# Analysis of the Effect of Nux Vomica on Three Models of Chondrocyte Apoptosis

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**Liu *et al.*: To Explore the Effect of Nux Vomica on Chondrocyte Apoptosis**

**To explore the effect of nux vomica on three kinds of chondrocyte apoptosis models. In the rabbit chondrocyte culture system, the rabbits were divided into normal group, sodium nitroprusside group, all-trans retinoic acid group, tumour necrosis factor alpha group, nux vomica+sodium nitroprusside group, nux vomica+all-trans retinoic acid group, nux vomica+tumour necrosis factor alpha group on the basis of sodium nitroprusside group, all-trans retinoic acid group and tumour necrosis factor alpha group, respectively. After 24 h of culture, the apoptosis of chondrocytes was quantitatively detected by Annexin V/propidium iodide two-parameter method, and the deoxyribonucleic acid content and the function of collagen and proteoglycan synthesis were measured by isotope labeling 3H thymidine, 3H-Pro and 35S-sodium sulfate incorporation method. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide assay was used to observe the proliferation changes of three kinds of chondrocyte apoptosis models. Western blotting was used to observe the effect of nux vomica on second mitochondria-derived activator of caspase and B-cell lymphoma 2 proteins in three apoptosis models of chondrocytes. The apoptosis rate of sodium nitroprusside group, all-trans retinoic acid group and tumour necrosis factor alpha group was raised than that of control group, while this in nux vomica+sodium nitroprusside group and nux vomica+all-trans retinoic acid group was reduced than that in sodium nitroprusside group and all-trans retinoic acid group, respectively. The synthesis of deoxyribonucleic acid, proteoglycan and collagen in sodium nitroprusside group, all-trans retinoic acid group and tumour necrosis factor alpha group were decreased than those in normal group. Nux vomica can inhibit the apoptosis of sodium nitroprusside and all-trans retinoic acid chondrocytes, and this mechanism may be realized by up-regulating the expression of second mitochondria-derived activator of caspase and B-cell lymphoma 2 to regulate mitochondrial related pathways.**

**Key words: Nux vomica, chondrocytes, apoptosis, mitochondria, caspase, transmission electron microscope**

Nux vomica is a common herb, which is widely used in traditional herbal medicine to treat a variety of diseases. In recent years, researchers have paid more and more attention to the active components of nux vomica and their effects on the process of cell biology. Especially in the model of chondrocyte apoptosis, the role of nux vomica has attracted much attention. Cartilage is an important structural tissue in joints, which has the functions of absorbing impact, cushioning pressure and protecting joint surface[1]. However, due to a variety of reasons, such as trauma, inflammation and osteoarthritis, apoptosis of chondrocytes can lead to joint tissue destruction and dysfunction. To find a way to regulate the apoptosis of chondrocytes is of great significance for the treatment of bone and joint diseases. Therefore, in this experiment, Sodium Nitroprusside (SNP), All-Trans Retinoic Acid (ATRA) and Tumor Necrosis Factor-Alpha (TNF-α) were added to the chondrocyte culture system to construct three different apoptosis models[2]. This experiment is through the construction of apoptosis model, focusing on the analysis of the specific effects of nux vomica on three kinds of chondrocyte apoptosis models, reported as follows. The 3 d old New Zealand rabbits were killed, and the cartilage was cut from

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the distal femur, proximal tibia and hip joint under aseptic conditions. The cartilage particles were added with 0.25 % trypsin, bathed at 37°, centrifuged, and then 0.1 % type II collagen prepared with Dulbecco's Modified Eagle Medium (DMEM) was washed with DMEM for 3 times. Finally, chondrocyte suspension was prepared with chondrocyte culture medium. SNP (National Medicine Standard H20143166) specification 50 mg/branch, ATRA is provided by Sigma Company, TNF-α is provided by Maixin Company, connexin V-fluorescein thiocyanate labeling (Annexin V-Fluorescein Isothiocyante (FITC)) model Kit Protocol ctNO2375 and Propidium Iodide (PI) reagent are provided by Suzhou Kurt immune Technology Company, German toluidine blue stain[3]. Brucine 20 mg is provided by the Institute of Pharmaceutical and Biological products identification in China. 50 ml/l Carbon dioxide ($CO_2$) incubator (BB5060, United States of America (USA)), flow cytometry (EPICSXLMCL), transmission electron microscope (Japanese Electron, JEM-1230) and inverted microscope (Japanese CK, Olmpus Tokyo) were used. In experimental grouping, chondrocytes were implanted on a 24-well plate at the concentration of $5\times10^4$ cells per well, and the culture medium for each well was 1 ml. Experimental groups; normal group (rabbit chondrocytes), SNP group, ATRA group, TNF-α group, nux vomica+SNP group, nux vomica+ATRA group, nux vomica+TNF-α group. 2 mmol/l SNP, $1\times10^{-4}$ mol/l ATRA and 30 ng/l human TNF-α were added into the culture system to construct SNP group, ATRA group and TNF-α group. On the basis of SNP group, ATRA group and TNF-α group, the dosage of nux vomica (250 mg/l) was added at the same time to construct nux vomica+SNP group, nux vomica+ATRA group and nux vomica+TNF-α group. The co-incubation was terminated for 24 h. Collect cells and set them aside. In quantitative detection of apoptosis by Annexin V/PI two-parameter method; first, the cultured chondrocytes were collected. The chondrocytes in the culture plate were collected after digestion, the supernatant was removed by centrifugation, washed twice with Phosphate Buffer Solution (PBS), and about 100 µl of cell suspension was retained. Then, 1 µl annexin V and 2.5 µl PI were added to the cell suspension and incubated in the dark for 15 min. Next, add enough buffer to make the total volume reach 500 µl for flow cytometry analysis. The light temperature is 488 argon particle laser. FITC emits green fluorescence and PI emits red fluorescence after laser. $5\times10^4$ cells were collected from each sample and analyzed by system software. Each group was incubated with 3H-TdR, 3H-Pro and 35S-Sodium sulfate ($Na_2SO_4$) at radiation concentration of 20 µCi/ml for 24 h, and washed with PBS for 3 times. The supernatant and rinse solution were filtered by negative pressure, and the cells were collected on 49 glass fiber filter paper. After drying, 5 ml scintillation solution was added and the pulse number per minute (cpm) was measured by liquid scintillation counter. Proliferation ability was detected by 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyl Tetrazolium Bromide (MTT). Cells of each group were inoculated in 96-well plate and cultured in 5 % $CO_2$ at 37° cell incubator. The absorbance of cell suspension at 490 nm wavelength was measured by automatic fluorescence enzyme labeling instrument. The cells of each group were fully lysed, centrifuged, the supernatant was prepared, the Bicinchoninic Acid (BCA) working solution was mixed, and the total protein content was determined by Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), membrane transfer, sealing, adding primary antibody and Tris-Buffered Saline with 0.1 % Tween® 20 (TBST) detergent cleaning, shaking the bed at 4°, adding secondary antibody and rinsed Polyvinylidene Difluoride (PVDF) membrane for antibody incubation, dripping Enhanced Chemiluminescence (ECL) developer, and using gel image processing system to analyze the strip optical density. All the research data were analyzed by Statistical Package for the Social Sciences (SPSS) 20.0 software package, and all the measurement data in accordance with normal distribution were compared by (variance, x±s), single factor analysis of variance was used for comparison among groups, and SNK-q test was used for pairwise comparison. Compared with normal group, $^ap<0.01$; compared with SNP group, $^bp<0.01$ and compared with ATRA group, $^cp<0.05$. When SNP, ATRA and TNF-α were added to the chondrocyte culture system, the apoptosis rate of SNP group, ATRA group and TNF-α group was raised than that of the control group. The apoptosis rate in nux vomica+SNP group and nux vomica+ATRA group was reduced than that in SNP group and ATRA group, respectively (Table

1). It was found that the synthesis of DNA, proteoglycan and collagen in SNP group, ATRA group and TNF-α group were reduced than those in normal group when chondrocytes were apoptotic by isotope labeling 3H-TdR, 3H-Pro and 35S-$Na_2SO_4$. The levels of 3H-TdR, 3H-Pro and 35S-$Na_2SO_4$ in nux vomica+SNP group and nux vomica+ATRA group were raised than those in ATRA group and SNP group, respectively (Table 2). Compared with the control group, the cell proliferation rate of SNP group, ATRA group and TNF-α group decreased, while this in nux vomica+SNP group and nux vomica+ATRA group was raised than that of SNP group and ATRA group, respectively (Table 3). The expressions of Second Mitochondria derived Activator of Caspase (SMAC) and Bcl-2-Associated Protein X (BAX) proteins in SNP group, ATRA group and TNF-α group were increased than those in control group. The expressions of SMAC and BAX protein in nux vomica+SNP group and nux vomica+ATRA group were reduced than those in SNP group and ATRA group (Table 4). The main pathological features of arthritis are the damage of articular cartilage caused by age, sex, genetic factors, obesity, changes of mechanical stress on the articular surface, joint deformities, injuries and metabolic disorders. The central link of the pathogenesis is the degenerative changes of articular cartilage. Recently, it has been found that the apoptosis of chondrocytes in osteoarthritis is abnormal[4], and apoptosis may play an important role in the pathogenesis of osteoarthrosis and is closely related to the prognosis of osteoarthrosis. The clinical manifestations of osteoarthritis include slowly developing joint pain, stiffness, swelling and limited movement. Lesions can be seen in many joints throughout the body, sometimes mild and sometimes severe, difficult to cure, and a long course of disease[5]. Its clinical manifestation is in line with the characteristics of collateral disease, i.e., long time, blood stasis (pain), stubbornness, and miscellaneous. Whether it is positive deficiency or evil invasion, the final pathogenesis of osteoarthritis is related to collaterals. Therefore, the use of collaterals dredging drugs should be strengthened in the treatment of osteoarthritis. The study found that nux vomica has an obvious effect on osteoarthritis, with the effect of warming the kidney and dispelling cold, dredging collaterals and relieving pain[6]. The chemical constituents of nux vomica contain a variety of bioactive substances, such as brucine and so on. Studies have shown that nux vomica has certain pharmacological activities and may affect biological processes such as apoptosis[7]. Apoptosis is an important process in regulating tissue development and disease progression in cartilage. The effect of nux vomica on three models of chondrocyte apoptosis will be discussed below. Some components of nux vomica are considered to have anti-apoptotic effects[8]. Three different apoptosis models were established by using SNP, ATRA and TNF-α in chondrocyte culture[9]. SNP is a kind of Nitric Oxide (NO) donor, which can release NO molecules. In chondrocyte culture, adding appropriate amount of SNP can simulate the excessive production of NO and lead to apoptosis[10]. NO is considered to be an important signal molecule to regulate the balance between cell survival and death in cartilage. SNP model can be used to study the mechanism of the effect of NO on chondrocyte apoptosis and related signal pathways[11]. ATRA is a derivative of vitamin A, which plays an important role in cell development and differentiation. In the culture of chondrocytes[12], adding a certain concentration of ATRA can induce the apoptosis of chondrocytes. ATRA model can be used to study the regulation of retinoic acid signaling pathway in chondrocyte apoptosis and its effect on chondrocyte development and differentiation. TNF-α is an important cytokine[13], which is involved in the regulation of biological processes such as inflammation and apoptosis. In chondrocyte culture, the addition of appropriate amount of TNF-α can induce chondrocyte apoptosis. This model is often used to study the regulatory mechanism of TNF-α signaling pathway in chondrocyte apoptosis and its role in arthritis and other diseases[14]. The results showed that the apoptosis of human embryonic chondrocytes induced by ATRA mainly occurred in the early stage, while the apoptosis induced by TNF-α mainly occurred in the late stage. The main purpose of this study is to study the effect of nux vomica on chondrocyte apoptosis induced by different drugs. 24 h after the serum containing nux vomica was added to the above apoptosis induction system, the apoptosis rate of each group was compared with the control group, there was significant difference between nux vomica+SNP group and

SNP group, there was significant difference between nux vomica+ATRA group and ATRA group, but there was no significant difference between nux vomica+TNF-α group and TNF-α group. Therefore, the experimental results show that nux vomica can reduce the early apoptosis of chondrocytes induced by SNP and ATRA, but has no significant inhibitory effect on the late apoptosis of chondrocytes induced by human TNF-α, and nux vomica can improve the anabolism function of chondrocytes to some extent. According to the experimental results, the proliferation rate of chondrocytes in the normal group was higher than that in the experimental group, which proved that brucine had a certain effect on the proliferation of chondrocytes. There is a sharp contrast between the proliferation rate and the apoptosis rate, which shows that nux vomica and brucine can inhibit the early apoptosis of chondrocytes, but have little effect on the late apoptosis of chondrocytes, or the protective effect of nux vomica on chondrocytes is slow, or the force on some specific receptors of chondrocytes is less than that of TNF-α. Nux vomica may have a positive effect on these three models of chondrocyte apoptosis by inhibiting the occurrence of apoptosis and protecting chondrocytes from injury[15]. The establishment of the three models provides an important tool for the study of chondrocyte apoptosis. By comparing the characteristics and regulatory mechanisms of apoptosis in different models, we can further understand the process of chondrocyte apoptosis and reveal the pathogenesis of related diseases. In addition, different drugs or interventions can be used in these models to evaluate their effects on chondrocyte apoptosis, so as to provide a theoretical basis for the study of intervention strategies and new drug development. However, it should be noted that these models can only simulate part of the cartilage tissue *in vitro*, and the results need to be verified in *in vivo* experiments or clinical studies. To sum up, *Strychnos nux-vomica* has a significant inhibitory effect on apoptosis of SNP and ATRA chondrocytes, and this mechanism may be realized by up-regulating the expression of SMAC and BAX to regulate mitochondrial related pathways.

**TABLE 1: EFFECTS OF NUX VOMICA ON THREE APOPTOSIS MODELS OF CHONDROCYTES (n=5)**

| Group | Apoptosis rate (%) |
|---|---|
| Normal | 2.71±2.49 |
| SNP | 65.09±5.66[a] |
| ATRA | 45.88±12.98[a] |
| TNF-α | 82.14±3.46[a] |
| Nux vomica+SNP | 32.30±10.22[ab] |
| Nux vomica+ATRA | 27.43±6.64[ac] |
| Nux vomica+TNF-α | 80.04±3.42[a] |

Note: Compared with SNP, [a]p<0.01; compared with nux vomica+SNP group, [b]p<0.01 and compared with ATRA group and [c]p<0.05

**TABLE 2: EFFECTS OF NUX VOMICA ON DNA, PROTEOGLYCAN AND COLLAGEN SYNTHESIS OF CHONDROCYTES DURING APOPTOSIS**

| Group | 3H-TdR | 3H-Pro | 35S-$Na_2SO_4$ |
|---|---|---|---|
| Normal | 3917±742 | 4227±391 | 1772±343 |
| SNP | 1614±450[a] | 2435±443[a] | 808±232[a] |
| ATRA | 1157±222[a] | 1720±495[a] | 886±157[a] |
| TNF-α | 986±186[a] | 1275±324[a] | 745±126[a] |
| Nux vomica+SNP | 3035±651[ab] | 2740±580[ab] | 2663±421[ab] |
| Nux vomica+ATRA | 2164±636[ac] | 2670±340[ac] | 2189±328[ac] |
| Nux vomica+TNF-α | 1024±217 | 1358±346 | 768±149 |

Note: Compared with SNP, [a]p<0.01; compared with nux vomica+SNP group, [b]p<0.01 and compared with ATRA group and [c]p<0.05

**TABLE 3: EFFECT OF NUX VOMICA ON THE PROLIFERATION OF CHONDROCYTES ($\bar{x}$±s)**

| Group | OD value of cell proliferation |
|---|---|
| Normal | 0.21±0.04 |
| SNP | 0.11±0.03[a] |
| ATRA | 0.10±0.03[a] |
| TNF-α | 0.11±0.02[a] |
| Nux vomica+SNP | 0.15±0.02[ab] |
| Nux vomica+ATRA | 0.16±0.03[ac] |
| Nux vomica+TNF-α | 0.12±0.02 |

Note: Compared with SNP, [a]p<0.01; compared with nux vomica+SNP group, [b]p<0.01 and compared with ATRA group and [c]p<0.05

**TABLE 4: EFFECTS OF *Strychnos nux-vomica* ON SMAC AND BAX PROTEINS IN THREE APOPTOSIS MODELS OF CHONDROCYTES (n=5)**

| Group | SMAC | BAX |
|---|---|---|
| Normal | 0.35±0.02 | 1.02±0.09 |
| SNP | 2.37±0.32[a] | 2.15±0.23[a] |
| ATRA | 2.46±0.35[a] | 2.26±0.25[a] |
| TNF-α | 2.94±0.42[a] | 3.08±0.32[a] |
| Nux vomica+SNP | 0.93±0.22[ab] | 1.46±0.15[ab] |
| Nux vomica+ATRA | 1.12±0.21[ac] | 1.57±0.14[ac] |
| Nux vomica+TNF-α | 2.49±0.38[a] | 2.78±0.38[a] |

Note: Compared with SNP, [a]p<0.01; compared with nux vomica+SNP group, [b]p<0.01 and compared with ATRA group, [c]p<0.05

## Conflict of interests:

The authors declared no conflict of interests.

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