Original Article
Diazoxide inhibits hydrogen peroxide induced apoptosis and oxidative damage in rat chondrocytes

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Abstract: Objective: The goal of this study was to investigate the effect of diazoxide on hydrogen peroxide induced chondrocytes. Method: Chondrocytes were obtained from the SD neonatal rat articular cartilages and then divided into five groups: the control group (A), the hydrogen peroxide (H_2O_2) group (B), H_2O_2 + 0.1 μmol/l diazoxide (DZ) group (C), H_2O_2 + 1.0 μmol/l diazoxide group (D), H_2O_2 + 10 μmol/l diazoxide group (E). Cells were analyzed for proliferation by MTT assay, apoptosis by flow cytometry and ELISA, and reactive oxygen species by 2',7'-dichlorofluorescin diacetate (DCFH-DA) staining. Results: compared with control group, H_2O_2 caused chondrocytes death, apoptosis, and excessive reactive oxygen species (ROS) production. But DZ (0.1-1.0 μM) inhibited the effect of H_2O_2 on chondrocytes. DZ (0.1-1.0 μM) pretreatment reduced the H_2O_2-induced apoptosis of chondrocytes by preventing cytochrome C release and caspases 3 activation. But 10 μM DZ did not abolish the effect H_2O_2 on chondrocytes. Conclusion: Diazoxide can effectively abrogate H_2O_2-induced apoptosis and ROS production of chondrocytes, suggesting that diazoxide may be a potential agent in the treatment of OA.

Keywords: Diazoxide, chondrocytes, reactive oxygen species, apoptosis

Introduction

Osteoarthritis (OA) is a common and progressive chronic disease leading to impaired joint function and can result in immobility mostly in elderly people. OA affects not only the articular cartilage but also the entire joint, including subchondral bone, ligaments, synovial membrane, menisci, and even periarticular muscles [1, 2]. An important factor, involved in the development of OA, is excessive reactive oxygen species (ROS). High levels of ROS damaged not only the cellular membrane and nucleic acids but also the associated extracellular matrix, resulting in chondrocytes death and cartilage degradation [3-6]. On the other hand, ROS can react with DNA, protein, and lipids, to disrupt the normal structure of these molecules, impair function, and promote progress of OA [7-10]. Therefore, ROS are toxic and play an important role in the initiation and pathophysiology of OA.

In various types of cells, diazoxide (DZ), opening of the mitochondrial ATP-sensitive potassi-

um channel (mK_{ATP}), leads to enhanced resistance to subsequent injury, e.g. induction of apoptosis, caused by different stimuli [11-16]. Pharmacological activation of mK_{ATP} (DZ) has an acute protective effect in several models of brain ischemia [17]. DZ protected skeletal myoblast from apoptosis caused by hydrogen peroxide (H_2O_2). But physiological ROS have been identified as pivotal signaling molecules in the cellular response to DZ-induced cellular protection [18-20]. A possible mechanism is that DZ can lead to mitochondrial membrane hyperpolarization, slightly inhibited the respiratory chain, resulting in physiological ROS, activating the ROS signaling pathways. Physiological ROS is hypothesized to upregulate uncoupling protein. These in turn induce modest mitochondrial uncoupling which protects against the subsequent pathological ROS generation [21, 22]. Much attention has been focused on the preconditioning effect of diazoxide on neuron and heart [16-18, 23-29]. But the protection of DZ on chondrocytes has not reported. Recently, Mobasheri B.Sc. et al. [30] demonstrated that

functional mK_{ATP} channels were present in chondrocytes in superficial and middle zones of cartilage. However, it is not known whether the mK_{ATP} channels may have an important role in metabolic regulation in chondrocytes. Therefore, in the present study, the effects of diazoxide on activating mK_{ATP} channels was investigated, as well as the changes of chondrocyte function and oxidative modifications after exposure to hydrogen peroxide.

Materials and methods

Cell culture

Murine chondrocytes were cultured at 37°C in 5% CO_2 atmosphere in culture medium (DMEM, Gibco, USA). The medium contained 10% (V/V) fetal bovine serum (FBS; Gibco), 1% (V/V) penicillin and streptomycin (Gibco). When the medium was full of cells, they were sub-cultured using 0.02% EDTA-0.05% trypsin solution. Other reagents were of the highest commercial grade available and purchased from Sigma Chemical (St. Louis, MO, USA).

Cell viability

Cells were suspended in medium supplemented with 10% FBS, and cell suspension containing 5×10^3 cells was added to the individual wells of 48-well microplates. The plates were incubated at 37°C in a CO_2 incubator for 24 hours. After discarding the culture medium and washing the cells with phosphate-buffered saline (PBS), a serum-free medium containing 0.3% bovine serum albumin (BSA) and samples at appropriate concentrations was added to the cell culture and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 4 hours. Surviving cells were counted by the 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) method. MTT 20 µl in 7.2 mM phosphate buffer solution, pH 6.5 (5 mg/ml), was added to each well, and the plates were incubated for an additional 2 hours. After the removal of solutions in the well, dimethyl sulfoxide was added to dissolve formazan products, and the plates were shaken for 5 minutes. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

Apoptotic rate assay by flow cytometry

Annexin V, an FITC conjugate of the PS-binding protein, is capable of detecting PS externaliza-tion in early apoptotic cells. Chondrocytes apoptosis was estimated using the Annexin-V Fluorescein (FITC) apoptosis staining kit (Nanjing KeyGen Biotech. Co. Ltd, China). The cell samples were analyzed by flow cytometry (BD Co., USA). Annexin V binds to phosphatidylserine that is translocated during apoptosis from the inner to the outer leaflet of the plasma membrane. Normal cells with intact membranes are distinguished by their ability to exclude propidium iodide (PI), which readily penetrates dead or damaged cells. Dual analysis was introduced using a quadrant dot plot, in which early apoptotic cells were annexin V-FITC-positive only, necrotic cells and late apoptotic cells were recognized as double-positive for annexin V-FITC and PI. Cells that stained negative for both annexin V-FITC and PI were classified as normal cells. Finally, the number of cells in each category was expressed as a percentage of the total number of stained cells counted. Each experiment was repeated three times [31].

Measurement of caspase-3 and cytochrome C activity

Measurement of caspase-3 activity. Proteolytic activities of caspase-3 were measured by colorimetric assay kits (Sigma-Aldrich St. Louis, US) according to the manufacturer's instructions. Briefly, cells were seeded in a dish at a seeding density of 1×10^5 and after reaching confluence. DMEM was aspirated and cocultured with various concentrations of DZ in culture medium for 30 minutes and then cocultured with 0.25 mmol·L^{-1} H_2O_2 for 4 hours. Cell pellets were suspended in a cell lysis buffer and incubated on ice for 10 minutes. The lysate was vortexes every 15 minutes. After centrifugation at 11,000 g for 15 minutes, 20 µL of cell lysate were added immediately to buffer containing p-nitroaniline (pNA)-conjugated substrate for caspase-3 (Ac-DEVDpNA). The samples were incubated for 1 hour at 37°C. Sample fluorescence was detected in a microplate reader (Spectra Fluor, TECAN, Sunrise, Austria). Concentration of the released pNA was calculated from the absorbance values at 405 nm and caspase activities are expressed as fold increases over the nontreated cells control.

Enzyme-Linked Immunosorbent Assay (ELISA Abnova HmbH, Heidelberg, Germany). Cytochrome C protein activity was evaluated. The cells
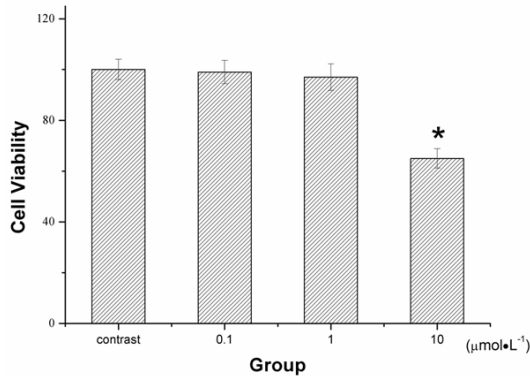
Figure 1. Cell viability of chondrocyte following DZ exposure. Chondrocytes plated on 6-well plate were treated with different concentrations of DZ for 30 minutes. Cell viabilities were measured with MTT assay as described in the text. All values are relative to the control cells and represent mean and 95% CI (error bar) from three independent experiments. Statistical difference from control was set to P < 0.05. Compare with control group, *P < 0.05.
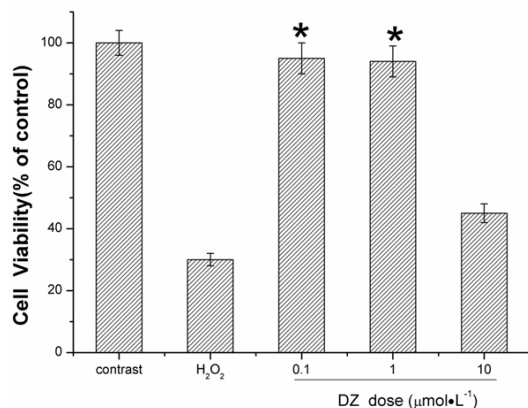

Figure 2. Chondrocytes plated on 6-well plate were pretreated with different concentrations of DZ for 30 minutes. Then all groups' cells were treated with 0.25 mmol/l H_2O_2 for 4 hours expect control group. Cell viabilities were measured with MTT assay as described in the text. All values are relative to the control cells and represent mean and 95% CI (error bar) from three independent experiments. Statistical difference from control was set to P < 0.05. Compare with H_2O_2 group, *P < 0.05.

were seeded at a density of 1×10^6 per dish and incubated overnight at 37°C, then the cells were treated with various concentrations of DZ in culture medium for 30 minutes and then cocultured with 0.25 mmol·L^{-1} H_2O_2 for 4 hours. Total protein was extracted from the cells using lysis buffer-containing protease inhibitor cocktail set III and phosphatase inhibi-

tor cocktail set I (Calbiochem, EMD Biosciences, San Diego, CA). The protein concentrations of all the samples were determined with A Bicinchoninic Acid Protein Assay Kit (Invitrogen Carlsbad, CA, USA).

Measurement of intracellular reactive oxygen species

Relative changes in intracellular reactive oxygen species in chondrocytes was monitored using a fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA diffuses through the cell membrane readily and is hydrolyzed by intracellular esterases to non-fluorescent 2',7'-dichlorofluorescein (DCFH), which is then rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of reactive oxygen species. The DCF fluorescence intensity is proportional to the amount of reactive oxygen species formed intracellularly. Cells were washed twice and resuspended at a concentration of 1×10^5 cells/ml in cell culture. For loading DCFH-DA into the cells, cells were incubated with the dye for 2 hours at a final concentration of 5 µM at 37°C. Fluorescence was monitored at 530 nm with excitation wavelength of 485 nm in a stirred quartz cuvette.

Image analysis

Fluorescent and immunohistochemical images were analyzed using Image J analysis software (Wayne Rasband, NIH, USA). For fluorescent images, individual cells from random fields were selected and the average red (MitoSOX Red) and/or green pixels present in the cell calculated after average background subtraction.

Statistical analysis

All experiments were performed independently at least three times and data are expressed as mean ± standard deviation (SD). Results from each experiment are expressed as mean ± SD. Statistical data of both cell viability experiments and cell apoptosis rate experiment were assessed by student's t test and the data of caspase-3 and cytochrome c experiments was assessed by one-way analysis of variance (ANOVA) and Tukey's post hoc analysis. All data analysis used SPSS for Windows statistical
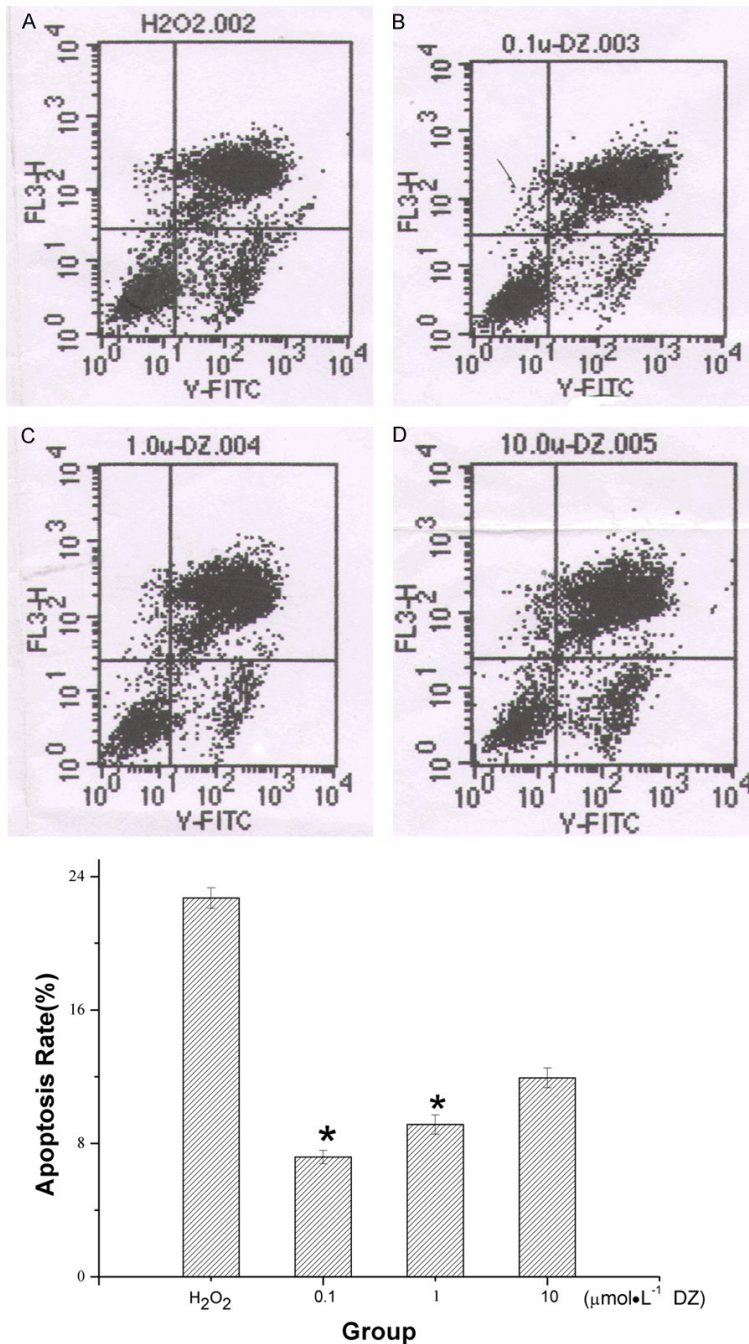
Figure 3. The diazoxide protect the chondrocytes against apoptosis induced by H_2O_2. The chondrocytes groups (A-D) were separately treated with (0, 0.1, 1.0, 10) $\mu mol \cdot L^{-1}$ DZ + 0.25 $mmol \cdot L^{-1}$ H_2O_2. Cell apoptosis was determined by Hoechst33342/PI staining. The different labeling regions in this quadrant represent the different cell populations. The number of cells undergoing apoptosis was increased significantly by H_2O_2. The groups pretreated by 0.1-1.0 $\mu mol \cdot L^{-1}$ DZ significantly decreased the extent of cell apoptosis, compare to H_2O_2 group. Each experiment was repeated three times (n = 3). At least 1×10^5 cells were used for each analysis. Compare with H_2O_2 group, *P < 0.01.

software (version 16.0). A *P*-value of less than 0.05 was considered as significant.

Results

Cytotoxicity

The MTT assay was used to determine cytotoxicity of diazoxide (DZ) on articular chondrocytes induced by Hydrogen peroxide (H_2O_2). As shown in **Figure 1**, chondrocytes viability was 60%±2% after 0.5 hours of exposure to 10 µM DZ. But the chondrocyte viability 0.1 µM and 1.0 µM DZ groups was not different with the contrast group. These data suggest that high concentrations of DZ (> 10 µM) have toxicity on chondrocytes. In the **Figure 2**, 0.1 µM and 1.0 µM DZ increased chondrocyte viability after exposure to 0.25 mM H_2O_2. But high concentration DZ did not protect chondrocytes from exposure to 0.25 mM H_2O_2 for 4 hours. These data suggest that DZ (0.1-1.0 µM DZ) can protect chondrocytes against the toxicity of H_2O_2.

The protective effect of diazoxide on H_2O_2 induced-chondrocytes apoptosis

Chondrocytes were pretreated with DZ under different dosage for 30 minutes before 0.25 mmol/L H_2O_2 treatment for 4 hours. chondrocytes apoptosis rates were measured by flow cytometry, to determine whether the DZ can protect chondrocytes treated by H_2O_2. As shown in **Figure 3**, chondrocytes exposed to H_2O_2 have high apoptosis rate (12.71%, *P* < 0.01). But DZ (0.1-1.0 µM) decreased the apoptosis rate to 7.18% of chondrocytes treated by H_2O_2 (*P* < 0.01). However, the chondrocytes pretreated with 1.0 µM DZ apoptosis rate have no significant difference with H_2O_2 group. These results suggest that 0.1-1.0 $\mu mol \cdot L^{-1}$ DZ reduces H_2O_2 induced-chondrocytes apoptosis.
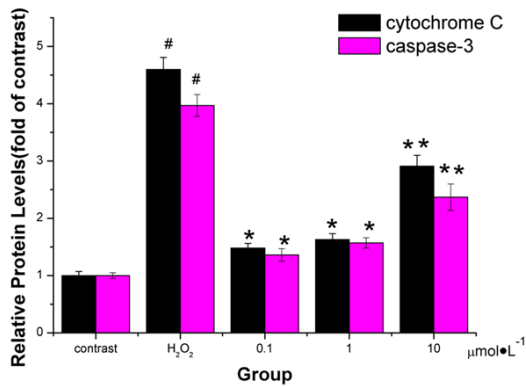
Figure 4. DZ inhibited H_2O_2-induced apoptosis in chondrocytes. Before exposure to H_2O_2, cells were incubated with the indicated concentrations of DZ. Cell proteins were obtained and analyzed with ELISA. The photograph shows that DZ (0.1, 1.0 $\mu mol \cdot L^{-1}$) inhibited caspased-3 activation and release of cytochrome c in chondrocytes. Each experiment was repeated three times (n = 3). *P < 0.05 VS H_2O_2 group, #P < 0.01 VS contrast group, **P > 0.05 VS H_2O_2 group.

Effect of DZ on H_2O_2-induced chondrocytes apoptosis in caspase-dependent way

Caspase-3 is activated by two cleavage events that occur between the pro-domain and the large subunit (P17) and between the large subunit and the small subunit (P12). The activated (cleaved) caspase-3 cleaves death substrates and ultimately leads to chondrocytes death [32], therefore the active (cleaved) caspase-3 is detected by ELISA. After 4 hour exposure to H_2O_2, the chondrocytes cleaved caspase-3 was significantly increased compared to the control group (**Figure 4**). In contrast, the chondrocytes pretreated with DZ (0.1-1.0 μM), caspase-3 activity was blocked and the cleaved caspase-3 was less than the H_2O_2 group. However, compared with the H_2O_2 group, 10 μM DZ did not protect the chondrocytes by reducing cleaved caspase-3.

On the other hand, cytochrome C release also can induce chondrocyte death. Mitochondrial release of cytochrome C into the cytoplasm induces formation of an oligomeric complex. This complex activates a downstream caspase program [33]. In order to research cytochrome C release infected by DZ, ELISA was performed. As shown in **Figure 4**, cytochrome C was significantly increased in the H_2O_2 group, compared with the contrast group. However, the 0.1 and 1.0 μM DZ groups significantly decre-

ased cytochrome C protein compared with the H_2O_2 group (P = 0.025 and P = 0.03 respectively). Cytochrome C release had no difference between 10 μM DZ group and the H_2O_2 group. Therefore, a high dosage (> 10 μM) DZ group may not protect the chondrocyte against oxidative damage.

DZ suppress H_2O_2-induced ROS elevation in chondrocytes

The effect of H_2O_2 elevated rapidly ROS in chondrocytes, but if the cells were pretreated with a certain dosage DZ, the ROS level was suppressed. Different groups were pretreated with different concentration DZ for 30 minutes, following by treatment with H_2O_2 for 4 hours. The ROS levels were different. Form **Figure 5**, 0.1 μM DZ (P < 0.01) and 1.0 μM DZ (P < 0.01) significantly suppress the ROS level in the H_2O_2-induced chondrocytes. In the 0.1 μM DZ group and 1.0 μM DZ group there was a reduction of H_2O_2-induced ROS production in chondrocytes by 217% and 158%, however compared with the H_2O_2 group, the ROS levels of 10 μM were not different (P > 0.01).

Discussion

OA is common chronic disease leading to impaired joint function and results in immobility mostly in elderly people. The more elder people that suffer from OA, the more attention that should be paid to this disease. But at present, the efficacy of OA therapy is not satisfying. The main cause of OA is due to oxidative damage and apoptosis in articular chondrocytes [8, 34, 35]. In this study, the mK_{ATP} opener diazoxide is shown to hinder oxidative damage of chondrocytes treated by H_2O_2 in vitro. Additionally, DZ suppressed H_2O_2-induced apoptotic chondrocyte death. DZ exerted protection against H_2O_2-induced decrease in cell viability in chondrocytes.

DZ is a mitochondrial ATP-sensitive K channel opener, which increases membrane permeability to potassium ions [36, 37]. Choi EM found that diazoxide was able to recover the H_2O_2-induced dysfunction in the differentiation of osteoblast. DZ exerted concentration-dependent protection against H_2O_2-induced decrease in cell viability in osteoblasts [38]. A similar result was reported here. DZ (0.1~1 μM) decreased H_2O_2-induced ROS generation and oxi-
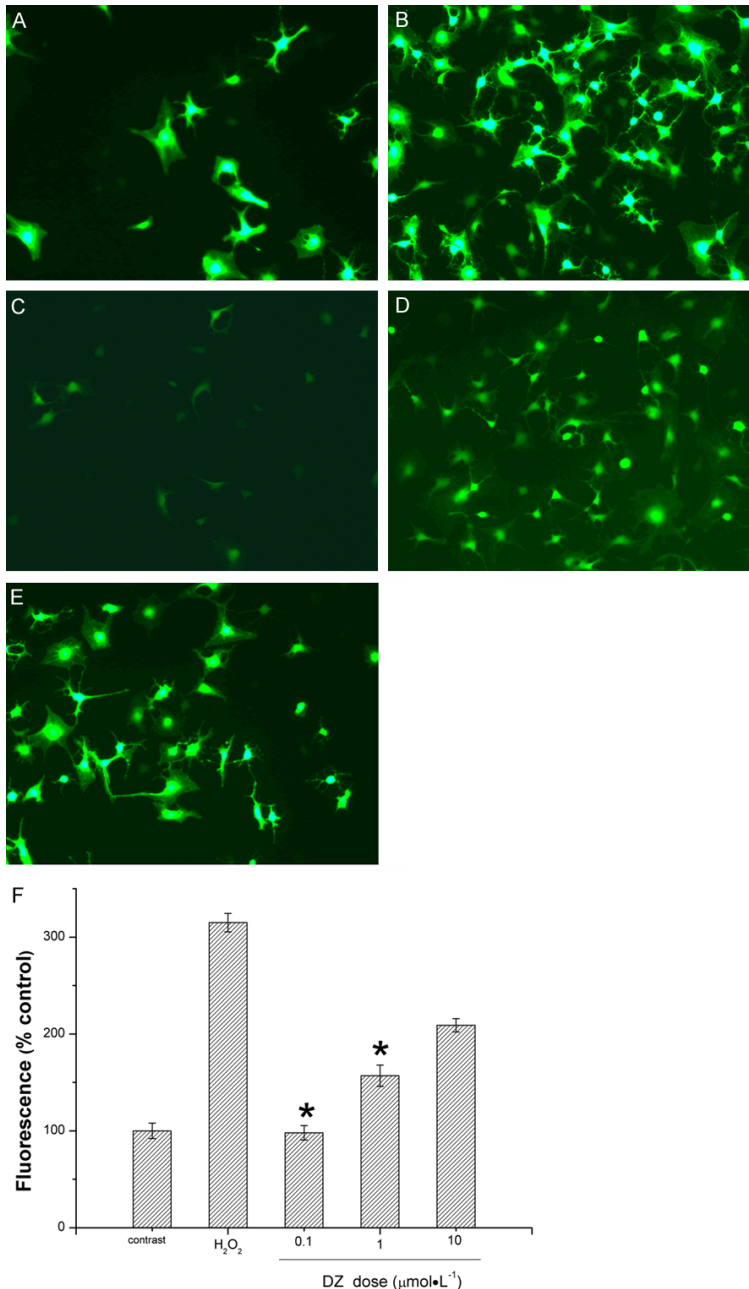
Figure 5. DZ decreased production of intracellular ROS in H_2O_2-induced chondrocytes. The chondrocytes group (A) was treated with 0 μmol·L⁻¹ DZ + PBS. The chondrocytes groups (B-E) were separately treated with (0, 0.1, 1.0, 10) μmol·L⁻¹ DZ + 0.25 mmol·L⁻¹ H_2O_2. Group A was treated with the PBS (Phosphate Buffered Saline). DCFH-DA fluorescence (green) imaging of chondrocytes and quantification of DCFH-DA fluorescence intensity were detected. Chondrocytes are pretreated with different concentrations DZ for 30 minutes, followed by treatment with 0.25 mmol·L⁻¹ H_2O_2 for 4 hours and DCFH-DA was used to detect the volume of intracellular ROS in chondrocytes. All the results are indicated in the (F) chart. The group B has highest volume of ROS. Compare other groups with the H_2O_2 group (group B), *P < 0.01.

cell viability in chondrocytes. The protection of DZ may be mediated by activation of mK_{ATP}. DZ increased mitochondrial membrane permeability and calcium overload [21, 38-42]. It protected oxidative stress-induced cell injury and improved the mitochondrial function.

Apoptosis, which is also termed as programmed cell death, plays a critical role in the progression of OA [43, 44]. There are two main pathways can lead to apoptotic process, that is, the receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. In the extrinsic pathway, the stimulus of cell death receptors (Fas, TNF-α, etc.) directly triggers caspase-8 initiating the following cascade reactions. In the intrinsic pathway, the apoptosis is initiated by response to a variety of stress signals. The classic signs of cell apoptosis are preceded by mitochondrial alterations including a decrease in energy production, a loss of Δφm, an increase in the permeability of the mitochondrial membrane and a release of pro-apoptotic factors such as cytochrome c [45]. In this study, DZ (0.1~1 μM) reduced the H_2O_2-induced chondrocytes apoptosis (P < 0.05) (**Figure 4**) and prevented cytochrome C release from the mitochondria (P < 0.05). DZ could also inhibit indirectly the activation of caspase-3 in H_2O_2-induced chondrocytes (P < 0.05). The active caspase-3 cleaved substrates (e.g PARP) and it ultimately lead to cell death [46]. Above all, DZ promoted potassium inflow into mitochondria from cytoplasm, resulted in the reduction of Δφm, reduced the release of cytochrome C from the mitochondri-

dative damage in culture chondrocytes and protected against H_2O_2-induced decrease in

on into the cytoplasm, blocked the activation of caspase-3, and finally decreased chondrocyte apoptosis [32].

Free radical damage induced cell apoptosis, which provides a strategy to protect the organism by scavenge of damaged cells. Also plenty of free radical could damage the normal functional cells. In this study, H_2O_2-induced chondrocytes had high apoptosis rate (**Figure 3A**) because much more free radicals (ROS) were produced in chondrocytes (**Figure 5B**). Diazoxide (0.1~1 μM) significantly inhibited ROS production (**Figure 5**) in chondrocytes and reduced chondrocyte apoptosis rate and protected chondrocytes function against H_2O_2 toxicity. Some research showed that free radical species have been shown to promote apoptosis [47, 48] by activation of c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein (MAP) kinases via apoptosis signal-regulating kinase (ASK)1 [36]. Mitochondrial damage by ROS/RNS has also been addressed to be an important factor for chondrocyte functional compromise and apoptosis induction [32, 49, 50].

In summary, diazoxide can decrease H_2O_2-induced chondrocytes apoptosis rate and hinder oxidative damage in chondrocytes. It protected chondrocytes function against H_2O_2-induced cytotoxicity via reducing of ROS production. However, the protection mechanism of diazoxide has not been reported. In the future, the protection mechanism of diazoxide on H_2O_2-induced chondrocytes will be a major focus.

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Disclosure of conflict of interest

None.

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