Effects of Icariin on Hypothalamic-Pituitary-Adrenal Axis Action and Cytokine Levels in Stressed Sprague-Dawley Rats

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Icariin is one of the major active flavonoids constituents of Epimedium brevicornum Maxim (Berberidaceae). Icariin and Epimedium brevicornum have a wide range of pharmacological activities. Abnormality in the hypothalamic-pituitary-adrenal (HPA) axis is considered to be a key neurobiological factor in major depression, and cytokines have a close relationship with the activation of the HPA axis. In the present study, the aim was to determine whether icariin possesses an antidepressant-like activity, and to explore the effects of icariin on the HPA axis and cytokine levels in chronic mild stress (CMS) model of depression in Sprague-Dawley rats. Icariin significantly increased the sucrose intake of CMS-treated rats from week 3. It not only attenuated the CMS-induced increases in serum corticotropin-releasing factor (CRF) and cortisol levels, but also reversed the abnormal levels of serum interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) to the normal in the stressed rats. These results suggested that icariin possessed an antidepressant-like property that was at least in part mediated by neuroendocrine and immune systems.

Key words icariin; chronic mild stress model; interleukin-6; tumor necrosis factor-alpha (TNF-α); corticotropin-releasing factor; cortisol hypothalamic-pituitary-adrenal axis activity

It is well known that abnormality of neuroendocrine function plays an important role in the occurrence of depression. The hypothalamic-pituitary-adrenal (HPA) axis is one of the important neuroendocrine axes, and enhanced activity of this axis is considered a key neurological alteration in major depression. Corticotropin-releasing factor (CRF), the major physiological regulator of the HPA system, is believed to contribute to several depressive symptoms, and augmentation of the pituitary, and elevated cortisol levels in serum have been observed in depressive disorders. Recent studies have showed there is a close relationship between activation of the cytokine system and the HPA axis in major depression. Cytokines are intercellular messengers in the immune system, they regulate the inflammatory response by interaction with specific receptors and are capable of triggering cellular responses. Cytokines regulate immune responses, possibly through activation of the HPA axis, while the HPA axis activity is related to behavioral lateralization and brain asymmetry. Some researchers reported that interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) regulated not only the inflammatory process, but also mediated a variety of CNS-mediated responses and neuroendocrine activity.

Icariin (Fig. 1) is one of the major active flavonoids isolated from Epimedium brevicornum Maxim (Berberidaceae). Icariin and Epimedium brevicornum have a wide range of pharmacological activities, including regulating the cardiovascular system, stimulating neurite growth and enhancing the estrogenic activity which possess the osteogenic differentiation. Clinical evidence suggested that Epimedium brevicornum could improve depressive symptoms after stroke. Recently, our laboratory demonstrated antidepressant actions of icariin and total flavonoid extracts from Epimedium brevicornum in animal behavior despair models of mice. Furthermore, icariin administration attenuated the swim stress-induced elevation in serum CRF and cortisol levels in mice. The aim of this study was to further confirm the antidepressant-like effects of icariin in a chronic mild stress (CMS) model of depression in Sprague-Dawley rats, which causes behavioral changes in rodents that parallel symptoms of depression, and to investigate whether icariin influences the HPA axis function and serum cytokine levels in the stressed animals in order to discuss its antidepressant mechanism.

MATERIALS AND METHODS

Chemicals Icariin was purchased from Bio-sep Bio-technique Stock Co., Ltd. Xi’an Jiaotong University (P.R. China). Its purity was at least 98% as checked by high-performance liquid chromatography (HPLC). Fluoxetine hydrochloride was from Changzhou Siyao Pharmaceuticals Co., Ltd. (P.R. China). Other reagents were analytical grades made in P.R. China.

Animals Male Sprague-Dawley (SD) rats (220—250 g), purchased from the Laboratory Animal Center, Jiangsu Province, P.R. China, were used in the experiments. Except as described below, the animals were singly housed and were kept on a 12 h light/12 h dark cycle under controlled temperature 22±2°C. They were allowed free access to a laboratory chow diet and, ad libitum, to water. Animal handling procedures were approved by the University Committee for Animal Experiments and performed strictly following the People’s Republic of China legislation on the use of laboratory animals.

Sucrose Solution Intake Baseline Test All the animals...
were first trained to consume a 1% sucrose solution after the 3-d adaptation period. The test of sucrose solution intake baseline was performed 9 times (once every three days) for each animal. The test involved an 18 h period of food and water deprivation followed by offering the 1% sucrose solution for 1 h. Sucrose-intake was measured by weighting bottle weights containing sucrose solution before and after the test respectively. The sucrose intake was expressed in relation to animals’ body weights (g/kg).

**CMS Procedure** After the sucrose intake baseline test, the animals were divided into two groups with similar average intake. Twenty-one rats in one group were housed under normal condition (as non-stressed animals) and twenty-eight rats in the other group were subjected to CMS procedure (as CMS-treated animals) throughout. Subsequently, sucrose consumption was monitored weekly for the next experimental period (12 weeks). The CMS procedure was carried out for 6 weeks using the method described by Willner et al. with small modification.19,20) Each experiment cycle extended 7 d and included the following steps:

- Low intensity stroboscopic illumination (150 flashes/min) for 6 h; tilted cage (45°) for 14 h; intermittent illumination (2 h light/2 h dark cycle) for 12 h; random partnership for 12 h; wet cage (200 ml water in 100 g sawdust bedding) for 12 h; continuous light for 12 h; low intensity stroboscopic illumination (150 flashes/min) for 10 h; water and food deprivation for 14 h; noise (6300 Hz tone, approx. 90 dB) for 6 h; tilted cage (45°) for 14 h; low intensity stroboscopic illumination (150 flashes/min) for 12 h; continuous light for 12 h; noise (6300 Hz tone, approx. 90 dB) for 9 h; water and food deprivation for 18 h; sucrose test for 1 h.
- All of the stressors were applied individually and continuously, day and night. Non-stressed rats were housed in separate rooms away from CMS-stressed animals. Food and water were freely available in the home cage except that they were deprived of both before each sucrose test.

**Drug Administration** Normal control rats and stressed rats were further divided into subgroups on the basis of sucrose intake scores after the CMS procedure. The non-stressed animal groups were divided into three subgroups and they received normal saline (1 ml/kg, normal control, n = 7), icariin (35 mg/kg, n = 7), icariin (70 mg/kg, n = 5) and fluoxetine (7 mg/kg, n = 7), respectively. The CMS-treated animal groups were further divided into four subgroups (7 rats/subgroup) and they received normal saline (1 ml/kg, stressed control), icariin (35 mg/kg), icariin (70 mg/kg), and fluoxetine (7 mg/kg), respectively. All drugs were suspended in 0.9% normal saline, and were orally administered once daily for 6 weeks.

**Blood Collection** After 6-week administration and the last sucrose intake test, all animals were left without any treatment until the following morning. The next day, they were etherized and decapitated for bleeding between 09:00 a.m. and 09:30 a.m. Blood was collected on ice and separated in a refrigerated centrifuge at 4 °C. Serum was stored at −80 °C until assays were performed for CRF, cortisol, IL-6 and TNF-α.

**CRF Determination** Serum CRF levels were measured using a commercially available radio-immunoassay kit (Techni-Que Center of Radioimmunity of the Navy in Beijing P.R. China) in the Immunoassay Department of Inspection Department, Nanjing General Hospital of the Nanjing Military Command, P.R. China. The sensitivity of the assay was 0.2 ng/ml. Intra- and inter-assay variabilities for these assays were less than 8%.

**Cortisol Determination** Serum cortisol levels were determined using Enzyme Immunoassay (magnetic solid phase) kits (Beijing Bio-Ekon Biotechnology Company Limited, Beijing). The sensitivity of the assay was 1.0 ng/ml. Intra-assay and inter-assay coefficients of variation were less than 4.85% and 6.08%, respectively.

**IL-6 and TNF-α Determination** Serum levels of IL-6 and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D SYSTEMS), following the manufacturer’s instructions, respectively. The minimum detectable concentrations were <14—36 pg/ml (mean 21 pg/ml) for IL-6 and <5 pg/ml for TNF-α, respectively.

**Statistical Analysis** All data are expressed as mean±S.M.E. The statistical significance was analyzed by Student’s t-test or analysis of variance with Bonferroni for post hoc comparison. p value of less than 0.05 was accepted as statistically significant.

**RESULTS**

**Effects of Icariin on Sucrose Intake** As shown in Fig. 2, there was no difference between vehicle group and drug (icariin or fluoxetine)-treated groups in non-stressed rats. In the CMS-treated groups, icariin at 35 mg/kg significantly elevated the sucrose intake of stressed rats after 3-week treatment (p <0.05). There also existed a difference between vehicle-stressed animals and icariin-treated stressed animals at week 4 (p <0.01), week 5 (p <0.05), and week 6 (p <0.01). Icariin at 70 mg/kg increased the sucrose intake in the CMS-treated rats at week 4 (p <0.05), week 5 (p <0.05) and week 6 (p <0.01). Fluoxetine remarkably reversed CMS-induced sucrose intake decrease from week 2 (p <0.05).

**Effects of Icariin on Serum CRF Levels** As shown in Fig. 3, the CMS procedure induced serum CRF levels of saline-treated rats a slightly higher. Icariin at 35 mg/kg sig-

![Fig. 2. Effects of Icariin (Ica) and Fluoxetine (Flu) on the Sucrose Intake in Non-stressed (Non-str) and CMS-Treated (CMS-tre) SD Rats](image-url)
significantly reduced serum CRF levels \((p<0.05)\) in stressed animals, but at 70 mg/kg it failed to significantly alter serum CRF levels. There was no significant change of CRF levels in the non-stressed rats after icariin treatment. In this study fluoxetine showed no changes in serum CRF levels both in two groups.

**Effects of Icariin on Serum Cortisol Levels**  As shown in Fig. 4, there was a significant increase in cortisol levels in saline-treated CMS rats \((p<0.01)\). Icariin at 35 mg/kg slightly but not remarkably reduced serum cortisol levels in the CMS-treated rats, and icariin at 70 mg/kg attenuated the CMS-induced increase in these levels \((p<0.05)\). Fluoxetine exhibited a tendency to decrease these levels of CMS-treated animals. In the non-stressed groups, icariin had no significant effect on the cortisol levels. However, significant increase in these levels was observed after administration of fluoxetine \((p<0.01)\).

**Effects of Icariin on Serum IL-6 Levels**  As shown in Fig. 5, a significant increase in serum IL-6 levels was observed after the CMS procedure \((p<0.05)\). Icariin at 35 mg/kg markedly reduced these levels in CMS-treated rats \((p<0.01)\), however, neither icariin at 70 mg/kg nor fluoxetine had a significant effect on serum IL-6 levels in this study. In the non-stressed groups, icariin was without significant effect. However, fluoxetine produced a significant elevation in serum IL-6 levels \((p<0.01)\).

**Effects of Icariin on Serum TNF-\(\alpha\) Levels**  As shown in Fig. 6, the CMS procedure evoked a significant increase in serum TNF-\(\alpha\) levels of saline-treated rats \((p<0.05)\). Icariin at 35 mg/kg significantly prevented the CMS-induced changes in serum TNF-\(\alpha\) levels of animals, but at 70 mg/kg failed to significantly alter the levels. Fluoxetine decreased these levels of stressed animals \((p<0.05)\). While in the non-stressed groups, icariin had no significant effect on the levels. In contrast, fluoxetine significantly elevated serum TNF-\(\alpha\) \((p<0.05)\).

**DISCUSSION**

The present study demonstrates that administration of icariin has an antidepressant-like activity in the CMS model of depression in Sprague-Dawley rats. This behavioral effect
was accompanied by icariin-induced modulation of both the HPA axis and cytokine response to the CMS procedure in these animals.

Chronic mild stress reduced the sucrose intake of rats in accordance with other results. Icariin significantly increased sucrose intake in the CMS model rats, and did not change the intake in normal rats. As previously noted, icariin was active in pre-clinical mouse models of behavioral depression with effects comparable to known antidepressants. Thus, the behavioral results from this current study confirmed and extended previous findings that icariin had an antidepressant-like effect in the CMS rat model.

The HPA axis plays a key role in the physiological response to various stressful situations. Long-lasting activation of the HPA axis, especially abnormally increased cortisol levels and CRF response, lead to disturbed hormonal balance and even to severe diseases such as depression. On the other hand, HPA axis hyperactivity is dependent on a depressed status and it tends to be normalized following alleviation of clinical symptoms after antidepressant treatment. Certain cytokines are potent modulators of corticotropin-releasing hormones such as CRF, which produces higher HPA axis activity characterized by increases in cortisol. Peripheral IL-6 and TNF-α predominantly produced by monocytes and macrophages act in the brain binding to cytokine receptors on the surfaces of glial cells and neurons of the hippocampus and hypothalamus. Alterations in the levels of circulating IL-6 and TNF-α have been linked to central nervous system depression. Interestingly, recent clinical studies found that IL-6 and TNF-α concentrations were significantly increased in depressed patients. Present results confirmed that there was a close relationship between stress and IL-6 or/and TNF-α which were overproduced in stressed animals. E. brevicornum regulated cytokine secretion. Recently, icariin was confirmed to reduce mRNA expression of TNF-α and IL-6. In the present study, icariin elicited a marked diminution in serum IL-6 and TNF-α levels in the CMS model of rats, suggesting that it could be beneficial in stress-related psychiatric disorders associated with an oversecretion of the peripheral cytokine system.

In conclusion, the present study demonstrated that icariin has potentially antidepressant-like activity in CMS model of depression in rats. The mechanism involved can be mediated by the HPA axis and cytokine systems. Icariin may be a favorable alternative to currently available antidepressant drugs. Further preclinical and clinical experiments are needed to clarify the role of icariin in contributing to the treatment of depressive disorders.

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REFERENCES

35) Kahl K. G., Bester M., Greggersen W., Rudolf S., Dibbelt L., Stoeck-


