Effect of Brucine on Three Chondrocyte Apoptosis Models

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Abstract: With the development of people's application technology of Strychnine, Strychnine is a relatively common type of medicine in the current clinical treatment of arthritis patients. And in the actual treatment, because strychnine has an important effect on the apoptosis of three kinds of chondrocytes. Better results can often be achieved through the use of horse money, which is of great significance to patients. The analysis of the influence of strychnose on the apoptosis of the three types of chondrocytes has become the focus of research in the academic and industry circles. The purpose of this article is to analyze the impact of Strychnoma chinensis on three chondrocyte apoptosis models. In this paper, by establishing a system for culturing rat chondrocytes in vitro, using nitric oxide in vitro cultured rat chondrocytes to induce their apoptosis, and observing the effect of Strychnine, the main monomer component of Chinese medicine Strychnine, on the cellular and molecular levels. The mechanism of SD rat chondrocyte apoptosis is to explore the establishment of a system for culturing rat chondrocytes in vitro. In this paper, rat chondrocytes cultured in vitro were induced by nitric oxide to induce their apoptosis, and the mechanism of strychnine, the main monomer component of Strychnine, was observed in order to explore the mechanism of strychnine on the apoptosis of SD rat chondrocytes in vitro. The experimental results showed that strychnine induced apoptosis of chondrocytes, and the survival rate of cells during 36h and 48h after administration was 99% and 97%, respectively. Its apoptotic rate decreases with the increase of the dose and time of strychnine.

Key words: Three Kinds of Chondrocytes, Apoptosis Model, Cell Apoptosis
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The traditional Chinese medicine brucine (also known as Panmuchi) has anti-inflammatory and pain relief, softening and dispelling knots, reducing swelling and promoting blood circulation, and activating the channels and collaterals. The indole-type alkaloids extracted from brucine are called brucine. Alkali is one of the important effective ingredients [1]. In clinical practice, strychnine is widely used in rheumatic arthralgia, tumor suppression and arthritis treatment [2]. However, it should be noted that the effect of chrysalis on the apoptosis and proliferation of osteoarthritis chondrocytes is actually not clear. Therefore, it is not widely used in the treatment of osteoarthritis. Under such a premise, it is very important to study the effect of strychnine on three chondrocyte apoptosis models and find out the specific ways and mechanisms of its influence.

The biopharmacological effect of strychnine has become the current medical industry. And an important topic of academic research.

Many scholars have conducted in-depth discussions on the analysis of the influence of strychnine on three chondrocyte apoptosis models, and have achieved very good results. For example, Tao T et al. proved that strychnine exerts an inhibitory effect. The important target protein is cyclooxygenase (COX-2), which can inhibit the expression of COX-2 and the release of prostaglandin E2 (PGE2) and transcription activation in A549 cells. COX-2siRNA can significantly increase the induction of brucine

Apoptosis [3]. Zhong X et al. found that when brucine acted on multiple myeloma (U266) cells, the expression of the pro-apoptotic gene Bax increased over time, while the expression of the anti-apoptotic gene Bcl-2 gradually decreased, indicating that horses Cicine is simultaneously acting on Bax and Bcl-2 to induce apoptosis [4].

In this paper, from the perspective of the impact of strychnine on three chondrocyte apoptosis models, RT-PCR was used to detect the expression of strychnine on the apoptosis-related genes Bcl-2, Bax and Caspase-3 mRNA in each group of cultured cells in vitro The real-time fluorescent quantitative PCR method (qRT-PCR) was used to detect the expression levels of cell cycle-related genes CyclinD1 and CyclinEmRNA in each group, and Western blotting was used to detect cell apoptosis and cycle-related genes Bcl-2, Bax, and Caspase -3. At the same time, this article prepares strychnine by reflux, acid extraction, extraction, purification and crystallization, and uses high performance liquid chromatography (HPLC) for qualitative and quantitative analysis of strychnine. Through the method of making rabbit knee osteoarthritis model, the specific effect of strychnine on the apoptosis of osteoarthritis chondrocytes was analyzed.

EFFECT OF STRYCHNOMA ON THREE CHONDROCYTE APOPTOSIS MODELS

Brucine Induces Cell Line Apoptosis and Its Preliminary Mechanism

Apoptosis is an active cell suicide process determined by multiple genes. In many cases, apoptosis is also called programmed cell death (PCD), that is, at a certain time cells die according to certain procedures, and this cell death has very strict genetic timing and selectivity [5]. It is a death that is different from cell necrosis, and is a form of active cell death. The apoptosis of each cell is a complex life process involving multiple genes. Among them, the release of cytochrome C and the Caspases cascade are the key links in the apoptosis process. This series of reactions is called the mitochondrial apoptotic pathway. The change of mitochondrial membrane potential can stimulate the release of cytochrome C, and cytochrome C is through Caspase-9 activates caspase-3 and induces cell apoptosis [6]. The Bcl-2 family proteins play an extremely important role in the regulation of mitochondrial apoptosis.

(1) The effect of Strychnoma on the expression of Fas/Fas L in synovial tissue

Fas is a receptor that exists on the surface of cell membranes. FasL is the ligand of Fas. The combination of Fas and FasL can cause T cells to kill target cells and cause target cell apoptosis. Apoptosis between target cells can also cause Local inflammation of the body [7]. In addition, the apoptosis mechanism initiated by Fas/FasL can lead to the dysfunction of the autoimmune system and the excessive secretion of a variety of inflammatory factors. It can also lead to the death of one or both of the cells, which can play a role in the immune down-regulation response; this may be caused by the synovial membrane of the joints. Tissue hyperplasia is one of the main reasons for the pathological changes of typical RA.

(2) The influence of Strychnoidea on the expression of Bcl-2/Bax in synovial tissue

Bcl-2 is the earliest researched proto-oncogene related to apoptosis. It mainly exists in vascular endothelial cells and lymphocytes, and can play an anti-apoptotic effect by preventing the release of mitochondrial cytochrome C. Therefore, this gene has the function of protecting cells, but its overexpression can inhibit the normal apoptosis of cells, thus easily Lead to the occurrence of tumor diseases; the physiological function of Bcl-2 is to inhibit cell apoptosis and prolong cell life. The accumulation of nuclear glutathione (GSH) is caused by the overexpression of Bcl-2, which leads to the imbalance of redox in the nucleus and inhibits the activity of Caspase, thereby hindering the normal apoptosis of cells [8]. Bax mainly activates the Caspase system through Caspase3 through mitochondria and nucleus, and makes it express at a high level, thereby inducing cell apoptosis [9]. There is a large amount of Bcl-2 in the synovial cells of RA patients and there is no apoptosis, which may be related to the inflammatory response and chondrocyte proliferation caused by the expression of Bcl-2. It can be seen that Bcl-2 can effectively protect sliding Membrane cells are protected from all kinds of important factors that induce apoptosis.

(3) The effect of Strychnoma on the expression of p53 in synovial tissue

The p53 gene is a tumor suppressor gene, and its overexpression in the synovial cells of RA patients may be a precursor to joint damage [10]. The p53 gene includes wild type and mutant type. The normal p53 gene, namely wild-type p53, can stop cell development in the G1 phase. It can not only inhibit cell proliferation, but also induce apoptosis of susceptible cells. Its main function is to promote cell apoptosis, regulate cell life cycle, and play a role. To the role of "molecular police" to control the integrity of genes. Activated p53 induces the release of cytochrome C through mitochondria, and finally activates the Caspase system to trigger cell apoptosis [11]. At the same time, p53 can directly down-regulate Bcl-2 and up-regulate the activity of Bax, ultimately leading to the release of cytochrome C. It can be seen that p53 interferes with cell apoptosis through a variety of ways.

(4) The effect of Strychnoidea on the expression of Caspases-3 in synovial tissue

Caspase3 plays an irreplaceable role in cell apoptosis. It can activate Caspase6, Caspase7, poly-ADP-ribose polymerase and other apoptotic factors. Caspase6 can activate Lamin A, etc., and Caspase7 can activate poly-ADP-ribose polymerization. Enzymes, etc., causing cell shrinkage, DNA degradation, etc. It can be seen that the expression level of Caspase3 affects the occurrence of apoptosis, and it is a key indicator for detecting apoptosis [12].

(5) Apoptosis model

In human joints, NO is the main chondrocyte apoptosis inducer, rather than oxygen free radicals. There is a time-effect relationship between NO and chondrocyte apoptosis; oxygen free radicals can cause chondrocyte necrosis, but NO induces chondrocyte apoptosis, chondrocyte apoptosis is determined by oxygen free radicals and balance, when NO is in the main position, Cells are dominated by apoptosis. When oxygen free radicals are in the dominant position, the balance shifts to necrosis[13].

Effect of Brucine on Chondrocyte Apoptosis and Mitochondrial Signal Transduction in SD Rats

(1) The relationship between mitochondria, CytC, Smac and apoptosis

Mitochondria are important organelles in all eukaryotic cells, are the main part of ATP production, and play an important role in maintaining cell energy metabolism and normal functional activities [14]. The mitochondria contains some substances closely related to cell apoptosis: such as pro-caspase, Cyt C, Smac/Diablo, AIF, etc. Under the stimulation of certain factors, the permeability of the mitochondrial membrane increases, and these substances can be released by the mitochondria. Therefore, mitochondria play an important role in the occurrence of apoptosis and are vividly called the "combustion chamber" of apoptosis.

In the mitochondrial-mediated apoptosis pathway, the release of CytC is a key step.

The release of CytC causes Apaf-1, aspase-9 and other factors to assemble into a caspase activation complex (apoptosome). The formation of apoptosome is a landmark molecular event initiated by the cell death pathway controlled by mitochondria, and its function is similar to non-mitochondrial apoptosis. The death-inducing signal complex composed of death receptors, linker molecules, and procaspase-8 in the pathway [14]. CytC is both an essential molecule for cell survival and one of the initiating factors of cell death. When mitochondria release CytC into the cytoplasm, it binds to Apaf-1, the apoptotic protease activator, and changes the conformation of Apaf-1 under the action of ATP/dATP to activate caspase-9, which then activates the apoptotic effector caspase- 3. The activated caspase-3 acts on the substrate protein, polyadenosine triphosphate ribose polymer (PARP), etc., and finally leads to cell apoptosis.

Unprocessed Smac is confined to the mitochondrial membrane. Mature Smac exists in both the membrane and the cytoplasm. In normal cells, only the processed form is found. The pro-apoptotic activity of Smac depends on its location rather than processing. Smac does not exert apoptosis normally, but only works in damaged cells. When a cell undergoes apoptosis, it is released into the cytoplasm. Smac itself does not work directly, it must work indirectly in conjunction with the IAPs gene. IAP protein is the only endogenous caspase effector enzyme inhibitor,

which limits the caspase cascade reaction. IAPs play an inhibitory role through their baculovirus IAP repeat sequence (BIR) structural region [15].

Like CytC, Smac is located in the mitochondria under normal conditions. The pro-apoptotic activity of Smac depends on its location rather than processing. Smac does not undergo apoptosis normally and only works in damaged cells. The cDNA encoding Smac is composed of 719 bp and encodes a reading frame of 239 amino acid residues, of which 1 to 55 amino acid residues are used as the target signal peptide, which is located in the mitochondria. Only the signal peptide part located in the mitochondria is separated from Smac. Apoptotic activity. When a cell undergoes apoptosis, it is released into the cytoplasm. As the second mitochondrial-derived caspase activator discovered, Smac itself cannot act directly, and must be combined with IAPs to act indirectly. IAP protein is the only endogenous caspase effector enzyme inhibitor, which limits the caspase cascade reaction. IAPs play an inhibitory role through the structural region of the baculovirus IAP repeat sequence (BIR) [16].

IAPs are important regulators of apoptosis, which can inhibit apoptosis caused by cell surface death receptors and CytC[17]. Human IAPs have a unique molecular structure, and their family members contain 1 to 3 baculovirus IAP repeat regions, and their anti-apoptotic activity originates from this repeat region. After the cell receives the apoptosis stimulating signal, the mitochondria not only release CytC to form an apoptotic complex, activate the caspase family, but also release Smac, which promotes apoptosis by resisting IAPs.

(2) The effect of brucine on the changes of HUVECs cell apoptosis

With the increase of the concentration of brucine, the number of HUVECs decreased significantly, and the apoptotic cells in nuclear condensed and fragmented morphology increased significantly, indicating that the concentration of brucine increased cell apoptosis in a dependent manner. Quantitative analysis of the number of apoptotic cells by flow cytometry showed that the brucine treatment group significantly increased cell apoptosis, and with the increase of the concentration of brucine, the HUVECs that had undergone apoptosis increased significantly. Therefore, within a certain concentration range, brucine may inhibit HUVECs cell proliferation by inducing HUVECs apoptosis.

The effect of strychnine on the vascularization of HUVECs strychnine can cause slow blood flow, pericardial edema, decreased heart rate, and other symptoms [18]. As a more toxic drug, from this point of view, it is necessary to confirm whether the cytotoxicity and the anti-angiogenic effect of strychnose are related, and to propose a new direction for the second use of toxic traditional Chinese medicine. Strychnine and strychnine are the main components of strychnine, both of which are toxic and effective. They also have better analgesic and anti-tumor growth effects. Experiments have shown that their mechanism may be more effective than preventing cancer. Programmed cell death causes cancer cell differentiation, tumor invasion and delay of migration, and is related to the inhibition of tumor cell neovascularization.

Brucine blocks the G0/G1 cell cycle and inhibits the proliferation of human lung cancer cell line PC-9. The expression of cyclin D1 and cyclin E is down-regulated. At the same time, studies have confirmed that brucine can induce human liver cancer. HepG2 cell proliferation and apoptosis, the mechanism is to increase Ca2+, and reduce Bcl-2 protein expression by damaging the mitochondrial membrane potential pathway [19].

Brucine also down-regulates the mRNA levels of transcriptional activator-3 and transcriptional activator-5 in the JAKSTAT pathway, which can increase the expression of caspase-8 and Bax protein in human myeloma U266 cells, thereby triggering apoptosis Induction mechanism plays a role.

Strychnine regulates the migration, invasion and neovascularization of HUVEC from the activation and suppression of the VEGF/ VEGFR signaling system [20]. EphA2 is highly expressed in many tumor cells, triggering tumor progression through signal transduction pathways. The promising new targets for tumor therapy are tumor invasion, metastasis and angiogenesis. However, it is highly expressed in a variety of malignant tumors or cells. The protein and mRNA of EphA2 receptors are one of the common events that

indicate tumors is high expression of EphA2, which indicates low survival rate, poor differentiation, high metastasis rate, poor prognosis, and strong invasiveness. It is also one of the prognostic judgments of malignant tumors.

EphA2 can promote the formation of new blood vessels, and high levels of EphA2 are expressed in cancer cells. Strychnine inhibits the overexpression of EphA2, has an activating effect on the VEGF/ VEGFR signaling pathway, and has a reversal effect on the upregulation of EphA2 and VEGF by the VEGF/ VEGFR signaling pathway system. The target of brucine acting on the cell upstream of HUVEC may be EphA2, and brucine has the ability to inhibit the migration and invasion of HUVEC cells and angiogenesis.

Cell Movement Imm Filter Algorithm

(1) Dynamic random walking model

The random walking model (RW model) regards the target's motion state as Brownian motion, that is, the target's motion state is only represented by the information of the target's position, and the target's speed is expressed in the form of random interference. Then the time domain model of the RW model can be expressed as formula (1):

$$X=x+w(t) \quad (1)$$

In formula (1), x is the position of the target, w(t) represents the system noise with a mean value of 0 and a variance of σ^2, then the state matrix of the target is:

$$X（t）=x(t) \quad (2)$$

The state transition process of the cell tracking system is expressed as formula (3):

$$S_k=F_kP_{k-1}+w_k(3)$$

(2) Time update

State prediction:

$$S_{k|k-1}=F_kS_{k-1|k-1}(4)$$

State prediction covariance:

$$P_{k|k-1}=F_kP_{k-1}F_k^T+Q_{k-1}(5)$$

(3) Status update

Innovation:

$$v_k=Z_k-Z_{k|k-1}(6)$$

Innovation covariance:

$$S_k=H_k-P_{k|k-1}H_k^T+R_k \quad (7)$$

Kalman gain:

$$K_k=P_{k|k-1}H_k^TS_k^{-1}(8)$$

Status update:

$$S_{k|k}=S_{k|k-1}+K_kv_k \quad (9)$$

Status covariance update:

$$P_{k|k}=P_{k|k-1}(1-K_kH_k)(10)$$

EXPERIMENTAL STUDY ON THE EFFECT OF BRUCINE ON THREE CHONDROCYTE APOPTOSIS MODELS

Experimental Materials

Materials and instruments

The experiment uses sodium nitroprusside, the specification is 50mg/bottle. 2 days old New Zealand rabbits, 3-4 days old SD rats. Toluidine blue produced in Germany. Strychnine produced by China Pharmaceutical and Biological Products Appraisal Institute, the specification is 20mg/bottle. The instrument is the American BB5060 carbon dioxide incubator with a specification of 50mL/L. EPICSXLMCL flow cytometer, Japan JEM-1230 transmission electron microscope.

Experimental Method

The preparation method of Brucine drug:

Use a small amount of dimethyl sulfoxide (DMSO) to dissolve brucine powder to prepare a mother liquor of 150mmol/L, and store at -20°C for later use (protect from light). Dilute with RPMI-1640 culture solution to the required concentration before the experiment.

Isolation and culture of chondrocytes:

SD rats of 3-4 days old were put to death by necking and soaked in alcohol for 4-5 minutes. Under aseptic conditions, cartilage was cut from the distal femur, proximal tibia and hip joint surface, and cut into particles of relatively uniform size. About 1mm. Rinse 3 times with Hank's solution in the small flask, transfer the cartilage particles into the digestion vial, add 6ml 0.25% trypsin, keep in a constant temperature water bath at 37°C for 30 minutes, centrifuge (1200r/min) for 5 minutes, and then add 0.1 prepared with DMEM Put 6ml of %II collagenase in a digestion flask and bath in water for 60 minutes. The chondrocyte suspension obtained by digestion was gently pipetted, collected in a centrifuge tube, centrifuged at 1500r for 5 minutes, the supernatant was discarded, and washed 3 times with DMEM.

Finally, chondrocyte culture medium (low-sugar DMEM containing 100U/ml penicillin, 100U/ml Streptomycin) was made into a chondrocyte suspension. Chondrocytes were inoculated into the culture flask at 2.5×106 cells/bottle, and the culture flask was placed in a CO2 incubator. The culture conditions are 37°C, 5% CO2 concentration and saturated humidity. After 24 hours of inoculation, the culture medium was replaced every day. Observe the growth of the cells with an inverted microscope and take pictures. The primary cells form a monolayer. After observing about 80% of the cells fused under a microscope, they are passaged and digested with 0.25% trypsin. The cells are collected to prepare a cell suspension and seeded in a culture flask at a density of 2×105.

Isolation, culture and passage of chondrocytes

SPF SD rats were sacrificed by intraperitoneal injection anesthesia, and the knee joints were cut out, placed in sterile alcohol for 10 minutes, and the articular cartilage of the femoral condyle and tibial plateau was scraped off on a clean bench, washed with PBS for 3 times, and cleaned with a clean scalpel blade. Cut into small pieces of about 1 mm, add 4 mL of 0.2% n-collagenase with a mass concentration of 0.2%, and place it in a C01 incubator at 37°C for digestion. Collect the supernatant every 2 hours, discard the supernatant, and add 10% FBS and DMEM. Resuspend the cell culture medium in LOW medium and transfer it to a culture flask. Repeat 4-5 times until the tissue mass is completely digested. At this time, the cells are marked as primary chondrocytes, and the medium is replaced every two days. When the cells grow to about 80-90% of the bottom area of the culture flask, wash 3 times with PBS, discard the PBS, add 500% trypsin containing 0.25% EDTA, place in a CO2 incubator at 37°C for 2 minutes, inverted under a phase contrast microscope When observing that the cell morphology becomes round and slightly floating, immediately add 4 mL of cell culture medium containing 10% FBS and DMEM-LOW medium to terminate the digestion, and pipette gently and repeatedly to blow the chondrocytes at the bottom of the culture flask until they are almost suspended, 1000r/ Centrifuge for 5 minutes for 5 minutes, discard the supernatant, add cell culture medium containing 10% FBS, DMEM-LOW medium to resuspend, pipette evenly, and inoculate them in two new culture flasks. At this time, the cells are the first generation chondrocytes. .

Identification of primary chondrocytes

The chondrocytes were inoculated on the treated coverslip, the cell clone slide was fixed with 4% polyaldehyde for 15 minutes, and freshly prepared 0.5% H2O2 formaldehyde was treated for 30 minutes to inactivate endogenous peroxidase for detection. The positive control was articular cartilage tissue, and the negative control was replaced by PBS.

Add normal goat serum blocking solution dropwise to the chondrocyte climbing sheet, at room temperature for 20 minutes, shake off the excess liquid without washing;

Add rabbit anti-human type II collagen clone antibody dropwise, in a 37°C incubator for 20 minutes, wash with PBS for 2 minutes × 3 times;

Add biochemical goat anti-mouse I antibody dropwise, 37°C incubator for 20 minutes, wash with PBS for 2 minutes × 3 times;

Add SABC dropwise, 37°C thermostat for 20 minutes, wash with PBS for 2 minutes × 4 times;

DAB color development, hematoxylin counterstaining, full washing;

Alcohol dehydration, transparent xylene, mountable.

Toluidine blue staining identification

Inoculate the second-generation chondrocytes in a 6-well plate containing cell slides at an appropriate concentration. After the cells adhere to the wall, add 2 mL of 10% FBS and DMEM-LOW culture medium for culture. After 72 hours, the culture medium was discarded, washed with PBS 3 times, fixed with ice methanol for 30 min, washed 3 times with PBS, stained with 0.5% toluidine blue for 30 min, rinsed quickly with anhydrous alcohol, dried, mounted, and observed under an optical microscope.

Cultivation of chondrocytes in vitro and establishment of OA model

Articular cartilage is composed of chondrocytes and extracellular matrix. As the only type of cell in

articular cartilage, chondrocytes, their structure and function changes are an important cause of OA. Traditional chondrocyte separation and acquisition mainly use the method of combining pancreatin with n-type collagenase, but pancreatin has certain damage to the cells, and the dosage and time are not easy to control.

In the experiment, the mechanical-n-type collagenase digestion method can not only efficiently separate and obtain chondrocytes in large numbers and with high purity. On the other hand, the successful establishment of an in vitro OA chondrocyte model is the prerequisite for this experiment. LPS is often used as an inducer of in vitro articular chondrocyte OA model. Mainly because LPS can promote the expression of inflammatory cytokines such as 1L-1P and TNF-a in chondrocytes. And 1L-1P and TNF-a play an important role in the occurrence and development of OA. Both 1L-1P and TNF-a can promote the expression of MMPs. MMPs are the main enzymes that cause matrix degradation, and MMP=3, 9, 13 are the most typical ones.

Establish an apoptosis model

Inoculate the third-generation cells in a six-well culture plate and a culture flask with a built-in glass slide. After the cells adhere to 80% and fuse, add 2mmol/LS-nitroso-N-acetylpenicillamine to the culture system. S-nitroso-N-acetylpenicillamine, SNP), after 20 hours of incubation, the cells were collected for detection of apoptosis rate and apoptosis-related proteins. In the chondrocyte apoptosis system induced by the addition of 2mmol/LSNP, the high-dose (500mg/L), medium-dose (250mg/L), and low-dose (125mg/L) of strychnine were added to incubate for 20 hours, each group had 3 Hole, collect cells, detect changes in apoptosis rate and apoptosis-related proteins.

Analyze the flow cytometer

Apoptosis detection adopts AnnexinV early detection method for apoptosis. Digest cells with 0.25% trypsin respectively, centrifuge at 1000r/min×5 minutes, discard the supernatant, wash the cells twice with PBS, centrifuge at 1000r/min×5 minutes, and use 100μl binding buffer Suspend in the liquid, add 5μl AnnexinV-FITC and 10μ PI, mix and incubate for 15 minutes at room temperature and dark in the dark. After incubation, 400ulPBS was added and analyzed immediately on the flow cytometer.

CytC detection by immunohistocytochemistry

After the cells in each group were cultured and treated with drugs, the culture medium was aspirated and washed once with 0.1% mol/L PBS. The cells were fixed with 4% paraformaldehyde for 30 minutes, and the paraformaldehyde fixative was replaced at the 15th minute. Wash with PBS three times for 2 minutes each time. Add PBS containing 0.1% TritonX-100 for 10 minutes to increase membrane permeability.

The PBS was aspirated and treated with PBS containing 4% H2O2 for 15 minutes to block the endogenous peroxidase. Wash with PBS three times for 5 minutes each time. Add non-immune serum to block for 10 minutes. Add anti-cytochrome C antibody in 0.1 mol/L PBS containing 0.1% Triton-X-100 and incubate at 37°C for 1 hour. Wash with PBS 5 times, 5 minutes each time. Add the substrate AEC and incubate for 2 minutes. Wash twice with double distilled water for 2 minutes each time. GVA water-based mounting tablets were mounted, inspected under microscope, and photographed and recorded. Choose more than 5 high power fields (400 times) and count no less than 500 chondrocytes for scoring.

Result criteria: positive cells are brown-yellow in cytoplasm, and they are divided into four levels according to the percentage of positive cells: 0 is no positive cells, and 0 is scored; 1 is that the number of positive cells accounts for less than 25% of the total number of cells in the visual field, and 1 is scored ; Level 2 means that the number of positive cells accounts for 26%-50% of the total number of cells in the visual field, which counts 2 points; grade 3 means that the number of positive cells accounts for >50% of the total visual field cells and counts 3 points.

Western blotting to determine the expression of Smac protein

Lyse the cells with cell lysate (for cytoplasmic protein extraction) and homogenize the lysed cells with a Dounce homogenizer. Centrifuge the cell

lysate at 4°C and 12000r for 5 minutes to precipitate the nucleus, and then centrifuge at 4°C and 14000r for 5 minutes. Separate the supernatant, determine the total protein concentration of the sample with BCA analysis reagent, and store it in aliquots at -80°C for later use. Take 100μg of total protein from each group and separate with sodium dodecyl sulfonate (SDS)-polyacrylamide gel electrophoresis, electrotransfer to NC membrane, and incubate the anti-mouse Smac antibody (1:500-800) with the membrane for 1 hour , Wash the membrane with PBS-T for 20 minutes × 5 times. The corresponding secondary antibody labeled with HRP was incubated with the membrane for 1 hour, and the membrane was washed with PBS-T for 15 minutes × 4 times. Use ECL kit to display protein bands. X-ray film exposure in a dark room, conventional development and fixation, image grayscale scanning analysis. Actin protein was used as sample loading control[21].

Statistical Processing

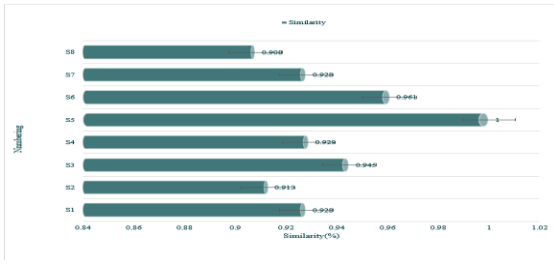
The SPSS statistical software package was used to analyze and compare the apoptosis rate of chondrocytes and the number of CytC-positive cells between different groups by T test. All data are expressed as $\bar{x}\pm s$, and the comparison of the mean of the data between each group adopts the variance test. Take $\alpha=0.05$ as the significance test level.

4. Data Analysis of the Impact of Strychnoma on the Apoptosis Model of Chondrocytes

Study on the HPLC Characteristic Spectrum of Brucine

The 8 batches of the total alkaloids of Strychnoma chinensis in CDF format were imported into the "Chinese Medicine Chromatographic Fingerprint Similarity Evaluation System" software for similarity evaluation. The specific results are shown in Figure 1.

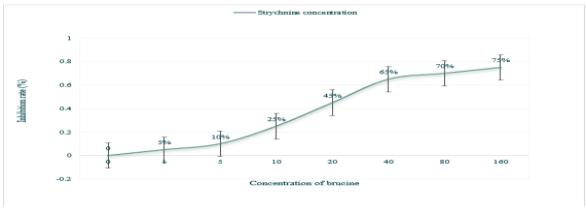
Figure 1.
Similarity of the HPLC feature maps of brucine



It can be clearly seen from figure 1 that the similarity of the total alkaloids of the 8 batches of Strychoma serrata is greater than 0.9, indicating that the similarity is good, and the characteristic map of the total alkaloids of Strychtenspp can be used for the chemical composition of the alkaloids. Analysis and quality rating and control.

Effect of Different Concentrations of Brucine on the Proliferation of Huvecs

Figure 2.
CCK-8 method to detect the effects of strychnine on HUVECs proliferation



As shown in picture 2. Different concentrations of strychnine can inhibit the growth of HUVECs. As the dose of strychnine (1, 5, 10, 20, 40, 80, 160 μmol/L) increases, it has a significant inhibitory effect on the proliferation of HUVECs Calculated with the inhibition rate of the blank control group being 0, with the increase of the concentration of strychnine in each treatment group, the proliferation inhibition effect gradually increased ($P<0.05$, $P<0.01$), and the IC50 of the 90% confidence interval was (29.2 ±3.5) μmol/L.

Effect of Brucine on the Apoptosis Rate of Three Kinds of Chondrocytes

It can be seen from table 1 that brucine has a significant pro-apoptotic effect on the three types

of chondrocytes. As the concentration increases, the cell apoptosis rate also shows an increasing trend. Compared with the blank group, the early apoptosis rate, late apoptosis rate and total apoptosis rate of each group were significantly different (**p<0.01).

Table 1.
The effect of brucine on the apoptosis rate of chondrocytes ($\bar{x}$ ±s)

Group	Early apoptosis rate%	Late apoptosis rate%	Total apoptosis rate%
Blank control group	1.43±0.50	1.61±0.60	2.71±0.74
125	18.59±1.53**	15.54±1.77**	33.06±0.76**
250	24.94±1.71**	19.45±0.41**	43.47±2.09**
500	26.31±5.90**	47.65±3.21**	70.96±2.94**

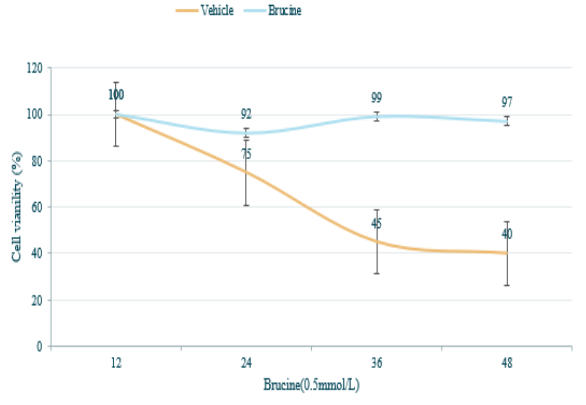
Note: Compared with the blank group, ** p <0.01

Brucine Inhibits Cell Survival in a Time and Dose-Dependent Manner

In this paper, the tetramethylazolium salt colorimetric method was used to observe the cell survival method to observe the cytotoxicity of brucine to A549. It can be seen from figure 3 that the survival inhibitor of A549 at 0.5 mmol/L of brucine reached 45% (**P<0.01). The same dose of strychnine has little toxicity to MDCK cells, showing that strychnine can selectively inhibit the survival of A549 cells.

Effect of Strychnine at Different Times on Cell Viabilit

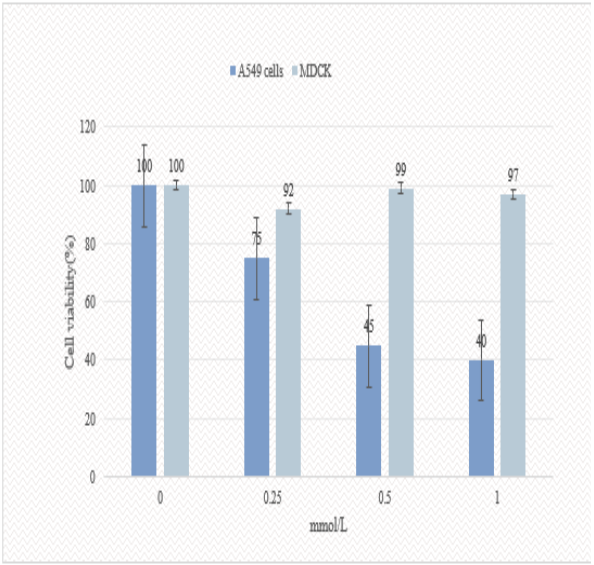
Figure 4.
Effect of Strychnine with Different Treatment Time on the Survival Rate of A594 Cells



In order to study the time-effect relationship of brucine to A549 cytotoxicity, the mesocells were treated with 0.5 mmol/L brucine for 12, 24, 36, 48h. It can be seen from Figure 4 that after A549 received different doses of brucine for 36 hours, the cell survival rate decreased in a dose-dependent manner. This shows that strychnine can also reduce the survival rate of chondrocytes in a time-dependent manner. The survival rates of the cells after administration are 100%, 92% (**P<0.01), 99% ((**P<0.01), 97% (***P<0.001)). Brucine has obvious cell killing effect on non-small cell lung cancer cell line A549.

Brucine Inhibits Cyclooxygenase 2 Mrna and Protein Expression Levels in A549 Cells

Figure 3.
The effect of different concentrations of brucine on the survival rate of A594 cells



In order to study the mechanism of brucine-induced apoptosis of A549 tumor cells, this article investigated the effect of brucine on cyclooxygenase 2, because more and more studies have shown that cyclooxygenase 2 is involved in the pathogenesis of non-small cell lung cancer. It can be seen from Table 2 that brucine can reduce the expression of cyclooxygenase 2 mRNA and protein in A549 cells in a dose-dependent manner, which is consistent with the above-mentioned brucine-induced apoptosis of A549 cells. At the same time, it was found by ELISA that brucine can inhibit the PGE_2 content in A549 cells in a dose-dependent manner.

Table 2.
Brucine inhibits the release of cyclooxygenase 2mRNA and PGE_2 in cells

Time（h）	mRNA	PGE_2
0	100	100
0.25	80	92
0.5	50	99
1	25	97

Effect of Brucine on Chondrocyte Mitochondrial Membrane Potential

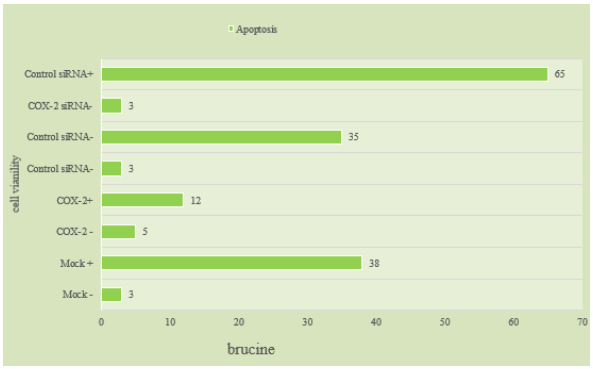
It can be seen from Table 3 that compared with the blank group, the changes in mitochondrial membrane potential of each group are statistically significant (**P<0.01). As the concentration of brucine increases, the mitochondrial membrane potential of chondrocytes gradually decreases.

Table 3.
The effect of brucine on chondrocyte mitochondrial membrane potential

Group	UR (%)	UL（%）	UL/UR
Blank control group	4.39±0.83	95.56±0.83	22.20±4.66
125μmol-L^{-1}Brucine	35.91±1.91	64.08±1.88**	1.79±0.15**
250μmol-L^{-1}Brucine	44.50±2.05	55.61±2.01**	1.24±0.10**
500μmol-L^{-1}Brucine	65.05±2.23**	34.87±2.16**	0.54±0.05**

Cyclooxygenase 2 Specifically Involved in Brucine-Induced Apoptosis of Three Kinds of Chondrocytes

Figure 5.
The effect of overexpression of cyclooxygenase 2 and interference with intracellular cyclooxygenase 2 expression on the apoptosis of A549 cells induced by brucine`



In this paper, a eukaryotic expression plasmid of cyclooxygenase 2 (pRK5-cyclooxygenase 2) was constructed, and cyclooxygenase 2 and control plasmids were overexpressed in three kinds of chondrocytes by transfection reagent. The purpose is to study cyclooxygenase. 2 In the molecular mechanism of brucine-induced apoptosis of A549 cells, the experimental results are shown in figure 5.

As shown in figure 5, the overexpression of cyclooxygenase 2 can significantly inhibit the chondrocyte apoptosis induced by brucine, but the inhibitory effect does not seem to be complete due to the problem of transfection efficiency. At the same time, we designed and synthesized cyclooxygenase 2 specific interference siRNA, and transfected it into three kinds of chondrocytes, so that the expression of cyclooxygenase 2 was insufficient. The results showed that compared with the control siRNA, because of the low expression level of cyclooxygenase 2 in the interfered chondrocytes, brucine can significantly enhance the induced apoptosis of the three chondrocytes. All these results indicate that cyclooxygenase 2 is the target protein for brucine to induce apoptosis of three chondrocytes.

CONCLUSION

SNP induces the apoptosis of chondrocytes in SD rats, and brucine can significantly reduce the apoptotic rate, and with the increase of the dose of brucine, the apoptotic rate gradually decreases, especially the early apoptotic rate; CytC and Smac It is basically not expressed in the plasma of normal chondrocytes. When apoptosis occurs, the expression increases significantly. While brucine inhibits apoptosis, as the dose increases, it gradually blocks its release from mitochondria. This article analyzes the effects of Strychnoides on the apoptosis and proliferation of three kinds of chondrocytes. Experimental studies have shown that after the treatment of three chondrocyte apoptosis models with brucine, it is found that the apoptotic rate of the models has been significantly reduced. This trend is intensified with the increase in the measurement of brucine. It can be seen that the use of large doses of strychnine can have a better protective effect on human bones and joints. To sum up, in the clinical treatment of patients with osteoarthritis, the use of strychnine can achieve safe and effective therapeutic effects, can effectively inhibit cell apoptosis and promote cell proliferation, and has high use value.

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