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Protective effects of Asiatic acid against pelvic inflammatory disease in rats

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Abstract. Asiatic acid (AA) is one of the major components of the Chinese herb *Centella asiatica* and exerts a variety of pharmacological activities. However, the pharmacological effects of AA on pelvic inflammatory disease (PID) remain unknown. The purpose of the present study was to investigate the therapeutic efficacy and potential mechanisms of AA on PID in rats. A total of 75 female Sprague Dawley rats were randomly divided into the following five groups: A control group; a PID group; a PID + AA 5 mg/kg group; a PID + AA 35 mg/kg group; and a PID + AA 75 mg/kg group. Changes in cytokine and chemokine levels, myeloperoxidase (MPO) activity, nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome and nuclear factor-κB (NF-κB) activation, oxidative stress and cleaved caspase-3 were measured. AA treatment significantly decreased the excessive production of cytokines and chemokines and suppressed MPO activity and the activation of NLRP3 inflammasome, NF-κB and caspase-3, as well as oxidative stress. These results suggest that AA exhibits potent anti-inflammatory and antioxidant effects in rats with pathogen-induced PID and that the mechanism of these anti-inflammatory effects may be associated with the suppression of NLRP3 inflammasome activation and the NF-κB pathway.

Introduction

Pelvic inflammatory disease (PID) is a gynecological disease that is common among young and sexually active females with upper genital tract infections, including endometritis, salpingitis, tubal ovarian abscesses or pelvic peritonitis (1). Clinical features of PID include adnexal pain, abnormal vaginal discharge, fever, menstrual irregularities and dyspareunia (2). Long-term and repeated infections lead to chronic pelvic pain, tubal infertility and ectopic pregnancy, which have a serious impact on health and quality of life (3,4).

The pathophysiological mechanism of PID includes the inflammatory response (5,6). The inflammatory response causes the release and maturation of interleukin (IL)-1β (5,6), which is mediated by nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome (7,8). NLRP3 inflammasome is composed of sensor NLRP3, adaptor protein apoptosis-associated speck-like protein and procaspase-1 (7,8). The nuclear factor-κB (NF-κB) pathway comprises important transcription factors that regulate inflammatory cytokines (9,10) and chemokines (11,12), including IL-1β, IL-6, tumor necrosis factor-α (TNF-α) and monocyte chemotactic protein 1 (MCP-1), which are associated with the inflammatory responses of PID (13,14). The clinical use of antibiotics is the preferred choice for PID treatment according to the Centers for Disease Control and Prevention guidelines in the United States (15); however, the majority of patients often experience subsequent bacterial drug-resistance (13). Therefore, the development of effective natural drugs for the prevention of PID is of great importance.

Asiatic acid (AA) is a natural triterpenoid extracted from *Centella asiatica* (16,17). It has many beneficial properties, including anti-inflammatory (16) and antioxidant (18) effects. Recently, AA has been demonstrated to control inflammation and exert protective effects via inhibiting NLRP3 inflammasome activation (16) and the NF-κB (19) pathway. However, the effects of AA on PID remain unknown. Therefore, the aim of present study was to investigate the protective effects and underlying mechanisms of AA in a rat model of PID.

Materials and methods

Chemicals and reagents. AA was purchased from Sigma-Aldrich (Merck KGaA; Darmstadt, Germany). Pentobarbital was purchased from Chengdu XiYa Chemical Technology Co., Ltd. (Chengdu, China). Progesterone injections were obtained from Zhejiang Xianju Pharmaceutical Co., Ltd. (Taizhou, China). Absorbable gelatin sponges were purchased from Jinling Pharmaceutical Co., Ltd. (Nanjing, China). The

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pathogenic *Escherichia coli* strain and *Ureaplasma urealyticum* strain (t-strain mycoplasma) were obtained from Nanjing Bianzhen Biology Science and Technology Co., Ltd. (Nanjing, China).

Animal selection and group allocation. A total of 75 female specific pathogen-free Sprague Dawley rats (aged 9 weeks; weighing 220-240 g; Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China), which were maintained under the controlled conditions at 23˚C with a 12-h light/dark cycle (60% humidity) and access to food and water *ad libitum*, were assigned randomly to the following five equal groups: A control group, consisting of rats that received vehicle only (propylene glycol, 1.0 mg/kg) via intragastric gavage once daily; a PID group consisting of rats that underwent the model of PID and received an equal volume of vehicle (propylene glycol, 1.0 mg/kg) via intragastric gavage once daily; a PID + AA 5 group consisting of rats that underwent the model of PID and received 5 mg/kg AA in vehicle (1.5 ml) via intragastric gavage once daily; a PID + AA 35 group consisting of rats that underwent the model of PID and received 35 mg/kg AA in vehicle via intragastric gavage once daily; and a PID + AA 75 group consisting of rats that underwent the model of PID and received 75 mg/kg AA in vehicle via intragastric gavage once daily. All procedures were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University (Hangzhou, China).

Establishment of the PID model. A PID model in rats was established based on previously published protocols (13,14). Rats in the study were immediately administered intramuscular injections of buprenorphine (0.01 mg/kg; bid; Tianjin Medicine Research Institute Pharmaceutical Co., Ltd., Tianjin, China) to relieve pain following infection (17). All rats were acclimated for 7 days and subcutaneously injected with 45 mg/kg progesterone (Zhejiang Xianju Pharmaceutical Co., Ltd., Taizhou, China) prior to infection. An absorbable gelatin sponge was saturated with microbe-mixing solution (Nanjing Bianzhen Biology Science and Technology Co., Ltd., Nanjing, China) with *U. urealyticum* ($1x10^8$ cfu/ml) and pathogenic *Escherichia coli* ($1x10^8$ cfu/ml). The upper genital tract of each rat in the PID group was then implanted with a microbe-containing gelatin sponge and rats were inverted for 3 min. The cervixes of rats in the control group were implanted with microbe-free gelatin sponges. A total of four infections were performed every 2 days. Following the first infection, the experimental groups were administrated with AA via intragastric gavage and the PID and control groups were gavaged with an equal volume of vehicle. At 8 days following the first infection, rats were intravenously anesthetized with pentobarbital at a dose of 30 mg/kg. Following anesthesia, the right fallopian tube and uterus were harvested and stored at -80˚C. When rats exhibited severe symptoms e.g. convulsion for a period of >10 min, they were euthanized with via intravenous administration of pentobarbital at a dose of 140 mg/kg as previously described (20).

Western blot analysis. The right fallopian tube and right uterine horn (n=5 from each group) were used for western blotting. Samples were homogenized and extracted using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Nanjing, China), and then centrifuged at 5,000 x g at 4˚C for 30 min. The protein concentration in the supernatant was measured using a bicinchoninic acid assay kit. Soluble lysates (30 μg/lane) were resolved using 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat dry milk at 25˚C for 2 h and incubation was performed with the following specific primary antibodies: Anti-NLRP3 (1:1,000; sc-34410), anti-caspase-1 (1:1,000; sc-1597 all Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-p-NF-κB p65 (1:500; ab10859369), anti-p-inhibitor of NF-κB (IκB)-α (1:500; ab331284), anti-caspase-3 (1:1,000; ab9664; all Abcam, Cambridge, UK) and anti-β-actin (1:1,000; sc-1616; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Membranes were then rinsed with Tris-buffered saline with Tween and further incubated with the secondary antibody (goat anti-mouse IgG; 1:500; bs12478; Bioworld, Biogottechnology, Co., Ltd., Nanjing, China) for 1 h at room temperature. Detection of specific proteins was performed with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The levels of protein were analyzed using ImageJ (v2.1.4.7, National Institutes of Health, Bethesda, MD, USA) software.

Biochemical analysis. Samples of the right uterus and fallopian tube were collected and homogenized, and then centrifuged at 5,000 x g at 4˚C for 30 min. The concentration of IL-1β (ERC007.96; Neobioscience, Beijing, China), IL-6 (ERC003.96; Neobioscience), TNF-α (ERC102a.96; Neobioscience), MCP-1 (ERC113.48; Neobioscience), chemokine C-X-C motif ligand 1 (CXCL-1; ab219044, Abcam) and chemokine C-C motif ligand 5 (RANTES; ERC105.96; Neobioscience,) supernatants was determined using ELISA kits according to the manufacturer's protocol. Malondialdehyde (MDA; S0131) production and superoxide dismutase (SOD; S0060) activity was measured by commercial kit (Beyotime Institute of Biotechnology) following the manufacturer's protocol. Myeloperoxidase (MPO) activity was assessed using an MPO assay kit (A044; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis. Data are expressed as the mean + standard error of the mean and were analyzed through SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using a one-way analysis of variance followed by Dunnett's post hoc test. $P<0.05$ was determined to indicate a statistically significant difference.

Results

AA reduces the level of inflammatory cytokines and chemokines. Levels of proinflammatory cytokines IL-1β (Fig. 1A), IL-6 (Fig. 1B) and TNF-α (Fig. 1C), as well as chemokines MCP-1 (Fig. 1D), RANTES (Fig. 1E) and CXCL-1 (Fig. 1F) were detected. The results indicate that IL-1β, IL-6, TNF-α, CXCL-1, MCP-1 and RANTES expression was significantly increased following infection in the PID group compared with the control group, whereas AA significantly decreased the expression of inflammatory cytokines and chemokines in

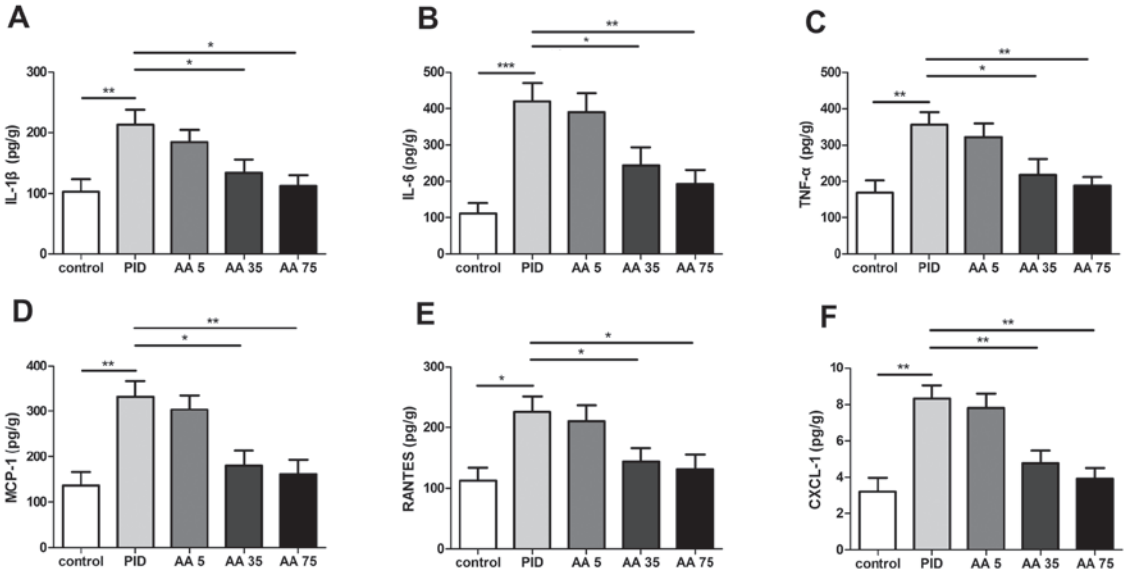

Figure 1. AA administration reduces the level of inflammatory cytokines and chemokines following the establishment of a PID model in rats. Effects of AA on the levels of (A) IL-1β, (B) IL-6, (C) TNF-α, (D) MCP-1, (E) RANTES and (F) CXCL-1. *P<0.05, **P<0.01, ***P<0.001. n=5. AA, Asiatic acid; PID, pelvic inflammatory disease; IL, interleukin; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemotactic protein 1; RANTES, chemokine C-C motif ligand 5; CXCL-1, chemokine (C-X-C motif) ligand 1; AA 5, PID + 5 mg/kg AA group; AA 35, PID + 35 mg/kg AA group; AA 75, PID + 75 mg/kg AA group.

the PID + AA 35 and PID + AA 75 groups compared with the PID group (Fig. 1). No significant differences were observed between the PID and PID + AA 5 groups, PID + AA 35 and PID + AA 75 groups, PID + AA 35 and control groups, and PID + AA 75 and control groups.

AA inhibits neutrophil infiltration. MPO activity was assessed, as it is an oxidative enzyme in neutrophils and acts as a specific marker of neutrophil infiltration (16,17,21) (Fig. 2). The results demonstrated that MPO activity was significantly increased in the PID group compared with the control group, whereas AA significantly inhibited MPO activity in the PID + AA 35 and PID + AA 75 groups compared with the PID group. The differences observed between the PID and PID + AA 5 groups, PID + AA 35 and PID + AA 75 groups, PID + AA 35 and control groups, and PID + AA 75 and control groups were not significant.

AA suppresses NLRP3 inflammasome activation and the NF-κB pathway. To determine the potential mechanisms of AA, the expression of NLRP3, caspase-1 p-NF-κB p65, p-IκB-α, and cleaved caspase-3 were measured. PID induced a significantly higher expression of NLRP3, active caspase-1, p-NF-κB p65, p-IκB-α and cleaved caspase-3 protein in the PID group compared with the control group (Fig. 3). By contrast, these changes were significantly reversed in the PID + AA 35 and PID + AA 75 groups compared with the PID group. However, the levels of active caspase-1, p-NF-κB p65, p-IκB-α and cleaved caspase-3 (only in the PID + AA 35 group) were significantly increased in the PID + AA 35 and PID + AA 75 groups relative to control animals. Furthermore, the differences observed between PID + AA 35 and control groups, and PID + AA 75 and control group on the level of NLRP3 were not significant. No significant differences were observed between the PID and PID + AA 5 groups or the PID + AA 35 and PID + AA 75 groups.

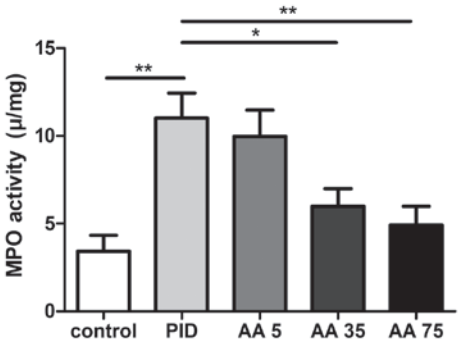

Figure 2. AA administration inhibits neutrophil infiltration. Effect of AA on MPO activity. *P<0.05, **P<0.01. n=5. AA, Asiatic acid; MPO, myeloperoxidase; PID, pelvic inflammatory disease; AA 5, PID + 5 mg/kg AA group; AA 35, PID + 35 mg/kg AA group; AA 75, PID + 75 mg/kg AA group.

AA attenuates oxidative stress. The effects of AA on SOD activity and MDA production were assessed (Fig. 4). There was a significant decrease in SOD activity in the PID group compared with the control group, whereas the MDA level was significantly increased. However, treatment with AA in the PID + AA 35 and PID + AA 75 groups significantly increased the SOD activity and decreased the production of MDA compared with the PID group. However, the levels of MDA were significantly increased in the PID + AA 35 group relative to control animals. No significant differences were observed between the PID and PID + AA 5 groups, the PID + AA 35 and PID + AA 75 groups, and PID + AA 75 and control groups.

Discussion

AA is derived from a Chinese herbal plant, which has a long medical history in China (16,17). Due to its low side-effect profile and range of biological properties, it has been used to treat a wide range of diseases (22-24). However, the effects of

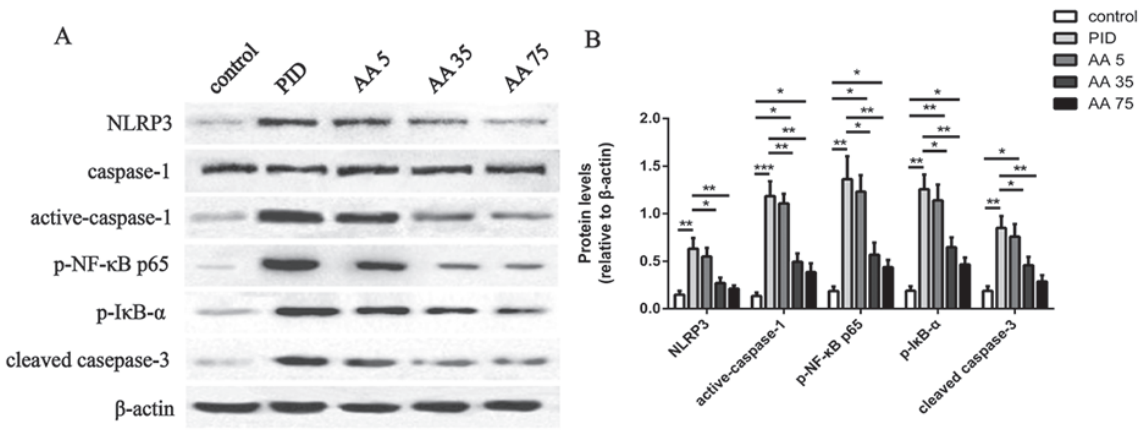

Figure 3. AA administration suppresses activation of NLRP3 inflammasome, the NF-κB pathway and caspase-3. (A) Levels of NLRP3, caspase 1, active-caspase-1, p-NF-κB p65, p-IκB-α, cleaved caspase-3 and β-actin indicated by western blotting. (B) Quantification of western blotting. *P<0.05, **P<0.01, ***P<0.001. n=5. AA, Asiatic acid; NLRP3, nucleotide-binding domain-like receptor protein 3; p-NF-κB p65, phosphorylated-nuclear factor-κB p65; p-IκB-α, phosphorylated- inhibitor of NF-κB; PID, pelvic inflammatory disease; AA 5, PID + 5 mg/kg AA group; AA 35, PID + 35 mg/kg AA group; AA 75, PID + 75 mg/kg AA group.

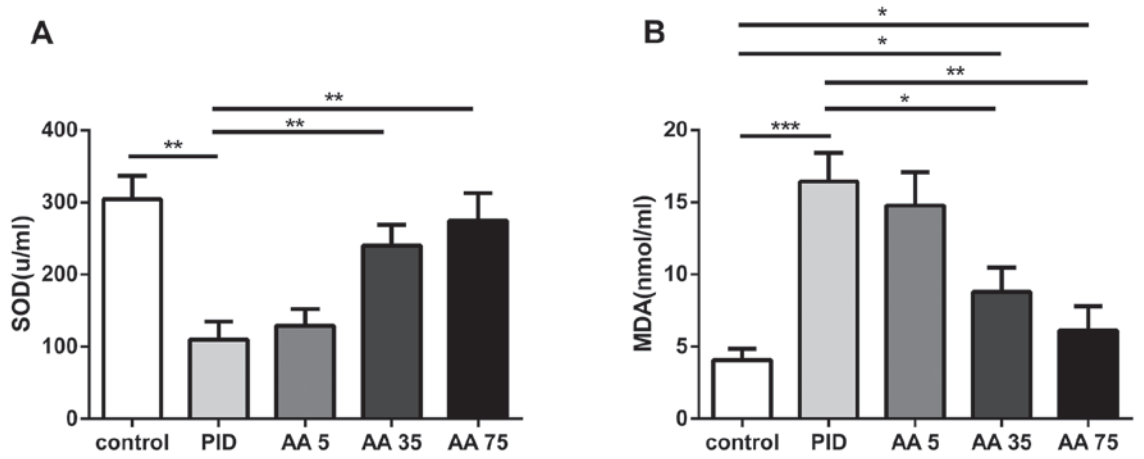

Figure 4. AA administration attenuates oxidative stress. Effects of AA on (A) MDA production and (B) SOD activity. *P<0.05, **P<0.01, ***P<0.001. n=5. AA, Asiatic acid; MDA, malondialdehyde; SOD, superoxide dismutase; PID, pelvic inflammatory disease; AA 5, PID + 5 mg/kg AA group; AA 35, PID + 35 mg/kg AA group; AA 75, PID + 75 mg/kg AA group.

AA on PID remain unknown. The results of the present study demonstrated that AA decreases the levels of inflammatory cytokines and chemokines, inhibits neutrophil infiltration, suppresses the activation of NLRP3 inflammasome, caspase-3 and the NF-κB pathway, and attenuates oxidative stress.

Inflammation is a primordial defense against infection (25); however, an excessive inflammatory response may induce tissue damage and cause physiological dysfunction (16,17). IL-1β is a subtype of IL-1 and a pivotal inflammatory cytokine that increases levels of proinflammatory cytokines, including IL-6 and TNF-α, amplifies the inflammatory response and induces apoptosis (26). IL-6 is another principal proinflammatory cytokine that serves an important role, regulating the release of chemotactic mediators and cell adhesion molecules (27). Furthermore, IL-6 directly affects tubal transport (28). TNF-α also contributes to cell death and tissue injury (28,29). In addition, chemokines CXCL-1, MCP-1 and RANTES serve a role in the recruitment and activation of inflammatory cells, and activated neutrophils promote the inflammatory response (13). In the present study, levels of proinflammatory cytokines, chemokines and neutrophils were significantly increased following pathogen infection and AA treatment markedly reversed these changes.

NLRP3 inflammasome serves an important role in inflammation (7,8,15). Aberrant NLRP3 inflammasome activation is deleterious and conducive to the development of a number of inflammatory diseases (30). The aim of the present study was therefore to evaluate its relevance during PID. To the best of our knowledge, the current study is the first to demonstrate that PID induces NLRP3 inflammasome activation, while AA administration suppresses it.

Oxidative stress, including reactive oxygen species (ROS), contributes to cell and tissue damage; furthermore, ROS regulate NLRP3 inflammasome activation (31). SOD is an important antioxidant enzyme that clears oxygen radicals and inhibits tissue damage (32). MDA is the final product of lipid peroxidation, which represents the oxidative stress intensity and oxygen free radical levels (17). The results of the present study suggest that

PID reduces SOD and increase MDA, whereas AA treatment markedly inhibits these changes and attenuates oxidative stress.

In conclusion, the results of the present study suggest that AA treatment has potent anti-inflammatory and antioxidant effects in rats with pathogen-induced PID and the mechanism of this action may be associated with suppression of NLRP3 inflammasome activation and the NF-κB pathway. The present study provides a basis for further research into the potential use of AA as a clinical treatment for patients with PID.

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Availability of data and materials

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

Authors' contributions

DK and PF were responsible for the experimental design and data analysis. DK drafted the manuscript. QZ and XM were responsible for the experiments. PJ analyzed amd interpreted the data and revised the manuscript critically for important intellectual content. All authors checked and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures and care were approved by the guidelines of Animal Ethics Committee of Zhejiang Chinese Medical University and were in compliance with the relevant laws and institutional guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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