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Icariin from *Epimedium brevicornum* attenuates chronic mild stress-induced behavioral and neuroendocrinological alterations in male Wistar rats

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Abstract

Chronic mild stress (CMS) is suggested to produce abnormalities in the hypothalamic–pituitary–adrenal (HPA) axis and hypothalamus–pituitary–thyroid (HPT) axis. Therefore, compound that attenuates the neuroendocrinological alterations may have potential as antidepressant. The behavioral and neuroendocrinological effects of icariin, a major constituent of flavonoids isolated from *Epimedium brevicornum*, were investigated in the CMS model of depression in male Wistar rats. CMS procedure caused an anhedonic state in rats resulted in increased corticotropin-releasing factor (CRF) concentrations in dissected brain regions and serum, decreased total triiodothyronine (tT_3) in serum with no significant changes in serum adrenocorticotrophic hormone (ACTH) and thyroxine (tT_4). Administration of icariin reversed CMS-induced sucrose intake reduction and CRF elevation. These results suggested that icariin possessed potent antidepressant-like activities which were at least in part mediated by improving the abnormalities in the HPA axis functions. However, we did not find a clear correlation between the HPT axis and icariin treatment in the CMS-treated rats.

Keywords: Icariin; Chronic mild stress; Hypothalamic–pituitary–adrenal axis; Hypothalamus–pituitary–thyroid axis

1. Introduction

Depression involves pathophysiological changes in neuroendocrinological function (Musselman and Nemeroff, 1996; Helmreich et al., 2005; Tichomirowa et al., 2005). The most frequently occurring neuroendocrinological abnormality in depressed subjects is hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis characterized by hypersecretion of corticotropin-releasing factor (CRF), which stimulates adrenocorticotrophic hormone (ACTH) release (DeMoranville and Jackson, 1996; Tsigos and Chrousos, 2002; Barden, 2004). Although, in contrast to the HPA axis system, overt hypothalamus–pituitary–thyroid (HPT) dysfunction is not common in depression (Fountoulakis et al., 2004; Schule et al., 2005a,b), thyroid hormones have a profound influence on behavior and appear to be capable of modulating the phenotypic expression of major affective illness (Musselman and Nemeroff, 1996; Bauer and Whybrow, 2001; Fountoulakis et al., 2004). Significant reduction in serum total triiodothyronine (tT_3) concentrations but not in total thyroxine (tT_4) concentrations was observed in depressed patients (Rubin et al., 1987; Sakaue, 1990). However, increased serum tT_3 and tT_4 levels were found in chronic mild stress-induced Sprague–Dawley and Wistar rats with no any change in plasma ACTH levels (Kioukia-Fougia et al., 2000). In addition, there was a close interrelationship between the HPA and the HPT axes in depression (Baumgartner et al., 1990; Helmreich et al., 2005). CRF suppressed thyroid functions (Tsigos and Chrousos, 2002), resulting in T_3 content reduction. Conversely, CRF release in the hypothalamus increased in hypothyroid animals (Tohei et al., 1998). Elimination of thyroid hormones caused a marked reduction in transcription of CRF gene in the paraventricular nucleus of male rats, suggesting that the hormones of the HPT axis had a major effect on the central

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regulation of the HPA axis (Shi et al., 1994). It is important to note that the complex regulation of the HPA and HPT axes highlights organism's complicated adjustability to stressful situations (Helmreich et al., 2005). The neuroendocrinological abnormalities of the HPA and HPT axes, although not observed in all patients with depression (Fava et al., 1995; Watson et al., 2002; Fountoulakis et al., 2004), have been identified as a useful diagnostic tool. Clinical studies also provided evidence that normalization of the HPA axis or the HPT axis abnormalities preceded successful treatment with antidepressants (Joffe, 1992; Bschor et al., 2003; Young et al., 2004; Nikisch et al., 2005; Rao et al., 2005; Schule et al., 2005a,b), indicating that future antidepressants might target the neuroendocrinological systems by regulating either the HPA axis or the HPT axis.

Icariin (Fig. 1) is a major constituent of flavonoids isolated from *Epimedium brevicornum* Maxim (Berberidaceae), which is used in traditional Chinese medicine to nourish the *kidney* and reinforce *yang*. Clinical evidences suggested that *E. brevicornum* and its decoction could improve depressive symptoms after stroke (Lai, 2001; Ma, 2003). *E. brevicornum* decreased plasma ACTH and corticosterone concentrations and possessed protective effects on hypothalamus–pituitary–adrenal–thymus axis induced by exogenous glucocorticoid in clinical and experiments (Cai et al., 1998). The total flavonoids reversed the attenuations of monoamine neurotransmitters and regulated neuroendocrine–immunological network in hypothalamus of the old rats (Shen et al., 2004). Recently, our laboratory demonstrated antidepressant actions of total flavonoid extracts and icariin from *E. brevicornum* in the forced swimming test (FST) and the tail suspension test (TST) in mice (Pan et al., 2005; Zhong et al., 2005). Subsequent study exhibited that icariin administration attenuated the swim stress-induced elevation in serum CRF concentrations (Pan et al., 2005). Thus, these findings might provide some supports for the hypothesis that icariin could modulate abnormal neuroendocrinological function in depressed animals.

Chronic mild stress (CMS) model of depression in animal is accepted as a valuable method for predicting potential antidepressant actions of compounds in humans. The CMS regimen altered behavioral parameters consistent with a loss of responsiveness to reward, such as decreased sucrose consumption, a specific hedonic deficit (Willner et al., 1987; Willner, 1997, 2005). The stress-induced anhedonic-like state in rats

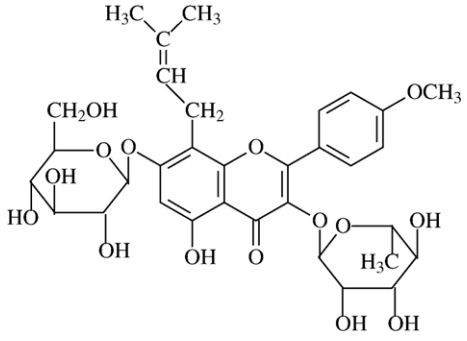

Fig. 1. Structure of icariin.

gradually developed over several weeks and could be prevented or reversed by chronic administration of antidepressant drugs (Griebel et al., 2002; Papp et al., 2003; Willner, 2005). In addition, the CMS produced several neurohormonal changes in rodents that are similar to those found in human depression (Azpiroz et al., 1999; Bratt et al., 2001; Kioukia-Fougia et al., 2002; Grippo et al., 2005a). However, few studies have simultaneously examined behavioral and neuroendocrinological changes in the HPA and the HPT axes during the CMS exposure in rats. The present study explores the possible relationship of the HPA axis and the HPT axis in the CMS model of depression in male Wistar rats, and simultaneously examines the effects of icariin and known antidepressant fluoxetine on neurohormonal mediators of the HPA axis (circulating CRF and ACTH), as well as the central nervous system (CRF in the dissected brain) and the HPT axis (circulating tT_3 and tT_4). These results firstly demonstrate that the CMS induces a profile of the two axes alterations in rats and provide a basis for examining the neurohormonal pathways directly and interactions that underlie the link between depression and icariin treatment.

2. Materials and methods

2.1. Animals

Male Wistar rats (Laboratory Animal Center, Jiangsu Province, China), weighing 220–250 g, were brought into the laboratory 3 weeks before the experiment started. The animals were individually housed, with food and water freely available, and maintained on a 12 h dark–light cycle (with the lights on at 07:00 h locate time) under regulated temperature conditions (22 ± 2 °C), except as described below. The study was approved by the institutional Animal Care Committee at the Nanjing University, or the China council on Animal Care at Nanjing University.

2.2. Drugs

Icariin was purchased from Bio-sep Bio-technique Stock Co., Ltd. Xi'an Jiaotong University (P. R. China). The purity of icariin was checked by high-performance liquid chromatography to be at least 98% pure (Wang et al., 2003). Fluoxetine hydrochloride was from Changzhou Siyao Pharmaceuticals Co., Ltd. (P. R. China). Other reagents were analytical grades made in P. R. China.

2.3. Chronic mild stress (CMS)

Before CMS procedure, rats were trained to consume a 1% sucrose solution. Training consisted of initial 72 h sucrose solution exposure without any food or water available. After the period of adaptation, animals were distributed into two subgroups and sucrose solution intake baseline tests were performed 6 times over 14 days for all subjects. Sucrose intake tests took place once a week at regular times. The intake was expressed in relation to the animal's body weight (g/kg).These tests involved a 14-h period of food and water deprivation

followed by the offering of a sucrose solution for 1 h. At the end of each test, sucrose intake was measured by weighting pre-weighed bottles containing the sucrose solution. Subsequently, sucrose consumption was monitored, under similar condition, in 1 h tests (11:00–12:00 h), at weekly intervals for the next 10 weeks.

On the basis of their sucrose intakes in the final baseline test, the animals were randomly divided into two groups ($n=40$ in every group) having similar average intake. One group was subjected the chronic mild stress (CMS-treated animals) procedure. The CMS procedure was slightly modified from that previously described by Willner et al. (1987) and Papp et al. (2002). The weekly stress regime consisted of one period (12 h) of paired caging, two periods (14 and 18 h) of tilted cage (45°), two periods of water and food deprivation (14 and 18 h), one 12-h period with wet cage (200 ml water in 100 g sawdust bedding), and two periods (12 and 12 h) of continuous light, three periods (6, 10 and 12 h) of low intensity stroboscopic illumination (150 flashes/min), one 12-h period of intermittent illumination (2-h/2-h light/dark cycle), two periods of noise (6300 Hz tone, 10 and 12 h). All of the stressors were applied individually and continuously, day and night. The other group was housed in separate rooms and had no contact with the stressed animals. These rats were deprived of food and water for the 14 h preceding each sucrose test, but otherwise food and water were freely available in the home cage.

On the basis of sucrose intake scores following 5 weeks of the stress, both stressed and unstressed animals were further divided into matched subgroups. Different groups of animals ($n=8$ in every group) were administered with vehicle (saline 1 ml/kg per day), icariin (15, 30 and 60 mg/kg per day), and fluoxetine (10 mg/kg per day), respectively. All drugs were suspended in a 0.9% normal saline, and were administered by gavage once daily at 13:00 h following the weekly sucrose intake test (approximately 1 h later) for the subsequent 5 weeks. Stress was continued throughout the entire treatment period.

2.4. Collection of blood

After the CMS period and post-CMS sucrose intake test, rats were left without any treatment until the following morning. To avoid fluctuations on hormone concentrations due to circadian rhythms, animals were sacrificed via decapitation between the hours of 09:00 and 10:00 h on two consecutive days. Blood was collected in pre-iced tubes with protease inhibitor aprotinin and centrifuged at 3000 rpm at 4 °C for 20 min. The separated serum samples were stored at −80 °C until the assay of CRF, ACTH, tT_3 and tT_4, respectively.

2.5. Collection of brain tissues

Immediately following decapitation, the cortex, hippocampus, corpus striatum and medulla oblongata in animal brain were dissected out, and placed into pre-weight plastic chilled tubes treated with aprotinin. The wet weight of the organs was then determined by subtraction of the weight boat alone, and represented as wet weight. Tissues (about 30 mg) were boiled for 3 min in 1 ml saline solution, and then homogenized in 0.5 ml of 1 M acetic acid at 4 °C using an ultrasonic cell disrupter, set at 4 °C for 1 h. 0.5 ml of 1 M NaOH was added and centrifuged at 15,000 rpm for 20 min at 4 °C (Vale et al., 1983; Tang et al., 1995). Clear supernatants were collected. Recovery ranged between 80 and 90% for extracted CRF. The supernatant was stored at −80 °C until assayed for CRF. The measured immunoreactive values for CRF in brain regions were expressed in ng/g wet tissue weight (ng/g ww).

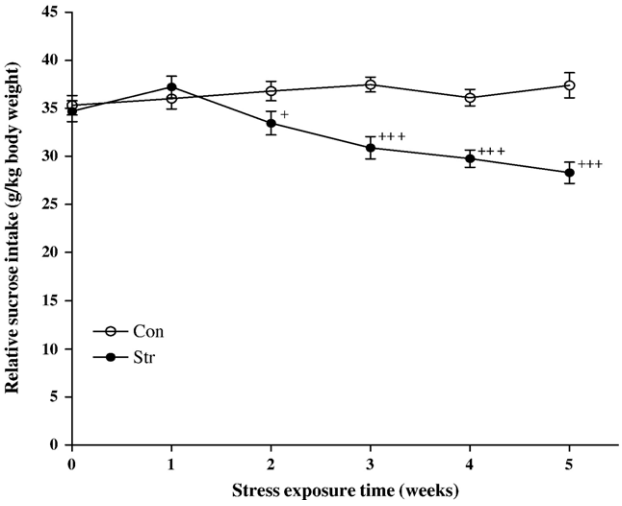

Fig. 2. Stress-induced anhedonia in chronic mild stress (CMS) rats. Variations in sucrose intake in stressed and unstressed animals are shown as a function of stress exposure time (weeks). Mean intake SEM in 1-h sucrose tests is shown for weekly test sessions ($N=40$). $^{+}P<0.05$, $^{+++}P<0.001$ compared to non-stressed animals at the same time point.

2.6. Neurohormone assay

CRF, serum ACTH, serum tT_3 and serum tT_4 concentrations were determined in duplicate by radioimmunoassay (RIA), using commercially available RIA kits which were manufactured by Technique Center of Radioimmunity of Navy in Beijing, P. R. China. The sensitivities of these assay kits were 0.2 ng/ml for CRF, 5 pg/ml for ACTH, 0.25 ng/ml for tT_3 and 3 ng/ml for tT_4, the intra- and inter-assay coefficients of variation were less than 8% and 12% for CRF, less than 6% and 12% for ACTH, less than 10% and 15% for tT_3, less than 10% and 15% for tT_4. The RIA procedure was performed as described by the kit's manufacturer, respectively.

2.7. Data analysis

Values were presented as mean ± standard error of the mean (SEM) for the indicated analyses. Sucrose intake data were analyzed by multiple analyses of variance (ANOVA) with drug treatments in stress and unstressed groups as between-subjects factors and sucrose test week as within-subject factor. Data for biochemical parameters also used ANOVA with drug treatment in stressed and unstressed groups as between-subjects factors. In all analysis the LSD test or Dunnett's T3 test was used for post-

Table 1
Effect of icariin and fluoxetine on sucrose intake in control, non-stressed rats

Group	Dose (mg/kg)	Relative sucrose intake (g/kg body weight) at drug treatment time (week)					
		0	1	2	3	4	5
Control rats							
Vehicle	–	36.72±1.69	37.30±3.31	36.52±3.17	36.59±3.73	36.86±3.33	34.91±2.98
Icariin	15	37.76±4.44	37.26±3.65	38.24±3.02	39.67±2.60	38.30±3.11	38.18±3.64
	30	37.94±2.64	38.38±3.04	39.75±3.54	40.55±4.62	38.63±3.91	39.73±2.31
	60	36.64±3.22	35.41±2.81	36.03±1.89	38.57±3.79	36.62±4.01	37.45±3.83
Fluoxetine	10	37.93±2.72	40.85±3.36	36.58±2.57	39.63±1.39	38.54±3.20	37.29±2.96

Mean intake SEM in 1-h sucrose tests is shown for weekly test sessions ($n=8$).

hoc comparisons of means. P-values lower than 0.05 were considered to be statistically significant.

3. Results

3.1. Sucrose intake

The effects of the CMS regimen on intake were shown in Fig. 2. Before stress, intake values for control and stressed groups were 35.33 and 34.70 g/kg body weight, respectively. Comparisons of data obtained in stressed and non-stressed animals revealed a significant stress effect [week 2: $F(1, 78)=4.446$, $P=0.038$; week 3: $F(1, 78)=22.280$, $P=0.000$; week 4: $F(1, 78)=26.321$, $P=0.000$; week 5: $F(1,78)=27.918$, $P=0.000$] from week 2 to week 5 of the experiment. In stressed animals, intake progressively decreased over a period of about 2 weeks of CMS and then remained consistently low throughout the rest stress period. At week 5 of stress regimen, intake values for control and stressed animals were 37.40 and 28.30 g/kg body weight, respectively, resulting in a significant Group (control/stressed) effect [$F(1, 78)=33.256$, $P<0.001$]. Such a difference between control and stressed animals treated with vehicle, persisted for the remainder of the 5-week treatment period.

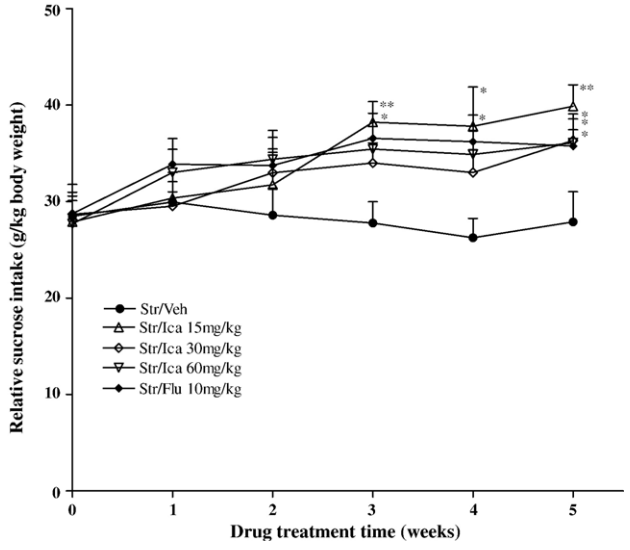

Fig. 3. Effect of icariin and fluoxetine on CMS-induced hedonic deficits measured as reduced intake of sucrose solution. Mean intake ±SEM in 1-hour sucrose tests is shown for weekly test sessions (n=8). *$P<0.05$, **$P<0.01$, compared to vehicle-treated stressed animals at the same time point.

In unstressed animals, the sucrose intake was not changed by treatments of three doses of icariin [$F(3, 168)=0.904$, $P=0.853$] or by treatment of fluoxetine [$F(1, 84)=1.375$, $P=0.244$] (Table 1).

In stressed animals, icariin caused a gradual increase of sucrose intake, resulting in a significant effect of icariin treatment [$F(3, 28)=3.438$, $P=0.030$] and Week [$F(4, 112)=2.548$, $P=0.044$] (Fig. 3). Compared to week 0 scores (the last scores before drug treatment), increase in sucrose intake was apparent during the first 2 weeks of treatment, and reached statistical significance at week 3 in stressed animals receiving 15 mg/kg of icariin [$F(1, 14)=11.070$, $P=0.005$]. This increase effect was maintained, and at week 5 the sucrose intake by these animals was comparable to that of vehicle-treated controls, and significantly higher than that of vehicle-treated stressed animals [$F(1, 14)=9.848$, $P=0.007$]. For the 30 and 60 mg/kg icariin-treated stressed groups, some significant effects appeared after 5 weeks of drug treatment [30 mg/kg: $F(1, 14)=5.107$, $P=0.041$; 60 mg/kg: $F(1, 14)=5.028$, $P=0.043$]. Compared to week 0 scores, the sucrose intake in fluoxetine-treated stressed animals was significantly increased after 3 weeks of treatment [$F(1, 14)=5.208$, $P=0.039$] and after 5 weeks of treatment there were no significant differences between drug-treated stressed and vehicle-treated control animals [$F(1, 14)=0.202$, $P=0.660$].

3.2. Body weight

Table 2 showed that at the end of the treatment period the vehicle-treated stressed animals were smaller in body weight than the vehicle-treated control animals but this difference was not significant [$F(1, 14)=3.428$, $P=0.085$]. As compared to vehicle-treated groups, body weights of control and stressed animals were not significantly affected by icariin [Control: $F(3, 28)=0.229$, $P=0.876$; Stressed: $F(3, 28)=0.229$, $P=0.841$]

Table 2
Body weight (g) measured at the end of drug administration ($n=8$)

Group		Unstressed	Stressed
Vehicle	–	514.4±9.2	482.8±14.3
Icariin	15	496.5±20.0	481.6±8.3
	30	506.9±15.2	487.8±17.9
	60	500.1±19.3	497.5±12.6
Fluoxetine	10	496.5±14.4	479.5±18.9

while fluoxetine caused a slight but insignificant decrease in stressed animals [$F(1, 14)=1.091$, $P=0.314$].

3.3. Neurohormonal concentrations

3.3.1. CRF

Following exposure to the CMS procedure, CRF concentrations were markedly increased compared with vehicle-treated control rats in dissected brain regions and serum [cortex: $F(1, 14)=20.158$, $P<0.001$; hippocampus: $F(1, 14)=20.158$, $P<0.001$; corpus striatum: $F(1, 14)=8.032$, $P=0.013$; medulla oblongata: $F(1, 14)=21.041$, $P<0.001$; hypothalamus: $F(1, 14)=29.820$, $P<0.001$; serum: $F(1, 14)=11.648$, $P=0.004$]. The effects were reduced by treatment with icariin or fluoxetine in animals subjected to the CMS procedure (Fig. 4).

3.3.1.1. Cortex. Icariin-treated stressed subjects showed a significant decrease in CRF [$F(3, 28)=25.341$, $P<0.001$]. Icariin treatment at 60 mg/kg decreased CRF concentrations below that of the unstressed control. However, icariin-treated control subjects showed a significant increase in CRF [$F(3, 28)=3.303$, $P=0.035$]. Icariin at 30 and 60 mg/kg significantly increased CRF compared to the vehicle control group. Fluoxetine treatment showed a significant decrease in CRF in stressed animals [$F(1, 14)=12.019$, $P=0.004$ *vs.* vehicle-treated stressed animals], but no change in control animals [$F(1, 14)=1.942$, $P=0.185$ *vs.* vehicle-treated control animals] (Fig. 4a).

3.3.1.2. Hippocampus. Icariin-treated stressed subjects showed a significant decrease in CRF compared with vehicle-treated stressed animals [$F(3, 28)=6.942$, $P<0.001$]. Control animals only receiving 30 mg/kg dose of icariin significantly elevated CRF [$F(1, 14)=5.870$, $P=0.030$]. Fluoxetine elicited to decrease CRF in stressed animals [$F(1, 14)=9.817$, $P=0.007$] but failed to affect the control rats [$F(1, 14)=1.429$, $P=0.252$] (Fig. 4b).

3.3.1.3. Corpus striatum. Icariin-treated stressed subjects showed a significant decrease in CRF [$F(3, 28)=13.865$, $P<0.001$]. A slight positive dose-dependent decrease in CRF was observed in the icariin-treated stressed animals. Icariin treatment at 60 mg/kg reduced CRF below that of the control.

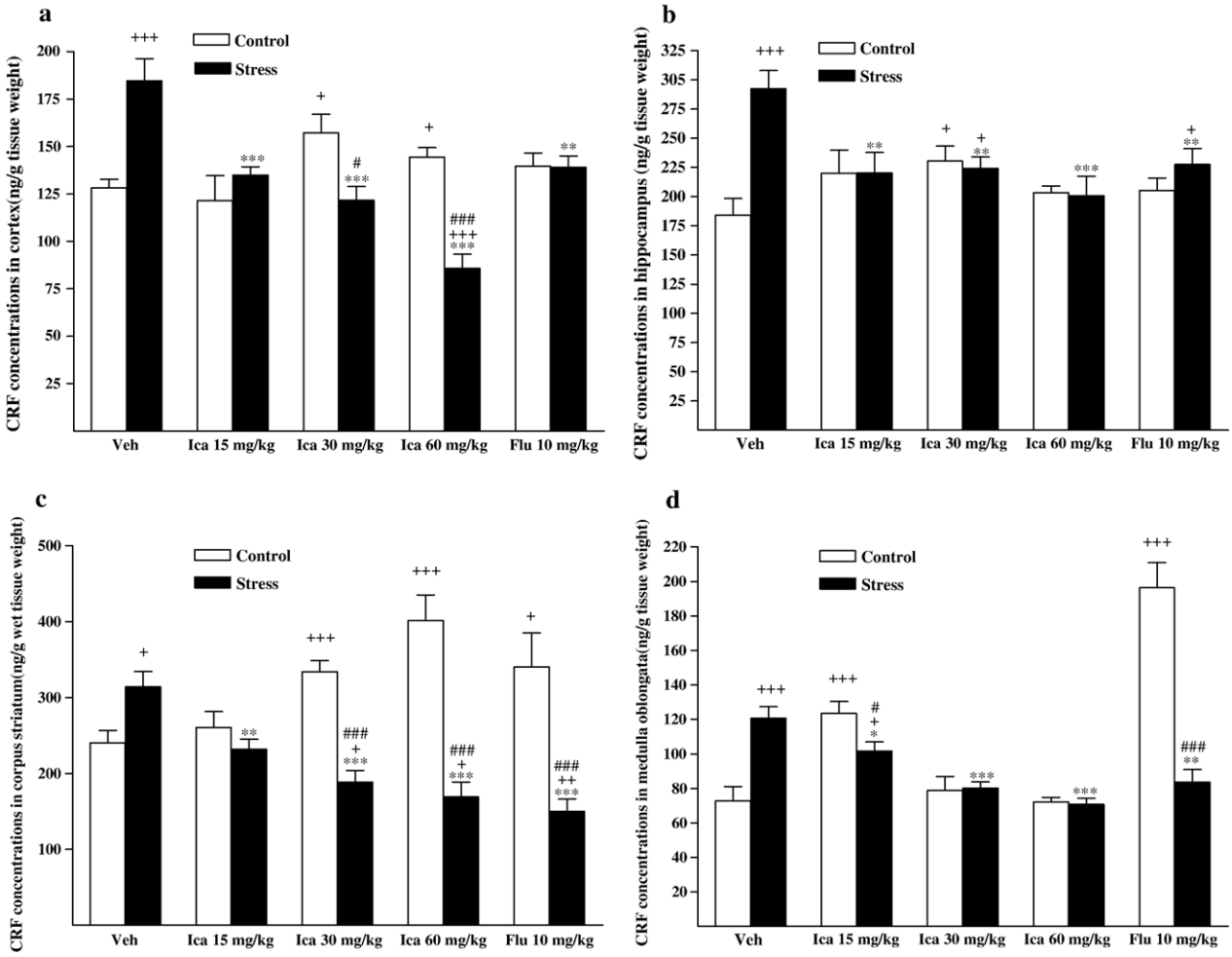

Fig. 4. Effects of icariin (Ica, 15, 30 and 60 mg/kg) and fluoxetine (Flu, 10 mg/kg) on CRF concentrations of dissected brain regions and serum in unstressed and CMS-treated rats. a) In cortex, b) in hippocampus, c) in corpus striatum, d) in medulla oblongata, e) in hypothalamus, f) in serum. Values are means ±SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared to vehicle-treated stressed animals; $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$, compared to vehicle-treated control animals. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared to appropriate drug-treated control animals.

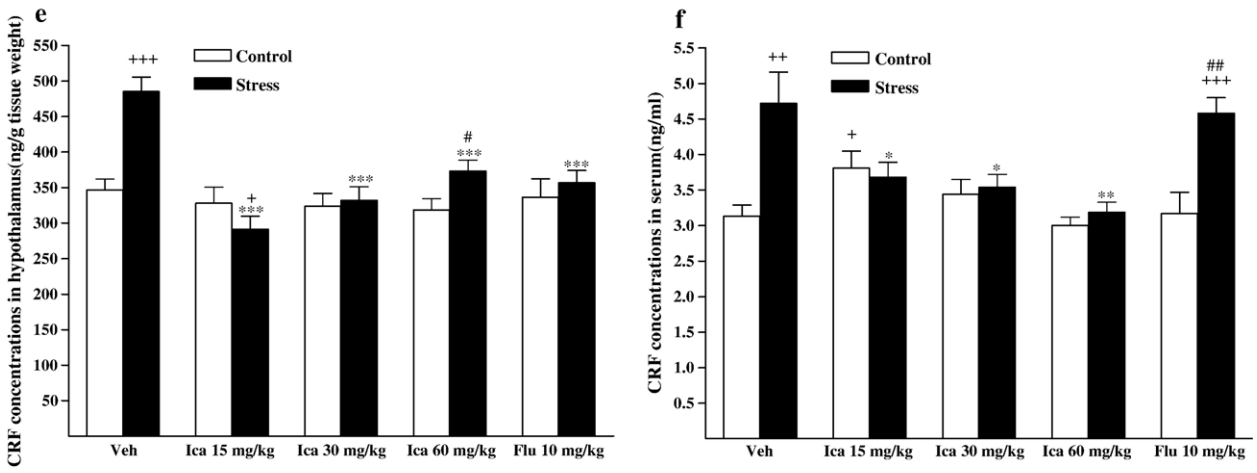

Fig. 4 (*continued*).

However, icariin-treated control subjects showed a significant increase in CRF [$F(3, 28)=10.607$, $P<0.001$]. The positive dose-dependent increase was also observed in the icariin-treated control animals. Fluoxetine-treated stressed rats revealed a decrease in the concentrations below the vehicle-treated stressed rats [$F(1, 14)=40.036$, $P<0.001$]. Fluoxetine treatment induced CRF rise in control rats [$F(1, 14)=4.393$, $P=0.055$] (Fig. 4c).

3.3.1.4. Medulla oblongata. Icariin-treated stressed subjects showed a significant decrease in CRF [$F(3, 28)=20.516$, $P<0.001$]. Stressed animals receiving 30 and 60 mg/kg of icariin could reverse CMS-induced CRF increase to the normal. However, icariin-treated unstressed subjects showed a significant increase in CRF [$F(3, 28)=13.011$, $P<0.001$]. The unstressed animals only receiving a dose of 15 mg/kg icariin significantly increased the CRF compared with the vehicle control group. Fluoxetine treatment significantly decreased the concentrations in stressed rats [$F(1, 14)=14.201$, $P=0.002$]. In contrast, it remarkably elevated CRF in control animals [$F(1, 14)=54.348$, $P<0.001$] (Fig. 4d).

3.3.1.5. Hypothalamus. Icariin-treated stressed subjects showed a significant decrease in CRF compared with vehicle-treated stressed animals [$F(3, 28)=20.665$, $P<0.001$]. Icariin treatment at the lowest dose decreased CRF below the unstressed control. There was no statistical significance of icariin treatment on CRF in unstressed animal. Fluoxetine significantly reduced the concentrations in stressed animals [$F(1, 14)=23.256$, $P<0.001$] but did not alter that in control animals [$F(1, 14)=0.112$, $P=0.742$] (Fig. 4e).

3.3.1.6. Serum. Icariin-treated stressed subjects showed a significant decrease in CRF [$F(3, 28)=6.047$, $P=0.003$]. In unstressed animals icariin merely at 15 mg/kg significantly increased CRF compared to vehicle-treated control [$F(1, 14)=5.675$, $P=0.032$] (Fig. 4f). However, fluoxetine failed to be

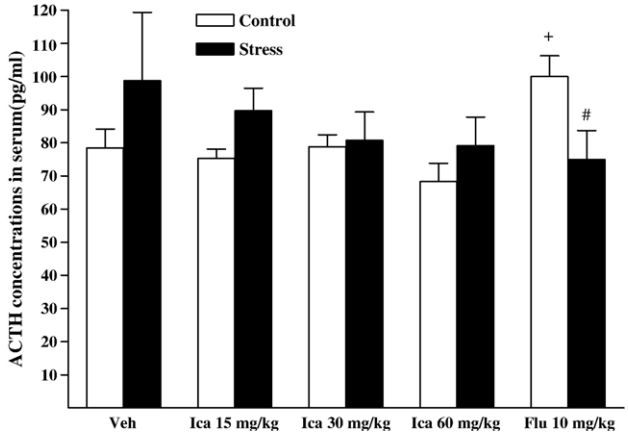

Fig. 5. Effects of icariin (Ica, 15, 30 and 60 mg/kg) and fluoxetine (Flu, 10 mg/kg) on ACTH concentrations in serum in unstressed and CMS-treated rats. Values are means ±SEM. $^{+}P<0.05$, compared to vehicle-treated unstressed control. $^{\#}P<0.05$, compared to appropriate drug-treated control animals.

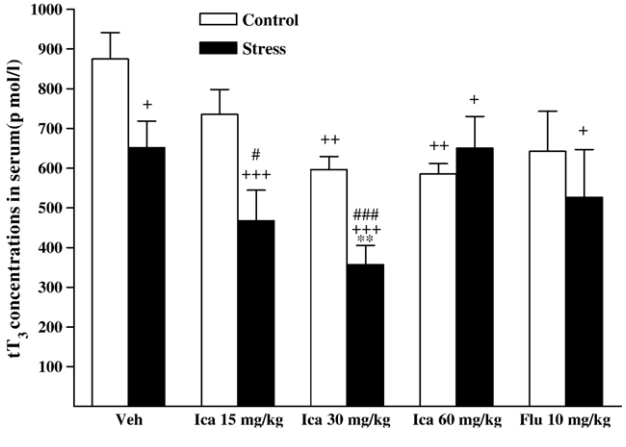

Fig. 6. Effects of icariin (Ica, 15, 30 and 60 mg/kg) and fluoxetine (Flu, 10 mg/kg) on tT3 concentrations in serum in unstressed and CMS-treated rats. Values are means ±SEM. $^{**}P<0.01$, compared to vehicle-treated stressed control. $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$, compared to vehicle-treated unstressed control. $^{\#}P<0.05$, $^{\#\#\#}P<0.001$, compared to appropriate drug-treated control animals.

effect both in stressed and unstressed animals compared to the corresponding vehicle groups.

3.3.2. ACTH. No stress effect was observed in serum ACTH concentrations ($F(1, 14)=0.899$, $P=0.359$). There was no significant interaction between icariin treatment and group [$F(3, 56)=0.334$, $P=0.801$). However, fluoxetine produced a significant increase in ACTH in unstressed groups but no change in stressed animals compared to the corresponding vehicle groups (Fig. 5).

3.3.3. tT_3

As shown in Fig. 6, rats subjected to the CMS procedure, showed a statistically significant decrease in serum tT_3 compared to the unstressed vehicle groups [$F(1, 14)=5.573$, $P=0.033$]. The significant interaction between icariin treatment and group was observed [$F(3, 56)=3.302$, $P=0.027$]. Icariin-treated stressed subjects revealed a significant reduction in serum tT_3 concentrations [$F(3, 28)=4.401$, $P=0.012$]. Icariin treatment at 30 mg/kg decreased tT_3 below the vehicle-treated stressed control [$F(1, 14)=12.954$, $P=0.003$]. Additionally, serum tT_3 in stressed animals receiving icariin at all doses tested were significantly lower than those of vehicle-treated controls [15 mg/kg: $F(1, 14)=16.018$, $P<0.001$; 30 mg/kg: $F(1, 14)=40.973$, $P<0.001$; 60 mg/kg: $F(1, 14)=4.667$, $P=0.049$].

Icariin-treated unstressed subjects revealed a significant reduction in serum tT_3 concentrations. The unstressed animals receiving icariin treatment at 30 and 60 mg/kg significantly reduced tT_3 concentrations compared to the vehicle control group. It was noted that there was no significant difference between control and stressed animals receiving a 60 mg/kg dose of icariin. Fluoxetine showed a slight decrease in both stressed and unstressed groups compared to the corresponding vehicle groups (Fig. 6).

3.3.4. tT_4

Serum tT_4 concentrations (data not shown) did not show any differences between stressed and unstressed control groups [$F(1, 14)=0.110$, $P=0.745$]. Serum tT_4 remained unaffected by icariin treatments both in stressed [$F(3, 28)=0.447$, $P=0.721$] and unstressed [$F(3, 28)=1.246$, $P=0.312$]. Fluoxetine also exhibited no effect (data not shown).

4. Discussion

The present study was focused on the changes that CMS model induced on a specific hedonic deficit, the HPA axis and HPT axis. In addition, the influences of icariin and fluoxetine treatments on CMS-related effects were examined.

Exposure to 5 weeks of CMS produced a gradual decline in sucrose intake per unit body weight, relative to baseline sucrose intake and intake of the respective unstressed control group. The present result on sucrose consumption was in agreement to the findings of similar studies (Willner et al., 1996; Li et al., 2003; Stathis et al., 2005; Gronli et al., 2005a,b). Noticeably, the sucrose consumption test is usually conducted by presenting the animals of a choice of solutions to drink during the test session–both water and the sucrose–and then the preference to chose sucrose over water is quantified. Simple tests of sucrose drinking may not be a valid measure of hedonic state when exposure to CMS in animals. Although water intake was not measured in the present study, some of recent studies have demonstrated that water intake was unaffected by the CMS procedure (Grippo et al., 2002, 2004, 2005b). In addition, neither CMS nor fluoxetine treatment affected water intake after 4 weeks of CMS (Grippo et al., 2006), suggesting that the changes in sucrose consumption might not be accompanied by nonspecific changes in ingestive behavior, such as no change in water intake.

Chronic administration of icariin in Wistar rats treated with CMS procedure, elicited a significant rise in sucrose intake with a time–effect course. 3–5 weeks of icariin treatment was required to reverse the deficit in sucrose consumption of CMS-treated rats. However, no significant changes were observed in control animals treated with icariin. To our knowledge, this is the first study that confirms antidepressant-like activity of icariin in the CMS rats. A recently published study demonstrated that icariin treatment reduced immobility in the FST and TST in mice (Pan et al., 2005). These behavioral data might confirm previous clinical reports that *E. brevicornum* extracts improved depression symptoms after stroke (Lai, 2001; Ma, 2003).

CMS induced important changes in the HPA axis (Azpiroz et al., 1999; Bratt et al., 2001; Kioukia-Fougia et al., 2002; Grippo et al., 2005a,b). In the present study, the observation of the significant CRF increases in dissected brain regions of the CMS-treated animals compared with the controls was consistent with the hypothesis that brain CRF was up-regulated in major depression (Nemeroff, 2000). Therefore the central CRF systems in particular might have a role in the response of rats to the CMS procedure. The abnormalities in the HPA axis of CMS-treated rats might relate to a hypersecretion of central CRF. In addition, increased CRF concentrations in serum were found in the CMS-treated rats, which agreed with previous studies (Widerlow et al., 1986; Catalan et al., 1998; Galard et al., 2002) that reported increased plasma CRF concentrations in depressed disorders, but differed considerably from another work, which showed unchanged neuropeptide concentrations (Charlton et al., 1986). Although generally thought of as a brain CRF regulating pituitary–adrenal function, CRF immunoreactivity has been observed in a number of peripheral tissues (Owens and Nemeroff, 1991). The hypothalamic or peripheral origin of plasma CRF concentrations is controversial. Since CRF reaches the pituitary through the hypothalamo–hypophyseal portal circulation, alterations in PVN-CRF neural activity might be affected in the peripheral circulation. CRF immunoreactivity has also been observed in peripheral plasma of patients with HPA disorders (Suda et al., 1987; Cunnah et al., 1987; Ellis et al., 1990). At present, serum CRF levels increased in parallel to the CRF hypersecretion of hypothalamic and other dissected brain regions in response to the CMS, suggesting that peripheral CRF might be secreted mainly co–by the hypothalamus and extrahypothalamus sources. On the other hand, CRF is the most potent ACTH secretagogue. However, in the present study, the CMS procedure rendered no relative differences on serum ACTH concentrations in Wistar rats, which was in line with ACTH concentrations found in Sprague–Dawley rats

exposed to CMS (Grippo et al., 2005b). ACTH did not correlate significantly with CRF in CMS-treated rats. This dissociation between CRF and ACTH observed by us and other authors (Catalan et al., 1998; Pignatelli et al., 2000; Kioukia-Fougia et al., 2002) did not rule out a hypothalamic origin for peripheral CRF concentrations. In this study, the origin of serum CRF was a confounding factor in these determinations, hence peripheral concentrations of CRF were difficult to interpret.

Normalization of the HPA axis system was hypothesized to play an important role for mediating the antidepressant activity in patients (Bschor et al., 2003; Nickel et al., 2003; Nikisch et al., 2005). In this study, we tested the hypothesis that icariin administration would modulate CRF in brain regions of rats. Our data elicited that icariin was capable of attenuating the CMS-induced increases in CRF of different brain regions in rats, suggesting central CRF system in stressed animals was sensitive to icariin treatment. The normalization of elevated serum CRF concentrations in stressed animals treated with icariin was also observed. In addition, icariin administration did not affect serum ACTH concentrations in stressed rats. Although measurement of CRF concentrations alone is insufficient to determine whether changes in synthesis, release or storage or degradation, are responsible, differences between treatment groups clearly represent alterations in brain CRF system in function of icariin. The fact that CRF changes accompanied with icariin-induced changes in behavior during the CMS procedure was interesting. Taken together the above studies indicated that CRF appeared to play a role in the interaction of CMS with icariin response, and activation of brain CRF might be involved in the behavioral cross-sensitization between CMS and icariin.

Fluoxetine reduced CRF in dissected brain regions of the CMS-treated rats without changing the serum CRF concentrations. There were no effects of fluoxetine administration on basal, median eminence CRF concentration, on depletion of the peptide after chronic stress (Stout et al., 2002). It was plausible that a blunted effect of stress on CRF might contribute to normalization of the HPA axis activity by fluoxetine observed in the CMS-treated rats.

In unstressed rats, icariin increased CRF both in serum and brain except in hypothalamus, however, these effects were partly dependent on icariin doses tested. Fluoxetine also produced a remarkably elevation of CRF in corpus striatum and medulla oblongata, ACTH concentrations in serum, which were partly consistent with an early report that fluoxetine caused a significant increase in plasma ACTH concentrations as well as in CRF concentrations of hypophysial portal plasma (Gibbs and Vale, 1983).

In the present study, we also sought to characterize stress regulation of the HPT axis. We firstly demonstrated that 5 weeks of exposure to the CMS procedure caused a significant decrease in serum tT_3 concentrations, without a significant change in tT_4 concentrations in Wistar rats. These findings partly supported previous studies' findings that chronic stress to mice (Cremaschi et al., 2000; Silberman et al., 2002), inescapable foot-shock stress to Sprague–Dawley rats caused a decrease in peripheral thyroid hormone concentrations (Helmreich et al., 2005). It is well known that brain CRF systems play a role in mediating the neuroendocrine. Thyroid T_3 contents were significantly decreased, but T_4 contents were not modified by CRF treatment (De Pedro et al., 1995), suggesting that CRF suppressed thyroid functions (Tsigos and Chrousos, 2002). Therefore we speculated that the brain CRF might be more relevant for regulation of thyroid hormones and play a role on thyroid gland activity in the CMS rats. Further evidence for regulation of the HPT axis during CMS comes from studies demonstrating that gluococorticoids (corticosterone in rats) exert negative effects on the HPT axis (Morley, 1981). Stress-induced corticosterone suppressed the peripheral conversion of T_4 to T_3, resulting in serum T_3 decrease (Bianco et al., 1987). Although corticosterone was not measured in the present study, most previous studies have demonstrated that CMS caused an increase in plasma corticosterone concentrations (Ayensu et al., 1995; Konkle et al., 2003; Grippo et al., 2005a,b), indicating that product of the HPA axis might induce serum tT_3 decrease observed in stressed animals. The negative correlation of tT_3 to CRF in the CMS model of rats in this study is new. The observed pattern of alterations in these measures of the HPA axis and the HPT axis activities suggested that there were close interrelations between the HPA and the HPT axes, which were possibly affected during the CMS procedure. This area requires further investigation.

In the present study, the failure of icariin to reverse CMS effects on tT_3 was observed. Icariin showed a reduction in serum tT_3 contents without any changes in tT_4, indicating that there was not a clearly closed interaction of icariin treatment with thyroid function in the CMS rats. In an early study, small decreases in plasma T_3 occurred after antidepressant desipramine administration for 14 days in rats (Atterwill et al., 1989). It was of considerable interest to notice that icariin altered serum tT_3 concentrations in unstressed and stressed animals in different pattern. Thus, there may appear to be more complex in the thyroid hormone response to the HPA axis. As mentioned above, CRF suppressed the T_3 release, we questioned whether there was a negative correlation between CRF and tT_3 in unstressed and stressed animals treated with different dosages of icariin. In unstressed animals, significant increases in CRF in cortex, hippocampus and corpus striatum induced by 30 mg/kg icariin treatment, or in cortex and corpus striatum induced by 60 mg/kg icariin treatment have been tightly correlated to a significant decrease in serum tT_3 concentrations, respectively. In stressed animals, 15 mg/kg icariin-induced decreased CRF concentrations in hypothalamus seemed to be slightly lower than that induced by 30 mg/kg icariin treatment, while 15 mg/kg icariin-induced tT_3 concentrations was higher than that induced by 30 mg/kg icariin treatment. Also in stressed animals, the significant decreased CRF concentrations (below the normal values) in cortex and corpus striatum induced by 60 mg/kg icariin treatment might enhance the ability of the same dose of icariin to block CMS-induced tT_3 decrease, resulting in no significant reduction in T_3 concentrations, which was in contrast to other findings in 15 and 30 mg/kg icariin-treated groups, where similar reductions in tT_3 and CRF in cortex and corpus striatum were simultaneously found. These observations indicated that the negative correlation between changes in some brain region CRF and serum tT_3 concentrations appeared to be

dependent on the condition of the animals and icariin dosages. More specifically, CRF in cortex and corpus striatum may be an important factor regulating serum tT_3 concentrations both in unstressed and stressed animals treated with 60 mg/kg icariin. However, in some icariin-treated animals, there was no significant correlation between brain CRF and serum tT_3. As an example, in control animals treated with 15 mg/kg icariin, CRF in cortex, hippocampus and corpus striatum may not reflect serum T_3 concentrations, for no change in CRF or tT_3 was observed. These findings indicated that the neuroendocrinological effects of icariin might depend on the drug used, the duration of administration and methodological criteria.

Serum tT_4 concentrations were unaffected in the stressed and unstressed animals after fluoxetine treatment in this study. However, fluoxetine showed a slight decrease both in stressed and unstressed animals. Early report showed that acute administration of fluoxetine (2 and 10 mg/kg ip) to normal rats elicited a decrease only in T_4 concentrations, whereas chronic administration resulted in a decrease in both T_3 and T_4 concentrations in rats (Golstein et al., 1983). Recently, it was found that serum T_4 but not T_3 concentrations were significantly lowered in normal rats after administration of fluoxetine at 15 mg/kg for 14 day (Eravci et al., 2000). The impact between unstressed and stressed animals treated with fluoxetine was also observed when examining the relationship between serum CRF and tT_3. Although in unstressed animals, fluoxetine treatment showed no significant changes in serum CRF and tT_3 concentrations, higher concentrations of CRF associated with lower concentrations of tT_3 were observed in stressed animals, suggesting that there might be in concordance with negative regulation of the HPA and the HPT axes in fluoxetine-treated stressed animals.

In summary, we have found that a chronic mild stress procedure generating an anhedonic state in rats resulted in increased CRF concentrations in dissected brain regions and serum, decreased tT_3 with no significant changes in serum ACTH and tT_4. This CMS provided a model to explore mechanisms of the HPA and HPT axes-mediated events relevant to human depression. Icariin reversed CMS-induced sucrose intake reduction and CRF elevation. These results suggested that the behavioral profile of icariin acted predominantly on the HPA axis system. In addition, the tT_3 changes in stressed and unstressed animals indicated a complex response to icariin treatment. Further preclinical and clinical experiments are required to clarify the role of icariin in contributing to treatment of depressive disorders.

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