Inhibitory effect of *Saposhnikovia divaricate* polysaccharide on fibroblast-like synoviocytes from rheumatoid arthritis rat *in vitro*

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Abstract: To study the mechanism and inhibitory effect of *Saposhnikovia divaricata polysaccharide* (SDP) on fibroblast-like synoviocytes (FLS) isolated from rheumatoid arthritis rat model. Rheumatoid arthritis rat model was established by the classical composite factors including wind, cold, damp plus biological agents. The synovial tissues were digested with trypsin to isolate FLS cells. The different dosage of SDP was applied in culture. The cell viability was evaluated by MTT assay and the apoptosis was determined by analytic flow cytometry. The expression change of p53 gene was monitored by RT-PCR method. The production of secretory inflammation factors TNF-α and IL-1β were determined by ELISA. The proliferative and apoptotic proteins such as Bcl-2, Bax, Caspase-3, MMP-1, MMP-3, P53 were measured by western blotting. Our data demonstrated that treatment with high concentration of SDP could enhance the expression of P53 at both mRNA ($P<0.05$) and protein ($P<0.05$) level, inhibit the secretion of TNF-α ($P<0.05$) and IL-1β ($P<0.05$). Simultaneously, the Bcl-2/Bax ratio and level of MMP-1, MMP-3 was significantly decreased, and apoptotic marker caspase-3 protein was increased. In addition, the FACS analysis consistently consolidated the apoptosis-inducing effect of SDP on RAFLS. SDP could significantly inhibit dysplasia of RAFLS via modulation of p53 expression and suppression of inflammatory factors, which suggested a potential therapeutic value for rheumatoid arthritis.

Keywords: p53, Apoptosis, MMPs, RAFLS, Inflammatory factors.

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common chronic inflammatory diseases worldwide with progressive damage, which imposes heavy burden to both patients and community (Smolen *et al*., 2016). RA is a heterogeneous and complicated disease, and systematic syndrome associated with RA primarily involves musculoskeletal deficits and rare inflammation in blood vessel, lung and heart (Singh *et al*., 2016). Previous studies indicated that various populations were in poor health, suffering from it (Liang *et al*., 2015; Liang, 2016). The epidemiological investigation suggested an incidence of 0.5~1%, which is higher in north versus south hemisphere (Gibofsky, 2014). The accurate etiology of RA is still elusive currently, but supposedly closely related to both genetic and environmental factors (Klein and Gay, 2015; Catrina *et al*., 2016).

RA is empirical defined by tender and swollen joint, morning stiffness and pain (Catrina *et al*., 2016). Some laboratory test such as elevated C-reactive protein and presence of antibodies against citrullinated proteins (ACPAs) and/or rheumatoid factors (RF) could assist diagnosis but with limited accuracy and specificity (Nell-Duxneuner *et al*., 2010). For example, circulating ACPAs can be detected in blood of RA patients 10 years before clinical onset (Nielen *et al*., 2004). Early diagnosis is crucial for to optimal therapy and becomes the focus of research in this field (Nielen *et al*., 2004).

Clinical management of RA has dramatically changed over the past two decades. Since inflammation is the driving event in progression of RA (Smolen *et al*., 2007), its reversal is the primary therapeutic target. The rapid accumulation of guideline and therapy for clinical practice includes disease-modifying anti-rheumatic drugs (DMARDs) (Kalden, 2016) targeting inflammation and reversing structural damage; non-steroidal anti-inflammatory drugs (NSAIDs) (Inotai and Meszaros, 2012) relieving stiffness/pain and glucocorticoids with quick response but long-term side-effect (WJ and Buttgereit, 2016). Clinical strategy adaptation is based on regular assessment in accordance with American Rheumatology Associations (ARA) guideline (Singh *et al*., 2016).

With advent of next generation of sequencing technology, our understanding of etiology of RA greatly advanced in the recent decade. Genome-wide association studies have identified hundred loci linked to rheumatoid arthritis risk, and some of them are common in other chronic inflammatory disease (Roberson and Bowcock, 2010). The dysfunction of HLA system and costimulatory system (e.g., CD28, CD40) are the dominated genetic events in RA (Lenz *et al*., 2015). In addition, epigenetics was increasingly realized significantly contributing. To pathogenesis of RA, with integration of both genetic and

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environmental influences. However, accurate molecular subtype and underlying mechanism warranted further investigation.

Many cell types are involved in pathogenesis of RA including T cells, B cells, macrophage and fibroblast-like synoviocytes (FLS) (Bartok and Firestein, 2010). FLS cells locate inside joints in the synovium and play a crucial physiological function in reduction friction between the joint cartilages during movement. Synovial hyperplasia is characteristic feature in the course of RA by producing cytokines and proteases. The effect of FLS cells in RA pathogenesis and progression please refer to the authoritative review (Bartok and Firestein, 2010).

In comparison with chemicals, the Traditional Chinese Medicines (TCMs) have unique advantages in combination, hypo-toxicity and cost (Normile, 2003). The dried root of *Saposhnikovia divaricata* (Turcz.) Schischk, a perennial herb of carrot family, is also well-known as Fang Feng in TCMs (Liang, 2015). It's one of the most important ingredients in many TCMs formulae to dispel "wind" and alleviate rheumatic conditions. However, the underlying mechanism of its therapeutic efficiency is still obscure, which prompt us to investigate the effect of SDP on FLS cells from RA model rat *in vitro.*

MATERIAL AND METHODS

Animal model

All rats were housed in a pathogen-free environment and experimental protocols were approved by the Committee of Animal Care and Use. All animal work was performed in strict accordance with the approved protocol of The Affiliated Hospital of Qingdao University. In total, 15 male SPF-grade Wistar rats, with average body weight 180~220g and 3~4 months of age were adopted first for one week of adaptive phase. Among which, 5 were picked randomly as normal control for the successive regular raising, and all the others were subjected to RA modeling.

In order to simulate the formation of RA as much as possible, the RA rat model was established by combination of environmental factors including wind, cold, damp and biological agents. The rats were cultured for 20 days (12 hours per day, 20:00 pm-8:00am) in home-made Perspex box made with aluminum alloy. The humidity and temperature (6±2°C) were maintained by iced ultrasonic atomizer and forced ventilation with electric fan with highest speed. The inflammation was induced on sole of the foot by injected with complete Freund's adjuvant to recapitulate RA at day 21. The model was assessed by the emergence of acute inflammatory ankle swelling 24 hours late and secondary systematic polyarthritis 48 hours late, and manifestation of redness/swelling or inflammatory nodules of fore- or contra lateral limb ear and tail.

Isolation and culture of FLS

The FLS were digested with typs in from synovial tissues. First, the synovial tissue was peeled, minced and incubated in DMEM media supplemented with 0.15 mg/ml DNase, 0.15 mg/ml hyaluronidase (type I-S), and 1 mg/ml collagenase (type IA) at 37°C for 1 hour. The cell then was washed and re-suspended in DMEM media containing 10% FBS, 30mg/ml glutamine, 250 μg/ml amphotericin B (Sigma-Aldrich) and 20μg/ml gentamicin (Invitrogen). The suspension cell was discarded and adherent cell was kept as FLS (purity>95%) after overnight culture.

SDP preparation

The root of *saposhnicovia divaricata* was first washed and rinsed thoroughly and dried at 60°C. After pulverization and screening with 40-mesh sieve, the powder product was dissolved in 40 times volume of distilled water. The solution was boiled at 90°C for 4 hours and filtered with 6 layers of gauze. The filtrate was centrifuged (4 000 r /min, 20 min) and supernatant was 4 times concentrated. The condensate was then precipitated with 3 times volume of pre-chilled absolute ethanol, and the supernatant was discarded after brief centrifuged (3000 r/min, 15 min). The precipitate was dissolved in distilled water and the residual protein was removed with filter paper, followed by the second precipitation. The final product was air dried for future use (purity > 90%). Model.

MTT assay

The cellular viability was determined by MTT assay (Sigma, CA) (Dong *et al*., 2018). FLS cells isolated from control and model group respectively in the logarithmic phase were seeded in 96-well plates for 24h culture and then were treated with varying doses (0, 5, 10, 15mg/ml) of SDP for another 24 h. Then two-hundred microliters of MTT (5 mg/ml; Sigma) was added to each well and incubate for4h at 37°C. The color formation was quantified by a spectrophotometric plate reader (Versa Max; Molecular Devices) at 490 nm wavelength after solubilizing in 200ul of dimethyl sulfoxide.

ELISA

The cells from both groups were treated as aforementioned in MTT assay and the culture supernatant was collected. The concentration of TNF-α (Thermo Fisher Scientific, Yokohama, Japan) and IL-1β (Thermo Fisher Scientific) were determined by ELISA in according to the manufacturer's instruction.

RT-PCR

The primers used in this study listed as below:

P53 Forward primer: 5'-CCATCTACAAGAAGTCAC AACAC-3'

P53 Reverse primer: 5'-CCCAGGACAGGCACAAAC-3'

GAPDH Forward primer: 5'-CAGTGCCAGCCTCGTCT CAT-3'
GAPDH Reverse primer: 5'-AGGGGCCATCCACAGT CTTC-3'

The total RNA was extracted with Trizol reagent and the first strand cDNA was synthesized with PrimeScript RT reagent kit with gDNA Eraser following the manufacturer's instruction (TaKaRa Bio, Co. LTD, Dalian, China). The Q-PCR was conducted with CFX96 Real Time PCR detection system (Bio Rad, Hercules, CA, USA) with SYBR green II, and the reaction was set up in according to the directions. The PCR conditions were as following: 35°C pre-reaction for 15min, 95°C denature for 10 s, 62°C annealing &extension for 30 s by 36 cycles, 62°C extra extension for 45 s. The melting curve was plotted post-amplification and the relative expression was calculated by the 2-ΔΔ Ct method and normalized to GAPDH. Each measurement was triplicated independently.

Annexin V/PI staining assay

The cell apoptosis was determined by Annexin V/PI double staining method with FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Diego, USA) (Li *et al*., 2018). The FLS cells isolated from control and model rats were cultured in 6-well plate for 24h and subjected to 24 hours of SDP treatment. The cells were washed with PBS and digested with trpsin. After re-suspension in calcium-enriched HEPES buffer, the cells were stained with Annexin V-FITC and PI for 15 min and analyzed with flow cytometry (CyAn ADP9, Beckman Coulter, Fullerton, USA).

Western blot

The FLS cells from control and RA model rates were treated with SDP for 24h first. The total protein was extracted and quantified by Coomassie brilliant blue assay. Approximately 15μg of total protein was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes 2h on the ice. The membranes were blocked with 5% milk and hybridized with antibodies against Bax, Bcl-2(Dallas Area Kitefliers Organization), Caspase-3(Santa Cruz, USA), P53 (Santa Cruz), MMP-1(Santa Cruz), MMP-3(Santa Cruz), β-actin (Santa Cruz) overnight at 4°C. The membranes were washed 6 × 5min with TBST (Tris-buffered saline plus 0.05% Tween-20) at room temperature and incubated with secondary anti-rat IgG-horseradish peroxidase (1:2000; Santa Cruz Biotechnology) for 1h at room temperature. The membranes were washed 6×5min with TBST at room temperature and visualized by enhanced chemiluminiscence reagent according to the manufacturer's instructions (Thermo Scientific). The intensity of the individual bands was quantified by densitometry (Bio-Rad) and normalized to the corresponding input control (β-actin) bands (Dong *et al*., 2018).

STATISTICAL ANALYSIS

The Feature Extraction Software was employed for preprocessing analysis. The differential expression and statistical significance P value was calculated with Gene Spring GX Software. The P values were determined using T-test and $p<0.05$ was considered as significant difference.

Data from three independent experiments were subjected to variance analysis using SPSS19.0 software, and all the results were presented as Mean ± standard deviation (SD). One-way ANOVA method was employed for comparison analysis among multiple groups and S-N-K method for pair wise comparison. The statistical significances between data sets were expressed as p values and $p<0.05$ was considered statistically different.

RESULTS

The effect of SDP on proliferation and apoptosis of synovial fibroblast

We examined the cell viability by the classical MTT assay. In comparison with FLS, the proliferation rate of synovial fibroblast cells was much higher (fig. 1, $p< 0.01$). Notably, the synovial fibroblast cells were sensitive to SDP treatment. The relative cell viability was calculated as the percentage of control. When challenged with low, medium and high dose of SDP, the cell viability significantly declined from 231.29±21.02 to 208.37± 18.24, 177.19±15.89 and 152.44±11.35 respectively. The inhibitory effect manifested in a dose-dependent manner.

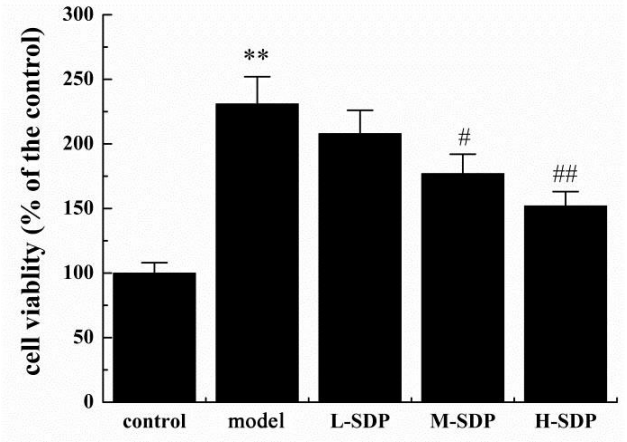

Fig. 1: Effect of SDP on proliferation of RAFLS.
The relative cell viability was determined by MTT assay in control and treatment groups. The proliferation of RAFLS cells was compared with FLS cells under normal culture conditions (**$p < 0.01$). In respect to treatment, three different dosages of SDP (L as Low, M as Medium, H as High) were applied. The medium (#$p < 0.05$) and high ((#$p < 0.05$) concentration of SDP treatment resulted in significant growth inhibition. Each bar is representative of at least three independent experiments.

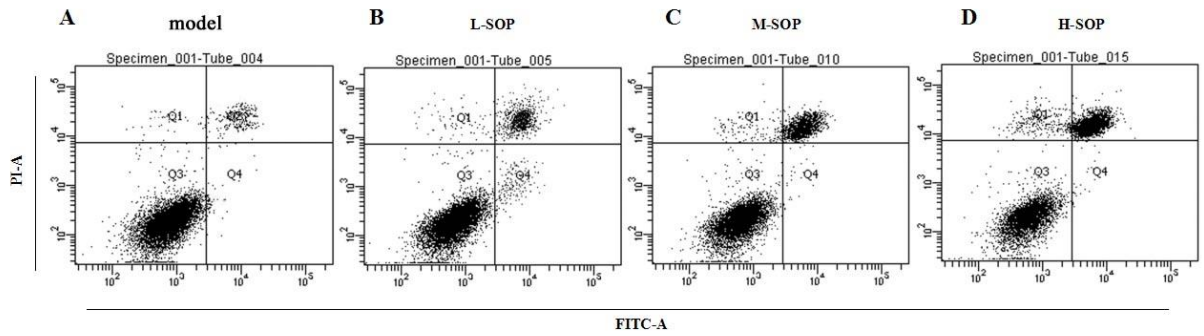

Fig. 2: Effect of SDP on apoptosis of RAFLS.
Representative scatter plots of bivariate Annexin-V/PI analysis of RAFLS cells dosed with SDP for 24h. Quadrant % gated in these plots identified different cell populations, i.e. section UL: Annexin-V-negative/PI-positive; UR: double positive, LL: double negative; LR: Annexin-V-positive/PI-negative. A, B, C, D indicated different concentrations of SDP respectively (blank, low, medium, high).

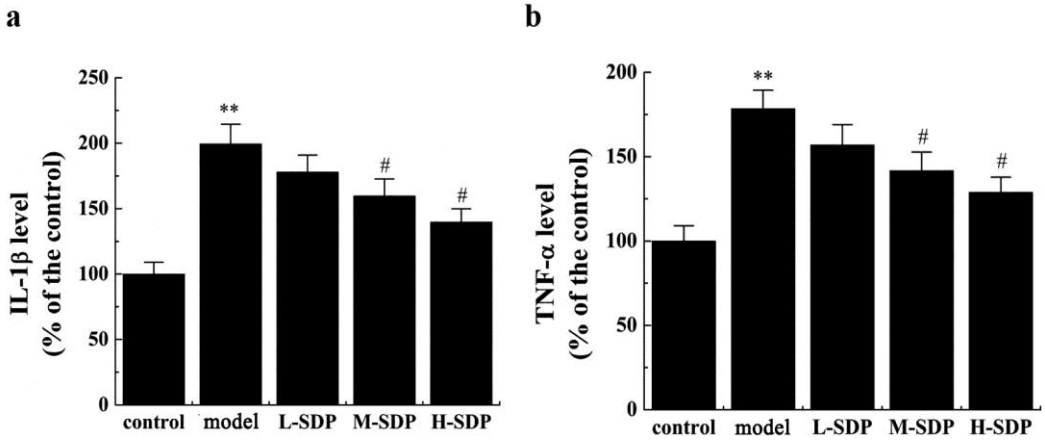

Fig. 3: SDP treatment affected secretion of inflammatory cytokines.
The contents of TNF-α and IL-1β in cell culture medium were determined by ELISA colorimetric method and relative concentrations were calculated. FLS and RAFLS cells were seeded in 96-well plates, followed by mock or SDP treatment for 24 hours. The supernatants were aspirated and subjected to ELISA reaction. Each bar represented at least three independent experiments. **p < 0.01 RAFLS vs. control; #p < 0.01 SDP vs. RAFLS.

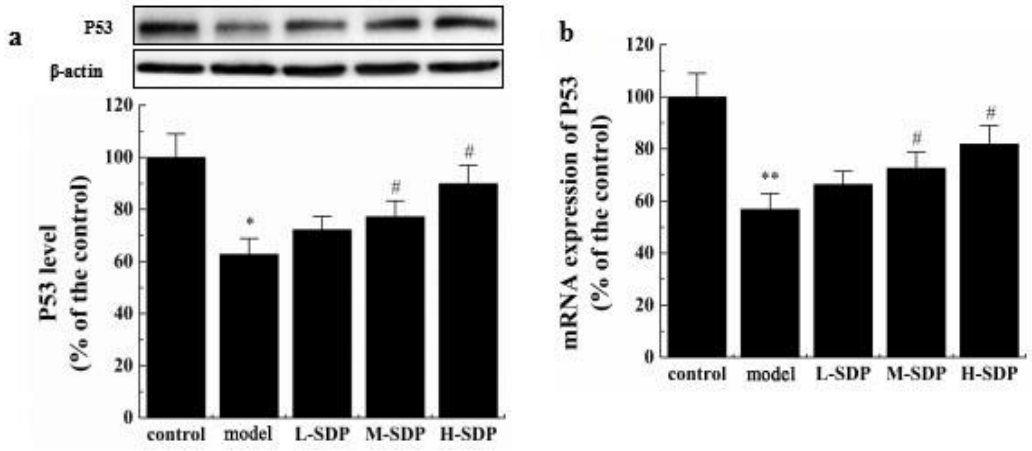

Fig. 4: Effect of SDP on the expression of p53.
The protein level of p53 was detected by western blotting. Total cell lysates were prepared properly from FLS and mock or SDP (Low, Medium, High dose for 24 h) treated RAFLS cells. The level of p53 was quantified by densitometry. Results are representative of at least three independent experiments. *p < 0.05 RAFLS vs. control; #p < 0.01 M-SDP/H-SDP vs. RAFLS.
Quantitative real-time PCR analysis of p53 and GAPDH in FLS and mock or SDP treated RAFLS cells. The relative expression was calculated by $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH. Data was presented as Mean ± SD from at least three independent experiments.

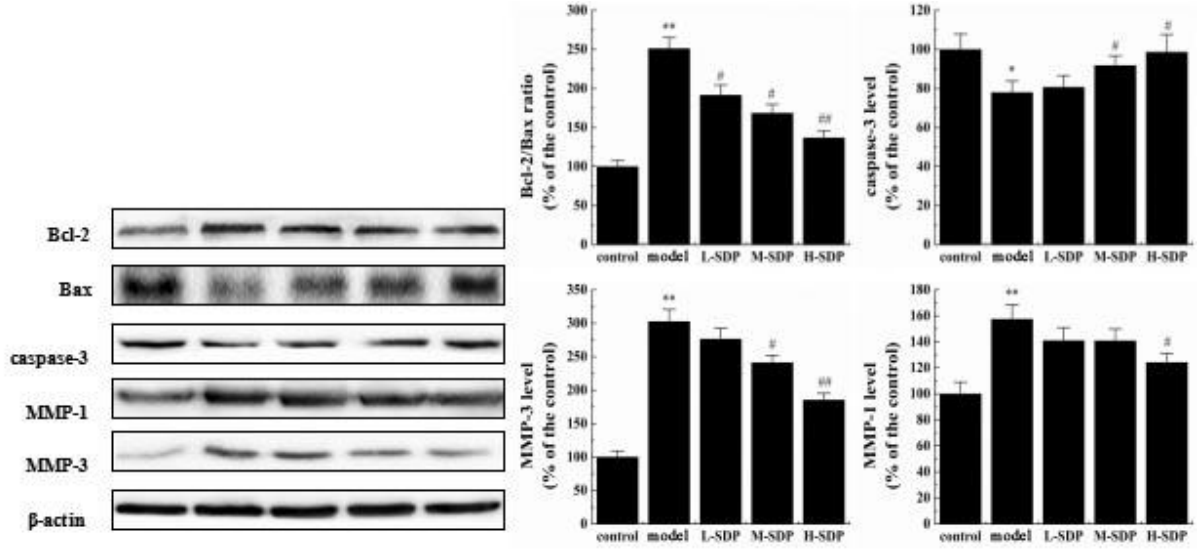

Fig. 5: SDP treatment affected expression of related factors in cell apoptosis and inflammation pathway.
The RAFLS cells were treated as mentioned before. The total cell lysates were prepared properly and analyzed with indicated antibody by western blotting. β-actin was included as internal reference. The band intensity was scanned and analyzed with ImageJ software. The relative expression was calculated and normalized, presented in bar plots. Each bar represented as Mean ± SD of at least three independent experiments. **p < 0.01 RAFLS vs. FLS; #p < 0.05 and ##p < 0.01 treated vs. mock in RAFLS cells.

We also analyzed the apoptosis induced by SDP in synovial fibroblast by flow cytometry. Representative data of Annexin V/PI staining results was shown in fig. 2 as scatter plots. The viable cells were Annexin-V and PI double negative while apoptotic cells were double positive. Our results demonstrated that high dose of SDP induced significant apoptosis, which is consistent with previous cytotoxicity data.

Effect of SDP on inflammatory cytokines

Next, we measured secretory inflammatory cytokines in the culture medium of synovial fibroblast versus FLS cells (fig. 3). The concentrations of TNF-α and IL-1β were detected by commercially available ELISA kit. In comparison to normal FLS, the cells from RA rat model produced more cytokines (**p<0.01). Treatment with SDP reduced the secretion of TNF-α and IL-1β in synovial fibroblast cells to different extent. In addition, the suppression effect was positively correlated with dosage of SDP. In our experiment, the level of TNF-α and IL-1β in cell-culture medium nearly dropped back to normal level with high concentration of SDP treatment.

SDP induced expression of p53

We monitored the expression of p53 given that SDP could induce significant cell death. Both transcript and protein level of p53 were measured and normalized to β-actin (fig. 4). The endogenous expression of p53 in synovial fibroblast cells was much lower than FLS cells, which is consistent with its active proliferation. However, the expression of p53 was significantly induced upon SDP treatment in a dose-dependent manner. Especially the high dosage of SDP elevated p53 to a comparable level with control. It's highly likely that SDP enhanced transcription of p53 through currently unknown mechanism, which warranted further investigation.

SDP treatment impact expression of related factors in apoptosis and inflammation pathway

Our previous results showed that SDP treatment elicited extreme suppression on synovial fibroblast via induction of apoptosis and inhibition of inflammation. Here we sought to examine the expression of related factors in the aforementioned pathways. The Bcl-2, Bax, Caspase-3, MMP-1 and MMP-3. The FLS and synovial fibroblast cells were isolated from control and RA model rats respectively. The latter was exposed to different dosage of SDP and total protein was extracted and quantified. The immunoblotting results in fig. 5 showed that either the ratio between bcl-1/bax and endogenous expression of MMP-1/3 was higher in synovial fibroblast in comparison to normal FLS cells (p<0.01). Rather, the apoptosis executive protein caspase-3 was much lower (p<0.05), which was in agreement with its high proliferative rate. Post-treatment with SDP, the expression of caspase-3 was significantly elevated, while bcl-2/bax ratio and MMP-1/3 level dramatically declined. The altered expression of these factors indicated that SDP could induce intensive apoptotic signal in synovial fibroblast.

DISCUSSION

In this study, we established and characterized RA model in rats with classical composite method. FLS cells were isolated from both normal and RA rats. The inhibitory effect and associated underlying molecular mechanism of

home-prepared SDP on synovial fibroblast cells were investigated. Our results unambiguously demonstrated that SDP treatment suppressed RA proliferation and induced apoptotic pathway activation. Exposure to SDP increased p53 expression at both transcript and protein level. Concomitantly, apoptotic indicators such as endogenous caspase-3 and bax/bcl-2 ratio were significantly increased upon SDP treatment. The physiological secretion of inflammatory factors TNF-α and IL-1β were determined in the culture medium by ELISA method from RLS. Beyond induction of apoptosis, our results indicated that SDP treatment could inhibit inflammation pathway by reduction TNF-α and IL-1β production simultaneously. In addition, extra cellular metal proteases MMP-1/3 was dramatically decreased. Annexin-V/PI double staining followed by flow cytometry analysis showed SPD greatly promoted cell death in synovial fibroblast cells. Thus, SDP suppressed dysplasia of synovial fibroblast cells by activation of apoptotic signaling. At the same time, aberrant activity of inflammation response was inhibited through modulating production of key factors.

RA is one of the most prevalent chronic inflammatory disease. Although not immediately lethal to the patients, it's a severe threat to life quality related to inconvenience and disability. Various vulnerable groups suffered from it (Tai and Cheung, 2007; Liang and Zhu, 2015). The chronic disease also decreased the life expectance significantly, which usually associated with comorbidity. Thus, exploitation of cost-effective and hypotoxic therapeutics is still the intensive field of research. Limitation of clinical management also involves the lack of definite prognostic criteria and specific early biomarker, which greatly delayed timely intervention and therapeutic efficacy. Clinically RA is regarded as autoimmunity disease. It's reported that autoantibodies presented in blood 10 years before manifestation of noticeable symptoms, which suggests that long period of development phase and wide window for early diagnosis and intervention. Development of highly specific and sensitive methods for gold standard of diagnosis is in urgent need.

Pathologically RA is heterogeneous and numerous lymphocytes involved in pathogenesis of RA, such as T cells, B cells, macrophages and especially fibroblast-like synoviocytes in the synovial intimal lining. Dysplasia and hypercellularity in joints are majorly due to over-proliferation of both macrophage- and fibroblast-like cells. The latter was activated by the signal molecules secreted by the former and then produced own repertoire of effectors. The intertwined autocrine and paracrine network exacerbated synovitis. Hyperplasia of FLS cells participate in local destruction either by activation of osteoclasts or direct production of proteases. Some Disease-modifying anti-rheumatic drugs DMARDs drugs targeting aberrant signaling pathway in FLS cells have been explored for clinical applications such as Janus kinases (JAK) inhibitors such as ofacitinib or baricitinib (Eli Lilly, USA) (Kremer *et al*., 2009; Lee *et al*., 2014), spleen tyrosine kinase (Syk) inhibitor fostamatinib (Rigel Pharmaceuticals, USA) (Weinblatt *et al*., 2008). The biological and targeted synthetic DMARDs hold more therapeutic promising, while induced more adverse effects than conventional synthetic DMARDs. Complications related such as serious infection and tuberculosis imposed extra medical care.

SDP is an essential ingredient for many TCMs formulae (Chun *et al*., 2016). Tai and Cheung have reported anti-proliferative and antioxidant activities of SDP in cancer cells K562, HL60, MCF7 and MDA-MB-468 in vitro and implicated potential therapeutic value of SDP in tumor patients. However, this study was limited by in vitro culture, animal tumor model was definitely necessary for further investigation. Wang's study demonstrated that SDP could inhibit production of NO by modulating expression of iNOS protein (Wang *et al*., 1999). In view of the key role of NO in inflammation conditions (Predonzani *et al*., 2015), it's highly likely that SPD functioned in our study mediated by the same mechanism, which warranted further characterization. Moreover, SDP treatment could potentiate reticuloendothelial function based on another two independent studies, suggested a broad pharmaceutical spectrum of SDP (Shimizu *et al*., 1989). SDP was used to dispel "wind" for a long history. However, the molecular mechanism underlying its clinical effectiveness is really elusive, which impede further optimization and development in the context of TCM modernization (Zhang and Zhang, 2015).

CONCLUSION

It is demonstrated that SDP induced apoptosis and suppressed proliferation of FLS cells from RA model rat in vitro, and reduced production of both inflammatory factors and metalloproteases simultaneously. In view of long history of TCMs in treatment of RA, our study elucidated a potential mechanism in support of clinical usage of SDP for this disease.

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