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Asiaticoside ameliorates uterine injury induced by zearalenone in mice by reversing endometrial barrier disruption, oxidative stress and apoptosis

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Abstract

Zearalenone (ZEA) is a mycotoxin produced by *Fusarium fungi* that has been shown to have adverse effects on human and animal health, particularly on the fertility of females. As a saponin derived from the medicinal plant *Centella asiatica*, asiaticoside (AS) has multiple bioactivities. This study aimed to investigate the protective effects of AS on ZEA-induced uterine injury and the underlying mechanism. In the present study, we demonstrated that AS could rescue ZEA-induced uterine histopathological damage and modulate the secretion of sex hormones, including progesterone (P4), luteinizing hormone (LH), and estradiol (E2), in ZEA-treated mice. Moreover, AS alleviated ZEA-induced damage to endometrial barrier function by upregulating the expression of tight junction proteins (ZO-1, occludin, and claudin-3). Further mechanistic investigations indicated that ZEA reduces the antioxidant capacity of uterine tissues, whereas AS improves the antioxidant capacity through activating the Nrf2 signaling pathway. Most notably, the protective effect of AS was blocked in Nrf2 gene knockout ($Nrf2^{-/-}$) mice. Moreover, the p38/ERK MAPK pathway has been implicated in regulating ZEA toxicity and the beneficial effect of AS. Additionally, an Nrf2 inhibitor (ML385) weaken the suppressive effect of AS on the oxidative stress and MAPK pathway. AS also inhibits ZEA-induced apoptosis in uterine tissues via the PI3K/Akt signaling pathway. However, when the PI3K small molecule inhibitor LY294002 was co-administered, the ability of AS to suppress the expression of apoptosis-related proteins and inhibit ZEA-induced apoptosis decreased. Collectively, these findings reveal the involvement of multiple pathways and targets in the protective effect of AS against ZEA-induced uterine injury, providing a new perspective for the application of AS and the development of a ZEA antidote.

Keywords Zearalenone, Asiaticoside, Uterine injury, Nrf2, Apoptosis

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Introduction

Zearalenone (ZEA), also known as F-2 toxin, is a non-steroidal mycotoxin derived from certain species of the *Fusarium* genus [1]. It is commonly found in human food and livestock feed globally, including wheat, corn, cereals, and their processed products [2]. Exposure to ZEA can result in severe damage to various organs in both humans and animals, such as liver damage, intestinal toxicity, and reproductive toxicity [3–5]. Studies have shown that ZEA can induce irreversible toxic injury during the development of embryos, leading to reproductive disorders [6]. In addition, a previous study revealed that the uterus is an important target organ affected by ZEA, posing a threat to female reproductive health [7]. Therefore, investigating the effects of ZEA on the uterus and finding methods or agents to mitigate ZEA poisoning are essential.

Centella asiatica, which belongs to the Apiaceae family, is widely used in traditional medicine in Southeast Asian countries [8]. In addition, it is eaten fresh as a vegetable in Malaysia and Indonesia [9]. Asiaticoside (AS) (Fig. 1A) is a triterpenoid saponin, the major bioactive constituent extracted from *Centella asiatica*. AS has been reported to exhibit potent pharmacological activity and extensive effects, such as antioxidative, antiapoptotic and anti-inflammatory effects [10]. Moreover, AS can exert anti-inflammatory and antioxidant effects on hyperoxia-induced lung injury and spinal cord injury [11, 12]. Previous studies have demonstrated that AS can also regulate the phosphatidylinositol 3-kinase (PI3K) pathway and inhibit the mitogen-activated protein kinase (MAPK) pathway [13, 14]. In addition, Dang et al. reported that AS alleviated hyperoxia-induced lung injury in premature rats and that this protective effect was mediated by the Nrf2/HO-1 signaling pathway [11]. However, the roles of AS in ZEA-induced uterine injury have yet to be elucidated.

There are many preventive agents that utilize various mechanisms and pathways to treat and block ZEA-induced toxicity, such as activating the Nrf2/HO-1 signaling pathway, repairing barrier function, and inhibiting apoptosis [5, 15, 16]. Nrf2 is a pivotal regulatory transcription factor involved in oxidative stress and serves as a master signaling pathway for antioxidant responses. Additionally, Nrf2 can relieve uterine damage and inflammation caused by ZEA [7]. The epithelial barrier plays an important role in protecting health and resisting disease. Tight junction (TJ) proteins, including ZO-1 and occludin, are major components of the endometrial barrier. Previous studies have shown that ZEA can decrease the expression of ZO-1, claudin-3, and occludin in intestinal tissue. Increasing TJ protein levels might be a mechanism to counter ZEA-induced damage [17].

Previous studies have extensively reported that ZEN can damage the reproductive system, especially the uterus, by increasing apoptosis and activating the mitogen-activated protein kinase (MAPK) signaling pathway [7, 18]. Studies have shown that activation of the PI3K/Akt signaling pathway is involved in regulating cell events (including metabolism, proliferation, and apoptosis) and physiological processes in various tissues [19]. Importantly, ZEA has been shown to induce both cell apoptosis and proliferation through the PI3K/Akt pathway [20]. Regulation of the PI3K/Akt pathway is therefore an important target for ZEA treatment. Moreover, the MAPK signaling pathway (ERK, JNK, and p38) plays a vital role in the inflammatory response, cell apoptosis, and cell proliferation [21]. In addition, numerous studies have shown that the MAPK pathway is crucial for ZEA-induced tissue damage [22].

Thus, we explored the possible mechanisms underlying the observed effects, focusing on endometrial barrier function, oxidative stress and apoptosis. Additionally, numerous studies have shown that AS possess powerful antioxidant properties and exert several beneficial effects through the activation Nrf2 [11, 13]. Therefore, we conducted an investigation to evaluate the effects of AS after exposure to ZEA and explored the possible mechanism from the lens of Nrf2.

Materials and methods

Reagents and chemicals

Zearalenone (ZEA) powder was acquired from Sigma–Aldrich (St. Louis, MO, USA). Asiaticoside (AS) and LY294002 were purchased from Glpbio Technology Inc. (Montclair, CA, USA). The assay kits for malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The primary antibodies used in this study were obtained from Abcam (Cambridge, UK) or Cell Signaling Technology (Boston, MA, USA). All other chemicals were obtained from Sigma–Aldrich unless otherwise indicated.

Animals and experimental design

Female C57BL/6 mice (7 weeks old, 20–21 g) were procured from Liaoning Changsheng Biotechnology Co. Ltd. C57BL/6 Nrf2 knockout ($Nrf2^{-/-}$, strain Nrf2tm1Ywk/J) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All the mice were maintained on a 12-h light/dark cycle at a temperature between 22 °C and 24 °C. All the animals were given a normal chow diet and drinking water. After seven days of adaptive feeding, they were randomly allocated to four treatment groups ($n=8$ per group) (Fig. 1B): the healthy control (Ctrl), AS treatment (AS), ZEA treatment (ZEA), and AS+ZEA treatment (AS_ZEA) groups. ZEA was dissolved in 5% ethanol, and

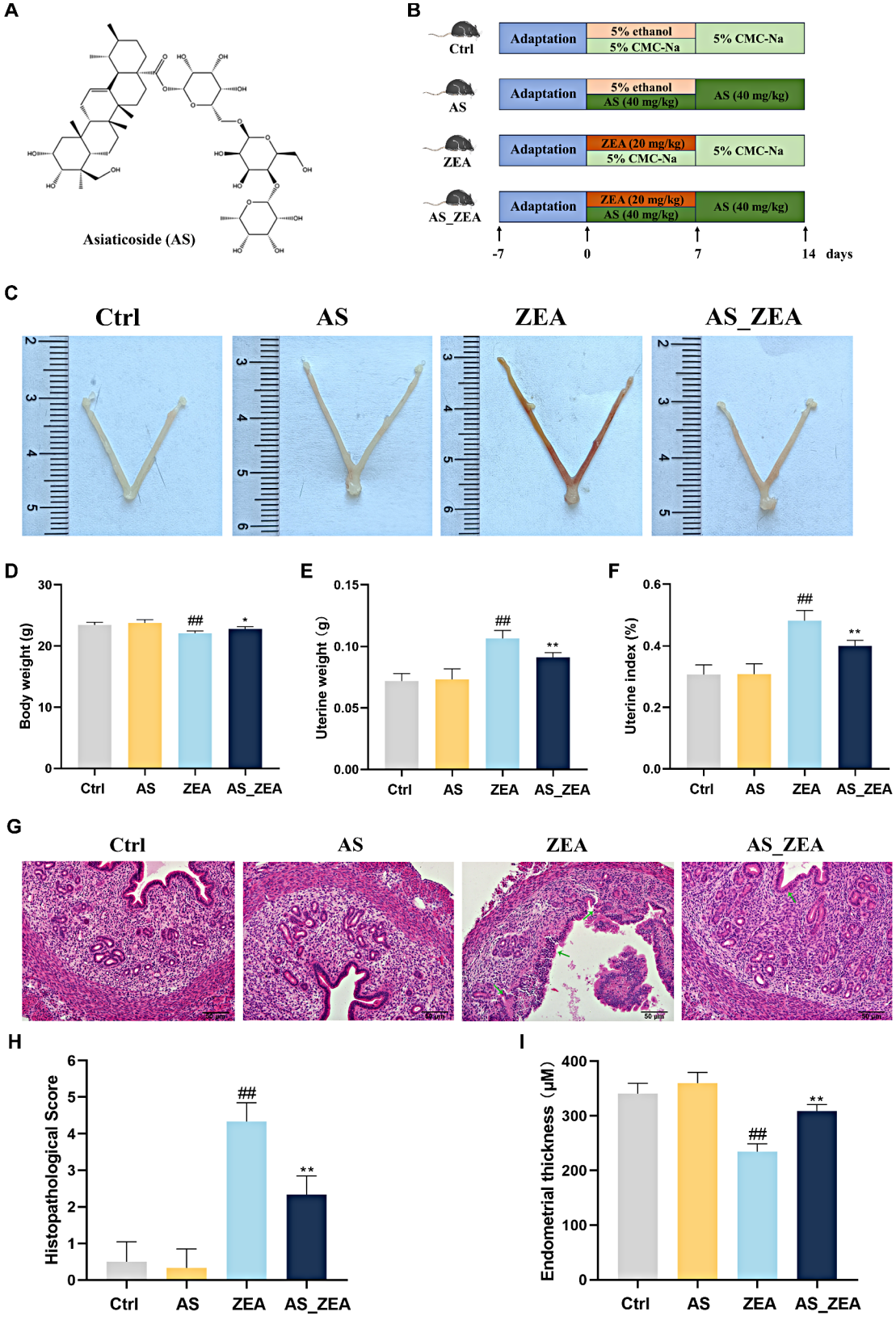

Fig. 1 The effects of AS on ZEA-induced uterine injury. **(A)** The chemical structure of AS. **(B)** Scheme of the drug delivery procedure. **(C)** Uterine morphology observation. Body weight **(D)**, uterine weight **(E)** and uterine index analysis **(F)**. **(G)** Histopathologic section of the uterus (the scale bar represents 50 μm). **(H)** Histopathological scores of uterine sections. **(I)** The average endometrial thickness in each group. ($n = 8$, the values are presented as the means ± SEMs). Compared with the Ctrl group: $^{\#\#}p < 0.01$; compared with the ZEA group, $^{*}p < 0.05$, $^{**}p < 0.01$

AS was dissolved in 5% CMC-Na. The Ctrl and AS groups were intragastrically administered 5% ethanol within 1–7 days, whereas the other groups were orally administered ZEA (20 mg/kg BW) once a day. The AS and AS+ZEA groups were treated with AS (i.e., 40 mg/kg) within 1–14 days, whereas the other groups were given 5% CMC-Na orally once a day. To explore the connection between the PI3K/Akt signaling pathway and apoptosis, an inhibitor of PI3K, LY294002 (30 mg/kg), was injected intraperitoneally 2 h before AS treatment once a day for 14 days (Fig. 6A). To investigate the effect of the Nrf2 pathway on the MAPK pathway and oxidative stress, an Nrf2 inhibitor (ML385) was used in subsequent experiments. ML385 (30 mg/kg) pretreatment was administered intraperitoneally 1 h before the administration of AS (Fig. 4F). To explore the role of the Nrf2 pathway in protecting against ZEA-induced uterine injury, we conducted Experiment 3 with $Nrf2^{-/-}$ mice (four groups, $n=8$ per group) (Fig. 5A). Both wild-type (WT) and $Nrf2^{-/-}$ mice were treated with ZEA to induce uterine damage. During this time, half of the WT and $Nrf2^{-/-}$ mice received AS treatment. The dose and method of administration were the same as those described above. On the 15th day of the experiment, the body weights of the mice were measured. Blood and uterine tissue samples were subsequently collected after euthanasia, and the uterus was weighed. The uterine index was calculated according to the following formula: uterine index (%)=uterine weight/body weight × 100% [7]. All animal experiments in this study strictly abided by the principles of animal welfare and scientific ethics and were carried out under the approval and guidance of the Animal Protection and Use Committee of Jilin University (Permit number: SY202309056).

Assessment of serum reproductive hormones

The mice were euthanized, and the serum was collected by centrifugation. The contents of P4, LH, and E2 in the serum were detected via enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, Wuhan, China).

Histological analysis

After being fixed with 4% formaldehyde for 24 h, the uterine samples were embedded in wax blocks following dehydration. The samples were subsequently cut into 5 μm slices via a microtome. The uterine sections were subsequently stained with hematoxylin and eosin (H&E). Finally, the histopathology of these slices was observed via a light microscope (Olympus, Tokyo, Japan), and images were acquired. Histological scores were estimated as previously described with minor modifications [23]: (i) Hyperaemia/edema: 0, normal; 2, mild; 4, severe. (ii) The number of neutrophils in the visual field: 0, 0–1; 1, 2–5; 2, 6–10; 3, 11–15; 4, 16–20; 5, > 20. The endometrial thickness of each slide was measured from each cross-section under high magnification. In brief, a total of eight high-power fields with 45° intervals in each cross-section were selected, measured, and averaged for each HE-stained section.

Detection of oxidative stress

The appropriate amounts of extraction buffer were added to the uterine tissues, which were then homogenized via a homogenizer. The uterine tissue homogenates were centrifuged, and the protein concentration of the supernatants was determined via a BCA protein concentration assay kit. The activities of SOD and CAT, as well as the content of MDA in the uterine tissues, were measured via commercial kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

Determination of uterine apoptosis via TUNEL staining

Apoptosis in uterine tissue was detected via a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit according to the manufacturer's instructions (Yeasen Biotechnology, Shanghai, China). The sections were observed under a fluorescence microscope, and cells displaying green fluorescence were identified as TUNEL-positive apoptotic cells.

Immunohistochemistry (IHC)

The slicers of uterine tissues were heated in 0.01 M citric acid buffer for antigen retrieval. After incubation with 3% H_2O_2 at room temperature in the dark to remove endogenous peroxidase activity, the sections were blocked with 3% BSA. The tissue slides were incubated with primary antibodies at 4 °C overnight and then with a secondary antibody (HRP) for 1 h at room temperature. Finally, the sections were treated with a DAB chromogenic reagent kit (Solarbio, Beijing, China) and counterstained with hematoxylin. The average optical density (AOD) of the IHCs was calculated via ImageJ software (National Institutes of Health).

Western blot analysis

The uterine tissues were lysed in RIPA lysis buffer containing protease and phosphatase inhibitors (Roche AG, Mannheim, Germany). The nuclear proteins of the uterine tissues were extracted via a nuclear and cytoplasmic protein extraction kit (Yeasen Biotechnology). Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Next, immunoblotting was performed as previously described [24]. Finally, the intensity of the protein bands was measured with ImageJ software, and normalization was conducted with reference to GAPDH.

Statistical analysis

All the data are expressed as the means ± SEMs and were analyzed with GraphPad Prism version 8.0 (GraphPad software, San Diego, CA, USA). To compare normally distributed variables, an independent sample t test was performed. One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to compare data between three or more groups. Unless otherwise stated, the data shown in this study represent at least three independent experiments, and a p value < 0.05 was considered statistically significant.

Results

AS alleviates ZEA-induced uterine injury in mice

To investigate the ability of AS to alleviate uterine toxicity, body weight and uterine weight were measured at the end of the experiment. As shown in Fig. 1C, after the mice were exposed to ZEA, obvious hyperemia of the serosal layer, edema, and necrosis were observed. However, the change in the AS_ZEA group was not significant. In addition, the results indicated that the administration of AS markedly alleviated weight loss and suppressed uterine weight gain induced by ZEA exposure (Fig. 1D and E). Exposure to ZEA also increased the uterine index, which decreased after AS treatment (Fig. 1F). Significant histopathological changes, including the infiltration of a substantial number of inflammatory cells, endometrial epithelial shedding, and severe damage to the structure of uterine tissues, were detected in the ZEA group (Fig. 1G). In addition, ZEA treatment increased the histological score of the uterus (Fig. 1H). Conversely, AS treatment obviously reduced histopathological changes and histological scores. Compared with the Ctrl group, the ZEA group presented a thinner endometrium, and after AS treatment, the thickness increased significantly (Fig. 1I). Moreover, treatment with AS alone had no effect on the morphology or histology of the uterus.

AS reverses sex hormone secretion disorders induced by ZEA

Next, we measured the serum levels of P4, LH and E2 in each group of mice to investigate the effect of ZEA on sex hormone secretion. Compared with the Ctrl group, exposure to ZEA significantly increased the secretion of P4 and LH (Fig. 2A and B) and reduced the secretion of E2 (Fig. 2C), indicating abnormal levels of reproductive hormones following ZEA treatment. Treatment with AS reversed the disorder of sex hormones caused by ZEA. Furthermore, the addition of AS alone had no obvious effect on hormone secretion.

AS improves endometrial barrier function in mice exposed to ZEA

To explore the mechanism by which AS protects against ZEA-induced uterine injury, we further investigated whether ZEA could affect endometrial barrier function in mice. Compared with the Ctrl group, exposure to ZEA decreased the protein expression of TJ proteins (ZO-1, Occludin, and Claudin-3) in uterine tissues (Fig. 3A and B). Compared with ZEA alone, AS therapy significantly increased the protein levels of ZO-1, Occludin, and Claudin-3, indicating that AS could restore endometrial barrier function in ZEA-treated mice.

AS alleviates oxidative stress in the uterus by activating the Nrf2 pathway

We also assessed whether AS could relieve ZEA-induced uterine injury by suppressing oxidative stress. As shown in Fig. 4A-C, compared with that in the Ctrl group, the level of MDA was noticeably increased, while the activities of SOD and CAT were significantly decreased after ZEA exposure. However, AS treatment reversed the changes in SOD, CAT and MDA levels in ZEA-treated mice. Next, we explored the possible mechanism by which AS alleviated ZEA-induced oxidative stress in the uterus. Compared with ZEA treatment, AS treatment markedly upregulated the protein expression of Nrf2

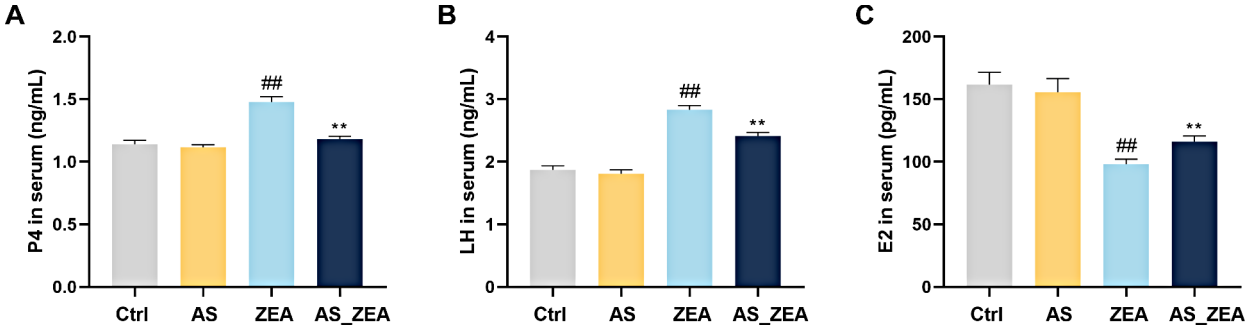

Fig. 2 Effects of AS on the serum levels of P4, LH and E2. **(A)** Serum P4 content. **(B)** Serum LH content. **(C)** Serum E2 content. ($n = 8$, the values are presented as the means ± SEMs). Compared with the Ctrl group: $^{\#\#}p < 0.01$; compared with the ZEA group, $^{**}p < 0.01$

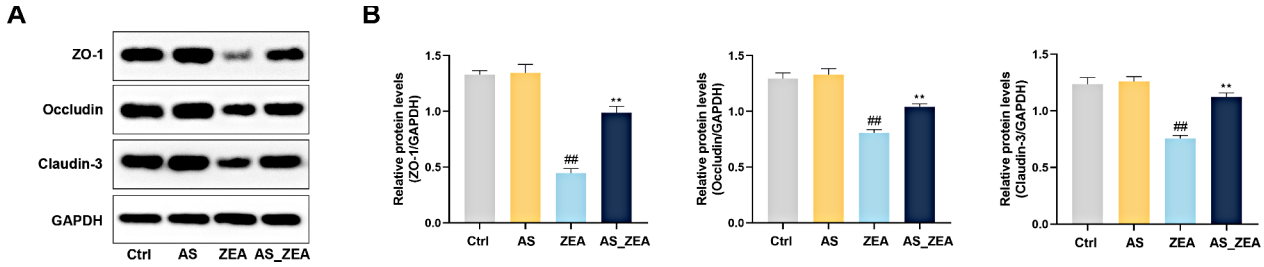

Fig. 3 AS upregulated the protein expression of TJ proteins in ZEA-treated mice. **(A)** Representative immunoblots of ZO-1, Occludin and Claudin-3. **(B)** Relative protein expression of ZO-1, Occludin and Claudin-3. ($n = 6$–8, the values are presented as the means ± SEMs). Compared with the Ctrl group: $^{\#\#}p < 0.01$; compared with the ZEA group, $^{**}p < 0.01$

pathway-related proteins (total Nrf2, nuclear Nrf2, HO-1, and NQO1) in the uteri of mice (Fig. 4D and E). To determine the mechanisms underlying the effects of AS on oxidative stress, an Nrf2 inhibitor (ML385) was used in the subsequent analysis. Notably, we found that ML385 effectively inhibited Nrf2 activity, and these effects of AS on oxidative stress in ZEA-treated mice were offset by pretreatment with ML385 (Fig. 4G-K). Thus, our results indicate that AS may suppress ZEA-induced oxidative stress in uterine tissues by activating the Nrf2 pathway.

Nrf2 is essential for AS to relieve ZEA-induced uterine injury

Next, we utilized Nrf2 knockout ($Nrf2^{-/-}$) mice to further validate the mechanism of the uterus-protective effect of AS in ZEA-treated mice. The experimental procedure and treatment of the mice are illustrated in Fig. 5A. As shown in Fig. 5B, compared with WT mice, $Nrf2^{-/-}$ mice exhibited more severe morphological changes in ZEA-induced uterine injury, along with dramatically suppressed protection of AS from ZEA-mediated uterine injury. H&E staining revealed that Nrf2 depletion dramatically promoted ZEA-mediated uterine damage, as evidenced by more uncertain boundaries of epithelial and stromal cells, increased inflammatory cell infiltration and decreased endometrial thickness (Fig. 5F-H). Moreover, in $Nrf2^{-/-}$ mice, AS did not alleviate ZEA-induced uterine damage or increase endometrial thickness. Unlike the effects of AS on body weight, uterine weight, and the uterine index in WT mice, Nrf2 deficiency eliminated the effects of AS on indicators associated with uterine disease (Fig. 5C-E). In addition, Western blot analysis revealed lower expression of HO-1 and NQO1 in $Nrf2^{-/-}$ mice than in WT mice (Fig. 5I and J). Nrf2 deficiency also abolished the effect of AS on Nrf2 pathway activation. Next, we evaluated oxidative stress levels in ZEA-treated WT and $Nrf2^{-/-}$ mice. AS markedly decreased the MDA content and increased the activity of SOD and CAT in ZEA-treated WT mice; however, it had no effect on $Nrf2^{-/-}$ mice (Fig. 5K-M). These findings suggest that the Nrf2 signaling pathway is essential for the protective and antioxidant effects of AS in ZEA-induced uterine injury.

Activation of the PI3K/AKT pathway by AS attenuates ZEA-induced uterine apoptosis

Several reports have shown that exposure to ZEA can cause apoptosis in uterine tissue [7]. Therefore, we analyzed the protective effects of AS against ZEA-induced uterine apoptosis. We found that the protein expression levels of p-PI3K/PI3K, p-AKT/AKT, and Bcl-2/Bax were significantly decreased and that the protein level of cleaved caspase 3/pro-caspase 3 was significantly increased in ZEA-treated uterine tissue (Fig. 6B and C). In addition, ZEA administration markedly reduced the expression of Ki67 (a proliferative marker) and increased the number of TUNEL-positive cells in uterine tissue (Fig. 6D-G), indicating that ZEA exposure could induce significant apoptosis in the uterus. However, AS treatment clearly reversed the changes caused by ZEA. To investigate the connection between apoptosis and the PI3K/AKT pathway, the mice were pretreated with LY294002 (a PI3K inhibitor) before undergoing AS treatment. LY294002 completely suppressed the increase in p-PI3K/PI3K and p-AKT/AKT caused by AS, indicating that LY294002 successfully inhibited the PI3K/AKT pathway (Fig. 6B and C). Notably, pretreatment with LY294002 partially reversed the inhibitory effects of AS on ZEA-induced apoptosis compared with those in the AS_ZEA group (Fig. 6B-G). These results suggest that AS attenuates ZEA-induced uterine apoptosis by activating the PI3K/AKT signaling pathway.

AS inhibits ZEA-induced MAPK activation by promoting the Nrf2 pathway

In the uteri of the mice, the Western blot results revealed that ZEA exposure increased the protein expression of MAPK pathway-related proteins (p-ERK, p-JNK, and p-p38) (Fig. 7A and B). However, in comparison with the group treated with ZEA alone, the group treated with AS presented significantly lower levels of p-ERK, p-JNK, and p-p38 in ZEA-treated mice. We have confirmed that AS can upregulate Nrf2 pathway in aforementioned results (Fig. 4). Then, we further examine whether the suppressive effect of AS on the MAPK pathway depends on Nrf2. Mice were co-treated with Nrf2 inhibitor ML385

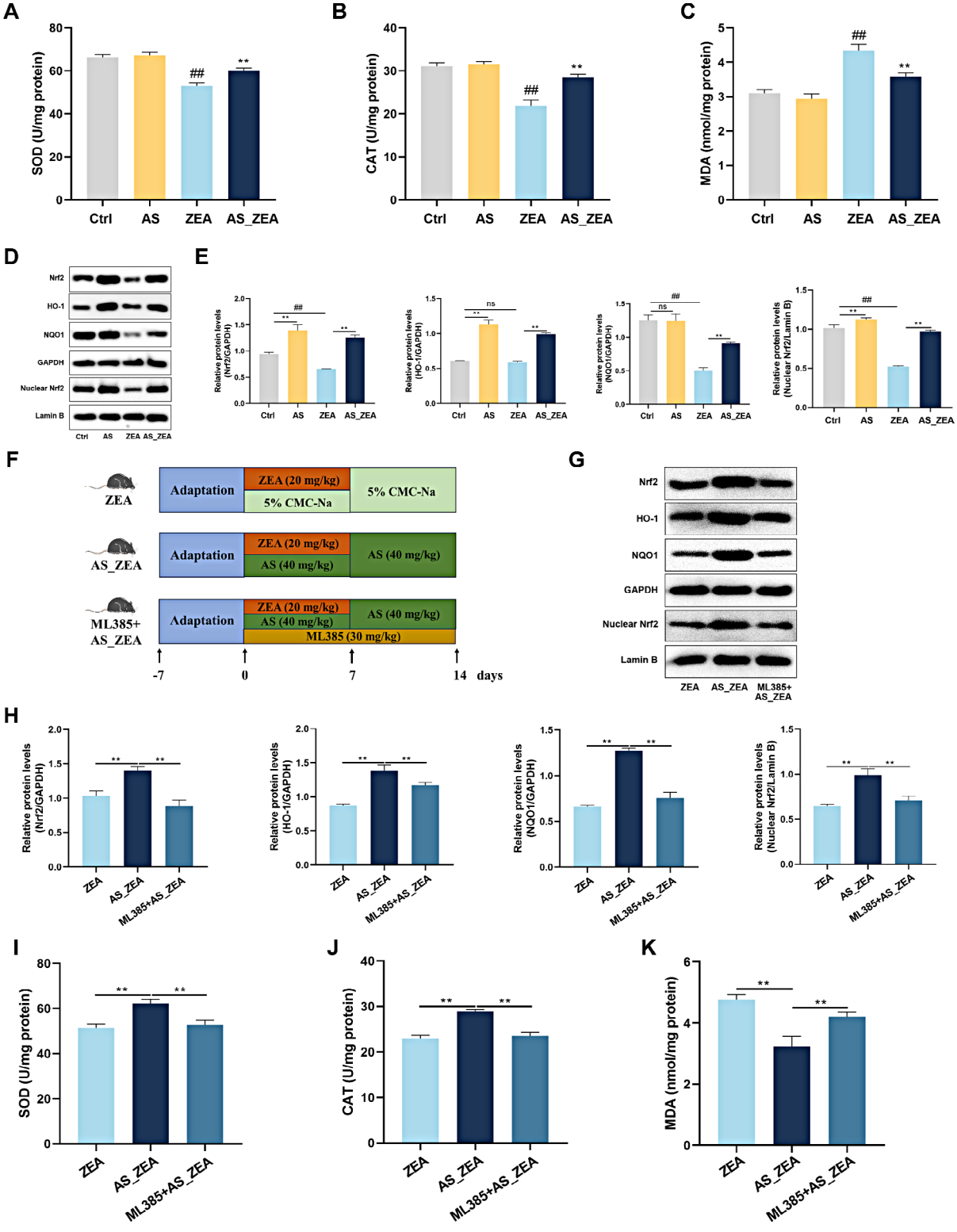

Fig. 4 AS inhibits ZEA-induced oxidative stress by activating the Nrf2 pathway. Mouse uterus **(A)** SOD activity; **(B)** CAT activity; **(C)** MDA content. **(D)** Representative immunoblots of Nrf2, HO-1, NQO1 and nuclear Nrf2. **(E)** Densitometric quantification of Nrf2, HO-1, NQO1 and nuclear Nrf2 expression levels. **(F)** Scheme of the drug delivery procedure. **(G)** Representative immunoblots of Nrf2, HO-1, NQO1 and nuclear Nrf2. **(H)** Relative protein expression of Nrf2, HO-1, NQO1 and nuclear Nrf2. **(I-K)** Uterine SOD, CAT and MDA levels in the mice. ($n = 6–8$, the values are presented as the means ± SEMs). Compared with the Ctrl group: $^{\#\#}p < 0.01$; $^{**}p < 0.01$; ns: not statistically significant

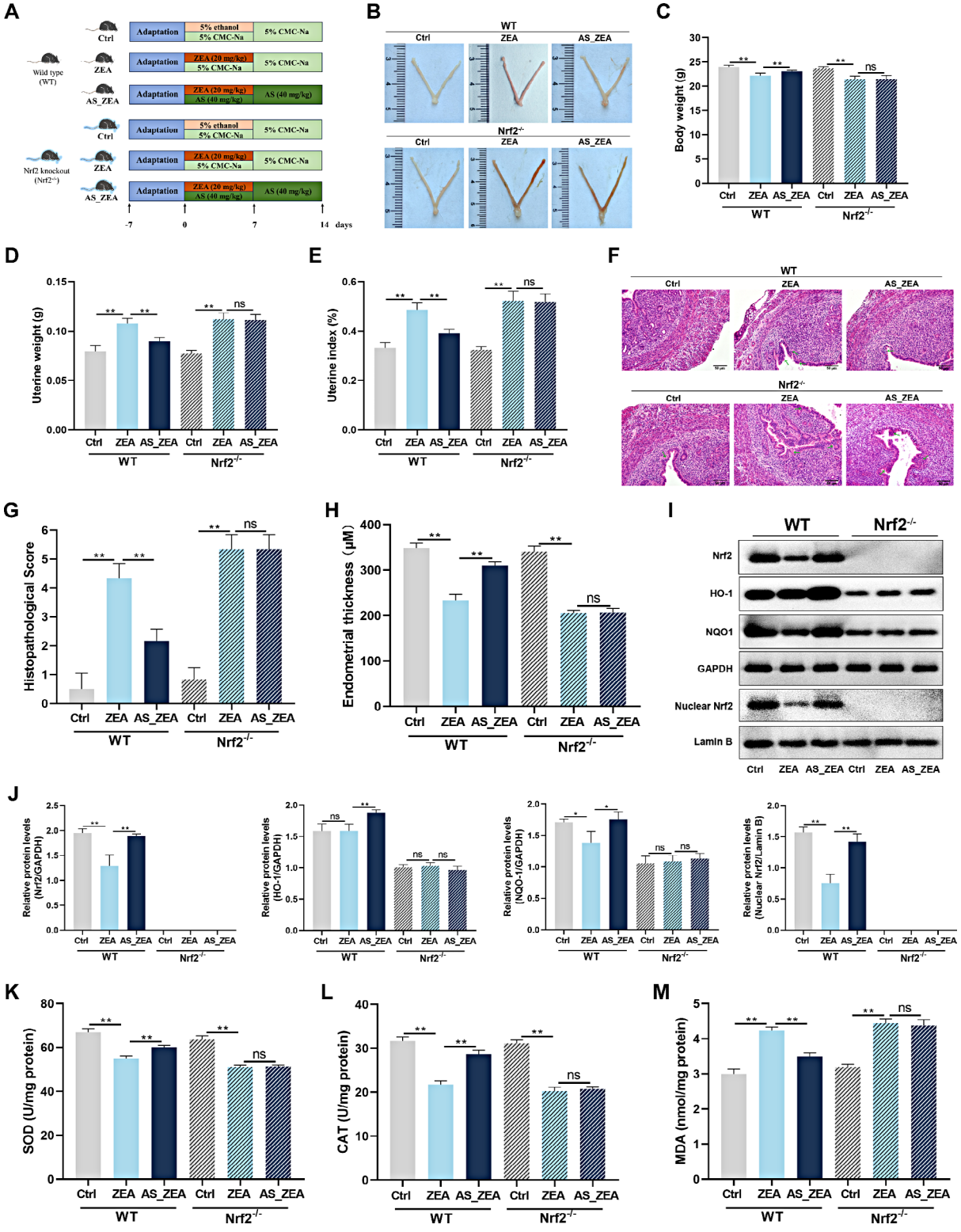

Fig. 5 Nrf2 knockout abolishes the effect of AS on attenuating ZEA-induced uterine injury. **(A)** Experimental design. **(B)** Uterine morphology observation. Body weight **(C)**, uterine weight **(D)** and uterine index analysis **(E)**. **(F)** Histopathologic section of the uterus (the scale bar represents 50 μm). **(G)** Histopathological scores of uterine sections. **(H)** The average endometrial thickness in each group. **(I, J)** The protein expression of Nrf2, HO-1 and NQO1 in the mouse uterus. **(K–M)** Uterine SOD, CAT and MDA levels in the mice. ($n = 6–8$, the values are presented as the means ± SEMs). $^{*}p < 0.05$, $^{**}p < 0.01$, ns: no statistical significance

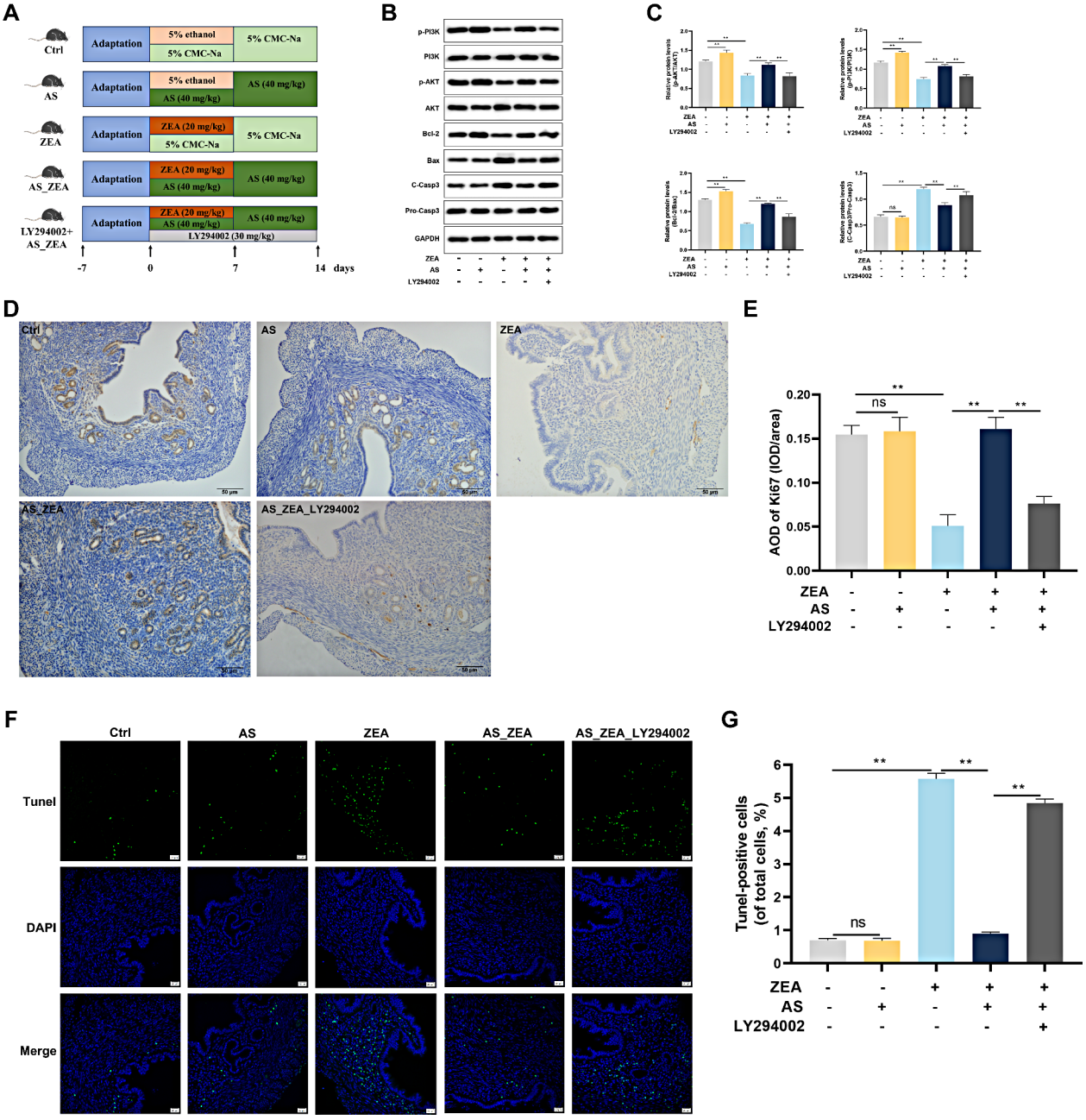

Fig. 6 AS inhibits ZEA-induced apoptosis by activating the PI3K/AKT signaling pathway. **(A)** Experimental design. **(B, C)** The protein ratios of p-PI3K/PI3K, p-AKT/AKT, Bcl-2/Bax and C-Casp3/Pro-Casp3 in the mouse uterus. **(D, E)** IHC staining of Ki67 in the uterine tissues of the mice. **(F, G)** Apoptosis levels were detected by TUNEL staining. ($n = 6–8$, the values are presented as the means ± SEMs). $^{**}p < 0.01$, ns: not statistically significant

in the presence of AS and ZEA (Fig. 4F). The expression of MAPK pathway-related proteins was evaluated. ML385 markedly decreased Nrf2 levels compared with AS_ZEA group (Fig. 7C and D). At the same time, we also observed the suppressive effect of AS on the MAPK-related proteins was strongly attenuated (Fig. 7C and D). These findings indicated that AS may partially block the activity of MAPK by activating the Nrf2 pathway in ZEA-induced uterine injury.

Discussion

ZEA, a mycotoxin produced from moldy grains, is widespread and has a negative impact on human and animal health [6, 25]. Studies have shown that ZEA can induce irreversible injury to the reproductive system of humans and animals, especially the uterus and ovaries [7, 26]. However, to the best of our knowledge, despite the severe uterine damage caused by ZEA contamination in both

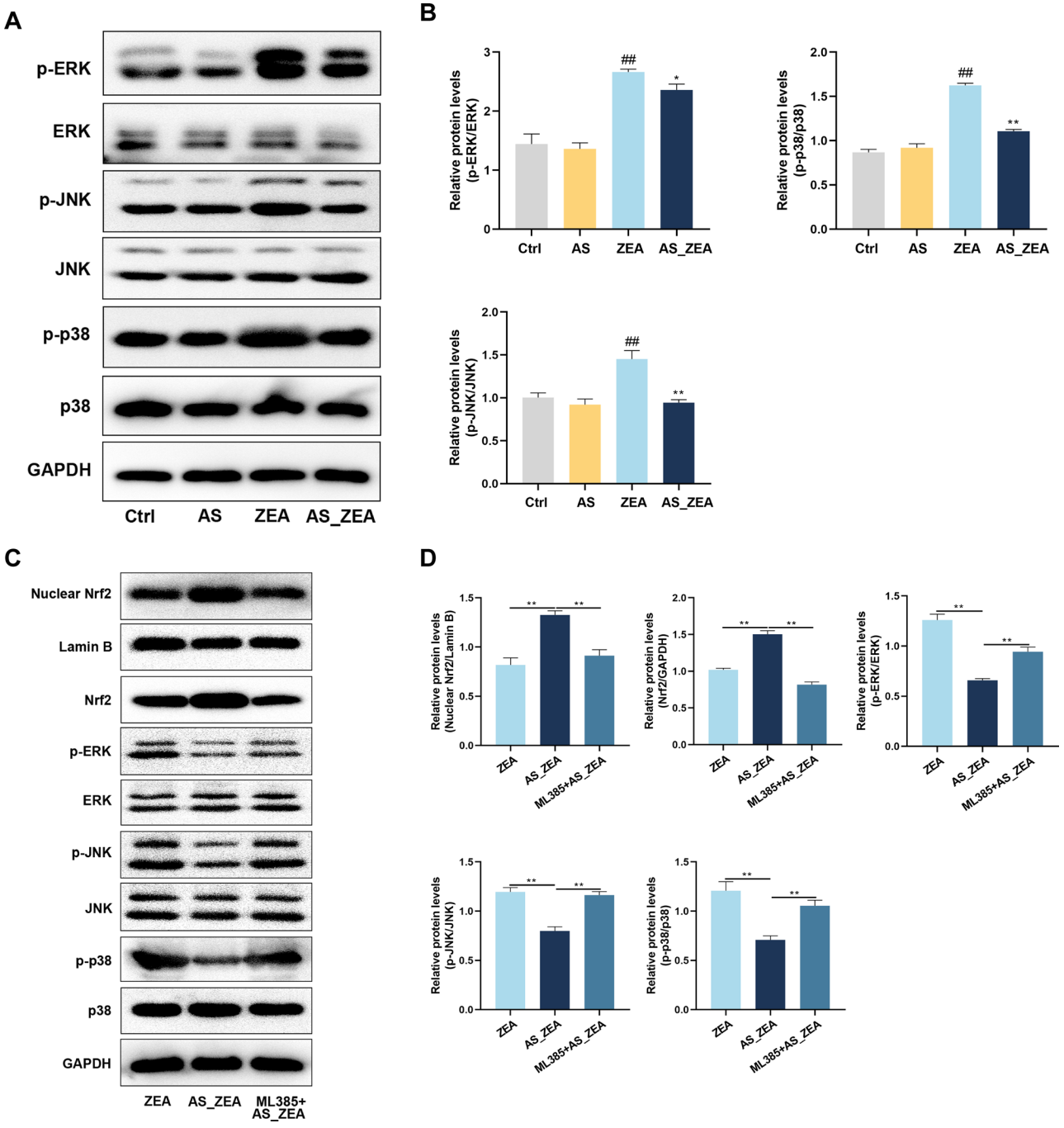

Fig. 7 AS suppresses the MAPK signaling pathway by promoting the Nrf2 pathway in ZEA-exposed mice. **(A, B)** The protein ratios of p-ERK/ERK, p-JNK/JNK and p-p38/p38 in the mouse uterus. **(C, D)** Western blot analysis of the expression of Nrf2, Nuclear Nrf2, p-ERK/ERK, p-JNK/JNK and p-p38/p38. ($n = 6–8$, the values are presented as the means ± SEMs). Compared with the Ctrl group: $^{\#\#}p < 0.01$; $^{*}p < 0.05$, $^{**}p < 0.01$

animals and humans, effective drugs to treat or prevent ZEA exposure are still lacking. Therefore, investigating safe and effective drugs to relieve the damage caused by ZEA to the uterus is highly important. The purpose of this study was to explore the protective effects and molecular mechanisms of AS on ZEA-induced uterine injury in mice. AS is the bioactive component of the plant *Centella asiatica* and has many biological functions, such as antioxidative stress, skin lesion treatment, anti-inflammatory, and antitumor promotion [13, 27, 28]. However, few in vivo and in vitro studies have investigated the effects of uterine-related diseases.

Oxidative stress is the most common cause of damage to uterine tissue. Nrf2 has been recognized as a master switch controlling the cellular redox status. Increasing evidence has shown that the activation of Nrf2 enhances oxidative metabolism and suppresses inflammation [3, 29]. In addition, Nrf2 and its downstream target genes (such as HO-1 and NQO1) provide inherent protective effects against oxidative stress [30]. A recent study showed that baicalin suppressed acute heat stress-induced uterine injury in mice by activating the Keap1/Nrf2 signaling pathway [31]. Our data revealed that AS increased SOD and CAT activity and decreased MDA levels in ZEA-induced uterine tissues, indicating that AS suppressed oxidative stress caused by ZEA. In our study, Nrf2 and Nrf2-responsive antioxidative enzymes (NQO1 and HO-1) were significantly upregulated after AS treatment in ZEA-induced mice. Next, we wanted to identify potential targets for AS to exert a protective effect. In addition, whether Nrf2 plays a key role in AS-mediated antioxidative stress against ZEA-induced uterine injury is unknown. To verify our inference, ML385, a special inhibitor of Nrf2, was used in our study. The results showed that ML385 significantly suppressed the activation of Nrf2 which was activated by AS, and also up-regulated the levels of SOD, CAT and MDA, which were reduced by AS, suggesting that AS inhibited oxidative stress by the Nrf2 pathway. Moreover, $Nrf2^{-/-}$ mice were used in our next study. The experimental data showed that $Nrf2^{-/-}$ mice with and without AS treatment presented no significant changes in Nrf2, NQO1, or HO-1 levels in uterine tissues. In addition, AS failed to reverse tissue damage and the inflammatory response in ZEA-treated $Nrf2^{-/-}$ mice, further supporting the notion that AS protects against ZEA-induced uterine damage through an Nrf2-dependent pathway.

The PI3K/AKT signaling pathway plays important roles in various physiological and pathological processes, including cell growth, survival, and differentiation [32]. Previous studies have highlighted the potential of targeting the PI3K/AKT pathway as a promising therapy for tissue damage caused by ZEA [3, 20]. In addition, activation of the PI3K/AKT signaling axis has been shown to play a vital role in the inhibition of apoptosis [33]. In the present study, mice treated with ZEA exhibited significant apoptosis in uterine tissue via downregulation of the expression of Bcl-2/Bax and upregulation of the expression of cleaved caspase-3. Treatment with AS reversed the ZEA-induced changes in the protein expression of apoptosis-related genes. Moreover, we also found significant activation of the PI3K/AKT pathway in AS-treated mice. Combined with existing research, we further investigated the relationship between apoptosis and the PI3K/AKT pathway. When the PI3K inhibitor LY294002 was used, the inhibitory effect of AS on ZEA-induced apoptosis in uterine tissue was reversed. Hence, we speculated that AS could inhibit uterine apoptosis caused by ZEA by activating the PI3K/AKT signaling pathway.

In the subsequent experiments, we confirmed that AS upregulated the expression of TJ proteins (ZO-1, Occludin, and Claudin 3) and suppressed the p38/ERK MAPK signaling pathway. The MAPK pathway is associated with apoptosis, oxidative stress, and inflammation, which are extremely important in ZEA-induced damage [12, 21]. Studies have shown that AS exerts therapeutic effects on skin diseases by inhibiting the NF-κB and MAPK signaling pathways [34]. In this study, the phosphorylation levels of ERK, JNK, and p38 were significantly decreased by AS treatment in ZEA-induced mice, indicating that inhibition of the MAPK pathway may be a potential mechanism involved in the protective effect of AS. Notably, in the present study, inhibiting Nrf2 using ML385 attenuated the suppressive effect of AS on MAPK activities. This study revealed that the inhibitory effect of AS on the MAPK pathway is partially dependent on activation of the Nrf2 pathway.

In this study, we found that AS can exert a protective effect on ZEA-induced uterine damage and identified possible mechanisms involved in the beneficial role of AS (Fig. 8). First, we observed that AS could maintain the normal secretion of sex hormones under ZEA exposure. Moreover, AS effectively mitigated the decrease in TJ protein expression caused by ZEA, indicating that AS enhanced endometrial barrier function in ZEA-treated mice. Our data confirmed that AS can inhibit oxidative stress by activating the Nrf2 pathway in mice treated with ZEA. Nrf2 plays an important role in the protective effect of AS on ZEA-induced uterine injury. Moreover, further results demonstrated that AS suppressed ZEA-induced apoptosis in uterine tissues by activating the PI3K/AKT pathway. Our data also revealed that AS inhibited the activation of the MAPK signaling pathway induced by ZEA, and that AS partially inhibited the MAPK pathway by triggering the Nrf2 pathway. However, there are still some limitations to the present study. One of the limitations of this study is the small sample size of mice in each group ($n=8$ per group) in our animal experiments, which diminishes the significance of the findings. Another limitation is that the blood concentration of AS in vivo during the animal experiments was not determined. Further research is needed to enhance our understanding of the mechanism of action of AS.

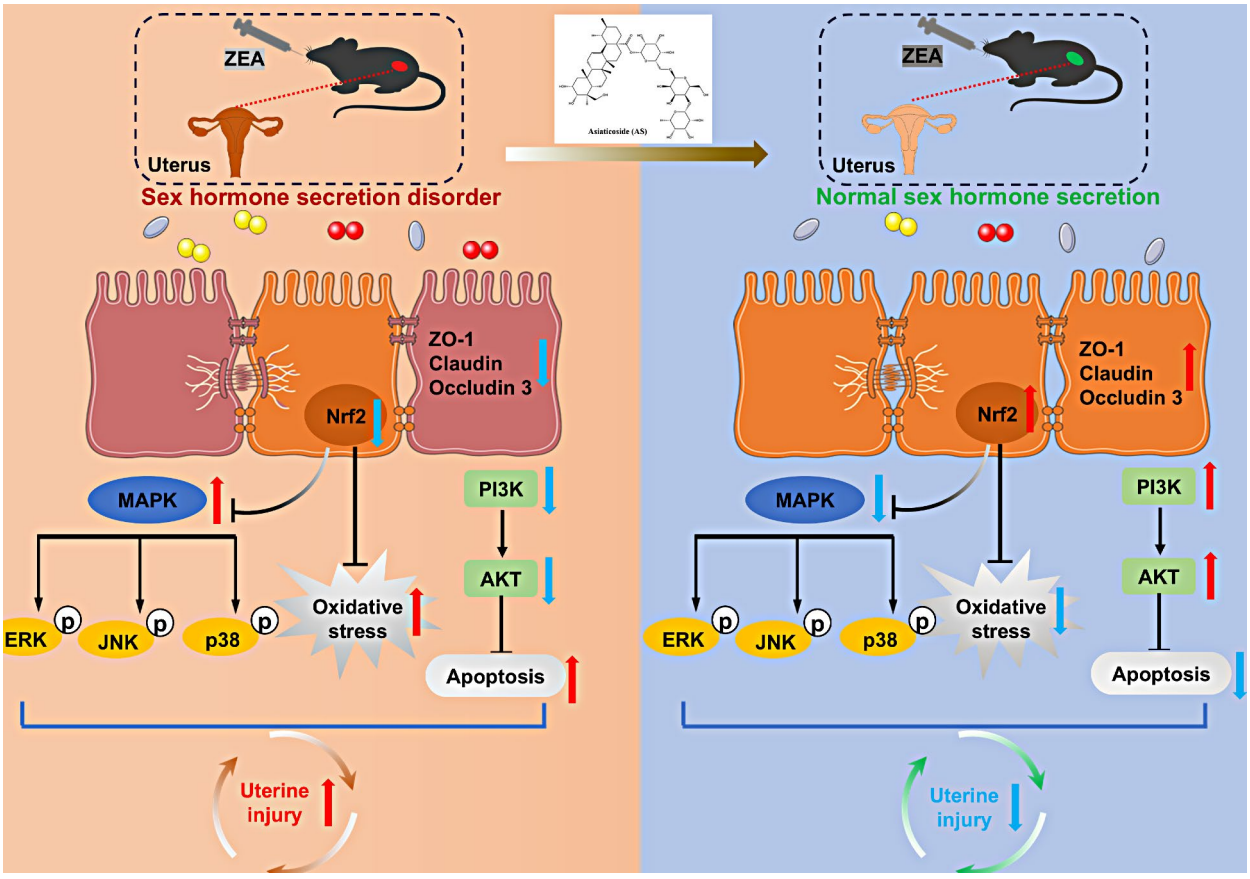

Fig. 8 A proposed signaling pathway involved in AS against ZEA-induced uterine injury in mice

Conclusions

In conclusion, our findings suggest that AS may alleviate ZEA-induced uterine injury in mice by regulating the secretion of sex hormones, enhancing endometrial barrier function, inhibiting oxidative stress, and preventing apoptosis. Mechanistically, AS is capable of suppressing oxidative stress and uterine damage through the activation of the Nrf2 pathway. The inhibition of apoptosis by AS may be related to the PI3K/AKT signaling pathways. Moreover, AS partially blocked MAPK in an Nrf2-dependent manner in ZEA-induced uterine injury. Taken together, our findings may provide a potential therapeutic strategy for ZEA-induced uterine injury.

Supplementary Information

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Supplementary Material 1

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Author contributions

GG and HYJ: conceptualization, visualization, writing, original draft preparation and software analysis. HL and KW: methodology, writing-original draft preparation and editing. HFY and KW: project administration and writing-review & editing. All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Jilin University (Permit number: SY202309056).

Competing interests

The authors declare no competing interests.

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