



The Anti-Inflammatory and Cytoprotective Efficiency of Curvularin, a Fungal Macrolactone against Lipopolysaccharide-Induced Inflammatory Response in Nucleus Pulposus Cells: An *In Vitro* Study

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Study Design: Developing an *in vitro* model for assessing the anti-inflammatory properties of curvularin.

Purpose: To evaluate the efficacy of natural fungal macrolactone as a therapeutic drug against lipopolysaccharide (LPS)-induced inflammation in primary human nucleus pulposus cells (NPCs) *in vitro*.

Overview of Literature: Lumbar disk disease is a common cause of lower back pain (LBP) and sciatica. It is an established fact that inflammation, rather than mechanical compression on the nerve root, plays a role in the cause of LBP and sciatica. Current treatment options for reducing inflammation are either nonsteroidal anti-inflammatory drugs or steroids, prolonged use of which can potentially lead to adverse effects such as gastrointestinal disturbances and renal and cardiac issues. Hence, there is a need for better anti-inflammatory drugs with no or minimal complications for treating inflammation-induced LBP and sciatica. Curvularin (Cur), a fungal macrolactone, is known for its anti-inflammatory activity, but nothing is known about its impact on inflammation due to disk pathologies.

Methods: Primary NPCs were cultured and characterized by flow cytometry and immunocytochemistry using the CD24 antibody and treated with 10 µg/mL LPS for 36 hours and then treated with Cur, betamethasone, and dexamethasone (10 µg/mL) for 48 hours, after which cell cycle analysis, cell viability assay, and gene expression studies (quantitative polymerase chain reaction [PCR] and quantitative real-time-PCR) were conducted. The NPCs treated with Cur downregulated the expression of pro-inflammatory cytokines (tumor necrosis factor- α , interleukin [IL]-1 β , and IL-6); matrix metalloproteinases (MMPs; MMP-2 and MMP-3), ADAMTS; and apoptotic marker (cytochrome c).

Results: In our study, Cur-treated cells showed enhanced expression of collagen 9A1 and insulin-like growth factor receptor 1, indicating the recovery of NPCs from inflammatory assault.

Conclusions: Based on observations, the anti-inflammatory properties of Cur render it an excellent drug molecule for treating disk degeneration nonsurgically, by direct injection into spinal disks when treating LBP and sciatica.

Keywords: Intervertebral disc degeneration; Macrolactone; Curvularin; Lipopolysaccharide; Inflammation; Cytokines; Nonsteroidal anti-inflammatory drugs

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Introduction

One of the common causes of lower back pain (LBP) and sciatica is intervertebral disc degenerative (IVDD) pathologies [1,2], a multifactorial process with complex biochemical cascade, due to the involvement of genetic and environmental factors in its etiology [2]. Perineural inflammation, rather than mechanical compression, has proven to be the primary cause of LBP and sciatica [3-5]. Numerous anti-inflammatory drugs are used in the treatment of LBP and sciatica but with limited success, leading to a significant economic burden on the global population [6]. Continuing efforts are being made by various scientific groups to find alternative anti-inflammatory drugs with better efficacy and safety.

A focus on phytochemicals denotes good anti-inflammatory properties, which can potentially reduce LBP and sciatica. Known phytochemicals, such as curcumin [5], resveratrol (3,5,4'-trihydroxy-trans-stilbene) [6], naringin [7], naringenin [7], quercetin [7,8], simvastatin [9], atorvastatin, lovastatin [9], and others, are known for their anti-inflammatory properties that downregulate interleukins (IL-1 α , IL- β , IL-6, IL-18), tumor necrosis factor- α (TNF- α), and aggrecanases and collagenases (matrix metalloproteases [MMPs] and ADAMTS) in nucleus pulposus cells (NPCs) [10-16]. These flavonoid-based drugs can be used individually or in combinations to control inflammation and to potentially regenerate intervertebral discs.

The present study focuses on inflammation-induced LBP and sciatica, which are treated symptomatically rather than by regenerative or curative therapies. Globally, researchers have used different inflammatory cytokines

(i.e., IL-1 β , IL-1 α , TNF- α , and IL-6) to induce inflammation in human NPCs, reporting increased expression of MMP-1–MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 and decreased expression of aggrecan, collagen types I and II, and SOX6 and SOX9 [6,9,12-15,17-20] (Table 1). In the current study, we used LPS to induce inflammation in NPCs. Our study aims to evaluate the anti-inflammatory and regenerative efficiency of curvularin (Cur), a known natural fungal macrolactone [16,21,22], against LPS-induced inflammation in primary human NPCs, by altering the expression profiles of pro-inflammatory and metalloprotease genes *in vitro*. To our knowledge, no studies have been published on Cur that show it as having anti-inflammatory and cytoprotective properties for NPCs; hence, our study will be the first to evaluate Cur in this context.

Materials and Methods

To achieve the aims of this study, Cur (isolated from *Penicillium citrinum*, C16H20O5, cat no. SML0772) and lipopolysaccharide (LPS) (isolated from *Escherichia coli*, O111:B4, cat no. L3024) were purchased from Sigma-Aldrich (Bangalore, India). The purity of the compounds was $\geq 95\%$ (derived via high-performance liquid chromatography). Cur and LPS were reconstituted in dimethyl sulfoxide (Sigma-Aldrich) to yield a stock solution of 2 mg/mL. Dulbecco's Modified Eagle Medium/nutrient mixture F12 Ham (DMEM-F12, 1:1 mixture), 10% fetal bovine serum (FBS), 0.1% collagenase type I, an antibiotic antimycotic solution 100 \times with 10,000 units of penicillin, 100 mg streptomycin and 25 μ g amphotericin B per mL

Table 1. Overview of inflammatory mediators used for inducing inflammation in varied NPCs and expression profile of cytokines, metalloproteases, and so forth

Reference	Stimulator	Models	Expression
Wuertz et al. [6] (2011); Le Maitre et al. [12] (2005); Yu et al. [13] (2009); Wang et al. [14] (2011); Zhao et al. [15] (2011); Shen et al. [17] (2003)	IL-1 β	Human NPC	Upregulated MMP's (1, 3, 9, and 13), ADAMTS (4 and 5) and down-regulated aggrecan, collagen type I & type II, SOX (6 and 9)
Shen et al. [18] (2018)	LPS	Rats NPC	Upregulated TNF- α , IL-1 β , PGE2, NO, iNOS, COX-2, p-IkB, p-P65; down-regulated collagen II and aggrecan
Mancino et al. [19] (2011)	LPS	Bovine IVD cells	IL-1 β , IL-6, and IL-10 upregulated; collagen 1 and 2 down-regulated
Li et al. [9] (2015)	LPS	Rat NPC	Upregulated ADAMTS4, ADAMTS5, MMP-3, MMP-13, PGE2, COX-2, and iNOS; down-regulated aggrecan and collagen II
Kong et al. [20] (2018)	LPS	Rat NPC	Upregulated MMP-3, MMP-13, ADAMTS4, ADAMTS5, TNF- α , IL-1, IL-6, and PGE2; down-regulated miR-194, aggrecan, and collagen II

NPCs, nucleus pulposus cells; IL, interleukin; MMPs, matrix metalloproteinases; LPS, lipopolysaccharide; TNF, tumor necrosis factor; PGE2, prostaglandin E2; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; IVD, intervertebral disc.

in 0.9% saline, 0.25% trypsin-ethylenediaminetetraacetic acid, centrifuge tubes (Tarsons, Kolkata, India; cat no. 546041), 100 µm nylon mesh (Himedia Laboratories, Mumbai, India; reference TCP183), and all other chemicals and reagents were analytical or culture grades, purchased from Himedia Laboratories and Sigma-Aldrich.

1. *In vitro* studies: culture of human nucleus pulposus cells

Intervertebral disc tissue from the lumbar spine was collected from patients undergoing interbody fusion surgery for degenerative disk disease (institutional ethical approval was secured prior to collection—SS/2015/IEC127). Disk tissue was immediately collected in 20 mL saline supplemented with an antibiotics solution and transported to a laboratory at 4°C. All procedures were conducted in a sterile environment. Tissue samples were repeatedly washed in a phosphate buffer saline (PBS) solution, and then the NPCs were separated by slicing the disk tissue into 1 mm³ segments in a moist environment and digested with 0.1% collagenase type I at 37°C for 4 hours, with intermittent shaking at 30-minute intervals. Following this, the reaction was stopped with FBS. The digested mixture was then filtered through a sterile 100 µm nylon mesh and centrifuged at 1,500 rpm for 5 minutes to collect cell pellets. The cells were repeatedly washed in a PBS solution and resuspended in a DMEM-F12 medium containing 10% FBS and antibiotics (prostate specific antigen) and seeded with a cell concentration density of 1×10⁵/mL into a 75 cm² flask. Cells were maintained in culture flasks at 37°C in a 5% CO₂ atmosphere, and culture media was changed 2 to 3 times a week. NPCs were used for further experimentation at passage three [23,24].

2. Characterization of human nucleus pulposus cells by flow cytometry

Degenerated human NPCs were cultured in a T75 culture flask in the presence of DMEM-high glucose and 10% FBS; 1× penicillin-streptomycin antibiotics were allowed to grow to 70% to 80% confluence, trypsinized, and resuspended in 300 µL PBS. The cells were characterized by examining the presence of CD24 markers (positive markers) using standard flow cytometry methods [23].

3. Treatment with lipopolysaccharide and curvularin

Cultured NPCs (5×10⁴/mL) were seeded in three 12-well cell culture plates for up to 72 hours prior to experimentation. The control group received no treatment (Table 2). The NPCs were treated with 10 µg/mL LPS for 36 hours before cells were treated with 10 µg/mL Cur, and beta-methasone (Beta) and dexamethasone (Dexa) was individually added to respective group wells and in combination (Cur, Beta, Dexa in a 1:1:1 ratio) for 48 hours before harvesting total RNA [24] (Table 2).

4. Cell cycle studies by flow cytometry

Degenerated human NPCs were cultured in 60 mm dishes and initially treated with LPS (10 µg/mL) for 24 hours. Later, anti-inflammatories Cur (10 µg/mL) and Dexa (10 µg/mL) and a combination of Cur and Dexa (10 µg/mL) were added to cells, harvested after 36 hours, prepared for cell proliferation probing with propidium iodide for 15 to 20 minutes in the dark at room temperature, and estimated using an Attune NxT flow cytometer (Applied Biosystems, Foster City, CA, USA) as per manufacturer's instructions [25,26].

5. Cell viability assay/MTT assay

An MTT assay was conducted to establish the cytotoxicity of drugs and was measured based on the number of viable cells. Normal cells (i.e., human embryonic kidney [HEK] cell lines) were plated in 96 well plates. Cells were individually treated with vehicle control or Cur, Beta, or Dexa and in combinations (Cur, Beta, Dexa) for 36 hours. The reason for this selection was to avoid unwanted cytotoxicity in normal cells. After 36 hours, cell viability was analyzed using the MTT assay, based on published protocols, and absorbance was recorded at 475 nm, with reference to

Table 2. Experimental groups

Groups	Treatment
Control (negative control)	No treatment
Lipopolysaccharide	10 µg/mL
Cur	10 µg/mL
Beta	10 µg/mL
Dexa	10 µg/mL
Combination	Cur-Bet-Dexa in 1:1:1 ratio

Cur, curvularin; Beta, Betamethasone; Dexa, Dexamethasone.

660 nm [25,26].

6. Gene expression studies: RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction

After 48 hours, NPCs were trypsinized, and total RNA was isolated using the Nucleopore Mini Kit (Genetix Biotech, New Delhi, India). The concentration of RNA was determined using NanoDrop; 50 ng/mL of total RNA was used for cDNA synthesis using a commercial cDNA synthesis kit (Verso cDNA Synthesis Kit; Thermo Fischer Sci-

entific, Waltham, MA, USA), and gene expression studies (quantitative polymerase chain reaction [PCR]) were conducted using a SimpliAmp Thermal Cycler (Applied Biosystems) and real-time PCR (QuantStudio 5, Applied Biosystems, Thermo Fischer Scientific).

All PCR products were visualized on a 1.5% agarose gel with 0.5 mg/mL ethidium bromide. Photographs were taken using the Gel Documentation System (E Gel Imager; Life Technologies, Carlsbad, CA, USA). The cDNAs were used for studying the gene expression of interleukins (IL-1 β , IL-6, IL-2, IL-10), TNF- α , MMPs (MMP-2, MMP-3, ADAMTS-5), mineralization and adhesion markers

Table 3. List of primer sequences used for gene expression studies

Serial no.	Gene name	Sequence (5'–3')	Time (sec)	Functions
1	TNF- α	Forward: AAGCACACTGGTTTCCACACT Reverse: TGGGTCCTGCATATCCGTT Product size: 74 bp	58	Inflammatory cytokine
2	IL-1 β	Forward: AACAGATGAAGTGCTCCTTCCAGG Reverse: TGGAGAACACCACTTGT GCTCCA Product size: 390 bp	61	Inflammatory cytokine
3	IL-6	Forward: ACTCACCTCTTCAGAACGAATTG Reverse: CCATCTTTGGAAGGTCAGGTTG Product size: 159 bp	61	Pro-inflammatory cytokine
4	IL-2	Forward: CATGTACAGCATGCAGCTCGCATCC Reverse: CCACCACAGTTGCTGGTCATCATC Product size: 390 bp	66	Anti-inflammatory cytokine
5	IL-10	Forward: GACTTTAAGGGTTACCTGGGTTG Reverse: TCACATGCGCCTTGATGTCTG Product size: 390 bp	62	Anti-inflammatory cytokine
6	MMP-2	Forward: GATACCCCTTTGACGGTAAGGA Reverse: CCTTCTCCAAGGTCATAGC Product size: 111 bp	62	Degradation of the disc matrix
7	MMP-3	Forward: CGGTTCCGCTGTCTCAAG Reverse: CGCCAAAAGTGCCTGTCTT Product size: 285 bp	64	Breakdown of extracellular matrix
8	ADAMTS-5 ^{a)}	Forward: GAACATCGACCAACTCTACTCCG Reverse: CAATGCCACCGAACCATCT Product size: 106 bp	64	Pathogenesis of human osteoarthritis
9	IGF1R	Forward: AGGATATTGGGCTTTACAACCTG Reverse: GAGGTAACAGAGGTCAGCATTTT Product size: 79 bp	61	Hypertrophy of muscle and tissue
10	CYT-C	Forward: TTCCGGGCAAAATGGCGAT Reverse: CAAGGAAGAGGTAGTGTGGGC Product size: 179 bp	58	Apoptosis
11	COL9A1	Forward: GGCAGTAGAGGAGAATTAGGACC Reverse: GTTCACCGACTACACCCTG Product size: 141 bp	64	Delay onset of osteoarthritis
12	GAPDH	Forward: GGCTCTCCAGAACATCATCCCTGC Reverse: GGGTGTCTGCTGTTGAAGTCAGAGG Product size: 260 bp	66	House keeping gene

TNF, tumor necrosis factor; IL, interleukin; MMPs, matrix metalloproteinases; IGF1R, insulin-like growth factor 1 receptor; CYT-C, cytochrome c; COL9A1, collagen 9 alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

^{a)}A disintegrin and metalloproteinase with thrombospondin motifs 5.

(insulin-like growth factor 1 receptor [IGF1R]), death markers (cytochrome c [CYT-C]), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene [24] (Table 3).

Results

1. Culturing of degenerated human nucleus pulposus cells

Degenerated human NPCs were successfully isolated from the disk, propagated, cultured for P0 to P3 generation, and characterized in the study after passage 3. The cells were polygonal in shape, similar to chondrocytes (Fig. 1).

2. Characterization of nucleus pulposus cells by flow cytometry and immunocytochemistry studies

Fig. 2A illustrates the CD24-positive population (NPCs), where the M1 peak denotes unstained cells that were not positive for CD24, and the M2 peak denotes the cell population positively stained by CD24. Fig. 2B also demonstrates CD24-positive cells under fluorescence, which indicates that most of the population in isolated cells were NPCs.

3. Treatment with curvularin, betamethasone, and dexamethasone

Cultured NPCs (5×10^4 cells/mL) were seeded in 12 well plates for up to 72 hours prior to the experiment. Once cell confluence reached 80%, these cells were treated with LPS ($10 \mu\text{g/mL}$) for 36 hours; subsequently, NPCs were treated with Cur, Beta, and Dexa ($10 \mu\text{g/mL}$) for 48 hours individually and also in combination (Cur-Beta-Dexa in a 1:1:1 ratio). Treatments were effected in duplicate.

NPCs treated with LPS were lower in number and ex-

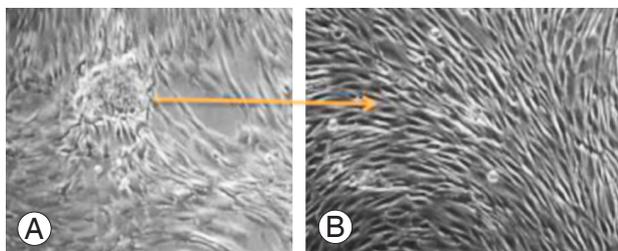


Fig. 1. (A, B) Culture of degenerated human nucleus pulposus cells.

hibited degenerative morphology, whereas NPCs treated with anti-inflammatories or cytoprotectants showed better cell proliferation and cell morphology when individual and combined treatments were effected. Cur exhibited protective effects similar to commercial drugs.

4. Cell cycle assay

Degenerated human NPCs were initially treated with LPS ($10 \mu\text{g/mL}$) for 24 hours; later, the anti-inflammatories (Cur, $10 \mu\text{g/mL}$; Dexa, $10 \mu\text{g/mL}$; and a combination of Cur and Dexa, $10 \mu\text{g/mL}$) were added to cells and harvested after 36 hours. Untreated NPCs were considered a control. The cell proliferation analysis showed decreased cell proliferation in LPS-treated NPCs and enhanced cell proliferation in NPCs treated with anti-inflammatories. NPCs that received combination treatment showed more cell proliferation compared to individual anti-inflammatory treatment.

5. Cell proliferation assay (MTT)

We carried out MTT assay studies for the assessment of cell viability and cell proliferation efficiency of the HEK-293 cell line; simultaneous treatment with LPS, Cur, Beta,

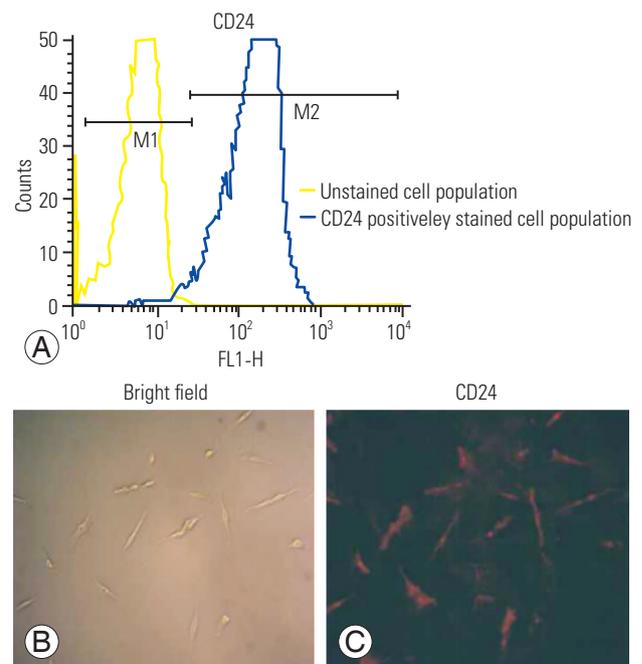


Fig. 2. (A–C) Flow cytometry and immunocytochemistry studies for the characterization of primary human nucleus pulposus cells using the CD24 antibody.

and Dexamethasone individually; and combinations of these after 36 hours of exposure (Fig. 3). Results of the MTT assay suggest that Curcumin, Beta, and Dexamethasone did not induce cytotoxicity in the HEK-293 (normal cell line), and cells proliferated normally when treated with similar dosages used during our study. No cytotoxicity was observed in any of the protective groups, but a lower number of cells was observed in the LPS-treated group. The 10 µg/mL Curcumin, Beta, and Dexamethasone appeared to be effective and were found to be an optimum dose for promoting cell proliferation.

6. Gene expression studies (real-time quantitative polymerase chain reaction)

The gene expression of TNF- α , IL-1 β , IL-6, MMP-2, MMP-3, ADAMTS, CYT-C, IL-10, IL-2, IGF1R, collagen 9A1, and GAPDH was studied in a primary culture of NPCs, with induced inflammation using LPS. The NPCs were treated with LPS, LPS-Curcumin, LPS-Beta, LPS-Dexamethasone, and with a combination (LPS-Curcumin-Beta-Dexamethasone).

TNF- α , IL-1 β , and IL-6 are major pro-inflammatories, and their gene expression was significant in LPS-treated NPCs. NPCs treated with Beta and Dexamethasone showed reduced expression of pro-inflammatories. The Curcumin and combination-treated NPCs showed no expression compared to LPS-treated NPCs (positive group). The untreated NPCs (control group) showed no expression of metalloproteases. Collagenases MMP-2 and MMP-3 degraded the extracellular matrix (ECM) and caused the loss of NPCs. MMP-2 and MMP-3 gene expressions were higher in NPCs treated with LPS compared to treatment with protective agents. It was observed that cells treated with Beta and Dexamethasone alone and in combination showed a lower expression of MMP-2 compared to LPS-treated cell. Curcumin was less

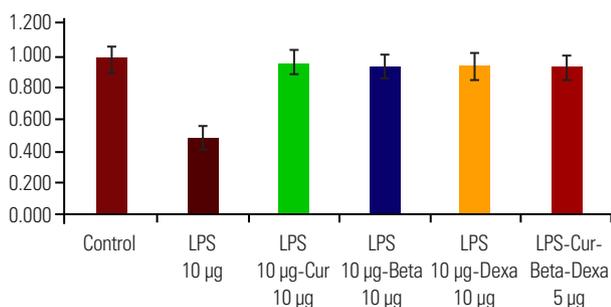


Fig. 3. MTT assay for assessing the cytotoxicity of individual and combined macrolactone and other steroid treatments on a normal cell line (human embryonic kidney-293). LPS, lipopolysaccharide; Cur, curcumin; Beta, betamethasone; Dexa, dexamethasone.

effective at controlling MMP-2 and MMP-3 expression in NPCs compared to other protectants. NPCs treated with Beta, Dexamethasone, and combinations showed variable expression of MMP-3.

ADAMTS are known as aggrecanases responsible for the degradation of ECM and loss of NPCs. ADAMTS-5 gene expression was significant in LPS-treated NPCs compared to Beta- and Dexamethasone-treated NPCs; however, NPCs treated with Curcumin and combinations showed no expression of ADAMTS-5. The observed results indicate that Curcumin had been effective for controlling the actions of ADAMTS on aggrecanases in the ECM of NPC.

CYT-C is known as a death marker. CYT-C gene expression was observed for LPS treatment, at comparatively lower levels in Beta and Dexamethasone treatment, and significantly lower in the combination treatment (Curcumin, Beta, and Dexamethasone) groups. However, the expression of CYT-C was not observed in the control and Curcumin-treated groups. These results indicate the cytoprotective properties of Curcumin, Beta, and Dexamethasone against LPS-induced cell death. Combined (Curcumin, Beta, and Dexamethasone) treatment functioned synergistically with other treatments.

An anti-inflammatory response was initiated in LPS treatment of NPCs. As a result, we were able to observe the expression of anti-inflammatory molecules being activated or expressed. IL-2 and IL-10 are anti-inflammatory molecules, the expression of which was significant in LPS-treated NPCs, signifying their role in reducing LPS-induced effects [8-11]. The expression of IL-10 was not observed in the negative control nor in any of the other treated groups (Curcumin, Beta, and Dexamethasone and their combination). The observations made in this experiment clearly indicate the efficiency of Curcumin, Beta, and Dexamethasone at controlling LPS-induced inflammation, individually and in combination. Our results are supported by reported studies [8-11].

LPS induction initiates inflammation in NPCs. As a result, we observed the expression of repair genes being activated or overexpressed. IGF1R is an anti-injury or repair gene, the expression of which was significant in the LPS-treated NPCs, signifying its role in reducing LPS-induced effects. The expression of IGF1R was not observed in the negative control, and also in NPCs treated with Curcumin, Beta, and Dexamethasone individually, and in combination. The observations made in this experiment clearly indicate the efficiency of Curcumin, Beta, and Dexamethasone at controlling LPS-induced inflammation, individually and in combination.

7. Expression of collagen genes in the experimental groups

Collagen is part of the ECM. Collagen gene expression was lower in the LPS-treated group than in the other experimental groups, which indicated a loss of collagen. It was observed that Cur and combination treatments effected higher expression of collagen compared to steroid treatment (Fig. 4).

8. Real-time polymerase chain reaction studies

At a concentration of 10 $\mu\text{g/mL}$, Cur and combination treatments downregulated the LPS-induced expression of proteolytic enzymes and pro-inflammatory markers in NPCs. As such, real-time quantitative-PCR results suggest that the upregulated expression of MMPs (MMP-2 and MMP-3) and ADAMTS (ADAMTS-5), induced by LPS, was downregulated by Cur and combination treatments. Similarly, LPS evidently increased the expression of pro-inflammatory markers (IL-1 β , IL-6); this increase was appreciably inhibited by Cur and combination treatments. A partial reversal of LPS-induced expression of collagen 9 by Cur and combination treatments was observed. Notably,

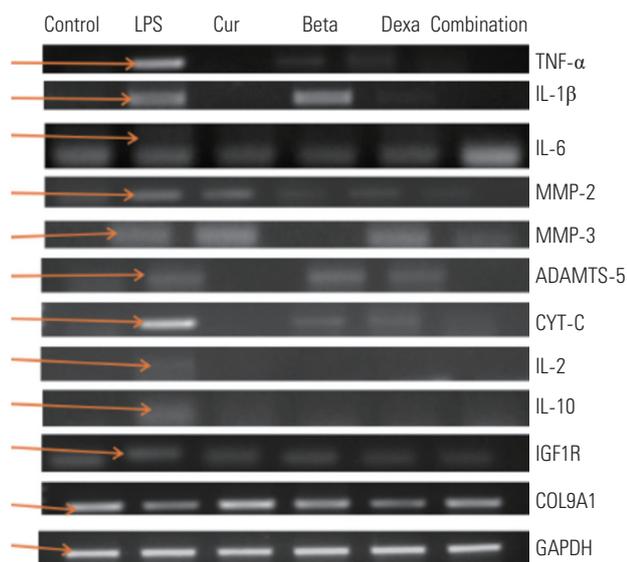


Fig. 4. Gene expression of pro-inflammatories (TNF- α , IL-1 β , and IL-6), metalloproteases (MMP-2, MMP-3; ADAMTS-5), anti-inflammatories (IL-2 and IL-10), cell death marker (CYT-C), stress-cum-repair marker (IGF1R), collagen 9A1, and endogenous control (GAPDH) in all experimental groups. TNF, tumor necrosis factor; IL, interleukin; MMPs, matrix metalloproteinases; CYT-C, cytochrome c; IGF1R, insulin-like growth factor 1 receptor; COL9A1, collagen 9 alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenas.

the overexpression of IGF1R induced by LPS was also significantly suppressed by Cur and combination treatments.

The above results indicate the cytoprotective and anti-inflammatory properties of Cur, Beta, and Dexa against LPS-induced cell death. Combination treatment worked synergistically with others (Cur, Beta, and Dexa).

Discussion

Perineural inflammation is a significant contributor to LBP and sciatica in patients with degenerative pathologies. Inflammation initiates the expression of pro-inflammatory cytokines, proteolytic enzymes (MMPs; i.e., MMP-2 and MMP-3), and ADAMTS (ADAMTS-5), contributing to disc pathologies [2,3]. Increased levels of inflammatory cytokines such as IL-1 β , IL-6, and TNF- α , which are associated with IVDD in human and animal models, are believed to compromise the biomechanical properties of intervertebral discs, thereby promoting a degenerative state [2]. On one hand, IL-1 β and TNF- α , as two key mediators involved in degenerative changes in intervertebral discs, can bind to their membrane receptors and induce the downregulation of B-cell lymphoma 3-encoded protein, thus promoting NPC apoptosis. On the other hand, IL-1 β and TNF- α promote the expression of MMPs and inhibit the synthesis of collagen and proteoglycan as well as the proliferation of NPCs [12-15]. Corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs) are often used to treat patients with LBP and sciatica [27]. Studies conducted by Benyamin et al. [28] in 2012 and Mailis and Taenzer [29] in 2012 reported that repeated administration of epidural injections affected bone mineral density, which leads to osteoporosis [30]. Additionally, NSAIDs inhibit the activity of cyclooxygenase-2 and the synthesis of prostaglandin, as they play a role in the upregulation of inflammation [29,30]. Overexpression of TNF- α and IL-6 by herniated discs was observed, which can induce the production of prostaglandin E2 [28]. The prolonged use of NSAIDs can induce gastrointestinal disturbances and renal and cardiac issues [30].

Cur is a novel bioactive fungal macrolactone (secondary metabolite) that can effect potent anti-inflammatory action; hence, it indicates importance for medicine and agriculture [22]. Schmidt et al. [21] in 2012 investigated the anti-inflammatory activity of (S)-Cur in mice and conducted microarray analyses of human C28/I2 chon-

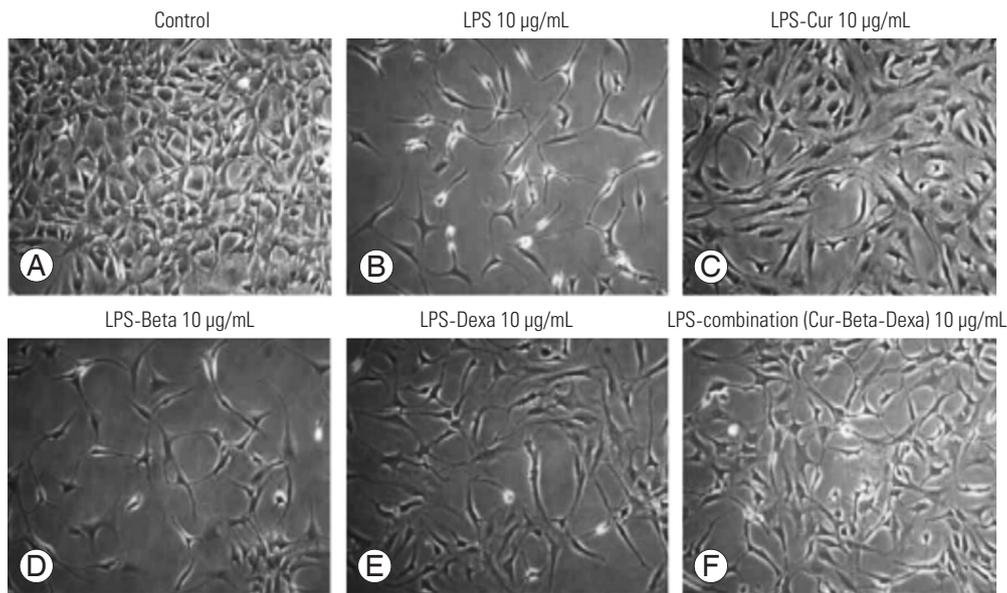


Fig. 5. (A–F) The effect of Cur, Beta, and Dexa, and their combination, on LPS-induced inflammation and loss of NPCs, compared to the untreated control group. We examined the growth enhancement and protective effects of Cur, Beta, and Dexa on NPCs. Cur, curvularin; NPCs, nucleus pulposus cells; LPS, lipopolysaccharide; Beta, betamethasone; Dexa, dexamethasone.

drocyte cells, by which they reduced the expression of pro-inflammatory genes. Nitric oxide (NO) plays a pivotal role in osteoarthritis and rheumatoid arthritis; (S)-Cur inhibited the production of NO by downregulating inducible NO synthase [22]. Rudolph et al. [16] in 2012 found that (S)-Cur and dehydrocurvularin can be used as anti-cancer drugs as they are able to hinder the cellular effects of transforming growth factor-beta, which is a key mediator in the cell cycle process.

The present study systematically evaluated the efficacy of Cur against LPS-induced inflammation in NPCs. Furthermore, we attempted to better understand the anti-inflammatory mechanism of Cur by studying the expression profile of inflammatory cytokines, metalloproteases, and apoptotic and structural genes in this *in vitro* research.

The fluorescence-activated cell sorting analysis conducted on isolated degenerated disk cells identified these cells as being NPCs, characterized using the CD24 marker (Fig. 2). The NPCs treated with LPS showed decreased cell numbers as well as cells that appeared to be blebbing or that were necrotic. However, cells that received Cur, Beta, and Dexa showed cytoprotective properties against LPS-induced cytotoxicity. NPCs that received Cur individually and in combination treatments exerted enhanced cytoprotective properties compared to individual Beta and Dexa treatments, which indicated that is a better cytoprotective agent (Figs. 5, 6). Similar findings were observed for

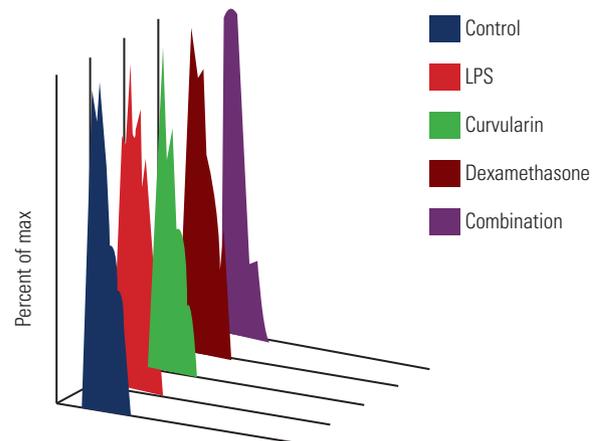


Fig. 6. The of individual and combinatorial anti-inflammators against LPS treated degenerated human NPC using cell proliferation assay. LPS, lipopolysaccharide.

the MTT assay, where treatment with LPS decreased cell viability but achieved the reversed result following treatment with protective agents (Cur, Beta, and Dexa), which indicated the cytoproliferative properties of these agents. The cytoprotective ability of Cur was found to be better compared to steroids (Fig. 3).

Gene expression studies (quantitative PCR and real-time quantitative-PCR) revealed the overexpression of pro-inflammatory cytokines (interleukins [IL-1 β and IL-6] and TNF- α), MMPs (MMP-2 and MMP-3), anti-inflammatory (IL-2 and IL-10), and cell repair responses

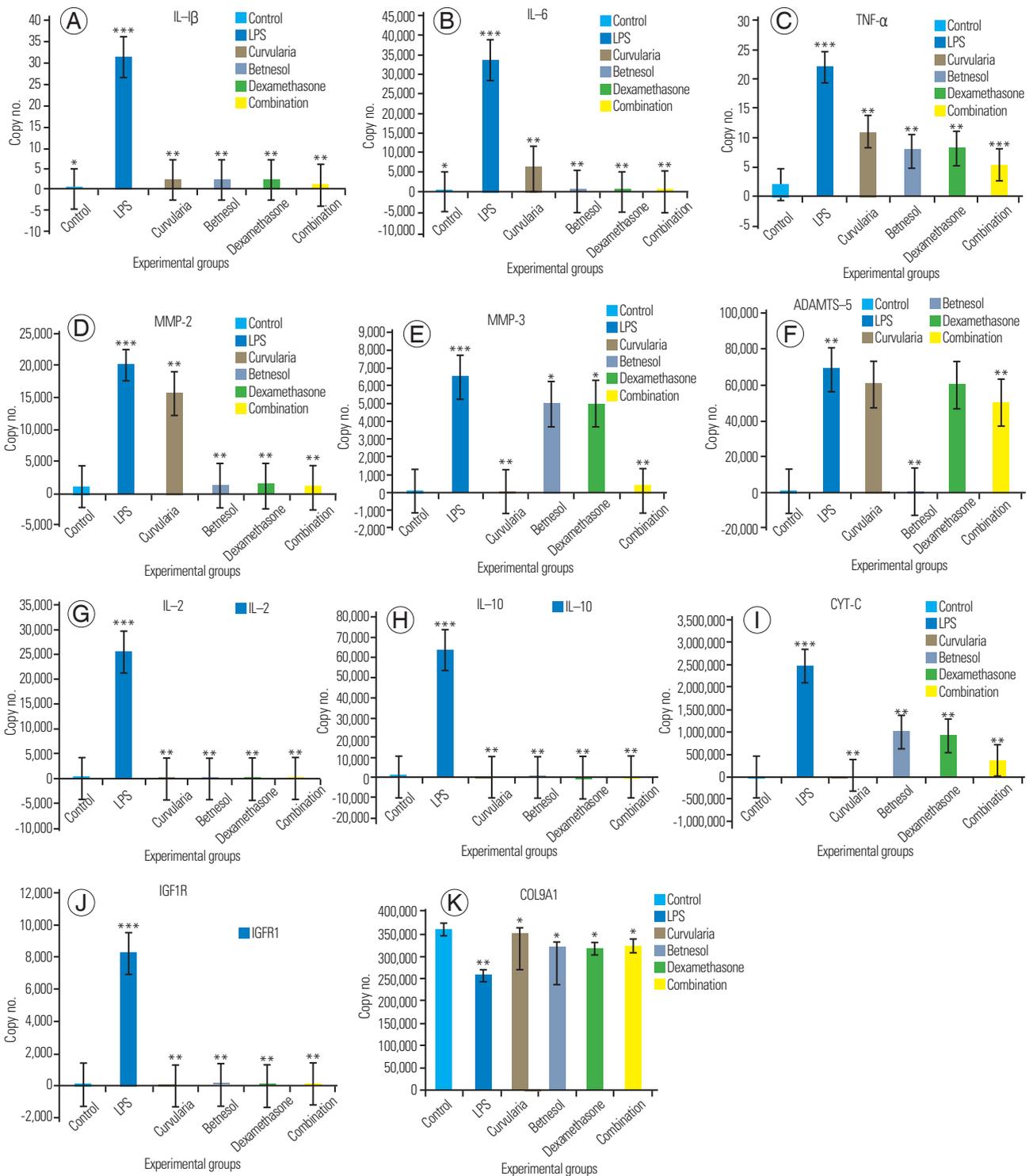


Fig. 7. (A–K) Graphical representation of varied gene expressions using real-time polymerase chain reaction. LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; MMPs, matrix metalloproteinases; CYT-C, cytochrome c; IGF1R, insulin-like growth factor 1 receptor; COL9A1, collagen 9 alpha 1. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

(IGF1R); additionally, downregulation of the structural gene (collagen 9A1) of the ECM was observed in the degenerated human NPCs upon LPS treatment. Treat-

ments with protective agents mediated the expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), metalloproteases (MMP-2 and MMP-3), IGF1R, and anti-

Table 4. Real-time polymerase chain reaction studies showing the gene expression of various genes when treated with LPS and anti-inflammatories/cytoprotectants

	IL-1 β	IL-6	TNF- α	MMP-2	MMP-3	ADAMTS-5	IL-2	IL-10	CYT-C	IGF1R	COL9A1
Control	0.0068	0.63	2.04	1,000.34	0.68	0.27	0.04	0.14	0.05	1.63	35,997.90
LPS	2,002.069	33,456.5	2,142.0562	19,483.96	4,544.79	68,556.22	254,975.65	63,303.6	2,476,721.12	8,220.44	256,789.00
Curvularin	0.097	6,555.08	11.05	15,545.16	0.00	60,500.13	0.00	0.00	0.00	0.00	348,942.77
Betamethasone	0.085	2.63	7.87	1,300.01	5,001.87	0.01	0.01	0.32	990,000.19	0.03	318,326.58
Dexamethasone	0.080	0.92	8.21	1,350.13	5,000.71	60,000.34	0.08	0.05	900,000.03	0.08	316,970.68
Combination	0.070	0.15	5.28	1,000.20	0.25	50,000.11	0.29	0.08	350,000.01	0.24	32,894.56

LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; MMPs, matrix metalloproteinases; CYT-C, cytochrome c; IGF1R, insulin-like growth factor 1 receptor; COL9A1, collagen 9 alpha 1.

inflammatory genes (IL-2) and downregulated collagen 9A1 expression. The expression of IL-2 and IL-10 was only observed in the LPS-treated group, which indicated inflammatory stress. However, its absence clearly suggested the anti-inflammatory efficacy of protective agents (Fig. 6) [21]. Real-time PCR studies showed a higher copy number of pro-inflammatory cytokines (i.e., IL-1 β , IL-6, TNF- α) and metalloproteases (MMP-2, MMP-3, and MMP-9) in the LPS-treated group (Fig. 7, Table 4). Gene expression studies have shown that Cur (10 μ g/mL) exerts good anti-inflammatory and cytoproliferative properties on degenerating human NPCs when treated with 10 μ g/mL LPS *in vitro*. Treatments with Cur, Beta, and Dexa, individually and in combination, showed declined expression of inflammatory cytokines and metalloproteases in LPS-treated NPCs. The anti-inflammatory response of Cur was significantly better compared to steroid treatment.

Conclusions

In summary, the present study demonstrated that Cur exhibited anti-inflammatory, cytoprotective, and regenerative effects against LPS-stimulated NPCs by inhibiting inflammatory and metalloprotease mediators such as TNF- α , IL-1 β , IL-6, MMP-2, MMP-3, and ADAMTS-5. Furthermore, Cur also showed signs of cell repair or regeneration, as it enhanced the expression of collagen 9A1 and IGF1R. Furthermore, we hypothesize that Cur may inhibit LPS-induced inflammatory and metalloprotease mediator production by obstructing the nuclear factor- κ B/mitogen-activated protein kinase signaling pathway. Our study, for the first time, suggests the potential clinical application of Cur (i.e., an alternative to steroids and NSAIDs in pain management).

Future directions are as follows: (1) *in vivo* study followed by a large animal study; (2) potential for the development of a Cur-based formulation for epidural usage in the management of LBP and sciatica.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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