

Apoptotic effect of pyrroloquinoline quinone on chondrosarcoma cells through activation of the mitochondrial caspase-dependent and caspase-independent pathways

RONGHUAN WU, JUN PAN, MIAODA SHEN and CHUNYANG XING

Department of Orthopedics, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, P.R. China

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Abstract. Chondrosarcomas are malignant tumors of the bone that exhibit resistance to chemotherapy and radiation. Pyrroloquinoline quinone (PQQ) is a bacterial redox co-factor and antioxidant that has been found to induce apoptosis in various cancer cells. This study investigated the role of PQQ in cell apoptosis of chondrosarcoma cells and the underlying pathways involved. We confirmed that PQQ was cytotoxic to chondrosarcoma SW1353 cells by a cell cytotoxicity assay. Furthermore, flow cytometry showed that the number of apoptotic cells increased in a concentration-dependent and time-dependent manner following PQQ treatment, but this effect was not significant in normal cells. Co-immunoprecipitation assays showed that the binding of Smac to X-linked inhibitor-of-apoptosis protein (XIAP) was significantly increased and the binding of XIAP with caspase-3 was significantly decreased following PQQ treatment. This was accompanied by a decrease in the levels of caspase-1 and procaspase-3, as demonstrated by western blot analysis. Western blotting also showed that the level of cytochrome *c* in the mitochondria was decreased and its level in the cytoplasm was increased. These findings indicate the role of caspase-dependent apoptotic pathways in the effect of PQQ. Furthermore, the cytoplasmic and nuclear levels of apoptosis-inducing factor (AIF) were increased and its mitochondrial levels were decreased, and similar results were obtained for endonuclease G. Thus, the role of caspase-independent pathways was also demonstrated. Finally, *in vivo* tumor implantation experiments showed that PQQ was able to inhibit tumor growth in mice with chondrosarcoma. These findings demonstrated that PQQ induced apoptosis in

human chondrosarcoma cells by activating mitochondrial caspase-dependent and caspase-independent pathways. Thus, the proteins involved in these pathways may have potential as antitumor treatment targets for chondrosarcoma.

Introduction

Chondrosarcoma (CHS) is the second most common primary bone malignancy; it accounts for 40% of all primary bone malignancies and is characterized by a series of clinicopathological signs (1,2). It usually affects adults between the ages of 20 and 60 years (3,4). Although chemotherapy and radiation have been investigated for their efficacy against CHS, they are not used as active treatments since these tumors are notoriously resistant to both chemotherapy and radiation (3,5). Therefore, there is an urgent need to identify new drugs and therapeutic approaches to improve the clinical management of CHS and prevent its recurrence.

Pyrroloquinoline quinone (PQQ) was first discovered as a natural synergistic redox agent and a novel enzymatic co-factor in bacteria. PQQ, as an essential nutrient, is a small molecule that is water soluble and thermally stable and is present in all types of plant and animal cells (6,7); in particular, it is extensively distributed in mammalian cells as well (8,9). It has been reported that PQQ can inhibit the formation of peroxynitrite (an oxidant) and has anti-lipid peroxidation and antioxidant properties (10-12). Studies have also demonstrated that PQQ can protect nucleus pulposus cells from hydrogen peroxide-induced apoptosis and oxygen/glucose deprivation-induced apoptosis by suppressing the mitochondrial-mediated apoptotic pathway and activating the PI3K/AKT cell proliferation pathway in cardiomyocytes, respectively (13,14). Moreover, PQQ was found to induce apoptosis in multiple types of human cancer cells, such as promonocytic leukemia U937 and lymphoma EL-4 cells, and induce Jurkat cell programmed death (12) and lung cancer cell apoptosis through mitochondrial-dependent pathways, as well as decreased expression of the Bcl-2 protein (involved in regulating cell death) (15). In addition, PQQ was found to have little effect on normal cells, which means that it may be an ideal drug for cancer therapy in the future (12,15,16). In the case of CHS, similar results were also obtained. Our recent study showed that the cell death rate of

Correspondence to: Dr Chunyang Xing, Department of Orthopedics, The First Affiliated Hospital, School of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China
E-mail: xing@zju.edu.cn

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CHS cells increased with an increase in PQQ concentration (at concentrations $<120 \mu\text{M}$), and that PQQ did not show significant toxicity against normal cells (17). However, the underlying molecular mechanism of PQQ-induced apoptosis in CHS cells remains to be elucidated.

In multicellular organisms, apoptosis plays a crucial role in embryogenesis and homeostasis and is also associated with neurodegenerative disorders and cancer (18,19). There are two types of apoptosis pathways: caspase-dependent and caspase-independent pathways (20). Caspases, which comprise a family of cysteine-dependent aspartate-guided proteases, play vital roles in the development of apoptosis and its initiation and execution (21). The caspase-dependent apoptotic pathways include caspase-3, procaspase-3, Smac and X-linked inhibitor-of-apoptosis protein (XIAP), among others. In addition, some of the proteins that are a part of the non-caspase-dependent mitochondrial pathways are pro-caspase, endonuclease G (Endo G), Apaf-1, apoptosis-inducing factor (AIF) and cytochrome *c*. Based on previous studies that have shown the role of the mitochondrial apoptosis pathways in the apoptotic mechanisms of PQQ in various cancers, in the present study, we explored the level of these proteins in CHS cells treated with various concentrations of PQQ.

In the present study, we investigated the effect of PQQ on the apoptosis of CHS cells and explored the potential apoptotic pathways that may be involved. The findings indicated that the apoptotic mechanism of PQQ involves caspase-dependent as well as non-caspase-dependent pathways. Furthermore, PQQ was shown to have *in vivo* effects as well on the growth of CHS.

Materials and methods

Cell culture and reagents. The chondrosarcoma cell line SW1353, osteosarcoma cell line Saos-2 and 293 cells from the American Type Culture Collection (ATCC; Manassas, VA, USA), as well as human XJH B lymphocytes from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing Gibco® 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The cells were grown in a 37°C incubator containing 5% CO₂. PQQ was purchased from Merck KGaA.

Cytotoxicity-induced cell death analysis. Cytotoxic death was assessed using a CytoTox-Glo™ Cytotoxicity Assay (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Briefly, the cells were seeded at a density of 1×10^4 cells/well in 3 ml of DMEM and incubated at 37°C in 5% CO₂ for 6 h. Then, different concentrations of PQQ (0, 40, 80, 120 and 200 μM) were added to the culture and the cells were incubated for 24 h under the same conditions. Following this, 50 μl of CytoTox-Glo™ reagent (Promega) was added, and the cells were incubated at 37°C for 15 min. Luminescence was then measured as an indicator of the percentage of dead cells. Finally, the cells were incubated with lysis reagent for 15 min at room temperature and total cell luminescence was measured by a microplate reader (ELx800; BioTek Instruments, Inc., Winooski, VT, USA).

Flow cytometric analysis. Flow cytometry combined with JC-1 staining was used to detect the mitochondrial membrane potential. The chondrosarcoma cell line SW1353 and osteosarcoma cell line Saos-2 were seeded in 6-well plates with complete medium and incubated for 24 h. After incubation, the cells were treated with 120 μM PQQ or control (PBS) for 48 h at 37°C in a 5% CO₂ incubator. Then, SW1353 cells were treated with a trypsin-EDTA (0.25%) solution and centrifuged at 1,000 \times g for 5 min. Finally, the cells were washed three times with PBS, and apoptosis was assessed using Mitochondrial membrane potential detection kit (JC-1) staining according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China) and flow cytometry with a FACSCalibur system equipped with Cell Quest software (version 5.1; BD Biosciences, Franklin Lakes, NJ, USA). FLH2/FLH1 ratio represented the changes in the apoptotic cells. When apoptosis was increased, FLH2/FLH1 decreased.

Western blot analysis. The cells were treated with PQQ or caspase inhibitor Z-VAD-fmk for 48 h. For obtaining the whole-cell extract, the cells were lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitors (Sigma-Aldrich; Merck KGaA). Then, the cells were centrifuged at 12,000 \times g for 5 min at 4°C, and the supernatant was collected and assessed using a BCA Protein assay kit (Sigma-Aldrich; Merck KGaA) to determine the protein content. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) with 20 mg of protein per lane and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, we used Tris-buffered saline (TBS) and 0.1% Tween-20 (TBS/T) containing 5% bovine serum albumin (BSA) to block the PVDF membranes at room temperature for 1 h. Incubation with the primary antibodies was performed overnight at 4°C with antibodies against caspase-3 (cat. no. ab13847), procaspase-3, Smac (cat. no. ab8115), and XIAP (cat. no. ab137392; Abcam, Cambridge, MA, USA) diluted to 1:1,000 in TBS/T. After the incubation, the membranes were washed three times for 5 min each with TBS/T, and then incubated with the corresponding horseradish peroxidase-labeled secondary antibodies (1:2,000; cat. no. ab97051; Abcam) for 2 h at 37°C. The membranes were again washed three times with TBS-T, and the protein bands formed were detected with the enzyme-catalyzed chemiluminescent (ECL) kit (GE Healthcare, Piscataway, NJ, USA) and quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Co-immunoprecipitation assay. Cells were collected and lysed in 50 μl of cell lysis buffer (Cell Signaling Technology) after treatment under different conditions such as H₂O₂, cisplatin and TNF- α for 48 h. A small portion of the cells was lysed in lysis buffer containing SDS buffer solution, and the rest were lysed by incubation in cell lysis buffer containing the appropriate antibodies or beads at 4°C for 24 h. The immuno-precipitate was separated by SDS-PAGE and transferred to PVDF membranes. Specific signals were detected using anti-Smac, anti-caspase-3 or anti-XIAP antibodies (Abcam). Protein expression was detected with the ECL kit (cat. no. ab65623; Abcam).

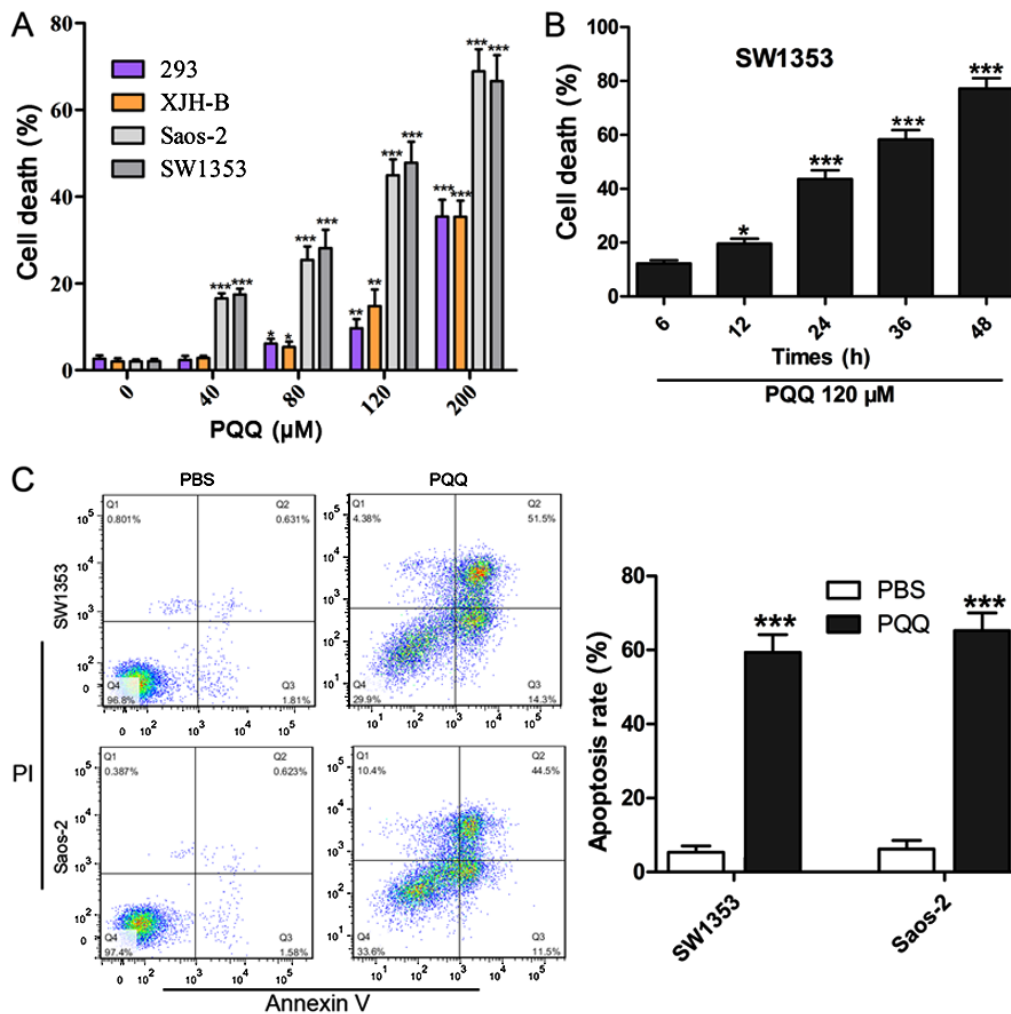


Figure 1. Cytotoxic and apoptotic effects of PQQ on CHS and normal cells. (A) Cytotoxicity of PQQ against SW1353, Saos-2, 293 and XJH B cells according to the results of the cell cytotoxicity assay. All cell types were incubated with PQQ (0, 40, 80, 120, or 200 μM) for 48 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0 μM PQQ. (B) Cytotoxic effect of 120 mM PQQ on SW1353 cells at different time points (6, 12, 24, 36, and 48 h). * $P < 0.05$, *** $P < 0.001$ vs. 6-h treatment. (C) Rate of cell apoptosis in SW1353 and Saos-2 cells after treatment with 120 μM PQQ for 24 h, according to flow cytometry results. *** $P < 0.001$ vs. PBS treatment for 24 h. PQQ, pyrroloquinoline quinone.

Tumor xenograft implantation. All experiments on animals were performed according to the Animal Care Guidelines of the First Affiliated Hospital of Zhejiang University School of Medicine and were approved by the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications, No. 8023, revised 1978). The animals experiments comply with the ARRIVE guidelines and the AVMA euthanasia guidelines 2013. A total of 10 female BALB/c nude mice (4-5 weeks old) were purchased from Shanghai Experiment Animal Centre (Shanghai, China). They were fed an irradiated pathogen-free diet and were housed in a specific pathogen-free (SPF) environment (a laboratory animal room maintained at $25 \pm 1^\circ\text{C}$ with $65 \pm 5\%$ humidity on a 12-h light/dark cycle). We used SW1353 cells to establish the mouse model. Briefly, 1×10^6 cells/mouse were injected subcutaneously into the left flanks of BALB/c nude mice. The mice were given a daily abdominal injection of 50 mg/kg PQQ (17) or PBS for 10 days. The mice were then sacrificed by CO_2 inhalation and the tumor xenografts were collected. The tumor volume (V) was calculated using the formula $V = (\text{width}^2 \times \text{length})/2$ and the maximum tumor size did not exceed a diameter of 2.0 cm.

Statistical analysis. All experiment results are expressed as mean and standard deviation (SD) values. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for analysis of the data. Statistical differences between two groups were analyzed with the Student's t-test, and multiple group comparisons were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. P-values > 0.05 were considered to indicate significant differences.

Results

PQQ promotes cell death in CHS cells. Following treatment of the cell lines SW1353, Saos-2, 293 and XJH-B with 0, 40, 80, 120, or 200 μM PQQ for 24 h, the cell death rate was higher in the SW1353 and Saos-2 cells than the rate in normal human cell lines 293 and XJH-B. Although 80 μM PQQ also induced normal cell 293 and XJH-B cell death, the cell death was $< 10\%$, and in Saos-2 and SW1353 cells, the cell death was $\sim 30\text{-}40\%$ following treatment with 80 μM PQQ (Fig. 1A). Furthermore, the cell death rate was higher with higher PQQ

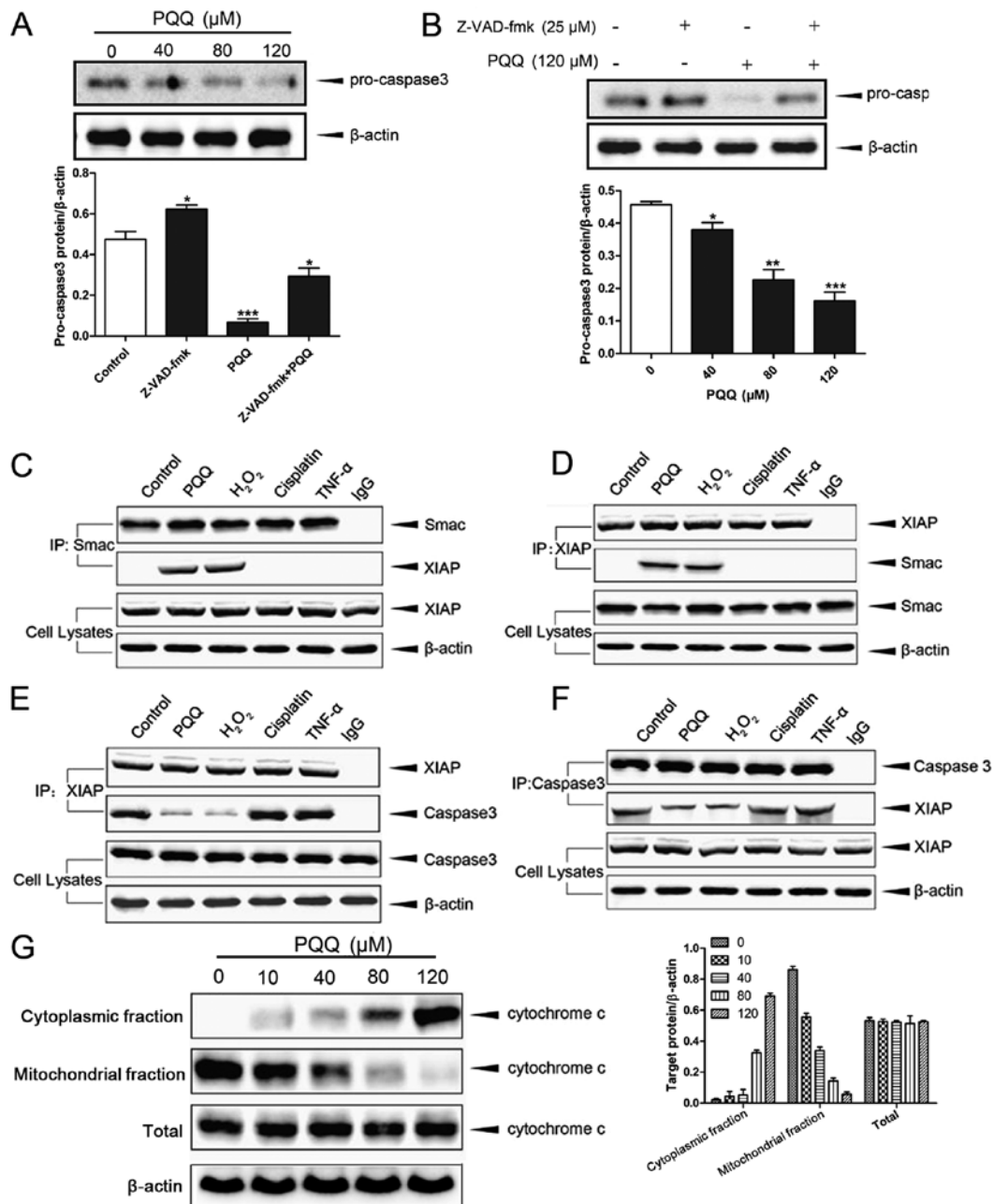


Figure 2. Effect of PQQ on the level of mitochondrial caspase-dependent apoptosis pathway proteins. (A) Western blot analysis showing the level of procaspase-3 in SW1353 cells treated with different concentrations of PQQ for 24 h. β -actin was used as a control. * $P < 0.05$, *** $P < 0.001$ vs. control. (B) Western blot analysis showing procaspase-3 expression after combined treatment with Z-VAD-fmk and PQQ. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0 μM PQQ. (C-F) Co-immunoprecipitation assay (IP) results showing the interaction between Smac and XIAP and between XIAP and caspase 3 after treatment with PQQ, H_2O_2 , cisplatin, TNF- α and IgG. (G) Western blot analysis showing the level of cytochrome *c* in the cytoplasm, mitochondria and total protein fraction after treatment with different concentrations of PQQ for 24 h. PQQ, pyrroloquinoline quinone.

concentrations. With regard to the effect of treatment time, we found that treatment with 120 μM PQQ significantly enhanced cell death in a time-dependent manner (Fig. 1B). Flow cytometric analysis indicated that the percentage of apoptotic cells was higher among the PQQ-treated SW1353 and Saos-2 cells than the PBS-treated SW1353 and Saos-2 cells (Fig. 1C). These findings demonstrate that the cell death rate of the CHS cells showed a dose- and time-dependent increase with PQQ treatment, while the effect on normal cells was relatively small.

PQQ induced apoptosis of CHS cells by activation of the mitochondrial caspase-dependent pathway. Western blot

analysis showed that the level of caspase-3 in the chondrosarcoma cells gradually decreased with an increase in the concentration of PQQ (Fig. 2A). Upon combined treatment with Z-VAD-fmk and PQQ, the level of procaspase-3 was significantly inhibited in the chondrosarcoma cells (Fig. 2B). Co-immunoprecipitation analysis showed that the binding of Smac to XIAP was significantly increased and the binding of XIAP with caspase-3 was significantly decreased in the combined PQQ- and H_2O_2 -treated cells compared with the control cells. This difference between the control and treatment groups was not observed in the case of treatment with only H_2O_2 , only TNF- α or only cisplatin (Fig. 2C-F).

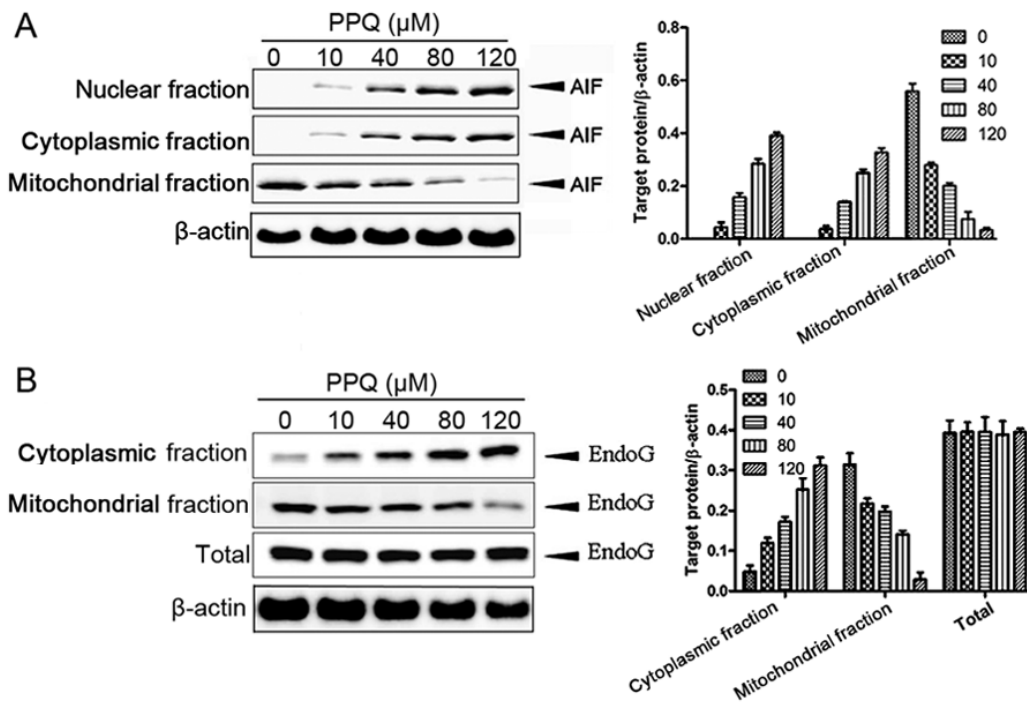


Figure 3. Effect of PQQ on the level of mitochondrial caspase-independent apoptosis pathway proteins. (A) Western blot analysis showing the level of AIF in the mitochondria, cytoplasm and nucleus after treatment of SW1353 cells with different concentrations of PQQ for 24 h. (B) Western blot analysis showing the level of EndoG in the mitochondria, cytoplasm and total protein fraction after treatment of SW1353 cells with different concentrations of PQQ for 24 h. PQQ, pyrroloquinoline quinone.

Furthermore, we found that the protein level of cytochrome *c* in the cytoplasm gradually increased with an increase in the concentration of PQQ, while its level in the mitochondria gradually decreased. Notably, the amount of total cytochrome *c* protein was not affected by the increased PQQ concentration (Fig. 2G). These results demonstrate that PQQ can induce apoptosis of CHS cells by activating the mitochondrial caspase-dependent apoptosis pathway.

PQQ induces the apoptosis of CHS cells by activating the mitochondrial non-caspase-dependent pathway. Western blot analysis showed that the level of AIF protein in the cytoplasm and nucleus was gradually upregulated with an increase in the concentration of PQQ, while the level of AIF protein in the mitochondria was gradually decreased (Fig. 3A). Furthermore, the level of the EndoG protein was gradually increased with an increase in the concentration of PQQ in the cytoplasm, but its level showed a gradual decrease in the mitochondria (Fig. 3B). However, the total amount of EndoG did not change in correspondence with the PQQ concentration. These results indicate that PQQ could induce apoptosis in the CHS SW135 cells by activating mitochondrial non-caspase-dependent pathways.

PQQ reduces the membrane potential of CHS cells and inhibits the growth of CHS cell xenografts. We used JC-1 staining combined with flow cytometry to detect mitochondrial membrane potential in response to treatment with 120 μM PQQ. The results indicated that PQQ reduced the membrane potential compared with the PBS group (Fig. 4A and B). To investigate whether PQQ had similar effects in *in vivo* conditions, SW1353 cells were xenografted into BALB/c nude mice. The tumor volume was significantly smaller after treatment with PQQ

than after control treatment (Fig. 5A and B). These findings indicate that PQQ can inhibit the growth of chondrosarcoma cell xenografts by inhibiting proliferation and promoting apoptosis of these tumor cells.

Discussion

In the present study, we provide initial evidence for the role of mitochondrial apoptotic pathways in the antitumor effect of pyrroloquinoline quinone (PQQ) against chondrosarcoma (CHS).

Reduction in mitochondrial membrane potential is considered to be an indicator of mitochondrial damage and early apoptosis. In the present study, the mitochondrial membrane potential was significantly reduced with PQQ treatment in comparison with the control groups. These findings indicate that PQQ treatment causes a change in mitochondrial membrane permeability, which is one of the key events in initiating mitochondrial pathways. Mitochondrial apoptosis is a well-known apoptotic signaling pathway that is accompanied by mitochondrial depolarization, cytochrome *c* over-release and caspase-3 activation (22), and the mitochondrial apoptotic pathways can be caspase-dependent or caspase-independent apoptotic pathways (23). In the caspase-dependent cytochrome *c* pathway, mitochondrial instability results in the redistribution of cytochrome *c* into the cytoplasm, which triggers apoptosis via the continuous activation of caspase-9 and caspase-3 (24,25). In the caspase-dependent Smac pathway, Smac is released into the cytoplasm during the onset of programmed apoptosis, where it specifically binds to XIAPs and prevents the inhibitory effect of XIAP on caspase precursors (26). In the present study, the level of caspase-3 in the

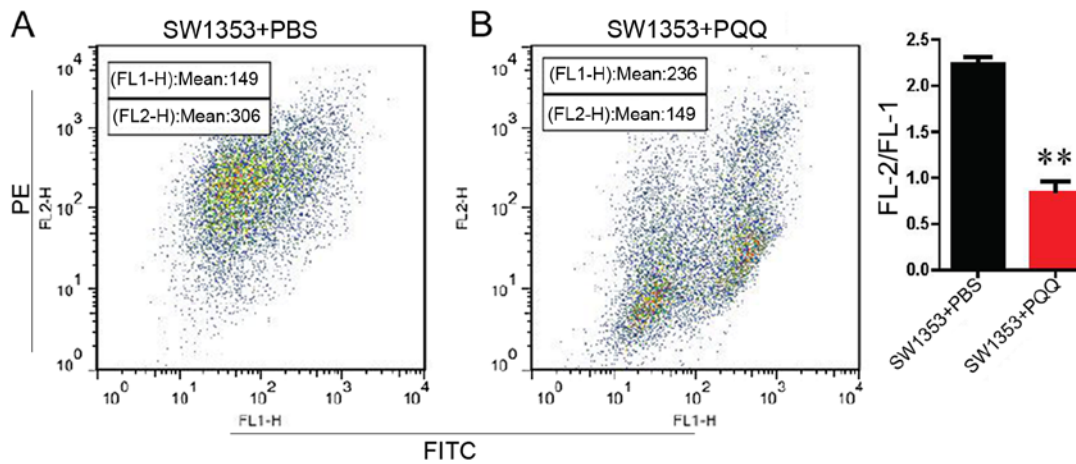


Figure 4. Effect of PQQ on the mitochondrial membrane potential of SW1353 cells. (A) Mitochondrial membrane potential in SW1353 cells treated with or without 120 μ M PQQ for 24 h. (B) FLH2/FLH1 ratio in SW1353 cells treated with or without 120 μ M PQQ for 24 h. ** $P < 0.01$ vs. PBS treatment. PQQ, pyrroloquinoline quinone.

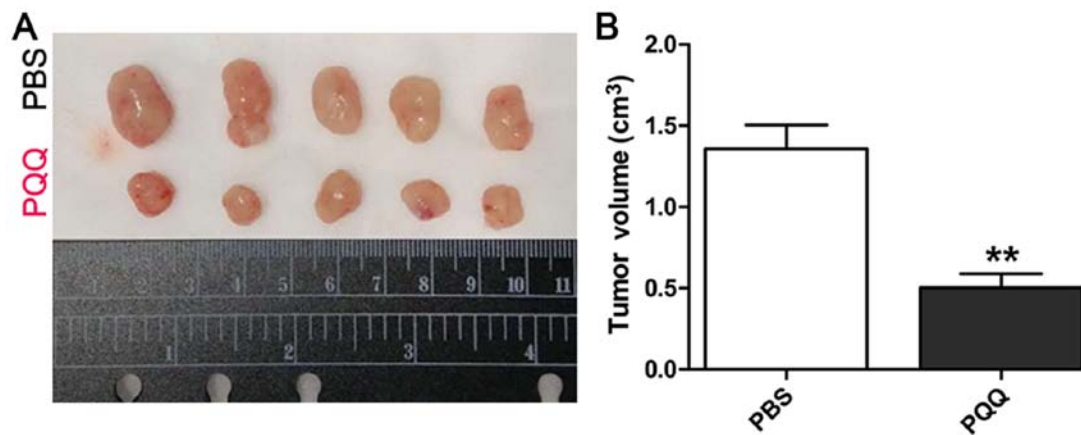


Figure 5. *In vivo* effect of PQQ on CHS growth. (A) After 10 days of PQQ treatment, the mice with the implanted tumors were euthanized and their tumors were extracted. (B) Tumor volume was calculated and found to be lower in the PQQ-treated group than that in the control group. ** $P < 0.01$ vs. PBS treatment. PQQ, pyrroloquinoline quinone.

chondrosarcoma cells was gradually decreased with increase in the concentration of PQQ. Further, the binding of Smac to XIAP was significantly increased while the binding of XIAP with caspase-3 was significantly decreased. Finally, the level of cytochrome *c* in the cytoplasm was gradually increased with an increase in the concentration of PQQ, and it was accompanied by a decrease in the mitochondrial cytochrome *c* level. All the findings indicate that the anticancer effects of PQQ in chondrosarcomas may involve the induction of apoptosis via regulation of caspase-dependent pathways.

Caspase-independent mitochondrial apoptotic pathways mainly include AIF- and EndoG-induced apoptosis pathways. AIF is a flavoprotein which is usually confined to the mitochondrial intermembrane space. New evidence indicates that the transport of mitochondrial AIF into the cytoplasm and then the nucleus is an indicator of caspase-independent apoptosis (27). In this study, PQQ treatment was found to result in increased levels of AIF protein in the cytoplasm and nucleus and decreased levels in the mitochondria. EndoG, another mitochondrial factor, is also transported from the mitochondria to the cytoplasm and then the nucleus upon

induction of apoptosis (28). Similar to the results for AIF, the levels of EndoG protein were also increased in the cytoplasm and decreased in the mitochondria with an increase in the concentration of PQQ. In the case of EndoG, the total amount in the cells did not change with PQQ treatment; this confirms that the higher cytoplasmic levels observed were a result of the transport of this protein from the mitochondria and not an increase in its expression. Thus, these findings indicate that PQQ induces apoptosis of the CHS cell line SW1353 by activating mitochondrial caspase-independent pathways.

Finally, we confirmed the *in vitro* effects by showing that PQQ had *in vivo* inhibitory effects on tumorigenesis and caused a decrease in tumor size. Thus, PQQ may be able to inhibit the proliferation of CHS cells. The *in vivo* mechanisms are probably similar to the *in vitro* ones, but they need to be studied in future investigations.

In conclusion, we established that the mechanism underlying PQQ-induced cancer cell apoptosis in CHS involves the activation of mitochondrial caspase-dependent as well as caspase-independent pathways. Thus, the proteins identified in

these pathways could be potential targets for the treatment of chondrosarcoma.

Acknowledgments

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CX and RW conceived the research idea; JP and MS performed the experiments; CX, RW and JP analyzed the data; CX wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experiments on animals were performed according to the Animal Care Guidelines of the First Affiliated Hospital of Zhejiang University School of Medicine and were approved by the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications, No. 8023, revised 1978). The animals experiments comply with the ARRIVE guidelines and the AVMA euthanasia guidelines 2013.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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