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Experimental Study on Inhibitory Effect of Niacinamide on Tumor Necrosis Factor-alpha-induced Matrix Degradation of Annulus Fibrous Tissue *in vitro*

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Summary: The inhibitory effect of niacinamide on tumor necrosis factor-α (TNF-α) induced annulus fibrous (AF) degradation was assessed, and the mechanism of the inhibition was investigated. Chiba's intervertebral disc (IVD) culture model was established. Forty-eight IVDs from 12 adult Japanese white rabbits were randomly divided into 4 groups (12 IVDs in each group), and various concentrations of niacinamide and TNF-α were added to the medium for intervention: negative control group, niacinamide control group (0.5 mg/mL niacinamide), degeneration group (10 ng/mL TNF-α), and treatment group (0.5 mg/mL niacinamide and 10 ng/mL TNF-α). After one week's culture, AFs were collected for glycosaminoglycan (GS) content measurement, safranin O-fast green staining, and immunohistochemical staining for type Ⅰ, Ⅱ collagen and cysteine containing aspartate specific protease-3 (Caspase-3). It was found that the GS content in treatment group was increased by about 48% as compared with degeneration group (t=16.93, P<0.001), and close to that in niacinamide control group (t=0.71, P=0.667). Safranine O-fast green staining exhibited higher staining density and better histological structure of AF in the treatment group as compared with the degeneration group. Immunohistochemical staining for both Type Ⅰ and Ⅱ collagen demonstrated that lamellar structure and continuity of collagen in treatment group were better reserved than in degeneration group. Positive staining rate of Caspase-3 in AFs of negative control group, niacinamide control group, degeneration group and treatment group was 3.4%, 4.3%, 17.9% and 10.3% respectively. The positive rate in treatment group was significantly lower than in degeneration group (P<0.01). It was concluded that niacinamide could effectively alleviate TNF-α induced destruction and synthesis inhibition of matrix ingredients in AFs. The inhibition may be related with reduction of expression of Caspase-3. Thus, niacinamide is of potential for IVD degeneration clinical treatment.
Key words: intervertebral disc degeneration; niacinamide; tumor necrosis factor-alpha

Intervertebral disc (IVD) related diseases, which are usually caused by IVD degeneration are common in orthopedics and produce severe work disability in many cases. Excessive degradation of extracellular matrix plays an important role in mechanism of IVD degeneration. The degradation of matrix results from a series of factors, including over secretion of proinflammatory cytokines (tumor necrosis factor-α, TNF-α) and over apoptosis of the cells[1]. It is reported that low levels of TNF-α, comparable to those present physiologically, is capable of inducing degradation in nucleus pulposus [2]. TNF-α is also a strong inducer of cell apoptosis, which may lead to significant decrease of cell number in IVDs [3]. Thus, inhibition the negative effects of proinflammatory cytokines like TNF-α is an indispensable part of IVD degeneration therapy. Niacinamide is a strong anti-inflammatory vitamin, which can promote energy metabolism of IVD cells as well. We have reported that niacinamide can promote quantity and quality of proteoglycan in both normal and degenerated IVDs[4, 5]. In the present study the inhibitory effects of niacinamide on TNF-α induced degradation of AF matrix were investigated *in vitro*.

1 MATERIALS AND METHODS

1.1 Animals

The experiment was carried out in the Center Laboratory and the Laboratory of Department of Orthopedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (China) from October to December, 2005. IVDs were collected from 12 adult Japanese white rabbits (4–5 months age, weighing 2.5–3.0 kg, obtained from the Experimental Animal Center of Tongji Medical College).

1.2 IVD Tissue Culture and Grouping

The rabbits were executed by air embolism. Lumbar vertebras of L_2–L_6 segments were obtained under the sterile conditions. The IVDs washed by phosphate buffered solution (PBS) and cultured in alginate gel in

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12-well plate as described by Chiba [6]. In each well of the plate, 1 mL of DMEM/F12 medium (Gibico, USA), containing 20% fetal bovine serum (FBS) (Sijiqing Co., China), 25 μg/mL vitamin C (Amresco Co., USA) and 100 μg/mL gentamicin (Amresco Co., USA) was added. The IVDs were cultured at 37°C, in 5% CO_2. And the medium was replaced every 1–2 days. Since the second day of culture, various concentrations of niacinamide and TNF-α were added to the mediums for intervention.

The IVDs were randomly divided into 4 groups: negative control group without any drug; niacinamide control group, with 0.5 mg/mL niacinamide; degeneration group, with 10 ng/mL TNF-α; and treatment group with both 0.5 mg/mL niacinamide and 10 ng/mL TNF-α. The IVDs were collected after 1 week's culture for further investigation.

1.3 Modified Uronic Acid Carbazole Reaction

Since uronic acid is an important ingredient of glycosaminoglycan (GS), uronic acid content assessment is frequently used to reflex content of GS. AFs of 5 IVDs from each group were carefully separated from nucleus pulposus and randomly selected for a modified uronic acid carbazole reaction. In short, 20 mg of defatted AFs fragments was smashed to obtain sample solution as described[4]. One mL attenuated sample solution (or standard uronic acid solution, Lancaster Co. USA) was added into 0.95% borax-sulfuric acid solution (W/V) at 4°C and incubated in boiling water for 10 min. The solution was then added with 0.125% carbazol-alcohol solution (W/V) after cooled down for another 15 min boiling water bath. Absorbance (*A*) value at 530 nm was measured after the solution cooled down. Linear regression analysis was used to obtain a standard curve of standard uronic acid concentration- *A* value. Content of uronic acid was calculated with *A* value according to the curve.

1.4 Safranine O-Fast Green Staining

The IVDs were fixed with 10% formalin, embedded with paraffin and sliced into 5 μm-thick sections. Then Safranine O-Fast Green staining was carried out as described with 1% Safranin O-Fast Green solution for 7 min[4]. The sections were photographed immediately after the staining with HMIAS-2000 system.

1.5 Immunohistochemical Staining

The sections of IVDs were deparaffinized to water and incubated with rabbit anti-human type Ⅰ, Ⅱ collagen or Caspase-3 multiclonal antibody (diluted 1:80, Boster Co., China) respectively for 30 min. An immunohistochemical S-P kit (Zhongshan Co., China) was then used for further staining according to the instructions until the sections were counterstained with hematoxylin. The sections were then photographed with HMIAS-2000 analysis system. Ten random visual fields of each Caspase-3 stained sections were observed and the positive cell rate was calculated (Positive cell rate=Positive cells/Total cells×100%).

1.6 Major Indexes

Content of GS (uronic acid), the staining density and AF structure revealed by safranine O-fast green staining and type Ⅰ, Ⅱ collagen staining, and the positive cell rate of Caspase-3 staining.

1.7 Statistical Analysis

When the positive and negative cell rate of type Ⅰ, Ⅱ collagen, Caspase-3 staining was summed up, SPSS12.0 software was applied to perform chi square test. Ratio of average *A* values of type Ⅰ, Ⅱ collagen was calculated.

2 RESULTS

2.1 Uronic Acid Content

The uronic acid content (μg/mg, $\bar{x}\pm s$) of uronic acid in the four groups was 41.59±1.90, 52.87±2.90, 35.23±1.02 and 52.16±2.75 respectively. The uronic acid content in niacinamide control group was increased by 27.1% as compared with control group (t=11.28, P<0.001). The content in degeneration group was decreased significantly as compared with control group (t=6.36, P<0.001). And the content in treatment group was increased by about 48% as compared with degeneration group (t=16.93, P<0.001), which was close to that in niacinamide control group (t=0.71, P=0.667).

2.2 Safranine-O-fast Green Staining

Safranine O-fast green staining reveals the quantity and distribution of GS chains in proteoglycan, which is an important matrix ingredient of AF. There was opulent orange in the AFs from niacinamide control group, and the lamellar structure was clear and complete. The AFs in degeneration group exhibited disordered and discontinuous lamellar structure, and an obviously decreased staining intensity (fig. 1A). However, in treatment group, the destruction of AF structure was obviously alleviated as compared with degeneration group, and the staining density was also remained higher (fig. 1B).

2.3 Type Ⅰ and Ⅱ Collagen Staining

Immunohistochemical staining showed that AF structure in both negative control group and niacinamide control group was integrated. Type Ⅰ and Ⅱ collagen distributed along lamellar structure orderly. The lamellar structure of AF in degeneration group was obscure, with the collagen lines turned discontinuous (fig. 2A and 2C). On the contrary, the lamellar structure in the treatment group was better maintained as compared with that in degeneration group. And the collagen lines were more regular as well (fig. 2B and 2 D).

2.4 Caspase-3 Staining

The positive staining rates of both negative control group and niacinamide control group were low (3.4% and 4.3% respectively). The positive rate of degeneration group was increased to 17.9%, which was significantly higher than that in control group (χ^2=19.746, P<0.001) (fig. 3A). The positive rate in treatment group was 10.3%, which was significantly lower than that in degeneration group (χ^2=5.081, P=0.024), but still higher than that in negative control group (χ^2=7.207, P=0.007) (fig. 3B).

3 DISCUSSION

During the long and complicated IVD degeneration procedure, extrinsic factors, like over loading, disturb biological behaviors of IVD cells primarily, then cause degradation of extracellular matrix, and lead to irreversible degeneration eventually. Pro-inflammatory factors participate in the whole procedure of degeneration: they influence quantity and quality of extracellular matrix by regulating synthesis rates of matrix ingredients and activating metalloproteinases; on the other hand, they regu-

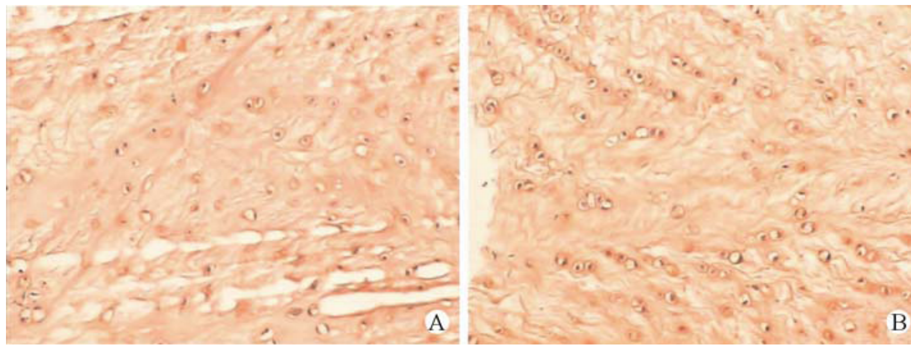

Fig. 1 Safranine-O-fast green staining (×100)

A: The AFs in degeneration group exhibited disordered and discontinuous lamellar structure, and an obviously decreased staining intensity; B: After treatment with niacinamide, the destruction of AF structure was obviously alleviated as compared with degeneration group, and the staining density was also remained higher

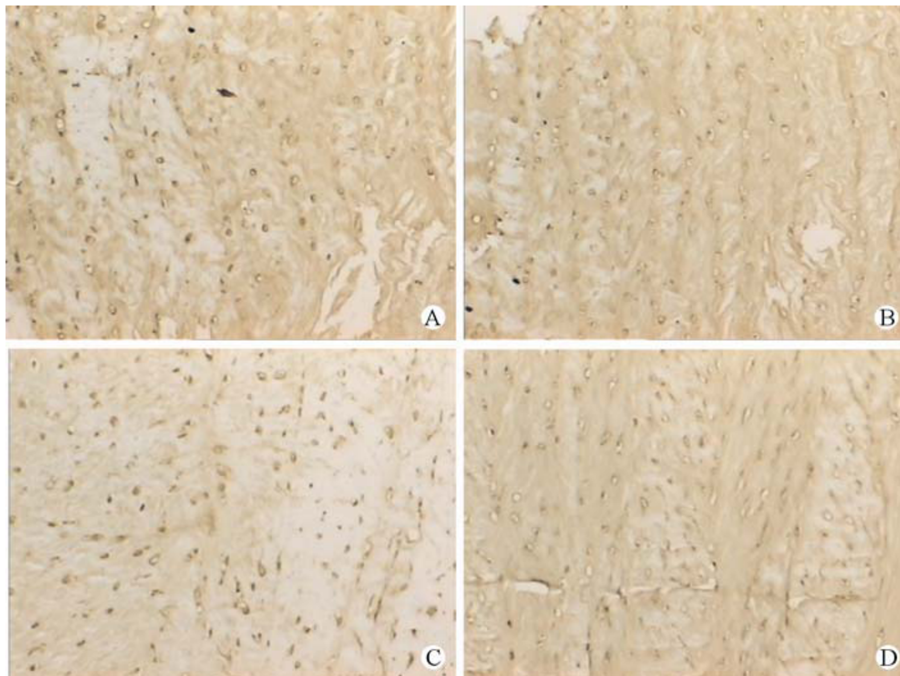

Fig. 2 Type Ⅰ and Ⅱ collagen immunohistochemical staining (×100)

A, C: The lamellar structure of AF in degeneration group was obscure, with the collagen lines turned discontinuous. B, D: The lamellar structure in the treatment group was better maintained as compared with that in degeneration group. And the collagen lines were more regular as well

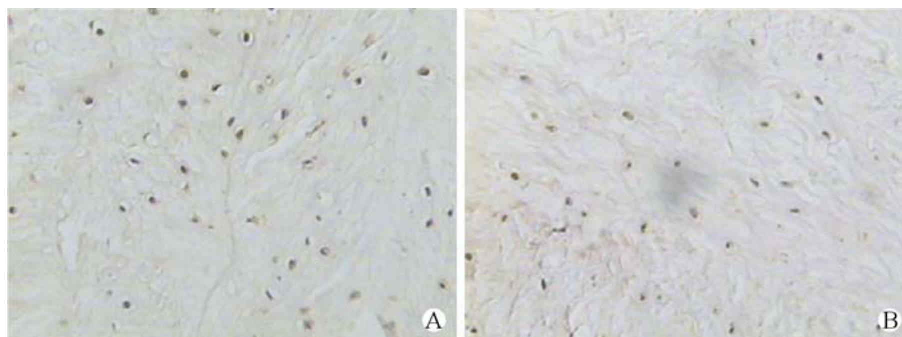

Fig. 3 Caspase-3 immunohistochemistry staining (×200)

A: The positive rate of degeneration group was increased up to 17.9%, which was significantly higher than that in control group (χ^2=19.746, P<0.001). B: The positive rate in treatment group was 10.3%, which was significantly lower than that in degeneration group (χ^2=5.081, P=0.024), but still higher than that in negative control group (χ^2=7.207, P=0.007)

late proliferation, differentiation and apoptosis of the cells *via* cytokine networks. TNF-α is a kind of pro-inflammatory cytokine which can not only interfere metabolism of matrix, but also initiate apoptosis[2, 3]. In previous study, Hu, who participated in the current study reported establishment of a novel rabbit IVD degeneration model by TNF-α intradisc injection[7]. Thus, in this study, TNF-α is adopted to induce degeneration.

Niacinamide is the precursor of nicotinamide adenine dinucleotide, coenzyme Ⅰ. It is able to inhibit side effects of inflammatory factors in various tissues, including nervous system, pancreatic islet, blood and skin. Niacinamide is able to promote proliferation and matrix synthesis of IVD cells by promoting cell metabolism significantly[4].

Proteoglycan is essential for IVD structure and function. Over-loss of proteoglycans is an important part of IVDs degeneration mechanism, and it is an unavoidable part of degeneration therapy. GS chain is an important structural fundament of proteoglycan's hydrophilicity and viscoelasticity. And uronic acid is the main component of the GS chain[8]. In this study, both the safranine O staining density and the uronic acid content in degeneration group coincidently decreased as compared with that in negative control group. The decrease indicated an insufficient content of proteoglycans that was offering biomechanical function in AFs. In Niacinamide control group, the uronic acid content was obviously higher than that in negative control group, which was probably related to niacinamide promoted cell energy metabolism. The uronic acid content and safranine O staining density in treatment group were significantly increased as compared with degeneration group and control group, suggesting that niacinamide could protect the AFs from TNF-α induced proteoglycan reduction.

There are 2 main types of collagens in AF, type Ⅰ and Ⅱ collagen. Type Ⅰ collagen provides tensile strength of AF, while type Ⅱ collagen supplies pressure strength. In degenerated IVDs, the content of type Ⅰ collagen could increase while the content of type Ⅱ collagen decrease sharply. In degeneration group, with disturbance by TNF-α, structure of the both type Ⅰ and Ⅱ collagen was broken severely, which was consistent with the results reported[7]. The coincident decrease suggest that TNF-α could strongly affect both of the two collagens. In treatment group, the destruction of collagens lines along the lamellar structure was obviously inhibited as compared with degeneration group. The inhibition may result from not only reserved synthesis of collagens, but also decreased metalloproteinase activities.

Apoptosis plays an important role in IVD degeneration, and Caspase family takes an important part in apoptotic procedure. Activation of Caspase-3 is the key point of the effective phase of apoptosis[9]. In this study, the positive cell rate was significantly reduced in treatment group as compared with degeneration group, suggesting that niacinamide could inhibit the TNF-α- induced apoptosis in AF cells. However, this inhibitory effect could not reduce the positive cell rate to the level of negative control group. The incomplete inhibition might result from diversity of biological function of TNF-α, including initiating a variety of apoptosis pathways.

The excessive degradation of the extracellular matrix is the key mechanism of IVDs degeneration and closely related with the apoptosis and excessive secretion of inflammatory factors. In this study, niacinamide displayed satisfactory effects to inhibit the TNF-α induced AF matrix degradation *in vitro*. In combination with the previous study, it is believed that niacinamide possesses favorable potentials for IVD degeneration therapy.

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