Oligomeric proanthocyanidins inhibit apoptosis of chondrocytes induced by interleukin-1β

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Abstract. Oligomeric proanthocyanidin (OPC) is a water-soluble plant polyphenolic compound known for its cytoprotective effects in various tissue types. However, its effect on chondrocytes has not been well characterized. The present study aimed to investigate the effect of OPC on interleukin-1β (IL-1β)-induced apoptosis in chondrocytes, and to determine the mechanisms underlying the protective effects of OPC. Knee articular chondrocytes obtained from 6-week-old SPF Kunming mice were cultured and serially passaged. First-generation chondrocytes were selected for subsequent experiments following toluidine blue staining. Subsequent to IL-1β and OPC administration, an MTT assay was performed to examine the viability rate of chondrocytes, and the optimal drug concentration was determined. The fluorescence dye 2',7'-dichlorofluorescein diacetate was used to determine the intracellular content of reactive oxygen species (ROS). Mitochondrial membrane potential (MMP) was measured using a 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) assay. The apoptosis rate of chondrocytes was assessed using an Annexin V-FITC/PI assay and ultrastructural changes were observed under an electron microscope. The results demonstrated that OPC increased the survival rate of chondrocytes against IL-1β-induced apoptosis. The most significant protective effect of OPC was observed at the concentration of 0.050 mg/ml. OPC reversed the increased ROS content and MMP levels, and inhibited IL-1β-induced apoptosis in chondrocytes. In addition, OPC was revealed to protect the ultrastructural integrity of chondrocytes. Taken together, the results of the present study suggest that OPC protects chondrocytes against IL-1β-induced damage by decreasing ROS content and MMP levels.

Introduction

Osteoarthritis (OA) is an age-related disease of joints characterized by degeneration of articular cartilage and deranged chondrocyte metabolism. In a healthy state, milder injuries to the articular cartilage are gradually compensated by the dynamic metabolic balance (1). However, due to the imbalance in chondrocyte metabolism in pathological states, the healing of articular cartilage does not take place.

Chondrocytes are present throughout the cartilage tissue in a low density and help maintain its integrity. Many soluble proteins and cytokines are known to affect the anabolic and catabolic processes in chondrocytes (2). Patients with OA experience a gradual reduction in chondrocytes due to apoptotic cell death (3,4). Studies have documented a significantly higher number of apoptotic cells in the articular cartilaginous tissue of OA patients as compared to that in healthy subjects (5). Thus, degeneration of cartilage in inflammatory joint disease might be indirectly related to the apoptosis of chondrocytes (6,7). This may be mediated by secretion of several cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) in the synovial fluid.

Studies have shown an increased level of these cytokines in the articular synovial fluid of OA patients during episodes of acute exacerbation (8). Studies have also shown that these cytokines can inhibit synthesis of cartilage matrix and induce degradation of cartilaginous tissue (9,10). Furthermore, these proinflammatory factors have been shown to induce production of reactive oxygen species (ROS) such as peroxynitrite, superoxide, nitric oxide and hydrogen peroxide, in the articular tissues (11-13).

Production of ROS serves as a physiological defence mechanism against microbial infection. In addition, it is also involved in the maintenance of normal cellular functions and intracellular signal transduction. However, excess ROS are capable of damaging lipid, protein, DNA and the extracellular matrix. IL-1β was shown to mediate apoptosis of chondrocytes

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by combining with ROS scavengers or by inducing activation of CD95 (14). IL-1β-induced apoptosis was found to be ROS-dependent. Both spontaneous and induced lipid peroxidation has been demonstrated in chondrocytes during the initial stages of OA (15).

Oligomeric proanthocyanidin (OPC), a polyphenolic compound obtained from plants, has a powerful antioxidant property (16,17). Proanthocyanidins are extracted from various types of plants as complex mixtures of structurally related compounds such as catechin, epicatechin, and their gallic acid esters. Among these, catechin has a low biological activity owing to its poor bioavailability. However, binding of these three compounds as dimers and trimers renders them as oligomeric (OPC) with high bioavailability and beneficial health effects. The absorption of proanthocyanidins depends on their molecular weight. Most proanthocyanidins with large molecular weight are not excreted in urine, as its dimer has been found in mouse and human serum after oral ingestion (18,19). This indicates that OPC is liable to absorption in the intestines (20). OPC is widely studied owing to its high content in human diet and its powerful antioxidant properties (21).

Recent studies have demonstrated the relevance of OPC in the treatment of ulcers, allergic disorders, caries and tumors (22,23). OPC has also been shown to control the incidence of food allergy, stimulate hair follicle growth and protect cells against ultraviolet damage (24-30). However, to date few studies have investigated the potential role of OPC in protecting against OA. In this study, we sought to investigate the protective effects of OPC via inhibition of IL-1β mediated ROS-induced apoptosis of chondrocytes *in vitro*. Our results suggest that OPC may represent a novel therapy for the treatment of OA.

Materials and methods

Reagents and equipment. DMEM/F12 medium, recombinant mouse IL-1β was obtained from Bioengineering Co., Ltd. (Songjiang, Shanghai, China); HyClone™ fetal bovine serum was obtained from GE Healthcare (Logan, UT, US); type II collagenase was obtained from Worthington Biochemical Corp. (Lakewood, NJ, USA); oligomeric proanthocyanidins were purchased from Weikeqi Biological Ltd. (Chengdu, Sichuan, China); trypsin from Gibco (Grand Island, NY, USA); MTT kit from Amresco (Solon, OH, USA); reactive oxygen species detection kit (DCFH-DA), mitochondrial membrane potential assay kit (5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-cabocyanine iodide, JC-1) obtained from Beyotime Institute of Biotechnology (Songjiang, Shanghai, China); flow cytometry apoptotic detection kit from BD Biosciences (Lake Franklin, NJ, USA); microplate reader from Thermo Fisher Scientific Inc. (Waltham, MA, USA); flow cytometry from Beckman Coulter, Inc. (Brea, CA, USA); JEM-2000EX transmission electron microscope from JEOL Ltd. (Akishima, Tokyo, Japan).

Cell culture and characterization of chondrocytes from mice. Six-week-old Kunming mice (male or female), were obtained from SPF Experimental Animal Center at the Dalian Medical University, China. Articular cartilage was dissected, cut and grinded under sterile environment; digested with 0.2% type II collagenase for 6 h; and filtered using a 74 μm diameter mesh steel filter. The filtrate was centrifuged and the supernatant was discarded. Chondrocytes were re-suspended in DMEM/F12 (with 10% FBS) and cultured in 5% CO_2 at 37°C incubator. Staining of chondrocytes with 1% toluidine blue confirmed their first-generation lineage; these were used in the subsequent experiments after achievement of 90% confluence.

Cell viability detection. Chondrocytes were seeded onto 96-well plates at a concentration of 10^5 cells in each well. A total of 10 groups were made with 3 replicates in each group. The experimental groups (Group 2-10) were incubated with 2 μl of 1,000 μg/l IL-1β solution (to achieve a final concentration of 10 μg/l) and co-treated with OPC at final concentrations of 0, 0.001, 0.005, 0.010, 0.050, 0.100, 0.500, 1.000 and 5.000 mg/ml. Simultaneously, chondrocytes in the control group (Group 1) were treated with 2 μl PBS. Serum-free DMEM/F12 was added to all wells to obtain a total volume of up to 200 μl. After incubation for 24 h, cell viability was determined by MTT assay.

Chondrocytes were seeded onto 96-well plates at a concentration of 10^5 cells in each well. A total of 9 groups were made with 3 replica wells in each group. The nine groups were incubated with OPC at a concentration of 0, 0.001, 0.005, 0.010, 0.050, 0.100, 0.500, 1.000 and 5.000 mg/ml. Serum-free DMEM/F12 was added to all wells to obtain a total volume of up to 200 μl. After incubation for 24 h, cell viability was determined by MTT assay.

Reactive oxygen species (ROS) detection in chondrocytes after OPC and IL-1β treatment. Chondrocytes were seeded as mentioned above. After determining the optimal OPC concentration for cell survival, three groups (10 wells per group) were established: i) Control group: Treated with 2 μl 0.1 M PBS and 198 μl serum-free DMEM/F12; ii) IL-1β group: Treated with 10 μg/l IL-1β; and iii) OPC+IL-1β group: Treated with 10 μg/l IL-1β plus 0.050 mg/ml OPC.

After incubation for 24 h, ROS levels in the chondrocytes were determined using a ROS kit. Briefly, 100 μl of 10 μmol/l fluorescent probe DCFH-DA was added into each well, and incubated at 37°C for 20 min. After removal of the supernatant, the cells were washed with 200 μl serum-free DMEM/F12 and fluorescence was immediately determined using a microplate reader at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Mitochondrial membrane potential (MMP) in chondrocytes after OPC and IL-1β treatment. Chondrocytes were seeded and treated as mentioned in the above ROS experiment. After incubation for 24 h, mitochondrial membrane potential (MMP) in chondrocytes was determined using JC-1 kit. Briefly, the supernatant was removed, and 100 μl of serum-free DMEM/F12 was added per well, and subjected to 100 μl of JC-1 staining. After incubation at 37°C for 20 min, the supernatant was removed. The cells were treated with 200 μl DMEM/F12, and the fluorescence detected using a microplate reader. Red fluorescence is observed in the presence of normal MMP, while reduced levels of MMP is associated with green fluorescence. Therefore, we used the ratio of green and red fluorescence intensity as a measure of MMP.

Determination of apoptosis in chondrocytes after OPC and IL-1β treatment by Annexin V-FITC. Chondrocytes were

seeded onto nine 60-mm petridishes, which were then divided into 3 equal groups as described above: Control, IL-1β, and OPC+IL-1β. After incubation for 24 h, chondrocytes were trypsinized and re-suspended in 500 μl binding buffer mixed with 10 μl Annexin V-FITC and 10 μl PI and incubated for 15 min at room temperature in dark. Assessment of apoptosis was performed by flow cytometry.

Examination of chondrocytes ultrastructure by electron microscopy. Chondrocytes were seeded and treated as described above. After trypsinization and centrifugation at 400 x g for 5 min, cells were pre-fixed with glutaraldehyde at a volume fraction of 2.5% and post-fixed with 1 g/l osmic acid. The specimens were then immersed in propylene oxide after dehydration with gradient ethanol, embedded with epoxy resin and ultrathin sections prepared. The sections were stained with lead-uranium and the ultrastructural changes examined under a transmission electron microscope.

Statistical analysis. Data pertaining to variables with normal distribution are expressed as mean ± standard deviation. Inter-group differences were assessed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $P<0.05$ was considered as statistically significant. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

Results

The suitable concentration of OPC on IL-1β-induced death of chondrocytes. The effect of different concentrations of OPC on IL-1β-induced cell death in primary chondrocytes was examined by MTT assay. Results showed a significant decrease in viability of primary chondrocytes after treatment with IL-1β, as compared to that observed in the control group (Fig. 1). OPC treatment showed significant protective effects against IL-1β-induced cell death at concentrations of 0.005, 0.010, 0.050, 0.100, 0.500, and 1.000 mg/ml. Among these, a concentration of 0.050 mg/ml appeared to confer maximal protection and reversed the decrease in cell viability induced by IL-1β. Therefore, 0.050 mg/ml of OPC was used in the subsequent experiments.

Viability of chondrocytes with OPC only. Cell viability of chondrocytes after treatment with OPC was examined by MTT assay. Results showed that cell viability rate decreased significantly until the concentration of OPC approached 0.500 mg/ml (Fig. 2).

ROS levels in primary chondrocytes treated with IL-1β and OPC. ROS levels in three groups were assessed (control, IL-1β and IL-1β+OPC). IL-1β at a concentration of 10 μg/l induced significant increase in ROS levels in the chondrocytes. However, the effect could be reversed by co-treatment with 0.050 mg/ml OPC (Fig. 3), which indicates an antioxidant effect of OPC in chondrocytes.

The effects of OPC on mitochondrial membrane potential in primary chondrocytes. To further detect the effects of OPC on MMP, we used the JC-1 to detect the level of MMP with
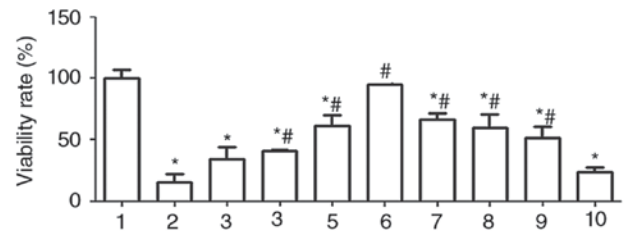

Figure 1. Results of MTT assay illustrating protective effects of OPC on primary chondrocytes against IL-1β-induced cell death. Chondrocytes were treated with 10 μg/l of IL-1β in Groups 2 to 10. OPC was administered at concentrations of 0, 0.001, 0.005, 0.010, 0.050, 0.100, 0.500, 1.000 and 5.000 mg/ml in Groups 3 to 10, respectively. Following incubation for 24 h, cell viability was assayed by MTT. *$P<0.05$ vs. Group 1; #$P<0.05$ vs. Group 2. MTT, 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide; OPC, oligomeric proanthocyanidin; IL-1β, interleukin-1β.
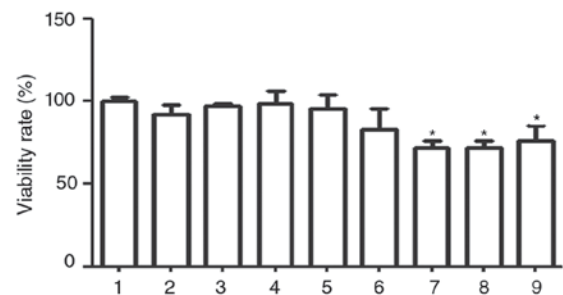

Figure 2. Viability of chondrocytes after treatment with OPC only. Chondrocytes were treated with OPC at concentrations of 0, 0.001, 0.005, 0.010, 0.050, 0.100, 0.500, 1.000 and 5.000 mg/ml in Groups 1-9, respectively. Following incubation for 24 h, cell viability was assayed by MTT. *$P<0.05$ vs. Group 1 (OPC 0 mg/ml).
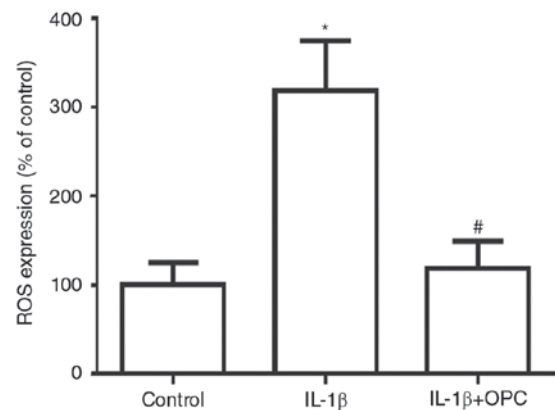

Figure 3. Effects of OPC on ROS level in primary chondrocytes by study group (Control, IL-1β and IL-1β+OPC groups). Control group received vehicle treatment. IL-1β+OPC and IL-1β groups received 10 μg/l IL-1β treatment with and without 0.050 mg/ml OPC, respectively. *$P<0.05$ vs. control; #$P<0.05$ vs. IL-1β. ROS, reactive oxygen species; OPC, oligomeric proanthocyanidin; IL-1β, interleukin-1β.

or without OPC treatment. The ratio of green and red fluorescence, which reflects the MMP level, was significantly higher in IL-1β group as compared to that in the control group; this finding was indicative of an enhanced expression of MMP. However, 0.050 mg/ml OPC treatment significantly reduced MMP expression in chondrocytes as compared to that in the IL-1β group, although not reaching the levels in the controls.
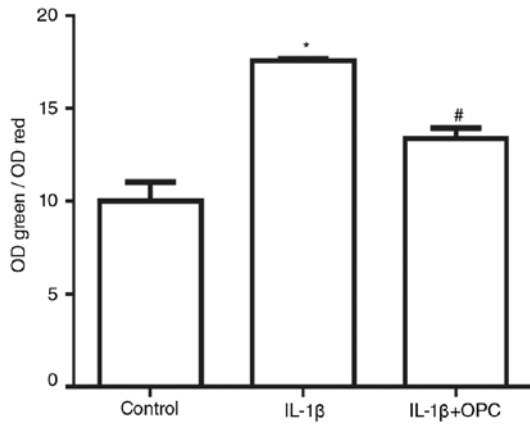
Figure 4. Effects of OPC on mitochondrial membrane potential (MMP) in primary chondrocytes by study group (Control, IL-1β and IL-1β+OPC groups). Control group received vehicle treatment. IL-1β+OPC and IL-1β groups received 10 μg/l IL-1β treatment with and without 0.050 mg/ml OPC, respectively. *P<0.05 vs. control; #P<0.05 vs. IL-1β.
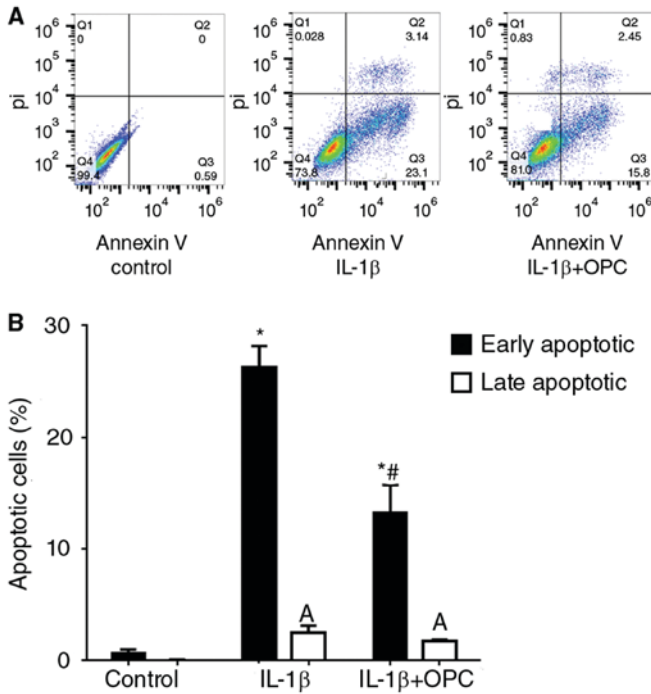

Figure 5. Effects of OPC on IL-1β-induced early and late apoptosis in primary chondrocytes by study group (Control, IL-1β and IL-1β+OPC). (A) Representative image of flow cytometry. (B) Statistical results of early and late apoptosis in chondrocytes in 3 groups. Control group received vehicle treatment. IL-1β+OPC and IL-1β groups received 10 μg/l IL-1β treatment with and without 0.050 mg/ml OPC, respectively. Inter-group differences assessed by one-way ANOVA followed by Tukey's post hoc test. *P<0.05 vs. early apoptosis control; #P<0.05 vs. early apoptosis IL-1β; AP<0.05 vs. late apoptosis control.
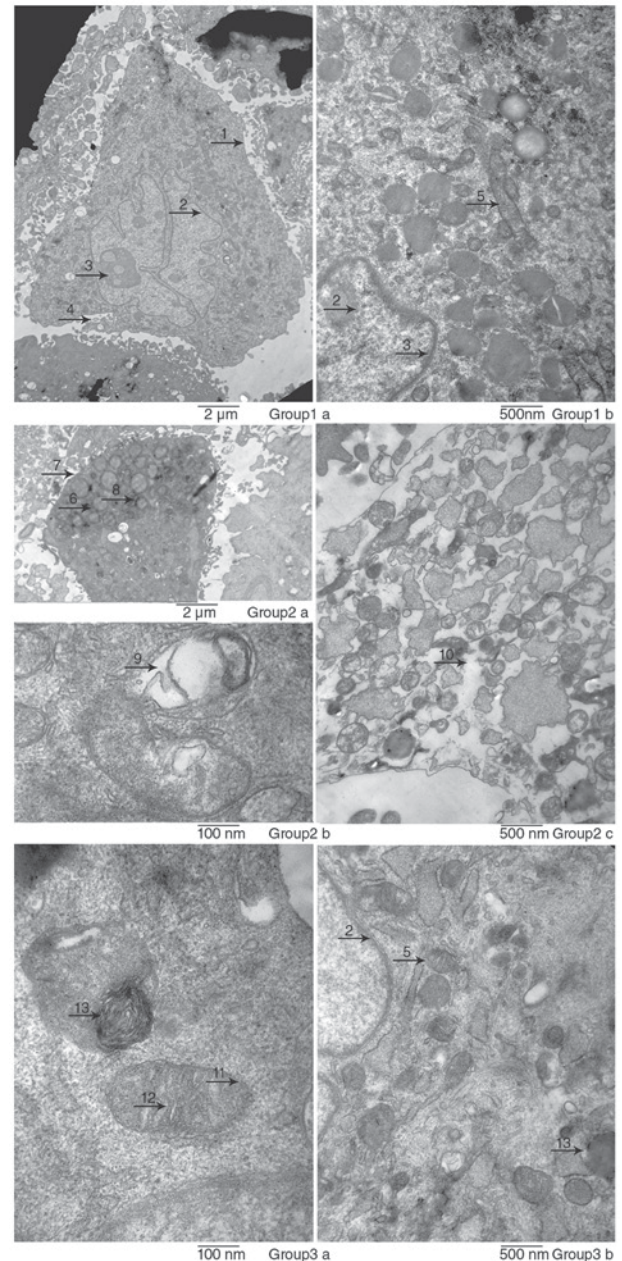

Figure 6. Effect of OPC on ultrastructure of chondrocytes observed by electron microscopy. Group 1: Control group chondrocytes (A) x6000 (B) x25,000; Group 2: IL-1β treated chondrocytes (A) x6000, (B) x10,000, (C) x20,000; Group 3: IL-1β+OPC group chondrocytes (A) x100,000 and (B) x40,000. Control group received vehicle treatment. IL-1β+OPC and IL-1β groups received 10 μg/l of IL-1β with and without 0.050 mg/ml OPC, respectively. 1) Intact plasma membrane; 2) intact nuclear membrane with ribosomes; 3) clear nucleolus; 4) intensive microvilli on plasma membrane surface; 5) clear mitochondria; 6) pyknosis in nucleolus; 7) reduced plasma surface microvilli; 8) abundant apoptotic bodies; 9) mitochondrial edema; 10) vacuolization of organelles; 11) intact mitochondrial membrane; 12) lamellar mitochondrial cristae; 13) scant apoptotic bodies.

The results indicate that OPC partially reversed the increase in MMP induced by IL-1β (Fig. 4).

The effects of OPC on IL-1β-induced apoptosis in primary chondrocytes. Quadrant 2 (Q2) represents late apoptosis and Quadrant 3 (Q3) represents early apoptosis. IL-1β treatment was associated with a significant increase in both early and late apoptosis in chondrocytes, as compared to that in the control group. OPC treatment markedly inhibited the early and late apoptosis, although it did not reach the levels observed in the control group. Thus, OPC appears to cause a partial inhibition of IL-1β-induced apoptosis in chondrocytes (Fig. 5).

Effects of OPC on chondrocyte ultrastructure. The effects of OPC on IL-1β-induced changes in chondrocyte ultrastructure were observed under transmission electron microscopy (TEM).

Chondrocytes in the control group showed active proliferation and intact bilayer cell membrane with intensive cell surface microvilli. Nuclear membrane structure was also found intact with a large number of ribosomes attached to it. Clearly visible nucleoli indicated active cell metabolism. Mitochondria were short and rod-shaped with clearly discernible intact bilayer membrane structure and lamellar mitochondrial cristae (Fig. 6, Group 1). Apoptotic chondrocytes observed in the IL-1β group were of small cell size, appeared to have concentrated cytoplasm, had reduced surface microvilli, with evidence of mitochondrial edema, and presence of several autophagic vesicles. Some chondrocytes showed swollen organelles and vacuolization (Fig. 6, Group 2). OPC-treated group showed intact plasma and nuclear membranes with attached ribosomes and visible nucleoli. Active cell metabolism, intensive microvilli as well as scattered autophagic vesicles were also observed. Mitochondria had intact bilayer membrane with lamellar mitochondrial cristae (Fig. 6, Group 3). These observations confirmed the occurrence of apoptosis in chondrocytes after IL-1β administration and also demonstrated the protective effects of OPC against IL-1β-induced cell damage.

Discussion

Osteoarthritis (OA) is a common disease in older age-groups caused due to an imbalance in cartilage degradation and synthesis. Chondrocyte apoptosis contributes to cartilage degradation and is a basic pathogenetic feature of degenerative joint diseases, including osteoarthritis and rheumatoid arthritis. As the only cells in articular hyaline cartilage, chondrocyte apoptosis leads to an imbalance between anabolic and catabolic metabolic pathways in the entire cartilage. This is thought to play an important role in the pathogenesis of OA. Inhibition of chondrocyte apoptosis is a potential target for developing preventive and therapeutic modalities for OA.

OA involves damage not only to the articular cartilage, but also to the subchondral bone, ligaments, synovial membrane and periarticular muscle tissue. Studies have shown a significantly increased levels of IL-1β in synovial fluid, synovial, cartilaginous tissue and subchondral bone in patients with OA (31-34), indicating that degradation and apoptosis of chondrocyte in OA patients may be related to the increased IL-1β levels (35). Therefore, in this study, we used IL-1β to induce apoptosis in chondrocytes to mimic the model of OA and further investigated the effects of OPC. IL-1β significantly reduced survival of primary chondrocytes, while co-administration of OPC appeared to reverse the IL-1β-induced cell death. Increased apoptosis after IL-1β administration appeared to have contributed to the loss of chondrocytes. OPC co-treatment with IL-1β appeared to reverse both early and late apoptosis, as well as ROS production in chondrocytes.

Oxidative stress is considered a key pathophysiological factor in the progression of OA. It refers to a microcellular milieu with enhanced production of ROS and/or decrease in the levels of antioxidants. Excessive levels of ROS have been shown to induce loss of superoxide dismutase *in vivo* (36), peroxide-induced damage of DNA and abnormal protein expression, which may in turn induce chondrocyte apoptosis, inhibit cartilage matrix synthesis and promote cartilage degradation. Previous studies have shown the beneficial therapeutic

effects of antioxidants such as vitamins E and C in clinical trials as well as in animal models of OA (37). Thus, we hypothesized that OPC as a powerful antioxidant may ameliorate the progress of OA by inhibiting the production of ROS and the apoptosis of chondrocytes. In our study, ROS level was found to increase significantly after IL-1β administration. However, the elevated levels were reversed by OPC treatment.

Our results are supported by those from other animal models of OA. Woo *et al* demonstrated protective effects of grape seed proanthocyanidin extract against joint damage in monosodium iodoacetate-induced OA (38). Procyanidin B3, a procyanidin dimer, has been shown to prevent degeneration of articular cartilage and heterotopic cartilage formation in a surgically-induced mouse model of osteoarthritis (39). IL-1β administration significantly increased the mitochondrial membrane potential in primary chondrocytes as compared to that in the control group. Co-treatment with OPC appeared to partially reverse this effect, which suggests an antioxidant effect of OPC.

The protective effect of OPC in IL-1β-induced apoptosis in primary chondrocytes was also substantiated by maintained ultrastructural integrity observed under electron microscope. Control group chondrocytes showed intact bilayer cytomembrane, nuclear and mitochondrial membranes with intensive microvilli, ribosomes and lamellar cristae. In contrast an increased number of apoptotic chondrocytes were observed in IL-1β group that were characterized by concentrated cytoplasm, reduced microvilli, swollen organelles and vacuolization. OPC treatment appeared to confer protection against IL-1β-induced damage, although a few of apoptotic chondrocytes were still observable.

Therefore, we found that OPC can inhibit apoptosis by protecting mitochondria. Similar results have been proven in other kinds of cells (39-43). In this study, we used chondrocytes derived from mice and did not compare with chondrocytes of other animals. This is a limitation of our experiment.

In conclusion, IL-1β induced injury to primary chondrocytes was reflected in the significant increases in ROS production, MMP levels and apoptosis, as compared to that in the control group. OPC treatment increased the survival rate of chondrocytes by reducing ROS content and MMP levels. The protective effect of OPC against IL-1β-inducd apoptosis and ultrastructural damage of chondrocytes was also demonstrated.

In this study, OPC appeared to protect chondrocytes against IL-1β-induced damage by reducing ROS production and MMP levels, which could potentially be a new way to prevent or treat OA.

Acknowledgements

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