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ORIGINAL PAPER

Interleukin-1β enhances the effect of serum deprivation on rat annular cell apoptosis

Chang-Qing Zhao · Da Liu · Hai Li · Lei-Sheng Jiang · Li-Yang Dai

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Abstract Excessive apoptosis of disc cells is believed to play an important role in intervertebral disc (IVD) degeneration. It has been shown that interleukin-1β (IL-1β) is involved in the failure of disc matrix by suppressing the synthesis of matrix components and stimulating the expression of matrix metalloproteinases. However, whether IL-1β induces disc cell apoptosis is still unclear. The objective of this study was to investigate the effect of IL-1β on the apoptosis of rat annular cells cultured with or without serum supplement. First-passage rat annular cells were cultured with 0% or 10% fetal bovine serum (FBS) supplement and stimulated with 0, 10, 20 or 50 ng/ml IL-1β for 12, 24 or 48 h. Apoptotic incidences were quantified by flow cytometry, morphologic changes in apoptotic cells were visualized by Hoechst 33258 staining and phase-contrast microscopy, and caspase-3 activity was also determined. When rat annular cells were cultured with 10% FBS supplement, no significant changes in apoptotic incidences, apoptotic morphology and caspase-3 activity were observed even when cells were stimulated with 50 ng/ml IL-1β for 48 h. In contrast, serum deprivation for 24 h led to an increase in apoptotic incidences, the number of apoptotic nuclei and caspase-3 activity, and IL-1β significantly increased the effects of serum deprivation in a dose-dependent manner. Our results indicate that IL-1β alone is not a sufficient stimulus to induce disc cell apoptosis and that in order to suppress disc cell apoptosis, improving the nutrient supply to the disc may be more effective than antagonizing the adverse effects of IL-1β.

C.-Q. Zhao · D. Liu · H. Li · L.-S. Jiang · L.-Y. Dai (✉)
Department of Orthopedic Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, 1665 Kongjiang Road, Shanghai 200092, China
e-mail: chinaspine@163.com

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Introduction

Disorders associated with intervertebral disc (IVD) degeneration are the leading causes of morbidity or life quality deterioration in the elderly [1]. However, the mechanism of IVD degeneration has not been fully elucidated. Because it is believed that cellular loss due to excessive apoptosis plays an important role in IVD degeneration [2–5], some authors [6–8] have suggested that suppression of apoptosis may be a potential strategy for retarding IVD degeneration. To develop such a therapy, identifying the causes of IVD cell apoptosis is crucial. To date, it has been demonstrated that abnormal mechanical stresses [3–5, 9–13], serum deprivation [6, 7, 14] and nitric oxide [15] can result in increased apoptosis of IVD cells in vivo and in vitro.

With degeneration and herniation, there is increased IL-1β production in the IVD tissue [16–19]. In vitro studies have shown that treatment with IL-1β suppresses the synthesis of Type II collagen and proteoglycan by IVD cells [18, 20], whereas it stimulates the expression of the enzymes responsible for IVD matrix degradation [18, 21]. It has also been reported that IL-1β sensitizes annulus fibrosus (AF) cells to fluid-induced shear stress [22].

Although the results were inconsistent, the effect of IL-1β on apoptosis of cartilage chondrocytes, which share a similar phenotype to IVD cells (especially those residing in the nucleus pulposus (NP) and inner AF), has been previously investigated [23–28]. However, the effect of IL-1β on IVD cell apoptosis has not been previously documented.

There is evidence showing that notochordal cells can persist in the NP through most of adult life in some species, including the rat [29], and an in vitro study has shown that notochordal cells can influence the function of other NP cells, for instance by stimulating them to synthesize more proteoglycan [30]. Thus, it is also possible that notochordal cells can influence the apoptosis of other NP cells in vitro. In contrast, cells residing in the outer AF share a similar phenotype. In order to maintain the homogeneity of cells cultured in vitro and exclude interactions between different cell types, only the cells from rat outer AF were used in the current study.

The objective of this study was to investigate the effect of IL-1β on the apoptosis of rat AF cells cultured with or without serum supplement. This may provide new insights on IVD degeneration and facilitate the development of potential therapies for regenerating denatured IVD.

Materials and methods

Isolation and culture of AF cells

Experimental studies were approved by the authors' institutional Animal Care and Use Committee. Eight male Sprague–Dawley rats, aged 3 months, were euthanized by intravenous administration of 150 mg/kg pentobarbital sodium. Lumbar spines including L1 to L6 levels were removed *en bloc* under aseptic conditions. The 5 lumbar IVDs (L1–L2, L2–L3, L3–L4, L4–L5 and L5–L6) were dissected free from their upper and lower vertebrae. The NP and inner AF as well as the surrounding ligament to which the AF is joined were discarded to ensure the identity of the tissue. AF tissues obtained from the same animal were pooled together, washed with Hank's balanced salt solution (HBSS, Gibco) and cut into small pieces (<1 mm^3). Minced AF tissues were agitated at 37°C for 90 min in an enzyme solution of 0.4% pronase (from *Streptomyces griseus*, Calbiochem) based on Dulbecco's modified Eagle medium/Ham's F-12 (DMEM/F-12, Gibco) supplemented with 5% FBS (Gibco), followed by overnight digestion with 0.025% collagenase Type II (from *Clostridium histolyticum*, Sigma), 0.01% hyaluronidase Type V (from *sheep testes*, Sigma) also based on DMEM/F-12 with 5% FBS at 37°C in a gyratory shaker (110 rev/min). Tissue debris was removed by filtering through a cell strainer with a pore size of 70 μm and isolated cells were rinsed 3 times with HBSS.

The resulting cells were seeded in 60-mm tissue culture dishes and grown with complete culture medium (DMEM/F-12 with 10% FBS, 100 μg/ml streptomycin and 100 units/ml penicillin (Gibco)) in a 37°C, 5% CO_2 environment. The medium was changed every other day. After 10–14 days, just before reaching complete confluence, the cells were harvested by brief exposure to 0.05% trypsin-ethylene diamine tetatacetic acid (Gibco), and re-plated into appropriate culture plates after washing with HBSS. First-passage cells maintained in a monolayer were used throughout the experiments.

Detection of apoptotic incidence by flow cytometry

Cells were sub-cultured in 6-well plates at 2×10^5 cells per well with complete culture medium. After reaching 90% confluence, the medium was changed to DMEM/F-12 containing 1% FBS and antibiotics for 12 h in order to synchronize the cells. The cells were then cultured in complete culture medium with 0, 10, 20 or 50 ng/ml recombinant rat IL-1β (Peprotech) for a further 12, 24 or 48 h, or were cultured in serum-free DMEM/F-12 containing antibiotics with 0, 10, 20 or 50 ng/ml IL-1β for 24 h (this period of culture time was applied to the rest experiments). Apoptotic incidence was detected by using the Annexin V-FITC apoptosis detection kit I (BD Pharmingen). Briefly, cells still attached to the plate as well as those present in the supernatant were collected together and re-suspended in 1× binding buffer at a concentration of 1×10^6 cells per ml. One hundred μl of solution containing 1×10^5 cells was incubated with 5 μl of AnnexinV-FITC and 5 μl of propidium iodide for 15 min at room temperature in the dark, followed by addition of 400 μl of 1× binding buffer. Samples were analyzed by a Fluorescence activated cell sorter (Beckman Coulter) within 1 h. Apoptotic cells, including those staining positive for annexin V-FITC and negative for propidium iodide and those that were double positive, were counted and represented as a percentage of the total cell count.

Hoechst 33258 staining

Cells were seeded onto sterile glass coverslips placed in 24-well plates at 5×10^4 cells per well and managed as described above. After treatment, the medium was removed and the cells were fixed with 4% paraformaldehyde for 15 min, washed with 0.15 M NaCl 3 times and stained with 2 μg/ml Hoechst 33258 (Sigma) in HBSS for 5 min. Following 2 washes with 0.15 M NaCl, the coverslips were mounted onto slides using anti-fade mounting medium (Beyotime). Morphologic changes in apoptotic nuclei were observed under a fluorescence microscope (Olympus IX50) with excitation at 350 nm and emission at 460 nm.

Caspase-3 activity assay

The first-passage AF cells were placed in 6-well plates at 2×10^5 cells per well and treated similarly. Caspase-3 activity was determined using a Caspase-3 activity kit (Beyotime), which is based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp *p*-nitroanilide into the yellow formazan product, *p*-nitroaniline. According to the manufacturer's protocol, treated cells were lysed with lysis buffer (100 µl per 2×10^6 cells) for 15 min on ice following washing with cold HBSS. After incubating the mixture composed of 10 µl of cell lysate, 80 µl of reaction buffer and 10 µl of 2 mM caspase-3 substrate in 96-well microtiter plates at 37°C for 4 h, caspase-3 activity was quantified in the samples with a microplate spectrophotometer (Biotek) at an absorbance of 405 nm. Caspase-3 activity was expressed as the fold of enzyme activity compared to that of synchronized cells.

Statistical analysis

Results were expressed as mean ± standard deviation. Statistical analyses were performed using the SPSS 11.5 statistical software program. The means of apoptotic incidences among groups receiving identical concentrations of IL-1β and identical concentrations of FBS for the same experimental duration, as well as the results for caspase-3 activity among groups receiving identical concentrations of FBS, were compared by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Caspase-3 activity results for cells treated with the same concentration of IL-1β with and without serum supplement were compared using the unpaired Student's *t* test. A P value < 0.05 was considered statistically significant.

Results

Effects of IL-1β and serum deprivation on apoptotic incidence

No significant changes in apoptotic incidence were observed when AF cells were cultured in complete culture medium with 0, 10, 20, 50 ng/ml IL-1β for 12, 24 or 48 h. In contrast, serum deprivation led to a slight but significant increase in apoptotic incidences after 24 h, and IL-1β significantly amplified the effect of serum deprivation, enhancing apoptosis of AF cells in a dose-dependent manner (Fig. 1A and B). Because our preliminary experiments showed that serum deprivation induced no significant increase in the apoptotic incidence of AF cells cultured for 12 h and there was a considerable increase in the apoptotic incidence of cells after 48-h-culture without FBS supplement, a period of 24 h was selected in order to avoid masking IL-1β stimulation.

Effects of IL-1β and serum deprivation on apoptotic morphology

Using phase-contrast microscopy, apoptotic AF cells exhibited plasma membrane blebbing (Fig. 2A), as previously described [31]. The nuclei of cells cultured in complete culture medium with or without IL-1β for 24 h had a homogeneous pattern of staining for Hoechst 33258. After 24-h of serum deprivation, the proportion of cells with brightly stained condensed, fragmented nuclei slightly increased. Treatment with IL-1β enhanced these changes in apoptotic nuclei morphology (Fig. 2B).

Effects of IL-1β and serum deprivation on caspase-3 activity

Similarly, IL-1β stimulation did not significantly change the caspase-3 activity of cells cultured in complete culture medium. Serum deprivation, however, led to an increase in caspase-3 activity, and IL-1β enhanced the effect of serum deprivation on caspase-3 activation (Fig. 3).

Discussion

Although IL-1β can induce apoptosis of several cell types including pancreatic beta cells [32], placental cells [33] and endothelial cells [34], its effect on the apoptosis of chondrocytes, which share a similar phenotype to IVD cells especially to those residing in the NP, is still controversial. Oliver et al. [23] showed that 1 ng/ml IL-1β administration did not induce apoptosis of immortalized human juvenile costal chondrocytes cultured with 10% FBS for 24 h. Lopez-Armada et al. [24] also reported that treatment with 5 ng/ml IL-1β did not reduce the viability of human articular chondrocytes cultured with 0.5% fetal calf serum (FCS) for 48 h, although it induced depolarization of the mitochondria. Moreover, the viability of chondrocytes from human osteoarthritic cartilage was not modified by stimulation with 50 ng/ml IL-1β even when cells were cultured with medium containing only 0.5% FCS for 72 h, and IL-1β did not increase chondrocyte apoptosis induced by actinomycin D [25]. Conversely, there is also evidence showing that chondrocytes cultured in medium supplemented with either 10% or 5% FCS undergo apoptosis after IL-1β stimulation in a dose- or time-dependent manner (1, 3, 10 ng/ml for 24 h [26] or 1 ng/ml for 24, 48, 72, 96 h

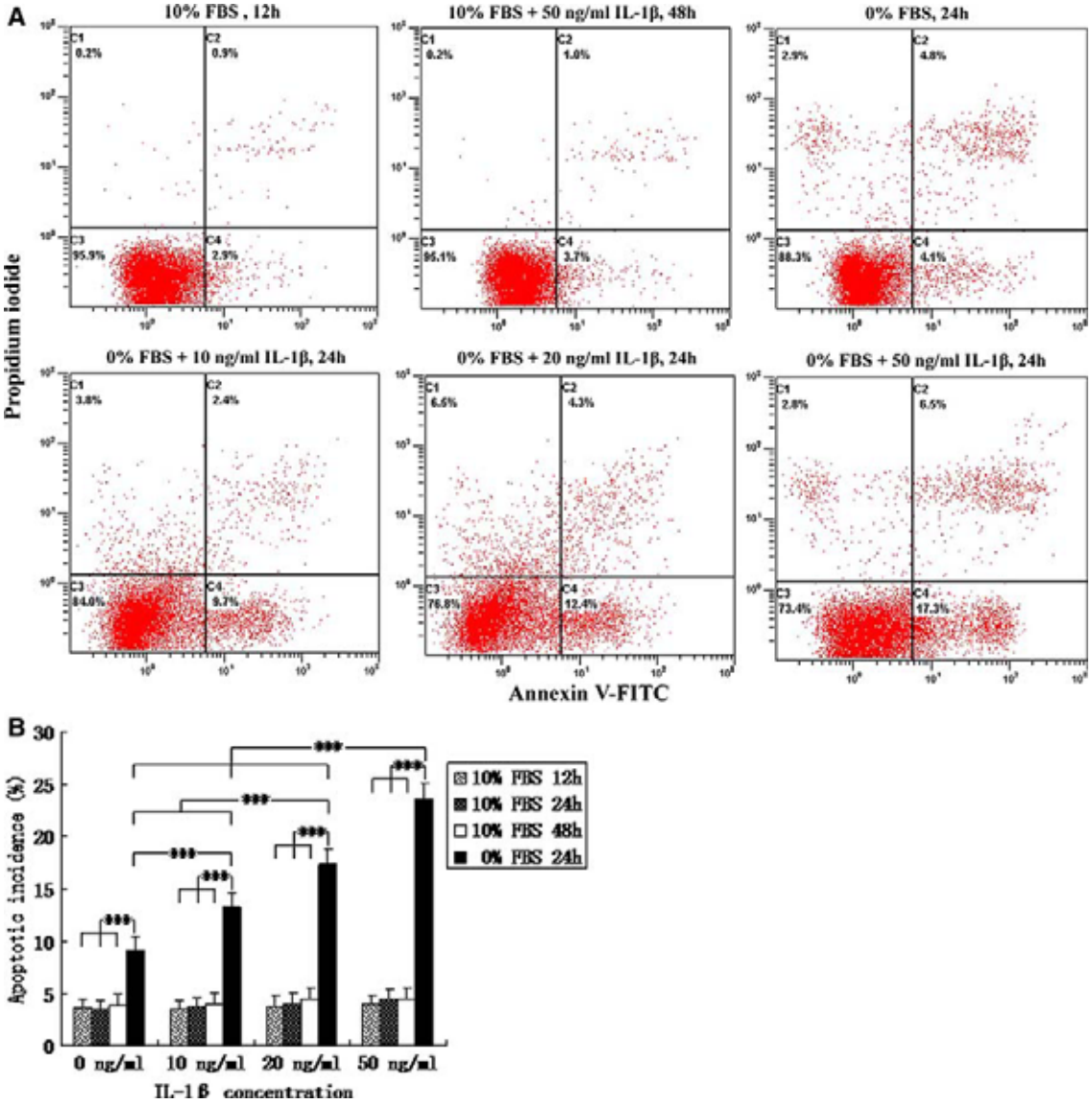

Fig. 1 Evaluation of apoptotic incidence. (**A**) Representative graphs obtained by flow cytometry analysis after double staining with annexin V-FITC and propidium iodide. (**B**) The apoptotic incidences of rat annular cells cultured with or without serum supplement and stimulated with various concentrations of IL-1β. ***$P < 0.001$. (**A**) and (**B**) show that IL-1β alone could not induce annular cell apoptosis in the presence of 10% FBS although serum deprivation led to a slight increase in the apoptotic incidence and IL-1β significantly amplified the effect of serum deprivation on AF cell apoptosis in a dose-dependent manner

[27]). In addition, Shakibaei et al. [28] demonstrated that treatment with 10 ng/ml IL-1β could induce apoptosis of serum-starved human articular chondrocytes within 30 min. However, whether IL-1β can induce disc cell apoptosis has not been reported.

The current study showed that IL-1β can not induce apoptosis of rat AF cells cultured in medium containing 10% FBS. However, when cultured in medium without FBS supplement for 24 h, AF cells exhibited a slight increase in apoptosis incidence, consistent with the findings of other authors [6, 7, 14]. Notably, IL-1β significantly amplified the effect of serum deprivation on AF cell apoptosis, as shown by the increased proportion of cells positively stained with Annexin V-FITC when examined by flow cytometry, the raised number of condensed and fragmented nuclei when stained with Hoechst 33258 and the enhanced caspase-3 activity, which is one of the most important events in the process of apoptosis.

As AF cells respond less well to IL-1β than articular chondrocytes with regards to production of factors implicated in tissue degradation [20], it is possible that AF cells may also be less responsive than articular chondrocytes to IL-1β-induced apoptosis. As shown in the present study, IL-1β did not compromise the viability of AF cells cultured

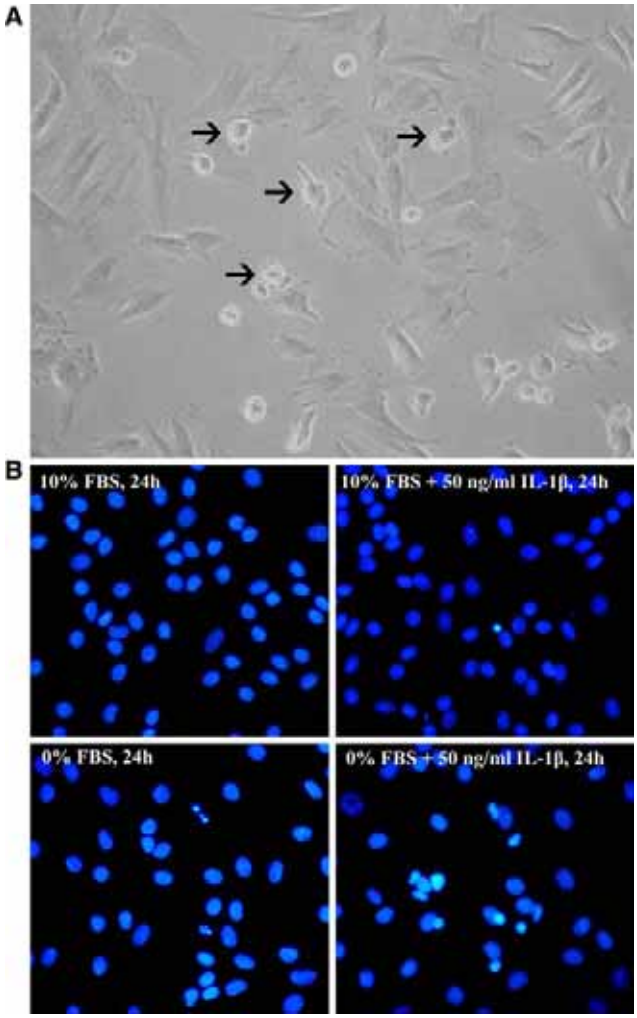

Fig. 2 Morphologic changes in apoptotic rat annular cells. (**A**) Phase-contrast photomicrograph of rat annular cells cultured in serum-free medium and stimulated with 50 ng/ml IL-1β for 24 h. Apoptotic cells were characterized by plasma membrane blebbing (*arrows*). Original magnification ×160. (**B**) Apoptotic nuclei were condensed or fragmented and brightly stained with Hoechst 33258. Original magnification ×200. IL-1β alone did not increase the number of apoptotic nuclei whereas serum deprivation resulted in an increased number of apoptotic nuclei. IL-1β enhanced the effect of serum deprivation

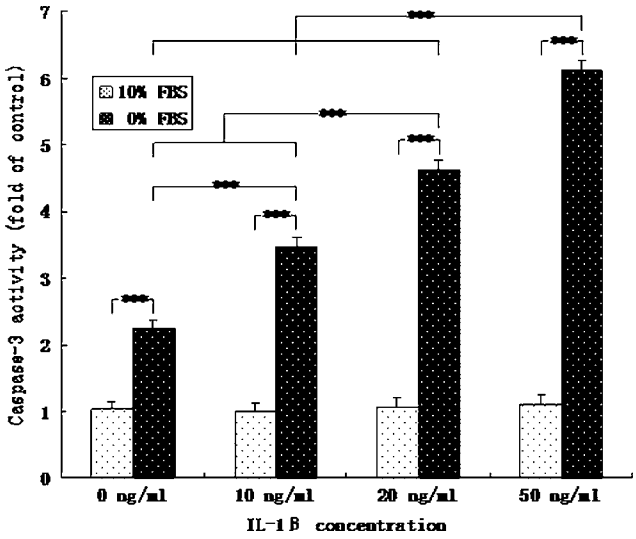

Fig. 3 Changes in caspase-3 activity. ***$P < 0.001$. In the presence of 10% FBS, IL-1β did not increase the activity of caspase-3. Serum deprivation led to a slight increase in caspase-3 activity, and IL-1β significantly amplified the effect of serum deprivation in a dose-dependent manner

with 10% FBS, although it can induce apoptosis of chondrocytes cultured in medium with the same concentration of FCS [26].

The serum contains various kinds of growth factors, some of which are responsible for cell survival, such as insulin-like growth factor-1 (IGF-1). It has been well recognized that IGF-1 inhibits serum deprivation-induced apoptosis in many cell types including IVD cells [6, 35–41], probably through the phosphatidylinositol 3'-kinase and mitogen-activated protein kinase pathways [35, 36, 38–41]. In addition, there is evidence showing that IGF-1 can also inhibit IL-1β-induced apoptosis in some cell types, such as growth plate chondrocytes from metatarsal bones [42] and pancreatic beta cells [43, 44], probably by preventing the Fas-mediated component. In this context, serum deprivation is expected to give rise to the withdrawal of some growth factors such as IGF-1, which in turn lead to not only a decreased trophism but also a compromised ability to resist pro-inflammatory cytokine counterattack of cells. Therefore, a significantly increased rate of apoptosis in AF cells cultured without serum supplement and simultaneously treated with IL-1β is reasonable.

It has long been recognized that there is a continuous increase in cell death during human IVD degeneration [2, 45, 46]. Meanwhile, the nutrient supply to human IVD deteriorates during degeneration [47], whereas the level of IL-1β increases [16, 18]. The relevance of our results to human IVD degeneration may be that IL-1β and serum deprivation synergize to increase apoptosis of human IVD cells in vivo.

There is evidence showing that IL-1β suppresses the synthesis of collagen Type II and proteoglycan by IVD cells [18, 20], whereas it stimulates the expression of the enzymes which are responsible for IVD matrix degradation [18, 21]. Thus, IL-1 receptor antagonist gene transfer, which inhibits IL-1β-mediated IVD matrix degradation, may be a potential therapy for retarding IVD degeneration [48]. However, it should be noted that IL-1β alone is not a sufficient stimulus to induce AF cell apoptosis. It acts to enhance the effect of serum deprivation, as shown by our results. This indicates that, in order to suppress IVD cell apoptosis, antagonizing the adverse effects of IL-1β might be less effective than improving the nutrient supply, which deteriorates during IVD degeneration. Indeed, Park et al. [7] have pointed out that even if IVD cells demonstrate

increased survival by means of suppressing apoptosis, they would inevitably die without an adequate nutrient supply.

Conclusions

Il-1β alone is not a sufficient stimulus to induce disc cell apoptosis. Our results suggest that, in order to suppress disc cell apoptosis, improving the nutrient supply to the disc may be more effective than antagonizing the adverse effects of IL-1β.

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