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Review article

Effects of Tongluo Zhitong formula on synovial fibroblast proliferation in human knee osteoarthritis☆,☆☆

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ABSTRACT

Objective: We aimed to investigate the effects of Tongluo Zhitong formula on synovial fibroblast proliferation in human knee osteoarthritis (KOA).
Methods: Discarded synovial tissue collected from patients undergoing total knee arthroplasty at our hospital was digested with type I collagenase. Primary culture was performed on three to four generations of fibroblasts, which were treated with high, medium, and low concentrations of Tongluo Zhitong formula. The KOA synoviocyte proliferation level was detected through applying the methyl-thiazol-tetrazolium (MTT) method, and cell morphology at each concentration was observed under inverted microscopy.
Results: Significant differences were observed between three concentration categories and a blank control ($p < 0.05$). MTT assay test results indicated that as the Tongluo Zhitong formula concentration decreased, the absorbance/optical density value of KOA synovial cells at 490 nm increased. As the concentration increased, a positive inhibition rate was observed in the high concentration category. As the concentration decreased, a negative inhibition rate was observed in the low concentration category. In phase S, the Tongluo Zhitong formula inhibited deoxyribonucleic acid replication, which became more pronounced at increasing concentrations. In the G2 phase, the proportion of cells in the mitotic preparation phase did not change in the low concentration category ($p > 0.05$). The proportion of cells in the mitotic preparation phase significantly increased in the high and medium concentration categories ($p < 0.05$).
Conclusion: High and medium concentrations of Tongluo Zhitong formula inhibited cell proliferation; therefore, the formula had an inhibitory effect on the cell cycle of synovial fibroblasts.

Knee osteoarthritis (KOA) is a common degenerative disease among older adults, which can seriously affect their quality of life. However, its etiology remains unclear, and efforts to prevent and treat KOA have had limited effect [1]. The synovial inflammatory response plays an important role in the progression of osteoarthritis (OA) [2]. Synovial cells are mainly divided into macrophage-like (type A cells), fibroblast-like (type B cells), and dendritic cells. The synovium is an important bridge connecting the internal structure of joints with musculoskeletal tissue. It secretes synovial fluid to reduce joint friction and cartilage loss, and is important in ensuring

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effective joint cartilage physiological function and nutritional supply. In the presence of OA lesions, pathological changes to the synovium directly affect subsequent OA progression. Healthy synovium has an inner layer (cell layer) and a lower layer (vascular layer). The inner layer lies close to the joint cavity and comprises 2–3 cell layers that include synovial macrophages (SM), fibroblast-like synoviocytes (FLSs), and synovial-derived mesenchymal stem cells (SDSCs). FLSs are a fibroblast subtype and comprise the main cellular components of joint synovium. They maintain cartilage structure stability through the synthesis of substances such as collagen, osteopontin, and hyaluronic acid. They also play an important role in the occurrence and development of synovitis, and are closely related to the degree of bone destruction and pain in patients with OA. As the major cells involved in mediating joint destruction, inhibiting the proliferation of fibroblasts can effectively alleviate the symptoms and progression of the osteoarthritic response. Using ultrasound-enhanced penetration, the use of Tongluo Zhitong formula to treat patients with KOA has achieved satisfactory efficacy [3,4]. This study aimed to clarify the effect of Tongluo Zhitong formula on the proliferation and cycle of synovial inflammatory cells in KOA using the methyl-thiazol-tetrazolium (MTT) method and flow cytometry (FCM) technology and explore its mechanism of action. The study was approved by the Ethics Committee of the Third Affiliated Hospital of Beijing University of Traditional Chinese Medicine (ID:BZYSY-2019KYKTPJ-26).

1. Materials and methods

1.1. Materials

1.1.1. Organization

Thirty patients with severe KOA (Kellgren Lawrence [KL] grade 4) who had undergone knee replacement surgery at our hospital were recruited from January 2022 to January 2024. Discarded synovial tissue was collected for cultivation during surgery. The synovial tissue was prepared by removing fat and fibers under aseptic conditions, washed twice with phosphate-buffered saline (PBS), placed in a sterile small penicillin vial, and crushed into a paste using anatomical shearing. Digestion was performed in 2 mL of 0.4 % type I collagenase in 25-cm^2 flasks for 2.5 h, which were shaken several times during this period. The tissue was then filtered through a 200-mesh metal sieve. Unadhered cells were centrifuged at 2000 rpm for 5 min, and the supernatant was discarded. A Roswell Park Memorial Institute (RPMI)-1640 medium containing 10 % fetal bovine serum was added, and the cells were inoculated in 25-cm^2 culture flasks after repeated blowing. Cells were incubated in 5 % CO_2 at 37 °C, and the medium was changed every one to two days. Three to four generations of cells with a purity >98 % were selected and used for further experiments after identification.

1.1.2. Reagents and instruments

Fetal bovine serum was purchased from Sijiqing Company. The RPMI-1640 medium, trypsin-ethylenediaminetetraacetic acid digestion solution (0.25 %), and type I collagenase were all purchased from Gibco. The MTT (thiazolium blue) and dimethyl sulfoxide (DMSO) were purchased from Sigma Corporation. The ELX800 enzyme-linked immunosorbent assay (ELISA) was purchased from Biotech Instruments, United States of America (USA), and the BH-2 inverted microscope was obtained from Olympus, Japan. The 96-well cell culture plate was purchased from Corning, USA. The FCM system was purchased from FACSCanto™ II, BD, and the vortex mixer (Vortex 5) from Zillingberg. The carbon dioxide (CO_2) incubator, 25-cm^2 culture bottles, and Sihuan freeze-drying machine were purchased from Thermo Scientific USA, NUCU Denmark, and Beijing Sihuan Scientific Instrument Factory Co., Ltd, respectively.

1.1.3. Drug preparation

Tongluo Zhitong formula, consisting of peach kernel, safflower, cinnamon stick, white peony, prepared grass crow, fine acrid, Sichuan pepper, hyssop, frankincense, and myrrh, was purchased from our hospital pharmacy. These ingredients were placed in a decoction container according to their proportion within the Tongluo Zhitong formula, soaked in water, and boiled for 30 min. The residue of the ingredients was discarded, and the remaining liquid was extracted, centrifuged at 2000 rpm, and then freeze-dried. The resulting product was powdered, dissolved in PBS, and filtered through a 0.22-μm micropore filter to remove bacteria. The product was formulated into high, medium, and low concentration categories. The high concentration category was arranged as follows: $10/4^2$, $10/4^3$, and $10/4^4$ mg/mL; the medium concentration category was $10/4^5$, $10/4^6$, and $10/4^7$ mg/mL; and the low concentration category was $10/4^8$, $10/4^9$, and $10/4^{10}$ mg/mL. A blank control with a concentration of 0 mg/mL was also set up. The formula was stored at −20 °C for future use.

1.2. Methods

1.2.1. Proliferative activity of KOA synoviocytes using an MTT assay

Fibroblasts with good growth conditions were obtained, digested with 0.25 % trypsin, and resuspended in RPMI-1640 culture medium containing 10 % fetal bovine serum under aseptic conditions to create synovial cell suspensions. The suspensions were inoculated in 96-well culture plates at 100 μL/well, with approximately 1000 cells per well. The side wells around each 96-well plate were filled with PBS to avoid edge effects. The cells were incubated in 5 % CO_2 at 37 °C for 24 h, allowing cells to adhere to the wall and enter the logarithmic growth phase.

The supernatant was discarded, and the configured different concentrations of the Tongluo Zhitong formula at 100 μL/well were added, with four duplicate wells for each concentration. This was incubated in 5 % CO_2 at 36 °C for 24 h.

After incubation, the drug was aspirated, discarded from each well, and washed three times with PBS. A 10 μL/well was replaced with a 5 mg/mL MTT-PBS solution and 100 μL of serum-free RPMI-1640 medium, and incubation was continued for 4–6 h. The culture

was then terminated. The supernatant was discarded and shaken with DMSO 100 mL/well for 10 min, and the absorbance optical density (OD) (A) at 490 nm was measured using an ELX800 enzyme labeler. The A-value was used to calculate the cell inhibition rate after drug intervention at each concentration according to the following equation:

$$\text{Inhibition rate}\ (\%) = \left(A_{\text{blank}} - A_{\text{drug solution}}\right) / A_{\text{blank}} \times 100\% \quad (1)$$

A positive inhibition rate indicated that the drug had an inhibitory effect on cell proliferation, whereas a negative inhibition rate indicated that the drug promoted cell proliferation.

1.2.2. FCM detection of the KOA synovial cell cycle

The cells from each concentration category were collected, washed, fixed with paraformaldehyde, preserved with ice ethanol as a medium, and washed before testing. Subsequently, propidium iodide/RNase staining solution was added according to the reagent instructions. The mixture was vortexed several times, briefly shaken, and incubated to allow RNA digestion and DNA staining. After approximately 15 min, the samples were ready for detection using FCM.

1.2.3. Morphological observations

The cell growth characteristics and morphology at different concentrations in each category were observed under inverted microscopy.

1.3. Statistical methods

The FCM data for synovial cell cycle detection were processed using the ModFit LT V3.3.11 software. SPSS17.0 software was applied for statistical processing, and a one-way analysis of variance was performed to analyze differences. Statistical significance was set at $p < 0.05$. Measurements are expressed as means ± standard deviations ($x \pm s$).

2. Results

2.1. Microscopic observation of synovial cell growth

After 4 h of primary culture, the cells adhered to the wall (Fig. 1) in the form of a stellate shape, and adhered to the culture bottle through 3–4 dendrites. The organelles were not evident, and the cell density was sparse. As the culture progressed to 3–4 generations of cells (Fig. 2), synovial fibroblasts appeared spindle-shaped and dendritic-like in shape, with a narrow and elongated nucleus, vigorous proliferation, and visible organelles. A low concentration of Tongluo Zhitong formula promoted the growth of human KOA synovial cells, substantially increasing cell numbers. A high concentration of the formula inhibited cell growth, with a small number of cells floating, tightly arranged, and irregularly shaped, and with their appearance mostly shrinking from being spindle-shaped to circular.

2.2. The MTT method for determining the proliferative activity of KOA synoviocytes in different concentration categories

The proliferative activity of KOA synoviocytes differed significantly between the concentration categories and the blank control ($p < 0.05$). The MTT assay showed that as the concentration of Tongluo Zhitong formula decreased, the absorbance OD values of KOA synovial cells at 490 nm increased (Table 1). High and medium concentrations of the Tongluo Zhitong formula inhibited the proliferation of KOA synovial cells. With increasing concentration, the inhibitory effect was enhanced, and the inhibition rate at high concentrations was significantly higher than that at medium concentrations. Furthermore, at high concentrations, the rate of inhibition accelerated with increasing concentration, whereas the Tongluo Zhitong formula promoted the proliferation of synovial cells in the

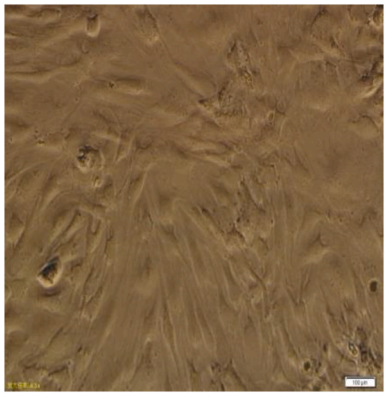

Fig. 1. Primary culture of 4-h adherent KOA synovial cells.

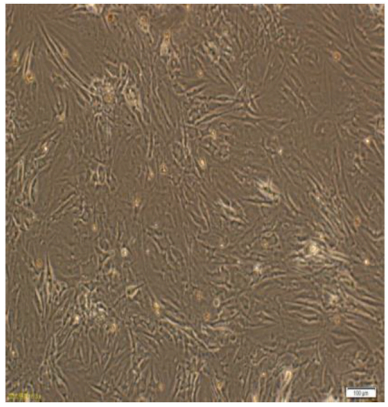

Fig. 2. KOA synovial membrane 3 to 4 generations of cellular cells.

Table 1
OD values of Tongluo Zhitong Formula intervention on the proliferation of KOA synovial cells.

grouping	Dosage of Tongluo Zhitong Formula(mg/mL)	OD value
	$10/4^2$	1.407 ± 0.042*
High concentration group	$10/4^3$	1.466 ± 0.032*
	$10/4^4$	1.623 ± 0.038*
	$10/4^5$	1.888 ± 0.070*
Medium concentration group	$10/4^6$	2.076 ± 0.059*
	$10/4^7$	2.412 ± 0.081*
	$10/4^8$	2.784 ± 0.075*
Low concentration group	$10/4^9$	3.005 ± 0.096*
	$10/4^{10}$	3.059 ± 0.114*
Blank group	0	2.656 ± 0.075

Note: * Compared to the blank group, $p < 0.05$.

low concentration category. As the concentration decreased, the promotion effect increased. At low concentrations, the enhancement amplitude of the promotion effect decreased (Table 2 and Fig. 3).

2.3. FCM determination of cell cycle of KOA synovium in different concentration categories

Data analysis and cell cycle distribution (Fig. 4) revealed that in the S phase, the Tongluo Zhitong formula inhibited DNA replication, and the inhibition became more pronounced with increasing concentration. In the G2 phase, the proportion of cells in the mitotic preparation phase did not change significantly in the low concentration category ($p > 0.05$). The high and medium concentration categories demonstrated a significantly increased proportion of cells in the mitotic preparation phase ($p < 0.05$) (Table 3). Therefore, the Tongluo Zhitong formula had a certain inhibitory effect on the synovial fibroblast cell cycle.

3. Discussion

KOA is a multifactorial chronic disease characterized by secondary osteophytic formation [1,2], and is the most common form of arthritis. Its prevalence is approximately 30.8 million among adults in China and 300 million worldwide [3,4]. It is the main cause of

Table 2
Inhibition rate of KOA synovial cell proliferation by Tongluo Zhitong Formula at different concentrations (%).

grouping	Dosage of Tongluo Zhitong Formula(mg/mL)	Inhibition rate
	$10/4^2$	47.02 %
High concentration group	$10/4^3$	44.82 %
	$10/4^4$	38.88 %
	$10/4^5$	28.92 %
Medium concentration group	$10/4^6$	21.82 %
	$10/4^7$	9.19 %
	$10/4^8$	−4.82 %
Low concentration group	$10/4^9$	−13.15 %
	$10/4^{10}$	−15.16 %
Blank group	0	–

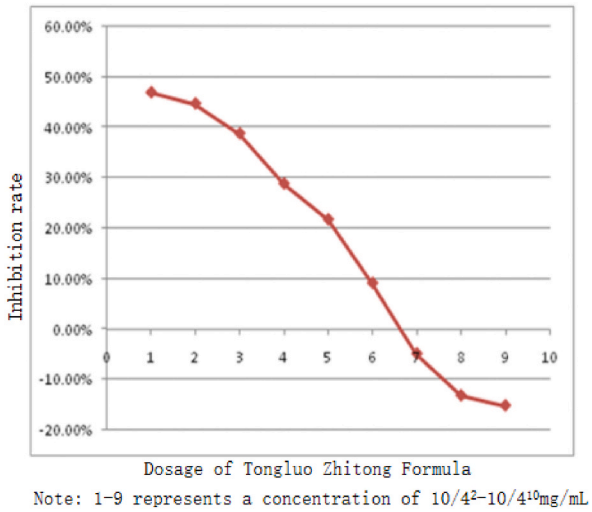

Fig. 3. Inhibition rate of KOA synovial cell proliferation by different concentrations of Tongluo Zhitong Formula (%).

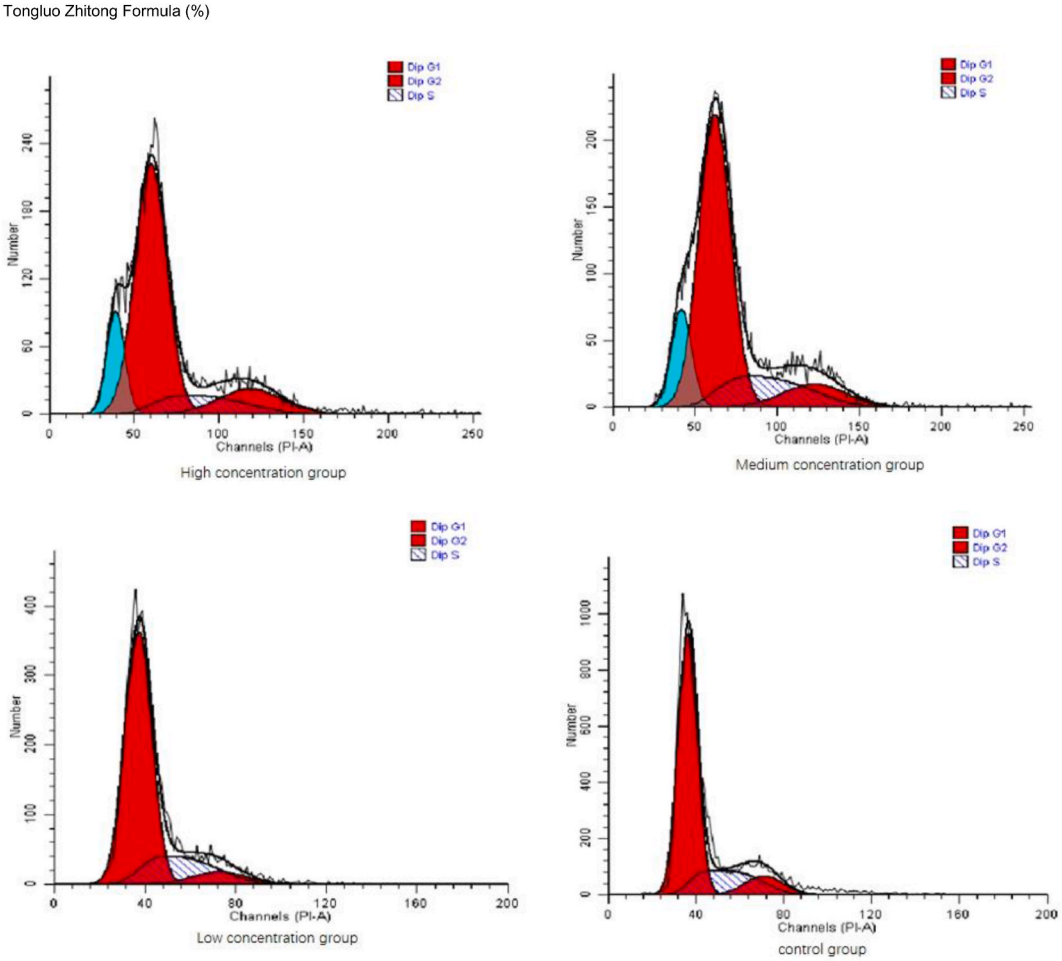

Fig. 4. Effect of different concentrations of Tongluo Zhitong Formula on the synovial cell cycle of KOA.

Table 3
Effect of Tongluo Zhitong formula on synovial fibroblast cell cycle (%).

grouping	G1	G2	S
High concentration group	72.24 ± 5.25	14.09 ± 1.95*	13.66 ± 3.31*
Medium concentrationgroup	70.40 ± 4.32	10.67 ± 1.38*	18.94 ± 2.97*
Low concentration group	72.91 ± 4.12	6.60 ± 1.11△	20.48 ± 2.33*
Blank group	69.60 ± 6.01	9.55 ± 2.77	20.85 ± 2.67

Note: * Compared with the control group, $p < 0.05$, △ Compared with the control group, $p > 0.05$.

disability in older adults, leading to pain, functional loss, and decreased quality of life. Currently, no treatment is available that can fundamentally prevent, alter, or stop KOA disease progression [5]. Ultimately, most patients with advanced KOA require knee replacement. However, many patients with KOA are unable to undergo knee replacement surgery owing to their physical conditions and limited financial capacity. Therefore, analyzing the pathogenesis of KOA and identifying effective therapeutic drugs and methods for preventing and treating KOA are urgently needed to enhance health, improve patient quality of life, and reduce the medical burden. KOA is generally considered a degenerative disease; however, the important role of synovitis in KOA pathology and clinical practice is gradually being recognized [6]. The etiology of OA mainly involves articular cartilage destruction, leading to changes in the biological characteristics of chondrocytes; and the synovial inflammatory response, causing synovial cells to release inflammatory mediators. Previous studies have indicated that in OA, pathogenic factors are involved in the destruction of joint cartilage, releasing fragments into the synovial fluid, stimulating the synovial membrane, and causing synovitis. Synovitis releases inflammatory mediators that further degrade the cartilage, resulting in a vicious cycle. Therefore, the presence of synovitis is an important cause of OA cartilage damage and chronic persistence of OA. KOA-related pain is related to the secretion of inflammatory mediators into the knee joint. These inflammatory mediators are closely related to the abnormal proliferation of synovial cells, inducing pain sensitization and stimulating the sensory nerves and surrounding tissues of the affected cartilage to produce inflammatory reactions, leading to pain [7]. Simultaneously, the abnormally proliferating inflammatory synovium stimulates sensory nerve sensitivity in the knee joint, increasing pain sensation. As the KL grade increases, inflammatory mediator expression in the joint cavity is more apparent, showing a positive proportional relationship [8]. Therefore, synovial fibroblast proliferation can reflect KOA severity and the degree of pain in patients, providing a guiding role in KOA treatment. In clinical practice, the Tongluo Zhitong formula concentration can be adjusted to adapt to treatment in line with different severity levels of KOA.

Although KOA is generally considered a degenerative disease, the important role of synovitis in KOA pathology and clinical practice has gradually been recognized. A significant inflammatory response is now known to occur in the disease progression of many patients with KOA, and the joint synovium exhibits non-infectious chronic inflammation characteristics. During the progression of KOA, the affected synovium exhibits different histological changes, ranging from an almost healthy joint synovium, with only slight thickening of the synovial tissue and proliferation of the synovial layer, to significant thickening of the synovial layer, formation of villous structures, neovascularization, and inflammatory changes. Several studies have reported that the progression of synovitis detected through arthroscopy, magnetic resonance imaging, and ultrasonography can indicate the severity of KOA lesions and is correlated with KOA imaging grading. However, the causes of KOA synovitis remain controversial. The more recognized mechanism involves activation of the KOA synovial inflammatory response, which is triggered by the intrinsic synovial immune response. Specifically, once the articular cartilage degenerates and the extracellular matrix is destroyed, debris shed into the joint comes into contact with the synovium, activating the intrinsic synovial immune response, after which synovial cells produce inflammatory mediators that are released into the synovial fluid. These mediators activate chondrocytes on the articular cartilage surface, leading to metalloproteinase synthesis and promoting cartilage degradation. Simultaneously, these mediators induce synovial angiogenesis and increase the synthesis of synovial cell auto-inflammatory factors and matrix metalloproteinases, which further destabilize intra-articular homeostasis and form a vicious cycle that continues to aggravate articular cartilage destruction. Therefore, identifying effective interventional drugs for synovitis is important for developing treatments and delaying KOA progression.

In cellular-level research, understanding the survival, cycle, and proliferation of cells is crucial. Currently, the main methods of detection include isotope doping and live cell counting. Isotope doping utilizes radioisotopes, but the relevant equipment are expensive and poses risks in terms of contamination. The results of live cell counting are readily influenced by subjective factors, involve cumbersome operations, and are prone to large errors. Alternatively, the MTT assay is based on the principle that the enzyme succinate dehydrogenase in mitochondria of living cells reduces exogenous MTT to water-insoluble blue-violet crystalline formazan, which deposits in the cells. Dead cells do not perform this function. The number of live cells can be indirectly reflected using DMSO to dissolve methylene chloride and then measuring its light absorption value at a specific wavelength using an ELISA reader. This method has high sensitivity and is economical. Moreover, it is widely used in the activity detection of bioactive factors, screening of antitumor drugs, cytotoxicity testing, and determination of tumor radiosensitivity. Regarding the experimental operation, the MTT method includes cell inoculation, addition of MTT solution, control of reaction time (generally 3–4 h), addition of DMSO to dissolve the formazan crystals, and measurement of the light absorption value at a specific wavelength (generally 490 nm) using an ELISA reader. During the experiment, the cell seeding density, amount of DMSO added, and selection of the detection wavelength should be monitored to ensure the accuracy of the experimental results. In addition, the MTT assay not only includes succinate dehydrogenase but also other substances, such as nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, which can also participate in the reduction process of MTT. It is widely used for its simplicity, low cost, and safety.

The MTT assay findings showed that as the Tongluo Zhitong formula concentration decreased, the absorbance OD value of KOA

synovial cells at 490 nm increased. The inhibition rate of the high concentration category was positive and increased with increasing concentrations. Meanwhile, the inhibition rate of the low concentration category was negative and decreased with increasing concentrations.

The FCM is a biological technique used for counting and sorting single cells or other biological particles in suspension by detecting labeled fluorescence signals. It enables the continuous analysis of multiple cell parameters passing through optical or electronic detectors, and is a high-speed and sequential quantitative analysis. Specifically, it can be used to quantitatively measure many important parameters such as cell DNA content, cell volume, protein content, enzyme activity, cell membrane receptors, and surface antigens through quickly measuring Kurt resistance, fluorescence, light scattering, and light absorption. Cells with different properties are separated based on these parameters to obtain pure cell populations for biological and medical research.

FCM can be used to analyze cells in the G1, G2, and S phases of the cell cycle, among which the G1 and S phases are important and highly representative. The S phase is the DNA synthesis phase, and the G2 phase, also known as the pre-DNA synthesis phase, is mainly responsible for the synthesis of RNA and proteins, and for generating material and energy reserves for the S phase. The amount of DNA is initially recorded as N, and as the number of actively dividing cells enter the S phase, this changes to 2N. If some cells do not enter during the S phase, they can enter the S phase after activation, while cells that are not activated or inhibited remain in the G2 phase to undergo senescence and apoptosis.

The Tongluo Zhitong formula was developed as a result of years of experience in treating KOA. The formula comprises peach kernel, safflower, cassia twig, Radix Paeoniae Alba, Aconiti Kusnezoffii Radix, *Asarum*, Sichuan pepper, *Achyranthes bidentata*, frankincense, and myrrh (patent application publication number: ZL201610903290. X). It mainly works to promote blood circulation, remove blood stasis, unblock collateral circulation, and relieve pain. Radix Paeoniae Alba and cassia twig are key medicinal compounds for unblocking collateral circulation and relieving pain. Cassia twig is sweet and warm and can unblock meridians, thereby assisting Yang and transforming qi. Radix Paeoniae Alba, which is bitter, sour, and slightly cold, can nourish the blood, restrain yin, soften the liver, and relieve pain. The two in combination can be used to relieve and disperse pain, replenish and open circulation, relieve muscle tension, induce sweating without damaging yin, and regulate qi and blood without damaging qi. This combination can also be used to treat limb soreness, numbness, and pain caused by qi and blood imbalance. Peach kernel, safflower, frankincense, and myrrh are used as medicinal herbs, which can promote blood circulation, remove blood stasis, promote qi circulation, relieve pain, and relax tendons and enhance collateral circulation. Safflower and peach kernel are commonly used medicinal pairs for promoting blood circulation and removing blood stasis. Safflower, which is warm and pungent, mainly enters the heart and liver meridians to disperse blood stasis and relieve pain. A previous study on the medical application of the combination of frankincense and myrrh indicated that such a combination promotes blood circulation in the organs and meridians [9]. This combination can also be used treat wind-cold-dampness, numbness, limb paralysis, and all sores. The adjuvants used in this formula include Aconiti Kusnezoffii Radix and *Asarum*, which can dispel wind and dampness, warm meridians and disperse cold, thus reducing swelling and pain. Sichuan pepper is another medicinal herb that can promote blood circulation, remove blood stasis, and dispel wind and dampness. *A. bidentata* has a good descending nature and meridian-guiding effect. It nourishes the liver and kidneys and strengthens the muscles and bones; the composition of the whole formula is rigorous, and the matching is exquisite.

Cassia twig is commonly used for the external treatment of pain. It is pungent, sweet, and warm, which can induce sweat and relieve muscles, warm the meridians, and promote veno-dilation, thus helping Yang to transform qi, dissipate cold, and relieve pain. It has been used by medical practitioners since ancient times for treating joint pain paralysis. Its main active ingredient is cinnamaldehyde. Modern pharmacological research has reported that cinnamaldehyde has an immunomodulatory effect, which can inhibit the secretion of various inflammatory factors such as tumor necrosis factor-α and interleukin-1β by macrophages [10]. In addition, it can induce specific and non-specific T regulatory cells. Previous studies have reported that cinnamaldehyde has an inhibitory effect on Toll-like receptor (TLR)-mediated inflammatory responses; however, only a few studies have analyzed its direct effect on KOA synovium. Our preliminary research confirmed that low-frequency ultrasound can efficiently promote the penetration of large molecules of the traditional Chinese medicine, cinnamaldehyde, through the skin, and achieve high drug concentrations in the joints, thereby exerting therapeutic effects [10].

Radix Paeoniae Alba, a commonly used traditional Chinese medicine in clinical practice, is another main component of the Tongluo Zhitong formula. It has a cool, bitter, and sour taste, and is slightly cold. It can nourish the blood, soften the liver, relieve pain slowly, restrain yin, and reduce sweat. Total glucosides of paeony (TGP) is the main active ingredient extracted from the dried roots of *Paeonia lactiflora*. It is a traditional Chinese medicine with bidirectional immunomodulatory effects and definite therapeutic effects for treating OA. Modern pharmacological studies have demonstrated its important role of TGP in inhibiting autoimmune reactions as well as its anti-inflammatory, analgesic, and other aspects, which are the basis for its clinical application in OA treatment [10]. Previous research has shown that TGP affects OA synovitis response by regulating the nuclear factor Kappa-B (NF-κB) signaling pathway.

To enhance the effective theoretical basis of this formula, we conducted a series of clinical and basic studies. Based on the clinical epidemiological efficacy evaluation system, a randomized, double-blind, controlled clinical trial was conducted to assess the efficacy of the ultrasound-enhanced transillumination using the Tongluo Zhitong formula for the external treatment of KOA. The external application of ultrasound to promote penetration, unblock collateral circulation, and relieve pain was more effective in relieving pain and improving joint function in KOA than external treatment techniques in traditional Chinese medicine. Subsequently, our research group conducted a series of studies on the mechanism of action of the Tongluo Zhitong formula for treating KOA using ultrasound. The experimental study confirmed that low-frequency ultrasound can penetrate not only the skin, but also the different tissue levels under the skin, including muscles, synovium, and synovial fluid. This study provides a pharmacological basis for the mechanism through which ultrasound promotes the penetration of the Tongluo Zhitong formula in treating KOA. The formula uses a traditional Chinese medicine extract as the main component, together with hydrophilic polymer materials, penetration enhancers, and humectants, to

form a gel, which plays the role of a coupling medium and carries and releases drugs during low-frequency ultrasonic drug penetration (patent application publication number: CN102091158A). This work also promotes the further promotion and application of this novel traditional Chinese medicine for external KOA treatment.

In an animal experiment, the Tongluo Zhitong formula was shown to significantly inhibit the generation of inflammatory factors in rabbit KOA synovial cells [11]. Reducing the levels of cartilage matrix-related cytokines, such as C-terminal crosslinking telopeptide of type II collagen (CTX-II), cartilage oligomeric matrix protein (COMP), and matrix metalloproteinase-3 (MMP-3) in serum, can effectively delay or prevent the degeneration of knee joint cartilage in rats with varying degrees of KOA [12]. Cinnamaldehyde, the active ingredient of cassia twig, was applied to human KOA synovial cells. The results showed that damage-associated molecular patterns (DAMPs)/TLR-mediated NF-κB inflammatory pathway play an important regulatory role in OA synovial inflammation, with many genes on the NF-κB pathway involved in OA synovial inflammatory responses [13]. However, owing to a lack of comprehensive studies, the Tongluo Zhitong formula has not been established as an effective treatment, resulting in an unclear mechanism of action and limiting its wide clinical applications.

In conclusion, an in vitro culture system for human KOA synovial fibroblasts was established. The MTT method and FCM were used to observe the effects of different concentrations of the Tongluo Zhitong formula on the proliferation and cell cycle of human fibroblasts. Suitable drug concentrations were selected to further explore the mechanism of action of the Tongluo Zhitong formula, providing valuable experimental parameters for the development of new drugs and the market entry of this formula. However, this study had some limitations. The study sample size was small, and further experimental research is needed to validate and supplement our study findings to provide further research data supporting application of the Tongluo Zhitong formula to treat KOA.

CRediT authorship contribution statement

Li-Li Yang: Writing – review & editing. **Qing-Fu Wang:** Data curation. **Xiao-Fang Ding:** Conceptualization. **Huan Liang:** Investigation.

Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Third Affiliated Hospital of Beijing University of Chinese Medicine.

Consent for publication

Not applicable.

Data statement

The data that support the findings of this study are available from the corresponding author, Yang Li-Li,upon reasonable request.

Data availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Statement of ethics

This study was carried out in accordance with the recommendations of tenets of the Declaration of Helsinki with written informed consent from all subjects. The study was approved by the Ethics Committee of the Third Affiliated Hospital of Beijing University of Traditional Chinese Medicine (ID:BZYSY-2019KYKTPJ-26).

Funding

This study was supported by the general program of the National Natural Science Foundation of China: Study on Mechanism of Tongluo Zhitong external therapy inhibiting osteoarthritis synovitis by regulating damps/TLRs/NF-κB pathway (no. 81373662).

Declaration of competing interest

I,YangLiLi, declare that there are no conflicts of interest in relation to the manuscript titled “Effects of Tongluo Zhitong formula on the proliferation of synovial fibroblasts in human knee osteoarthritis” submitted to Heliyon. I confirm that the results and

interpretations reported in the manuscript are original and have not been plagiarized.

I certify that I have read and understand the Heliyon conflict of interest policy,and I understand that failure to disclose a conflict of interest may result in the manuscript being rejected or retracted.

I also certify that I have disclose any financial or non-financial relationships that may be interpreted as constituting a conflict of interest in relation to this manuscript. I understand that this information will be subject to peer review,and I am willing to provide further information or clarification if required.

I confirm that I have no known conflicts of interest that would influence the results or interpretation of the data presented in this manuscript,and I understand that failure to disclose a conflict of interest is unethical and may result in sanctions being imposed on me.

References

[1] Heng Hu, Wei Peng, Zhang Zhong the therapeutic effect of Shuang Ju Rui Yin capsules combined with Erexib tablets on patients with knee osteoarthritis and their effects on COX-2, Wnt-3 α, and bone metabolism levels, J. Hebei Med. Univ. 43 (2) (2022) 223.
[2] Xiaoyun Zhang, Hao Zeng, Meng Lin Research progress on the pain mechanism and treatment of knee osteoarthritis [J] Chinese, Journal of Pain Medicine 29 (1) (2023) 50.
[3] A. Mahecha, M.J. Beers, H.C. Veen, et al., A review of the role of bioreactors for iPSCs-Based tissue-Engineered articular cartilage, Tissue Eng Regen Med 20 (7) (2023) 1041.
[4] J.C. Mora, R. Przkora, Y. Cruz-Almeida, Knee osteoarthritis: pathophysiology and current treatment modalities, J. Pain Res. 11 (2018) 2189.
[5] BondesonJ, et al., The role of synovial macrophages and macrophageproduced mediators in driving inflammatory and destructive responses in osteoarthritis, Arthritis Rheum. 62 (2010) 647–657.
[6] S. Chakrabarti, D.R. Jadon, D.C. Bulmer, et al., Human osteoarthritic synovial fluid increases excitability of mouse dorsal root ganglion sensory neurons: an in-vitro translational model to study arthritic pain, Rheumatology 59 (3) (2020) 662–667.
[7] Lulu Lin, Shi Guangxia, Tu Jianfeng, et al., Research progress on pain mechanism of knee osteoarthritis, J] Chinese Journal of Pain Medicine 28 (6) (2022) 454–459.
[8] Bo Wu, Ma Xu, Liu Ye, et al., The correlation between the expression of inflammatory factors in cartilage and the severity of knee osteoarthritis patients, Chinese Journal of Tissue Engineering 24 (2) (2020) 236–241.
[9] Lili Yang, Qingfu Wang, The effect of Tongluo Zhitong Formula on TLR in synovitis cells of human knee osteoarthritis, Journal of Tianjin University of Traditional Chinese Medicine 39 (6) (2020) 656–660.
[10] Huan Wang, Qingfu Wang, Rongluo Shi, et al., The effects of cinnamaldehyde and total paeoniflorin on synovitis response in osteoarthritis [J] Chinese, J. Tradit. Chin. Med. 33 (8) (2018) 3356–3360.
[11] Pu Chen, Anmin Ruan, Jun Zhou, et al., Tongluo Zhitong gel intervenes cartilage inflammation and degeneration of knee osteoarthritis model rabbits, J] Chinese Journal of Tissue Engineering 25 (17) (2021) 2670–2675.
[12] Chen Pu, Anmin Ruan, Jun Zhou, et al., Exploring the mechanism of paeoniflorin on LPS induced inflammation and degeneration of human chondrocytes based on NF kB signaling pathway, Journal of Beijing University of Traditional Chinese Medicine 43 (11) (2020) 903–909.
[13] Anmin Ruan, Qingfu Wang, Yufeng Ma, et al., The effect of electroacupuncture on NF -κB signaling pathway and synovitis in rabbits with knee osteoarthritis, Journal of Integrated Traditional Chinese and Western Medicine 30 (28) (2021) 3095–3099.