

Effects of L-NG-Monomethyl-Arginine on Apoptosis of Articular Chondrocytes

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Wang *et al.*: Effects of L-NG-Monomethyl-Arginine

We aimed to investigate the effects of L-NG-monomethyl-arginine on the apoptosis of articular chondrocytes. 30 rabbits were randomly divided into six groups ($n=5$). The pathological changes were detected by Hematoxylin and Eosin staining. The expression levels of synovial fluid and serum nitric oxide were measured. The apoptosis of chondrocytes was tested using the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. The expression levels of reconstituted B-cell lymphoma-2 and human B-cell lymphoma-2 associated X protein in cartilage tissues were detected by immunohistochemical assay. The Mankin score of the model group was significantly higher than that of the control group ($p<0.01$). The scores significantly decreased in L-NG-monomethyl-arginine treatment groups ($p<0.05$). The expression levels of B-cell lymphoma-2 associated X protein, apoptosis rate and index of chondrocytes, and those of synovial fluid and serum nitric oxide were markedly higher in the model group than those in the control group ($p<0.01$). However, the expression levels of B-cell lymphoma-2 in model and control groups were similar ($p>0.05$). With increasing L-NG-monomethyl-arginine treatment dose, the expression level of B-cell lymphoma-2 and B-cell lymphoma-2/B-cell lymphoma-2 associated X protein ratio were significantly elevated ($p<0.05$). L-NG-monomethyl-arginine reduced the expressions of synovial fluid and serum nitric oxide, and raised B-cell lymphoma-2/B-cell lymphoma-2 associated X protein ratio, thereby inhibiting chondrocyte apoptosis.

Key words: L-NG-monomethyl-arginine, knee joint osteoarthritis, articular cartilage, apoptosis

At present, the Chinese population is aging. Epidemiological investigations indicate that the incidence rate of degenerative osteoarthritis (OA) is increasing annually. It is expected that, in the near future, the disease will be one of the main causes of disability among adult males in China^[1,2]. The main pathological characteristics of degenerative OA include significant apoptosis of cartilage cells in joints and progressive degradation of the matrices outside articular chondrocytes^[3]. The expression levels of nitric oxide (NO) and its byproducts are significantly positively correlated with the number of apoptotic cells in articular cartilage and the severity of OA. In the early stage of the disease, NO and its byproducts stimulate and induce the apoptosis of articular cartilage cells, leading to the pathological degradation of articular cartilage^[4,5]. L-NG-monomethyl-arginine (L-NMMA), as a congener of L-arginine, competes with it at the binding sites of nitric oxide synthase (NOS). L-NMMA can inhibit three NOS's with low selectivity, resulting

in the irreversible inactivation of inducible nitric oxide synthase (iNOS) with extended time^[6]. Thereby motivated, we herein evaluated the influence of L-NMMA on the apoptosis of articular chondrocytes in OA rabbits.

MATERIALS AND METHODS

Materials:

L-NMMA was purchased from Sigma (USA). S-P kit was bought from Wuhan Boster Biological Technology Co., Ltd. (China). 3,3'-Diaminobenzidine was bought from Fuzhou Maixin Biotechnology Development Co.,

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Ltd. (China). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit was purchased from Nanjing KeyGen Biotech Co., Ltd. (China). NO detection kit was obtained from Nanjing Jiancheng Bioengineering Institute (China). Annexin A V-FITC apoptosis kit was obtained from Gibco (USA). Human Bone Morphogenetic Protein 2 (BMP2) and Transforming growth factor beta 1 (TGFβ1) serum ELISA kits, as well as caspases-3, B-cell lymphoma-2 associated X protein (Bax) and B-cell lymphoma-2 (Bcl-2) mouse anti-rabbit antibodies, were purchased from Beijing Zhongshan Technology Co., Ltd. (China).

Animal grouping and model establishment:

All animal experiments have been approved by the ethics committee of our hospital, and great efforts have been made to minimize their suffering. The experimental animals were provided by the Experimental Animal Center of Xuzhou Medical College. Thirty female or male Sun Protection Factor (SPF) grade New Zealand white rabbits aged about 8 mo and weighing 2.0~2.5 kg were selected. The 30 rabbits were randomly divided into 5 groups (n=6): normal group (Group A) without treatment; OA model group (Group B) established by the plaster immobilization method (fig. 1); low-dose L-NMMA treatment group (Group C), medium-dose L-NMMA treatment group (Group D) and high-dose L-NMMA treatment group (Group E): The articular cavities of OA model animals were injected with 0.5 ml of L-NMMA at 0.1 %, 0.25 % and 0.5 %, respectively, 7 times every 3 d. After each injection, infusion and withdrawal were repeated twice to ensure successful intra-articular injection.

Sampling of joint fluid and serum:

Arthrocentesis was performed on the right knee joint of each group. After the needle penetrated the joint cavity, 1 ml of 0.9 % normal saline solution was injected slowly



Fig. 1: Modeling of rabbit OA

through repeated infusion and withdrawal three times. Then the cavity liquid was collected and centrifuged at 1,000 r/min for 5 min, and finally the supernatant was collected and stored at -20°. Subsequently, 6 ml of blood was drawn from the ear vein and centrifuged at 3500 r/min for 10 min, and then the upper layer serum was collected and kept in a refrigerator prior to detection.

Detection of synovial fluid and serum NO:

The nitrate reductase method was used to detect NO expression levels in synovial fluid and serum. A standard curve was plotted by the following procedure. Seven clean dry 15 ml scaled test tubes were used to prepare 0-2.0 µg of standard nitrite nitrogen solutions. After being shaken well, the solutions were incubated in a 30° incubator or thermostatic water bath for 30 min, and then measured at 540 nm. The standard curve was plotted or regression equation was established with nitrite nitrogen level (µg) as the x-axis and optical density as the y-axis. Three portions of synovial fluids (1 ml) were weighed and put into 3 numbered test tubes. Afterwards, 9 ml mixture of Potassium Nitrate (KNO₃), isopropyl alcohol and phosphate buffer was added into each test tube, and 1.0 ml of trichloroacetic acid was immediately added into one of the three tubes and mixed as a control. Then all the tubes were connected to a vacuum pump in a vacuum dryer for air exhaust, and kept in dark at below 30° for 30 min for thermal insulation. Subsequently, 1.0 ml of trichloroacetic acid was added into the tubes and shaken well. After 2 min, 2 ml of the supernatant was collected and added into another group of test tubes, with the control tube as a reference. According to the standard curve, the enzymatic activity was calculated.

Sampling of cartilage tissues, Hematoxylin and Eosin (HE) staining and immunohistochemical assay:

Under anesthesia, the rabbits were killed by head knocking with a hard object. Immediately after their death, the right knee joint cavity was cut open to observe the lesions in articular cartilage, synovium and other parts with naked eye. Then cartilage tissues in the tibial plateau and the lower edge of femoral condyle were resected with a blade, fixed, dehydrated, transparentized, embedded into paraffin, sectioned, and subjected to Hematoxylin and Eosin (HE) staining and TUNEL assay. The lesions were graded according to the modified Mankin histologic/histochemical grading system^[7,8]. Bax and Bcl-2 expressions in cartilage tissues were detected by immunohistochemical assay,

and quantified with high-resolution pathological image analysis system (HPIAS-1000). The percentage (%) of positive area to total one in the entire tissue was calculated under a light microscope. Five visual fields were randomly selected for each section, and the results were averaged.

Statistical analysis:

All data were analyzed by Statistical Package for the Social Sciences (SPSS) 13.0 software and plotted by Excel 2003 software. The categorical data were expressed as $\bar{x} \pm s$. Multigroup comparisons were performed by one-way analysis of variance, intergroup comparisons were conducted by the Student-Newman-Keuls (SNK) method, and intragroup comparisons were carried out by the t test. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

In Group A, the HE staining was even without destaining. The articular cartilage surface was smooth and flat with uniformly distributed chondrocytes. In Group B, the HE staining was less even with obvious destaining. The articular cartilage surface became thinner with fractures deep in the subchondral bone. The chondrocytes were arranged disorderedly, and the tide line vanished. With increasing concentration of L-NMMA, the damage in articular cartilage tissue was significantly alleviated (fig. 2). There were significant differences in the Mankin scores between groups ($F=133.873$, $p < 0.01$) (Table 1). The Mankin scores of Group B-E were significantly

higher than that of Group A. Starting from Group B, the Mankin score significantly declined with increasing dose of L-NMMA ($p < 0.05$) (fig. 3).

The expression levels of NO in the synovial fluid of Group B was (90.55 ± 15.72) pg/ml which was significantly higher than that of Group A $[(15.33 \pm 3.21)$ pg/ml] ($p < 0.01$). With increasing concentration of L-NMMA, the expression level of NO in synovial fluid significantly decreased ($p < 0.05$) (Table 2 and fig. 4).

Before treatment, the expression levels of serum NO in all groups were similar ($p > 0.05$). After treatment, the expression level of serum NO was (21.54 ± 2.16) pg/ml in Group B, which was significantly higher than that of Group A $[(14.98 \pm 2.16)$ pg/ml] ($p < 0.01$). With increasing concentration of L-NMMA, the expression level of serum NO significantly decreased ($p < 0.05$) (Table 3).

The chondrocyte AI values of all groups were similar before treatment ($p > 0.05$). After treatment, AI of Group B was (25.15 ± 7.72) which was significantly higher than that of Group A $[(2.73 \pm 1.69)]$ ($p < 0.01$). With rising concentration of L-NMMA, the apoptosis rate and AI dropped significantly ($p < 0.05$) (Table 4).

In Group B, the expression level of Bax in articular cartilage was significantly higher than that of Group A ($p < 0.01$). With elevating concentration of L-NMMA, the Bax expression level significantly reduced ($p < 0.05$). The expression level of Bcl-2 was not significantly different from that of Group A ($p > 0.05$). However, with increasing concentration of L-NMMA, both Bcl-

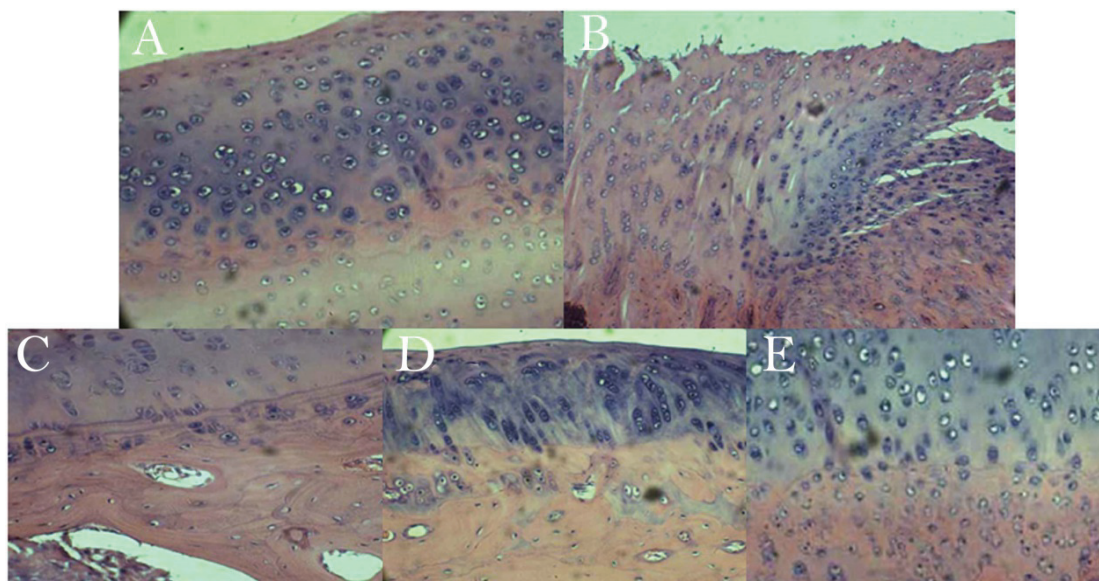
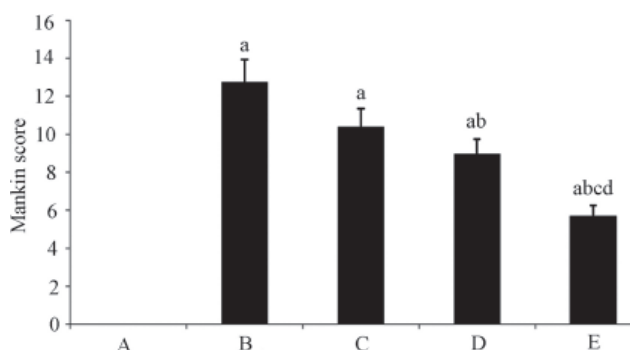


Fig. 2: HE staining results of articular cartilages (magnification: 200x)

A-E: Normal group, model group, low-dose L-NMMA treatment group, medium-dose L-NMMA treatment group and high-dose L-NMMA treatment group

TABLE 1: MANKIN SCORES OF ARTICULAR CARTILAGE (x±s)

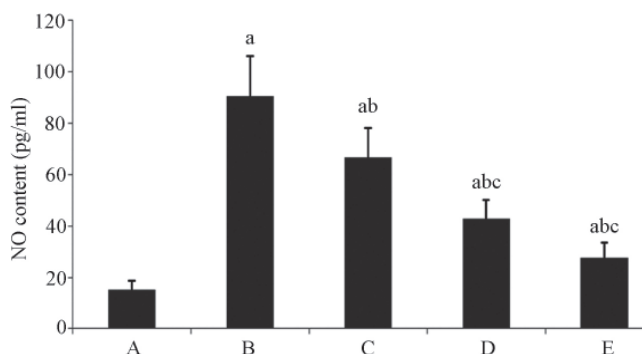
| Group | n | Mankin score | F | p |
|-------|---|---------------------------|--------|-------|
| A | 6 | 0.00±0.00 | | |
| B | 6 | 12.73±1.22 ^a | | |
| C | 6 | 10.38±0.97 ^a | 161.21 | <0.01 |
| D | 6 | 8.96±0.79 ^{ab} | | |
| E | 6 | 5.71±0.55 ^{abcd} | | |

^aCompared with Group A, p<0.05; ^bcompared with Group B, p<0.05;^ccompared with Group C, p<0.05; ^dcompared with Group D, p<0.05**Fig. 3: Mankin scores**

A-E: Normal group, model group, low-dose L-NMMA treatment group, medium-dose L-NMMA treatment group and high-dose L-NMMA treatment group. a: Compared with Group A, p<0.05; b: compared with Group B, p<0.05; c: compared with Group C, p<0.05; d: compared with Group D, p<0.05

TABLE 2: NO LEVELS IN JOINT FLUIDS (x±s)

| Group | n | NO content (pg/ml) | F | p |
|-------|---|---------------------------|---------|-------|
| A | 6 | 15.33±3.21 | | |
| B | 6 | 90.55±15.72 ^a | | |
| C | 6 | 66.87±11.13 ^{ab} | 412.451 | <0.01 |
| D | 6 | 42.71±7.54 ^{abc} | | |
| E | 6 | 27.56±5.91 ^{abc} | | |

^aCompared with Group A, p<0.05; ^bcompared with Group B, p<0.05;^ccompared with Group C, p<0.05**Fig. 4: NO contents**

A-E: Normal group, model group, low-dose L-NMMA treatment group, medium-dose L-NMMA treatment group and high-dose L-NMMA treatment group. a: Compared with Group A, p<0.05; b: compared with Group B, p<0.05; c: compared with Group C, p<0.05

2 expression and Bcl-2/Bax ratio were significantly elevated (p<0.05) (Table 5, fig. 5-8).

NO, which is a small molecule widely existing in the physiological and pathophysiological media of human body, also has a simple structure in the form of free radicals. This molecule is readily soluble in lipids and can easily spread to the internal and external cellular environments through the cell membrane barrier. Therefore, it has a wide variety of biological functions, playing crucial roles in the onset and progression of OA^[9,10]. NO in joint is mainly derived from the cells and chondrocytes of joint synovium. The secretion of inflammatory cytokines can significantly stimulate articular chondrocytes and synovial cells, so the expression level of iNOS is gradually elevated, promoting the production of NO molecules in joints^[11,12]. In addition, inflammatory cytokines and lipopolysaccharides *in vivo* can significantly stimulate the secretion of NO molecules in human body^[13,14]. In the case of OA, the interactions between these factors

TABLE 3: NO LEVELS IN SERUM (x±s, pg/ml)

| Group | n | Before | After | t | p |
|-------|---|------------|---------------------------|-------|-------|
| A | 6 | 13.38±1.98 | 14.98±2.16 | 0.447 | 0.665 |
| B | 6 | 13.19±2.12 | 21.54±2.16 ^a | 6.596 | <0.01 |
| C | 6 | 12.91±2.21 | 19.88±2.15 ^a | 5.914 | <0.01 |
| D | 6 | 12.91±2.32 | 15.99±2.01 ^b | 2.542 | 0.029 |
| E | 6 | 12.97±2.13 | 14.88±1.95 ^{abc} | 1.507 | 0.163 |
| F | - | 0.031 | 15.251 | | |
| p | - | 0.998 | <0.01 | | |

^aCompared with Group A, p<0.05; ^bcompared with Group B, p<0.05;^ccompared with Group C, p<0.05**TABLE 4: APOPTOTIC INDICES OF CHONDROCYTES (x±s)**

| Group | n | Before | After | t | p |
|-------|---|-----------|---------------------------|-------|-------|
| A | 6 | 3.75±1.01 | 3.84±0.99 | 0.091 | 0.929 |
| B | 6 | 3.81±1.12 | 28.21±6.12 ^a | 7.818 | <0.01 |
| C | 6 | 3.84±0.96 | 21.98±5.84 ^{ab} | 6.982 | <0.01 |
| D | 6 | 3.78±0.99 | 13.76±3.17 ^{abc} | 5.204 | <0.01 |
| E | 6 | 3.77±1.01 | 8.55±2.21 ^{bc} | 3.093 | 0.011 |
| F | - | 0.010 | 21.413 | | |
| p | - | 1.000 | <0.01 | | |

^aCompared with Group A, p<0.05; ^bcompared with Group B, p<0.05;^ccompared with Group C, p<0.05**TABLE 5: EXPRESSIONS OF BAX AND BCL-2 AND BCL-2/BAX RATIOS**

| Group | n | Bcl-2 | Bax | Bcl-2/Bax |
|-------|---|--------------------------|--------------------------|---------------------------|
| A | 6 | 4.59±1.48 | 5.97±1.58 | 0.77±0.19 |
| B | 6 | 4.86±1.53 | 22.79±5.87 ^a | 0.21±0.08 ^a |
| C | 6 | 7.97±3.39 | 18.95±5.22 ^a | 0.42±0.25 |
| D | 6 | 11.47±3.83 ^{ab} | 14.68±3.19 ^{ab} | 0.78±0.29 ^{bc} |
| E | 6 | 14.78±4.65 ^{bc} | 12.24±2.69 ^{bc} | 1.21±0.22 ^{abcd} |
| F | - | 11.774 | 16.176 | 15.549 |
| p | - | 0.000 | 0.000 | 0.000 |

^aCompared with Group A, p<0.05; ^bcompared with Group B, p<0.05;^ccompared with Group C, p<0.05; ^dcompared with Group D, p<0.05

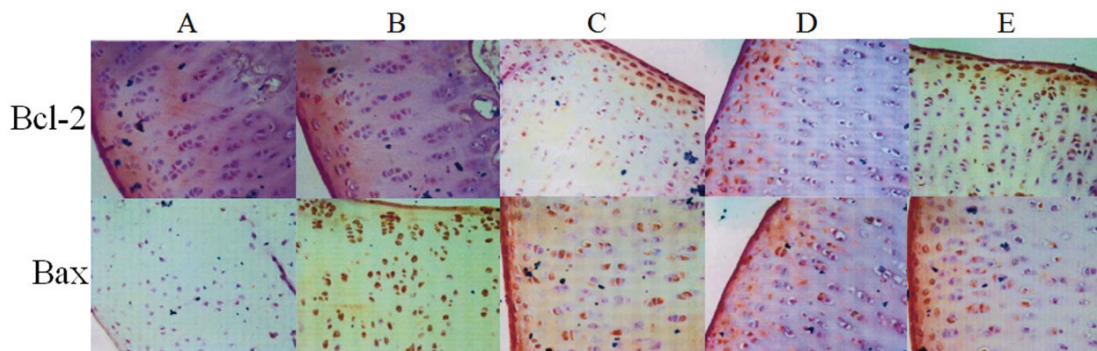


Fig. 5: Expressions of Bcl-2 and Bax (magnification: 400x)

A-E: Normal group, model group, low-dose L-NMMA treatment group, medium-dose L-NMMA treatment group and high-dose L-NMMA treatment group

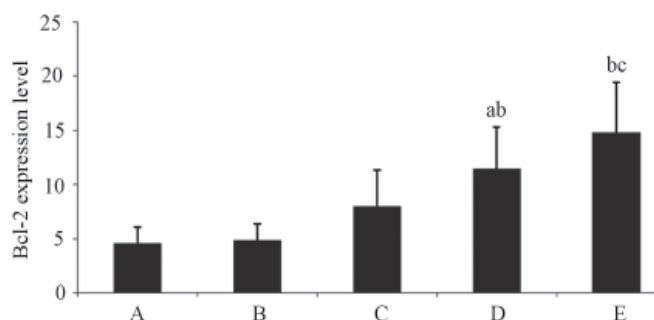


Fig. 6: Bcl-2 expressions

A-E: Normal group, model group, low-dose L-NMMA treatment group, medium-dose L-NMMA treatment group and high-dose L-NMMA treatment group. a: Compared with Group A, $p < 0.05$; b: compared with Group B, $p < 0.05$; c: compared with Group C, $p < 0.05$

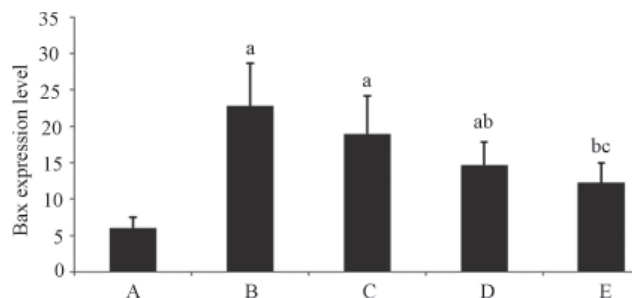


Fig. 7: Bax expressions

A-E: Normal group, model group, low-dose L-NMMA treatment group, medium-dose L-NMMA treatment group and high-dose L-NMMA treatment group. a: Compared with Group A, $p < 0.05$; b: compared with Group B, $p < 0.05$; c: compared with Group C, $p < 0.05$

gradually lead to aggravation. Accordingly, completely blocking this vicious cycle can drastically relieve damage to the articular cartilage, finally significantly alleviating OA.

NOS's consist of neuron NOS (nNOS), iNOS and endothelial NOS (eNOS), among which iNOS is expressed in the articular chondrocytes of human, rabbit and sheep. OA pathological changes can be induced by injecting collagenase into the articular cavity of normal mice and those with iNOS gene deficiency^[15], and the latter mice were less prone to these changes. NO and its

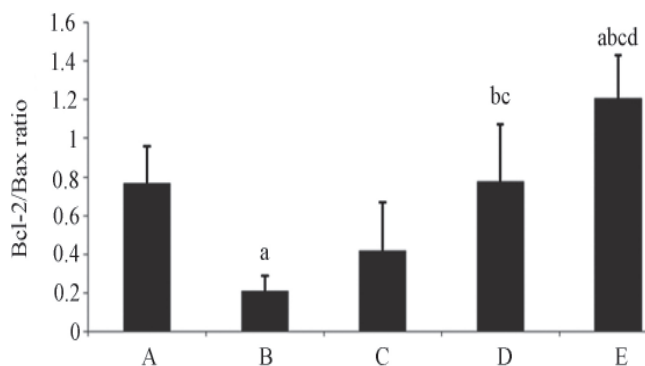


Fig. 8: Bcl-2/Bax ratios

A-E: Normal group, model group, low-dose L-NMMA treatment group, medium-dose L-NMMA treatment group and high-dose L-NMMA treatment group. a: Compared with Group A, $p < 0.05$; b: compared with Group B, $p < 0.05$; c: compared with Group C, $p < 0.05$; d: compared with Group D, $p < 0.05$

byproducts may stimulate and induce the apoptosis of articular chondrocytes, thus inhibiting the synthesis and facilitating the decomposition of substances such as protein polysaccharides and collagen^[16]. As the protein polysaccharide content plummets in joint, the water content of articular chondrocytes also reduces markedly, finally inducing abnormal drying of articular cartilage which gradually loses its original gloss^[17,18]. The obvious decline in collagen content may significantly reduce the elasticity of articular cartilage, so it lacks the normal stress and easily wears during movement, leading to apparent fracture^[19]. Afterwards, immune reactions occur, which generate more inflammatory mediators such as IL-1 β and TNF- α , activate iNOS in articular chondrocytes and finally produce numerous NO substances.

Bcl-2 gene, as a proto-oncogene located in the short arm of chromosome 18 of human beings, can be found in the breakpoints of lymphoma cells. It is capable of inhibiting cell apoptosis and prolonging the survival time^[20]. Bax gene, which is mainly located in the chromosome 11 of human beings, is one of the most

important genes in the Bcl-2 family that promotes cell apoptosis^[21,22].

The overexpression of Bcl-2 gene *in vivo* can evidently inhibit cell apoptosis mediated by Fas gene. This gene is expressed in the articular cartilage of OA patients^[23]. Bcl-2 messenger ribonucleic acid (mRNA) is highly expressed in articular chondrocytes close to the articular cartilage defect areas of OA patients. Moreover, the apparent increase of Bax mRNA in the articular chondrocytes of OA patients is one of the important factors for articular chondrocyte apoptosis, and the obvious up-regulation of Bcl-2 mRNA expression suggests that articular chondrocytes are protected from damage and apoptosis.

In summary, L-NMMA can significantly reduce the expression levels of NO in synovial fluid and serum, and may inhibit the apoptosis of articular chondrocytes by raising Bcl-2/Bax ratio. Therefore, L-NMMA is feasibly applicable to the clinical treatment of OA.

Acknowledgements:

None

Conflict of interests:

The authors declared no conflict of interest.

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