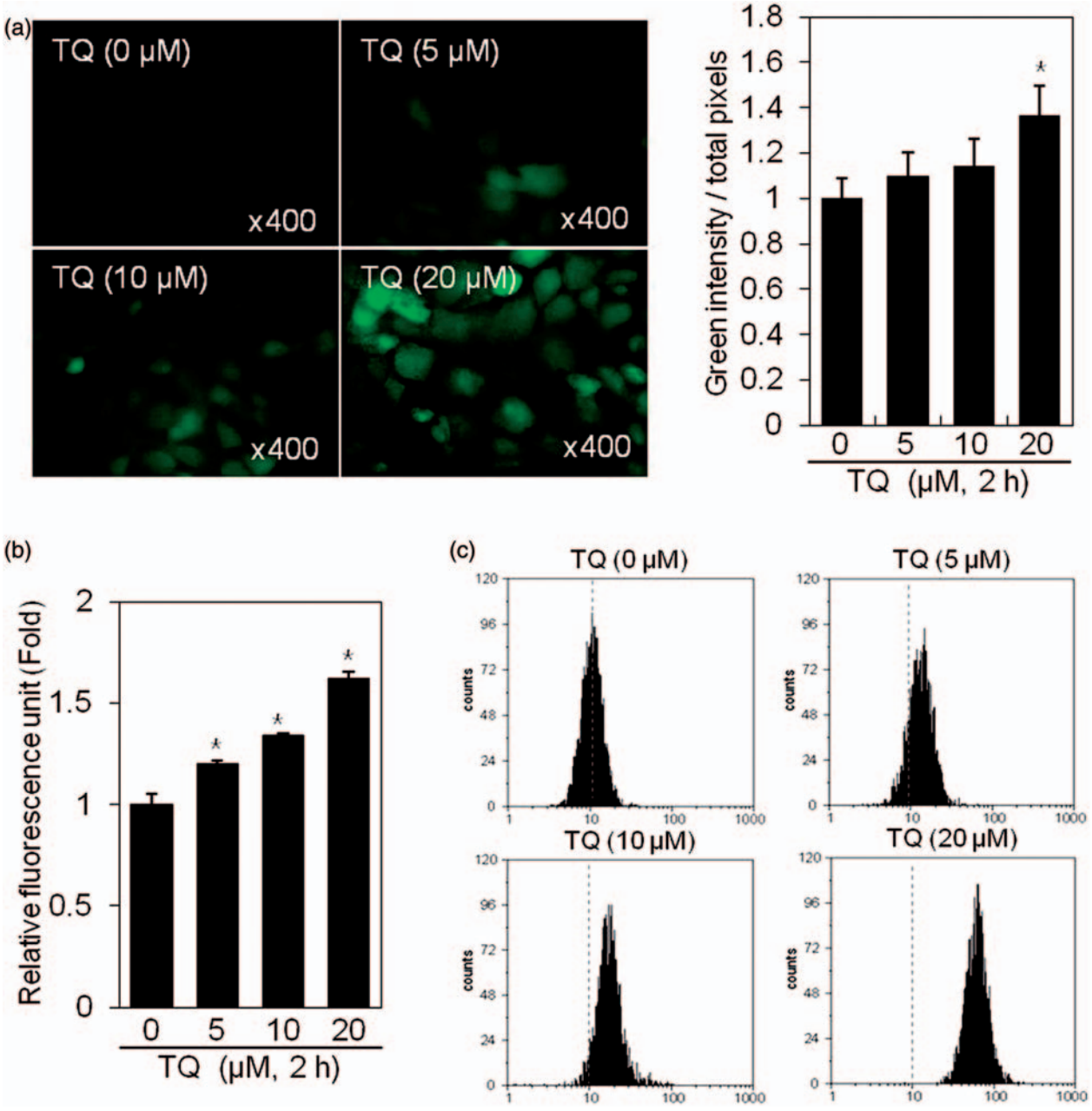

Figure 1 Effect of TQ on intracellular ROS generation in primary rabbit articular chondrocytes. (a) Chondrocytes were treated with various concentrations of TQ for 2 h. DCF fluorescence intensity was observed by converted fluorescence microscopy (left panel). DCF fluorescence intensity was measured by Image J (Vector Lab., USA; right panel). (b) Cells were treated with the indicated concentrations of TQ for 2 h. ROS fluorescence was measured by using an Flx 8000 Bio-Tek fluorometer (Biotek Instruments Inc., Winooski, VT, USA). (c) Articular chondrocytes were treated with an increasing dose of TQ for 1 h, and then ROS accumulation (shift to right) was determined by flow cytometry analysis. Data were given as mean ± SD with three independent experiments performed in triplicate. Compared with the control group: $^*P < 0.01$. TQ: thymoquinone; ROS: reactive oxygen species; DCF: dichlorofluorescein. (A color version of this figure is available in the online journal)

cycle arrest in sub-G1 phase and level of apoptotic fractions increased up to 34.5% (Figure 2d). ROS has been shown to cause apoptosis in chondrocytes through several important signal transmission steps, including accumulation of p53 and activation of caspase-3. We examined the changes in apoptosis-related proteins, p53, p21, and procaspase-3. We observed the accumulation of p53 and we observed that expression of p21 increased, whereas expression of procaspase-3 decreased in a dose- and time-dependent manner (Figure 2e). These results suggested that TQ inhibits proliferation and causes apoptosis via p53 and caspase-3 dependent pathways (Figure 2).

TQ-induced apoptosis is inhibited by NAC, an ROS scavenger

Numerous chemical treatments capable of causing apoptosis can also induce ROS production, suggesting a close relationship between ROS production and apoptosis.[23]

Therefore, the alteration of apoptosis was examined to determine the relationship between the accumulation of

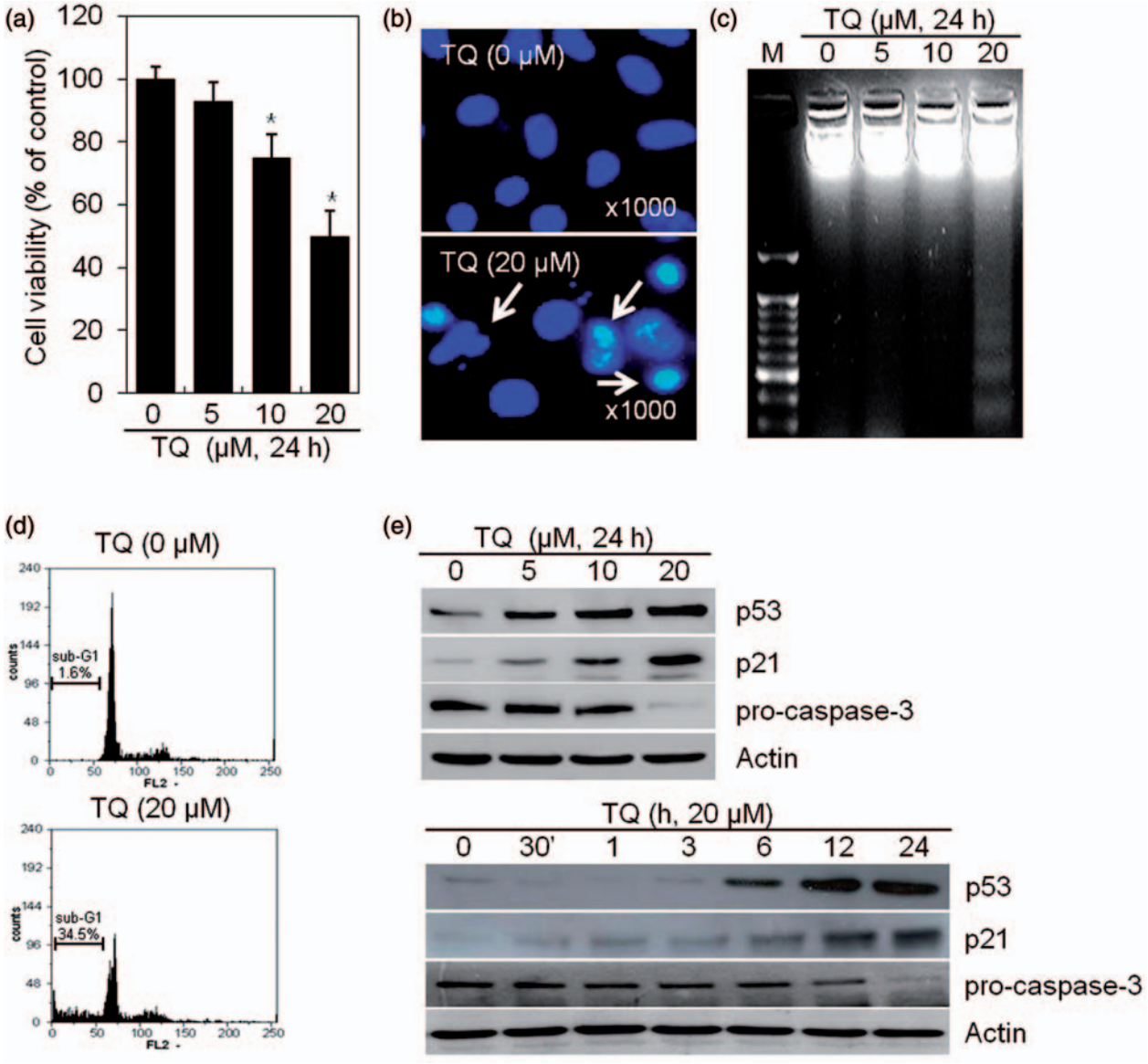

Figure 2 Effect of TQ on cell viability in rabbit articular chondrocytes. (a) Chondrocytes were treated with various concentrations of TQ for 24 h. Cell viability was assessed by MTT assay. (b) Cells were treated with 20 μmol/L TQ for 24 h. Apoptotic cells were determined by DAPI staining and fluorescence microscopy. Arrow point is the apoptotic body in apoptotic cells. (c) Primary cells were treated with various concentrations of TQ for 24 h. DNA Fragmentation was determined by DNA gel electrophoresis. Line M, 100 bp size markers. (d) Articular chondrocytes were treated with 20 μmol/L TQ for 24 h. Apoptosis of cells were quantified with the propidium iodide, using a flow cytometer. (e) Chondrocytes were treated with various concentrations of TQ for 24 h or 20 μmol/L TQ for the indicated time periods. Expression of p53, p21, procaspase-3, and actin was detected by Western blot analysis. Actin was used as a loading control. Data were given as mean ± SD with three independent experiments performed in triplicate. Compared with the control group: $^*P < 0.01$. TQ: thymoquinone; DAPI: 4,6-diamidino-2-phenylindole. (A color version of this figure is available in the online journal)

ROS and the induction of apoptosis (Figure 3). When cells were pretreated for 1 h with 5 mmol/L NAC, a ROS scavenger, and subsequently treated with TQ, cell viability was evaluated, indicating that the antioxidant NAC interfered with the TQ-caused apoptosis of chondrocytes (Figure 3). As expected, compared with the control cells, NAC did not affect the ROS level or the apoptotic rate (data not shown), but it dramatically reduced TQ-induced ROS accumulation in chondrocytes as determined by fluorescence microscopy and flow cytometry analysis (Figure 3a). Compared with the TQ-treated cells, the mean DCF fluorescence intensity of chondrocytes treated with NAC and TQ was markedly decreased to the level of control cells, as determined by fluorometry (Figure 3b). DAPI staining and flow cytometry analysis were performed to examine whether NAC improved cell survival rate by inhibiting apoptosis (Figure 3c). Treatment with NAC increased cell survival ratio by ~95% (Figure 3c, upper panel). The proportion of fragmented cells was dramatically lower in TQ-treated cells with NAC than in cells treated with TQ alone. The survival ratio was also significantly increased, from 50% to 95% (Figure 3d). To investigate the mechanisms underlying the anti-apoptotic effects of NAC, we analysed p53, p21, and procaspas-3 in NAC-treated and untreated chondrocytes. Consistent with the changes in the apoptotic rate and the ROS level, NAC considerably reduced the expression of p53

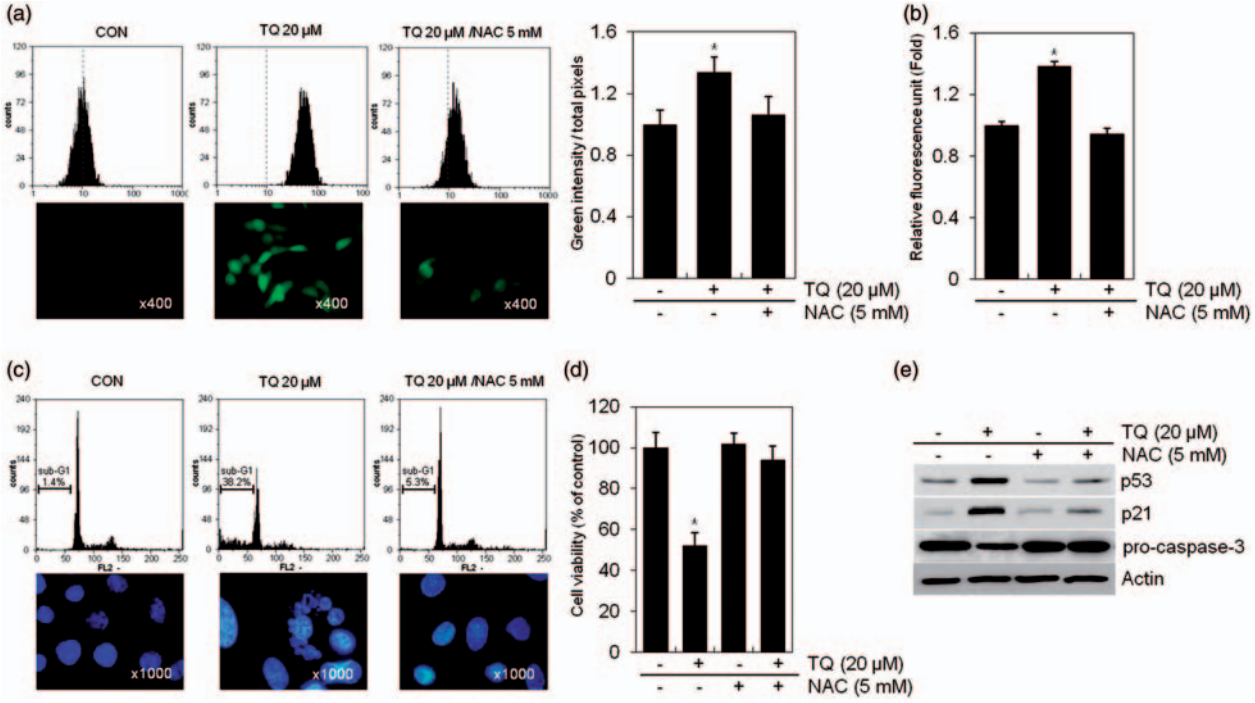

Figure 3 Effect of antioxidant, NAC, on TQ-caused ROS generation and apoptosis in chondrocytes. (a) Chondrocytes were treated with 20 μmol/L TQ in the absence or presence of 5 mmol/L NAC for 1–2 h. ROS accumulation (shift to right) was determined by flow cytometry analysis (left upper panel). DCF fluorescence intensity was observed by converted fluorescence microscopy (magnification, × 400, left lower panel). DCF fluorescence intensity was measured by Image J (Vector Lab., USA; right panel). (b) Cells were treated with 20 μmol/L TQ in the absence or presence of 5 mmol/L NAC for 2 h. ROS fluorescence was measured by using an Flx 8000 Bio-Tek fluorometer (Bio-tek Instruments Inc., Winooski, VT). (c–e) Chondrocytes were treated with 20 μmol/L TQ in the absence or presence of 5 mmol/L NAC for 24 h. (c) Nuclear damage in chondrocytes was assessed using nuclear stain DAPI (magnification, × 1000, lower panel). Apoptosis of cells were quantified with the propidium iodide, using flow cytometer (upper panel). (d) Cell proliferation was assessed by MTT assay. (e) Expression of p53, p21, procaspase-3, and actin was measured by Western blot analysis. Actin was used as a loading control. Data were given as mean ± SD with three independent experiments performed in triplicate. Compared with the control group: $^*P < 0.01$. NAC: *N*-acetyl-L-cysteine; TQ: thymoquinone; ROS: reactive oxygen species; DCF: dichlorofluorescein; DAPI: 4,6-diamidino-2-phenylindole. (A color version of this figure is available in the online journal)

and p21, and the cells with activated caspase-3 were treated with TQ (Figure 3e). These findings indicated that NAC inhibited TQ-induced apoptotic changes via suppression of apoptosis (Figure 3).

TQ activates PI3K/Akt and MAPKs

To determine whether ROS production could be responsible for the activation of PI3K/Akt and MAPKs by TQ, chondrocytes were treated with TQ in the absence or presence of NAC. Western blot analysis was used to detect the expression of PI3K/Akt and MAPKs, p38kinase, ERK-1/-2, and JNKinase, in cells treated with TQ. TQ was found to increase PI3K/Akt and MAPKs, p38kinase, ERK-1/-2, and JNKinase in a dose-dependent manner (Figure 4a). Compared with the control cells, treatment with NAC alone did not affect the expression of observed proteins (data not shown), whereas NAC significantly blocked TQ-activated PI3K/Akt and MAPKs (Figure 4c). These data indicate that TQ increases PI3K/Akt and MAPKs, p38kinase, ERK-1/-2, and JNKinase.

TQ-caused apoptosis was abolished by inhibitor of PI3K/Akt and p38kinase, SP600125 and SB203580

The PI3K/Akt and MAPKs, stress kinases activated under conditions of extreme oxidative stress, were examined in cells treated with TQ in the absence or presence of PI3K/Akt inhibitor, LY294002, and MAPKs inhibitors, SB203580, PD98059, and SP600125, for 24 h. To confirm the activation of PI3K/Akt and MAPKs on apoptosis caused by the production of ROS, levels of ROS and cell viability were monitored in chondrocytes that were pretreated with inhibitors for 1 h and then with TQ for 24 h, followed by determination of cell viability and ROS assay (Figure 5a and b). We clearly observed that pretreatment of inhibitors, SB203580 and LY294002, significantly inhibited TQ-induced apoptosis, whereas treatment of PD98059 and SP600125 did not show any significant changes in TQ-caused apoptosis compared with TQ-treated cells (Figure 5b). However, inhibitors in cells treated with TQ had no dramatic effects on ROS production (Figure 5a). Because inhibition of PI3K/Akt and p38kinase with LY294002 or SB203580 had effects on cell viability, it was therefore examined whether apoptosis by TQ-induced ROS production was regulated by PI3K/Akt and p38kinase pathway or not (Figure 5c). Chondrocytes were treated with TQ in the absence or presence of LY294002 or SB203580 and then the levels of apoptosis-related proteins, p53, p32, and pro-caspase-3, were analysed by Western blot analysis (Figure 5c). These data showed that apoptosis caused by TQ-induced generation of ROS was modulated by PI3K/Akt and p38kinase pathways (Figure 5).

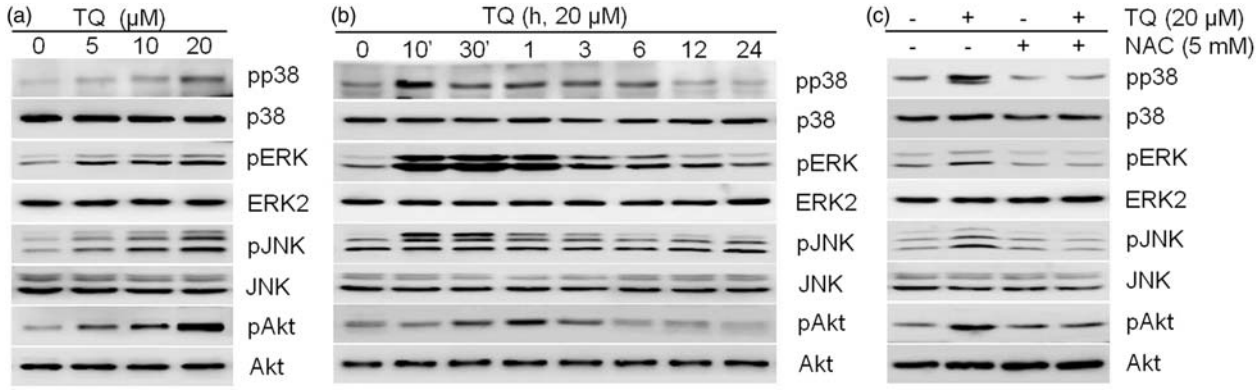

Figure 4 Effect of TQ on activation of PI3K/Akt and MAPKs in chondrocytes. (A) Rabbit articular chondrocytes were treated with various concentrations of TQ for specific time period. Expression of pp38, p38, pERK, ERK-2, pJNK, JNK, pAkt, and Akt was measured by Western blot analysis. (B) Chondrocytes were treated with 20 μmol/L TQ for indicated time periods. Expression of pp38, p38, pERK, ERK-2, pJNK, JNK, pAkt, and Akt was determined by Western blot analysis. (C) Cells were treated with 20 μmol/L TQ in the absence or presence of 5 mmol/L NAC for specific time period. Expression of pp38, p38, pERK, ERK-2, pJNK, JNK, pAkt, and Akt was determined by Western blot analysis. Data were given as mean ± SD with three independent experiments performed in triplicate. TQ: thymoquinone; PI3K/Akt: phosphatidylinositol 3-kinase/Akt; MAPKs: mitogen-activated protein kinases

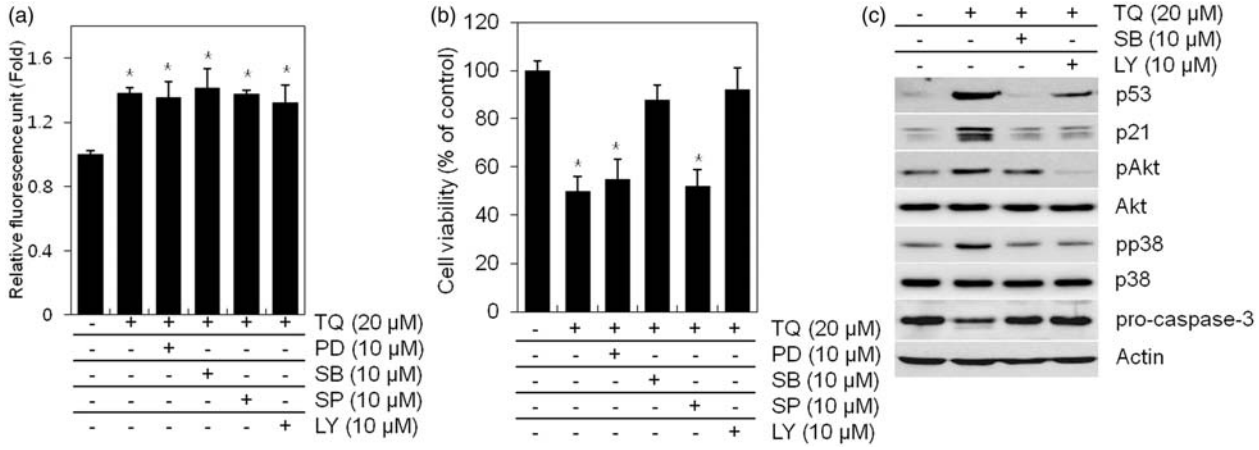

Figure 5 The relationship of PI3K/Akt and MAPKs on apoptosis by TQ-induced ROS in rabbit articular chondrocytes. (A) Chondrocytes were treated with 20 μmol/L TQ in the absence or presence of various inhibitors, PD98059, SB203580, SP600125, and LY294002, for 2 h. ROS fluorescence was measured by using an Flx 8000 Bio-Tek fluorometer (Bio-tek Insturments Inc., Winooski, VT). (B) Cell proliferation was assessed by MTT assay. (C) Rabbit chondrocytes were treated with 20 μmol/L TQ in the absence or presence of inhibitors, SB203580 or LY294002, for specific time periods. Expression of p53, p21, pAkt, Akt, pp38, p38, procaspase-3, and Actin was determined by Western blot analysis. Actin was used as a loading control. Data were given as mean ± SD with three independent experiments performed in triplicate. Compared with the control group: $^*P < 0.01$. PI3K/Akt: phosphatidylinositol 3-kinase/Akt; MAPKs: mitogen-activated protein kinases; TQ: thymoquinone; ROS: reactive oxygen species

TQ-induced apoptosis by ROS generation was blocked by DIDS, a mitochondrial anion channel inhibitor

Mitochondria are an important source of ROS within most mammalian cells.[24,25] Mitochondria-generated ROS is transported to the cytoplasm by voltage-dependent anion channels (VDAC)[26] and it is inhibited by DIDS, a mitochondrial anion channel inhibitor. DIDS protects cells by blocking the egress of ROS from the mitochondria.[27] To determine whether DIDS prevents TQ-induced apoptosis, chondrocytes were treated with DIDS for 1 h, and then cell viability was analysed by MTT assay (Figure 6a). DIDS completely inhibited TQ-induced apoptosis (Figure 6a). As shown in Figure 6(b), Western blot analysis also indicated that TQ-caused expression of p53 and p21 was reduced by DIDS (Figure 6b). These results collectively demonstrate that transport inhibition of ROS into the cytoplasm by DIDS recovered apoptosis by TQ-induced ROS production (Figure 6).

Taken together, our results indicated that TQ-caused ROS induces apoptosis through PI3K/Akt and p38kinase pathways in rabbit articular chondrocytes.

Discussion

ROS plays a critical role in the modulation of cellular responses, such as proliferation, differentiation, and immune regulatory responses.[28–31] ROS is produced via various stimuli and a variety of cellular events. A high level of generation of ROS results in oxidative stress, which contributes to abnormal events including organ

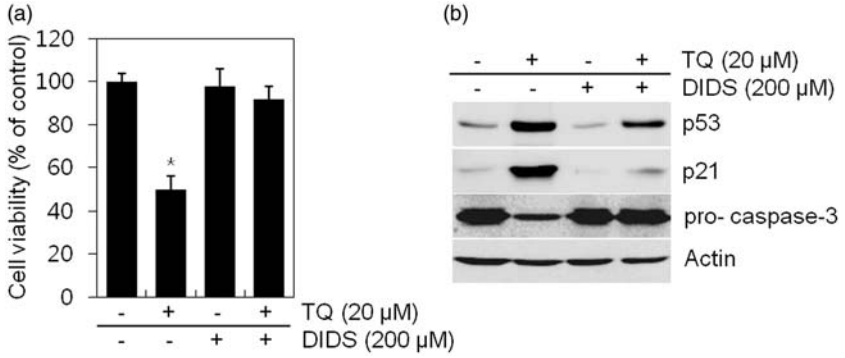

Figure 6 Effect of DIDS on TQ-induced apoptosis in chondrocytes. (A) Rabbit articular chondrocytes were treated with 20 μmol/L TQ in the absence or presence of 200 μmol/L DIDS for 24 h. Cell proliferation was assessed by MTT assay. (B) Expression of p53, p21, procaspase-3, and actin was detected by Western blot analysis. Actin was used as a loading control. Data were given as mean ± SD with three independent experiments performed in triplicate. Compared with the control group: *$P < 0.01$. DIDS: cyano-2,2′-stilbenedisulphonic acid, Si; TQ: thymoquinone

dysfunction and apoptosis of cells.[32,33] A lot of recent evidence demonstrates that the accumulation of ROS is related to the apoptotic response caused by several chemotherapy reagents.

Apoptosis has two major pathways: the extrinsic, also known as the death receptor pathway, and intrinsic, the so-called mitochondrial pathway, regulating apoptosis and cross-talk between pathways. In the death receptor pathway, the death receptors such as Fas or tumour necrosis factor-α receptors are activated by specific death ligands. The mitochondrial pathway is activated by various stimuli including ROS, DNA damage, calcium, and proapoptotic Bcl-2 family members. These stimuli cause an increase of permeabilization of the outer mitochondrial membrane, as determined by altering mitochondrial membrane potential, and activate caspase-9 and caspase-3.[6,34]

The intrinsic pathway has been described as an important downstream signal of ROS in apoptosis. The excessive generation of ROS can cause apoptotic cell death by accelerating mitochondrial permeability transitioning pore opening, releasing pro-apoptotic factors, and activating caspases, such as caspase-3 and caspase-9.[35,36] It has been suggested that ROS-triggered apoptosis involves the intrinsic pathway of apoptosis which involves the activation of caspase-3.[37]

TQ has been shown to inhibit proliferation and to induce apoptosis in various cancer cells, but the mechanism of TQ-induced apoptosis in rabbit articular chondrocytes has not been well defined.[38–40] Our study, in concert with previous publications, showed that treatment with TQ induced proliferation inhibition in the articular chondrocytes (Figure 2). Our data also showed that treatment with TQ resulted in significant up-regulation of ROS expression in chondrocytes (Figure 1). All of these findings of TQ imply that TQ-induced ROS may regulate apoptosis.

A number of previous publications suggest that preincubation with NAC is necessary to abrogate the cytotoxic effects of quinines because NAC can form adducts with quinines and reduce their toxicity.[41,42] These may be modulatory mechanisms for the potent effects of NAC in suppressing the apoptotic effects of TQ.

To confirm the role of ROS generation in TQ-induced apoptosis, we also used NAC to scavenge the ROS (Figure 3). Pretreatment of chondrocytes with NAC not only abrogated the induction of ROS by TQ, but it also significantly protected these cells against TQ-induced apoptosis (Figure 3). These findings clearly corroborated our hypothesis that TQ induces apoptosis in chondrocytes via the generation of ROS.

The elevated ROS levels have been shown to inhibit proliferation and to induce apoptosis in malignant cell types by activating both caspase and stress kinase pathways, including PI3K/Akt and MAPKs pathways.[43–46] Activation of caspase is a very common mechanism by which eukaryotic cells undergo apoptosis.[47] Our data indicated that the activation of caspase-3 is involved in TQ-induced cytotoxicity (Figures 2 and 5).

The apoptosis-inducing effect observed in *in vitro* systems as shown in this study may be pathologically implicated as inducers of joint diseases such as arthritis. *In vitro* responses are variable depending on the species, condition of the donor, and the culture conditions. Although the significance of our findings should be validated in subsequent studies using *in vivo* animal models, the study suggests an initial clue that quinines such as TQ may play a role in the aetiology of cartilage disease through an induction of chondrocyte apoptosis. Our results may support a new light in studying the role of quinones in the aetiology of arthritis.

In conclusion, our results demonstrate that TQ induces apoptosis in rabbit articular chondrocytes through the generation of ROS as depicted in Figure 7. Caspase-3 and p53, which are located downstream of ROS and perform apoptosis, were activated during the apoptosis of cells treated with TQ. Moreover, ROS caused by TQ increased the activation of PI3K/Akt and MAPKs, which is also relevant to apoptosis. We predict that TQ may have the potential not only to inhibit proliferation, but also to induce apoptosis through p38kinase and Akt pathways.

Author contributions: SMY designed experiments, conducted research, and wrote manuscript. SJK designed experiments, conducted research, analysed data, and wrote manuscript.

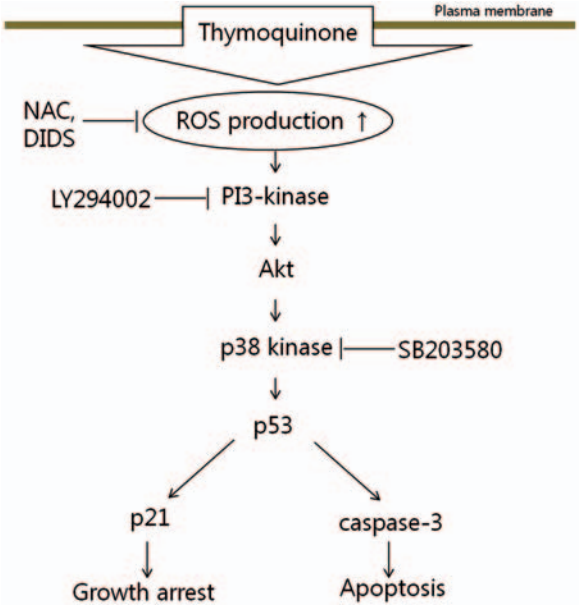

Figure 7 Schematic summary of TQ-induced apoptosis of chondrocytes. Production of ROS by TQ-caused apoptosis is due to activate PI3-kinase and p38kinase, p53 accumulation, and caspase-3 activation. The apoptosis by TQ are inhibited by treating NAC and DIDS. TQ: thymoquinone; ROS: reactive oxygen species; DIDS: cyano-2,2′-stilbenedisulphonic acid; NAC: *N*-acetyl-L-cysteine

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MEST) (2012-0004359).

REFERENCES

1. Loeser RF. Age-related changes in the musculoskeletal system and the development of osteoarthritis. *Clin Geriatr Med* 2010;**26**:371–86
2. Zamli Z, Sharif M. Chondrocyte apoptosis: a cause or consequence of osteoarthritis? *Int J Rheum Dis* 2011;**14**:159–66
3. Blanco FJ, Guitian R, Vazquez-Martul E, de Toro FJ, Galdo F. Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. *Arthritis Rheum* 1998;**41**:284–9
4. Malemud CJ, Islam N, Haqqi TM. Pathophysiological mechanisms in osteoarthritis lead to novel therapeutic strategies. *Cells Tissues Organs* 2003;**174**:34–48
5. Bortner CD, Cidlowski JA. Cellular mechanisms for the repression of apoptosis. *Annu Rev Pharmacol Toxicol* 2002;**42**:259–81
6. Ricci JE, Waterhouse N, Green DR. Mitochondrial functions during cell death, a complex (I-V) dilemma. *Cell Death Differ* 2003;**10**:488–92
7. Kim HA, Song YW. Apoptotic chondrocyte death in rheumatoid arthritis. *Arthritis Rheum* 1999;**42**:1528–37
8. Lee HG, Yang JH. PCB126 induces apoptosis of chondrocytes via ROS-dependent pathways. *Osteoarthritis Cartilage* 2012;**20**:1179–85
9. Casaccia-Bonnefil P. Cell death in the oligodendrocyte lineage: a molecular perspective of life/death decisions in development and disease. *Glia* 2000;**29**:124–35
10. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther* 2002;**1**:639–49
11. Bonekamp NA, Volkl A, Fahimi HD, Schrader M. Reactive oxygen species and peroxisomes: struggling for balance. *Biofactors* 2009;**35**:346–55
12. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 2000;**279**:L1005–28
13. Blanco FJ, Rego I, Ruiz-Romero C. The role of mitochondria in osteoarthritis. *Nat Rev Rheumatol* 2011;**7**:161–9
14. Steenvoorden MM, Huizinga TW, Verzijl N, Bank RA, Ronday HK, Luning HA, Lafeber FP, Toes RE, DeGroot J. Activation of receptor for advanced glycation end products in osteoarthritis leads to increased stimulation of chondrocytes and synoviocytes. *Arthritis Rheum* 2006;**54**:253–63
15. Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ. Role of quinones in toxicology. *Chem Res Toxicol* 2000;**13**:135–60
16. Way TD, Kao MC, Lin JK. Apigenin induces apoptosis through proteasomal degradation of HER2/neu in HER2/neu-overexpressing breast cancer cells via the phosphatidylinositol 3-kinase/Akt-dependent pathway. *J Biol Chem* 2004;**279**:4479–89
17. Mani AM, Fenwick MA, Cheng Z, Sharma MK, Singh D, Wathes DC. IGF1 induces up-regulation of steroidogenic and apoptotic regulatory genes via activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells. *Reproduction* 2010;**139**:139–51
18. Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 2004;**68**:320–44
19. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* 2011;**75**:50–83
20. Lee PJ, Camhi SL, Chin BY, Alam J, Choi AM. AP-1 and STAT mediate hyperoxia-induced gene transcription of heme oxygenase-1. *Am J Physiol Lung Cell Mol Physiol* 2000;**279**:L175–82
21. Aggeli IK, Gaitanaki C, Beis I. Involvement of JNKs and p38-MAPK/MSK1 pathways in H2O2-induced upregulation of heme oxygenase-1 mRNA in H9c2 cells. *Cell Signal* 2006;**18**:1801–12
22. Yoon YM, Kim SJ, Oh CD, Ju JW, Song WK, Yoo YJ, Huh TL, Chun JS. Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. *J Biol Chem* 2002;**277**:8412–20
23. Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* 2010;**48**:749–62
24. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol* 2003;**552(Pt 2)**:335–44
25. Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc)* 2005;**70**:200–14
26. Han D, Antunes F, Canali R, Rettori D, Cadenas E. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *J Biol Chem* 2003;**278**:5557–63
27. Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. *J Biol Chem* 2000;**275**:25130–8
28. Panasyuk A, Frati E, Ribault D, Mitrovic D. Effect of reactive oxygen species on the biosynthesis and structure of newly synthesized proteoglycans. *Free Radic Biol Med* 1994;**16**:157–67
29. Kamata H, Hirata H. Redox regulation of cellular signalling. *Cell Signal* 1999;**11**:1–14
30. Inoue M, Sakaguchi N, Isuzugawa K, Tani H, Ogihara Y. Role of reactive oxygen species in gallic acid-induced apoptosis. *Biol Pharm Bull* 2000;**23**:1153–7
31. Simamura E, Hirai K, Shimada H, Koyama J, Niwa Y, Shimizu S. Furanonaphthoquinones cause apoptosis of cancer cells by inducing the production of reactive oxygen species by the mitochondrial voltage-dependent anion channel. *Cancer Biol Ther* 2006;**5**:1523–9
32. Annunziato L, Amoroso S, Pannaccione A, Cataldi M, Pignataro G, D'Alessio A, Sirabella R, Secondo A, Sibaud L, Di Renzo GF. Apoptosis induced in neuronal cells by oxidative stress: role played by caspases and intracellular calcium ions. *Toxicol Lett* 2003;**139**:125–33
33. Monks TJ, Xie R, Tikoo K, Lau SS. Ros-induced histone modifications and their role in cell survival and cell death. *Drug Metab Rev* 2006;**38**:755–67
34. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;**281**:1309–12

35. Mates JM, Sanchez-Jimenez FM. Role of reactive oxygen species in apoptosis: implications for cancer therapy. *Int J Biochem Cell Biol* 2000;**32**:157–70
36. Zu K, Hawthorn L, Ip C. Up-regulation of c-Jun-NH2-kinase pathway contributes to the induction of mitochondria-mediated apoptosis by alpha-tocopheryl succinate in human prostate cancer cells. *Mol Cancer Ther* 2005;**4**:43–50
37. García-Berrocal JR, Nevado J, Ramírez-Camacho R, Sanz R, González-García JA, Sánchez-Rodríguez C, Cantos B, España P, Verdaguer JM, Trinidad Cabezas A. The anticancer drug cisplatin induces an intrinsic apoptotic pathway inside the inner ear. *Br J Pharmacol* 2007;**152**:1012–20
38. El-Mahdy MA, Zhu Q, Wang QE, Wani G, Wani AA. Thymoquinone induces apoptosis through activation of caspase-8 and mitochondrial events in p53-null myeloblastic leukemia HL-60 cells. *Int J Cancer* 2005;**117**:409–17
39. Roepke M, Diestel A, Bajbouj K, Walluscheck D, Schonfeld P, Roessner A, Schneider-Stock R, Gali-Muhtasib H. Lack of p53 augments thymoquinone-induced apoptosis and caspase activation in human osteosarcoma cells. *Cancer Biol Ther* 2007;**6**:160–9
40. El-Najjar N, Chatila M, Moukadem H, Vuorela H, Ocker M, Gandesiri M, Schneider-Stock R, Gali-Muhtasib H. Reactive oxygen species mediate thymoquinone-induced apoptosis and activate ERK and JNK signaling. *Apoptosis* 2010;**15**:183–95
41. Cornwell DG, Jones KH, Jiang Z, Lantry LE, Southwell-Keely P, Kohar I, Thornton DE. Cytotoxicity of tocopherols and their quinones in drug-sensitive and multidrug-resistant leukemia cells. *Lipids* 1998;**33**:295–301
42. Wang X, Thomas B, Sachdeva R, Arterburn L, Frye L, Hatcher PG, Cornwell DG, Ma J. Mechanism of arylating quinone toxicity involving Michael adduct formation and induction of endoplasmic reticulum stress. *Proc Natl Acad Sci U S A* 2006;**103**:3604–9
43. Onyango IG, Bennett JP Jr, Tuttle JB. Endogenous oxidative stress in sporadic Alzheimer's disease neuronal cybrids reduces viability by increasing apoptosis through pro-death signaling pathways and is mimicked by oxidant exposure of control cybrids. *Neurobiol Dis* 2005;**19**:312–22
44. Zanotto-Filho A, Delgado-Canedo A, Schroder R, Becker M, Klamt F, Moreira JC. The pharmacological NFkappaB inhibitors BAY117082 and MG132 induce cell arrest and apoptosis in leukemia cells through ROS-mitochondria pathway activation. *Cancer Lett* 2010;**288**:192–203
45. Kwon SH, Hong SI, Kim JA, Jung YH, Kim SY, Kim HC, Lee SY, Jang CG. The neuroprotective effects of Lonicera japonica THUNB. against hydrogen peroxide-induced apoptosis via phosphorylation of MAPKs and PI3K/Akt in SH-SY5Y cells. *Food Chem Toxicol* 2011;**49**:1011–9
46. Singh RK, Dorf L, DeMartino A, Illenye S, Koto K, Currier EA, Ashikaga T, Kim KK, Brard L, Sholler GL. Oral RKS262 reduces tumor burden in a neuroblastoma xenograft animal model and mediates cytotoxicity through SAPK/JNK and ROS activation in vitro. *Cancer Biol Ther* 2011;**11**:1036–45
47. Curtin JF, Donovan M, Cotter TG. Regulation and measurement of oxidative stress in apoptosis. *J Immunol Methods* 2002;**265**:49–72

(Received October 11, 2012, Accepted February 7, 2013)