L-NMMA delays apoptosis in the nucleus pulposus cells of intervertebral discs.

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Abstract

Apoptosis plays an important role in degeneration of Nucleus Pulposus (NP) cells. L-Monomethyl-Arginine (L-NMMA) is an analogue of L-arginine and can be inactivated irreversibly by induced NOS (iNOS). L-NMMA can also compete with L-arginine for binding sites on iNOS molecules. In addition, L-NMMA has been reported to influence the apoptosis of hyaline cartilage cells in vitro. Thus, we hypothesized that L-NMMA would influence the apoptosis of NP cells in vitro. Rabbit primary NP cells were cultured and subjected to flow cytometry to determine the levels of apoptosis in each group at each time point. Western blotting was performed to detect the Bcl-2/Bax ratio, as well as protein levels of caspase-3 and caspase-9. A significant increase in apoptosis was observed between the NP cells cultured with 1% FBS medium versus without L-NMMA (P<0.01). Likewise, the Bcl-2/Bax ratio was also increased in NP cells cultured in 1% FBS with L-NMMA, versus those cultured without L-NMMA (P<0.05). Meanwhile, the activity levels of both caspase-3 and caspase-9 were reduced in the presence of L-NMMA, compared with levels observed in the absence of L-NMMA (P<0.01). This study showed that L-NMMA treatment delayed the senescence and apoptosis of NP cells in vitro, raising the possibility that L-NMMA may be a novel treatment to delay intervertebral disc degeneration.

Keywords: L-monomethyl-arginine, Nucleus pulposus cells, Apoptosis, Caspase-3, Caspase-9, Bcl-2, Bax.

Accepted on September 07, 2017

Introduction

The main clinical manifestation of degenerative disc disease includes severe pain in the back, neck, and shoulder. This pain has a relatively large impact on human spine function and can negatively affect both bones and organs [1]. To date, the exact pathogenesis of intervertebral disc degeneration remains elusive. Accumulating evidence suggests that Nitric Oxide (NO) may play a physiological role in neuronal signal transmission, and it is also involved in various disease processes [2,3]. Nitric Oxide (NO) was initially discovered as an endothelium-derived relaxing factor that contributes to regulating vascular tone. NO is formed from the amino acid precursor, L-arginine, by Nitric Oxide Synthase (NOS) [4]. Elevated NO production has been shown to play an important role in the pathogenesis of neural diseases such as Alzheimer’s, and excess levels of NO have been detected in the Cerebrospinal Fluid (CSF) of patients with degenerative lumbar disease compared with pain-free control patients [4-6].

The Nucleus Pulposus (NP) performs several critical functions, one of which is ensuring that compressive loads are evenly distributed and transferred between the vertebral bodies of intervertebral discs [7]. Intervertebral disc degeneration is characterized by a cascade of cellular, biochemical, and structural changes. This cascade begins in the central NP where decreasing proteoglycan content and an associated reduction in hydrostatic pressure impair the function of the NP, thereby leading to back pain [8,9]. NP cells only represent approximately 1% of the entire intervertebral disc tissue, yet they play an essential role in resisting mechanical loadings by synthesizing an Extracellular Matrix (ECM) to maintain the stability of intervertebral discs. The synthesized ECM also mediates an important protective effect on disc integrity [10,11]. Correspondingly, the loss of NP cells has been reported to closely correlate with the onset of intervertebral disc degeneration [12].

Numerous studies have revealed that NP cells may be induced to produce NO in response to certain stimuli [13,14]. In a study of chondrocytes by Maneiro et al., NO was found to activate mitochondria-dependent events and to induce mitochondrial pathways involved in mediating apoptosis [15]. For NP cells, recent studies have demonstrated that apoptosis plays an important role in their degeneration [16,17]. L-Monomethyl-Arginine (L-NMMA) is an analogue of L-arginine and can irreversibly inactivate induced NOS (iNOS) by competing with L-arginine for binding sites on iNOS molecules. This inactivation results in significantly lower levels of NO production and an inhibition of apoptosis. We hypothesize that NO-mediated apoptosis plays a critical role in the loss of NP...
cells in intervertebral disc degeneration. To test this hypothesis, primary NP cells were isolated and NO production was inhibited by L-NMMA to investigate the effects of NO on serum-induced apoptosis in NP cells cultured in vitro, and to study the mechanism(s) involved.

Materials and Methods

Equipment and reagents

A Nikon inverted microscope (Japan Nikon Corporation); an EPICS® ALTRA™ flow cytometer (Beckman Coulter, Inc.); a 72SS-visible spectrophotometer (Prism Optical Technology Co., Ltd., Shanghai, China); an Electrophoresis JY-300C unit (Beijing Liuji Instrument Plant); DMEM medium, an anti-collagen II antibody, ALP (C-8), and an immunocytochemistry kit (including goat anti-mouse and goat anti-rabbit secondary antibodies) (Ye Le Biotechnology Co., Ltd., Guangzhou, China) were used. An Annexin V-FITC PI apoptosis detection kit, a Caspase-3 Detection Kit and a Caspase-9 Detection Kit were all purchased from Pik-day Biotechnology Institute (-). L-NMMA was purchased from Sigma (St. Louis, MO, USA).

Isolation and culturing of NP cells

Twelve New Zealand white rabbits, independent of gender (weight 3 ± 0.5 kg), were anesthetized and sacrificed for the resection of lumbar discs 1 through 4 under sterile conditions. The NP of each disc was removed and rinsed in D-Hank’s solution. Then the NP tissues were cut into small pieces with ophthalmology scissors and were digested with trypsin (0.25%) and 1%FBS+L-NMMA (cultured in medium with 1% FBS and 50 μM L-NMMA). These three treatments groups were distributed in sheets, exhibited some refraction, and had a round morphology, exhibited strong refraction, and were slow to affix to the container wall when suspended in culture media, and to study the mechanism(s) involved.

Characterization of apoptosis and caspase activity

An Annexin V-FITC PI apoptosis detection kit, a Caspase-3 Detection Kit, and a Caspase-9 Detection Kit were all purchased from Pik-day Biotechnology Institute (Beyotime Institute of Biotechnology) and were used according to the manufacturer’s instructions.

Immunoblot detection of Bel-2 and Bax

Expression levels of Bel-2 and Bax expression were detected by Western blotting. Briefly, NP cells were lysed with a detergent buffer containing: 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.02% sodium azide, 100 μg/ml PMSF, 1 μg/ml aprotinin, and 1% Triton X-100 (or NP-40). The total protein concentration of each sample was determined by using the Bradford method. Western blotting was performed using Bel-2 and Bax primary antibodies (5 mg/1 mL; Ye Le Biotechnology Co., Ltd., Guangzhou, China). Detection of GAPDH was performed as a loading control. A horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody was diluted 1:5000 as the secondary antibody (Ye Le Biotechnology Co., Ltd.). Bound antibodies were detected using enhanced chemiluminescence reagents (Ye Le Biotechnology Co., Ltd.). Image analysis software was used to analyze band densities (Tian Neng Biotechnology Co., Ltd., Shanghai, China).

Statistical methods

Values represent means ± standard errors of 3-5 trials involving independent cell isolations from separate samples. Analyses of variance (ANOVAS) were followed up with Student-Newman-Keuls pair-wise post hoc tests [18-20]. For small sample sizes, confidence intervals were calculated based on t-distribution. All data analyses were conducted in SPSS 17.0 software (IBM Incorporated, Armonk, NY). P-values less than 0.05 and less than 0.01 were considered significant and very significant, respectively.

Results

Morphology of NP cells

Primary NP cells (monolayer primary cultures established with cells isolated from rabbit intervertebral discs) had a small, round morphology, exhibited strong refraction, and were slow to affix to the container wall when suspended in culture medium (Figure 1A). After 2-3 d in culture, the cells were predominantly elliptical or small and round in shape with large nuclei and small cell volumes; they were scattered or distributed in sheets, exhibited some refraction, and had reached 20-30% confluence (Figure 1B). After 5-7 d in culture, cell confluence had increased to about 40% and a majority of the cells were fusiform in morphology with a protruding appearance, a round central nucleus, and one or two nucleoli, though some cells remained round or oval (Figures 1C and 1D). After the cells had been in culture for 10 d, cell confluence had reached 60-70%, the cells had abundant cytoplasm, and they were mainly triangular, spindled, or small...
and round in shape (Figure 1E), which indicated that post-passage cell morphology could be used in downstream tests. After passage, the cells grew rapidly and exhibited complete adherence much faster (~3d) than the original-generation cells, but they had intracellular vacuoles. In the second generation after passage, signs of aging were apparent, with ~80% of the cells taking on long shuttle morphology (Figure 1F).

**Figure 1.** Representative images of NP cells isolated from rabbit intervertebral discs at: (A) 0 d; (B) 3 d; (C) 5 d; (D) 7 d; (E) 10 d in culture; (F) Image of passaged cells in culture. All six images show 100X magnifications.

**Identification of NP cells**

The primary NP cells exhibited strong toluidine blue staining near their nuclei (Figure 2A), consistent with the ability of NP cells to secrete proteoglycans. The NP cells were also immunopositive for type II collagen; the type II collagen staining was rather diffuse with stronger staining around nuclei (Figure 2B).

**Figure 2.** Representative images of: (A) Isolated NP cells in culture after toluidine blue staining; (B) Immunocytochemical detection of type II collagen. Both images show 400X magnification.

**L-NMMA reduced apoptosis of NP cells**

Flow cytometry showed that NP cells cultured with 1% FBS exhibited significantly higher levels of apoptosis than cells cultured with 10% FBS after 24 h, 48 h, and 72 h (Figure 3). Inclusion of 50 μM L-NMMA in NP cell cultures containing 1% FBS resulted in a significant reduction in cell death relative to 1% FBS alone (P<0.01, all three time points; Figure 3).

**L-NMMA increased Bcl-2: Bax ratio in NP cells**

Western blot analysis showed that, among the three NP cell culture groups, the 1% FBS and 10% groups had the lowest and highest Bcl-2: Bax ratios (Figure 4). In addition, a significant increase in the Bcl-2: Bax ratio was detected for NP cells cultured in 1% FBS containing 50 μM L-NMMA compared to that of cells cultured in 1% FBS alone (P<0.05). The increased ratio reflected an increased level of Bcl-2 expression in NP cells cultured in 1% FBS containing 50 μM L-NMMA that corresponded with the significant decline in cell death described above for the same experimental group of cells.

**Figure 3.** Flow cytometry detection of apoptosis in isolated NP cells cultured with 10% FBS, 1% FBS, or 1% FBS+L-NMMA (50 μM) for 24 h, 48 h, and 72 h. Representative images of the density plots obtained are shown. Mean apoptosis rates (± SDs) at 24 h, 48 h, and 72 h differed significantly between NP cells cultured in 1% FBS+L-NMMA and NP cells cultured in 1% FBS alone (*P<0.01 vs. 1% FBS).

**L-NMMA decreased caspase-3 and caspase-9 activity levels in NP cells**

Caspase-3 and caspase-9 activity levels per mg of caspase protein in NP cells cultured in 1% FBS were significantly greater than the levels observed for NP cells cultured in 10% FBS after 24 h, 48 h, and 72 h of culturing (P<0.01). However, the activity levels of both caspase-3 and caspase-9 were significantly decreased at each time point when 50 μM L-NMMA was added to 1% FBS cultures (all P<0.01; Figures 5A and 5B).

**Discussion**

Apoptosis is a normal physiological process that can be used to remove harmful or severely damaged cells. However, when this process becomes excessive, it represents a pathological phenomenon. Currently, the exact pathogenesis of intervertebral disc degeneration remains elusive, although the loss of NP cells due to apoptosis closely correlates with the onset of intervertebral disc degeneration and it has been shown to play an important role [18-20]. Therefore, over the past few years, the role of apoptosis in intervertebral disc degeneration has become an active area of research, and inhibition of apoptosis in NP cells may be an important strategy for delaying intervertebral disc degeneration.

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cultured NP cells with toluidine blue revealed that the isolated proteoglycans were performed to determine the purity of the present study, ICC detection of type II collagen and staining of proteoglycans and type II collagen [24, 25]. Therefore, in the cells were metachromatic and they had the capacity to synthesize proteoglycans [26, 27]. Detection of type II collagen further confirmed that the cultured cells were NP cells [26, 27].

After obtaining the isolated NP cells, L-NMMA was administered to investigate apoptosis induced by serum starvation. L-NMMA is a congener of L-arginine and has been used as an inhibitor of NO production based on its ability to compete with NOS for L-arginine. The present results show that L-NMMA can rescue serum starvation-induced apoptosis in NP cells and NO plays an important role in the apoptosis of NP cells.

The Bax gene, located in human chromosome 19, is one of the most important genes in the Bcl-2 gene family and it promotes apoptosis. Bax and Bcl-2 can form heterodimers or homodimers, and only the combination of Bcl-2 and Bax mediates anti-apoptotic effects. Furthermore, it has been confirmed that the Bcl-2/Bax ratio negatively correlates with apoptosis [28-30]. In the present study, L-NMMA treatment of serum starved NP cells led to a significant rescue of the decrease in the Bcl-2/Bax ratio due to serum starvation, thereby confirming that NO is involved in mediating apoptosis in NP cells, and it mediates this effect, in part, by regulating the Bcl-2/Bax ratio.

Caspases contribute to a variety of important biological functions during apoptosis, including the initiation, regulation, and enforcement of this process. However, in recent years, correlation study results have also shown that caspases have important roles in cell differentiation as well [31, 32]. Studies of the osteoblast differentiation process have showed that excessive activation and expression of caspases are involved in the induction of differentiation of MC3T3-E1 bone cells by Bone Morphogenetic Protein (BMP)-4, while apoptotic or necrotic cells were not observed [25, 31, 32]. Specific inhibitors of caspases 2, 3, and 8, as well as a broad-spectrum caspase inhibitor, have also been shown to inhibit the biological activity of alkaline phosphatase. In addition, parathyroid hormone levels were found to correlate with cAMP expression levels in MC3T3-E1 cells treated with BMP4, thereby revealing a possible mechanism for the observed inhibition of osteoblast function. Thus, caspase-3 appears to play a very crucial role in maintaining gene expression in osteoblasts.

In previous studies, L-NMMA was found to significantly reduce the expression level of caspase-3 in articular cartilage, to up-regulate the ratio of Bcl-2/Bax, and to inhibit the apoptosis of articular chondrocytes in a dose-dependent manner. In the present study, the effect of L-NMMA on the expression levels of caspase-3 and caspase-9 during apoptosis induced by serum starvation was investigated. In our preliminary experiments, 50 μM L-NMMA was found to significantly reduce the expression levels of caspase-3 and caspase-9 in articular cartilage. Subsequently, L-NMMA treatment was found to decrease the elevated expression levels of caspase-3 and caspase-9 due to apoptosis induced by serum starvation. Thus, it appears that caspase-3 and caspase-9 are apoptotic effectors of NO-mediated apoptosis in NP cells. This is consistent with the hypothesis that both Bax and caspase-3 have important roles in mediating apoptosis in NP cells in human cervical discs [33]. Furthermore, the observations that caspase-9 is an initiating factor for activation of the mitochondrial apoptotic pathway, is an essential factor in the death receptor-dependent pathway, and is a key protease in the mitochondrial apoptosis pathway, supports this hypothesis [34].

**Figure 4.** Western blot analysis of Bcl-2 and Bax protein expression in NP cells. Lanes 1-3 contain extracts that were collected after 24 h in culture, lanes 4-6 contain extracts that were collected after 48 h in culture, and lanes 7-9 contain extracts that were collected after 72 h in culture. Mean Bcl-2: Bax ratio (± SD) at 24 h, 48 h, and 72 h differed significantly between NP cells cultured in 1% FBS and NP cells cultured in 1% FBS alone (*P<0.01 vs. 1% FBS).

**Figure 5.** (A) Addition of L-NMMA to 1% FBS medium reduced mean active Caspase 3 protein levels; (B) Addition of L-NMMA to 1% FBS medium reduced mean active Caspase 9 protein levels. (*P<0.01 vs. 1% FBS).

Compared with other in vitro cell cultures, the culturing of NP cells is relatively difficult. Existing disc cell culture methods include organized block culture and enzymatic digestion [21-23]. For the former method, a longer adherent time is needed and the obtained cell number is limited. Cells have been isolated more thoroughly with enzyme digestion, and this method was successfully applied to the isolation and culturing of NP cells in the present study. Phenotypically, cartilage cells and NP cells are very similar and they both secrete proteoglycans and type II collagen [24,25]. Therefore, in the present study, ICC detection of type II collagen and staining of proteoglycans were performed to determine the purity of the primary NP cells that were isolated. Positive staining of the cultured NP cells with toluidine blue revealed that the isolated cells were metachromatic and they had the capacity to synthesize proteoglycans [26,27]. Detection of type II collagen further confirmed that the cultured cells were NP cells [26,27].
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NO generated by endogenous NOS enzymes or NO donors have been found to promote or prevent apoptosis induced by diverse pro-apoptotic stimuli in cell culture models [35]. In general, low doses of NO production mediate a protective effect in apoptosis, while high doses of NO production promote apoptosis. Both mitochondrial-dependent and mitochondrial-independent apoptotic signaling pathways are involved in mediating this dichotomous cellular response to NO and the molecular mechanisms responsible for these effects are complex [36]. Elevated levels of NO can induce apoptosis by mediating oxidative stress, by interfering with energy metabolism, by directly damaging DNA, by activating poly-ADP polymerase, or by inducing Ca$^{2+}$ disorder [35]. In the present study, inhibition of NO production by L-NMMA in NP cells led to a marked inhibition of the decrease in the Bcl-2/Bax ratio induced by serum starvation. Increases in caspase-3 and caspase-9 expression levels were also inhibited. Taken together, these results clearly indicate that NO regulates the expression of apoptotic proteins involved in mediating the apoptosis process in NP cells. However, the exact mechanism by which NO regulates the Bcl/Bax ratio and the expression levels of certain caspases remains to be elucidated.

In conclusion, the isolation and treatment of primary NP cells with L-NMMA was performed to inhibit NO production and to investigate the effects of NO, and the associated mechanism(s), on serum-induced apoptosis in NP cells cultured in vitro. When NO production was inhibited by L-NMMA, serum starvation-induced apoptosis was delayed in NP cells, concomitant with blocking of the decrease in the Bcl-2/Bax ratio and increased expression levels of caspase-3 and caspase-9. Thus, inhibition of apoptosis by NP cells may represent a novel treatment approach for delaying intervertebral disc degeneration.

Acknowledgement

This research was funded by Qingyuan People’s Hospital.

Disclosure of Conflict of Interest

The authors have no conflicts of interest.

References


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