International Journal of Nutritional Sciences

Open Access

Austin Publishing Group

Research Article

Anti-Rheumatoid Arthritis Effects of Dangshen Ejiao Pill Mediated through Nuclear Factor-κB Pathway

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Received: November 24, 2022; **Accepted:** January 02, 2023; **Published:** January 08, 2023

Abstract

Dangshen Ejiao (DE) pill, a traditional Chinese hospital preparation, has been used for the treatment of rheumatoid arthritis (RA) in China for decades with an unknown mechanism. This study aims to investigate the anti-RA and anti-inflammatory effect of DE using a Collagen-Induced Arthritis (CIA) model and explore its underlying mechanism of action. The quality control analysis of DE was performed by the phytochemical determination of 9 main monomers. The anti-RA effect of DE was validated by its reduction of inflammatory responses and CCP level in the CIA model. DE inhibited the production of pro-inflammatory cytokines IL-1β and IL-6 both in vivo and in vitro. Further mechanism study revealed that DE might alleviate inflammatory responses in the treatment of RA by blocking both canonical and noncanonical NF-κB pathways, and thus provided a powerful scientific foundation for its expanded application and approval as an anti-RA drug.

Keywords: Dangshen Ejiao Pill; Nuclear Factor-κB; Pro-Inflammatory Cytokines; Rheumatoid Arthritis

Introduction

Rheumatoid Arthritis (RA) is a chronic, disabling and systemic inflammatory disease, affecting approximately 1% of the population worldwide. Currently, there is still no effective cure or treatment [1,2], while the cause and pathogenesis of this disease remains unclear. Accumulating evidence indicates that certain cytokines such as tumor necrosis factor alpha (TNF-α) could be induced and synergized to promote the induction of interleukin (IL)-1β and IL-6 in target cells [3,4], eventually leading to arthritis. Nuclear factor-(NF)-κB signaling plays a crucial role in the expression of the pro-inflammatory cytokines, which leads to RA [5,6]. Therefore, inhibition of NF-κB-dependent pro-inflammatory cytokine production by the development of anti-RA candidates may have promising therapeutic potential.

NF-κB is a family of structurally related and evolutionarily conserved transcription factors, including RelA (p65) and NF-κB2 (p52 and its precursor p100), recognized as the most important factors [7,8]. Under normal conditions, NF-κB exists in the cytoplasm as an inactive complex by interacting with inhibitor of NF-κB (IκB) proteins [9]. Both canonical and noncanonical NF-κB pathways might be activated in RA. In canonical NF-κB signaling, stimuli such as TNF-α trigger a cascade of effects, including activation of transforming growth factor-activated kinase1(TAK1), phosphorylation of inhibitor of NF-κB (IκB) kinase (IKK)-β and degradation of IκBα, which liberates RelA (p65) for nuclear translocation and turns on several pro-inflammatory genes. Moreover, the alternative noncanonical NF-κB pathway depends selectively on the activity of IKKα and NF-κB-inducing kinase (NIK) [10]. Following pro-inflammatory cytokines stimulation, the IKKα subunit is phosphorylated by NIK to convert the NF-κB2 precursor p100 into p52. Consequently, p52 translocates into the nucleus and initiate the transcription of specific genes [11].

The treatment of RA has largely focused on cytokines inhibition and has faced many challenges [12]. Chinese traditional medicine could be more effective in treating RA because of its multi-target approach [13,14]. Dangshen Ejiao (DE) Pill has been applied for the treatment of RA patients in a local hospital

Citation: Liu T, Zhang L, Lan J, He K, Zhang D, et al. Anti-Rheumatoid Arthritis Effects of Dangshen Ejiao Pill Mediated through Nuclear Factor-κB Pathway. Int J Nutr Sci. 2023; 8(1): 1069.

in China for decades, with the effects of relieving joint swelling and pain [15]. DE pill, composed of 20 traditional Chinese herbs, is an improved version of Guipi Decoction (GD), a traditional Chinese prescription recorded as early as 1253 in "*Ji Sheng Fang*". GD prescription and its various improved versions have been used in RA treatment, and relieved RA symptoms effectively [16-18].

The main ingredients of DE were identified and quantified in our previous studies [19]. However, the quality control analysis has not been established, reports on the anti-RA effect of DE are very limited, and the mechanism is still unknown. In this study, quality control analysis was performed on the main ingredients of DE while their anti-RA activity was investigated for the first time to provide a pharmacodynamic basis for its use in RA. CIA rat model are used in this study to investigate the role of DE in treating RA, its modulation of pro-inflammatory cytokines and the NF-κB pathways and to understand the underlying anti-RA mechanism.

Materials and Methods

Materials and Reagents

The fine powder of DE pill (lot number: 20160301 was provided by Xianning Matang Fengshibing Hospital (Xianning, China). The powder contained a mixture of 20 Chinese traditional herbs (Table 1).

Table 1: 20 components of DE pill.

Name of Herbal Medicine	Parts of Plants	Name of traditional Chinese Medicine	Pharmacological Activities
Codonopsis Radix	dried root of *Codonopsis pilosula* (Franch.) Nannf.	Dangshen	anti-inflammatory [20]
Colla Corii Asini	glue made by boiling *Equus asinus* Linn's skins after depilation	Ejiao	anti-fatigue, anti-oxidation and hemostasis 21, 22]
Angelicae Sinensis Radix	dried root of *Angelica sinensis* (Oliv.) Diels	Danggui	bone protective and hemostasis [23, 24]
Astragali Radix	dried root of *Astragalus membranaceus* (Fisch.) Bunge	Huangqi	the treatment of skeletal muscle injury and relieves allergy [25, 26]
Salviae Miltiorrhizae Radix et Rhizoma	dried rootof *Salvia miltiorrhiza* Bunge	Danshen	hemostasis and anti-osteoporosis [19, 27]
Chaenomelis Fructus	dried fruit about to ripen of *Chaenomeles speciosa* (Sweet) Nakai	Mugua	anti-inflammatory and immunomodulatory [28]
Glycyrrhizae Radix Et Rhizoma	dried root and rhizome of *Glycyrrhiza uralensis* Fisch.	Gancao	anti-inflammatory, anti-RA and immunomodulatory
Aucklandiae Radix	dried root of *Aucklandia lappa* DC	Muxiang	anti-inflammatory and anti-tumor [29]
Arillus Longan	aril of *Euphoria longan* (Lour.) Steud.	Longyanrou	anti-aging, anti-tumor and immunomodulatory [30]
Macrocephalae Rhizoma	dried root and rhizome of *Atractylis macrocephala* (Koidz.) Hand.-Mazz.	Baizhu	anti-inflammatory, anti-tumor, immunomodulatory and analgesia [31]
Poria	sclerotia of *Poria cocos* (Schw.) Wolf	Fuling	anti-inflammatory, anti-tumor and immunomodulatory [32]
Semen Coicis	seed of *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf	Yiyiren	anti-tumor and immunomodulatory [33,34]
Rehmanniae Radices	fresh or dried tuberous root of *Rehmannia glutinosa* (Gaertn.) Libosch. ex Fisch. & C.A. Mey.	Dihuang	anti-oxidant [35]
Spatholobi Caulis	dried rattan of *Spatholobus suberectus* Dunn	Jixueteng	hematopoiesis, anti-inflammatory, anti-tumor and immunomodulatory [36]
Mori Ramulus	dried tender branch of *Morus alba* L.	Sangzhi	anti-bacterial and anti-inflammatory [37]
Stephaniae Tetrandrae Radix	dried root of *Stephania tetrandra* S.Moore	Fangji	anti-tumor, anti-bacterial and anti-inflammatory [38]
Cyathulae Radix	dried root of *Cyathula officinalis* K.C.Kuan	Chuanniuxi	hemostasis [39]
Pheretima	dried body of *Pheretima aspergillum* (E. Perrier)	Dilong	hypolipidemic [40]
Vinegar Trionycis Carapax	shell of *Trionyx sinensis* Wiegmann processed with vinegar	Cubiejia	anti-tumor and immunomodulatory [41]
Faeces Bombycis	Dry feces of *Bombyx mori* L. larvae	Cansha	analgesia, anti-bacterial and hemostasis [42]

Indomethacinenteric-coated tables were purchased from Shanxi Yunpeng Pharmaceutical Co., Ltd., (Linfen, China). Absolute alcohol and ether were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Bovine type II collagen (BC II) was purchased from Shanghai Yuanye Bio-Technology Co. Ltd., (Shanghai, China). Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvant (FICA), and pentobarbital sodium were obtained from Sigma-Aldrich (St. Louis, MO, USA). The 293T cell line was procured from American Type Culture Collection (Manassas, VA, USA).

Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were procured from GE Healthcare HyClone™ (Logan, UT, USA). The rat IL-1β, IL-6, and Cyclic Citrullinated Peptide (CCP) kits were purchased from R&D Systems, Inc., (Minneapolis, MN, USA). p5×NF-κB reporter plasmid, pRL-TK plasmid, and dual-luciferase reporter assay system was purchased from Promega Corporation (Madison, WI, USA). The nuclear/cytosol fractionation kit was purchased from BioVision Inc., (San Francisco, CA, USA). Antibodies against phosphorylated-p65 (p-p65), p65, p-IKKα/β, IKKα, p100/p52, and Histone3 (H3) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) and β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). The hemolytic agent was purchased from Lianke Biology (Hangzhou, China). Vigofect transfection reagent was purchased from Vigorous Biotechnology, Inc., (Beijing, China).

Quality Control Analysis of DE

Quality control studies of the main components of DE were performed using High-Performance Liquid Chromatography (HPLC, Supplementary Table 1) according to the US Pharmacopeia (USP40-NF35) [43], Japanese Pharmacopoeia (JP17), Chinese Pharmacopoeia (ChP Aucklandiae radix, 2015; ChP Chaenomelis fructus, 2015) [44], and the International Council for Harmonization (ICH) guidelines [45].

Effects of DE on CIA Rats

Induction of CIA and DE administration: Male Wistar rats (170±10 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., (Beijing, China). All animal experiments All animal experiments met the ethical requirements of laboratory animals and approved by the Animal Care and Use Committee, Safety Evaluation Center for Food and Drug, Hubei Provincial Center for Disease Control and Prevention (No. R2016005). All animals were kept in an air-conditioned room, and adequate standard pelleted forage and purified water are provided.

After 3 days of adaptive feeding, all rats were formed randomly into control group (Con) and model group. The CIA rat model was established by intradermal immunization of each rat at the base of the tail with 100 μg BC II emulsified with an equal volume of FCA on day 1, followed by a booster dose of intradermally injected BC II (100 μg emulsified with an equal volume of FICA) in the tail and right hind toe pad on day 7. Rats in the control group were similarly injected intradermally with equal volumes of saline [46]. All CIA rats were divided randomly into the following five groups of 10 each: CIA model (Mod), DE powder, 4.0 g/kg (DE-H), 2.0 g/kg (DE-M), 1.0 g/ kg (DE-L) and indomethacin, 6.25 mg/kg (Indo). Oral administration was given at the same time every day from day 21 to 30 after the establishment of the CIA model. Rats in the DE and Indo groups were treated with DE or Indo, respectively at the indicated doses, while those in the Con and Mod groups were treated with distilled water.

Determination of Paw Edema

The body weight and general condition of the rats were monitored on day 21, 28, 35, and 40. Furthermore, paw swelling was evaluated by measuring the hind paw volume using the YLS-7C plethysmometer (Jinanyanyi Science and Technology Development Co., Ltd., Jinan, China). The value was calculated as follows: paw edema value = Vt - Vn, where Vn were the volume of bilateral hind paws before induction and Vt were the volume of bilateral hind paws after induction [47].

Determination of Serumanti-CCP Antibody, IL-1β, and IL-6 Levels

Rats from the different groups were anesthetizedwith2% pentobarbital sodium1 h after the last treatment administration. Blood samples were collected from the aortaventralis of each rat and centrifuged at 2000 rpm for 10 min to obtain the serum, which was then stored at −80 ^{O}C. Serum CCP, IL-1β and IL-6 levels were subsequently detected by an Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions. For each group, plasma samples were obtained from 10 rats and analyzed in duplicate.

Effect of DE on Viability of 293T Cells

The 293T cell line was maintained in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. The cells were incubated at 37^{O}C in a humidified atmosphere of 5% CO_2. All cells used in this study were within 20 passages after receipt or resuscitation. At approximately 80% confluency, the cells were treated with the indicated concentrations of DE for various periods and the cell proliferation was detected in vitro using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [48]. Briefly, 100 μL of the 293T cell suspension was seeded at a density of 6×10^4 cells/mL into a 96-well plate and DEat concentrations of 0, 20, 80, 320, 1280, and 5120 μg/mL was added (with double distilled water [ddH_2O] as the diluent). Then, 20 μL MTT solutions at a concentration of 5 mg/mL was added to each well, followed by incubation for another 4 h, and then the culture medium was discarded. Then, 150 μL DMSO was added to each tested well and the absorbance was measured at 570 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific, MA, USA).

Effect of DE on TNF-α-induced Generation of IL-1β and IL-6

The 293T cells were incubated as described in section 2.4. The cells were seeded in 100 μL suspension at a density of 6×10^4 cells/mL in a 96-well plate, DE at a concentration of 160 or 320 μg/mL was added, followed by incubation for 2h, and then they were treated with TNF-α (20 ng/mL) for 24 h at 37 ^{O}C. The supernatants were collected for detection of the IL-1β and IL-6 levels using an ELISA as described above.

Effects of DE on Canonical NF-κB Pathway

Activation of canonical NF-κB pathway by DE: The NF-κB luciferase assay was performed as described previously [48]. Briefly, 293T cells were co-transfected with the p5×NF-κB reporter and pRL-TK plasmids using the Vigofect transfection reagent. After 24 h of transfection, the cells were pretreated with DE (80, 160, 320, and 640 μg/mL) or dimethyl sulfoxide (DMSO) for 10 h. Following DE treatment, cells were lysed, and the luciferase activity was determined using the dual-luciferase reporter assay system according to the manufacturer's protocols.

Mechanism of DE Inhibition of Transcriptional Activity via Canonical NF-κB Pathway

The 293T cells were treated with DE (160 and 320 μg/mL) or DMSO for 2 h, followed by TNF-α stimulation for 30 min, and then the nuclear and cytoplasmic fractions were separated from cell homogenates using a nuclear/cytosol fractionation kit according to the manufacturer's protocol. Briefly, cells were centrifuged at 600×g for 5 min at 4°C, Cytosol Extraction Buffer (CEB) was added to the pellet, followed by centrifugation at 16,000×g for 5 min at 4°C, and the resulting supernatant fraction was collected as the cytosolic fraction. The pellet fractions were further centrifuged and the final supernatant was the nuclear fraction as described in the procedure. Samples were then subjected to immunoblotting.

Immunoblotting was performed as described previously [49]. Briefly, 293T cells were grown in a six-well plate, treated with DE (80, 160, 320, and 640 μg/mL) or DMSO, the total cells were washed with PBS, and then they were lysed in M2 lysis buffer (20 mM Tris-hydrochloride, pH 7.5, 150 mM sodium chloride, 10 mM β-glycerophosphate, 5 mM ethylene glycol tetraacetic acid, 1 mM sodium pyrophoshate, 5 mM sodium fluoride, 1 mM

sodium orthovanadate, 0.5% Triton X-100, and 1 mM dithiothreitol) supplemented with protease inhibitor cocktail (Sigma P8340). The Bradford method was used to determine the protein concentrations, and the samples were denatured by adding Sodium Dodecyl Sulfate (SDS) loading buffer for 10 min at 100°C to prepare the samples for immunoblotting. Proteins were subsequently separated using SDS-Polyacrylamide Gel Electrophoresis (PAGE) and electrically transferred onto a polyvinylidene difluoride membrane. The membrane was probed with the appropriate primary antibody, followed by a Horseradish Peroxidase (HRP)-conjugated secondary antibody. Blots were visualized using the Tanon 5200 system (Shanghai Tanon Technology Co., Ltd., Shanghai, China).

Effects of DEon p-IκBα, IκBα, p-p65 and p65 in Canonical NF-κB Pathway

The 293 cells were treated with DE (80, 160, 320, and 640 μg/mL) or DMSO for 2 h, followed by TNF-α (20 ng/mL) stimulation for 30 min. The phosphorylation of TAK1 and IKKα/β (Ser180/181) were measured using immunoblotting with corresponding antibodies. The levels of p-IκBα, IκBα, p-p65 and p65were detected using immunoblotting as described above.

Effect of DE on Noncanonical NF-κB Pathway

The 293T cells were pretreated with DE (160 and 320 μg/mL) or DMSO, followed by TNF-α treatment for 30 min. The phosphorylation levels of IKKα, expression levels of NIK and p100/p52 were detected using immunoblotting as described above.

Statistical Analysis

The results were analyzed using the Statistical Package for the Social Sciences (SPSS) 15.0 statistical software. The data are expressed as the means ± Standard Deviation (SD) and one-way and repeated measurement analyses of variance (ANOVA) were used to perform the statistical analysis.

Results

Quality Control Analysis of DE

Multi-compound Chinese herbal medicine is a complex system homogeneous and stable quality is one of the key factors to ensure the study of formulation mechanism. In this study a new HPLC method for the simultaneous determination of four major components of DE was previously established and published [19], the authentic chromatographic profile for components of the DE was established to reduce the probable variation of DE ingredients in the course of each manufacturing procedure (Supplementary Figure 1a). Furthermore, the other five major components were identified by using pharmacopeia methods (Supplementary Figure 1b-d), and the main validation parameters of nine components, linear correlation coefficient conform to the pharmacopeia (r^2>0.99) (Supplementary Table 2). The mean contents of the nine components of the tested sample were determined, Ferulic Acid (0.13%), Calycosin-7-glucoside (0.03%), Liquiritin (0.23%), Glycyrrhizic Acid (2.16%), Tanshinone IIA (0.1%), Oleanolic Acid (0.42%), Ursolic Acid (0.05%), Costunolide (1.00%) and Dehydrocostus Lactone (1.57%) in the DE pill (n=3, Supplementary Table 3). These experiments achieved the quality control analysis of DE and demonstrated its fulfillment of the biological test requirements.

Effects of DE on CIA in Rats

DE alleviated inflammatory responses of CIA rat model: The

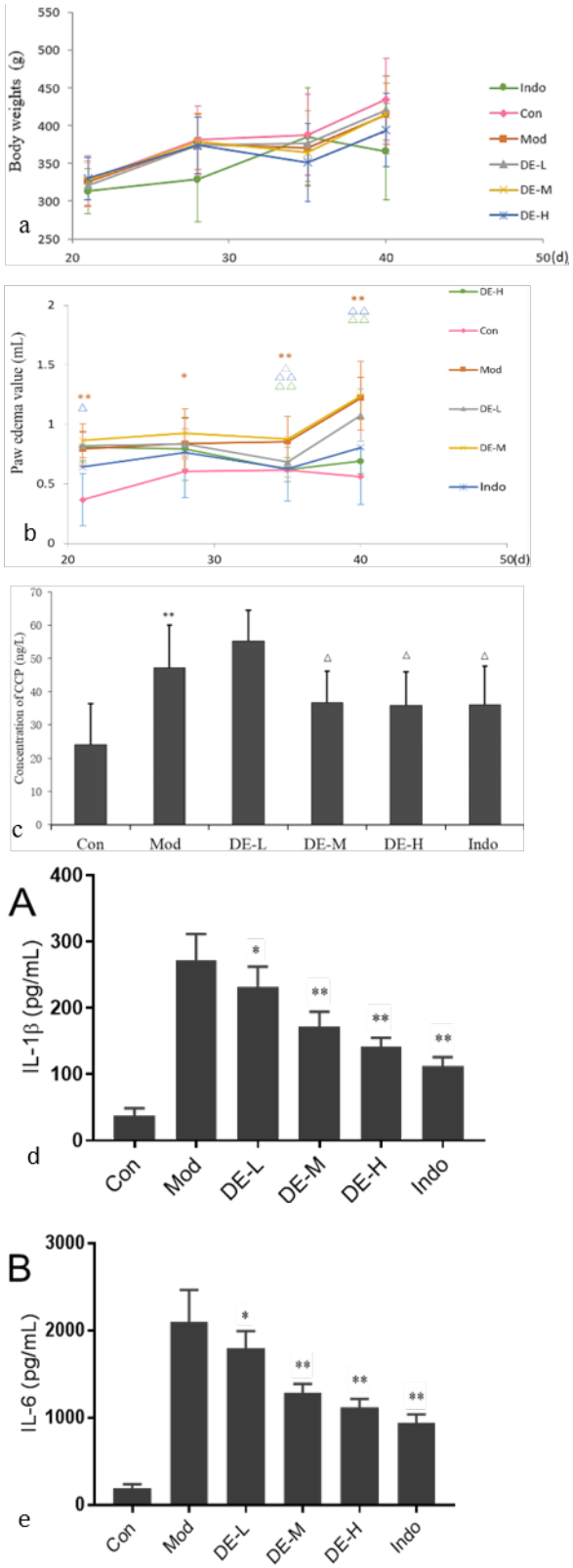

Figure 1: Effect of DE on body weights, paw edema, CCP, IL-1β and IL-6 of rats. Rats received an orally administration of DE (4.0 g/kg, 2.0 g/kg, 1.0 g/kg), indomethacin (Indo, 6.25 mg/kg), or distilled water daily after CIA modeling. Control and model groups were administered equal volumes of distilled water. Body weights (a) and paw edema (b) were measured on days 21, 28, 35, and 40. CCP (c), IL-1β (d) and IL-6 (e) were analyzed on day 40. Data are means ± standard deviation (SD, n = 10); *p< 0.05 and **p < 0.01, compared with normal group; $^{\triangle}p$< 0.05 and $^{\triangle\triangle}p$< 0.01, compared with model group.

in vivo efficacy of DE was first analyzed using a CIA model. CIA rats are an autoimmune type of arthritis, which displays many similar characteristics with RA in human [50]. CIA rats were initially immunized with type II collage, n and the subsequent booster dose was administered on day 7.

The autonomic activities paw edema values, and weights of rats in all groups were determined on days 21, 28, 35, and 40. During the experiment, there was no significant difference in body weight among groups (Figure 1a). Mental state, diet and defecation of rats in the control group were normal, and there was no abnormal behavior. There was no erythema and swelling of the joints in the control group. However, from day 9, CIA rats showed symptoms of arthritis, including paw swelling and erythema, accompanied by a decrease in spontaneous activity. Inflammatory symptoms gradually aggravated and the paw edemapeaked on day 35.

Treatment with DE significantly suppressed inflammation. Compared with the mean paw swelling values of 0.854 and 1.220 mL on day 35 and 40, respectively in the Mod group, and the counterpart values in the DE-H group were reduced very significantly to 0.620 mL ($p < 0.01$) and 0.690 mL ($p < 0.01$), respectively. The corresponding value on days 35 and 40 of DE-L group was reduced to 0.683mL ($p < 0.05$) and 1.076 mL, respectively. In contrast, the Indo group exhibited significantly lower mean values of 0.627($p < 0.01$) and 0.805 mL ($p < 0.01$), respectively (Figure 1b). These data indicated that DE exerted a dose-dependent suppression of inflammation, and it showed a higher anti-RA effect at the dose of 4.0 g/kg than that of Indo at the dose of 6.25 mg/kg.

DE Reduced Serum Concentrations of Anti-CCP Levels in CIA Rats

CCP is well recognized as an effective and specific indicator in the early diagnosis of RA [51]; therefore, the serum CCP in rats was investigated using ELISA [52]. As indicated in (Figure 2c), concentrations of CCP in samples from CIA rats were significantly higher than those of rats in the normal control group ($p<0.01$). The production of CCP in both DE-H and DE-M groups were significantly reduced ($p<0.05$), in a similar degree of Indo group, while no obvious suppression was observed in the DE-L group, indicating that DE might exert a therapeutic effect on CIA rats, in a dose-dependent manner.

DE Reduced Serum Concentrations of IL-1β and IL-6 Levels in CIA Rats

Considering the crucial roles of pro-inflammatory cytokines (IL-1β and IL-6) in the progression of RA, the effects of DE on serum levels of these cytokines were investigated. As illustrated in (Figure 1d and e), concentrations of IL-1β and IL-6 in samples from CIA rats were significantly higher than those from the normal rats ($p<0.01$). Significant dose-dependent decreases of both IL-1β and IL-6 levels were observed in all three DE treatments. The levels of IL-1β and IL-6 in serum were declined to as low as142 and 1121 pg/mL in the DE-H group, in a comparison of 112 and 942 pg/mL in the Indo group, and 272 and 2101 pg/mL in the model group, respectively. We have established in this experiment the dose-dependent down regulation of the serum levels of IL-1β and IL-6 by DE to be 4.0 g/kg compared to the 6.25 mg/kg of Indo. These *in vivo* data confirmed that DE exerted anti-RA effects by alleviating inflammatory responses and reducing CCP level, which might be related to pro-inflammatory cytokines.

DE Reduces Production of Pro-Inflammatory Cytokines

Since 293T cell is a well-characterized TNF-α response cell mode, the *in vitro* inhibitory effects of DE on the production of IL-1β and IL-6 were then evaluated on this cell. First, the cytotoxicity of DE in 293T cells was analyzed using the MTT assay. As indicated in supplementary (Figure 3), DE did not exert any significant effect on the cell viability at the concentration of 20, 80, 320, 1280, or 5280 μg/mL. These data demonstrated that DE possessed a high biological safety profile.

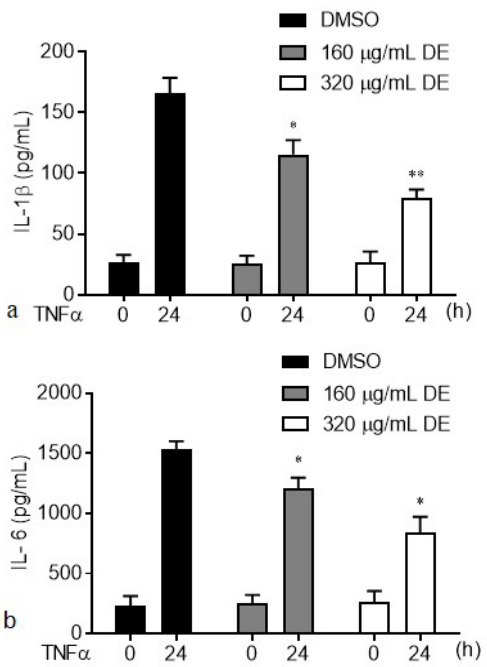

Figure 2: DE on TNF-α-induced IL-1β and IL-6. Following pretreatment with DE (160 and 320 μg/mL) for 2 h, 293T cells were treated with TNF-α (20 ng/mL) for 24 h. Supernatants were collected to determine (a) IL-1β and (b) IL-6 using ELISA. Data are means ± SD of three independent experiments; *$p < 0.05$ and **$p < 0.01$, compared with normal group.

To characterize the potential anti-inflammatory activities of DE, we assessed its effects on the production of IL-1β and IL-6 in TNF-α-stimulated 293T cells. As shown in (Figure 2a and b), TNF-α treatment induced the production of a large amount of IL-1β and IL-6; however, this was significantly inhibited by DE in a dose-independent manner. These data indicated that DE effectively inhibited the production of IL-1β and IL-6.

DE Suppressed NF-κB Pathway Activation

DE Suppressed Activation of Canonical NF-κB Pathway: NF-κB signaling was investigated. We first determined the effect of DE on a TNF-α-induced NF-κB-luciferase reporter. As shown in (Figure 3a), DE significantly reduced TNF-α-induced transcriptional activity of NF-κB in a dose-dependent manner.

Next, the translocation of p65 from the cytoplasm to nucleus was detected to explore the possible transcriptional inhibitory mechanism of NF-κB. As shown in (Figure 3b), DE treatment gradually decreased the nuclear and increased the cytoplasmic p65 levels in a concentration-dependent manner. This data suggests that DE suppressed TNF-α-induced transcriptional activity of NF-κB may through inhibition of the transfer of p65 from the cytoplasm to the nuclear.

Since IκBα degradation and p65 phosphorylation are crucial for activation of the NF-κB signal pathway, we next sought

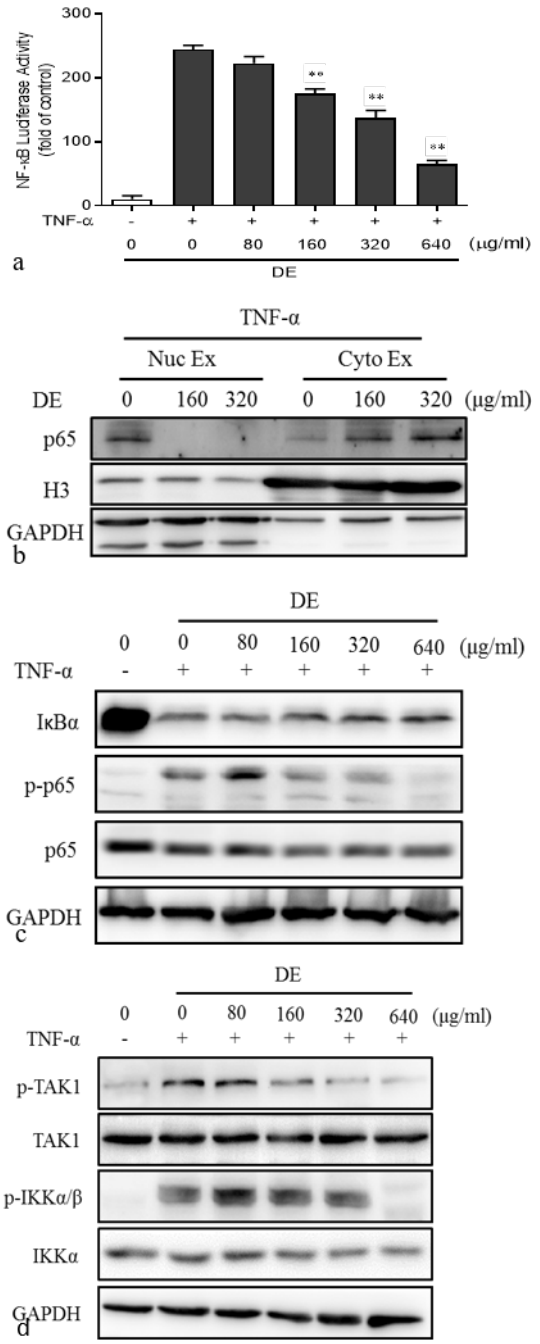

Figure 3: DE suppressed activation of canonical NF-κB signaling. **(a)** 293T Cells were transiently transfected with p5×NF-κB and renilla luciferase reporter constructs. Cells were incubated with or without DE (80, 160, 320, and 640 μg/mL) for 2 h followed by treatment with TNF-α (20 ng/mL) for 10 h. Luciferase activity was measured in triplicate and expressed as percentage (%) activity relative to control group; **p* < 0.05 and ***p* < 0.01, compared with control group. **(b)** 293T cells were pre-treated with DE (160 and 320 μg/mL) for 2h, following treatment with TNF-α (20 ng/mL) for 30 min, the nuclear and cytoplasmic fractions were prepared to detect p65 levels using immunoblotting. Histone 3 (H3) was a nuclear marker and GAPDH was a cytoplasmic marker. (c and d) Following treatment with indicated concentrations of DE for 2 h, 293T cells were treated with TNF-α (20 ng/mL) for 30 min. The phosphorylated-IκBα (p-IκBα), IκBα, phosphorylated-p65 (p-p65) and p65 levels were detected using immunoblotting **(c)**, and the phosphorylation of TAK1and IKKα/β (Ser180/181) were measured using immunoblotting with corresponding antibodies **(d)**.

to elucidate whether TNF-α-inducedp65 phosphorylation and IκBα degradation are affected by DE. The immunoblotting results showed that DE inhibited TNF-α-induced phosphorylation of IκBα and p65, as well as reduced the degradation of IκBα protein in a dose-dependent manner (Figure 3c). Moreover, TNF-α-induced phosphorylation of the upstream signaling factors TAK1 and IKK was also inhibited by DE treatment, as shown in (Figure 3d). These data collectively suggested that DE effectively inhibited canonical NF-κB transcriptional activity induced by TNF-α.

DE Suppressed Activation of Noncanonical NF-κB Pathway

In addition, we also investigated whether DE inhibits TNF-α-induced noncanonical NF-κB pathway, which relies on processing of NF-κB2 precursor protein p100 to p52 [53]. Since NIK over expression was shown to enhance p100 phosphorylation and processing [54], and IKKα is a pivotal component of noncanonical NF-κB activation [53], we next examined whether DE has any effects on NIK and IKKα activation. As shown in (Figure 4), DE treatment induced the degradation of NIK protein and reduced TNF-α-induced phosphorylation of IKKα. Moreover, DE suppressed the processing of p100 to p52. These data suggested DE also inhibited the noncanonical NF-κB pathway induced by TNF-α.

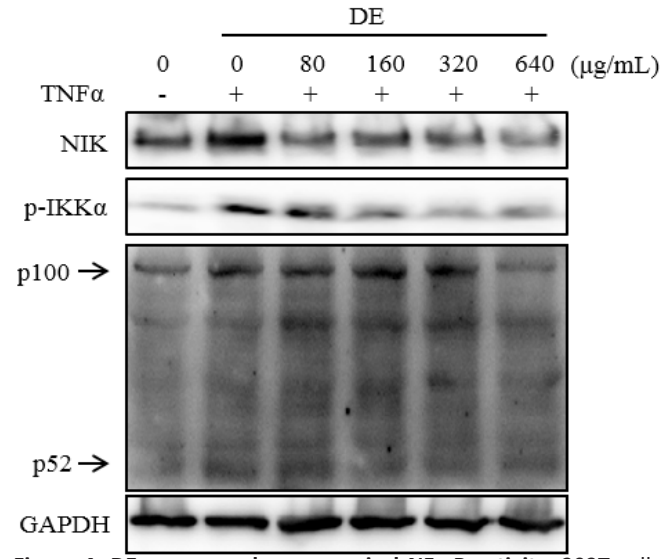

Figure 4: DE suppressed noncanonical NF-κB activity. 293T cells were pretreated with DMSO or DE at indicated concentrations, followed by TNF-α treatment for 30 min. The expression levels of NIK and p100/p52, phosphorylation level of IKKα were detected using immunoblotting.

To summarize these results, DE inhibited the p65-mediated canonical NF-κB pathway by inhibiting TNF-α-induced phosphorylation of TAK1 and IKKβ, which in turns suppressed the degradation of IκBα and phosphorylation of p65. On the other hand, DE also inhibited the p100-mediated noncanonical NF-κB pathway by triggering NIK degradation and inhibiting IKKα phosphorylation. DE represses both the canonical and noncanonical pathways might contribute to the reduced production of IL-1β and IL-6. The schematic illustration of the mechanism of action of DE is shown in (Figure 5), which might be one of the most important anti-RA mechanisms of DE.

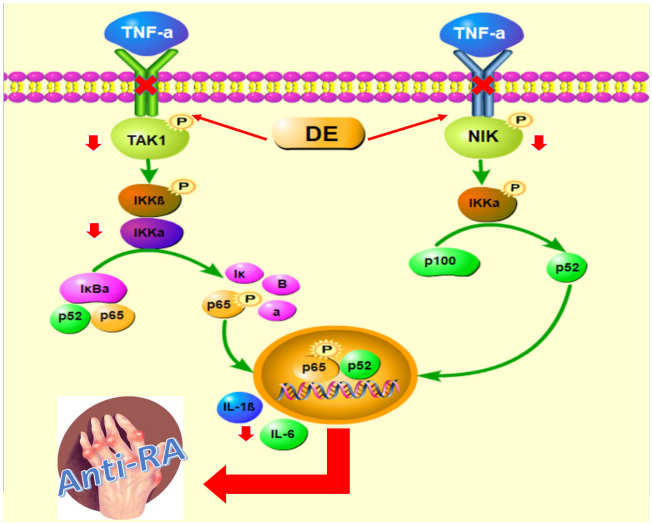

Figure 5: DE suppressed canonical and noncanonical NF-κB pathways.

Discussion

Recently, the use of traditional Chinese medicine in RA treatment has increased in China.DE has been extensively used in Chinese hospitals to treat of RA. However, the underlying mechanism remains unclear. This study investigated the pathways responsible for the potent anti-RA effects of DE. We provide evidence that DE significantly inhibits inflammatory responses and CCP level in the CIA model. In addition, DE inhibited the production of pro-inflammatory cytokines IL-1β and IL-6 both *in vivo* and *in vitro*. These results clearly demonstrate that DE alleviates inflammatory responses in RA.

The recommended oral dose of DE in human is 12 g/day, which is equivalent to 0.2 g/kg/day (body weight calculated as 60 kg). The doses used in this study were 1, 2, and 4 g/kg, which are 5, 10, and 20 times the recommended dose. We have shown that DE represses the inflammation responses in a dose-dependent manner. In the high dose treatment (4.0 g/kg), DE exhibited a comparable to or even better effect than Indo in reducing paw edema. Although DE-M treatment (2.0 g/kg) failed to reduce paw edema during administration, it showed comparable activities to that of DE-H treatment in reducing anti-CCP levels. DE-L treatment (1.0 g/kg) showed a reasonable effect in reducing paw edema after mid-term (day 35) administration, and no obvious effect after late-term (day 42) administration was observed. This may be because the given dose was not strong enough to control the development of inflammation. Combined with the dose-dependent reduction of IL-1βand IL-6 levels in rat serum, it is then speculated that the relief effect of DE on paw edema might be because of its reducing effect on the inflammation cytokines.

The pro-inflammatory cytokine TNF-α plays a crucial role in the development of RA [55]. High level of TNF-α is positively correlated with RA and several pro-inflammatory mediators are activated by transcription factors, such as AP-1 and NF-kappa B [56], hence we used TNF-αas a RA stimulus in our cell model. We found that TNF-α markedly increased the levels of IL-6 andIL-1β, which were subsequently repressed by DE treatment. It is generally known that IL-6 and IL-1βare strongly associated with the pathogenesis of RA [57]. Anti-RA treatment has been successfully achieved by using inhibitors of IL-6 and IL-1β [57,58]. Our results thus suggest that DE may alleviate inflammation by reducing the production of IL-6 and IL-1β.

Several pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β can be activate by NF-κB [59]. Inhibiting the activity of NF-κB has become an effective method to treat RA, and many chemical inhibitors have been used in clinic (www.nf-kb.org). In this study, we found that DE alleviates inflammatory responses at least partly through inhibition of NF-κB activity. DE treatment significantly reduced the phosphorylation of TAK1 and IKKα/β, two critical signaling steps in the canonical NF-κB pathway. Specifically, the nuclear translocation activity of p65 NF-κB was significantly reduced by DE via inactivation of IKKα/β. These results indicate that reduced IL-6 and IL-1β secretions by DE treatment are possibly mediated by preventing the canonical IKKα/β and p65 phosphorylation. In addition to inhibition of p65-mediated canonical NF-κB activity, DE also inhibited TNF-α-induced noncanonical NF-κB activity. This was demonstrated by triggering NIK degradation, inhibiting IKKα phosphorylation, and subsequently reducing p100 processing and nuclear accumulation of p52 upon DE treatment. Although the detailed mechanism of DE-induce TAK1 dephosphorylation and NIK degradation require further examination, the inhibition of both canonical and noncanonical NF-κB pathways may explain the anti-RA effect of DE, as described in (Figure 7).

To the best of our knowledge, we have identified the anti-RA activity of DE in animal model for the very first time. At a dose of 4.0 g/kg, DE displayed a comparable effect to Indo (6.25 mg/kg), and therefore, DE might benefit patients with indomethacin intolerance in the future. This study explored the pharmacodynamic basis for DE as a potential treatment option for RA.

Conclusions

DE might exert anti-RA effects through anti-inflammation-related mechanism, specifically, by suppressing the activation of both canonical and noncanonical NF-κB pathways. As a Chinese traditional medicine preparation, DE also has the advantage of satisfactory clinical efficacy and safety. Therefore, the expansion of its clinical use and further clinical approval can be greatly recommended.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Author Contributions

T.L. and L.Z performed the animal experiments and biological experiments and wrote the paper. J.L. performed data analysis. K.H. performed animal experiments. D.Z., M.X. and Y.L. conceived the experiments and revised the manuscript.

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