

Anti-inflammatory effect of Man-Pen-Fang, a Chinese herbal compound, on chronic pelvic inflammation in rats

Li-Jun Zhang^{a†}, Jian-Yong Zhu^{a†}, Meng-Yao Sun^a, Ya-Nan Song^a, Khalid Rahman^b,
Cheng Peng^c, Miao Zhang^a, Yu-Mei Ye^{d*}, Hong Zhang^{a*}

^a Central Laboratory, Seventh People's Hospital of Shanghai University of TCM, Shanghai 200137, China

^b School of Pharmacy and Biomolecular Sciences, Faculty of Science, Liverpool John Moores University, Liverpool L3 3AF, England, UK

^c Key Laboratory of Standardization of Chinese Herbal Medicines of Ministry of Education, Pharmacy College, Chengdu University of Traditional Chinese Medicine, Chengdu 610075, China

^d Department of Traditional Medical Science, Seventh People's Hospital of Shanghai University of TCM, Shanghai 200137, China

[†] These authors contributed equally to this work.

Corresponding authors

* Hong Zhang and Yu-Mei Ye, E-mail addresses: hqzhang51@126.com (HZ), yeyumei0206@163.com (YMY)

Abstract

Ethnopharmacological relevance: Traditional Chinese Medicine (TCM) has become the focus of research for the treatment of chronic pelvic inflammatory disease (CPID) based on unique medical theory system. Man-Pen-Fang (MPF), a Chinese herbal compound, which is composed of *Thlaspi arvense* L. (Cruciferae), *Gleditsia sinensis* Lam. (Leguminosae), *Smilax china* L. (Liliaceae), *Euonymus alatus* (Thunb.) Sieb. (Celastraceae) and *Vaccaria segetalis* (Neck.) (Caryophyllaceae) MPF has been used for the treatment of CPID and exerted significant clinical curative effects. However, the corresponding active principles and anti-inflammatory mechanism of MPF are still unknown.

Aim of the study: The objective of present study is to evaluate the effect of MPF on CPID in the chronic pelvic inflammation (CPI) rat model and elucidate its possible anti-inflammatory mechanism.

Materials and Methods: The CPI in rats was induced by administration with *E. coli*, *Staphylococcus aureus* and Beta-hemolytic streptococcus. MPF (8.112 g/(kg.d) (20 times of adult dosage), 4.056 g/(kg.d) (10 times of adult dosage) and 2.028 g/(kg.d) (5 times of adult dosage)) and Jingangteng Capsule 2 g/(kg.d) (20 times of adult dosage) were administered orally for 20 days. The serum levels of five inflammation-associated cytokines (IL-2, IL-6, IL-10, TNF- α and TGF- β_1) were determined by enzyme-linked immunoassay, and the mRNA expression levels of TGF- β_1 , P53, Fas, FasL and MMP-2 in the uterus tissue were measured by quantitative RT-PCR. Furthermore, the expression of NF- κ B p65 in uterus and ovary tissues was detected by immunohistochemistry assay and the pathological changes induced in the uterus and ovary tissues were observed by histology.

Results: MPF caused a reduction in serum levels of IL-2, IL-6, IL-10, TNF- α and TGF- β_1 . The expression of P53 mRNA, Fas/FasL mRNA and MMP-2 mRNA in the uterus tissue was significantly elevated after treating with MPF, in contrast the expression of TGF- β_1 mRNA was decreased. Furthermore, the expression of NF- κ B p65 in uterus and ovary tissue was inhibited after treating with MPF.

Conclusions: These results taken together suggest that MPF has a significant

anti-CPID effect, probably due to inhibition of the inflammation reaction by the promotion, and the induction of the apoptosis of inflammatory cells and downregulation of the serum levels of inflammation cytokines.

Key words: Chronic pelvic inflammatory disease, anti-inflammatory, apoptosis, mechanism, Man-Pen-Fang

1. Introduction

Pelvic inflammatory disease (PID) is an infection and inflammation of the uterus, ovaries, and other female reproductive organs and is one of the most common infections in non-pregnant women of reproductive age and remains an important public health problem (Loucks, 1983; Snaith, 1959). It can lead to long-term sequelae, such as infertility, ectopic pregnancy, pelvic pain, abscesses, and other serious problems (Cassell et al., 1997). These problems are called sequelae of pelvic inflammatory disease (SPID) or chronic pelvic inflammatory disease (CPID), which seriously influence the health of women and the quality of life and increases the economic burden of family and society (Barrett and Taylor, 2005; Gradison, 2012). The sexually transmitted organisms, *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are present in many cases and microorganisms comprising the endogenous vaginal and cervical flora are frequently associated with PID. These include anaerobic and facultative bacteria, similar to those associated with bacterial vaginosis (Burnakis and Hildebrandt, 1986; Duarte et al., 2015). Some women have no symptoms while others have pain in the lower abdomen, fever, foul smelling vaginal discharge, irregular bleeding, and pain during intercourse or urination (Haggerty et al., 2005). Early treatment is important as waiting too long increases the risk of infertility.

There are many disadvantages of antibiotics and surgical treatment for CPID, hence Traditional Chinese Medicine (TCM) has become the focus of research for the treatment of CPID. There is no definition of CPID in TCM. And in the theory of TCM, CPID is considered to be caused by cold and dampness damage to the uterus and

uterine collateral leading to Qi obstruction, stagnation of blood stasis, recurrent and refractory lingering (Chen, 2012; Liu et al., 2014; Bu et al., 2015). The main pathogenesis of CPID is stagnation of blood stasis. Thus, promoting and dredging the channel circulation and removing stasis are principles of the treatment for CPID (Liu et al., 2014).

A Chinese herbal compound, Man-Pen-Fang (MPF), which is composed of *Thlaspi arvense* L. (Cruciferae), *Gleditsia sinensis* Lam. (Leguminosae), *Smilax china* L. (Liliaceae), *Euonymus alatus* (Thunb.) Sieb. (Celastraceae) and *Vaccaria segetalis* (Neck.) (Caryophyllaceae), as shown in **Table 1** is effective in dispersing blood stasis and dredging collaterals. In this Chinese medicine formula, *Thlaspi arvense* L. is recorded in Shen Nong's Herbal Classic and is reported to clear heat, is an antitoxicant, activates blood, and resolves stasis. Compendium of Materia Medica indicates that *Gleditsia sinensis* Lam. has effect on removing toxicity for detumescence (Kim et al., 2015; Li et al., 2016). *Smilax China* L. is good at dispelling wind-damp, reducing swelling and activating blood circulation to dissipate blood stasis (Lee et al., 2001; Shah et al., 1962). *Euonymus alatus* (Thunb.) Sieb. activates blood to promote menstruation and *Vaccaria segetalis* (Neck.) has analgesic and anti-inflammatory effects. All these drugs have a role in activating blood circulation to dissipate blood stasis, clearing heat as an antitoxicant, reducing swelling and analgesia.

Table 1 The composition of Man-Pen-Fang (MPF)

Scientific name	Chinese name	Weight (g)	%
<i>Thlaspi arvense</i> L. (Cruciferae)	Baijiangcao	30	20
<i>Gleditsia sinensis</i> Lam. (Leguminosae)	Zaojiaoci	30	20
<i>Smilax china</i> L. (Liliaceae)	Baqia	30	20
<i>Euonymus alatus</i> (Thunb.) Sieb. (Celastraceae)	Guijianyu	30	20
<i>Vaccaria segetalis</i> (Neck.) (Caryophyllaceae)	Wangbuliuxing	30	20
Total amount		150	100

MPF has a history of clinical use for the treatment of CPID more than 20 years and has a significant curative effect (Ye, 2009). Herein, CPI model in rats was established to evaluate the anti-inflammatory effect of MPF on CPID. In order to explore the possible mechanism of MPF, the serum levels of five inflammatory cytokines (IL-2, IL-6, IL-10, TNF- α , and TGF- β_1) were measured by Elisa assay followed by the measurement of mRNA expression levels of TGF- β_1 , P53, Fas, FasL, and MMP-2 in uterus tissue by quantitative RT-PCR. Furthermore, the expression of NF- κ B p65 in uterus and ovary tissues was measured by immunohistochemistry assay and finally the tissues were examined histologically by staining with haematoxylin and eosin to ascertain the effect of MPF on CPID.

2. Materials and Methods

2.1 Bacterial strains and Animals

E. coli (ATCC25922) and *Staphylococcus aureus* (ATCC29213) were obtained from bacteriological laboratory of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine. Beta-hemolytic streptococcus (CMCC32210) was purchased from Beina Chuanglian biotechnology research institute. The three bacterial strains were cultured by the conventional bacterial cultivation method. Female Wistar rats, 6-8 weeks old were obtained from animal laboratory of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine and were kept at room temperature.

2.2 Preparation of Man-Pen-Fang

The crude drugs of Man-Pen-Fang were purchased from Shanghai Kangqiao Chinese herbal pieces co., LTD. All the herbs were prepared in proportion by soaking in 8 times the weights of pure water and were extracted twice by refluxing for 1 h. The filtrate was merged, concentrated under vacuum and the moisture was removed by placing the samples in an oven at 50 °C. The extract was then used for the following experiments.

2.3 HPLC/ESI-MS and Fingerprint Analysis

An Agilent 1100 HPLC system, equipped with a quaternary pump, an autosampler, a degasser, an automatic thermostatic column compartment, a DAD and an LC/MSD Trap XCT ESI mass spectrometer (Agilent Technologies, MA, USA), was used for the analysis of the herb. The separation was performed on a GS-120-5-C18-BIO chromatographic column (5 μ m, 250 \times 4.6 mm i.d.) with the column temperature set at 35°C. A linear gradient elution of A (0.1% formic acid water) and B (acetonitrile) was used with the gradient procedure as follows: 0 min, B 5%, to 60 min B 40% (v/v). The flow rate was 1.0 mL/min and the injection volume was 10 μ L. DAD was on and the target wavelength was simultaneously set at 210 nm. The split ratio to the mass spectrometer was 1:3. The acquisition parameters for negative ion mode were: collision gas, ultra high-purity helium (He), nebulizer gas (N₂), 35 psi, drying gas (N₂), 10 L/min, drying temperature, 350°C, HV, 3500 V, mass scan range, m/z 100-2200, target mass, 500 m/z, compound stability, 100%, trap drive level, 100%. All the data were analyzed by Chemstation software.

2.4 The establishment of CPID model in rats and Animal treatment

The oestrus of female Wistar rats was identified by vaginal smear for continuous 3 cycles. Only those with regular cycles were allowed to continue the next experiments. Suitable female Wistar rats were anesthetized with an intraperitoneal injection of 10 % chloral hydrate and the endometrial tissue was damaged with a syringe, followed by administration of a mixture of *E. coli*, *Staphylococcus aureus* and Beta-hemolytic streptococcus of 9×10^5 IFUs in 0.15 mL distilled water to the uterus on the right hand side (Tuffrey et al., 1992; Chen Ying, 2008;). *E. coli*, *Staphylococcus aureus* and Beta-hemolytic streptococcus were in a ratio of 2:1:1.

Seven days after infection, successfully infected rats were randomly divided into 5 groups (n=10) as following: the model group, positive group, and high-dose, mid-dose and low-dose MPF groups (MPF-H, MPF-M and MPF-L). 10 female Wistar rats did not receive any treatment and served as the control group. The drug was intragastrically administrated to the rats for 20 days, and the distilled water was used as control. The rats in the control group and the model group received distilled water in the same volume; in the positive group, Jingangteng Capsule 2 g/(kg.d) (20 times

of adult dosage) was dissolved in distilled water; in the MPF-H, MPF-M and MPF-L groups, MPF was dissolved at 8.112 g/(kg.d) (20 times of adult dosage), 4.056 g/(kg.d) (10 times of adult dosage) and 2.028 g/(kg.d) (5 times of adult dosage), respectively.

2.5 Elisa assay

The rats were anesthetized with an intraperitoneal injection of 1.5 % pentobarbital sodium (40 mg/kg), blood was collected by abdominal aortic method followed by centrifugation at 3000 rpm for 10 min. The plasma was analysed for interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor alpha (TNF- α) and transforming growth factor beta 1 (TGF- β ₁) level by using a commercially available enzyme-linked immunosorbent assay (ELISA). ALL the Elisa kits were purchased from Shanghai Maiye biological technology co., Ltd. From each plasma sample, 50 μ L was directly transferred to the microtest strip wells of the ELISA plate and then assayed according to the manufacturer's instructions. Absorbance was measured at 450 nm in a microtest plate spectrophotometer.

2.6 RNA extraction, reverse transcription and quantitative RT-PCR

The rats were anesthetized with an intraperitoneal injection of 1.5 % pentobarbital sodium (40 mg/kg). The uterus tissues were chosen for RNA extraction. Total RNA was extracted by TransZol Up reagent (TransGen Biotech, China) and 2 μ g of total RNA was reversely transcribed to cDNA with oligo (dT) primer using the TransScript First-Strand cDNA Synthesis Supermix Reagent Kit (TransGen Biotech, China). Quantitative RT-PCR was performed using a TransStart Top Green qPCR SuperMix Reagent Kit (TransGen Biotech, China) protocol on lightcycler96 (Roche, Switzerland) thermal cycler according to manufacturer's instruction. GAPDH mRNA levels were used for normalization. The primers sequences (sense/antisense) were provided by Sangon Biotech (Shanghai) Co., Ltd. and list in **Table 2**.

2.7 Histology

The uterus and ovary tissues were dehydrated and embedded in paraffin, and 4-7 μ m sections were deparaffinized and stained sequentially with haematoxylin and eosin (H&E, BASO, China). Stained tissue sections on slides were analyzed under identical light microscope (D5100, NIKON, Japan) at \times 400 magnifications.

Table 2 Primers sequences used in this study

Gene	Sequences (5'-3')
GAPDH-forward	GGTCGGTGTGAACGGATTTG
GAPDH-reverse	CGTTGAACTTGCCGTGGGTA
FAS- forward	TTGCTGTCAACCGTGTTCAGC
FAS- reverse	CCACTTCTAAACCATGCCCT
FASL- forward	CACCAACCACAGCCTTAGAG
FASL- reverse	CCAGAGATCAAAGCAGTTCC
MMP2- forward	AAGCTCATCGCAGACTCCTGG
MMP2- reverse	CAGCCAGTCCGATTTGATGC
P53- forward	ACCAGCACAAGCTCCTCTC
P53- reverse	TCTTGGTCTTCGGGTAGCTG
TGF- forward	AAGGAGACGGAATACAGGGC
TGF- reverse	CAGGTGTTGAGCCCTTTCCA

2.8 Immunohistochemistry

The uterus and ovary tissues were treated for deparaffinization and hydration and then heated in EDTA (pH = 8.0) and incubated with 3 % hydrogen peroxide for 10 min for antigen retrieval. The reaction of NF- κ B p65 rabbit monoclonal antibody (Abcam, USA) was allowed to take place for 1 h at room temperature, followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated IgG (Shanghai Long Island Biotec.Co., Ltd., China). Slides were stained with DAB (Shanghai Long Island Biotec.Co., Ltd., China) and hematoxylin staining (BASO, China). Immunohistochemical signals were calculated with the positive staining cells and stained tissue sections on slides were analyzed under identical light microscope at $\times 400$ magnifications.

2.9 Statistical analysis

All experiments were performed in triplicate and the data was expressed as mean \pm SD. Analysis of variance (ANOVA) and Student's t-test were used to determine the statistically significant difference among different groups where appropriate. All statistical analyses were two-sided and performed using the Statistical Package for the Social Sciences software version 16.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

2.10 Statement

The experiments were carried out in accordance with the guidelines issued by the Experimental Animal Ethical Committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine (EAEC/SPH#46/2015).

3. Results

3.1 Identification of Compounds

The aqueous extract of a mixture of five Chinese medicinal herbs was analysed by high-performance liquid chromatography coupled with electrospray mass spectrometry (HPLC/ESI-MS) in negative and positive-ion mode (**Figure 1**). Eight compounds **1 - 8** (**Figure S1 - S8**), with the retention time at 0.68 min, 4.54 min, 4.67 min, 4.90 min, 5.14 min, 5.49 min, 7.27 min, and 3.95 min, were identified as hydroferulic acid (**1**), kaempferol-3-O-rutinoside (**2**), (-)-catechin (**3**), vaccarin (**4**), isoorientin (**5**), isovitexin (**6**), segetalin A (**7**), and (+)-usnic acid B (**8**), on the basis of the observation of the pseudomolecular ion peak at m/z 195.0491 $[M-H]^-$ (**1**), m/z 593.1503 $[M-H]^-$ (**2**), m/z 289.0699 $[M-H]^-$ (**3**), m/z 725.1927 $[M-H]^-$ (**4**), m/z 447.0920 $[M-H]^-$ (**5**), m/z 431.0970 $[M-H]^-$ (**6**), m/z 608.3200 $[M-H]^-$ (**7**), m/z 367.1506 $[M+Na]^+$ (**8**), in HPLC/ESI-MS chromatogram, in accordance with the molecular weights of eight compounds (hydroferulic acid, kaempferol-3-O-rutinoside, (-)-catechin, vaccarin, isoorientin, isovitexin, segetalin A, and (+)-usnic acid B). A total of 8 compounds were unambiguously identified by comparing the retention times and the MS data with the reference standards.

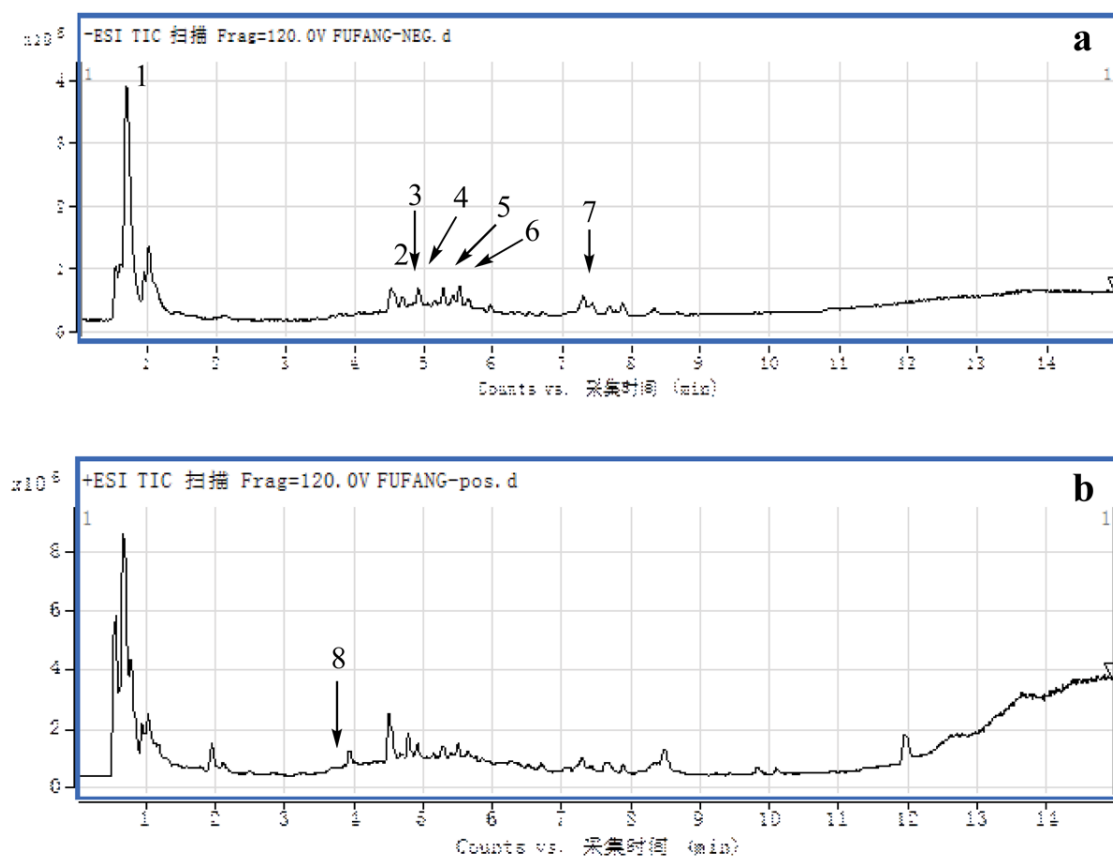


Figure 1 HPLC/ESI-MS chromatogram of the aqueous extract in negative and positive mode (a and b)

3.2 Effects of MPF on the serum levels of IL-2, IL-6, IL-10, TNF- α and TGF- β_1 of CPID in rats

In order to investigate the effect of MPF on the regulation of immune system, the levels of pro-inflammatory cytokines (IL-6 and TNF- α) and anti-inflammatory cytokines (IL-2, IL-10, and TGF- β_1) were analyzed in plasma samples by Elisa assay. The results are presented in **Figure 2** and **Table 3**. The serum levels of IL-2, IL-6, IL-10, TNF- α , and TGF- β_1 were significantly elevated in the CPID model group when compared with those in the control group ($P < 0.01$). These serum levels decreased in the positive, MPF-H, MPF-M, and MPF-L groups compared with those in the model group. These serum levels in MPF-H group were lower than those in the positive group, and similar to those in control group. In the MPF-H, MPF-M, and MPF-L groups, the significant differences were dependent on the dose of MPF. The higher the dose of MPF, the more significant was the differences.

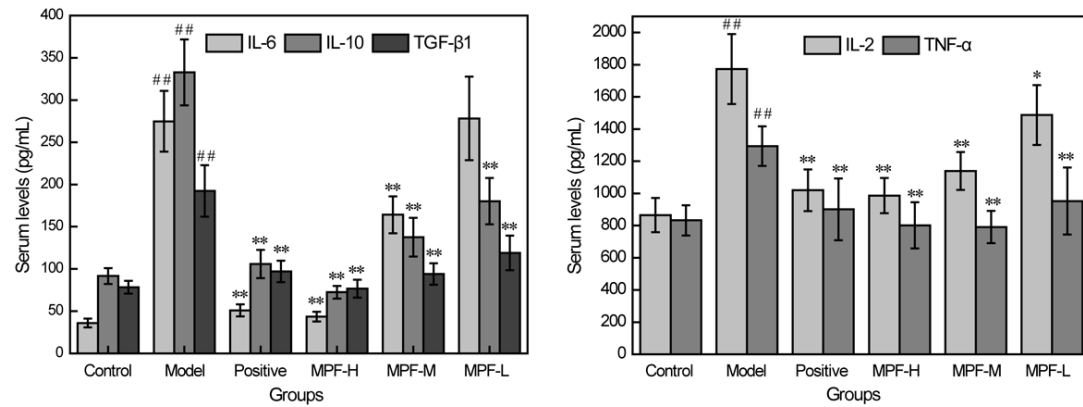


Figure 2 The serum levels of IL-2, IL-6, IL-10, TNF- α , and TGF- β_1 . The serum levels were determined by Elisa assay in the control, model, positive, MPF-H, MPF-M and MPF-L groups. The results represent means \pm SD of three independent experiments (n=10). ##P < 0.01, control versus model, **P < 0.01, *P < 0.05, model versus positive, MPF-H, MPF-M and MPF-L.

Table 3 The serum levels of IL-2, IL-6, IL-10, TNF- α , and TGF- β_1 (pg/mL)

Groups	IL-2	IL-6	IL-10	TNF- α	TGF- β_1
Control	864.73 \pm 106.30	35.96 \pm 5.24	91.65 \pm 9.41	832.45 \pm 94.64	78.40 \pm 7.60
Model	1773.26 \pm 217.84##	274.78 \pm 35.95##	332.77 \pm 39.06##	1294.00 \pm 122.44##	192.43 \pm 30.47##
Positive	1020.04 \pm 129.64**	50.96 \pm 7.21**	105.93 \pm 16.65**	900.76 \pm 192.14**	97.07 \pm 12.75**
MPF-H	986.31 \pm 109.87**	43.52 \pm 5.74**	72.39 \pm 7.44**	800.90 \pm 143.41**	76.59 \pm 10.65**
MPF-M	1139.47 \pm 117.80**	164.20 \pm 21.88**	137.53 \pm 22.93**	790.92 \pm 100.33**	93.97 \pm 12.78**
MPF-L	1487.33 \pm 185.93**	278.26 \pm 49.47	180.29 \pm 27.54**	952.47 \pm 208.52*	118.97 \pm 20.43**

Results represent means \pm SD of three independent experiments (n=10). ##P < 0.01, control versus model, **P < 0.01, *P < 0.05, model versus positive, MPF-H, MPF-M, MPF-L.

3.3 Effects of MPF on the expression of TGF- β_1 mRNA, P53 mRNA, Fas/FasL mRNA and MMP-2 mRNA in uterus of CPID rats

The relative mRNA levels of TGF- β_1 , P53, Fas/FasL, and MMP-2 in uterus were measured by quantitative RT-PCR to investigate the effect of MPF on the expression

of apoptosis gene. The results are presented in **Figure 3** and **Table 4**. The levels of TGF- β_1 mRNA, P53 mRNA, Fas/FasL mRNA, and MMP-2 mRNA as expected were higher in the CPID model group than those in the control group ($P < 0.01$ & $P < 0.05$). The expressions of P53 mRNA, Fas/FasL mRNA, and MMP-2 mRNA were increased in the positive, MPF-H, MPF-M, MPF-L groups when compared with those in the model group. The expression of TGF- β_1 mRNA was lower in the positive, MPF-H, MPF-M, MPF-L groups than the levels observed in the model group. The significant differences were more common in the MPF-H and MPF-M groups, and these differences were dose-dependent.

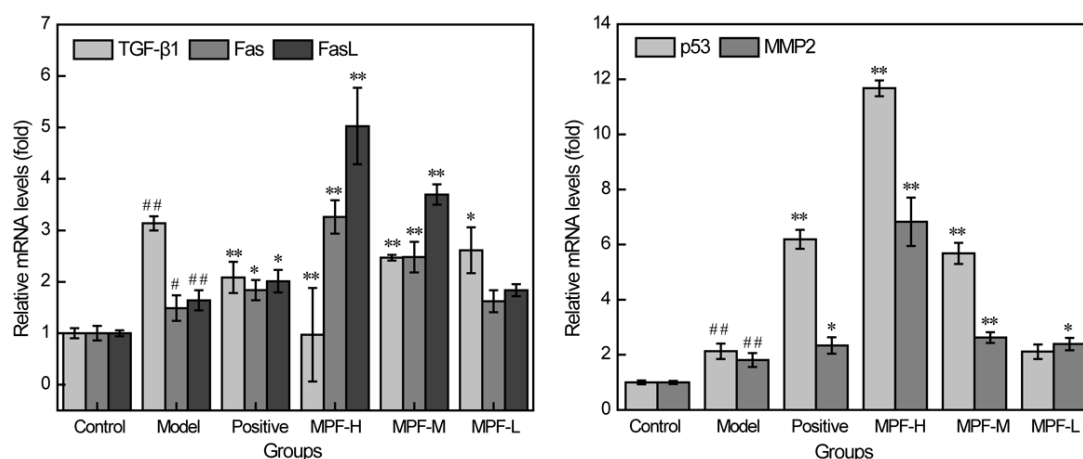


Figure 3 The relative mRNA levels of TGF- β_1 , P53, Fas, FasL and MMP2. These relative mRNA levels were detected by quantitative RT-PCR in uterus tissues from the control, model, positive (*Smilax china* L. Capsule), MPF-H, MPF-M and MPF-L groups. The results represent means \pm SD of three independent experiments ($n=10$). ## $P < 0.01$, control versus model, ** $P < 0.01$, * $P < 0.05$, model versus positive, MPF-H, MPF-M and MPF-L.

Table 4 Relative mRNA levels of TGF- β_1 , P53, Fas, FasL, and MMP2

Groups	TGF- β_1	P53	Fas	FasL	MMP2
Control	1.00 \pm 0.099	1.00 \pm 0.07	1.00 \pm 0.14	1.00 \pm 0.06	1.00 \pm 0.06
Model	3.14 \pm 0.14 ^{##}	2.13 \pm 0.28 ^{##}	1.49 \pm 0.25 [#]	1.64 \pm 0.20 ^{##}	1.81 \pm 0.25 ^{##}
Positive	2.084 \pm 0.304 ^{**}	6.20 \pm 0.34 ^{**}	1.84 \pm 0.20 [*]	2.01 \pm 0.22 [*]	2.34 \pm 0.30 [*]
MPF-H	0.97 \pm 0.91 ^{**}	11.67 \pm 0.29 ^{**}	3.26 \pm 0.32 ^{**}	5.03 \pm 0.74 ^{**}	6.83 \pm 0.88 ^{**}
MPF-M	2.47 \pm 0.06 ^{**}	5.68 \pm 0.38 ^{**}	2.48 \pm 0.30 ^{**}	3.70 \pm 0.20 ^{**}	2.63 \pm 0.19 ^{**}
MPF-L	2.62 \pm 0.45 [*]	2.14 \pm 0.26	1.62 \pm 0.21	1.84 \pm 0.12	2.39 \pm 0.22 [*]

Results represent means \pm SD of three independent experiments (n=10). ^{##} P < 0.01, control versus model, ^{**}P < 0.01, ^{*}P < 0.05, model versus positive, MPF-H, MPF-M, MPF-L.

3.4 Effects of MPF on the pathologic structures of uterus and ovary tissues of CPID rats

The changes of pathology of uterus and ovary tissues were observed by histology staining with H&E to confirm the effect of MPF on CPID. **Figure 4A** represents the histological images observed in the uterus tissues stained with H&E. In the control group, the chamber walls were clear, the uterus gland structure was normal, and there was no hyperemia and edema. While in the model group, the lamina propria became thinner or disappeared, the chamber wall structures were disordered, many chronic inflammatory cells were observed, and hyperemia and edema was obvious in the endometrium. In the positive group, there was still some chronic inflammatory cells present, and the endometrium was partly in hyperemia and edema. In the MPF-H group, the mucosal epithelial cells were ranked neatly, the hyperemia and edema in endometrium was significantly decreased, and the uterine lesions were almost reversed to normal. In the MPF-M and MPF-L groups, the uterine lesions were reversed back to normal to a certain extent. **Figure 4B** represents the histological images of ovary tissues stained with H&E. In the control group, the structure of ovary was clear, and the granular cells had normal morphology. In the model group, the atretic follicle was damaged, granular cells were ranked in disorder and the structure

was loose. In the positive, MPF-H, MPF-M, and MPF-L groups, the damage to the ovarian lesions was reversed to a certain extent. The structure of granular cells became close and the atretic follicle damage was reversed.

3.5 Effects of MPF on the expression of NF- κ B p65 in uterus and ovary tissues of CPID rats

To verify the changes of the protein levels, the expression of NF- κ B p65 in uterus and ovary tissues were measured by immunohistochemistry and the results were shown in **Figure 5**. The expression of NF- κ B p65 in uterus and ovary tissues in the model group was significantly higher than that in the control group ($P < 0.01$). An obvious reduction of NF- κ B p65 level was observed in the positive, MPF-H, MPF-M, and MPF-L groups. In the MPF-H, MPF-M, and MPF-L groups, the differences observed were dose-dependent.

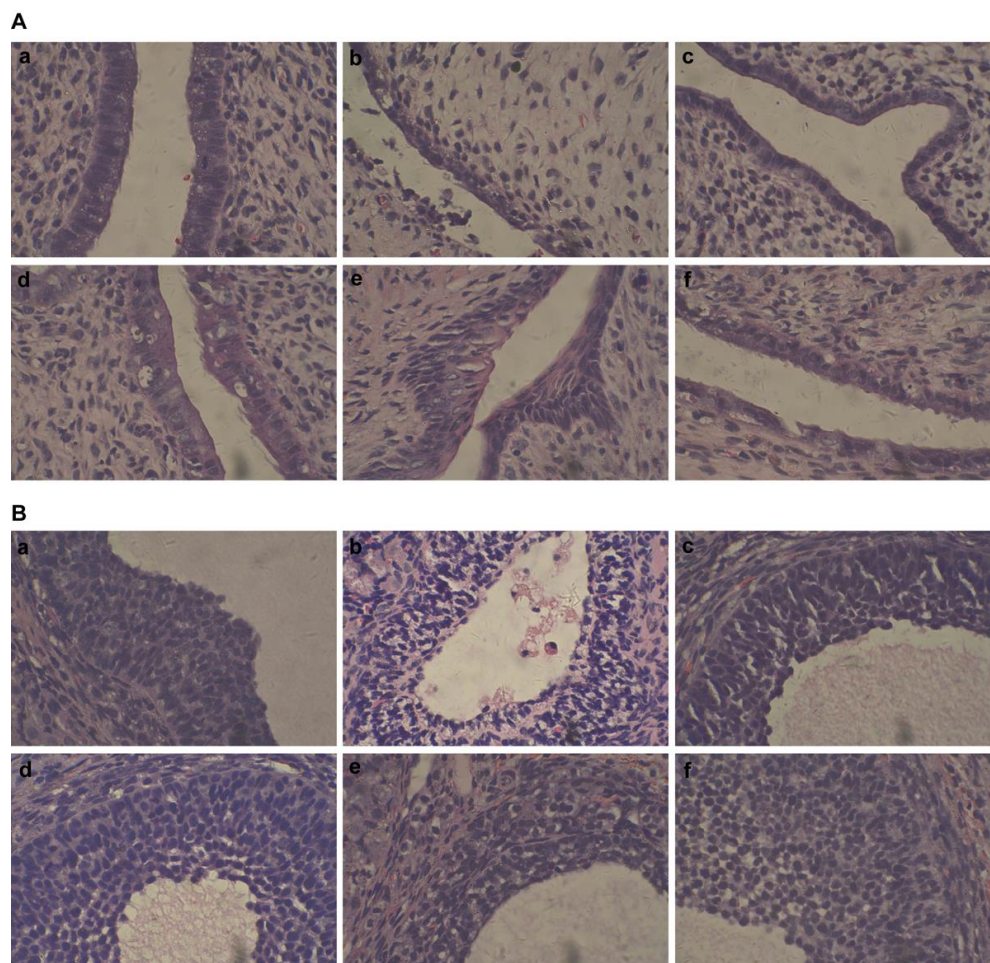


Figure 4 Histological images of uterus and ovary tissues stained with H&E. A. Histological images of uterus tissues staining with H&E. B. Histological images of

ovary tissues staining with H&E. a. Control group, b. Model group, c. Positive group, d. MPF-H group, e. MPF-M group, f. MPF-L group. (original magnification $\times 400$)

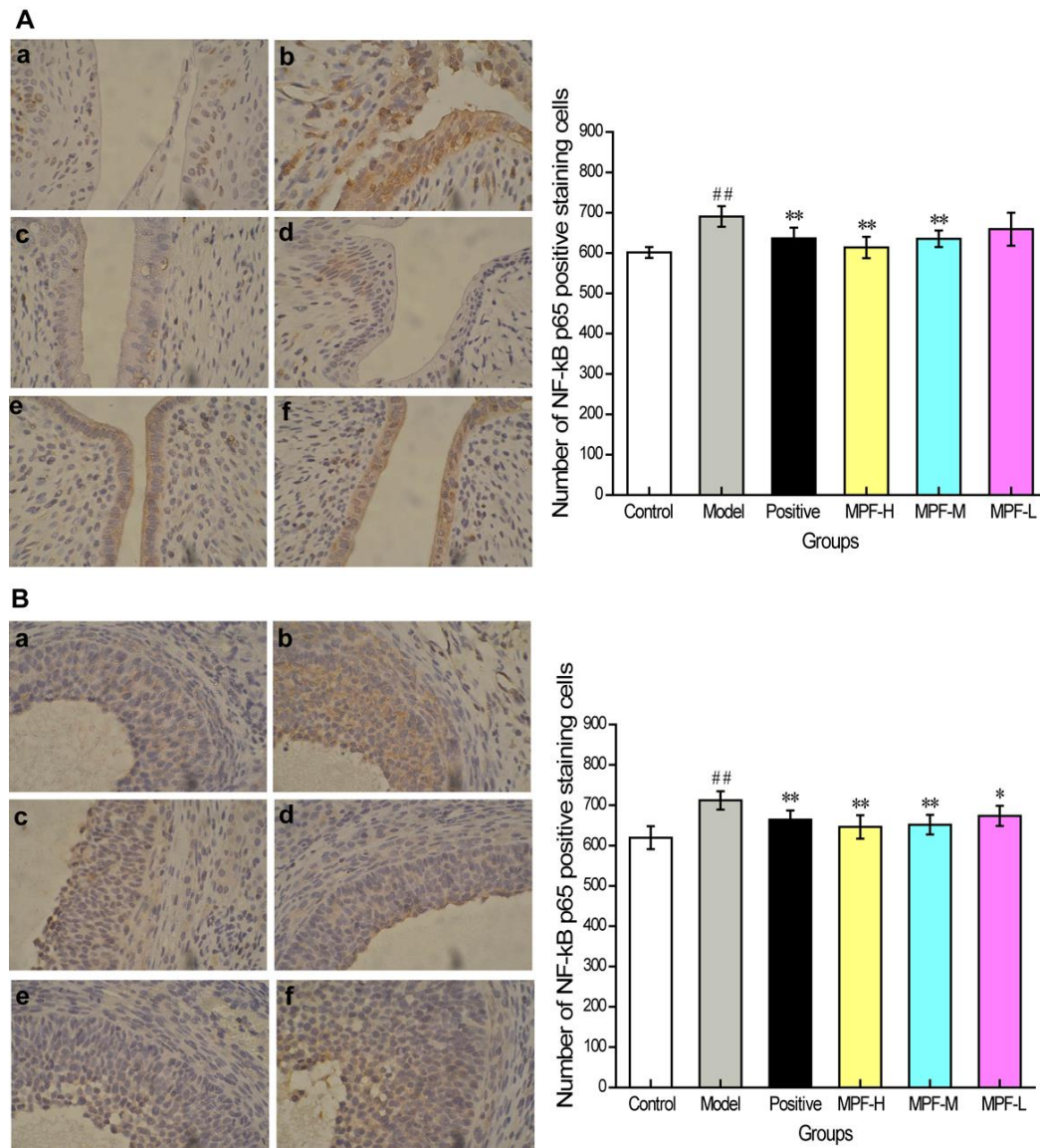


Figure 5 Expression of NF- κ B p65 in uterus and ovary tissues A. Expression of NF- κ B p65 in uterus. B. Expression of NF- κ B p65 in ovary tissues. a. Control group, b. Model group, c. Positive group, d. MPF-H group, e. MPF-M group, f. MPF-L group. (original magnification $\times 400$) Results represent means \pm SD of three independent experiments (n=10). ^{##}P < 0.01, control versus model, ^{**}P < 0.01, ^{*}P < 0.05, model versus positive, MPF-H, MPF-M, MPF-L.

4. Discussion

The CPID refers to chronic inflammation of the female internal reproductive organs, surrounding connective tissue and the pelvic peritoneum. This can be present due to untreated acute PID and it can manifest itself as a mild, moderate chronic pelvic inflammatory disease due to the patient's constitution or mild infection. In China, the rate of incidence of PID is very high due to personal health and medical conditions, indifferent aseptic concepts in minor gynecologic and family planning surgeries. Since there are many disadvantages in the use of antibiotics and surgical treatments of CPID, the use of traditional Chinese medicine has become significant in the treatment of CPID.

MPF, a Chinese medicine formula, was formulated in accordance with the principle and in combination of herbs according to the doctors' individual experience. MPF is composed of *Thlaspi arvense* L., *Gleditsia sinensis* Lam., *Smilax china* L., *Euonymus alatus* (Thunb.) Sieb., and *Vaccaria segetalis* (Neck.). Modern pharmacological studies have found that *Thlaspi arvense* L. has anti-bacterial and antiviral properties. *Gleditsia sinensis* Lam. has anti-bacterial, anti-inflammatory and anti-tumorigenic properties (Li et al., 2007; Zhou et al., 2007; Li et al., 2016;). And *Smilax china* L. has anti-inflammatory, anti-hyperuricemic and nephroprotective effects (Khan et al., 2009; Chen et al., 2011). Jingangteng is the root of *Smilax china* L., a traditional Chinese herb which can promote blood circulation by removing blood stasis. It is commercially available in capsules and has been available in the market for more than 10 years, and has remarkable curative effects. *Euonymus alatus* (Thunb.) Sieb. is reported to prevent hyperglycemia and hyperlipidemia (Park et al., 2005). Whilst *Vaccaria segetalis* (Neck.) can alleviate pain and has anti-inflammatory properties (Wang et al., 2015). All these drugs have a role as anti-inflammatory agents and are good at activating blood circulation by dissipating blood stasis, clearing eliminating heat as antitoxinants, reducing swelling and analgesia.

Evidences from clinical studies indicate that inflammation plays a critical role in the initiation and development of CPID (Zhou et al., 2007; Mitchell and Prabhu, 2013). And most researchers consider that inflammatory injury is the key pathological

change in CPID. Persistent activation of inflammatory factors and effusion of inflammatory cells lead to inflammatory autoimmune injury and subsequently indirectly or directly influence the initiation, progression and prognosis of SPID. Persistent inflammation results in blood stasis, and in turn, blood stasis promotes inflammation (Liu et al., 2014). In our previous study, it was revealed that MPF had remarkable anti-inflammatory and analgesic activities and is a promising herbal preparation which can be applied in the treatment of CPID (Ye et al., 2016). In this study, CPID model in rats was established in order to evaluate the effect of MPF on CPID and investigate its anti-inflammatory mechanism.

The changes of pathology of uterus and ovary tissues were observed by staining with H&E and it was confirmed that MPF had significant anti-inflammatory effect on CPID. It is known that the balance between pro- and anti-inflammatory cytokines is the key to maintain the body's normal immune status (Chou et al., 2013). When the balance is disturbed, the body will be prone to inflammatory diseases (Christensen et al., 1998; Basic Kes et al., 2008). The regulation of pro- and anti-inflammatory cytokines in inflammatory response is a very complicated process and almost all the anti-inflammatory cytokines have part to play in the induction of inflammation except IL-1 (Dinarello, 1997). TNF- α and IL-6 which are well-known pro-inflammatory cytokines (Alexander et al., 1998; Nadler et al., 2000). They are increased during inflammation, and the expression levels are positively correlated with the severity of inflammatory responses (Gao et al., 2016; Lin et al., 2016). IL-2, IL-10, and TGF- β_1 are three of the most important anti-inflammatory cytokines in the human immune response (Miller et al., 2002a, b; Curtis and Pavletic, 2016; Guo et al., 2016; Zhou et al., 2016). Our data indicated that the serum levels of TNF- α , IL-6, IL-2, IL-10, and TGF- β_1 were higher in the CPID model group than those in the control group. After treatment with MPF, the serum levels of these inflammatory cytokines decreased significantly and were similar to those in the control group, suggesting that MPF might play a positive role in the anti-inflammatory process by downregulating the serum levels of inflammatory cytokines to recover the balance of immune system.

Jingangteng capsules were chosen as a positive control for medicine treated CPID in

rats. Jinggangteng is the root of *Smilax china* L., which has anti-inflammatory effects and improves blood circulation (Khan et al., 2009; Luo et al., 2014). We observed that MPF had similar anti-inflammatory effects to Jinggangteng capsules on CPID in rats, and the high-dose MPF was in fact more effective than Jinggangteng capsules.

Apoptosis or programmed cell death (PCD) is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death (Elmore, 2007). Inappropriate apoptosis (either too little or too much) is a factor in many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. In recent years, the relationship between apoptosis and chronic inflammatory disease is receiving more and more attention. There is evidence to show that the blocking of inflammatory cells' apoptosis may be one of the pathogenesis of chronic inflammation (Pacova et al., 2009). Triggering the apoptosis of inflammatory cells is one of the mechanisms of improving the chronic inflammation. The results of quantitative RT-PCR showed that the expressions of P53 mRNA, Fas/FasL mRNA, and MMP-2 mRNA in uterus were significantly elevated after treatment with MPF and the expression of TGF- β_1 mRNA was reduced. The expression of P53 mRNA, Fas/FasL mRNA, and MMP-2 mRNA all have an effect to promote the apoptosis (Kitamura et al., 2001; Dmitrieva et al., 2003; Smane et al., 2013; Zhang et al., 2015; Umezawa et al., 2016). High expression of TGF- β_1 mRNA can induce the activation of anti-apoptotic cytokine NF- κ B, resulting in blocking of inflammatory cells' apoptosis. The immunohistochemistry analysis showed that the expressions of NF- κ B p65 in uterus and ovary tissues were inhibited after treatment with MPF. The results indicate that MPF may reduce the inflammatory reaction and eliminate the chronic pelvic inflammation by promoting and inducing the apoptosis of inflammatory cells.

5. Conclusions

MPF has noteworthy anti-inflammatory effect on CPID, and this effect is dose – dependent. It may play a positive role in the anti-inflammatory process by

downregulating the serum levels of inflammatory cytokines and at the same time it may reduce the inflammatory reaction and eliminate CPID by promoting and inducing the apoptosis of inflammatory cells. Our results indicate that MPF may have a role in the medical treatment of CPID.

Conflict of interest

None.

Acknowledgments

This work was supported by Traditional Chinese Medicine Development Special Funds ‘standardized clinical validation of hospital preparation and experience prescription’ of Shanghai Pudong new area (PDYNZJ2015-02), the Outstanding Leaders Training Program of Pudong Health Bureau of Shanghai (PWR12015-05), the State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources (ME2016012) and the Talents Training Program of Seventh People’s Hospital of Shanghai University of TCM (XX2017-01).

Li-Jun Zhang (E-mail: zhanglijun0407@163.com), who is contribute to the operation of molecular biology experiments and the writing of this manuscript. Jian-Yong Zhu (E-mail: jyzhu@foxmail.com), who is contribute to the identification of compounds in the Chinese herbal compound by high-performance liquid chromatography coupled with electrospray mass spectrometry (HPLC/ESI-MS). Meng-Yao Sun (E-mail: 1461293225@qq.com), Ya-Nan Song (E-mail: synabc.123@163.com) and Miao Zhang (E-mail: zhangmiao1083@163.com), who are contribute to the operation of molecular biology experiments and collecting the experimental data. Khalid Rahman (E-mail: K.Rahman@ljmu.ac.uk) and Cheng Peng (E-mail: Davor_1234@126.com), who are contribute to the language modification of this manuscript. Hong Zhang (E-mail addresses: hqzhang51@126.com) and Yu-Mei Ye (E-mail addresses: yeyumei0206@163.com), who are contribute to the design of the whole experiments and modification of this manuscript.

References

- Alexander, R.B., Ponniah, S., Hasday, J., Hebel, J.R., 1998. Elevated levels of proinflammatory cytokines in the semen of patients with chronic prostatitis/chronic pelvic pain syndrome. *Urology* 52, 744-749.
- Barrett, S., Taylor, C., 2005. A review on pelvic inflammatory disease. *Int. J. Std & Aids* 16, 715-720.
- Basic Kes, V., Simundic, A.M., Nikolac, N., Topic, E., Demarin, V., 2008. Pro-inflammatory and anti-inflammatory cytokines in acute ischemic stroke and their relation to early neurological deficit and stroke outcome. *Clin. Biochem.* 41, 1330-1334.
- Bu, X.L., Liu, Y.X., Lu, Q.D., Jin, Z., 2015. Effects of "Danzhi Decoction" on Chronic Pelvic Pain, Hemodynamics, and Proinflammatory Factors in the Murine Model of Sequelae of Pelvic Inflammatory Disease. *Evid-Based Compl Alt.* 2015, 1-12.
- Burnakis, T.G., Hildebrandt, N.B., 1986. Pelvic inflammatory disease: a review with emphasis on antimicrobial therapy. *Rev. Infect. Dis.* 8, 86-116.
- Cassell, J., Kell, P., Adler, M., 1997. Pelvic inflammatory disease: a review. *J. Int. Assoc. Physic. in Aids Care* 3, 20-23.
- Chen, K.J., 2012. Blood stasis syndrome and its treatment with activating blood circulation to remove blood stasis therapy. *Chin. J. Integr. Med.* 18, 891-896.
- Chen, L.Y., Yin, H.F., Lan, Z., Ma, S.W., Zhang, C.F., Yang, Z.L., Li, P., Lin, B.Q., 2011. Anti-hyperuricemic and nephroprotective effects of *Smilax china* L. *J. Ethnopharmacol.* 135, 399-405.
- Chen, Y., Tian, L., Wang, X., Wang, C.X., 2008. Drug Particles of FUYANNING to Cheonic Pelvic Inflammatory Disease Model of the Impact of Rats IL-2, IL-6. *Chin. Arch. Traditional Chin. Med.* 26(8).
- Chou, C.W., Lin, F.C., Tsai, H.C., Chang, S.C., 2013. The importance of pro-inflammatory and anti-inflammatory cytokines in *Pneumocystis jirovecii* pneumonia. *Medical Mycol.* 51, 704-712.
- Christensen, V.B., Toft, P., Tonnesen, E.K., 1998. Inflammatory response of the body

- to surgery illustrated by pro-inflammatory and anti-inflammatory cytokines. Ugeskrift for Laeger 160, 6951-6953.
- Curtis, L.M., Pavletic, S.Z., 2016. IL-2, the next best thing in chronic GVHD therapy? Blood 128, 13-15.
- Dinarelli, C.A., 1997. Role of pro- and anti-inflammatory cytokines during inflammation: experimental and clinical findings. J. Biol. Regul Homeost Agents 11, 91-103.
- Dmitrieva, E.V., Moskaleva, E., Kogan, E.A., Bueverov, A.O., Belushkina, N.N., Ivashkin, V.T., Severin, E.S., Pal'tsev, M.A., 2003. The role of Fas/FasL system in induction of hepatocyte apoptosis in chronic viral hepatitis. Arkh Patol 65, 13-17.
- Duarte, R., Fuhrich, D., Ross, J.D., 2015. A review of antibiotic therapy for pelvic inflammatory disease. Int. J. Antimicrob. Agents 46, 272-277.
- Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495-516.
- Gao, X., Li, Y., Wang, H., Li, C., Ding, J., 2016. Inhibition of HIF-1 α decreases expression of pro-inflammatory IL-6 and TNF- α in diabetic retinopathy. Acta Ophthalmol.
- Gradison, M., 2012. Pelvic inflammatory disease. Am. Fam. Physician 85, 791-796.
- Guo, W., Liu, Z.Y., Wei, Z.L., Lou, M.Q., Zhan, Q.B., Zhao, Y.D., 2016. TGF- β 1 polymorphism in glioma. J. Neurosurg. Sci. 60, 401-402.
- Haggerty, C.L., Peipert, J.F., Weitzen, S., Hendrix, S.L., Holley, R.L., Nelson, D.B., Randall, H., Soper, D.E., Wiesenfeld, H.C., Ness, R.B., 2005. Predictors of chronic pelvic pain in an urban population of women with symptoms and signs of pelvic inflammatory disease. Sex. Transm. Dis. 32, 293-299.
- Khan, I., Nisar, M., Ebad, F., Nadeem, S., Saeed, M., Khan, H., Samiullah, Khuda, F., Karim, N., Ahmad, Z., 2009. Anti-inflammatory activities of Sieboldogenin from *Smilax china* Linn.: experimental and computational studies. J. Ethnopharmacol. 121, 175-177.
- Kim, Y., Koh, J.H., Ahn, Y.J., Oh, S., Kim, S.H., 2015. The Synergic

- Anti-inflammatory Impact of *Gleditsia sinensis* Lam. and *Lactobacillus brevis* KY21 on Intestinal Epithelial Cells in a DSS-induced Colitis Model. Korean J. Food Sci. Anim. Resour. 35, 604-610.
- Kitamura, Y., Hashimoto, S., Mizuta, N., Kobayashi, A., Kooguchi, K., Fujiwara, I., Nakajima, H., 2001. Fas/FasL-dependent apoptosis of alveolar cells after lipopolysaccharide-induced lung injury in mice. Am. J. Respir. Crit. Care Med. 163, 762-769.
- Lee, S.E., Ju, E.M., Kim, J.H., 2001. Free radical scavenging and antioxidant enzyme fortifying activities of extracts from *Smilax china* root. Exp. Mol. Med. 33, 263-268.
- Li, K.K., Zhou, X., Wong, H.L., Ng, C.F., Fu, W.M., Leung, P.C., Peng, G., Ko, C.H., 2016. In vivo and in vitro anti-inflammatory effects of Zao-Jiao-Ci (the spine of *Gleditsia sinensis* Lam.) aqueous extract and its mechanisms of action. J. Ethnopharmacol. 192, 192-200.
- Li, W.H., Zhang, X.M., Tian, R.R., Zheng, Y.T., Zhao, W.M., Qiu, M.H., 2007. A new anti-HIV lupane acid from *Gleditsia sinensis* Lam. J. Asian. Nat. Prod. Res. 9, 551-555.
- Lin, C.C., Li, T.C., Liu, C.S., Yang, C.W., Lin, C.H., Hsiao, J.H., Meng, N.H., Lin, W.Y., Liao, L.N., Li, C.I., Wu, F.Y., 2016. Associations of TNF-alpha and IL-6 polymorphisms with osteoporosis through joint effects and interactions with LEPR gene in Taiwan: Taichung Community Health Study for Elders (TCHS-E). Mol. Biol. Rep. 43:1-13.
- Liu, B.Q., Gong, X., Jin, Z., 2014. Effect of Danzhi decoction on expression of angiogenesis factors in patients with sequelae of pelvic inflammatory disease. Asian Pac. J. Trop. Med. 7, 985-990.
- Loucks, A., 1983. Pelvic inflammatory disease: a review of therapy. The Nurse Pract. 8, 13-19, 23.
- Luo, Y., Ma, Y., Song, L., Luo, H., Hou, L., 2014. Effect of *Smilax china* bioactive fraction on tumor necrosis factor-alpha and interleukin-4 contents in uterine tissue of rats with chronic pelvic inflammatory disease. J. South. Med. Univ. 34,

236-240.

- Miller, L.J., Fischer, K.A., Goralnick, S.J., Litt, M., Burleson, J.A., Albertsen, P., Kreutzer, D.L., 2002a. Interleukin-10 levels in seminal plasma: implications for chronic prostatitis-chronic pelvic pain syndrome. *J. Urol.* 167, 753-756.
- Miller, L.J., Fischer, K.A., Goralnick, S.J., Litt, M., Burleson, J.A., Albertsen, P., Kreutzer, D.L., 2002b. Nerve growth factor and chronic prostatitis/chronic pelvic pain syndrome. *Urology* 59, 603-608.
- Mitchell, C., Prabhu, M., 2013. Pelvic inflammatory disease: current concepts in pathogenesis, diagnosis and treatment. *Infect. Dis. Clin. North. Am.* 27, 793-809.
- Nadler, R.B., Koch, A.E., Calhoun, E.A., Campbell, P.L., Pruden, D.L., Bennett, C.L., Yarnold, P.R., Schaeffer, A.J., 2000. IL-1beta and TNF-alpha in prostatic secretions are indicators in the evaluation of men with chronic prostatitis. *J. Urol.* 164, 214-218.
- Pacova, H., Astl, J., Martinek, J., 2009. The pathogenesis of chronic inflammation and malignant transformation in the human upper airways: the role of beta-defensins, eNOS, cell proliferation and apoptosis. *Histol. Histopathol.* 24, 815-820.
- Park, S.H., Ko, S.K., Chung, S.H., 2005. *Euonymus alatus* prevents the hyperglycemia and hyperlipidemia induced by high-fat diet in ICR mice. *J. Ethnopharmacol.* 102, 326-335.
- Shah, C.S., Sukkawala, V.M., Chavan, A.R., 1962. Pharmacognostic study of *Smilax china* L. rhizome. *J.Sci. Ind. Res. (C)*, 21C,19-21.
- Smane, L., Pilmane, M., Akota, I., 2013. Apoptosis and MMP-2, TIMP-2 expression in cleft lip and palate. *Stomatologija* 15, 129-134.
- Snaith, L., 1959. Chronic pelvic inflammation and infertility. *Clin Obstet Gynecol.* 2, 862-876.
- Tuffrey, M., Alexander, F., Woods, C., Taylor-Robinson, D., 1992. Genetic susceptibility to chlamydial salpingitis and subsequent infertility in mice. *J Reprod Fertil* 95, 31-38.
- Umezawa, Y., Kurosu, T., Akiyama, H., Wu, N., Nogami, A., Nagao, T., Miura, O., 2016. Down regulation of Chk1 by p53 plays a role in synergistic induction of

apoptosis by chemotherapeutics and inhibitors for Jak2 or BCR/ABL in hematopoietic cells. *Oncotarget* 7, 44448-44461.

Wang, L., Cui, D.G., Wang J.Z., Zhang, J.Y., Yang, Z.Q., Qin, Z. Kong, X.J., Wang, X.Z., Li, J.X., 2015. Analgesic and anti-inflammatory effects of hydroalcoholic extract isolated from *Semen vaccariae*. *Pak J Pharm Sci* 28, 1043-1048.

Ye, Y.M., Yang W., Zhang, X.D., Wu, W.W., Cai, Y., 2012. Clinical observation of Man-Pen-Fang for the treatment of pelvic inflammatory disease, *Proceedings of International conference on gynecology of TCM*, 220-222.

Ye, Y.M., Zhang, L.J., Xiao, L., Dai, J.J., 2016. Experimental study on anti-inflammatory and analgesic effect of ManPenFang. *Shanghai Journal of TCM* 50, 92-95.

Zhang, J.X., Han, Y.P., Bai, C., Li, Q., 2015. Notch1/3 and p53/p21 are a potential therapeutic target for APS-induced apoptosis in non-small cell lung carcinoma cell lines. *Int J Clin Exp Med* 8, 12539-12547.

Zhou, L.G., Li, D., Wang, J.G., Liu, Y.S., Wu, J.Y., 2007. Antibacterial phenolic compounds from the spines of *Gleditsia sinensis* Lam. *Nat Prod Res* 21, 283-291.

Zhou, X., Hopkins, J.W., Wang, C.K., Brahmakshatriya, V., Swain, S.L., Kuchel, G.A., Haynes, L., McElhaney, J.E., 2016. IL-2 and IL-6 cooperate to enhance the generation of influenza-specific CD8 T cells responding to live influenza virus in aged mice and humans. *Oncotarget* 7, 39171-39183.