



Icariin attenuates glucocorticoid insensitivity mediated by repeated psychosocial stress on an ovalbumin-induced murine model of asthma

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ABSTRACT

Evidence shows that psychosocial stress exacerbates asthma, but there is little intervention to alleviate negative effects of psychosocial stress on asthma. We investigated the role of icariin in anti-inflammation and anti-anxiety potential in a murine model combined psychosocial stress with allergic exposure. The results indicated that icariin administered remarkable increased activity in the center of the open field, reversed airway hyperresponsiveness, reduced inflammatory cytokine infiltration to the lung and whole body and also in part recovered glucocorticoid responsiveness. Furthermore, our data also showed that icariin significantly inhibited increases of corticosterone and markedly increased glucocorticoid receptor mRNA and protein expression in the lungs of mice exposed to both stress and allergen. Collectively, we speculate that inducing glucocorticoid receptor modulation might be the potential mechanisms of icariin to facilitate corticosteroid responsiveness of cytokine production.

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1. Introduction

Although physicians and scientists have long recognized and investigated the underlying mechanisms that chronic psychosocial stress contributes to exacerbations of asthma [1–5], there is little intervention to alleviate the negative effects of psychosocial stress on asthma. Evidences had showed that altered innate immune and inflammatory cell function through neural and hormonal pathways played an important role in how psychosocial stress aggravates asthma [6–9]. Experiment studies had demonstrated that physical and psychological stressors activated the hypothalamic–pituitary–adrenal (HPA) axis and increased levels of circulating glucocorticoid hormones [10,11]. However, raised endogenous circulating glucocorticoid had not eased the airway inflammatory, but enhanced and prolonged airway inflammation and altered corticosterone responsiveness [12]. Researchers speculated that these effects were mediated at least in part by impaired glucocorticoid receptor (GR) expression and function [13], which was also confirmed in our previous study [14]. In our previous study, we used social disruption, a well-characterized psychological stressor, to imitate psychosocial stress and established three murine models: ovalbumin (OVA)-induced only model, social disruption stress (SDR) model, combined OVA-induced and SDR model, and mice without any interference as the control. By comparison between models and the control mice in aspects of animal behaviors in the open field

test (OFT), airway hyperresponsiveness (AHR), airway inflammation, glucocorticoid sensitivity and the expression of GR in lung tissue, we found that SDR can promote anxiety behavior, activate the HPA axis, increase AHR and inflammation, and also impair glucocorticoid sensitivity and its function in a murine model of OVA-induced asthma. The down-regulation of GR expression induced by SDR is in part associated with glucocorticoid insensitivity, which leads to asthma exacerbation.

Icariin (2-(4'-methoxyphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3'-methyl-2-butenyl)-4-chromanone) (Fig. 1) is a flavonoid extracted from the traditional Chinese herb *Epimedium brevicornum* Maxim which has been used as a tonic, aphrodisiac and an anti-rheumatic in traditional Chinese medicine for centuries [15]. Numerous studies from our group and others showed that icariin exhibited a wide range of pharmacological and biological activities, including estrogenic activity [16], anti-tumor effects [17], immunoregulation [18] and protective effects against neurotoxicity [19,20] and it was widely used to treat various diseases, such as coronary heart disease, osteoporosis, menopause syndrome, rheumatism, bronchitis and hypogonadism [21]. Notably, we recently studied the mechanism of icariin's effect on rat asthmatic model [18]. The data showed that icariin could regulate the imbalance of helper T (Th) 1/Th2 cytokines and associated transcription factors T-bet and GATA-3 in asthmatic rat pulmonary tissue. Moreover, icariin could inhibit the activation of NF-κB p65 protein in asthmatic rat pulmonary tissue. Another study from our group demonstrated that icariin could attenuate social defeat-induced down-regulation of glucocorticoid receptor in mice and its derivative, ICT, exert anti-inflammatory, anti-tumor effects,

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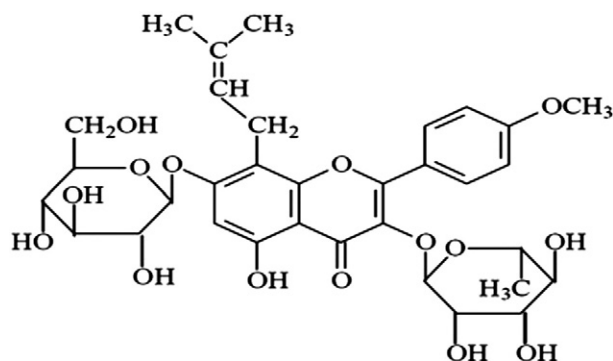


Fig. 1. Chemical structure of icariin (C33H40O15; molecular weight = 676.67).

and modulate myeloid derived suppressive cell (MDSC) functions [22,23].

In order to investigate the role of icariin in protective effect of psychosocial stress on asthma, we used a unique murine model that combined SDR and OVA challenge, which had been established in our previous study [14]. We hypothesized that icariin could alleviate the airway inflammation and anxiety behaviors in the murine model of combined SDR and OVA challenge by modifying HPA-axis function, regulating innate immune cell production of cytokines, and that this effect was mediated by an altered corticosteroid action.

2. Materials and methods

2.1. Animals

54 specific pathogen-free male BALB/c (test mice) and 20 male C57BL/6J (aggressors) [24] were purchased from Shanghai SLAC Co (Shanghai, China) and used in this study. BALB/c studied for their stress and allergic responses (aged 6–8 weeks, weighed 18–20 g, 3 per cage) were allowed to acclimate to their new environment for 1 week before experiment and were maintained on a 12-h light/dark schedule (lights on at 6:00 a.m.) with food and water available ad libitum. Then, the total of 54 mice were randomly assigned to six groups ($n = 9$ per group): A—no manipulation (control); B—sensitized, antigen challenged, and social disruption model (asthma-SDR); C—administered dexamethasone (DEX) 1 mg/kg and sensitized, antigen challenged, social disruption model (DEX 1 mg/kg-Asthma-SDR); D—administered icariin 10 mg/kg and sensitized, antigen challenged, social disruption model (Icariin 10 mg/kg-Asthma-SDR); E—administered icariin 50 mg/kg and sensitized, antigen challenged, social disruption model (Icariin 50 mg/kg-Asthma-SDR); F—administered icariin 100 mg/kg and sensitized, antigen challenged, social disruption model (Icariin 100 mg/kg-Asthma-SDR). In addition, C57BL/6J mice arrived in the animal facility at 8–9 weeks old and were allowed 1 week habituation before being singly-housed in cages for 4 weeks in order to reduce inter-individual variability and increase the likelihood of social interaction-induced stress without creating overt phenotypical changes. All animal experiments were approved by the Huashan Hospital, Fudan University Animal Care and Use Committee (permit number: 11-1002).

2.2. Selection of aggressive intruders

Prior to the social disruption procedure, C57BL/6J mice were screened in a preliminary study for aggressive behaviors towards a separate cohort of BALB/c mice to ensure the defeat of the resident experimental mice, as previously described [25]. The heaviest C57BL/6 mice were selected for the aggression test. Latency of first attack and dominance status of mice were visually determined by observing key behaviors such as tail rattling, chasing or biting for dominance and

escape and upright postures or immobility for submission [25–27]. Mice being aggressive, dominant and presenting the lowest latency to attack were then selected to be aggressive intruders for the stress procedure [28]. Five C57BL/6J mice were excluded in this study.

2.3. Social disruption model

Social disruption model has been extensively used and has been previously described by our laboratory [14,22] and others with slight modulation in this study. Briefly, an older and larger male C57BL/6J (the intruder) was placed into the cage containing an established social hierarchy of three young male BALB/c (the residents) for a 2-h confrontation beginning at 4:30 p.m. Animals underwent six SDR cycles over a week: three nightly cycles, one night off, and three more cycles. Observations were made to ensure that the aggressors defeated the experimental subjects. To avoid habituation to the intruder, aggressors were rotated daily. An intruder displaying no aggressive behavior within the first 5 min towards resident mouse was replaced by another one. Different aggressors were used on consecutive nights. To make sure that mice in the control groups were left undisturbed, they were put in another room during the SDR cycles. Researchers observed carefully in order to avoid serious injury to happen. In the whole experiment process, no severe injury happened.

2.4. Allergic sensitization and challenge

Asthma-SDR mice were sensitized and challenged by OVA according to the modified protocol reported previously [29]. Briefly, the mice were immunized at 1 and 8 days (7 days apart) by a peritoneal injection of sterile saline 0.5 ml containing OVA 10 μ g (Grade V, Sigma, St. Louis, MO, USA) and aluminum hydroxide 4 mg. Fourteen days after the first sensitization, the mice were challenged continuously for 2 weeks (30 min each day) by an inhalation of 1% OVA through an ultrasound aerification inhaler (Buxco, USA). Half an hour before each challenge, DEX (Roche Pharmaceutical Ltd., Shanghai, China) at a dose of 1 mg/kg, icariin (Sichuan Chengdu Pharmaceutical Co., Chengdu, China) at the dose of 10 mg/kg, 50 mg/kg, 100 mg/kg were orally administered into the stomachs of mice in the DEX-Asthma-SDR group, Icariin 10 mg/kg-Asthma-SDR group, Icariin 50 mg/kg-Asthma-SDR group and Icariin 100 mg/kg-Asthma-SDR.

2.5. Sensitization, antigen challenge, and social stress schedule

The challenge schedule is presented in Fig. 2 and briefly summarized below: day 1 and day 8—sensitization (ip); days 15–28—antigen challenge (inhalation); days 22–24—psychosocial stress; and days 26–28—psychosocial stress; and days 15–28—icariin or DEX administration (orally).

2.6. The open field test

Spontaneous activity under novelty stress was measured for 5 min, approximately on the morning immediately following the final cycle of social disruption. As previously described [30], all mice were acclimated to the test room for 1 h and thereafter placed in a clear Plexiglas chamber (Med Associates Inc., St. Albans, VT, USA) measuring 270 $\times$ 270 $\times$ 200 mm with arrays of 16 $\times$ 16 photo detector, positioned 2.5 cm and 10 cm above the floor of the chamber. The software (Activity Monitor version 5, Med Associates) allowed a distinction to be made between repetitive interruptions of adjacent photo beams. As indexes of ambulatory counts (number of individual horizontal movements registered when the mice walked on all four feet) and vertical counts (rearing counts registered when mice's body inclined vertically with hind paws on the floor and forepaws on the wall of the activity cage) were evaluated. The time spent at the center of the chamber served as an index of anxiety [31]. The equipment allowed four mice to test at

one time. Moreover, the number of fecal boli was counted at the end of each trial. In order to reduce anxiety caused by the environment, a low-level illumination was used throughout the experiment. After the test of one mouse, the chamber was wiped by 30% alcohol in order to avoid the interference between different mice.

2.7. Measurement of AHR by Buxco's modular and invasive system

AHR was assessed more directly by measuring changes in pulmonary resistance (RL) in response to increasing concentrations of inhaled methacholine (Mch). RL is calculated according to the method of Amdur and Mead [32,33]. Amdur and Mead were two researchers who firstly introduced the technique used to calculate of the pulmonary flow-resistance and compliance for an experimental animal. Before Mch challenge and measurement of AHR, the mice were anesthetized with pentobarbital sodium (60–70 mg/kg ip). Then a tracheostomy was made; the trachea was cannulated; the pleural spaces were opened; and the mice were placed in a whole-body plethysmography (WBP) chamber for anesthetized animals [34]. After a stable baseline airway pressure (<5% variation over 2.5 min) is reached, Ca- and Mg-free phosphate buffered saline (PBS) and increasing concentrations of Mch (3.125, 6.25, 12.5 mg/ml) in succession were administered via a jet nebulizer into the head chamber. Minimum values for RL were determined and expressed as percent change from the baseline value as previously described [35].

2.8. Collection and analysis of bronchoalveolar lavage fluid (BALF)

After Mch challenge and measurement of AHR, the mice's tracheas were cannulated, and their chests were opened. The right lungs were ligated at the right main branch of the trachea using surgical forceps, and the left lungs were slowly lavaged twice with 0.8 ml of PBS [36]. The retrieved lavage aliquots were pooled and centrifuged. The supernatant was stored at -20°C for cytokine detection. The levels of interleukin (IL)-4, IL-5, IL-6, IL-13, and TNF- α in BALF were detected by ELISA kits following the manufacturer's instructions.

2.9. Measurement of OVA-specific immunoglobulin E (OVA-IgE) and Th2 cytokines in serum

Levels of OVA-IgE and Th2 cytokines IL-4, IL-5, IL-13 in serum were measured using ELISA kits which were obtained from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

2.10. Histological analysis

After the left lung was being lavaged, the middle lobe of the right lung tissues excised from each mouse were fixed in 4% formalin and embedded in paraffin. Sections 3–4 μm thin from blocks were stained with hematoxylin–eosin (H&E). Histopathological assessment (light microscopy) was performed blind on randomized sections. Inflammatory changes were graded by two separate researchers using a semi-quantitative scale of 0–5 for perivascular eosinophilia, peribronchiolar eosinophilia, epithelial damage and edema [37].

2.11. Serum corticosterone assay

Blood was collected by means of eyeball and separated in a refrigerated centrifuge at 4°C . Serum was stored at -80°C until assays were performed. For corticosterone detection, 10 μl of serum and 0.5 μl steroid displacement reagent were diluted in 990 μl of assay buffer, completing a 100-fold dilution. Plates were coated with 100 μl of anti-corticosterone overnight at 4°C and then blocked with assay buffer for 2 h at room temperature. After two washes with PBS-Tween, 100 μl of standards and supernatant samples were added and incubated overnight at 4°C . Plates were washed four times before 100 μl /well of biotinylated anti-corticosterone was added. After 45 min at room temperature, plates were washed six times with PBS-Tween. After addition of 100 μl /well of a 1:1000 dilution of avidin–peroxidase, plates were incubated at room temperature for 30 min. After eight washes, 100 μl of substrate solution was added per well, and the enzymatic reaction was allowed to develop at room temperature. OD was measured at 405 nm on an ELISA plate reader. Corticosterone concentrations were quantified by comparison to the standard curves. Samples were analyzed

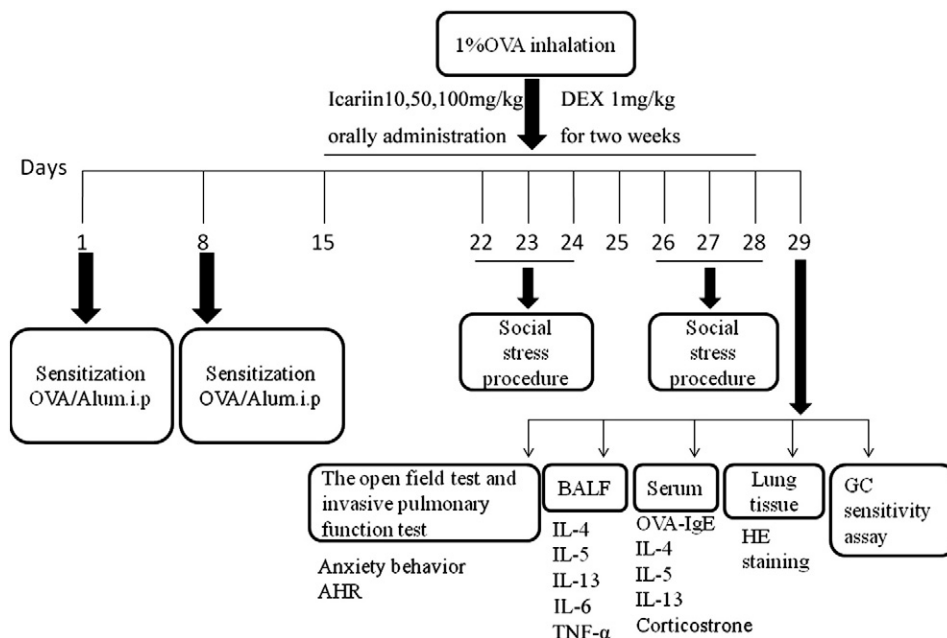


Fig. 2. Mice model of combined psychosocial stress with allergy sensitization and challenge as described in the Materials and methods section.

in duplicate in a single assay; threshold detection = 150 pg/ml; coefficient of variation limit = 9.6%; concentration expressed in pg/ml.

2.12. Splenocyte isolation and corticosterone sensitivity assay

Glucocorticoid-induced cell death was quantified, as described previously [13,14,38–40]. A single-cell splenocyte suspension was prepared, and added in triplicate (2.5×10^5 cells/well) to flat-bottom 96-well plates at a volume of 100 μ l/well for cell proliferation assays and 300 μ l/well to flat-bottom 48-well plates for cytokine analysis. Cells were stimulated with 1 μ g/ml LPS and corticosterone (dose range 0.01–5 μ M) at 37 °C and 5% CO₂. After 18 h of incubation, cell supernatants were harvested and frozen at –20 °C for cytokine analysis. Other cultures were incubated for 44 h, at which time cell viability was determined by Cell Counting Kit-8 assay (CCK-8, Japan), and read at 450 nm by an ELISA plate reader [41]. Cell viability was expressed as a percentage of OD of the no-corticosterone control.

2.13. RNA extraction, cDNA synthesis, and quantitative real-time PCR

The total RNA was extracted with TRIZOL (Invitrogen Corporation, Carlsbad, CA, USA). For cDNA synthesis, total RNA (2 μ g) was used to generate cDNA in each sample by using SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) with 0.5 μ g oligo (dT) primers. The OD₂₆₀ of prepared total RNA was measured with spectrophotometer and then used to determine RNA concentration. One hundred nanograms of total RNA from each sample was applied to 1% agarose gels to determine the purity and quantity of RNA by ethidium bromide stain. The intensity of 28S rRNA bands, determined by densitometry, was used to control RNA amounts used in real-time PCR analysis. One microliter of cDNA was amplified in each PCR reaction mixture containing 200 nM of sense and antisense primers of selected genes. The internal control gene used was GAPDH. For PCR amplification, the following mouse-specific sense and antisense primers were used: GR, 5' GGAATA GGTGCAAGGTCT 3' (forward) and 5' GAGCACACCAGGCAGAGTTT 3' (reverse); GAPDH, 5'-GGGGAGCCAAAAGGGTCATC-3' (forward) and 5'-

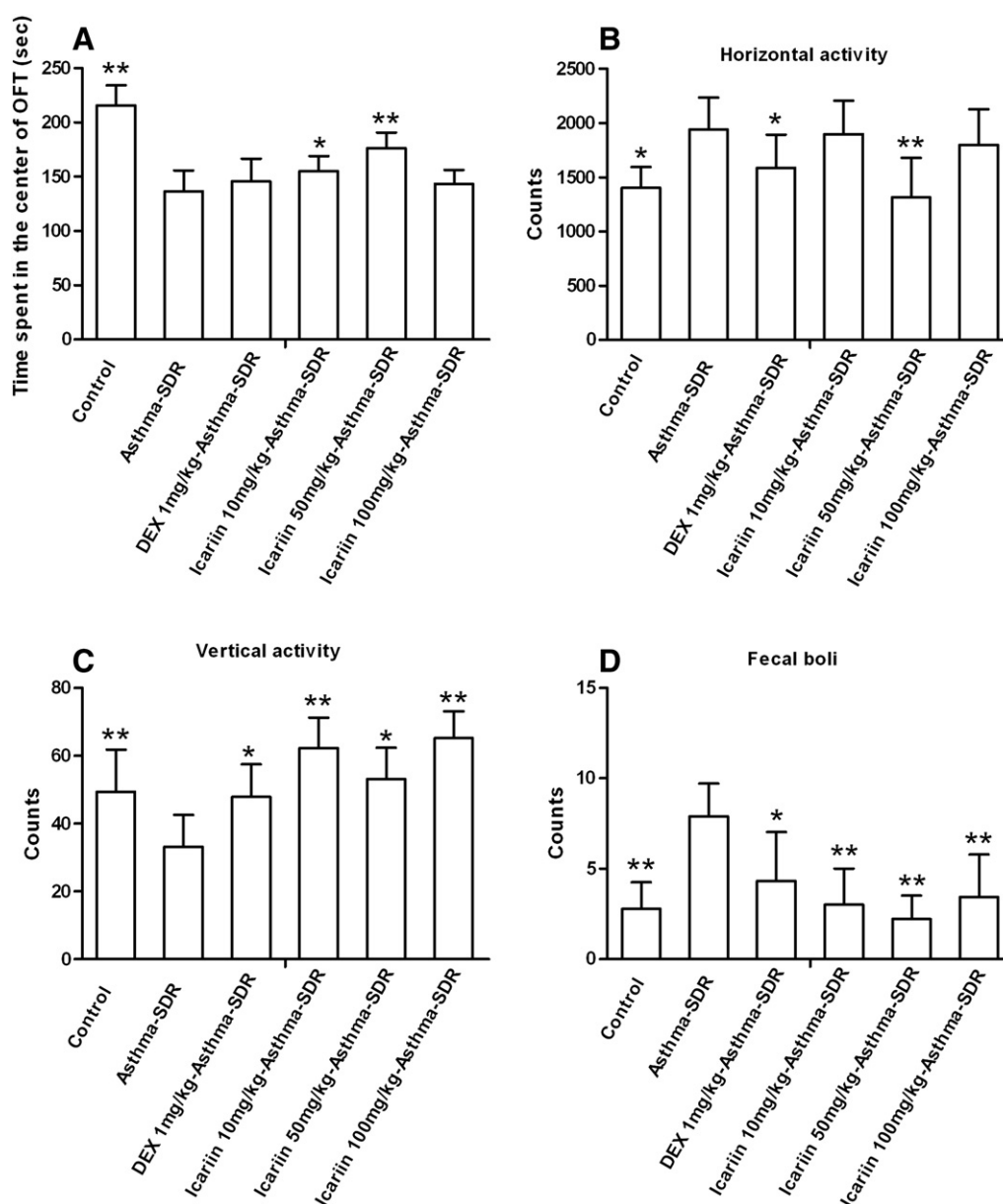


Fig. 3. The open field test. Anxiety behaviors were induced by repeated psychosocial stress on ovalbumin sensitivity and challenge murine and icaritin administration reversed the negative effect of social stress on the mice asthmatic model. ** $P < 0.01$, * $P < 0.05$, vs the asthma-SDR group.

GACGCCTG CTTACACCTTCTTG-3' (reverse). PCR amplification was performed on Light Cycler FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany). The amplification was performed in a Roche Light Cycler 2.0 instrument under the following conditions: initial denaturation at 95 °C for 10 min, 40 cycles of amplification at 95 °C for 20 s, annealing at 56 °C for 15 s, and then extension at 72 °C for 20 s.

2.14. GR Western blot analysis

Lungs were homogenized in lysis buffer and incubated for 30 min at 4 °C. Cell debris was removed by micro-centrifugation, and supernatants were quickly frozen. The protein concentration was determined by BSA method. 30 µg of proteins was electrophoreted onto a PVDF membrane, following the separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated 1 h with 5% milk at room temperature, and then incubated overnight at 4 °C with a 1:1000 dilution of anti-GR antibody (Millipore) and 1:10,000 dilution of anti-GAPDH antibody (MultiSciences Biotech Co., Ltd.), respectively. Blots were washed three times with Tween-20/Tris-buffered saline (TTBS) and then incubated with a 1:1000 dilution of HRP-conjugated secondary antibody for 2 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence. Band intensities were quantified using UN-SCAN-IT gel analysis software (version 6). The optical density for GR was shown as a proportion of GAPDH optical density.

2.15. Statistical analysis

Data are expressed as mean ± standard error (S.E.M.). SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Differences between groups were determined by one-way ANOVA followed by post hoc Dunnett's tests. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of icariin and DEX on the anxiety behaviors in the OFT

The pilot experiment had demonstrated that SDR and asthma-SDR mice markedly shortened the amount of time the mice spent in the center of the open field, decreased vertical activity, increased ambulatory activity and the count of fecal boli [14]. As illustrated in Fig. 3, icariin and DEX administration could reverse the negative impact of SDR on the

mice anxiety behaviors. Treatment of icariin and DEX prolonged the amount of time the mice spent in the center of the open field (Fig. 3A), decreased ambulatory activity (Fig. 3B), increased vertical activity (Fig. 3C) and reduced the count of fecal boli (Fig. 3D).

3.2. Effects of icariin and DEX on AHR

The asthma-SDR group significantly enhanced AHR compared with the control group at Mch concentrations of 3.125 mg/ml–12.50 mg/ml ($P < 0.01$). These increases in AHR were significantly suppressed by icariin and DEX. Treatment with 50 mg/kg icariin once daily was more potent than treatment with DEX at any concentration of Mch (Fig. 4).

3.3. Effects of icariin and DEX on pro-inflammatory cytokines in BALF

The levels of IL-4, IL-5, IL-6, IL-13, and TNF- α in BALF were significantly increased in asthma-SDR mice than those in control as showed in Fig. 5A, B, C, D and E. Administration of icariin and DEX significantly inhibited the increase of IL-4, IL-5, IL-6, IL-13 and TNF- α in BALF. The IL-5 level in BALF was significantly reduced in animals treated 50 mg/kg icariin compared with 10 mg/kg icariin and 100 mg/kg icariin as seen in Fig. 5B ($P < 0.05$). The TNF- α level in BALF in the 50 mg/kg icariin group was significantly lower than those in the 10 mg/kg icariin group as showed in Fig. 5E ($P < 0.05$).

3.4. Effects of icariin and DEX on OVA-IgE and Th 2 cytokines in serum

Levels of IL-4, IL-5, and IL-13 in serum showed in Fig. 5F, G and H were significantly increased in asthma-SDR group compared with those in control. Serum OVA-IgE level, which was barely detectable in control mice, was increased by OVA-sensitization and challenges (Fig. 5I, $P < 0.05$). Treatment with icariin significantly attenuated the increase in IL-4, IL-5, IL-13 and OVA-IgE levels in serum compared with asthma-SDR group ($P < 0.05$), similar to DEX treatment. At the dose of 50 mg/kg icariin was more potent than other two doses of icariin in the level of IL-5 in serum seen in Fig. 5G.

3.5. Effects of icariin and DEX on corticosterone level in serum

The results presented in Fig. 5J demonstrated that the level of corticosterone in asthma-SDR group was significantly higher than that in control group ($P < 0.05$), whereas DEX and icariin (50 mg/kg) administration remarkably inhibited the production of corticosterone ($P < 0.01$).

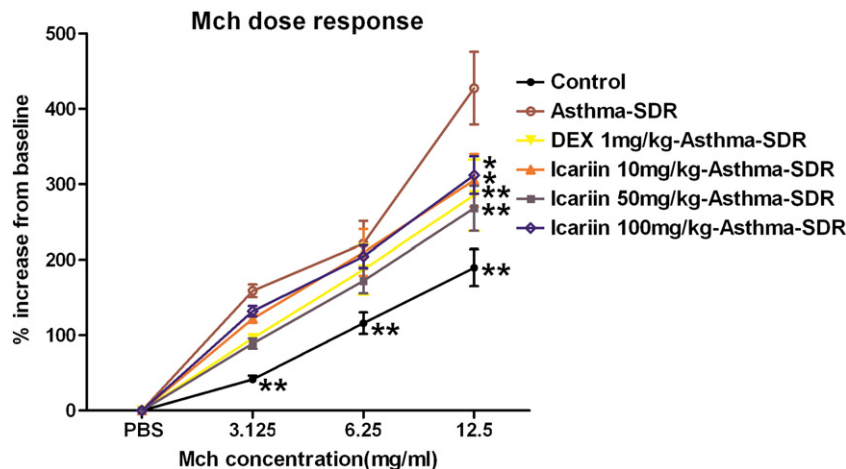


Fig. 4. Airway responsiveness to aerosolized methacholine (Mch) was evaluated by a Buxco's modular and invasive system, as described in the Materials and methods section. Allergy exposure combined with social stress significantly increased AHR response to every concentration of Mch compared to the control group. Administration of DEX and icariin at 50 mg/kg significantly attenuated the AHR. ** $P < 0.01$, * $P < 0.05$, vs the asthma-SDR group.

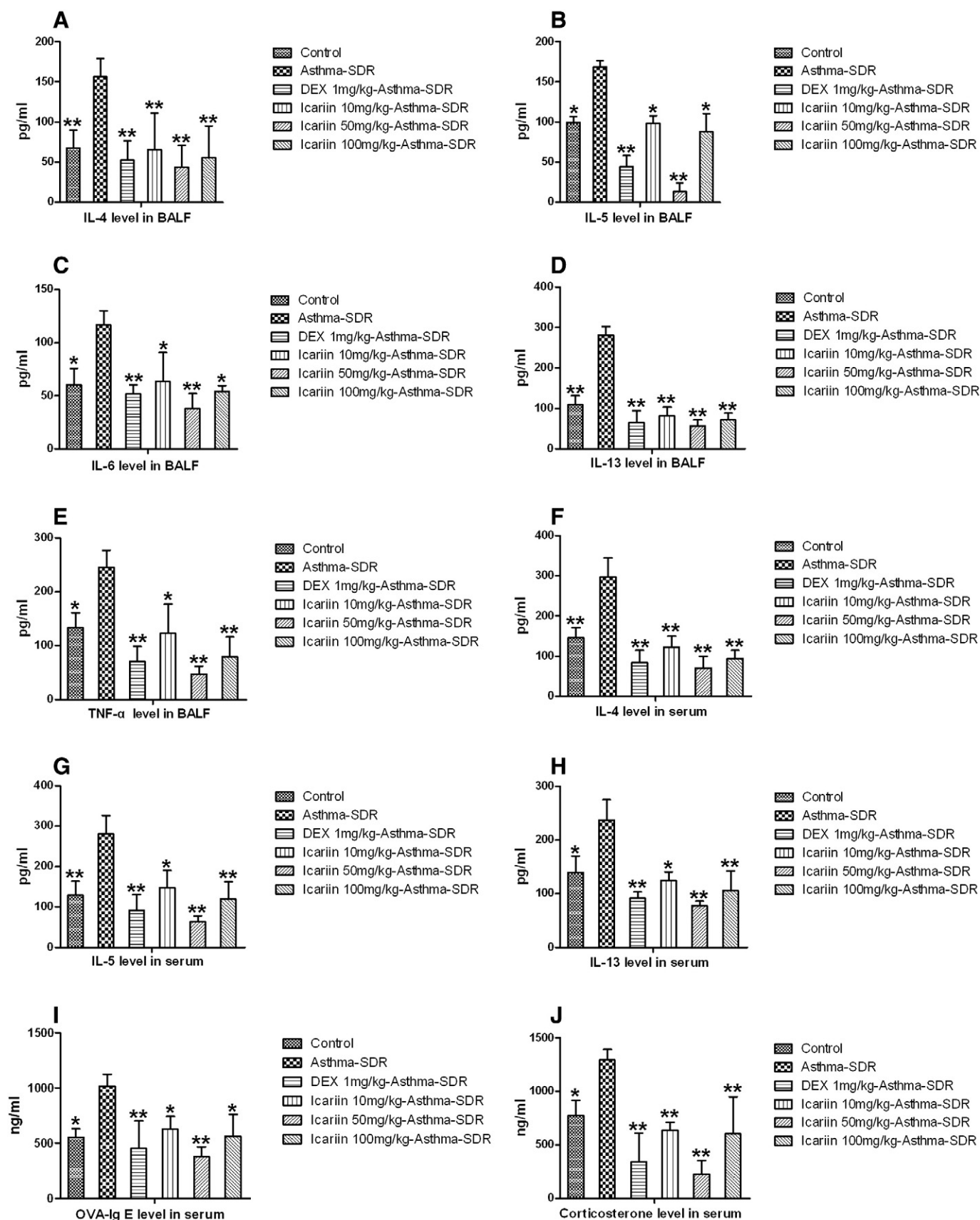


Fig. 5. The effect of icariin on cytokine secretion and corticosterone level in a rat model of asthma-SDR. (A), (B), (C), (D) and (E) showed that combined stress and asthma significantly increased the levels of IL-4, IL-5, IL-6, IL-13, and TNF- α in BALF compared to the control group. (F), (G), (H), (I) and (J) demonstrated that despite of increasing level of serum corticosterone, the levels of OVA-IgE and Th2 inflammatory cytokines (IL-4, IL-5, IL-13) in asthma-SDR group were significantly increased in serum. And the increased cytokines in serum and BALF were significantly reduced by DEX and icariin treatment. ** $P < 0.01$, * $P < 0.05$, vs the asthma-SDR group.

Table 1

Effects of icariin on reduction of the increased scores represented perivascular eosinophilia, peribronchiolar eosinophilia, epithelial damage and edema in Asthma-SDR mice. ** $P < 0.01$, * $P < 0.05$, vs the Asthma-SDR group.

Group	Perivascular eosinophilia	Peribronchiolar eosinophilia	Edema	Epithelial damage
Control	1(1–2)**	0(0–1)**	1(1–1)**	1(1–1)**
Asthma-SDR	4(3–4)	3(2–4)	5(4–5)	4(3–5)
DEX 1 mg/kg-Asthma-SDR	2(2–3)**	2(1–2)**	3(3–3)*	3(3–3)
Icariin 10 mg/kg-Asthma-SDR	3(3–4)	2(2–3)*	3(2–3)*	2(2–3)*
Icariin 50 mg/kg-Asthma-SDR	2(1–2)**	2(1–3)*	2(1–3)**	1(1–2)**
Icariin 100 mg/kg-Asthma-SDR	3(2–3)*	1(1–2)**	1(1–2)**	2(2–2)*

3.6. Effects of icariin and DEX on lung histology

According to the analysis of HE stain in lung tissues, there were more severe perivascular eosinophilia, peribronchiolar eosinophilia, epithelial damage and edema in asthma-SDR group than the control group (Table 1 & Fig. 6B). Icariin and DEX significantly reversed the airway inflammation (Table 1 & Fig. 6C–F). The suppression effect of 50 mg/kg icariin (Fig. 6E) was greater than DEX and other two doses of icariin.

3.7. Effects of icariin and DEX on splenocyte corticosterone sensitivity

Splenocyte function, including the release of cytokines, should normally be significantly inhibited by corticosteroids. Such dose-dependent inhibition is clearly shown in Fig. 7A (dot) in the cells derived from control mice. However, in splenocytes derived from the asthma-SDR group, the inhibitory effect of corticosterone was completely abolished. The levels of IL-6, TNF- α in supernatant demonstrated that corticosteroid responsiveness of immune cells in the spleen of mice that were subjected to Asthma-SDR was significantly impaired (Fig. 7B & C). The combination of stress and allergy exposure appeared synergistic in reversing the inhibitory effects of corticosterone. Moreover, treatment of icariin but not DEX facilitated corticosteroid responsiveness of cytokine production (Fig. 7A). As shown in Fig. 7B and C, at the dose of 10 mg/kg and 50 mg/kg icariin markedly ameliorated glucocorticoid sensitivity in inhibition of inflammatory cytokine secretion.

3.8. Effects of icariin and DEX on expression of GR protein and GR mRNA in the lung tissue

To confirm the effects of OVA-exposed-SDR on GR expression locally in the lung, we studied GR gene activation in the whole lung tissue of mice. As showed in Fig. 8B, asthma-SDR mice significantly inhibited GR mRNA expression in the lung ($P < 0.01$). Furthermore, Western blot analysis of lung extracts from the mice showed that both sensitization and challenge with OVA and experience social stress significantly reduced GR expression compared with the control mice, as demonstrated in Fig. 8A and C. Icariin 50 mg/kg and 100 mg/kg administration remarkably increased the expression GR mRNA and GR protein, compared to asthma-SDR group ($P < 0.01$). However, treatment of DEX did not show the effect of elevating GR expression. Instead, the expression of GR mRNA was decreased due to administration of DEX.

4. Discussion

Although stress-induced enhancement of allergic airway inflammation has been noted in different models [13,42–44], also confirmed in our pre-test [12,14], there is little intervention to inhibit stress-induced exacerbation of airway inflammation. This study demonstrated the role of icariin in reducing airway inflammation and anxiety behaviors in a murine model of combined SDR and asthma, and facilitating corticosteroid responsiveness of cytokine production. Treatments with icariin increased the time spent in the center of OFT, attenuated AHR response

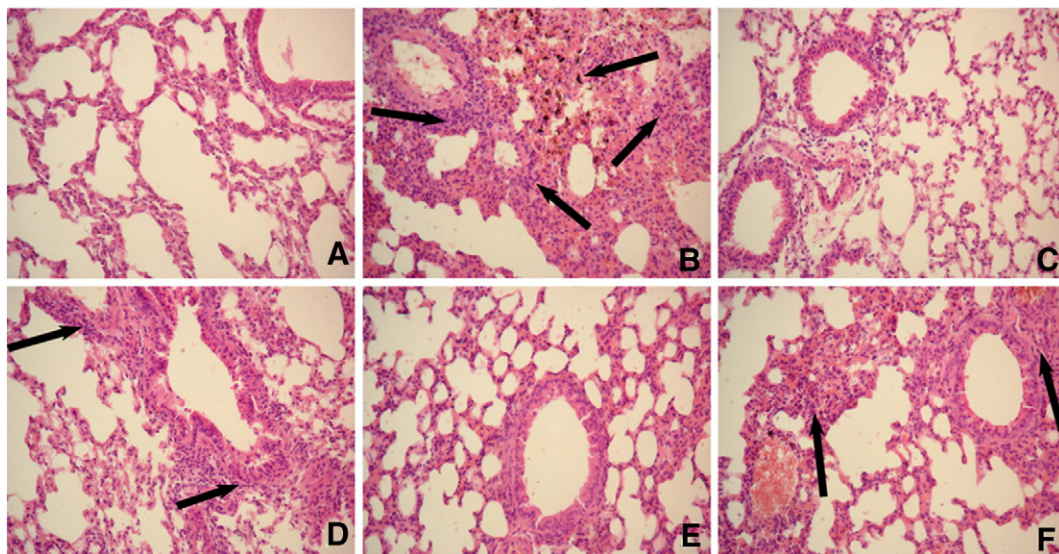


Fig. 6. The effect of icariin on airway inflammation in the lung tissue (HE staining, original magnification $\times 200$). In Asthma-SDR group the inflammatory cell infiltration into the perivascular and peribronchiolar was more severe than those in control group. C–F showed that DEX and icariin treatment alleviated the inflammatory cell immersion into the lung. Black arrows pointed to the place in which was the infiltration of inflammatory cells. A: Control group; B: Asthma-SDR group; C: DEX 1 mg/kg-Asthma-SDR; D: Icariin 10 mg/kg-Asthma-SDR; E: Icariin 50 mg/kg-Asthma-SDR; F: Icariin 100 mg/kg-Asthma-SDR.

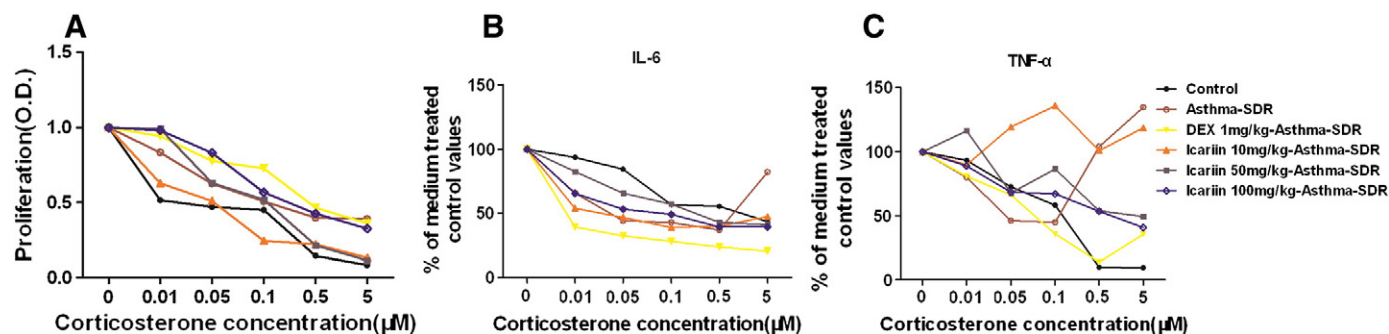


Fig. 7. The effect of icariin on corticosterone sensitivity. Data are presented as the percentage of cell viability or cytokine concentration of control values measured in the absence of corticosterone. Treatment of icariin but not DEX facilitated corticosteroid responsiveness. The dose of 10 mg/kg and 50 mg/kg icariin markedly ameliorated glucocorticoid sensitivity in inhibition of inflammatory cytokine secretion.

to Mch, reduced inflammatory Th2 cytokine infiltration to the lung and whole body, significantly inhibited psychosocial stress-induced increases of corticosterone and markedly increased GR mRNA and protein expression in the lungs of mice exposed to both stress and allergen.

In present study, we used a unique murine model in which repeated psychosocial stress and allergen exposure provided a platform to investigate the psycho-endocrine-immunological axis that regulates airway inflammation. Therefore, whether the model successfully made or not was extremely important. Social disruption, a well-characterized psychological stressor, had been used by our laboratory [22] and others to assess the impact of a seminatural stressor on immune function. In this model, a previously established social hierarchy of a stable cohort of younger male mice was disrupted by a large, aggressive male that confronted and repeatedly defeated the cage residents. Previous studies demonstrated that repeated defeat led to physiological and behavioral responses (collectively referred to as the stress response), such as development of anxiety-like behaviors [14,31] and glucocorticoid release [45,46], which were showed in the present study. Thus, the model of SDR was successfully made in the study. Moreover, administration of icariin relieved anxiety-like behaviors in OFT and inhibited the increase in serum corticosterone.

Despite the increased circulating corticosteroid, the levels of OVA-IgE and Th2 cytokines in serum and lung significantly rose in asthma-SDR group. Treatment with icariin increased the inhibitory effect of corticosterone on airway inflammation response.

In order to explain the mechanisms of synergistic effects between repeated psychosocial stress and the allergic immune response and the effect of icariin, we employed the means which was widely reported by others to evaluate corticosteroid sensitivity [39,47,48]. Briefly, we quantified GR binding to GRE in the nuclear fractions of splenocytes stimulated with corticosterone (a steroid that binds both the glucocorticoid and mineralocorticoid receptor with high affinity). In our pilot experiment study [14], we found that stress by itself markedly decreased the inhibitory effect of GR to immune cells and cytokine production, but additional OVA exposure of the stressed mice did not enhance this effect, confirming other previous studies [13,47]. Moreover, we also showed that GR mRNA and protein expression in the lung was significantly inhibited in the asthma-SDR group. Our study demonstrated that treatment with icariin in part restored corticosteroid sensitivity and recovered GR translocation and expression in the lung.

Icariin is a major constituent of flavonoids isolated from herb *Epimedium* [49]. It displays as an anti-inflammatory activity in a rat

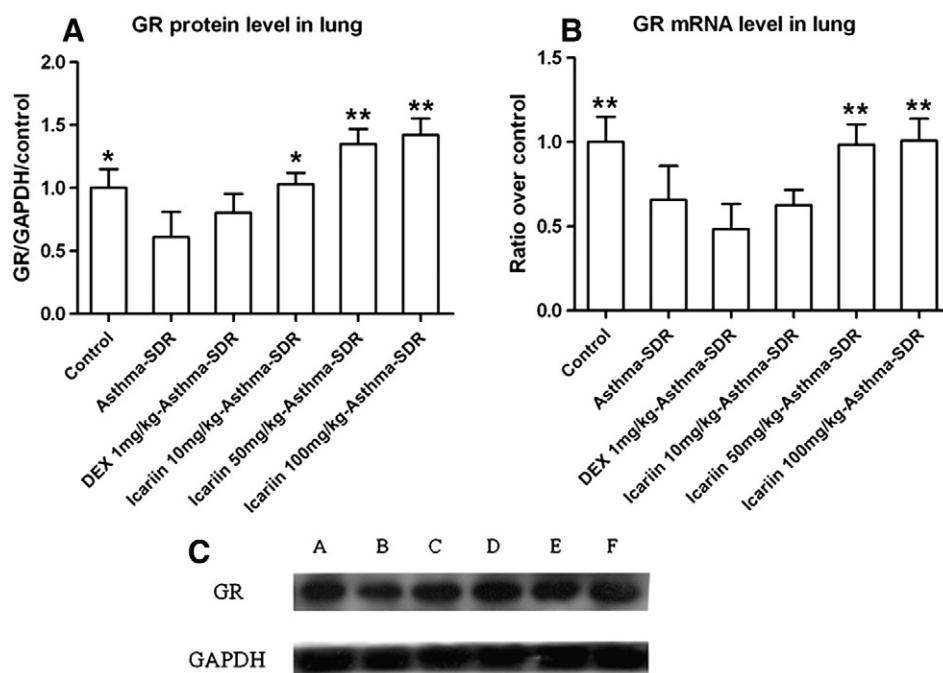


Fig. 8. The effect of icariin on expressions of glucocorticoid receptor (GR) and GR mRNA in the lung tissue. Total protein and mRNA were isolated from the lungs of mice. Western blot and RT-PCR were performed. Expression was assessed using densitometric analysis and GAPDH as control ($n = 4-6$). ** $P < 0.01$, * $P < 0.05$, vs the Asthma-SDR group. A: Control; B: Asthma-SDR; C: DEX 1 mg/kg-Asthma-SDR; D: Icariin 10 mg/kg-Asthma-SDR; E: Icariin 50 mg/kg-Asthma-SDR; and F: Icariin 100 mg/kg-Asthma-SDR.

asthmatic model [18]. It was reported that icariin could regulate the imbalance of Th1/Th2 cytokines in asthmatic rat pulmonary tissue, recover the imbalance of Th1/Th2 associated transcription factors T-bet and GATA-3 in asthmatic rat pulmonary tissue and spleen lymphocytes and inhibit the activation of NF- κ B p65 protein in asthmatic rat pulmonary tissue. Furthermore, Xu et al. [50] studied that activation of the PI3K/Akt pathway and the inhibition of NF- κ B were involved in the protective effects of icariin on LPS-induced acute inflammation responses according to the results in vitro and in vivo. In our previous study [22], we found that GR binding affinity and protein expression in the social defeat model were remarkably decreased and icariin administration attenuated social defeat-induced GR down-regulation. And it also showed that icariin administration significantly inhibited social defeat-induced increases of corticosterone and IL-6 levels, which were confirmed in our present research. Besides, our preliminary study also showed that icariin had the protective effects on apoptosis in hippocampal neuronal cells, potentially mediated through blockade of p38 MAPK phosphorylation [20]. Therefore, we speculated that icariin had anti-inflammation, anti-tumor, anti-depression and neuroprotective effects.

In the present study, DEX as one of glucocorticoids is used as a positive control drug, which is an effective treatment for patients with asthma [51]. However, as a double-edged sword, the use of glucocorticoids is limited by unwanted severe side effects, such as osteoporosis, disorders of glucose and lipid metabolism, and hypertension [52,53]. Although using inhaled glucocorticoids have diminished the negative side effects, there is recent case reports of symptomatic adrenal suppression in asthmatic children treated with moderate to high dose inhaled glucocorticoid [54–56]. Therefore, the goal to discover a compound that has anti-inflammatory properties, but lacks the negative side effects seen with glucocorticoids, has attracted researchers' attention. Recently, Juan Du et al. [57,58] have discovered that Ginsenoside Rg1 which is the most abundant and active ingredient of *Panax ginseng* can effectively inhibit acute and chronic inflammation in vivo, but it does not cause hyperglycemia or osteoporosis as seen with DEX and suggest that ginsenoside Rg1 may serve as a novel anti-inflammation agent and may exhibit a potential profile for therapeutic intervention in inflammatory diseases. Although there was little evidence that long-time usage of glucocorticoid led to the GR down-regulation in clinics [5], our data showed that DEX administration had no effect of restoring corticosteroid sensitivity and increasing GR expression in asthma-SDR mice. It might be the reason why the efficacy of anti-inflammatory of icariin was more effective than DEX on the SDR-Asthma murine model.

Although reduced glucocorticoid sensitivity widely exists in airway disease such as: severe asthma, chronic obstructive pulmonary disease and cystic fibrosis [59], there is no effective therapeutic approach to restore the anti-inflammatory action of glucocorticoids. Undoubtedly, the identification of novel therapeutic targets and drugs to overcome stress-mediated glucocorticoid insensitivity can offer substantial value in the management of asthma [60]. Our study is the first demonstration that icariin, a novel and promising therapeutic approach of plant origin, increases the efficacy of glucocorticoid, but the mechanism how icariin modulates GR expression and function needs further study. However, there also exist some unsolved problems in our study. One of the problems is why icariin at 50 mg/kg is the most effective dose to overcome glucocorticoid insensitivity compared to the lower or higher doses. In our previous study, we used icariin to treat a mouse model for depression and the data indicated that icariin administered at 25 mg/kg and 50 mg/kg for 28 consecutive days produce a remarkable effect on improving depression situation. The dose of 50 mg/kg icariin was more efficient than the dose of 25 mg/kg icariin [22]. And other studies about the effect of icariin on stressed rats found that the best effective dose of icariin was 60 mg/kg [61,62]. However, another study by our team investigated the mechanism of icariin on asthmatic rats and the data showed that the dose of 20 mg/kg icariin had the best anti-inflammatory effect [18]. Therefore, in the present study, we set the 50 mg/kg icariin as the middle dose and doubled middle

dose icariin was the high dose. The data showed that icariin at 50 mg/kg was the most effective dose in the present study which was consistent to our previous study [22]. These results reminded us that icariin at 50 mg/kg was the most effective dose in depression mouse model and asthma-SDR model. The potential mechanism needs deeper research in the future. Besides, physicians and scientists have recognized the anti-inflammatory effect of herb medicine, especially in asthma [63–65], but deeper and more extensive studies are needed to fully understand its functional profiles.

5. Conclusions

From this study, it was suggested that icariin, which had been demonstrated to be anti-inflammation and up-regulation GR, showed a better therapeutic index in comparison with DEX in a mice model of allergy and stress exposure. Icariin could ameliorate anxious behaviors in OFT, reverse AHR, reduce inflammatory cytokine infiltration to the lung and whole body and also in part recover glucocorticoid responsiveness. Icariin could significantly inhibit psychosocial stress-induced increases of corticosterone and markedly increase GR mRNA and protein expression in the lungs of mice exposed to both stress and allergen. However, in this study, airway inflammation was induced by OVA-challenged and strengthened by SDR (from our previous results), but we don't know the anti-inflammatory effect of icariin against the asthma or SDR or both, which need further study.

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