Plumbagin exerts protective effects in nucleus pulposus cells by attenuating hydrogen peroxide-induced oxidative stress, inflammation and apoptosis through NF-κB and Nrf-2

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Abstract. Plumbagin, one of the constituents responsible for the various biological activities of Plumbago zeylanica has been demonstrated to possess antioxidant activity, which may inhibit lipid peroxidation in a dose- and time-dependent manner. In the present study, we aimed to examine the protective effects of plumbagin as well as the underlying mechanisms through which plumbagin attenuates hydrogen peroxide (H2O2)-induced oxidative stress in nucleus pulposus cells (NPCs). For this purpose, the NPCs were incubated with fresh medium containing H2O2 (200 µM) at 37˚C in a humidified 5% CO2 atmosphere for 6 h, and cultured with various concentrations of plumbagin (0, 0.5, 1, 2, 5, 10 and 20 µM). Treatment with plumbagin significantly increased the viability of the H2O2-exposed NPCs in a dose-dependent manner. Moreover, plumbagin significantly reduced the generation of reactive oxygen species, lipid peroxidation, as well as the levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 in the H2O2-exposed NPCs. Glutathione (GSH) content, as well as the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were increased. We found that the administration of plumbagin significantly inhibited the activity of caspase-9 and -3, and downregulated NF-κB expression and upregulated Nrf-2 expression in the H2O2-exposed NPCs. Taken together, these findings suggest that plumbagin exerts neuroprotective effects in NPCs by attenuating H2O2-induced oxidative stress, inflammation and apoptosis through mediating the expression of NF-κB and Nrf-2.

Introduction

Population aging is a major problem in China and it is also a global issue. Degenerative spinal disorders are caused by pathological changes resulting from lumbar disc degeneration, such as lumbar spondylolisthesis, lumbar spinal stenosis and lumbar disc disease, and have gained increasing attention from researchers (1). Lumbar disc degeneration in the early stages, does not cause pain in the waist or leg; it is only when lumbar disc degeneration has progressed to a particular point that spine-related diseases develop, and pressure on the spinal cord or nerve root results in symptoms including paraplegia, bladder dysfunction, lumbar scoliosis, back pain, sciatica, loss of bilateral or unilateral lower limb muscle strength and paresthesia (2). Approximately 90% of the worldwide population has suffered from pains in the waist or leg, and some individuals may experience permanent incapacitation from the pain (3). Epidemiological data has also shown that the incidence of lumbar disc degeneration is rising, and it is developing in younger patients (4). A high morbidity as well as a trend towards lumbar disc degeneration in younger patients has gained the attention of experts, and research into the etiology, pathogenesis and treatment of disc degeneration is ongoing (5).

A number of degenerative spinal diseases and secondary lesions caused by lumbar disc degeneration are commonly seen in clinical practice, and the cause and the exact mechanisms responsible for these conditions remains unclear. The excessive apoptosis of disc cells directly leads to a reduction in the number of disc cells, resulting in lumbar disc degeneration (6). Nucleus pulposus cells (NPCs) play an important role in maintaining and repairing the environment within the normal intervertebral disc (7). Thus, the excessive apoptosis of NPCs is a direct cause of lumbar disc degeneration (8). Apoptosis may be triggered by the mitochondrial-dependent and the non-mitochondrial-dependent apoptotic signaling pathways (9). The mitochondrial-dependent pathway may be activated by hydrogen peroxide (H2O2)-mediated oxidative stress, leading to apoptosis (10).

Plumbago zeylanica has been used in traditional Chinese medicine, as it possesses various pharmacological activities including anti-inflammatory, antibacterial and antitumor effects, as well as the ability to inhibit glycolysis and to...
stimulate the central nervous system (3,11). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is one of the active constituents responsible for the various biological activities of *P. zeylanica* (chemical structure shown in Fig. 1). Plumbagin belongs to the class of compounds known as naphthoquinones, which are phenolic compounds, and a phenolic hydroxyl group with a quinone ring structure is the principal active antioxidant group (12). As the phenolic hydroxyl group is an active hydrogen donor, it provides a hydrogen atom to the peroxide free radical of unsaturated fatty acids, and thereby prevents the formation of new radicals, and interrupts the process of fat oxidation (13). The present study was designed to determine whether plumbagin exerts protective effects against oxidative stress in H$_2$O$_2$-exposed NPCs as well as to elucidate the underlying mechanism responsible for these effects.

**Materials and methods**

*Animals and cell culture.* Male Sprague-Dawley (SD) rats (8 weeks of age) weighing 200±30 g were housed in animal quarters at a temperature of 23±1°C and 60-70% relative humidity on a 12 h light/dark cycle (8:00; 20:00). The experimental study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Ethics Committee of The 100th Hospital of Chinese People's Liberation Army (Suzhou, China). The SD rats were injected with an overdose of chloral hydrate, and then the L1-L6 lumbar intervertebral discs were collected from the spinal column. The gel-like nucleus pulposus (NP) tissues were harvested from the discs and immediately placed into 25 cm$^2$ plastic bottles containing Dulbecco's modified Eagle's medium (DMEM)/F-12, and 10% fetal bovine serum (FBS). The NP tissue samples were cultured for 1-2 h and then digested with 0.01% trypsin (Beyotime Institute of Biotechnology, Haimen, China) for 30 min at room temperature. Following the removal of trypsin, the NP tissue samples were washed twice with phosphate-buffered saline (PBS) and digested with 0.5% collagenase II (Beyotime Institute of Biotechnology) for 4 h at room temperature. The NPCs were then filtered through a 200-µm mesh strainer and incubated with DMEM/F-12, 10% FBS, and antibiotics (1% penicillin/streptomycin) at 37°C in a humidified 5% CO$_2$ atmosphere. After 2 days of incubation, the NPCs were washed with PBS and incubated with fresh medium containing H$_2$O$_2$ (200 µM) at 37°C in a humidified 5% CO$_2$ atmosphere for 6 h.

*Cell viability.* The NPCs (5,000 cells in 100 µl of medium) were seeded in 96-well plates and cultured with plumbagin (Sigma-Aldrich China, Inc., Shanghai, China) at various concentrations (0, 0.5, 1, 2.5, 10 and 20 µM) at 37°C in a humidified 5% CO$_2$ atmosphere for 24 h. The glutathione (GSH) content, as well as the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were detected using a Labsystems Multiskan MS Plate Reader (Synergy2; BioTek Instruments) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

*Detection of lipid peroxidation.* The NPCs (5,000 cells in 100 µl of medium) were seeded in 96-well plates and cultured with plumbagin (0, 2, 5 and 10 µM) at 37°C in a humidified 5% CO$_2$ atmosphere for 24 h. Lipid peroxidation was examined as previously described by Pi et al (14) using a lipid peroxidation kit (Beyotime Institute of Biotechnology) and measured using Labsystems Multiskan MS Plate Reader (Synergy2; BioTek Instruments) at 532 nm and the results are expressed as nM thiobarbituric acid reactive substances (TBARS)/mg of protein.

*Detection of oxidative stress.* The NPCs (5,000 cells in 100 µl of medium) were seeded in 96-well plates and cultured with plumbagin (0, 2, 5 and 10 µM) at 37°C in a humidified 5% CO$_2$ atmosphere for 24 h. The glutathione (GSH) content, as well as the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were detected using commercial kits (Beyotime Institute of Biotechnology) and absorbance was measured using a Labsystems Multiskan MS Plate Reader (Synergy2; BioTek Instruments).

*Detection of inflammatory cytokines.* The NPCs (5,000 cells in 100 µl of medium) were seeded in 96-well plates and cultured with plumbagin (0, 2, 5 and 10 µM) at 37°C in a humidified 5% CO$_2$ atmosphere for 24 h. The levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were detected using commercial kits (Beyotime Institute of Biotechnology) and absorbance was measured using a Labsystems Multiskan MS Plate Reader (Synergy2; BioTek Instruments).

*Detection of apoptosis.* The NPCs (1x10$^5$ cells in 100 µl of medium) were seeded in 6-well plates and cultured with plumbagin (0, 2, 5 and 10 µM) at 37°C in a humidified 5% CO$_2$ atmosphere for 24 h. The NPCs were washed twice with cold PBS, and resuspended in 500 µl of binding buffer (BestBio, Inc., Shanghai, China). Thereafter, 5 µl of

![Figure 1. The chemical structure of plumbagin.](image_url)
Annexin V-FITC was added and incubated for 30 min at 4°C in the dark. Then, 5 µl of PI was added in the dark and immediately analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA).

Detection of caspase-9 and -3 activity. The NPCs (1x10^5 cells in 100 µl of medium) were seeded in 6-well plates and cultured with plumbagin (0, 2, 5 and 10 µM) at 37°C in a humidified 5% CO₂ atmosphere for 24 h. The NPCs were harvested and resuspended using lysis buffer (Beyotime Institute of Biotechnology) for 30-60 min on ice. The NPCs were harvested by centrifugation (10,000 x g for 10 min at 4°C), and the protein concentration was determined using a bicinchoninic acid protein assay (BCA; KeyGen, Nanjing, China). The activity of caspase-9 and -3 was detected at a wavelength of 405 nm using caspase-3 and -9 colorimetric assay kits (Beyotime Institute of Biotechnology) on a FACScan flow cytometer (BD Biosciences).

Western blot analysis of nuclear factor-κB (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf-2). The NPCs (1x10^5 cells in 100 µl of medium) were seeded in 6-well plates and cultured with plumbagin (0, 2, 5 and 10 µM) at 37°C in a humidified 5% CO₂ atmosphere for 24 h. The NPCs were harvested and resuspended using lysis buffer (Beyotime Institute of Biotechnology) for 30-60 min on ice. The NPCs were harvested by centrifugation (10,000 x g for 10 min at 4°C), and the protein concentration was determined using a bicinchoninic acid protein assay (BCA; KeyGen, Nanjing, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with approximately 50 µg of sample proteins which were loaded onto 12% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories GmbH, Munich, Germany). The nitrocellulose membrane was blocked with Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TBST) and 5% non-fat milk for 2 h. The membrane was then incubated with chemiluminescence reagent (ECL Plus Western Blotting Detection system; GE Healthcare, Waukesha, WI, USA). The relative quantity of protein was measured using AlphaEase FC (FluorChem FC2) software (Cell Biosciences Inc., Santa Clara, CA, USA).

Results

Protective effect of plumbagin increases the viability of NPCs. We demonstrated that the viability of NPCs was increased by treatment with plumbagin in a dose-dependent manner. Particularly, cell viability was significantly increased following treatment with plumbagin at concentrations of 2 to 20 µM, compared with that in the 0 µM plumbagin-treated group (Fig. 2).

Protective effect of plumbagin decreases the generation of ROS in NPCs. We next examined whether plumbagin exerted a protective effect against the H₂O₂-induced generation of ROS in the NPCs. The generation of ROS was highest in the 0 µM plumbagin-treated group and there was a significant reduction in ROS levels in the plumbagin-treated groups (2-10 µM) (Fig. 3).

Protective effect of plumbagin decreases lipid peroxidation in NPCs. We examined whether plumbagin exerted a protective effect against lipid peroxidation in the H₂O₂-exposed NPCs. The results showed that treatment with plumbagin (2-10 µM) significantly inhibited H₂O₂-induced lipid peroxidation in the NPCs compared with that in the 0 µM plumbagin-treated group (Fig. 4).

Protective effect of plumbagin decreases oxidative stress in NPCs. We examined whether plumbagin exerted a protective effect against the H₂O₂-induced oxidative stress in the NPCs. As compared with the 0 µM plumbagin-treated groups, GSH content as well as the activity of CAT, SOD and GSH-Px in the plumbagin-treated groups (2-10 µM) was significantly increased in the H₂O₂-exposed NPCs (Fig. 5).
Protective effect of plumbagin decreases the levels of inflammatory cytokines in NPCs. In order to further explore the protective effects of plumbagin, we examined whether plumbagin decreased the levels of inflammatory cytokines in the H$_2$O$_2$-exposed NPCs. The administration of plumbagin (2-10 µM) significantly decreased the levels of TNF-α, IL-1β.
and IL-6 in the H₂O₂-exposed NPCs compared with those in the 0 µM plumbagin-treated groups (Fig. 6).

Protective effect of plumbagin decreases the apoptosis of NPCs. In the next experiment, we examined the anti-apoptotic effect of plumbagin in the H₂O₂-exposed NPCs. As shown in Fig. 7, plumbagin treatment (2-10 µM) significantly decreased the cell apoptosis rate in the H₂O₂-exposed NPCs, compared with that in the 0 µM plumbagin-treated group (Fig. 7).

Protective effect of plumbagin decreases the activity of caspase-9 and -3 in NPCs. In order to further examine the anti-apoptotic effect of plumbagin in the H₂O₂-exposed NPCs, the activity of caspase-9 and -3 was also measured. Caspase-9 and -3 activity was significantly inhibited in the plumbagin-treated groups (2-10 µM), compared with those in the 0 µM plumbagin-treated groups (Fig. 8).

Protective effect of plumbagin decreases NF-κB protein expression in NPCs. To explore the anti-inflammatory effect of plumbagin in the H₂O₂-exposed NPCs, NF-κB protein expression was evaluated using western blot analysis. NF-κB protein expression was significantly suppressed by the administration of plumbagin (2-10 µM), compared with that in the 0 µM plumbagin-treated group (Fig. 9).

Protective effect of plumbagin increases Nrf-2 protein expression in NPCs. To examine the anti-inflammatory effect of plumbagin in the H₂O₂-exposed NPCs, Nrf-2 protein expression was evaluated using western blot analysis. When compared with the 0 µM plumbagin-treated group, Nrf-2
protein expression was significantly increased by treatment with plumbagin at concentrations of 2-10 µM (Fig. 10).

**Discussion**

Approximately 90% of the worldwide population has suffered from pains in the waist or leg to varying extents, and some individuals may experience permanent incapacitation from the pain (15). Epidemiological data also shows that the incidence of lumbar disc degeneration is rising and it is developing in younger patients (16). With the changes in human longevity and lifestyle, degenerative spinal disorders caused by lumbar disc degeneration, such as lumbar spondylolisthesis, lumbar spinal stenosis and lumbar disc disease have gained increasing attention (15, 17). However, it is proving difficult to treat intervertebral disc degeneration in clinical practice (18). In the present study, we found that treatment with plumbagin significantly increased the viability of H₂O₂-exposed NPCs in a dose-dependent manner. Zhang et al suggested that plumbagin protects against spinal cord injury through the suppression of oxidative stress and inflammation through Nrf-2 upregulation (19). Wang et al reported that plumbagin inhibits
lipopolysaccharide-induced inflammation in RAW 264.7 cells (20).

Oxidative stress refers to the process of tissue damage caused by ROS accumulation in the body or cells as well as the cell toxicity that results from a serious imbalance between free radicals production and the antioxidant defenses when the cells are subjected to harmful stimuli (21). ROS are a class of free radicals which cause oxidative stress, (including O$_2^-$; OH, H$_2$O$_2$ and NO, etc.) and may be produced by mitochondria in human cells, and also induced by environmental stresses (22). Oxidative stress damage in the body is revealed by lipid peroxidation, intracellular protein and enzyme denaturation as well as DNA damage, resulting in abnormal cell function, which ultimately leads to cell death or apoptosis (23). ROS may cause degradation of the extracellular matrix through the inhibition of proteoglycan synthesis and the destruction of extracellular matrix protein structure and protease activity. With increasing age, oxidative stress levels in the body gradually increase as ROS accumulate, causing the destruction of the extracellular matrix in disc tissues and the death or apoptosis of NPCs, thus causing disc degeneration (24).

In the present study, treatment with plumbagin significantly reduced the H$_2$O$_2$-induced generation of ROS as well as lipid peroxidation. GSH content as well as the activity of CAT, SOD and GSH-Px were increased in the NPCs. Zhang et al suggested that plumbagin protects against spinal cord injury through the suppression of oxidative stress, as well as the inhibition of ROS and lipid peroxidation in Wistar rats (19). Checker et al demonstrated that plumbagin inhibits lipopolysaccharide-induced oxidative stress and inflammation (25).

Evidence indicates that the essential pathological changes responsible for lumbar disc degeneration include not only morphological and histological changes, but also a series of changes in biochemical properties (26). Trauma may cause early disc degeneration, which in turn induces the expression of cytokines; the secondary inflammation induces further damage, and moreover increases the severity of lumbar disc degeneration (27). IL-1β, IL-6 and TNF-α are important cytokines, which may be involved in the pathogenesis of lumbar disc degeneration (26). The results of this study showed that the administration of plumbagin significantly decreased the levels of TNF-α, IL-1β and IL-6 in the H$_2$O$_2$-exposed NPCs. A study by Sandur et al indicated that plumbagin abrogated the expression of NF-κB-regulated gene products which led to the potentiation of apoptosis induced by cytokine and chemotherapeutic agents (28). Luo et al demonstrated that plumbagin inhibits NF-κB activation and therefore has the potential to be developed as a novel anti-inflammatory agent (29).

Apoptosis may occur under physiological and pathological conditions and circumstances, subject to a variety of factors including caspases. Caspases belong to a family of cysteine hydrodases, which play a central role in apoptosis. Caspase-3 is an effector of apoptosis (30). NPCs exposed to H$_2$O$_2$ were used in the present study, and researchers have found that oxidative stress leads to the activation of apoptotic signaling pathways, which include not only the intrinsic apoptotic pathway (such as caspase-9), but also includes the extrinsic apoptotic pathway (caspase-8) as well as the common pathway of both channels (such as caspase-3) (31). This evidence further proves that oxidative stress accelerates the apoptosis of NP cells and causes disc degeneration (32). We found that plumbagin significantly inhibited the activity of caspase-9 and -3 in the H$_2$O$_2$-exposed NPCs. Checker et al demonstrated that plumbagin inhibits lipopolysaccharide-induced oxidative stress, inflammation and caspase-3 (25).

The Nrf-2-antioxidant response element (ARE) signaling pathway regulates the encoding of antioxidant proteins through various interactions, and it is the central regulator of cellular antioxidant responses (33). The ARE, which is a cis-acting enhancer sequence of many antioxidant enzyme/protein gene upstream, is one of the defense mechanisms which enables the body to deal with oxidative stress (34). Previous findings have shown that Nrf-2 adjusts the encoding of antioxidant genes by interacting with ARE. Under normal conditions, Nrf-2 is localized in the cytoplasm, combined with the cytoplasmic protein Keap1; when subjected to attack from ROS, Nrf-2 dissociates from Keap1, translocates into the nucleus, binds to Maf protein in order to form a heterodimer, which binds to ARE in the gene to activate targeted gene expression as well as to regulate antioxidant enzyme/protein transcription (34,35). Thus, Nrf-2 is a receptor for oxidative stress, which plays an important role in the protection of cells against oxidative stress and it is one of the principal defense mechanisms induced by exposure to exogenous toxic substances (19). The findings of the present study revealed that plumbagin significantly downregulated NF-κB expression and upregulated Nrf-2 expression in the H$_2$O$_2$-exposed NPCs. Zhang et al suggested that plumbagin protects against spinal cord injury through the suppression of oxidative stress and inflammation through Nrf-2 upregulation in Wistar rats (19).

In conclusion, our findings have demonstrated that plumbagin exerts neuroprotective effects in NPCs by attenuating H$_2$O$_2$-induced oxidative stress, inflammation and apoptosis. Moreover, we have provided evidence that NF-κB/Nrf-2 are potential targets for plumbagin in intervertebral discs. These findings suggest that plumbagin may be of potential therapeutic value in the treatment of various neurological diseases.

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