Xuebijing inhibit inflammation, oxidative stress and promote apoptosis in human synovial cells via inhibition of MEK1/2 and NF- κ B pathway

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ABSTRACT

Rheumatoid arthritis (RA) is categorized as an autoimmune disease that leads to bone or joint deformity due to altered immune response. Studies have concluded the role of inflammation and oxidative stress in the progression of RA and agents inhibiting these processes showed beneficial effect against the disease. Xuebijing (XBJ) injection is an intravenous patent preparation made from five-traditional Chinese medicines. Previous studies showed its excellent pharmacological activities, such as against sepsis, inflammation, and oxidative stress which has encouraged us to investigate the protective effect of XBJ against rheumatoid arthritis cell line (MH7A). For this purpose, the effect of XBJ was quantified on several parameters on the human synovial MH7A cell line activated with tumor necrosis factor- α (TNF- α). The results of the study showed that the level of tested interleukines (IL-1 β , IL-6, IL-8) and collagenases 1, and 13, and matrix metallo-proteinases 1, and 13 (MMP-1, and MMP-13) were found significantly reduced in XBJ treated group as compared to TNF- α treated MH7A cells. The XBJ treated group showed reduction in mRNA protein expression of COX-2 and iNOS in RT-qPCR assay. The rate of cellular apoptosis was found increased in XBJ treated group with reduction of cell viability of MH7A cells. The XBJ also showed attenuation of the expression of p-MEK1/2 and p-p65 in MH7A cells in a western blot analysis. Our results demonstrated that XBJ significantly inhibits the inflammatory response, prevents cell viability, and induces apoptosis in human RA synovial cells by preventing the activation of the MEK/NF- κ B pathway.

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Introduction

Traditional Chinese medicine (TCM) has evolved over thousands of years. TCM practitioners use various mind and body practices (such as acupuncture and tai chi) as well as herbal products obtained from plants to address health problems (Wang et al., 2011; Yuan et al., 2016). Among them, Traditional Chinese herbal medicine therapy is a mixture of Chinese herbs prescribed by Chinese herbalists depending on the differentiation of the patient's syndrome according to Chinese diagnostic patterns (inspection, listening, smelling, inquiry, and palpation). They have been used all over the world for hundreds or even thousands of years, and now have blossomed into orderly-regulated systems of medicine (Arikawa et al., 2002; Marsella and De Benedetto, 2017; Thomsen, 2014). The uniqueness of the

natural product lies in their chemical skeleton, which results in diversity in their pharmacological activities against numerous diseases or ailments.

Various studies have documented the effectiveness of TCM for the benefit against rheumatoid arthritis (RA) (Zhang et al., 2010). It is categorized as an autoimmune disease that leads to bone or joint deformity due to altered immune response. This altered immune response causes inflammation and destruction of tendons or ligaments where the immune system attacking its healthy tissues (Firestein and McInnes, 2017; Smolen et al., 2018). Synovial fibroblasts (SFs), also known as fibroblast-like synoviocytes, have been demonstrated to serve an important role in the pathogenesis of RA, and their tumor-like proliferation leads to the development of synovial hyperplasia. During RA, activated SFs accumulate in the hyperplastic synovium of patients

with RA. In recent years, RA has become a grave worry affecting more than 1.5% of the population across the global (Huber et al., 2006; Ospelt, 2017; Wei et al., 2020). The majority of the drugs in a clinical trial for rheumatoid arthritis have immunomodulatory properties and the clinical approach to managing RA has been greatly relying on the use of disease-modifying anti-rheumatic drugs (DMARDs). The development of novel drugs for RA is impending and scientists are exploring new strategies through various innovative approaches for RA drug development.

Xuebijing (XBJ) injection is an intravenous patent preparation made from five traditional Chinese medicines, namely, Chishao (*Radix Paeoniae Rubra*), Danggui (*Radix Angelica Sinensis*), Chuanxiong (*Rhizoma Chuanxiong*), Honghua (*Flos Carthami*), and Danshen (*Radix Salviae Miltiorrhizae*). It consists numerous bioactive compounds, such as safflower yellow A, ligustrazine, danshensu, ferulic acid, paeoniflorin, and protocatechualdehyde (Long et al., 2020). It dissipates blood stasis by activating blood circulation, thus eliminating pathogenic heat from the blood and degrading toxins. It also improves coagulation dysfunction, inhibiting platelet adhesion and aggregation via inducing microcirculation (Hou et al., 2015; Jin et al., 2018; Li et al., 2014). Clinically, it is mainly used for the treatment of sepsis, infection-induced systemic inflammatory response syndrome (SIRS), and multiple organ dysfunction syndromes (MODS), and patented for use in severe pneumonia (Chen et al., 2009; He et al., 2015; Song et al., 2019; Wang et al., 2016). It also attenuates pulmonary injury by reducing oxidative stress and pro-inflammatory damage in rats with heat stroke (Chen et al., 2017). In a recent study, it showed beneficial effect against inflammatory markers and disease outcome of coronavirus disease 2019 (Guo et al., 2020; Long et al., 2020). The main pharmacological effects of XueBiJing might be summarized as follows: (1) antagonizing endotoxin; (2) inhibiting inflammatory cytokines; (3) decreasing oxidative stress; (4) regulating immune function; (5) improving the balance of coagulation; (6) protecting organ damage. Considering the significant benefit of anti-inflammatory and antioxidants in RA and strong antiinflammatory and antioxidant effect of XBJ, in the present study, we intend to scrutinize whether XBJ has any protective role against RA by examining its effect against TNF- α -treated human RA synovial cells.

Material and methods

Cell culture

The human RA synovial MH7A cell line was obtained from Shanghai Guandao Biological Engineering Co., Ltd. (Shanghai, China; cat no. C0878). Cells were grown in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and incubated at 37°C with 5% CO₂.

Cell treatment

The MH7A cells were pre-treated with various concentrations of XBJ (10, 15 and 30 ml/kg) for 2 h, then the cells were subjected to treatment with TNF- α (10 ng/ml; Sigma-Aldrich; Merck KGaA) at room temperature for 24 h to establish the in vitro model of RA. Cells in the sham group were treated with normal RPMI-1640 medium with.

MTT assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to detect cell viability. The MH7A cells were seeded into each well (5×10^5 cells per well) and incubated with various concentrations of XBJ (10, 15, and 30 μ M) for 24 h at 37°C. Then, 0.5 mg/ml MTT (in PBS) was added to every well, and the cells were incubated for an additional 3 h at 37°C with 5% CO₂. Dimethyl sulfoxide was used to dissolve the formazan crystals. Finally, absorbance was detected at 590 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell apoptosis assay

The MH7A cells were treated with 10, 15, and 30 ml/kg of XBJ and 10 ng/ml TNF- α . Then, MH7A cell apoptosis was analyzed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit [cat no. 70-AP101-100; Hangzhou MultiSciences (Lianke) Biotech, Co., Ltd., Hangzhou, China]. Briefly, MH7A cells (5×10^5 cells per well) were dyed with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's protocol. Flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was performed to analyze cell

apoptosis, and data were analyzed using Version 2.5 WinMDI software.

ELISA analysis

To detect the production of interleukin (IL-1 β), IL-6, IL-8, matrix metallo-proteinases (MMP) MMP-1, and MMP-13, the MH7A cell culture medium was collected by centrifugation at 4°C and 1,048 $\times$ g for 10 min. ELISAs were performed according to the manufacturer's protocol of each ELISA kit (all Abcam, Cambridge, UK).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from MH7A cells was isolated using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was generated using the PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. qPCR was performed by using the SYBR® Premix Ex Taq™ II (Takara Bio Inc.). Amplification conditions were as following: 95°C for 10 min, followed by 37 cycles at 95°C for 15 sec and 72°C for 30 sec and 78°C for 1.5 min, following which samples were stored at 4°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Relative gene expression was analyzed by performing the $2^{-\Delta\Delta C_q}$ method.

Western blot analysis

The MH7A cells were treated with 10, 15, and 30 ml/kg of XBJ and 10 ng/ml TNF- α . Then, total proteins from MH7A cells were isolated using radioimmunoprecipitation assay lysis buffer (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). The quantification of the proteins was measured by BCA assay (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Equal amounts of protein samples (25 μ g/lane) were separated on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride membranes. The membranes were firstly blocked with 5% non-fat milk in PBS with 0.1% Tween-20 for 2 h at room temperature and then incubated with primary antibodies (all Cell Signaling Technology, Inc., Danvers, MA, USA) or β -actin at 4°C overnight. Subsequently, the membranes were incubated with the horseradish peroxidase-conjugated anti-rabbit IgG secondary

antibody for 2 h at room temperature. Finally, a chemiluminescence detection kit (Cell Signaling Technology, Inc.) was used to visualize the blots according to the manufacturer's protocol. Gel-Pro Analyzer densitometry software (version 6.3; Media Cybernetics, Inc., Rockville, MD, USA) was used for band density quantification.

Statistical analysis

The data were articulated as mean $\pm$ standard deviation (SD). The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc multiple comparison test (Graph-Pad Prism 5.0, USA). The P-value < 0.05 was considered statistically significant.

Result

Effect of XBJ on inflammatory markers

Initially, the effect of XBJ in different doses was estimated on the level of various pro-inflammatory cytokines (IL-1 β , IL-6, and IL-8) and MMPs (MMP-1, and MMP-13) to assess inflammatory response in the human RA synovial MH7A cell line. As shown in Fig. 1, the level of the cytokines and MMPs were found elevated in TNF- α treated group as compared to sham. Moreover, upon treatment with XBJ the level of these cytokines as well as MMPs were found significantly lowered in a dose-dependent manner as compared to TNF- α .

Effect of XBJ expression on iNOS and COX-2

An RT-qPCR study was conducted to determine the effect of XBJ on iNOS and COX-2. As shown in Fig. 2, the mRNA expression of iNOS and COX-2 was found significantly elevated in TNF- α treated group as compared to sham. However, the elevated level of these markers were significantly reduced to approximately 50% in XBJ treated group, as compared to TNF- α treated group.

Effect of XBJ on cell viability

The effect of XBJ was also investigated on the viability of the human RA synovial MH7A cell line by MTT assay. As shown the Fig. 3, XBJ showed a significant reduction in the cell viability of MH7A cells which was found highly elevated after induction with TNF- α .

Figure 1. Effect of XBJ on the level of various pro-inflammatory cytokines (A) IL-1β, (B) IL-6, and (C) IL-8, and MMPs (D) MMP-1, and (E) MMP-13. Values represent the mean ± SD. ***P < 0.05 vs sham; **P < 0.05 vs TNF-α group, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

Figure 2. Effect of XBJ on the mRNA expression of (A) iNOS and (B) COX-2. Values represent the mean ± SD. ***P < 0.05 vs sham; **P < 0.05 vs TNF-α group, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

Effect of XBJ on apoptosis and apoptotic biomarker

The effect of XBJ was investigated on the apoptosis of human RA synovial MH7A cells via flow cytometry analysis. As shown in Fig. 4A and B, the number of apoptotic cells in the TNF-α treatment group was found significantly decreased as compared to the sham group. On the other hand, the number of apoptotic cells found significantly increased in XBJ treated group in a dose-dependent manner suggesting its apoptosis promoting effect. The effect of XBJ was further investigated on the mRNA expression of Bax and Bcl2, apoptosis biomarkers by RT-qPCR analysis. As shown in Fig. 4C and D, The results suggested that TNF-α treatment significantly increased Bcl-2 expression and inhibited Bax expression

in MH7A cells. Results showed that the level of Bcl-2 was notably decreased and Bax was increased in XBJ treated group as compared to TNF-α treated group with maximum modulation was achieved in 30 ml/kg group.

Effect XBJ on MEK1/2 and NF-κB pathway

Lastly, we intend to investigate the mechanism behind the effect of XBJ. Therefore, the effect of XBJ was investigated on the MEK/NF-κB pathway. As shown in Fig. 5, the TNF-α group showed increased expression of p-MEK1/2 and p-p65 as compared to sham. However, upon treatment with XBJ, the level of these proteins was found significantly decreased in dose-dependent manner.

Figure 3. Effect of XBJ on the cell viability of human RA synovial MH7A cell line. Values represent the mean $\pm$ SD. *** P < 0.05 vs sham; ** P < 0.05 vs TNF- α group, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

Figure 4. Effect of XBJ on the (A) apoptosis of MH7A cells via flow cytometry analysis, (B) apoptosis rate, and mRNA level of (C) Bcl-2, and (D) Bax. Values represent the mean $\pm$ SD. *** P < 0.05 vs sham; ** P < 0.05 vs TNF- α group, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

Discussion

In the present study, our results showed that XBJ in the tested dose causes a significant reduction in the level of various pro-inflammatory cytokines induced by TNF- α . It was also found that XBJ significantly inhibited the cell viability and promoted the apoptosis of MH7A cells in a concentration-dependent manner possibly via attenuation of the MEK/NF- κ B pathway. The mRNA expression level of iNOS and COX-2 was also found reduced in XBJ treated group. Thus, it could be indicated

that XBJ could serve as a capable adjuvant for therapeutic treatment of RA. At present, there is no current therapeutic option to treat RA, the current therapeutic modality to manage RA only provides symptomatic relief. Thus, many efforts are currently underway to find an effective agent to curb the menace of RA (Guo et al., 2018; Kourilovitch et al., 2014).

In RA, Synovial fibroblasts due to its tumor-like proliferation leads to the development of synovial hyperplasia and further aggravate the disease condition. In the present manuscript, we have shown significant

Figure 5. Effect of XBJ on (A) p-MEK1/2 and NF- κ B expression via western blot analysis, representative bargraph of protein level of (B) p-MEK1/2, and (D) p-p65. Values represent the mean $\pm$ SD. *** P < 0.05 vs sham; ** P < 0.05 vs TNF- α group, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

inhibitory effect of XBJ against synovial fibroblasts via reducing cell viability and induction of apoptosis. Initially, the effect of XBJ was investigated in various pro-inflammatory cytokines. Studies have shown that these inflammatory cytokines are responsible for joint destruction after accumulation in the synovium (Choy and Panayi, 2001; Littlejohn and Monrad, 2018). Moreover, it has been also found that neutralizing anti-TNF- α monoclonal antibodies have an inhibitory effect on the elevated level of these cytokines and thus have protective effect against RA (Feldmann, 2002; Venkatesha et al., 2015). In the present manuscript, we have shown that XBJ significantly reduces the level of cytokines that were found elevated in response to TNF- α . Thus, it could be suggested that XBJ might exert protective effect against RA via reducing the inflammation in MH7A cells. Inflammatory stimuli including endotoxin and numerous cytokines induce the expression of both COX-2 and iNOS at sites of inflammation or tissue injury. The prostaglandins and nitric oxide generated by these enzymes contribute

to the pathology of arthritis including pain, swelling and joint destruction (Hochberg, 2005; Praveen Rao and Knaus, 2008). Accumulating shred of pieces of evidence suggests that, attenuation of iNOS and COX-2 have a significant impact on RA and able to reduce the pathological changes in the affected joints (Hochberg, 2005; Jin et al., 2010; Simon et al., 1999). In the present manuscript, to further enlarge the spectrum of analysis, we have determined the mRNA expression of iNOS and COX-2 in MH7A cells. It has been found that XBJ significantly reduces the elevated level of these inducible enzymes after induction with TNF- α . Apoptosis is a key mechanism that regulates tissue composition and homeostasis. The SF cells in RA are the most affected prominently because of their resistance to apoptosis which leads to the progressive destruction of articular cartilage (Firestein et al., 1995; Huber et al., 2006; Liu and Pope, 2003; Pap et al., 2000; Turner and Filer, 2015; Wei et al., 2020). Thus, in the next instance, we intend to scrutinize the effect of XBJ on the apoptosis of MH7A cells. For this, we had first identified the effect of

XBJ on the cell viability of MH7A cells in MTT cells. The result suggested that XBJ significantly reduces the proliferation of SF cells possibly via induction of apoptosis. Studies have shown that Bax is essential for apoptosis in normal cells. However, the overexpression of Bcl-2 enhances cell survival by suppressing apoptosis in cells subjected to apoptosis-inducing stimuli (Basu and Haldar, 1998; Dai et al., 2016). To understand the mechanism behind the pro-apoptotic effect of XBJ, a study was conducted to determine the expression of Bcl2 and Bax in MH7A cells after induction with TNF- α in XBJ treated group. The results of the present study indicated that XBJ significantly decreases Bcl-2 and increases Bax in a dose-dependent manner. The effect of XBJ was further investigated on the MEK1/2 and NF- κ B pathway in MH7A cells to understand the plausible mechanism for antiinflammatory effect on human synovial fibroblasts. NF- κ B is a collective name for dimeric transcription factors comprised of the Rel family of proteins that include RelA (p65), c-Rel, RelB, NF- κ B1 (p50), and NF- κ B2 (p52) (T Liu et al., 2017; Oeckinghaus et al., 2011). A component of NF- κ B, RelA (also known as p65 proto-oncogene NF- κ B subunit) reportedly regulates the expression of a disintegrin and metalloproteinase with thrombospondin motifs 5 (Adamts5) in chondrocytes (YX Liu et al., 2017; Shukla et al., 2004; Wang et al., 2006). Moreover, p65/RelA has been implicated in osteoarthritis development. In murine experimental models, NF- κ B activity was correlated with the degree of pain induced by osteoarthritis (Giridharan and Srinivasan, 2018; Handel et al., 1995; Luo et al., 2014; Simmonds and Foxwell, 2008; Sun et al., 2017). On the other hand, the mitogen-activated protein kinase (MAPK) pathway in the regulation of key cellular processes, including cell survival/apoptosis, proliferation, and differentiation, as well as cellular stress and inflammatory responses in RA. MEK1 and MEK2 belong to the family of MAPKKs (also known as MEK's or MKKs), which are dual-specificity enzymes that phosphorylate threonine and tyrosine residues within the activation loop of their MAP kinase substrates (Cuadrado and Nebreda, 2010; Kim and Choi, 2010; Seger and Wexler, 2016). A selective MEK1/2 inhibitor, U0126, was found to be effective on IL-1 β , IL-8, TNF- α , and prostaglandin E2 (PGE2) production in lipopolysaccharide (LPS)-stimulated monocytes (Scherle et al., 1998). U0126 also blocked matrix metalloproteinase 2 (MMP2) and MMP-9 secretion from a rat fibroblast cell line (Liu et al., 2000). These findings strongly suggest

that suppression of MEK-ERK activity would be efficacious in RA. In the present study, XBJ treated group showed significant attenuation of MEK/NF- κ B pathway as confirmed by reduced expression of p-p65 and p-MEK 1/2 in MH7A cells, which might be suggested as a possible mechanism for anti-inflammatory effect of XBJ against human fibroblast cells. The iNOS and COX-2 was considered as a downstream mediators of MEK/NF- κ B pathway, thus inhibition of this pathway by XBJ might be the possible reason for the reduction of iNOS and COX-2 in MH7A cells (Kim et al., 2011).

Conclusion

Our study demonstrated that Xuebijing injection inhibited inflammatory response, prevented cell viability, and induced apoptosis in TNF- α -treated human RA synovial cells by preventing the activation of the MEK/NF- κ B pathway. However, this is a preliminary study examining the effect of Xuebijing on RA and requires additional study to confirm its pharmacological benefit.

Conflict of Interest

None

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