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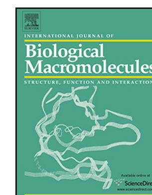

C-phycocyanin alleviates osteoarthritic injury in chondrocytes stimulated with H_2O_2 and compressive stress

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ABSTRACT

During the progression of osteoarthritis (OA), dysregulation of extracellular matrix anabolism, abnormal generation of reactive oxygen species (ROS) and inflammatory cytokines have been shown to accelerate the degradation process of cartilage. The potency of c-phycocyanin (C-PC) to protect cellular components against oxidative stress, along with its anti-inflammation and anti-apoptosis effects, are well documented; however, effects of C-PC on OA are still unclear. In this study, we aimed to investigate the effects of C-PC on OA using H_2O_2 or compression-stimulated OA-like porcine chondrocyte models. The results showed that C-PC had the ability to inhibit ROS production, reverse caspase-3 activity, and reduce apoptosis cell population. C-PC also reversed aggrecan and type II collagen gene expressions after stimulation with 1 mM H_2O_2 or 60 psi of compression. Inhibition of IL-6 and MMP-13 genes was observed in compression-stimulated chondrocytes but not in H_2O_2-treated cells. In dimethylmethylene blue assay and alcian blue staining, C-PC maintained the sulfated-glycosaminoglycan (sGAG) content after stimulation with compression. We concluded that C-PC can prevent early signs of OA caused by compressive stress and attenuate H_2O_2-induced oxidative stress. Therefore, we suggest that C-PC can be used as a potential drug candidate for chronic OA treatment.

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1. Introduction

C-phycocyanin (C-PC) is one of the major biliproteins purified from blue-green algae, such as *Spirulina platensis* [1]. In addition to its health benefits, C-PC has been used as a dietary nutritional supplement and natural colorant in nutraceutical, cosmetics, and pharmaceutical industries [2]. Numerous beneficial effects of C-PC have been demonstrated, including anti-inflammatory, anti-apoptosis, and antioxidant properties, all of which are critical properties to prevent further progression of the inflammatory process [1,3–5]. Thus, C-PC is used as a potential therapeutic agent in oxidative stress-induced disease [6]. Therefore, the use of C-PC as a potential compound for inflammatory disease prevention and symptom alleviation of affected tissue, including osteoarthritic cartilage, has recently raised considerable interests.

Osteoarthritis (OA) is the most common chronic condition of the joints and a leading cause of disability in people >65 years of age [7,8]. It primarily affects the weight-bearing joints, including hip, knee, and spine joints, and involves joint inflammation, degeneration of articular cartilage, and related changes of subchondral bone, ligaments, periarticular muscle, and synovium [8]. Articular cartilage is composed of extensive amounts of extracellular matrix (ECM), mainly consisting of type II collagen, hyaluronic acid, and sulfated-glycosaminoglycan (sGAG) [9]. Chondrocytes are the unique cellular components of cartilage, which are responsible for the synthesis and degradation of ECM. In OA cartilage, several events have been shown to accelerate the degradation process of cartilage, such as the dysregulation of ECM anabolism, abnormal generation of reactive oxygen species (ROS), and overproduction of proteolytic enzymes and inflammatory cytokines [10,11]. Disruption of ECM homeostasis also decreases type II collagen and sGAG content and leads to chondrocyte apoptosis, which is associated with the caspase proteolytic cascade. All these events are present in OA cartilage [12].

Multiple risk factors are associated with OA, most notably age, obesity, local inflammation, and mechanical stresses [13,14]. Excessive compressive stress can trigger the production of ROS from chondrocytes, which may lead to hyaluronic acid depolymeriza-

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tion or even cell death [15,16]. In response to compressive stress, chondrocytes exhibit the same changes in gene expression as those during OA, such as upregulation of the matrix metalloproteinase 13 gene (MMP-13) and the gene family of disintegrin and metalloproteinase with thrombospondin motifs. Therefore, cartilage explants stimulated with mechanical stresses have been used as typical experimental models [17]. In addition to compressive stress, increasing evidence has shown that oxidative stress is also responsible for osteoarthritic development [18]. Articular chondrocytes actively produce numerous forms of ROS, including superoxide, hydroxyl radical, hydrogen peroxide (H_2O_2), and reactive nitrogen species [19]. H_2O_2 is a potent mediator of membrane lipid peroxidation. The mitochondrial membrane disruption caused by H_2O_2 affects cell viability and contributes to the disruption of extracellular matrix homeostasis [18,20]. Thus, H_2O_2 is used as an inducer of oxidative stress to examine the potency of antioxidants in mitigating cellular injury [4].

In the present study, we introduced H_2O_2 or external compression to porcine chondrocytes to generate an OA-like cell model to examine the effects of C-PC on OA-related signs. The effects of C-PC on the following gene expressions were investigated: ECM-related genes (type II collagen and aggrecan), catabolic matrix MMP-13 gene, and the proinflammatory cytokine interleukin-6 (IL-6) gene. Production of ROS, caspase-3 activity, chondrocyte apoptosis, cell proliferative activity and cytotoxicity, and sGAG content were evaluated to assess the impact of C-PC on chondrocytes. Finally, we discuss the potential utility of C-PC in prevention of OA in light of our findings.

2. Materials and methods

2.1. Isolation of chondrocytes

Chondrocytes were isolated from macroscopically normal cartilage of the femoral condyles of porcine [21]. Finely diced cartilage pieces were treated with 10% antibiotic PBS solution (Gibco, Waltham, MA, USA) at 37 °C for 10 min. Then, these were resuspended in Dulbecco's modified eagle's medium (DMEM; Sigma, St. Louis, MO, USA), containing 10% fetal bovine serum (SAFC, USA), 1% penicillin, and 0.05% L-ascorbic acid (Sigma, St. Louis, MO, USA) with 0.2% collagenase (Sigma, St. Louis, MO, USA) at 37 °C for 18 h. Chondrocytes were collected and cultured in DMEM.

2.2. Cell proliferation and cytotoxicity of C-PC on chondrocytes

Chondrocytes were seeded in 96-well cell culture plates at a density of 1×10^4 cells/well and cultured in DMEM for 18 h. Cells were then cultured in a medium containing 3, 10, 30, and 100 μg/ml C-PC. To evaluate the cell proliferation of C-PC on chondrocytes, WST-1 (Cell Proliferation Reagent WST-1; Roche, Mannheim, Germany) was measured on days 1 and 3. The OD value was measured at 450 nm using the enzyme-linked immunosorbent assay (ELISA) reader (Sunrise Remote; Tecan, Durham, NC, USA).

The LDH (CytoTox96 Non-Radioactive Cytotoxicity Assay; Promega, Madison, WI, USA) assay was used on days 1 and 3 to evaluate the cytotoxicity of C-PC-treated chondrocytes. LDH released in the culture supernatants was measured with a 30-min coupled enzymatic assay and measured using the ELISA reader at a wavelength of 490 nm. The percentage of cytotoxicity was calculated using the following equation:

$$\text{Cytotoxicity}\,(\%) = \frac{\text{OD}_{\text{exp}} - \text{OD}_{\text{medium}}}{\text{OD}_{\text{total lysis}} - \text{OD}_{\text{medium}}} \times 100$$

2.3. C-PC pretreatment and induction of oxidative stress

C-PC was gifted to us by Far East Bio-Tec Corporation, Taiwan. The purity of C-PC was analyzed using A620/A280 absorbance ratio detection. In this study, we used C-PC with a purity of 4.07, which is qualified as analytical grade with A620/A280 >4.0 [2].

The chondrocytes were seeded in six-well cell culture plates at a density of 1×10^5 cells/well, cultured in DMEM with 10% fetal bovine serum (Gibco, Waltham, MA, USA), and incubated overnight. Cells were pretreated with C-PC 100 μg/ml for 24 h. After being washed with PBS, the cells were subjected to oxidative stress induced by stimulation with 1 mM H_2O_2 for 30 min, followed with another PBS wash. Chondrocytes were then cultured in fresh medium at 37 °C for 24 h before further experiments.

2.4. ROS scavenging effect

The ROS production was evaluated using the Total ROS/Superoxide Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA). Chondrocytes were pretreated with 0, 3, 10, 30, and 100 μg/ml of C-PC for 0.5, 1, 4, and 24 h. Chondrocytes were then stimulated with 1 mM H_2O_2 for 30 min. After H_2O_2 treatment and 24 h incubation, chondrocytes were collected, washed twice with PBS, and stained with 500 μl of the ROS detection mix for 30 min in the dark. They were then analyzed using the ELISA reader (Molecular Devices, USA). The fluorescence of ROS was also examined using a confocal laser scanning biological microscope (Olympus, Japan).

2.5. Caspase-3 activity

Chondrocytes were pretreated with C-PC 100 μg/ml for 24 h and then stimulated with 1 mM H_2O_2 for 30 min. After 24-h incubation, the total protein content was determined through a BCA protein assay kit (Pierce, Rockford, IL, USA). Caspase-3 activity was evaluated using the CaspACE assay system (Promega, Madison, WI, USA). Total protein (30 μg) from each sample was mixed with a reaction buffer containing 2 μl DMSO, 10 μl of 100 mM DTT, and 32 μl caspase assay buffer in a 96-well microplate. The colorimetric substrate, DEVD-pNA (2 μl; Asp-Glu-Val-Asp-*p*-nitroaniline), was then added and incubated at 37 °C for 4 h. The absorbance was measured at a wavelength of 405 nm using the ELISA reader (TECAN, Zürich, Switzerland).

2.6. Chondrocyte apoptosis

Chondrocyte apoptosis was evaluated using the Annexin V-FITC apoptosis detection kit (abcam, Cambridge, MA, USA). After C-PC pretreatment, H_2O_2 stimulation and 24-h incubation, chondrocytes were collected, washed twice with cold PBS, and stained with 500 μl of Annexin V-FITC for 30 min in the dark. Chondrocytes were then analyzed using flow cytometry (Beckman, Atlanta, GA, USA).

2.7. Induction of compressive stress

Compressive stress was applied using the homemade compressive device. We incorporated chondrocytes with chitosan–gelatin–glycerol phosphate hydrogel as cell carrier [22]. The hydrogel-incorporated chondrocytes were seeded into the wells of the compression device (200 μl/well) and cultured at 37 °C. After 24-h incubation, wells with cells were covered with a polydimethylsiloxane membrane. The compression was applied via nitrogen gas with a pressure of 60 psi, compressing the cells through the structural depression of the membrane. Chondrocytes

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