1 Introduction

Osteoarthritis is a degenerative disease of the joint characterized by progressive damage and erosion of the articular cartilage, destruction of the articular surface, exposure of the subchondral bone, and long-term wear and tear, secondary bone hyperplasia, formation of callus and cartilage lower bone sclerosis, joint deformity, chronic pain, limited mobility, and reduced quality of life, which will eventually lead to long-term illness and disability (Riddle & Stratford, 2014; Kato et al., 2014). The earliest lesions of osteoarthritis occur in articular cartilage. Under normal conditions, articular chondrocytes maintain a dynamic balance between the synthesis and degradation of extracellular matrix (ECM) components, including n-type collagen and proteoglycans in articular cartilage, the most abundant proteoglycan (PG) (Wojdasiewicz et al., 2014; Yang et al., 2014).

Due to the excessive production of inflammatory cytokines and matrix degrading enzymes, the chondrocyte metabolism is imbalanced, and synergistic with the decrease of anabolic signals, eventually leading to the destruction of extracellular matrix and consequent cartilage degeneration. Reactive oxygen species (ROS)-induced oxidative stress further disrupts cartilage homeostasis and promotes catabolism through inducing apoptosis and matrix component destruction, up-regulating potential matrix degrading enzymes, and inhibiting matrix synthesis as well as intracellular and cellular oxidation of external molecules (Tuli et al., 2020).

Baicalin significantly inhibits the expression of inflammatory mediators in chondrocytes of human osteoarthritis and regulates the JNK/ERK/p38 MAPK and PBK/Akt signaling cascades. The experiment evaluated the anti-inflammatory and anti-osteomyelitis effects of Baicalin in order to discover new treatment strategies. 

2 Methods

2.1 Chondrocyte separation and cell culture

The experiment was approved by the hospital ethics committee (No.: 20B1K0J50). Twenty five informed and consenting patients underwent total knee arthroplasty, with a definitive diagnosis of osteoarthritis/osteodystrophy and exclusion of other diseases. Diagnostic criteria for knee osteoarthritis refer to the American College of Rheumatology diagnostic criteria for knee osteoarthritis/osteodystrophy.Articular cartilage is obtained from the primary cartilage cells acquired from the knee joint in patients with knee osteoarthritis. The cartilage tissue was cut into pieces, and digested with 0.25% trypsin for 30 min, and then digested with 0.2% collagenase II and DMEM at 37 °C for 6 hours. The cells were resuspended in DMEM containing 10%
bovine serum albumin, 100 U/mL penicillin and 100 mg/mL streptomycin. The culture was carried out at 37 °C, and was simultaneously filled with 5% carbon dioxide (CO2).

2.2 MTT assay detecting cell viability

The obtained chondrocytes were seeded in a 96-well plate at a density of 6x10⁴ per well and cultured overnight, and then the cells were subjected to different concentrations of Baicalein for 24 hours in Experiment 1: 0 μg/mL, 10 μg/mL, 20 μg/mL, 40 μg/mL and 80 μg/mL.

In Experiment 2, the cells were divided into five groups. A, the Control group, the cells were treated without Baicalein or IL-1β. B, the Model group, the cells were treated with 5 ng/mL IL-1β for 24 hours. C, the low concentration group, the cells were treated with 10 μg/mL Baicalein for 4 hours, then with 5 ng/mL IL-1β for 24 hours. D, the medium concentration group, the cells were treated with 20 μg/mL Baicalein for 4 hours, then with 5 ng/mL IL-1β for 24 hours. E, the high concentration group, the cells were treated with 40 μg/mL Baicalein for 4 hours, then with 5 ng/mL IL-1β for 24 hours.

The medium was removed, 20 mL of MTT (5 mg/mL) was added to each well for 4 h incubation. The supernatant was discarded and 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance at 570 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

2.3 Annexin V staining to detect apoptosis

The cells were washed once with PBS and then digested with trypsin. After being washed with ice-cold PBS, the cells were resuspended in staining buffer. An appropriate amount of FITC-Annexin V staining solution was added according to the kit instructions. The cells were incubated in darkness.

2.4 Detection of interleukin (IL-6) and prostaglandin E2 (PGE2)

Chondrocytes were pretreated with different concentrations of Baicalein for 4 hours and then with IL-1β for 24 hours. The concentrations of IL-6 (MSK,NO.:69-23047) and PGE2 (MSK,NO.:69-80118) in the supernatant were examined by enzyme-linked immunosorbent assay.

2.5 Detection experiment of nitric oxide (NO)

The concentrations of NO in the supernatant of chondrocytes treated with Baicalein and IL-1 at various concentrations of 10 μg/mL, 20 μg/mL, and 40 μg/mL described above were detected using a Griess kit.

2.6 Western Blot detecting contents in apoptotic proteins and PI3/Akt, NF-kB and MAPK cascades

The cells were collected from each group, and washed twice with PBS. Each bottle was added with 400 μl of cell lysate, then with 40 μl of 10 mmol/L PMSF. The bottles were gently agitated, then placed on ice for 10 min to lyse the cells sufficiently. The cells were repeatedly aspirated with a sterile syringe. Following this, the lysed product was added to an EP tube, and the EP tube was ice-bathed for 30 min, then centrifuged at 12000 g for 15 min. The supernatant was transferred to a new EP tube, and the protein concentration was quantified by a protein standard BCA method. After this, 100 μl of each tube was added with 20 μl of protein 6x Buffer and boiled for 5 min, then stored at -80 °C for later use. The above samples were obtained, and proteins were separated by 12% SDS-PAGE electrophoresis, and the separated protein bands were transferred to PVDF membrane. The cells were blocked at room temperature for 1 h, then incubated with the primary antibody (concentration 1:1000) at 4 °C overnight. The cells were washed with PBST three times. The secondary antibody (1:1000) was added for 1 h incubation. The cells were washed with PBST three times. Color development and fixation were conducted by chemiluminescence. The expressions of each of the above proteins were determined.

2.7 Statistical approach

All data are expressed as mean ± standard deviation (mean ± SD). The data has passed the normal distribution assessment. The t-test was used for comparison between the two groups, and the comparison between multiple groups of data (>2) was performed using One-way ANOVA. There was a statistical significance at p<0.05. The data was analyzed using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA).

In reference to the “Diagnostic Criteria for Knee Osteoarthritis” of the American Academy of Rheumatology (ARA), the calculation for the sample size is selected in this study.

The clinical diagnostic criteria are as follows:

1. Recurrence of knee joint pain in the past month;
2. There is a bone friction sound when the knee joint moves;
3. Morning stiffness of the knee joint less than 30 min;
4. Hypertrophy of the knee joint with bone hyperplasia;
5. Age more than 40.

Those who meet the above (1), (2), (3) and (4) or (1), (2), (3) and (5) can be clinically diagnosed as knee osteoarthritis. The combined clinical and radiology diagnostic criteria are as follows:

1. Knee pain most of the time in the past month;
2. X-ray shows the formation of osteophytes on the edge of the knee joint;
3. Synovial fluid laboratory examination accords with osteoarthritis;
4. Age ≤ 40;
5. Morning stiffness of the knee joint < 30 minutes;
6. There is bone friction sound when the joints move.

Those who meet (1) and (2) or (1), (3), (5) and (6) or (1), (4), (5) and (6) can be diagnosed as knee osteoarthritis.
3 Results

3.1 The effect of Baicalein on cell viability

The chondrocytes treated with different concentrations of Baicalein were tested by MTT assay to detect whether the drug has cytotoxic effect. It is found that the viability of chondrocytes was almost unaffected regardless of the concentration of drug used, as shown in Figure 1. The results showed that Baicalein did not have a cytotoxic effect. After IL-1β stimulation, it can be seen that Baicalein improved the survival rate of chondrocytes, as shown in Figure 2.

3.2 Baicalein inhibited the production of NO and PGE2 induced by IL-1β stimulation

The results displayed that the production of NO and PGE2 increased significantly after chondrocytes were stimulated with IL-1β. Besides, the content of inflammatory cytokine IL-6 was also elevated. However, after treatment with Baicalein, the contents of NO and PGE2 were significantly decreased, suggesting that Baicalein is effectively anti-inflammatory, and the higher the concentration, the better the effect, as shown in Figure 3.

3.3 Western Blot detecting the contents of apoptotic proteins, as well as proteins in PI3K/Akt, NF-κB and MAPK cascades

The results showed that the expressions of proapoptotic proteins Bax and Bad increased in chondrocytes treated with IL-1β, while the expressions of anti-apoptotic proteins Bcl-2 and Bcl-xL decreased (Figure 4). The increase in the number of apoptosis may be due to an increase in the expression of pro-apoptotic proteins. The expressions of Bcl-2 and Bcl-xL were significantly elevated after the administration of Baicalein. At the same time, the expressions of Bax and Bad were significantly decreased. The above results indicated that Baicalein has an effect on the expressions of Bax, Bad, Bcl-2 and Bcl-xL in the apoptotic pathway, suggesting an anti-apoptotic effect.

Stimulation of chondrocytes by IL-1β significantly elevated the expressions of JNK, ERK and p38 MAPK (Figure 5). The treatment of Baicalein significantly decreased the expressions of JNK, ERK and p38MAPK. Phosphorylated JNK and p38 MAPK decreased more significantly than p-ERK. The expression of ERK also decreased under the administration of Baicalein, but the decrease was not as obvious as that of JNK and p38 MAPK. After the chondrocytes were treated with IL-1β, the expressions of p-NF-κB, p65, p-IκBα and TNF-α were significantly increased. However, after administration with Baicalein, the expressions of the above proteins were significantly reduced (Figure 6), indicating that the decrease of NF-κB signaling may lead to a decrease in the expression of inflammatory mediators, thereby reducing the inflammatory response. When chondrocytes were stimulated with IL-1β, high concentrations of Akt, phosphorylated Akt, phosphorylated GSK-3P and mTOR were produced in the cells, while PTEN expression was lower. However, after treatment with Baicalein, the expressions of phosphorylated Akt, GSK-3P and mTOR were significantly inhibited (Figure 7). In addition, Baicalein can elevate the expression of PTEN in a dose-dependent manner. Therefore, Baicalein can effectively regulate the PI3K/Akt signaling pathway, which also partially caused the reduce of NF-κB expression.

3.4 Effect of Baicalein on apoptosis

Annexin V / PI double staining showed that apoptosis was obvious in the control group, but it was significantly inhibited in the drug group, as shown in Figure 8.

4 Discussions

The NF-κB and MAPK signaling pathways play a crucial role in the regulation of inflammatory mediators (Li et al., 2011). Under physiological and pathological conditions, NF-κB can regulate various genes involved in immune function and inflammation. Inflammation is a reaction mechanism of the body against pathogen invasion. (Wang & Dong, 2021) It is not a simple but a complex biological process. F-κB is involved in the inflammatory response, and regulates many molecules involved in the early immune response and various stages of the inflammatory
response. It is highly active in the inflammatory sites of various inflammatory diseases (Zhu & Liu, 2020). NF-κB can efficiently induce inflammatory cytokines (IL-1, IL-6, and TNF-α), adhesion molecules (ICAM-1 and VCAM-1), inflammatory enzymes (iNOS and COX-2), and chemokines, etc. NF-κB amplifies the inflammatory cascade, making it a transcription factor of many inflammatory cytokines in inflammation (Kato et al., 2014; Sun et al., 2017).

**Figure 3.** Baicalein inhibiting production of NO and PGE2 induced by IL-1β stimulation.

**Figure 4.** Effect of drugs on various apoptotic proteins.

**Figure 5.** Effect of drugs on various proteins in the MAPK cascade.
PTEN induces dephosphorylation of inositol 3-phosphate and negatively regulates the PI3K pathway. PI3K and Akt are upstream molecules of the NF-κB signaling pathway and play a crucial role in the activation of NF-κB (Yang et al., 2013; Wang et al., 2014). When chondrocytes were stimulated with IL-1β, high concentrations of Akt, phosphorylated Akt, phosphorylated GSK-3β, and mTOR were produced in the cells, while PTEN expression was lower. However, after treatment with Baicalein, the expressions of phosphorylated Akt, GSK-3β, and mTOR were significantly inhibited (Figure 7). In addition, Baicalein can elevate the expression of PTEN in a dose-dependent manner. Therefore, Baicalein can effectively regulate the PI3K/Akt signaling pathway, which also partially caused the reduction of NF-κB expression.

To investigate the effect of Baicalein on PI3K/Akt signaling, the expression of proteins in the above pathways was examined. IL-1β can induce several times the expression of phosphorylated Akt and GSK-3β, indicating that it can activate the pathway. The pretreatment of Baicalein significantly inhibited this pathway, showing a significant decrease in the phosphorylation level of Akt. These results suggest that the inhibition of NF-κB activation by Baicalein may be involved in the PI3K/Akt cascade. After the chondrocytes were treated with IL-1β, it was found that the expressions of related proteins JNK, ERK, and p38 in the MAPK pathway increased, while Baicalein inhibited the expressions of these proteins. The mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in the regulation of inflammatory mediators (Fu et al., 2016; Akhtar et al., 2017). P38 is the most important member of the MAPK family to control inflammation, and its activation can be caused by lipopolysaccharide, physiological stress, ultraviolet radiation, and osmotic stress (Valdes & Spector, 2011). Key enzymes of the P38 pathway include ASK, MLK, and TAK of the MAPKK class, as well as MKK6 and MKK3 of the MAPKK class. TAK binding protein (TAB) activates TAK and mediates signal transduction of transforming growth factor. TAK also activates MKK4, which in turn activates P38 (Liu et al., 2020). Nuclear translocation occurs after P38 activation, causing phosphorylation and activation of many protein kinases and transcription factors (Jeong et al., 2016; Zhou et al., 2016; Wang et al., 2015).

The result of PI3K activation is the production of the plasma membrane. The two messengers PIP3 and HP3 bind to the PH domain–containing signaling proteins Akt and PDK1, and cause the phosphorylation of Ser308 of the Akt protein by PDK1, as well as the activation of Akt. Akt can also be activated by the phosphorylation of Thr473 by PDK2. The activated Akt activates or inhibits its downstream target proteins through phosphorylation of Bad, Caspase9, NF-κB, GSK-3, and FKHR, thus regulating cell proliferation, cell differentiation, apoptosis, and cell migration. The product of the tumor suppressor gene PTEN induces dephosphorylation of inositol 3-phosphate and negatively regulates the PI3K pathway. PI3K and Akt are upstream molecules of the NF-κB signaling pathway and play a crucial role in the activation of NF-κB (Yang et al., 2013; Wang et al., 2014)). When chondrocytes were stimulated with IL-1β, high concentrations of Akt, phosphorylated Akt, phosphorylated GSK-3β, and mTOR were produced in the cells, while PTEN expression was lower. However, after treatment with Baicalein, the expressions of phosphorylated Akt, GSK-3β, and mTOR were significantly inhibited (Figure 7). In addition, Baicalein can elevate the expression of PTEN in a dose-dependent manner. Therefore, Baicalein can effectively regulate the PI3K/Akt signaling pathway, which also partially caused the reduction of NF-κB expression.
Mechanism of Baicalein in treatment of arthritis

Ethical approval

This study is approved by the Ethics Committee of Wendeng Orthopedic and Traumatic Hospital.

Conflicts of interest

None.

Availability of data and material

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Author contribution

Xiaobo Wang contributed to research concept and design. Jing Yang contributed to collection and/or assembly of data. Hongguo Liu contributed to data analysis and interpretation. Jiandong Liu contributed to writing the article. Lianjia Wang contributed to critical revision of the article. Lianjia Wang and Jiandong Liu helped to the final approval of the article.

References


Fu, Y., Lei, J., Zhuang, Y., Zhang, K., & Lu, D. (2016). Overexpression of HMGB1 A-box reduced IL-1β induced MMP expression and the

** p < 0.01 vs Control  *** p < 0.01 vs 10μg/mL  &&& p < 0.01 vs 20μg/mL & p < 0.05 vs 20μg/mL.

!! p < 0.01 vs 40μg/mL ! p < 0.05 vs 40μg/mL.

Figure 8. Effect of Baicalein on apoptosis.

JNK, ERK and p38 MAPK, indicating that Baicalein can inhibit the activation of MAPK. There was no significant change in the expression of ERK compared to changes in the expressions of JNK and p38 MAPK, although some significant changes were observed. Baicalein can effectively inhibit the MAPK pathway in chondrocytes treated with IL-1β, which may partly explain the observed decrease in inflammatory mediator levels.

5 Conclusion

Although great research work and efforts have been made in recent years, the treatment of osteoarthritis is still experimental and unknown. There is a wide range of interaction among various cytokines to maintain cartilage metabolism balance in vivo. Any drug can not affect a single cytokine, and it is impossible to completely cause complete cartilage repair or affect arthritis joint in clinic. On the contrary, joint interaction is needed.

In summary, inflammatory mediators play a key role in the pathogenesis of osteoarthritis. Despite tremendous research efforts in recent years, the treatment of osteoarthritis is still experimental and unknown. There are a wide range of interactions between various cytokines that maintain the balance of cartilage metabolism in the body. It is impossible for any drug to affect a single cytokine, it is even less likely to cause complete cartilage repair or clinically affect arthritic joints. Baicalein can significantly inhibit the expression of inflammatory mediators in human osteoarthritic chondrocytes and regulate the JNK/ERK/p38 MAPK and PKB/Akt signaling cascades.

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