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# Soy isoflavones ameliorate experimental colitis by targeting ERα/NLRP3 inflammasome pathways☆

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**Abstract**

Soy isoflavones (SIFs) are selective estrogen receptor modulators (SERMs) that have anti-inflammatory activities. Our previous study found that estrogen receptor α (ERα) directly regulates the NLRP3 transcription and NLRP3 inflammasome assembly. Therefore, we hypothesized that SIFs alleviate colitis *via* an ERα-dependent mechanism by targeting the NLRP3 inflammasome. The influence of SIFs on colitis and the potential mechanisms were thoroughly determined in this study. The results suggested that SIFs ameliorated dextran sodium sulfate (DSS)-induced body weight loss, reduced disease activity index and promoted the recovery of colon pathological damage in mice. Moreover, expression of the NLRP3 inflammasome was significantly inhibited, and the release of IL-1β and IL-18 was suppressed by SIFs. Furthermore, ERα blockade ameliorated DSS-induced inflammatory responses in the intestine, and SIFs markedly suppressed the expression of ERα in a dose-dependent manner. Our study demonstrated that the protective therapeutic action of SIFs on DSS-induced colitis depended on inhibition of ERα and subsequent NLRP3 inflammasome activation, and SIFs are promising therapeutic agents for the treatment of colitis.

*Keywords:* Colitis; ERα; Inflammation; NLRP3 inflammasome; Soy isoflavones

## 1. Introduction

Inflammatory bowel disease (IBD) is chronic, relapsing inflammatory disorder of the gastrointestinal tract that is a global health problem with sustained increasing incidence [[1],2]. The precise pathogenesis of IBD has not yet been ascertained, but breakdown of the epithelial barrier followed by aberrant immune responses of the host immune system is widely considered to be the basis of IBD [3]. As an important part of the host immune system, inflammasomes are involved in inflammatory gut diseases [4]. To date, many inflammasomes have been identified; among them, the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is the most fully characterized inflammasome and can assemble into large cytosolic complexes and promote the maturation and secretion of the proinflammatory cytokines IL-1β and IL-18 upon cellular infection or stress [5]. A study demonstrated that NLRP3-deficient mice had attenuated colitis and reduced mortality [6]. Further, in our previous study, we also found that the NLRP3 inflammasome played an important role in IBD [7,8]. Thus, inhibition of NLRP3 inflammasome activation may be beneficial for the treatment of IBD.

In our previous studies, we proved that the nonsteroidal estrogenic mycotoxin zearalenone relieved the inflammatory reaction in colon tissue due to its estrogenic activity [7]. Furthermore, we confirmed that estrogen receptor α (ERα) and NLRP3 were differentially expressed in

*Abbreviations:* IBD, Inflammatory bowel disease; SIFs, Soy isoflavones; DSS, Dextran sodium sulfate; ERα, Estrogen receptor α; ERβ, Estrogen receptor β; NLRP3, NOD-like receptors family pyrin domain containing 3; IL-1β, Interleukin 1β; IL-18, Interleukin 18; ASC, Apoptosis-associated speck-like protein containing CARD; FBS, Fetal bovine serum; PPT, Propyl pyrazole triol; SERMs, Selective estrogen receptor modulators

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normal colon and cancer cells [9]. More importantly, we identified that ERα was the transcriptional regulator of NLRP3, and both regulated NLRP3 expression and promoted NLRP3 inflammasome colocalization [10]. Similar results were obtained in other studies. Armstrong et al. found that epithelial cells within tumors had dramatically increased ERα mRNA and protein expression compared with that of nondiseased mice [11]. ERα-deficient mice showed a significantly reduced incidence of severe disease in dextran sodium sulfate (DSS)-induced colitis [12]. Therefore, we hypothesized that ERα regulates inflammatory pathways, and inhibiting ERα might be a possible strategy for IBD treatment.

Soy foods have been consumed for centuries in Asian countries and are especially in demand in China [13]. Soybeans contain many natural active substances, and they are the richest sources of SIFs in the human diet. Recent studies revealed that SIFs has many benefits, such as effects against cancer, cardiovascular disease, hyperlipidemia and osteoporosis [14,15]. In particular, studies have proven that SIFs have positive effects in the prevention and treatment of inflammatory disorders and diarrheal diseases [16–18]. For example, dietary isoflavones are helpful for individuals with ulcerative colitis who are in remission [19] and alleviate DSS-induced inflammation in mice by inhibiting TLR4/MyD88 signaling [20]. SIFs are typical phytoestrogens that compete with endogenous estrogens to bind with estrogen receptors and exert their effects [21]. However, whether SIFs can alleviate colitis by regulating estrogen receptors and whether the NLRP3 inflammasome plays a key role in this process remain unknown. In this study, we investigated the influence of SIFs on experimental colitis, and the results showed that SIFs ameliorated DSS-induced experimental colitis by targeting ERα/NLRP3 inflammasome pathways. Our study provides new insights into the mechanisms underlying the pharmacological effects of SIFs on colitis, which contribute to novel strategies for the management of colitis.

## 2. Experimental section

### 2.1. Reagents and antibodies

AZD9496 (Cat. HY-12870) was purchased from MCE (New Jersey, USA). SIFs (Cat. SI8910, >95%) was obtained from Solarbio (Beijing, China). Propyl pyrazole triol (PPT, Cat. ab120161) was purchased from Abcam (Cambridge, UK). The compounds in SIFs extracts, as quantified by HPLC, are shown in Table S1. DSS (molecular weight 36–50 kDa, Cat. 60316ES25) was provided by Yeasen (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β (Cat. ml063132) and IL-18 (Cat. ml063131) were purchased from Shanghai Enzyme-Linked Biotechnology Co., Ltd. (Shanghai, China). Total protein extraction kits (Cat. 20101ES60) and BCA protein assay kits (Cat. 20201ES76) were purchased from Yeasen (Shanghai, China). Primary antibodies against ASC (Cat. OM122624), Caspase-1 (p20) (Cat. OM122907), NLRP3 (Cat. OM285746), ERα (Cat. OM186318) and ERβ (Cat. OM252100) were obtained from Omnimabs (CA, USA). FITC-labeled pro-Caspase-1 (Cat. sc-392736) and PE-labeled ACS (Cat. sc-514414) were obtained from Santa Cruz (CA, USA). β-Actin antibodies (Cat. ABS830031SS) were obtained from Absin (Shanghai, China). Goat anti-rabbit HRP linked antibodies (Cat. 7074S) were purchased from CST (MA, USA).

### 2.2. Cell culture and treatment

NCM460 cells were purchased from GuanDao Biological Engineer Corporation (Shanghai, China). NCM460 cells were cultured in DMEM (Cat. C11995500BT, Gibco, Carlsbad, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Cat. F2442, Gibco, Carlsbad, USA). NCM460 cells were cultured in a humidified environment with 5% $CO_2$ at 37°C. NCM460 cells were incubated with 3% DSS for 24 h and administered with 100 nM AZD9496 for another 24 h.

### 2.3. Animal experimental design

Eight-week-old male C57BL/6 mice weighing 20–22 g were used in the study (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China). The animals were maintained under standard conditions (with a temperature of 25°C, a 12-h/12-h light/dark cycle and *ad libitum* access to food and water). All animal welfare and experimental procedures were in accordance with the approval of the ethical regulations of Nanjing Agricultural University (Permission number: SYXK (Su) 2017-0007).

Throughout the experiment, seven groups of mice were utilized; each group has at least six mice: control group, DSS group, DSS+SIFs (50 or 100 mg/kg body weight) group, DSS+AZD9496 (5 mg/kg body weight) group, DSS+PPT (5 mg/kg body weight) group and DSS+PPT (5 mg/kg body weight) + SIFs (100 mg/kg body weight) group. The mice were first challenged with 3% (w/v) DSS in drinking water for 7 consecutive days. The mice in the control group were first given water for 7 days. Then, SIFs, AZD9496, PPT and PPT+SIFs were administered to the mice for 5 days (SIFs were administrated by gavage, AZD9496 and PPT were administrated by intraperitoneal injection). SIFs was dissolved in DMSO and diluted to the corresponding concentration in physiological saline. The doses of SIFs, PPT and AZD9496 used in the study were in accordance with the previous studies [13,16,22]. The mice in the control and DSS groups were treated with the same volume of physiological saline for 5 consecutive days.

### 2.4. Clinical score and histological analysis of colonic lesions

The body weights of the mice were recorded individually, and the disease activity index (DAI) was calculated by the sum of the scores of mental status, appetite, body weight, diarrhea and the presence of occult or overt blood in the stool to evaluate the grade and extent of colitis (Table S2) [8,23]. At the end of the experiment, mice were all sacrificed by cervical dislocation, and the colon was excised and measured. The colon was washed with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde. Then, the fixed colons were embedded in paraffin and cut into 5-μm sections. The sections were stained with hematoxylin and eosin (H&E) for histopathological examination.

### 2.5. Cytokine quantification by ELISA

Colon tissues in each group were flushed with PBS, and the distal-most 1 cm colon was homogenized with PBS. The homogenate was centrifuged at 12,000*g* at 4°C for 15 min. The concentrations of IL-1β and IL-18 in homogenate supernatant were evaluated using ELISA kits. Simultaneously, IL-1β and IL-18 in cell culture medium were also analyzed.

### 2.6. Western blot analysis

The protocols for Western blotting have been reported previously [8]. Proteins for Western blotting were isolated by lysing the colonic tissue and NCM460 cells in RIPA buffer supplemented with PMSF (1:100). Protein concentration was measured by using a BCA kit, and 10 μg of proteins was loaded and separated on 10% SDS-polyacrylamide gels before being transferred onto nitrocellulose membranes (Cat. 10600002, GE Healthcare, MA, USA). The membranes were blocked with 5% nonfat milk for 1 h and incubated with primary antibodies (1:500) overnight at 4°C followed by horseradish -peroxidase-coupled secondary antibodies for 1 h at 37°C. Chemoluminescence was detected with an Amersham Imager 600 (GE Healthcare).

### 2.7. Immunofluorescence

For confocal imaging of fixed cells, after the appropriate treatment, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30

min and blocked with 3% BSA for 1 h. For colon tissue, immunofluorescence was performed on paraffin-embedded colonic tissue sections (5 μm). The sections were deparaffinized, rehydrated and washed in 1% PBS-Tween 20, treated with 3% hydrogen peroxide and blocked with 10% goat serum. Cells and colonic tissue sections were incubated for 2 h at 37°C with primary antibodies. Images were acquired by confocal laser-scanning microscopy (Olympus, Lake Success, NY, USA).

### 2.8. Statistical analyses

Statistical analysis was performed by using GraphPad Prism 7. The data are presented as the mean±S.E.M. One-way analysis of variance was utilized for comparisons between groups. A *P* value of less than .05 was considered significant and less than .01 was considered extremely significant.

## 3. Results

### 3.1. SIFs alleviated DSS-induced colitis

To investigate the effect of SIFs on colitis, we constructed a mouse model of experimental colitis induced by DSS. The results showed that, after treatment with DSS for 7 days, the mice lost approximately 25% of their initial body weight. In contrast, SIFs obviously promoted the recovery of colitis in a dose-dependent manner (Fig. 1A). The DAI score further confirmed the results (Fig. 1B). In addition, colonic

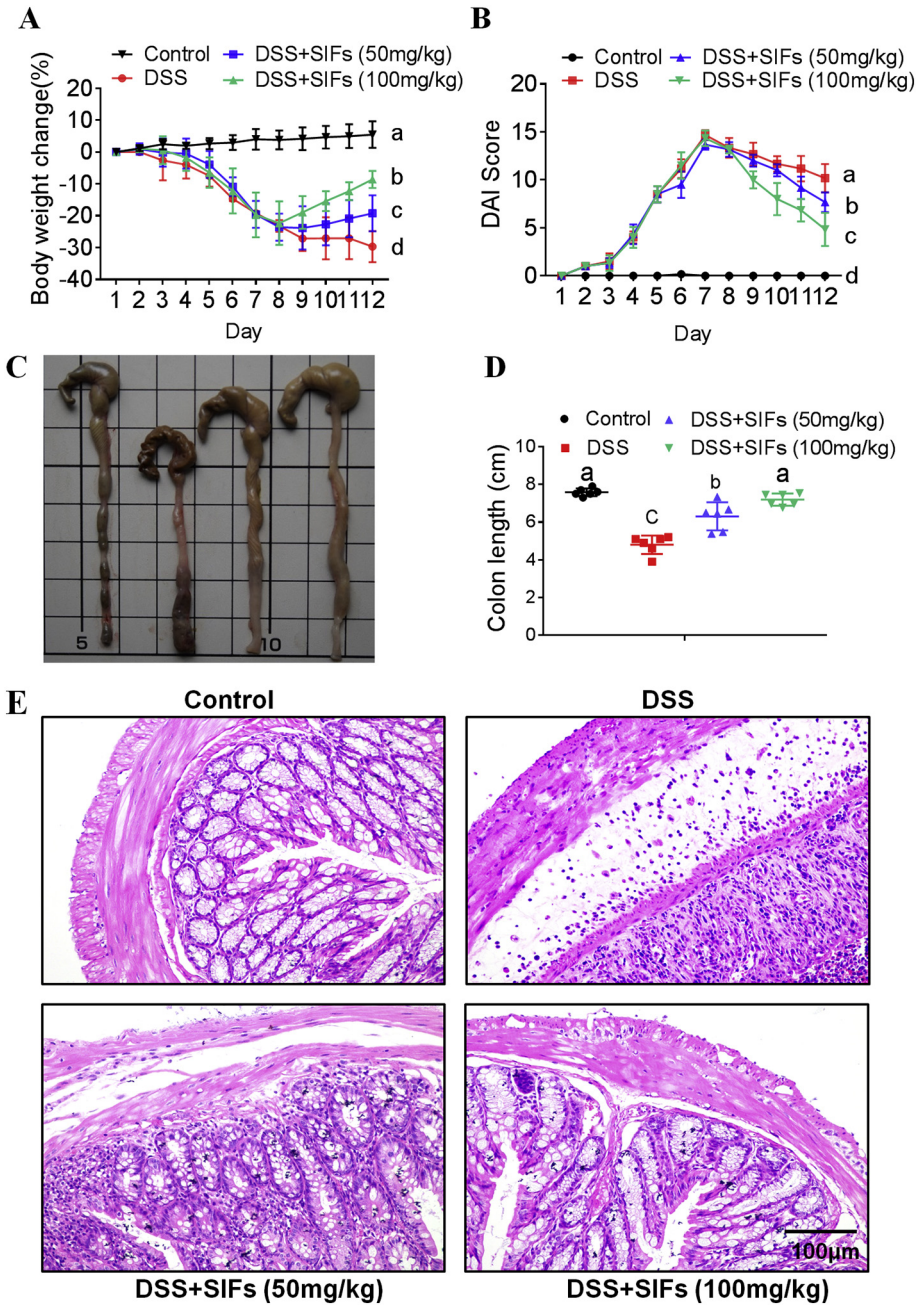


Fig. 1. SIFs alleviated DSS-induced colitis in mice. (A) Body weight change. (B) DAI score of mice. (C and D) The length of colons from each group of mice was measured. (E) The colons from each experimental group were processed for histological evaluation (H&E staining, 200×). The results are expressed as the mean±S.E.M. ($n$=3). Different letters mean significant difference between groups ($P$<.05); the same letters mean that there is no significant difference between groups ($P$>.05).

shortening caused by DSS was significantly reversed by SIFs (Fig. 1C and D). Subsequently, colonic inflammation was evaluated by histopathological analysis. As shown in Fig. 1E, DSS promoted the destruction of epithelial tissue, damaged the intestinal crypts and caused many inflammatory cell infiltrations, which were obviously improved by SIFs administration.

In this study, IL-1β and IL-18 levels in colon tissues of the different experimental groups were determined. SIFs obviously reduced the levels of IL-1β and IL-18 in colon tissues compared to those treated with DSS only (Fig. 2A and B). NLRP3 inflammasome activation is a checkpoint of intestinal inflammation in the colitis model [6]. We then further explored the expression NLRP3 inflammasome components, including NLRP3, ASC and Caspase-1, using Western blotting. As shown in Fig. 2C and D, the expression of NLRP3, Caspase-1 (p20) and ASC was significantly increased in DSS-treated mice and was markedly suppressed by SIFs. SIFs are natural SERMs, which have estrogen-like effects [24]. Therefore, we further investigated the expression of ERα and ERβ in this study. Interestingly, the protein expression of ERα was significantly increased in the DSS group, and SIFs markedly suppressed the expression of ERα in a dose-dependent manner. The expression of ERβ was the opposite that of ERα (Fig. 2E). Our previous study showed that although ERα and ERβ are both regulatory transcription factors of NLRs, ERα increases improve the expression of NLRP3 more significantly than ERβ [10]. Therefore, the protective role of SIFs might be due to inhibition of ERα activation.

### 3.2. ERα blockade protected against DSS-induced colitis

We then blocked ERα *in vitro* by using AZD9496, a specific ERα antagonist, to ascertain the role of ERα in DSS-induced colitis. As shown in Fig. 3A and B, the levels of IL-1β and IL-18 were enhanced by DSS and were markedly reduced by AZD9496 administration. In addition, the increased NLRP3, Caspase-1 (p20) and ASC levels in DSS-treated NCM460 cells were significantly suppressed by the addition of AZD9496 (Fig. 3C and D). We then investigated the effect of AZD9496 on NLRP3 inflammasome assembly by using immunofluorescence. The results demonstrated that, in response to DSS stimulation, NLRP3 fluorescence colocalization with ASC and pro-Caspase-1 with a higher colocalization index (Pearson's *r*), indicating successful NLRP3 inflammasome formation. However, with the administration of AZD9496, the colocalization of ASC, Caspase-1 and NLRP3 was obviously reduced (Fig. 3E).

The role of ERα in the colitis model was further assessed *in vivo* in this study. As seen in the figure, body weight loss, DAI score and colon shortening were obviously ameliorated in the AZD9496-treated group compared to those of the DSS group (Fig. 4A–D). The histopathological analysis indicated that the colon tissue of AZD9496-treated mice presented significantly less histological damage compared to that of DSS-treated mice, shown by intact crypts in large areas without extensive infiltration and mucosal damage (Fig. 4G). Additionally, IL-1β and IL-18 cytokine production in colonic tissues was measured. The results showed that the ERα antagonist AZD9496 significantly inhibited

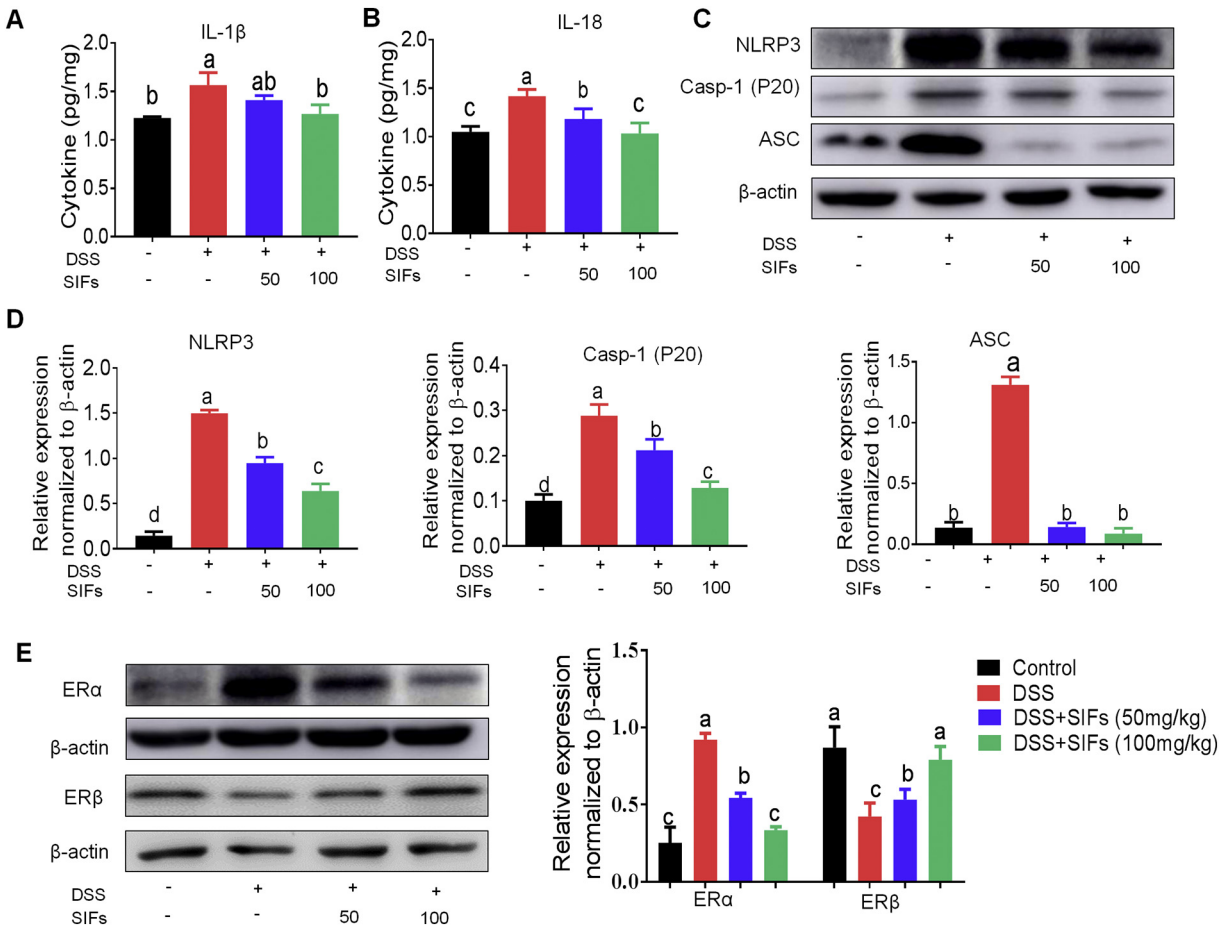


Fig. 2. SIFs reduced proinflammatory cytokine secretion and suppressed NLRP3 inflammasome activation in DSS- induced colitis mice. (A and B) The production of IL-1β and IL-18 in colon. (C) Protein levels of NLRP3, ASC and Caspase-1 in colon tissues. (D) The relative protein expression of NLRP3, ASC and Caspase-1 was normalized to β-actin. (E) Protein levels of ERα in the colon tissues. The relative protein expression of ERα was normalized to β-actin. The results are expressed as the mean±S.E.M. ($n$=3). Different letters mean significant difference between groups ($P$<.05); the same letters mean that there is no significant difference between groups ($P$>.05).

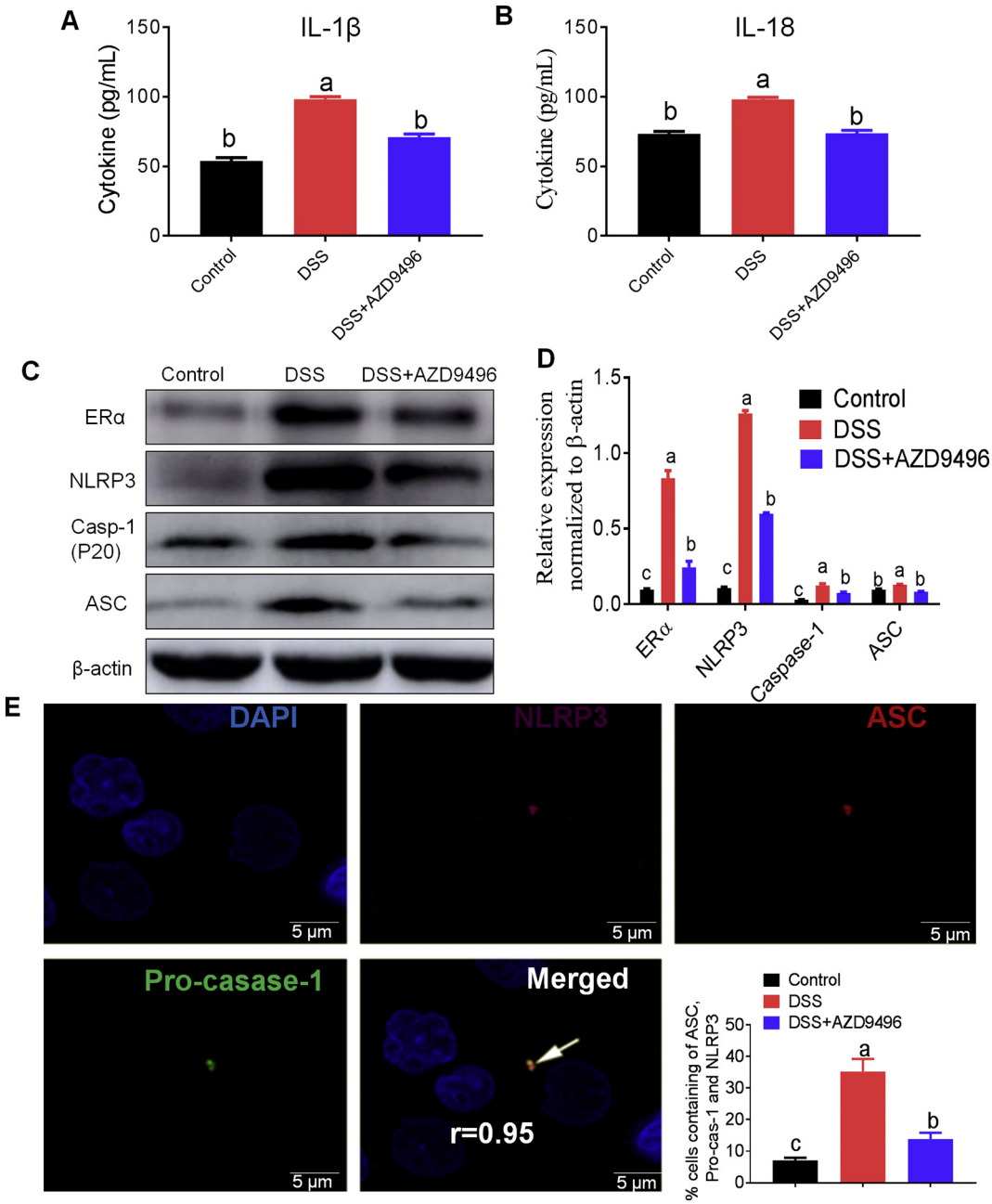


Fig. 3. The ERα antagonist AZD9496 suppressed NLRP3 inflammasome activation and assembly *in vitro*. (A, B) IL-1β and IL-18 in NCM460 cell supernatant were measured by ELISA. (C) Protein levels of NLRP3, ASC, Caspase-1 and ERα in colon tissues. (D) The relative protein expression of NLRP3, ASC and Caspase-1 was normalized to β-actin. (E) NCM460 cells were immunofluorescently stained with Cy5-NLRP3 antibody, PE-ASC antibody and FITC-pro-Caspase-1 antibody. NLRP3-containing aggregates are marked by arrowheads. Images were acquired at 60× magnification. The colocalization index (Pearson's *r*) is graphically represented. The results are expressed as the mean±S.E.M. ($n$=3). Different letters mean significant difference between groups ($P$<.05); the same letters mean that there is no significant difference between groups ($P$>.05).

the production of IL-1β and IL-18 to nearly half of that of DSS-induced group (Fig. 4E and F). Furthermore, NLRP3 translation and inflammasome assembly were studied. As shown in Fig.5, DSS enhanced NLRP3 activation and the NLRP3 inflammation complex assembly in colonic tissue; however, administration of AZD9496 obviously decreased the expression and colocalization of ASC, pro-Caspase-1 and NLRP3.

### *3.3. The ameliorating effect of SIFs in colitis was reversed by an ERα agonist*

To confirm that SIFs alleviated DSS-induced colitis by inhibiting the activation of ERα, we then studied the protective effects of SIFs in the presence of the ERα agonist PPT. As expected, the ERα agonist obviously exacerbated DSS-induced experimental colitis. In the presence of PPT, the therapeutic effect of SIFs on colitis was no longer obvious. Briefly, the body weights (Fig.6A) and colon length (Fig. 6C and D) of mice in the PPT group were significantly reduced compared to those of the DSS group, and the DAI scores (Fig.6B) of the PPT group were the highest. Furthermore, the mice treated with PPT manifested worse pathological changes in leukocyte infiltration and crypt integrity (Fig. 6E). Furthermore, IL-1β and IL-18 levels in colonic tissues were markedly increased in the DSS+PPT group, while, due to the presence of PPT, the administration of SIFs no longer dramatically suppressed the increased expression of these cytokines (Fig. 7A and B,

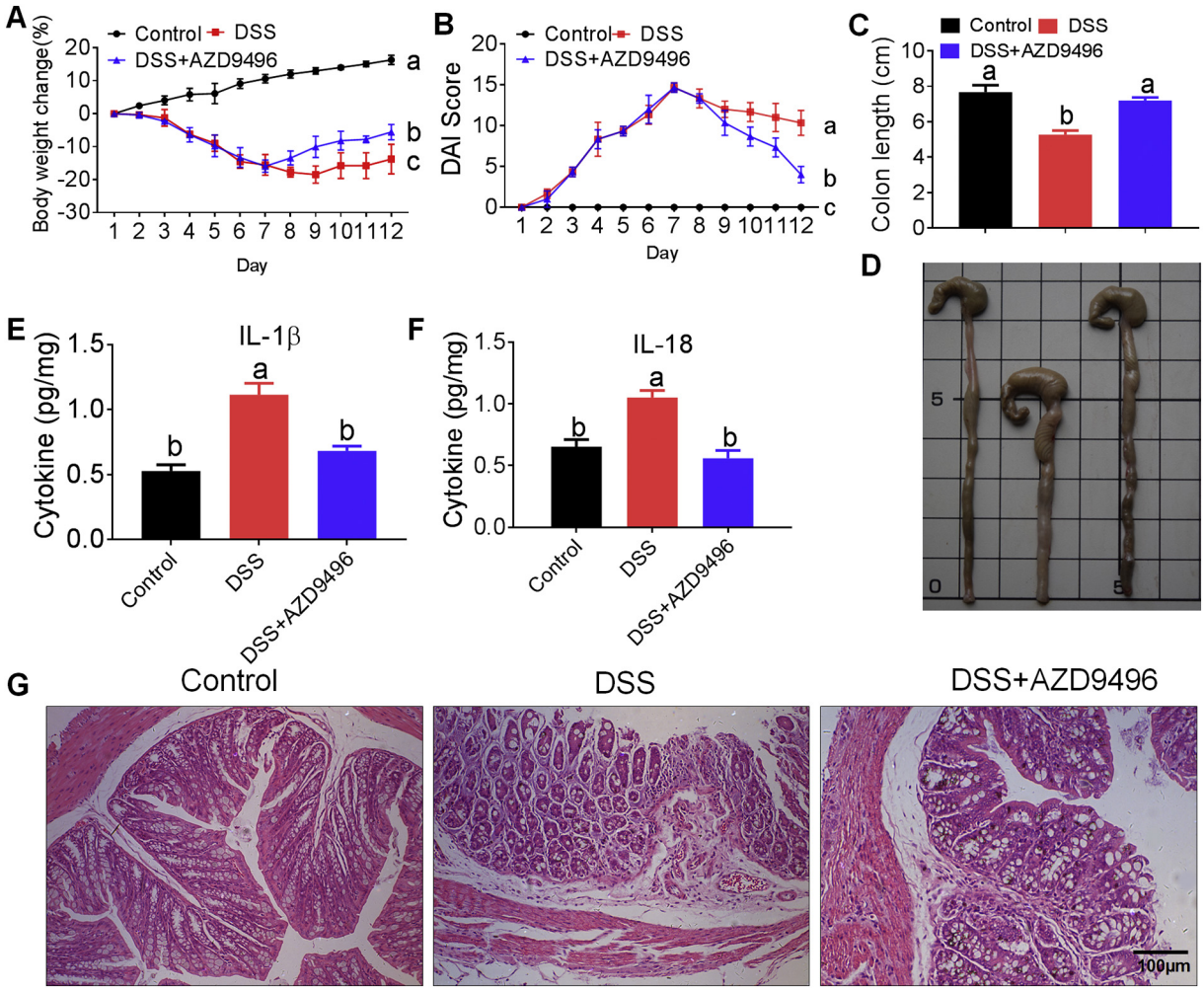


Fig. 4. The ERα antagonist AZD9496 protected against DSS-induced colitis. (A) Body weight change. (B) DAI score of mice. (C and D) The lengths of colons from each group. (E and F) Production of the inflammation-related cytokines IL-1β and IL-18 in colonic cultures. (G) The colons from each experimental group were processed for histological evaluation. The results are expressed as the mean±S.E.M. ($n$=3). Different letters mean significant difference between groups ($P$<.05); the same letters mean that there is no significant difference between groups ($P$>.05).

Fig. 2A and B). Similar results are shown in Fig. 7C and D; PPT increased the expression of NLRP3, ASC, Caspase-1 and ERα and decreased the expression of ERβ, and the protective role of SIFs was remarkably inhibited by PPT.

## 4. Discussion

IBD is a chronic and relapsing inflammatory disease of the gastrointestinal tract that seriously affects the quality of life and increases the risk of colon cancer [25]. At present, some agents, such as 5-aminosalicylic acid, corticosteroids, biological agents and immunosuppressants, have been used for the clinical treatment of IBD. However, treatments with these medicines are prone to increase the susceptibility to infection and lead to different adverse drug reactions in patients [26,27]. Therefore, finding new drugs against IBD with reduced toxicity and robust effectiveness is urgent and necessary.

SIFs in soy products have received high attention as a natural remedy for multiple diseases. Many studies have proven that dietary isoflavones are beneficial for individuals with colitis. For example, SIFs are capable of inducing macrophage polarization and systemic cytokines, which play important roles in the development of colitis [28]. Additionally, SIFs alleviate colitis by inhibiting the COX-2 and TLR4/MYD88 pathways [29]. SIFs are SERMs, and they down-regulate the expression of ERα in cholangiocarcinoma cells [30,31]. Furthermore, ERα is involved in regulating the expression of TLR4 and subsequently producing inflammatory mediators and cytokines [32]. In this study, we found that feeding colitis mice with SIFs inhibited ERα and the NLRP3 inflammasome, reduced the proinflammatory cytokines and alleviated colitis symptoms. Therefore, our study suggested that the anticolitis effect of SIFs was due to its modulation of estrogen receptors. It is worth noting that the main components of SIF in the study are daidzin, glycitin and genistin, of which daidzin and genistin can be hydrolyzed by bacterial β-glucosidase into daidzein and genistein, while glycitin is resistant to enzymatic hydrolysis [33]. Therefore, the main active ingredients of SIF are daidzein and genistein, similar to other SIFs.

Numerous studies have shown that ERα expression is dramatically improved in colon tumors, and ERα-deficient mice have a significantly lower incidence of IBD [11,12]. Furthermore, patients with clinical IBD activity showed reduced ERβ/ERα values [34]. In the present study, ERα protein expression was significantly increased in DSS-treated mice, and SIFs inhibited the expression of ERα in a dose-dependent manner. Furthermore, ERα antagonist AZD9496 alleviated DSS-induced colitis, while the ERα agonist PPT had the opposite effect. Therefore, we hypothesized that SIFs played an important role in alleviating colitis by inhibiting the expression of ERα. Although both

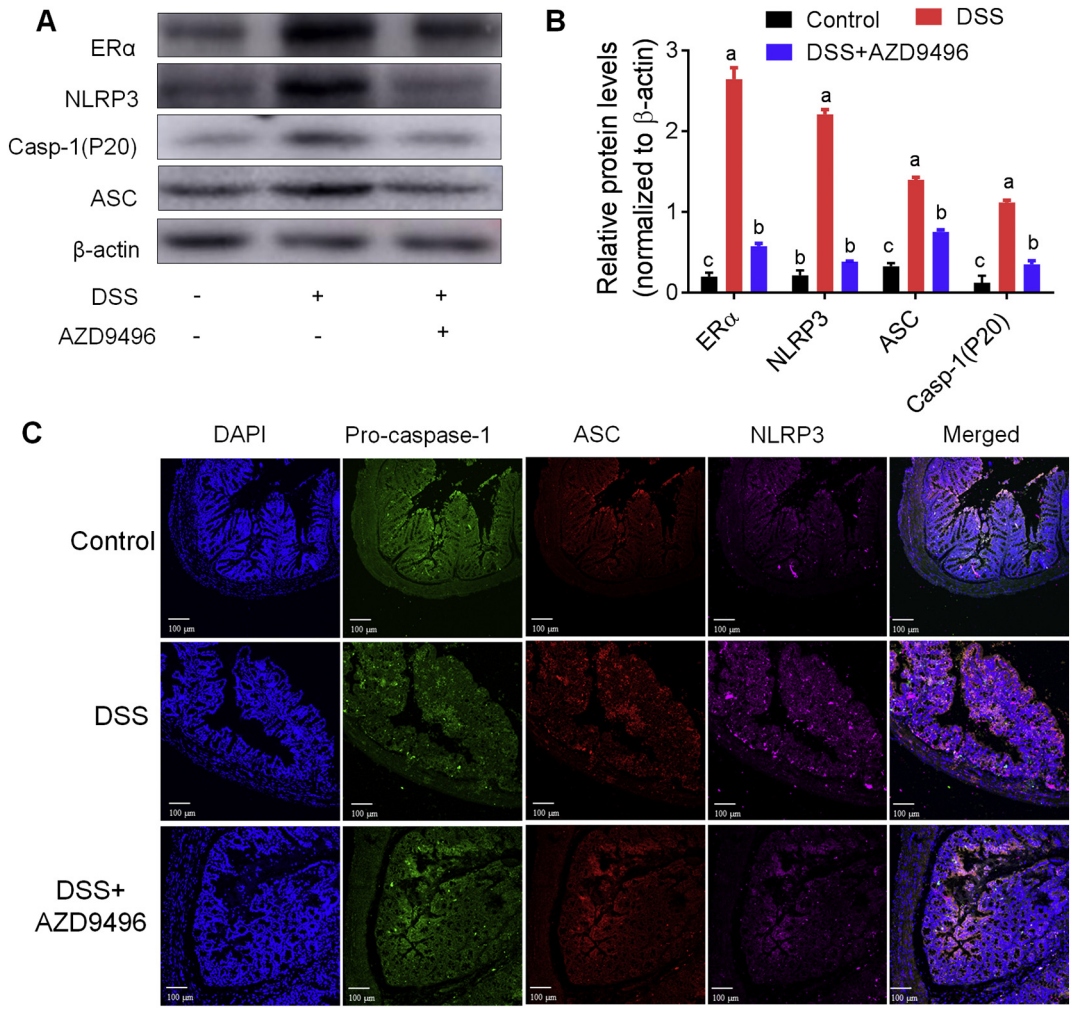


Fig. 5. The ERα antagonist AZD9496 suppressed NLRP3 inflammasome activation and assembly in DSS-treated C57BL/6 mice.(A) Protein levels of NLRP3, ASC and Caspase-1 in colon tissues. (B) The relative protein expression of NLRP3, ASC and Caspase-1. (C) Colon tissue was immunofluorescently stained with Cy5-NLRP3 antibody, PE-ASC antibody and FITC-pro-Caspase-1 antibody. Images were acquired at 60× magnification. The results are expressed as the mean±S.E.M. ($n$=3). Different letters mean significant difference between groups ($P$<.05); the same letters mean that there is no significant difference between groups ($P$>.05).

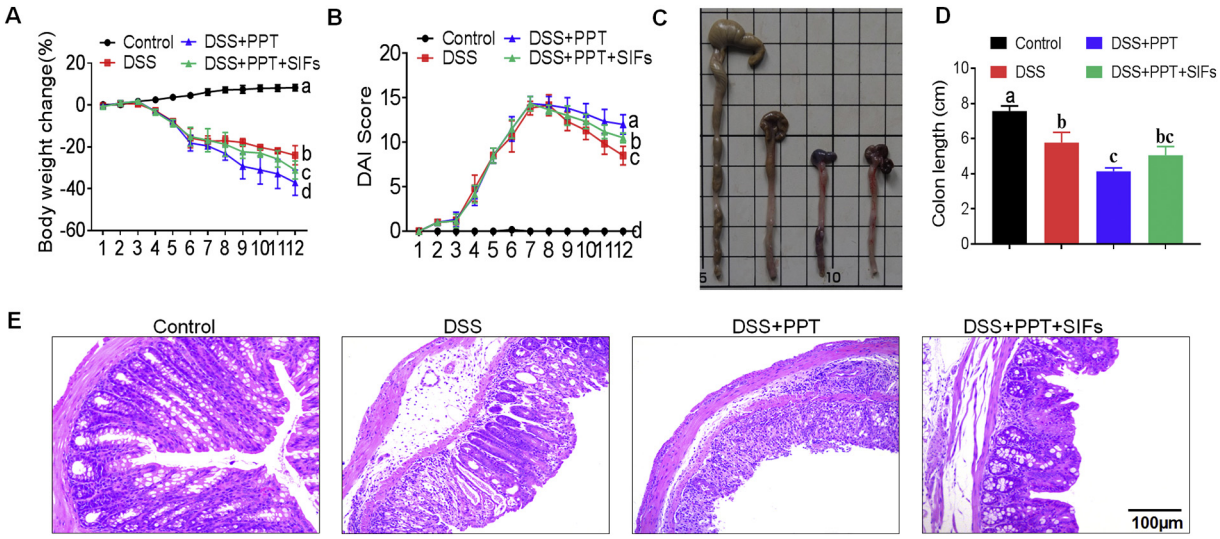


Fig. 6. The ameliorating effect of SIFs on colitis was reversed by the ERα agonist PPT. (A) Body weight change. (B) DAI score of mice. (C) The lengths of colons from each group. a: control group. (D) The colons of each group were processed for histological evaluation (H&E staining 100×). The results are expressed as the mean±S.E.M. ($n$=3). Different letters mean significant difference between groups ($P$<.05); the same letters mean that there is no significant difference between groups ($P$>.05).

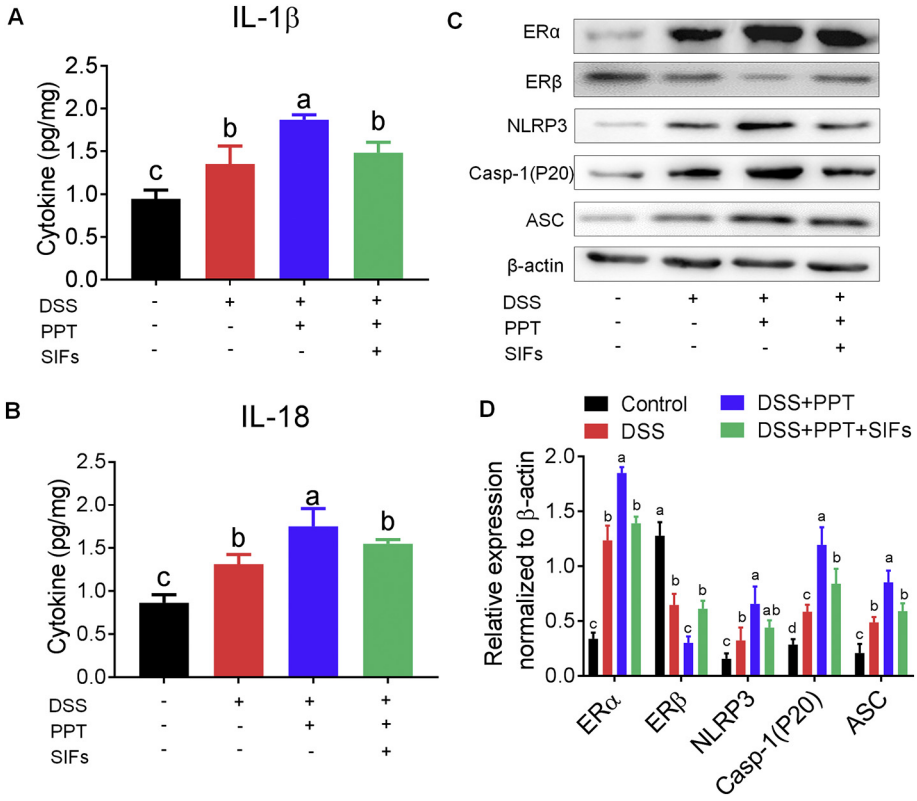


Fig. 7. The ERα agonist PPT induced NLRP3 inflammasome activation and cytokine release in DSS-treated C57BL/6 mice. (A, B) Protein levels of IL-1β and IL-18 in colon were measured by ELISA. (C) Protein expression of NLRP3, ASC, Caspase-1 and ERα in colon tissues. (D) The relative protein expression of NLRP3, ASC and Caspase-1. The results are expressed as the mean ±S.E.M. ($n$=3). Different letters mean significant difference between groups ($P$<.05); the same letters mean that there is no significant difference between groups ($P$>.05).

SIFs and AZD9496 inhibited the expression of ERα, the way each factor regulates ERα might be different [35,36]. Because SIFs are natural SERMs, their reduced toxicity deserves much more attention.

Data from human specimens and mouse colitis models indicate that excessive expression of IL-1β and IL-18 plays a key role in the pathogenesis of IBD [37,38], while the NLRP3 inflammasome is a critical regulator of intestinal homeostasis that processes and releases of active IL-1β and IL-18 [39]. Thus, understanding the molecular regulation of the inflammasome complex and its dysregulation during aberrant inflammation in IBD is an attractive avenue for therapeutic development. Our previous studies indicated that NLRP3 inflammasome, IL-1β, and IL-18 levels were significantly increased in DSS-induced colitis mice [7]. However, in the present study, these cytokines were successfully suppressed by SIFs and an ERα antagonist in the colon. In addition, the expression and colocation of NLRP3, ASC and pro-Caspase-1 in NCM460 cells and colon tissue in the colitis model were significantly reduced. Therefore, we inferred that SIFs and ERα antagonists had an anticolitis effect by regulating the NLRP3 inflammasome. In conclusion, our study demonstrated that the protective therapeutic action of SIFs on DSS-induced colitis depends on the inhibition of ERα and subsequent NLRP3 inflammasome activation, and SIFs are promising therapeutic agent for the treatment of IBD.

## Declaration of competing interest

There are no conflicts of interest to declare.

## Author statement

**Xiaona Gao** performed the research with the help of **Chenchen Ding**, **Shuhui Liu**, **Yan Luo** and **Yufan Miao**. **Lei Tan** and **Yuanguo Shi** participated in the sample collections and data processing. **Wentao Fan** and **Xiaona Gao** prepared the original draft. **Xizhi Shi** and **Sarah DeSaeger** revised the draft. **Suquan Song** conceived the experiments and wrote the paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2020.108438.

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