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Icariin attenuates social defeat-induced down-regulation of glucocorticoid receptor in mice

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ABSTRACT

Icariin is a major constituent of flavonoids isolated from the herb Epimedium. It displays antidepressant-like activity in mice behavioral despair models and chronic mild stress models. In this study, a chronic social defeat protocol is used as a mouse model for depression, and the social avoidance effects of icariin administration are investigated. The data indicate that social defeat significantly reduces mice social interaction time and that icariin administered at 25 mg/kg and 50 mg/kg for 28 consecutive days produce remarkable increases in social interaction time. Impaired glucocorticoid receptor (GR) function is related to depression and normalization of GR function is closely associated with the recovery from depression. In this study, GR binding affinity and protein expression were evaluated by radioactive ligand and western blot, respectively. Our results demonstrate that both GR binding affinity and protein expression in the social defeat model are remarkably decreased and that icariin administration attenuates social defeat-induced GR down-regulation. In the present study, our data also show that icariin administration significantly inhibits social defeat-induced increases of corticosterone and IL-6 levels. The potential mechanisms of icariin induced GR modulation, such as effects on HPA-axis function, proinflammatory signaling pathway and membrane steroid transporters, need further study.

1. Introduction

Depression has a huge impact on individuals and society. It will be the second leading illness in the world by 2020 based on a survey of World Health Organization.

One of the most consistent biological findings in patients with major depression is a hyperactivity of the hypothalamic-pituitary-adrenal (HPA) system, postulated to be caused by impaired glucocorticoid sensitivity (van Rossum and Lamberts, 2006). Up to 80% of patients with major depression have displayed cortisol non-suppression to dexamethasone in the dexamethasone-CRH (DEX-CRH) test (Holsboer, 2000). Following in vitro glucocorticoid exposure, peripheral blood mononuclear cells from depressed patients are less sensitive to dexamethasone-induced inhibition of proliferation, compared to healthy controls (Pariante, 2004).

Although the mechanism of impaired glucocorticoid sensitivity is poorly understood, the glucocorticoid receptor (GR) is postulated to be involved. Following exposure to acute immobilization stress, the GR heterozygous mice with GR down-regulation, exhibited depression-like neuroendocrinological abnormalities, such as higher levels of corticosterone and lower sensitivity to dexamethasone test in comparison to their wild-type littermates. Behaviourally, the mice demonstrated increased depressive like behaviors (learned helplessness) after stress exposure (Ridder et al., 2005). In contrast, animals overexpressing GR were resistant to the development of helplessness and demonstrated a heightened sensitivity to dexamethasone suppression of corticosterone levels, as well as reduced production of corticosterone after immobilization stress (Ridder et al., 2005). These findings suggest that impaired GR function is related to depression and that normalization of GR function maybe closely associated with the recovery from depression.

Data indicate that major depression is also associated with immune activation as reflected by increased plasma and cerebrospinal fluid (CSF) concentrations of a variety of proinflammatory cytokines including interleukin-1(IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) (Raison et al., 2006). These proinflammatory cytokines can decrease GR expression (Pariante and Miller, 2001; Miller et al., 1999) and inhibit GR function by decreased GR translocation and decreased activation of relevant GR-inducible enzymes or reporter gene constructs (Pariante and Miller, 2001; Miller et al., 1999).

Icariin (Fig. 1) is a major constituent of flavonoids isolated from the herb Epimedium (Ye and Chen, 2001). Icariin is used to treat various diseases, such as coronary heart disease, osteoporosis, menopause syndrome, rheumatism, bronchitis and hypogonadism (Li and Wang, 2008). Our group has demonstrated that it can

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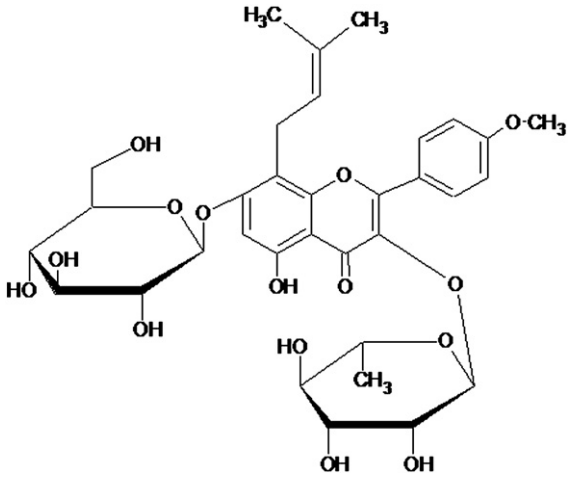

Fig. 1. Structure of icariin.

attenuate lipopolysaccharide (LPS)-induced pro-inflammatory activation, including IL-6 and TNF-α in murine macrophage cell lines and in mouse models of inflammation (Wu et al., 2009; Xu et al., 2010). A recent study reported that icariin displayed antidepressant-like activity in mice behavioral despair models and chronic mild stress models (CMS) (Pan et al., 2007; Pan et al., 2006).

In this study, a chronic social defeat protocol was used as mouse model for depression, which mimics many symptoms of depression in humans (Berton et al., 2006; Tsankova et al., 2006). We investigated the effects of icariin administration on social avoidance, GR protein expression, GR binding affinity, corticosterone and IL-6 serum levels.

2. Materials and methods

2.1. Reagents

Icariin was purchased from Shanghai Ronghe Co (Shanghai, China). Fluoxetine was obtained from Eli Lilly Co. (Suzhou, China). LPS was supplied by Sigma-Aldrich (St Louis, MO). ^{3}H labeled dexamethasone was supplied by Amersharm Pharmacia Biotech (Buckinghamshire, UK). Mouse Anti-Glucocorticoid Receptor Antibody was purchased from Santa Cruz (Santa Cruz, CA). Mouse IL-6 and corticosterone ELISA kits were obtained from R&D Systems (Minneapolis, MN).

2.2. Animals and drugs administration

Male C57BL/6J mice (8 weeks old) were purchased from Shanghai SLAC Co (Shanghai, China) and housed 4 per cage. Male CD1 mice (8 months old) were obtained from Beijing vitalriver Co (Beijing, China) and housed individually. All animals were maintained at constant temperature (23 °C) with a 12 h light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.) and had free access to water and food. All animal experiments were approved by the Fudan University Animal Care and Use Committee. Icariin and fluoxetine were dissolved or dispersed in saline, respectively.

Twenty-four hours after the last social defeat exposure, icariin (25 mg/kg and 50 mg/kg), fluoxetine (10 mg/kg), or vehicle (saline) were administered by intragastric (i.g.) in 0.2 ml volume for 28 consecutive days to different groups, respectively. The number of animals in each group was 12.

2.3. Social defeat model

Male C57BL/6J mice were used as experimental mice. Resident mice were CD1 retired breeders, selected for their attack latencies reliably shorter than 30 s. Every day, each experimental mouse was introduced into the home cage of a new resident aggressor for 5 min and was physically defeated. Then, the resident home cage was divided into two halves by using a perforated plexiglass partition. Residents and intruders were maintained in sensory contact for 24 h. Experimental mice were submitted to social defeat for 10 consecutive days. Control mice were housed in equivalent cages but with members of the same strain, which changed daily. The mice were individually housed without their CD1 partner for 28 days following the last social defeat (Berton et al., 2006).

2.4. Mice social avoidance evaluations

The social avoidance evaluations occurred 28 days after the last social defeat by a videotracking system (MED-OFA-RS, USA). Each experimental mouse was introduced into the open field (43.2×43.2 cm). During the first session ("no target"), the open field contained an empty wire mesh cage (10×6.5 cm) located at one end of the field. During the second session ("target"), the conditions were identical except that an unfamiliar CD1 male mouse had been introduced into the cage. Between the 2 sessions, the experimental mouse was removed from the arena, and was placed back into its home cage for approximately 1 min. The videotracking data from both the "no target" and "target" conditions were used to determine the time spent by the experimental mouse in the "interaction zone" (an 8 cm wide corridor surrounding the cage) (Berton et al., 2006).

2.5. Blood and livers collection

After the social avoidance evaluations were finished, experimental mice were placed back into their home cage. An unfamiliar CD1 social target was introduced in the cage on the opposite side of the partition. Experimental mice were killed 120 min after introduction of the social target (Berton et al., 2006). Blood was collected by intracardiac puncture and separated in a refrigerated centrifuge at 4 °C. Serum was stored at −80 °C until assays were performed. Following blood collection, livers were rapidly removed for GR protein assay and GR binding assay.

2.6. GR protein assay by western blot

Livers were homogenized in lysis buffer and incubated for 30 min at 4 °C. Cell debris was removed by microcentrifugation, and supernatants were quickly frozen. The protein concentration was determined by BSA method. 30 μg of proteins were electroblotted 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 and 1:2000 dilution of anti-β-actin antibody, 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 1 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 glucocorticoid receptors was shown as a proportion of β-actin optical density.

2.7. GR binding assay by radioactive ligand

Livers were homogenized in three volumes of the ice-cold buffer (50 mM Tris–HCl buffer that contained 250 mM sucrose, 50 mM KCl, 3 mM $MgCl_2$, 20 mM sodium molybdate, and 1 mM mercaptoethanol, pH 7.5). The homogenates were centrifuged at 114,500×*g* using an ultracentrifuge for 30 min at 4 °C. The cytoplasmic supernatant fraction was harvested. Protein determinations were performed by BSA method. Protein concentration was adjusted to 10 mg/ml. 250 μl

supernatant was incubated with ^{3}H labeled dxamethasone. The incubation was carried out at room temperature for 30 min, followed by 0 °C for 2 h. After incubation, 500 µl dextran-coated charcoal (500 mg dextran and 5.0 g charcoal suspended in 100 ml of 10 mM Tris–HCl buffer, pH 7.5) was added, and 10 min after incubation at 4 °C, the assay mixture was centrifuged at 8500×*g* for 3 min. The supernatant was harvested for liquid scintillation spectrometry. For determination of nonspecific binding, 1000-fold excess of unlabeled dexamethasone was added, and specific binding was determined by subtracting nonspecific binding from total binding.

2.8. Serum IL-6 and corticosterone determination by ELISA

For corticosterone detection, 10 µl of serum and 0.5 µl steroid displacement reagent was diluted in 990 µl of assay buffer, completing a 100-fold dilution. Plates were coated with 100 µl of anti-IL-6 or anti-corticosterone overnight at 4 °C 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-IL-6 or 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. IL-6 and corticosterone concentrations were quantified by comparison to the standard curves. Intra-assay variability for IL-6 ELISA was 6.2%, and inter-assay variability was 8.8%. Intra-assay variability and inter-assay variability for corticosterone ELISA was 5.4% and 9.6%, respectively.

2.9. Statistical analyses

Data were presented as the mean ± standard deviation (SD). Data analysis was performed by one-way analysis of variance (ANOVA). For comparison of two groups, a student's *t*-test was used. Differences with $P<0.05$ were considered to be statistically significant.

3. Results

3.1. Effects of icariin and fluoxetine on the time spent in the interaction zone

Videotracking pictures were captured from control and defeated C57BL/6J mice in the absence and presence of unfamiliar CD1 mice (Fig. 2). As shown in Fig. 3, in the absence of an aggressor, all the five groups of mice spent similar amounts of time in the interaction zone. When an aggressor was introduced into the cage, chronically defeated mice showed significant reduction in their interaction time ($P<0.001$), which implied the successful establishment of a social defeat model, whereas fluoxetine (10 mg/kg) and icariin (25 mg/kg and 50 mg/kg) administration could reverse the social defeat-induced decrease of interaction time.

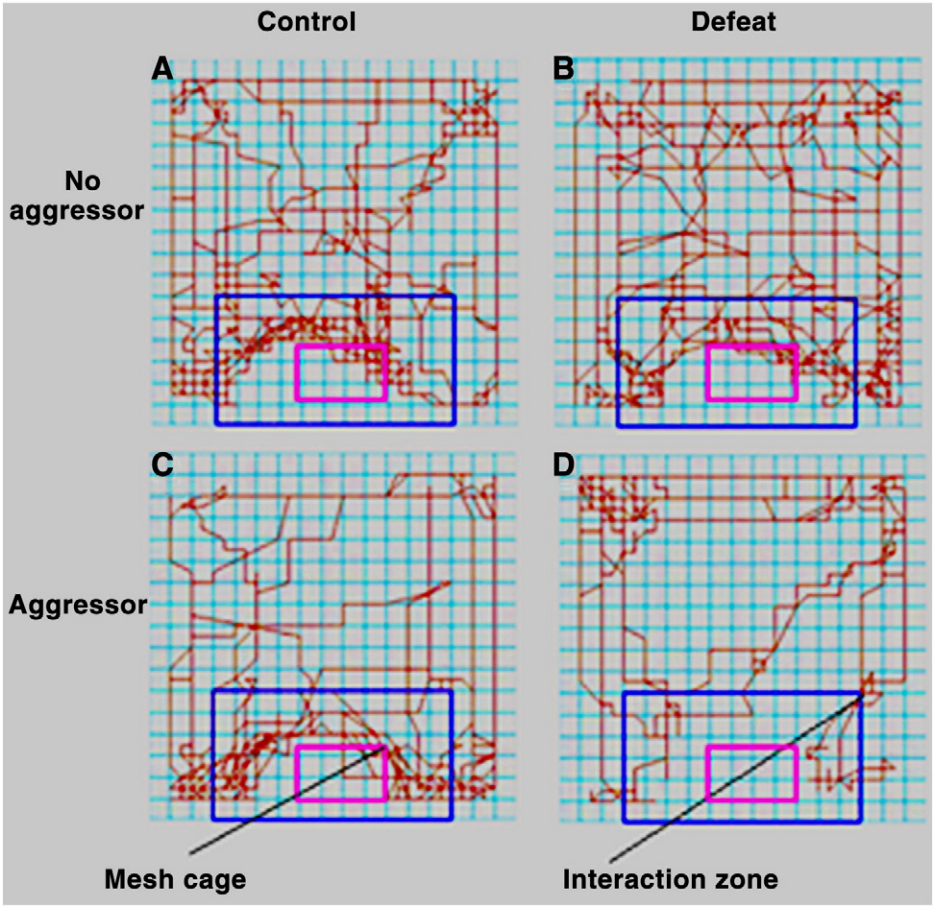

Fig. 2. Videotracking pictures from control and defeated C57BL/6J mice in the absence and presence of unfamiliar CD1 mice. The interaction zone was defined as 8 cm around a cage. A = normal control mice with no aggressor, B = social defeat mice with no aggressor, C = normal control mice with aggressor, and D = social defeat mice with aggressor.

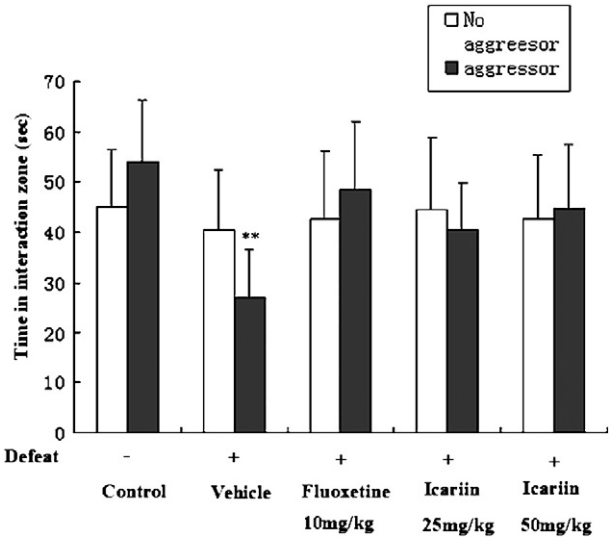

Fig. 3. Effects of icariin and fluoxetine on the time spent in the interaction zone. The social defeat mice were treated with vehicle alone, icarrin, or fluoxetine, respectively. The videotracking data were used to determine the time spent by the experimental mouse in the "interaction zone". In the absence of an aggressor, all five groups of mice spent similar amounts of time in the interaction zone. When an aggressor was introduced into the cage, the vehicle-treated social defeat mice showed significant reduction in their interaction time, whereas fluoxetine and icariin administration could effectively reverse social defeat-induced reductions of interaction time. $^{**}P<0.01$, vs when the aggressor was absent. The number of animals in each group was 12.

3.2. Effects of icariin and fluoxetine on GR protein expression and binding activity

The GR protein expression and GR binding affinity were detected by western blot and radioactive ligand, respectively. As shown in Fig. 4 and Fig. 5, the GR protein expression and GR binding affinity in social defeat group were both significantly lower than that in normal control ($P<0.001$), whereas fluoxetine(10 mg/kg) and icariin (25 mg/kg, 50 mg/kg) administration remarkably increased the GR protein expression and binding affinity, compared to social defeat group ($P=0.006$, $P=0.035$, and $P=0.016$).

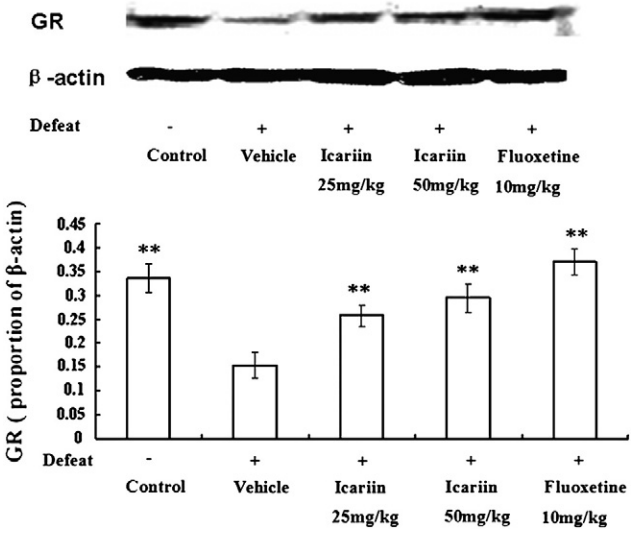

Fig. 4. Effects of icariin and fluoxetine on GR protein expression. The GR protein expression was detected by Western blot. All lanes contained 30 μg of protein in total. The GR protein in the vehicle-treated social defeat group was significantly decreased than that in normal control. Fluoxetine and icariin administrations could reverse social defeat induced decreases of GR protein. Band intensities were quantified using UN-SCAN-IT gel analysis software. The optical density for glucocorticoid receptors was shown as a proportion of β-actin optical density. $^{**}P<0.01$, vs. the vehicle-treated social defeat group.

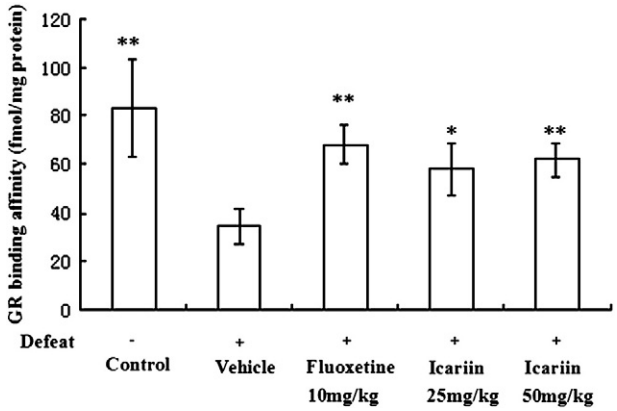

Fig. 5. Effects of icariin and fluoxetine on GR binding affinity. The GR binding affinity was detected by liquid scintillation spectrometry. The specific GR binding was determined by subtracting nonspecific binding from total binding. The GR binding affinity in the vehicle-treated social defeat group was significantly lower than that in normal control. Fluoxetine and icariin administration partly restored the GR binding affinity. $^{**}P<0.01$, $^{*}P<0.05$, vs. the vehicle-treated social defeat group.

3.3. Effects of icariin and fluoxetine on corticosterone and IL-6 level in serum

On the test day, experimental mice were killed 120 min after introduction of a new social target. The blood was collected and serum was analyzed for corticosterone and IL-6 by respective ELISA kit. The results presented in Fig. 6 and Fig. 7 demonstrated the levels of corticosterone and IL-6 in social defeat group were significantly higher than that in normal control ($P<0.001$ and $P<0.001$), whereas fluoxetine (10 mg/kg) and icariin (25 mg/kg, 50 mg/kg) administration remarkably inhibited the production of corticosterone ($P=0.002$, $P=0.038$, and $P=0.005$) and IL-6 ($P=0.004$, $P=0.033$, and $P=0.006$).

4. Discussion

Icariin is a major constituent of flavonoids isolated from herb Epimedium (Ye and Chen, 2001). It displays an antidepressant-like activity in behavioral despair models and chronic mild stress models. Icariin can significantly shorten immobility time in the forced swimming test (FST) and the tail suspension test (TST) (Pan et al., 2007; Pan et al., 2006). It decreases monoamine oxidase (MAO) A and

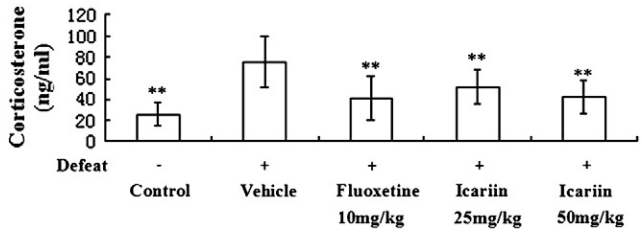

Fig. 6. Effects of icariin and fluoxetine on corticosterone level in serum. After 10 days of social defeat or control conditions, experimental mice were administered with icariin (25 mg/kg and 50 mg/kg, ig), fluoxetine (10 mg/kg, ig) and vehicle (saline, ig) for 28 consecutive days to different groups, respectively. On the test day, experimental mice were killed 120 min after introduction of a new social target. The blood was collected and serum was analyzed for corticosterone by ELISA. The level of corticosterone in the vehicle-treated social defeat group was significantly higher than that in normal control. Fluoxetine and icariin remarkably inhibited the production of corticosterone. $^{**}P<0.01$, vs. the vehicle-treated social defeat group.

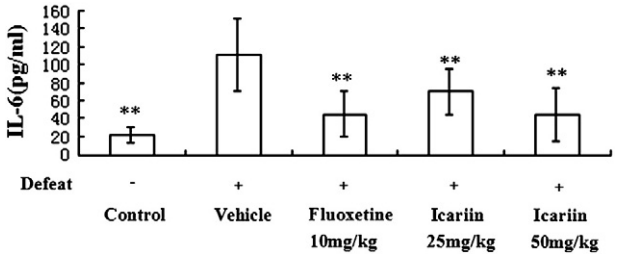

Fig. 7. Effects of icariin and fluoxetine on IL-6 level in serum. The experimental mice were treated as described in Fig. 6. The blood was collected and serum was analyzed for IL-6 by ELISA .The number of animals in each group was 12. The level of IL-6 in the vehicle-treated social defeat group was remarkable higher than that in normal control. Fluoxetine and icariin significantly inhibited the production of IL-6. $^{**}P<0.01$, vs. the vehicle-treated social defeat group.

B activities in brain, serum corticotropin-releasing factor (CRF) levels, as well as increases monoamine neurotransmitter levels in the brain of behavioral despair models (Pan et al., 2007; Pan et al., 2006). Icariin also significantly increased sucrose intake in animals exposed to chronic mild stress. It not only attenuates the chronic mild stress-induced increases in serum CRF and corticosterone levels, but also reverses the abnormal levels of serum IL-6 and TNF-α (Pan et al., 2007; Pan et al., 2006). In this study, a social defeat protocol is used as a mouse model of depression, which mimics many symptoms of depression in humans (Berton et al., 2006; Tsankova et al., 2006). Our study shows that chronic social defeat significantly reduces mice social interaction time, whereas icariin (25 mg/kg and 50 mg/kg) and fluoxetine (10 mg/kg) administration produced remarkable increases in social interaction time.

Impaired glucocorticoid sensitivity has been observed in social defeat models (Bailey et al., 2004; Merlot et al., 2004), and depressed patients (Holsboer, 2000; Pariante, 2004). Although the mechanism of impaired glucocorticoid sensitivity in depression is poorly understood, GR is believed to be involved (Buwalda et al., 1999; Buwalda et al., 2001; Spijker and van Rossum, 2009). GR is widely distributed in animal and human tissues. The link between impaired glucocorticoid sensitivity and major depression is based on GR in the hippocampus or hypothalamus (Buwalda et al., 1999; Buwalda et al., 2001; Spijker and van Rossum, 2009). Radioactive ligand and western blot are two classical methods for GR quality and quantity detection. The mouse hippocampus or hypothalamus tissue is not sufficient for these two methods. Because many investigations show similar changes in GR binding in the brain and liver (Taymans et al., 1997; Watzka et al., 2000), we used liver tissue instead of hippocampus and hypothalamus tissue to assay the quality and quantity of GR. Our results demonstrated that icariin administration effectively attenuated social defeat-induced GR down-regulation both in protein expression and binding affinity.

One of the most consistent biological findings in patients with major depression is a hyperactivity of the HPA-system, including elevated serum and 24 h urinary cortisol levels, a flattened diurnal cortisol rhythm, and adrenal gland hyperplasia (Schule et al., 2009). Glucocorticoids exert their effects via the cytoplasmic GR. There are reports that GR undergo down-regulation after exposure to high concentration of glucocorticoids in vitro, in animals and human (Knutsson et al., 1995; Silva et al., 1994). In the present study, animals that received chronic social defeat displayed high levels of serum corticosterone upon exposure to a social challenge. Icariin administration significantly inhibited this increase in serum corticosterone, which maybe contributes to GR up-regulation.

Depressed patients often exhibit higher plasma concentrations of proinflammatory cytokines such as IL-6 and TNF-α than that of non-depressed patients (Alesci et al., 2005; Humphreys et al., 2006; Maes et al., 1993). Proinflammatory cytokines and their signaling pathways may play key roles in altered GR function of major depression (Pace et al., 2007). Our data suggests that icariin administration remarkably attenuated the increases in serum IL-6 level that occur following re-exposure to a social target. Our previous study has shown that icariin displays anti-inflammatory potential (Wu et al., 2009; Xu et al., 2010), which was further confirmed by another study (Liu et al., 2010). We hypothesize that icariin induced GR up-regulation maybe partly due to its anti-inflammatory potential.

In the present study, fluoxetine was used as a positive control drug, and it effectively attenuated the social defeat induced GR down-regulation, elevated serum corticosterone, and elevated IL-6 levels. Several reports have shown that fluoxetine can up-regulate GR via modulating membrane (Okuyama-Tamura et al., 2003; Pariante et al., 2003; Pariante et al., 2001). Future research is necessary to determine whether icariin has a similar effect on GR via modulating membrane steroid transporters.

5. Conclusions

The present study suggests that icariin displays antidepressant-like activity in a social defeat model, and has the potential to become a useful antidepressant treatment. Our results suggest that the antidepressant effects of icariin are partly attributed to normalization of the GR expression and function. However, the potential mechanisms of icariin induced GR modulation, such as effects on HPA-axis function, proinflammatory signaling pathway and membrane steroid transporters, need further study.

Acknowledgements

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