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Investigation for effects of iNOS on biological function of chondrocytes in rats with post-traumatic osteoarthritis

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Abstract. – OBJECTIVE: Inducible nitric oxide synthase (iNOS) is one rate-limiting enzyme in nitric oxide (NO) synthesis, and plays a role in mediating cell proliferation, apoptosis, and inflammation. Post-traumatic arthritis (PTA) is secondary after osteoarthritis (OA). This study thus established rat PTA model, on which the role of iNOS in PTA pathogenesis was investigated.

MATERIALS AND METHODS: Chondrocytes and synovial fluids were collected for comparing NO content and iNOS activity. *In vitro* cultured PTA rat chondrocytes were tested for expression of iNOS, matrix metallopeptidase 13 (MMP-13), and alpha-1 chain of type II collagen (COL2A1) expression with or without Interleukin 1β (IL-1β) treatment. NO content and iNOS activity in the supernatant were measured, followed by flow cytometry for Ki-67 expression and apoptosis. IL-1β treated cells were transfected with small interfere iNOS (si-iNOS), followed by expressional assay of iNOS, MMP-13 and Col2A1, activity assay of NO and iNOS, plus Ki-67 level and apoptosis.

RESULTS: Compared to Sham group, PTA model rats had higher iNOS expression, NO content, and iNOS activity. IL-1β treatment remarkably elevated iNOS and MMP-13 expression in chondrocytes, it decreased COL2A1 expression, increased NO content and iNOS activity, whilst suppressed cell proliferation to facilitate apoptosis. Silencing of iNOS expression decreased iNOS or MMP-13 expression, increased COL2A1 expression, suppressed NO or iNOS content, potentiated proliferation and decreased apoptosis.

CONCLUSIONS: iNOS expression and NO content were elevated in PTA rat chondrocyte. iNOS and NO production can facilitate chondrocyte apoptosis and matrix degradation, and suppress chondrocyte proliferation, thus playing a role in PTA pathogenesis.

Key Words:
iNOS, PTA, Chondrocyte, MMP-13, Proliferation, Apoptosis.

Introduction

Post-traumatic arthritis (PTA) is one common type of osteoarthritis (OA), and is secondary to OA-induced joint injury[1]. PTA is commonly caused by trauma, bone fracture or overloading. With time elapses and disease progression, most PTA patients develop into OA[2]. Major pathological changes of PTA or OA include the destruction of joint cartilage tissues and reactive proliferation of sub-chondral bones, leading to joint pain, immobility or structural deformation, severely affecting patient labor ability and life quality[3]. Large amounts of cytokines and inflammatory mediators can be released from focal tissues of PTA chondrocytes. These factors may cause abnormal proliferation or apoptosis of joint chondrocytes, disrupt cartilage tissue metabolism, synthesis or degradation of extracellular matrix (ECM), denature cartilage matrix, eventually leading to change of joint cartilage structure or function, and interrupting joint activity[4]. Nitric oxide (NO) is one major component in body reactive nitrogen species (RNS) with multiple biological activities and is one free gaseous radical under catalyzing L-arginine substrate[5]. As one novel signal molecule and inflammatory mediator, NO participates in the regulation of various body pathology and physiology processes. During PTA pathogenesis, NO can mediate synthesis and release of an inflammatory factor, regulate proliferation or apoptosis of chondrocytes, inhibit ECM synthesis, and affect degradation enzyme of ECM[6]. Nitric oxide synthase (NOS is the most important rate-limiting enzyme inside the body to regulate NO content. It can be further classified into endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) subtypes. The former two species mainly exist in endothelial and neuronal cells, respectively,

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whilst iNOS can be expressed in chondrocytes, where it plays a critical role in mediating trauma, post-inflammatory chondrocyte damage and joint cartilage injury[5]. Therefore, this study established a rat PTA model by serving knee joint anterior cruciate ligament, medial collateral ligament, and medial meniscus. Moreover, siRNA was used as an intervention on iNOS expression of *in vitro* cultured PTA model rat chondrocytes, in order to investigate the role of iNOS in PTA pathogenesis.

Materials and Methods

Major Reagent and Material

Dulbecco's modified eagle medium F12 (DMEM/F12) culture medium, fetal bovine serum (FBS), trypsin, collagenase type II, 100 mg/ml streptomycin and 100 U/ml penicillin were purchased from Gibco BRL. Co. Ltd. (Grand Island, New York, USA). Hyaluronidase was purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). ReverTra Ace qPCR RT Kit and SYBR Green dye were purchased from Toyobo Co. Ltd. (Osaka, Japan). Cytokine interleukin 1β (IL-1β) was purchased from Beyotime Biotechnology (Shanghai, China). Rabbit anti-mouse alpha-1 chain of type II collagen (COL2A1) and anti-matrix metallopeptidase 13 (MMP-13) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-mouse iNOS and HRP-conjugated secondary antibody were purchased from Abcam Biotechnology (Cambridge, MA, USA). NO and NOS analyzing test kits were purchased from Jiancheng Biotech. (Nanjing, China). APC-Ki-67 antibody was purchased from Biolegend (San Diego, CA, USA). Annexin V-FITC/PI cell apoptosis kit was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China).

Generation of PTA Model

Male Sprague-Dawley (SD) rats (8 weeks old, body weight 220-250 g) were provided by Zhejiang University, Zhejiang, China. Rats were anesthetized by 10% hydrate chloral and were fixed in a supine position. After focal shaving, sterilization, and draping, a 2 cm incision was made on the inner side of the knee joint. Anterior and posterior cruciate ligament, medial collateral ligament, and medial meniscus were severed but leaving joint cartilage intact. The joint cavity was rinsed, followed by suture incisions. Penicillin (400000 U/kg/d) was intramuscularly injected for 7 days. After that, animals were forced to move for 60 min per day. OA model was obtained by 7 weeks of treatment. In the Sham group, the joint cavity was opened but without an incision.

This investigation was approved by the Ethics Committee of Ningbo No. 6 Hospital, Zhejiang, China.

Mankin Score

Total of 8 weeks after surgery, rat cartilage tissues were observed for intact and regular morphology, tissue cleft, change of cell number, distribution and arrangement, integrity of AB-PAS staining, and tidal line. Mankin score was evaluated.

NO Release and NOS Activity Assay

Total of 8 weeks after surgery, synovial fluids were collected from both groups of rats. After centrifugation at 2000 r/min for 10 min, the supernatant was collected and stored at -80°C for further use. When testing, the supernatant was slowly thawed and tested for NO release (in μmol/l) and iNOS activity (in U/ml) following manual instructions.

Chondrocyte Separation and Culture

PTA model rats were sacrificed 8 weeks after surgery. Knee joints were removed and severed for the tibia. Joint cartilage tissues were removed and were transferred for PBS washing and homogenization. Cartilage tissues were then digested in hyaluronidase, trypsin, and type II collagenase to obtain cell-containing lysate. Tissue debris was filtered out, and cells were re-suspended in DMEM/F12 medium containing 10% FBS and 1% streptomycin-penicillin and were incubated in a 37°C chamber with 5% CO_2. Culture medium was changed every other day. Cells were passed at 1:4 ratio after fully grown.

Chondrocyte Transfection and Grouping

In vitro cultured PTA model rat chondrocytes were stimulated by 0 or 10 ng/ml IL-1β to mimic body environment of arthritis. Moreover, cells after 10 ng/ml IL-1β stimulus were further divided into control, small interfere normal control (si-NC) transfection using scrambled negative control sequence, and small interfere iNOS (si-iNOS) transfection group using interference sequence targeting iNOS (si-iNOS sense: 5'-AATGT GGAGA AAGCC CCCTG-3'; si-iNOS anti-sense: 5'-CAGGG GGCTT TCTCC ACATT-3'; si-NC sense: 5'-GACTT CATAA GGCGT ATGC-

3'; si-NC anti-sense: 5'-GCATA CGCCT TATGA AGTC-3'.

Quantitative Real Time-PCR (qRT-PCR) for Gene Expression

Total RNA was extracted by the TRIzol method. Complementary DNA (cDNA) was synthesized from RNA by reverse transcription using ReverTra Ace qPCR Kit. cDNA products were used for PCR amplification with SYBR fluorescent dye. Data were collected and analyzed. PCR conditions were: 95°C 15 s, 60°C 30 s and 74°C 30 s. 40 cycles were performed on ABI ViiA7 fluorescent quantitative PCR cycler. PCR primer sequences for amplifying human genes were: iNOSP$_F$: 5'-GATCA ATAAC CTGAA GCCCG-3'; iNOSP$_R$: 5'-GCCCT TTTTT GCTCC ATAGG-3'; COL2A1P$_F$: 5'-TGGAC GCCAT GAAGG TTTTC T-3'; COL2A1P$_R$: 5'-TGGGA GCCAG ATTGT CATCT C-3'; MMP-13P$_F$: 5'-CCAGA CTTCA CGATG GCATT G-3'; MMP-13P$_R$: 5'-GGCAT CTCCT CCATA ATTTG GC-3'; β-actinP$_F$: 5'-GAACC CTAAG GCCAA C-3'; β-actinP$_R$: 5:-TGTCA CGCAC GATTT CC-3'.

Western Blot

Cartilage tissue homogenate was prepared and centrifuged at 10000 r/min for 10 min. Bicinchoninic acid assay (BCA) method was used to detect the quality and concentration of protein in the supernatant. Total of 40 μg supernatant was separated by SDS-PAGE and was transferred to the membrane, which was blocked and incubated using primary antibody (anti-iNOS at 1:200, anti-MMP-13 at 1:300, anti-COL2A1 at 1:300, and anti-β-actin at 1:600) under 4°C overnight. With phosphate-buffered saline Tween-20 (PBST) washing, HRP conjugated secondary antibody (1:5000 dilution) was added for 60 min incubation. With three times of PBST washing, enhanced chemiluminescence (ECL) substrate was added for detecting protein expression.

Flow Cytometry For Cell Apoptosis

Trypsin was used to digest cells, which were collected and re-suspended in binding buffer. 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) were added for incubation. Cell apoptosis was examined by flow cytometry.

Ki-67 Expression of Cells by Flow Cytometry

Trypsin was used to digest cells, which were collected and re-suspended in stain buffer. The APC-labelled Ki-67 antibody was added for 4°C incubation. Flow cytometry was used to measure Ki-67 for reflecting proliferation potency of chondrocytes.

Statistical Analysis

SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean ± standard deviation. The Student's *t*-test was used to compare the differences between the two groups. The Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data among multiple groups. A statistical significance was defined when $p<0.05$.

Results

Significantly Elevated NO and iNOS in Cartilage Tissues and Joint Fluids

In Sham group, rat joint cartilage tissues showed smooth and regular structures without cleft, plus a regular arrangement of cells. In PTA model, rat cartilage tissues had an unsmooth surface having more clefts, irregularly arranged cells with a fewer number, plus fiber-like coverage on cartilage surface. Mankin score in PTA model group was significantly higher than Sham group (Table I), indicating successful model generation.

Assays for NO and iNOS levels showed significantly higher NO release and iNOS activity in joint cavity fluids of PTA model rats compared to those of Sham rats (Table I). qRT-PCR showed remarkably elevated iNOS mRNA expression in cartilage tissues of PTA rats (Figure 1A). Western blot results showed significantly higher iNOS protein

Table I. Mankin score, NO release and iNOS activity in rats.

	Mankin score	NO release (μmol/l)	iNOS activity (U/ml)
Sham group	0±0	26.8±5.4	2.1±0.3
PTA group	7.1±0.6*	114.6±17.5*	4.7±0.5*

Note: *$p<0.05$ comparing between PTA and Sham group.

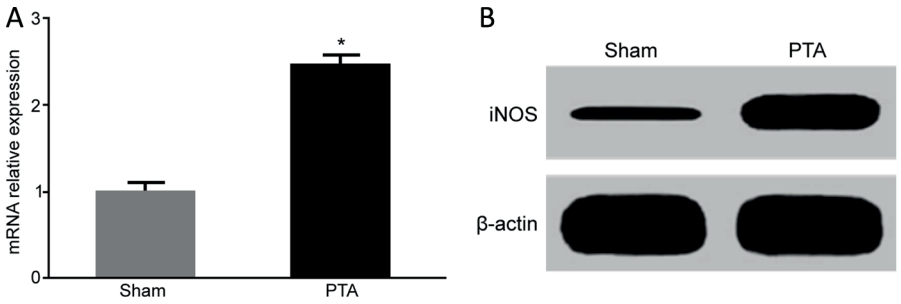

Figure 1. Significantly elevated iNOS expression in PTA rat cartilage tissues. ***(A)*** qRT-PCR for iNOS mRNA expression in cartilage tissues. ***(B)*** Western blot for iNOS protein expression in cartilage tissues. $^{*}p<0.05$ compared to Sham group.

expression of PTA model rat joint cartilage tissues compared to those of Sham group rats (Figure 1B).

IL-1β Induced iNOS Expression in Chondrocytes, Affected Cell Proliferation, Apoptosis, and MMP-13 Expression

NO and iNOS assay showed significantly higher NO release and iNOS activity in the supernatant of cultured chondrocytes after IL-1β treatment ($p<0.05$ compared to untreated group, Table II). qRT-PCR results showed significantly elevated iNOS and MMP-13 mRNA expression in IL-1β treated chondrocytes, plus lower COL2A1 mRNA expression (Figure 2A). Western blot showed higher iNOS and MMP-13 protein expression in IL-1β treated chondrocytes compared to untreated group, whilst COL2A1 protein expression was lower (Figure 2B). Flow cytometry results showed that IL-1β treatment significantly suppressed Ki-67 expression or cell proliferation in chondrocytes (Figure 2C), and facilitated chondrocyte apoptosis (Figure 2D).

Intervention on iNOS Expression Decreased NO Content or Chondrocyte Apoptosis and Facilitated Cell Proliferation

NO and iNOS assays showed that siRNA interference significantly decreased NO release amount and iNOS activity in the supernatant of chondrocyte culture medium (Table III). qRT-

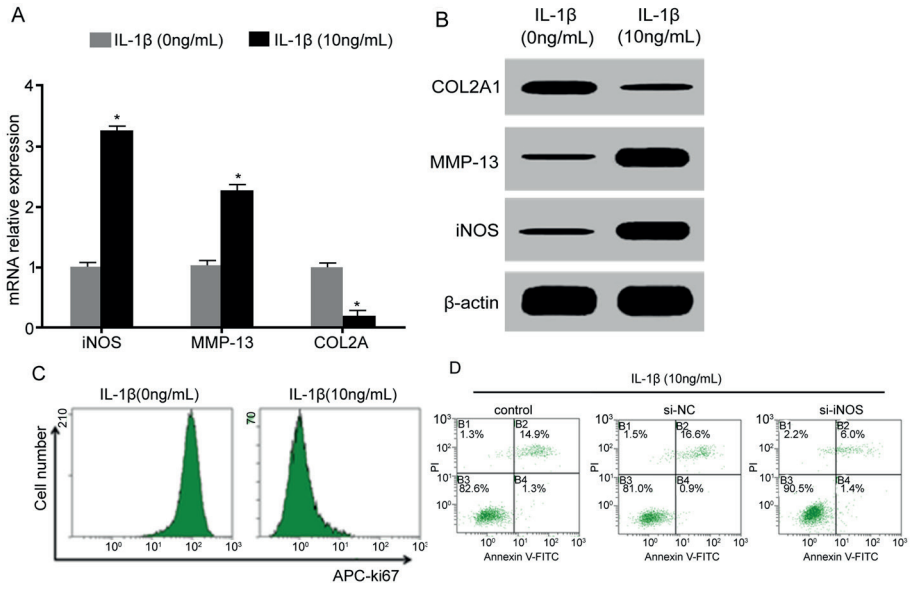

Figure 2. IL-1β induced iNOS expression in chondrocytes, affected cell proliferation, apoptosis, and MMP-13 expression. (A) qRT-PCR for mRNA expression in chondrocytes. (B) Western blot for protein expression in chondrocytes. (C) Flow cytometry for Ki67 expression; (D) Flow cytometry for cell apoptosis. $^{*}p<0.05$ compared to 0 ng/ml IL-1β group.

Table II. NO release and iNOS activity assay in chondrocytes.

	NO release (μmol/l)	iNOS activty (U/ml)
IL-1β (0 ng/ml)	37.2±6.1	1.7±0.2
IL-1β (10 ng/ml)	203.5±22.7*	5.1±0.6*

Note: *p<0.05 compared to 0 ng/ml IL-1β group.

Table III. NO release and iNOS activity in chondrocytes.

	NO release (μmol/l)	iNOS activty (U/ml)
Control	179.5±14.6	4.8±0.7
si-NC	181.3±16.7	5.0±0.8
si-iNOS	46.6±6.9*#	1.8±0.3*#

Note: *p<0.05 comparing between si-iNOS and control group, #p<0.05 comparing between si-iNOS and si-NC group.

PCR results showed significantly decreased iNOS, MMP-13 mRNA expression in si-iNOS transfected chondrocytes compared to control or si-NC group, whilst COL2A1 mRNA expression was potentiated (Figure 3A). Western blot results showed lower iNOS or MMP-13 protein expression in chondrocytes transfected by si-iNOS compared to control or si-NC group, whilst COL2A1 protein expression was higher (Figure 3B). Flow cytometry results showed higher Ki-67 expression and less apoptosis of chondrocytes in si-iNOS transfected group compared to control or si-NC group (Figure 3C and 3D).

Discussion

Normal cartilage tissues are composed of ECM and chondrocytes, the latter of which is a major cellular component of cartilage tissues and exert important roles maintaining metabolic homeostasis of cartilage matrix and biological functions of cartilage tissues. Under traumatic conditions, focal cartilage tissues can release large amounts of cytokines and inflammatory mediators, leading to abnormal survival or quantity of chondrocytes, imbalance of ECM synthesis or degradation, eventually causing structural or functional injury of cartilage tissues for PTA pathogenesis. NO is the major component of reactive nitrogen species (RNS) inside the body, and is one free gaseous free radical with pluripotent biological functions and is widely involved in cell proliferation[7], apoptosis[8], neuronal activity, blood pressure regulation, and inflammatory mediator release. NO is also important signal transduction and effector molecules of multiple cytokines, which can exert biological functions under the induction of NO[9]. As one important inflammatory mediator, NO within physiological level works as important host defense function, whilst over-production of NO may lead to chronic inflammation of body

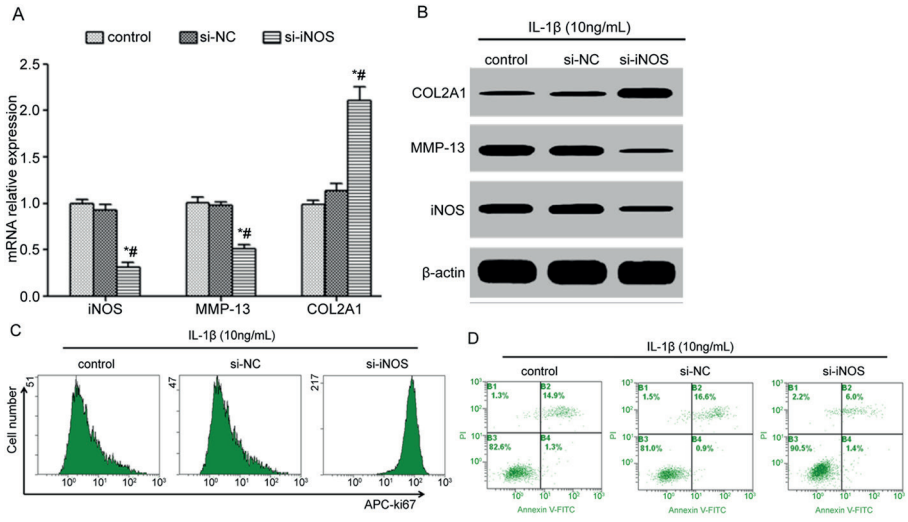

Figure 3. IL-1β induced chondrocyte apoptosis and suppressed their proliferation. ***(A)*** qRT-PCR for mRNA expression in chondrocytes. ***(B)*** Western blot for protein expression in chondrocytes. ***(C)*** Flow cytometry for Ki67 expression. ***(D)*** Flow cytometry for cell apoptosis. *p<0.05 comparing between si-iNOS and control group. #p<0.05 comparing between si-iNOS and si-NC group.

respiratory tract[10], cardiac muscles[11] or livers[12]. Under traumatic or inflammatory conditions, large amounts of inflammatory factors including IL-1β, TNF-α, IL-6, IL-17, and IFN-γ, all of which can stimulate iNOS synthesis and expression[13]. As iNOS activity is non-Ca^{2+}-dependent, enhanced iNOS synthesis and expression constitutively induce NO release, which can facilitate synthesis and release of other inflammatory factors via positive feedback regulation, regulate chondrocyte proliferation and apoptosis, inhibit ECM synthesis, and affect expression of ECM degrading enzymes, thus participating onset of PTA or OA[14]. Therefore, this study established a rat PTA model via severing rat knee joint anterior cruciate ligament, medial collateral ligament, and medial meniscus, followed by assay of chondrocyte iNOS expression by siRNA interference, in order to investigate the role of iNOS in PTA pathogenesis.

Our results showed significant injury of PTA rat joint cartilage, with higher Mankin score, indicating successful model generation for further experiments. NO and iNOS test showed significantly higher NO release and iNOS activity in PTA model rat joint cavity fluid than Sham group. Western blot results showed remarkably higher iNOS expression in PTA model rat chondrocytes than Sham group. All these results indicated the role of iNOS and inducible NO in PTA pathogenesis. Lotz et al[15] showed enhanced NO content and iNOS expression in traumatized joint cartilage tissues. Suantawee et al[6] showed significantly elevated NO and iNOS expression in joint synovial fluids of OA patients compared to normal people. Nakagawa et al[16] also showed more chondrocyte apoptosis in OA model rats plus higher NO release level. In this work, NO expression and iNOS enzyme activity were abnormally elevated in both joint synovial fluids and cartilage tissues of PTA model rats, as consistent with Lotz et al[15], Suantawee et al[6], Scher et al[17], and Nakagawa et al[16] results. Furthermore, this study separated chondrocytes of PTA model rats for *in vitro* culture, which contains certain concentrations IL-1β for mimicking PTA inflammation *in vivo*. Results showed that IL-1β pre-treated *in vitro* cultured chondrocytes had up-regulated iNOS activity and protein expression, plus more amounts of NO release in the supernatant of culture medium. El Mansouri et al[18] showed that IL-1 treatment significantly induced iNOS expression in *in vitro* cultured OA chondrocytes. Rasheed et al[5] showed that IL-1β treatment significantly potentiated iNOS expression in OA patient's chondrocytes. In this research, IL-1β treatment significantly facilitated iNOS expression in chondrocytes, as consistent with El Mansouri et al[18] and Rasheed et al[5]. Matrix metalloproteinase (MMPs) is one type of proteinase with critical roles in ECM degradation and can be secreted by fibroblast, synovial cells, and chondrocytes[19]. Flow cytometry results showed weakened proliferation potency in IL-1β treated chondrocytes, plus higher cell apoptosis compared to untreated group. MMP-13 belongs to collagenase family of MMP, and is one potent enzyme specific for type II collagen to degrade ECM, and can directly degrade type II collagen with most featured and abundant amounts[20]. The abnormally elevated MMP-13 expression can degrade type II collagen, which occupies more than 90% of all cartilage matrix, thus facilitating PTA or OA pathogenesis. In this study, IL-1β treated chondrocytes had potentiated MMP-13 expression, whilst COL2A1 expression was decreased, indicating the inflammatory mediator could facilitate cartilage matrix degradation via inducing iNOS expression and NO release. However, the direct regulatory role of IL-1β on chondrocyte iNOS expression and NO release cannot be ruled out. This study thus treated IL-1β induced chondrocytes with siRNA for iNOS to investigate its effect on chondrocytes. Our results showed that siNRA interference significantly decreased iNOS expression or NO content in the supernatant of the culture medium of chondrocytes. The silencing of iNOS expression potentiated chondrocyte proliferation potency, decreased cell apoptosis, decreased MMP-13 expression whilst enhanced COL2A1 expression. These results demonstrated that the effect of IL-1β treatment on proliferation, apoptosis and matrix degradation of chondrocytes via the induction by iNOS and released NO. Lotz et al[15] showed a significantly positive correlation between NO/iNOS expression and cartilage matrix degradation after OA cartilage injury. With higher NO and iNOS expression, the number of apoptotic chondrocytes was also increased, suggesting the role of iNOS in mediating chondrocyte apoptosis and matrix degradation. Scher et al[17] showed that abnormally elevated NO in OA chondrocytes significantly suppressed cartilage matrix synthesis and facilitated matrix degradation. Balaganur et al[21] found that treatment using iNOS inhibitor S-methylisothiourea (SMT) remarkably suppressed OA model rat joint cartilage tissues. Balaganur et al[22] found that SMT could significantly inhibit MMP-13 expression in OA model rat chondrocytes. In this study, blockade of

iNOS expression or inducible NO-related effects significantly suppressed MMP-13 expression or type II collagen degradation in chondrocytes under inflammatory environment, as consistent with Balaganur et al[21]. Nakagawa et al[16] found that antagonizing NO effects by applying N-acetylcysteine (NAC) significantly decreased chondrocyte apoptosis. This study observed that blockade of iNOS expression or related NO effects could decrease chondrocyte apoptosis, as consistent with Nakagawa et al[16]. This work transfected *in vitro* cultured chondrocytes by siRNA, and demonstrated the role of iNOS and induced NO synthesis in facilitating chondrocyte apoptosis or matrix degradation, plus inhibition of chondrocyte proliferation, under inflammatory conditions. This study may play a role in the understanding of the pathogenesis mechanism of post-traumatic arthritis. Certain weakness in this work resides in the lack of *in vivo* model rat experiments for replicating its *in vitro* role on chondrocytes, thus requiring further investigations.

Conclusions

We found that iNOS expression and NO release were significantly elevated in cartilage tissues of PTA rats. iNOS and inducible NO can facilitate apoptosis of chondrocyte and its matrix degradation, thus suppressing chondrocyte proliferation, thus playing a role in PTA pathogenesis mechanism.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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