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The antidepressant effect of ethanol extract of radix puerariae in mice exposed to cerebral ischemia reperfusion

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Abstract

In our pilot study, the depressive-like behaviors of mice exposed to cerebral ischemia reperfusion (CIR) were observed and the antidepressant effects of radix puerariae (RP; root of the *Pueraria* plant) extract in CIR mice were assessed because it was speculated that the neuronal damage caused by CIR played an important role in the development of poststroke depression (a common and severe complication after stroke) and the RP extract was reported to exhibit effect of neuronal protection from cerebral ischemia damage. Our studies above indicated that the RP extract markedly shortened the increased immobility time induced by CIR of male mice in the forced swimming test (FST) and tail suspension test (TST), indicating a possible antidepressant activity. Thus, the aim of the present study was to confirm the putative antidepressant effect of RP extract (75, 150, and 300 mg/kg, administered orally 24 h after the CIR) on reserpine-induced symptoms. To get further insight into the mode of antidepressant action of RP extract, biochemical examination was conducted concomitantly to examine possible involvement of the brain monoamine systems in the behavioral syndromes observed. In CIR mice, pronounced low levels of norepinephrine (NE) and 4-dihydroxyphenylacetic acid (DOPAC, a metabolite of dopamine) in the hippocampus or striatum were detected, which were reversed by RP extract, whereas no significant change of serotonin (5-HT) was detected in either CIR or RP extract-treated mice. The data suggested that the disturbance of NE and DA systems in hippocampus and striatum played more important roles in the development of depressive-like behavior of CIR mice than 5-HT system did, and RP extract ameliorated the abnormal symptoms caused by CIR, which may throw new lights on the treatment of poststroke depression.

Keywords: *Pueraria lobata*; Antidepressant activity; Cerebral ischemia reperfusion (CIR); Reserpine; Monoamine

1. Introduction

Pueraria lobata (Willd.) Ohwi is one of the earliest medicinal plants to be used in China. Radix puerariae (RP; root of the *Pueraria* plant) was first described in the *Chinese Materia Medica, Shen Nong Ben Cao Jing* (Anon., ca. 200 B.C.) and was used as an antipyretic, antidiarrhetic, diaphoretic, and antiemetic agent—a general antimicrobial agent in today's parlance. Hundreds of years later, medications based on RP were found useful in the treatment of alcohol-related problems, first as an amethystic (anti-intoxication) agent (Sun Simiao, ca. A.D. 600) and later as an antidipsotropic (antidrinking) agent (Li, Dongyuan, ca. A.D. 1200) (Duke and Ayensu, 1985). Recently, numerous investigations indicated that the extract of RP significantly ameliorated the microcirculation and protected the neurons from the damage of cerebral ischemia (Lai and Tang, 1989; Wang et al., 1997). Furthermore, scientists found that the extract of RP could influence β-adrenoreceptor (Lu et al., 1980) and the concentrations of monoamines (Zeng and Zhang, 1979). Since it was speculated that neuronal damage and altered concentrations of monoamines caused by cerebral ischemia reperfusion (CIR) play important roles in the development of poststroke depression (Francisco, 1993; Andersen et al., 1994; Gustafson et al., 1995), we assessed the antidepressant effect of RP extract in our pilot study and observed that the RP extract markedly shortened the increased immobility time induced by CIR of male mice in the

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forced swimming (FST) and tail suspension test (TST) in a dose range of 75–300 mg/kg, indicating a possible antidepressant activity. This effect was comparable to that of the tricyclic antidepressant clomipramine (17.5 mg/kg). Neither clomipramine nor RP extract in various doses produced any overt behavioral change or motor dysfunction in the open-field test confirming the assumption that the antidepressant effect of an RP extract in the FST and TST was specific. Based on the results, we assessed the antagonism of reserpine-induced symptoms (another animal behavior experiment for depression study) in mice to get further insight into the mode of antidepressant action of the RP extract.

In addition, although central norepinephrine (NE), dopamine (DA), and serotonin (5-HT) were suspected to play important roles in the etiology of poststroke depression, the extent of involvement of these neurotransmitters was not clearly understood nor was it known whether these amines were causally or secondarily related to the disorder (Delgado, 2000). Accordingly, the therapeutic effects of antidepressants were believed to be caused by central monoaminergic systems and in vivo studies examining the mechanism of antidepressant action of RP extract have not existed until now. Thus, in the present study, we reported on the effects of RP extract on CIR mice brain levels of NE, DA, and 5-HT and their metabolites after short-term treatment, measured by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD), to make initial study of the putative mechanism of action of RP extract.

2. Material and methods

2.1. Animals

Male ICR mice (18–22 g, Vital River Laboratory Animal Technology, Beijing, China) were housed in a 12-h light/dark cycle, with lights off at 18:00 h, at a constant temperature of 25 ± 1 °C and free access to food and tap water. Mice were randomly assigned to the various experimental groups ($n = 12$/group) and weighed daily. The experimental procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Animals were tested in reserpine experiment or sacrificed by decapitation for neurochemical measurement between 15:00 and 16:00 h after the last administration of drugs on day 3 postsurgery.

2.2. Substances and drug administration

For all experiments, RP extract (drug/extract ratio: 16:1 prepared by Phytochemical Laboratory, Institute of Medicinal Plant, Chinese Academy of Medical Sciences and Peking Union Medical College) was used and the voucher specimen (020807) was preserved in our laboratory for future reference. Clomipramine and other drugs was purchased from commercial sources: Clomipramine (from Beijing Novartis Pharm), NE, 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), DA, 4-dihydroxyphenylacetic acid (DOPAC), 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), and dihydroxybenzylamine-hydrobromide (DHBA) (from Sigma, St. Louis, MO), L-octanesulfonate sodium (from Nacalai Tesque, Kyoto, Japan). Clomipramine and RP extract were dissolved in deionized water. Sham-operated mice and one group of mice subjected to CIR received deionized water only, other groups of CIR mice received clomipramine (17.5 mg/kg) and RP extract (75, 150, and 300 mg/kg), respectively. All substances were administered orally by gavage in a final volume of 20 ml/kg body weight on days 2 and 3 postsurgery (twice per day).

2.2.1. Preparation of radix puerariae extract

RP (100 g) were refluxed for 1 h in aqueous ethanol (80% v/v, 60 ml) two times and the combined alcoholic extractive was evaporated to dryness (26 g). The extract was dissolved in hot water (400 ml), adjusted to pH 5.0 with sulfuric acid, and filtered. The filtrate was chromatographed on macroreticular resin D-101 (3.6 cm id × 18 cm) and eluted with water (400 ml) and then aqueous ethanol (70% v/v, 400 ml). We performed the experiential extract procedure to obtain a higher purity for the ingredient (puerarin) and to reduce the nonaction ingredients, such as glucose, protein, inorganic salt, etc. The aqueous ethanol fraction was collected and evaporated to dryness to obtain RP extract (6.4 g). The extract was standardized on an amount of 3.2% puerarin. HPLC analytical conditions were as follows: column: ZORBAX SB-C18 reversed-phase column, 46 mm id × 150 mm, detector at 252 nm, mobile phase: water/methanol (78:22, v/v), flow rate: 1.0 ml/min; equipment: Waters 515 with Waters 996 Photodiode Array (PDA) detector (Milford, MA). Puerarin appeared at 7.5 min. Authentic puerarin was purchased from National Chemicals and Biological Products Institute, with batch number: 0703-9914 (Beijing).

2.3. Cerebral ischemia reperfusion

Anesthesia was induced using 10% ethyl carbonate CP. CIR was induced by the isolation of the common carotid arteries through a ventral midline incision in the neck followed by bilateral occlusion of the arteries using vascular clamps for 5 min and the clamps were removed for 10 min (reperfusion), then the arteries were occluded for 5 min again. At the end of the occlusion, the clamps were removed, the arteries were visually inspected for reflow, and the midline incision was sutured (Jingtao et al., 1999). Sham-operated animal underwent exactly the same procedure except there was no arterial occlusion. All animals were maintained at normothermia using an electric radiator until they were able to regulate their own temperature 15–30 min postsurgery. Day of the surgery was defined as day

0. The animals were housed in their cages until the tests on day 3 postsurgery.

2.4. Forced swimming test and tail suspension test

FST was similar to that described by Porsolt et al. (1977). Briefly, mice were individually placed in 10 cm of ambient temperature water (25 ± 2 °C) in 2000-ml glass beakers and were allowed to swim for 6 min, and the durations of immobility were recorded during the last 240 s of the test. Duration of immobility is defined as the absence of active, escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving. Testing was performed 1 h after the administration of the tested drugs on day 3 postsurgery, after which mice were removed from the tank, dried with a towel, and returned to their home cages. TST was similar to that described by Steru et al. (1985). Mice were suspended on the edge of a shelf 58 cm above a tabletop by adhesive tape, placed approximately 1 cm from the tip of the tail. They were allowed to hang for 6 min, and the duration of immobility was recorded during the last 240 s of the test. Mice were considered immobile only when they hung passively and completely motionless. Testing was performed 1 h after the administration of the test drugs on day 3 postsurgery.

2.5. Antagonism of reserpine-induced symptoms in mice

This test was performed with modifications of Bourin et al.'s (1983) procedure. Test drugs were orally administered to groups of 12 mice simultaneously with reserpine (5 mg/kg ip) between 10:00 and 11:00 h on day 3 postsurgery. Ptosis and akinesia were evaluated 2 h after reserpine treatment. For the evaluation of ptosis, animals were placed on a shelf (20 cm above the tabletop) and were judged, on an all-or-none basis, to have ptosis if their eyes are half closed or completely closed within 30 s. For the evaluation of akinesia, animals were placed at the center of a white paper circle (9.5 cm in diameter) and were judged, on an all-or-none basis, to be akinetic if they remained within the circle for 15 s or more. Then, the animals were returned to their cages and the mortalities were recorded within 7 days after reserpine treatment. Data were expressed as a percent of positive symptoms.

2.6. High-performance liquid chromatography/electrochemical detection determination of monoamine and their major metabolites in different brain regions

Mice were sacrificed by decapitation between 15:00 and 16:00 h, 1 h after the last administration of drugs on day 3 postsurgery. Brains were removed rapidly and dissected and the various regions weighed. Each dissected brain region was immediately homogenized (4 °C) with 0.5 ml of 0.2 M perchloric acid containing 0.05 M glutathione as antioxidant. The mixture was centrifuged at 12,000 × *g* (4 °C) for 20 min, the pellet was discarded. The resultant supernatant was filtered (0.22 mm Nylon, Roth, Karlsruhe), and 20 μl was directly injected into the HPLC for the determination of monoamine analysis. The amounts of monoamines and their metabolites in various brain tissues were quantitatively measured by HPLC with ECD. The system for determining neurochemistry consisted of an ESA Coulochem II detector equipped with a model 5014 analytical cell and a 600E pump (Waters). The ZORBAX SB-C18 (5 mm, 4.6 mm id × 150 mm, Agilent, Palo Alto, CA) was kept at room temperature. The chromatographic mobile phase consisted of 0.05 M phosphate monobasic, 0.05 mM EDTA, 0.4 mM octyl sodium sulfate, 8% v/v acetonitrile, and 6% v/v methanol, brought to pH 3.2 with phosphoric acid. Tissue levels were determined by means of the internal standard DHBA and expressed in terms of nanograms per gram of tissue.

2.7. Data analysis and statistics

Statistical comparison between groups of control and drug-treated mice was calculated by use of SPSS software. χ^2 test was used to analyze the data from the reserpine experiment and analysis of variance with Dunnett's test was used to analyze the data from neurochemical and behavioral (TST and FST) measurements. Statistical significance was set at $P < .05$.

3. Results

3.1. Forced swimming test and tail suspension test

The effects of RP extract on CIR mice in FST and TST were assessed. As demonstrated in Table 1, CIR increased the immobility time, while RP extract and clomipramine in tested doses significantly decreased the duration of immobility.

3.2. Antagonism of reserpine-induced symptoms in mice

The effects of RP extract on reserpine-induced symptoms were assessed. As demonstrated in Fig. 1, the symptoms,

Table 1
Effects of short-term (2 days) administration of clomipramine (Clom, 17.5 mg/kg) and RP extract (75, 150, and 300 mg/kg) on immobility time of mice in TST and FST

Group	Dose (mg/kg)	TST (s)	FST (s)
Sham		68.25 ± 12.71	98.83 ± 30.77
CIR		115.42 ± 48.36 **	132.33 ± 22.19 **
CIR + RP	75	70.58 ± 30.18 ##	94.17 ± 33.69 ##
	150	67.25 ± 25.67 ##	88.17 ± 25.02 ##
	300	51.08 ± 23.13 ##	73.50 ± 27.26 ##
CIR + Clom	17.5	52.92 ± 15.19 ##	81.08 ± 35.46 ##

Values were mean ± S.E.M. expressed as the time (in seconds) of 12 animals in each group. Data analysis was performed using Dunnett's test.
** $P < .01$ vs. sham.
$P < .01$ vs. CIR.

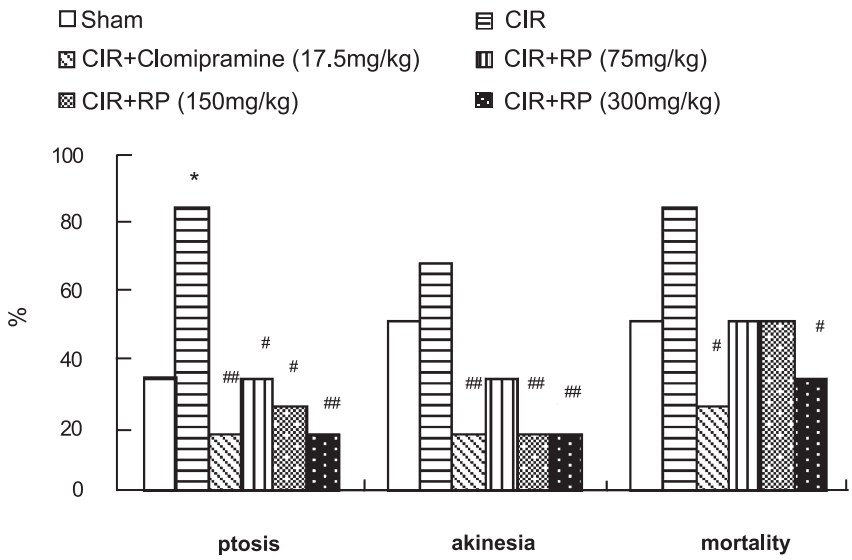

Fig. 1. Effects of clomipramine (17.5 mg/kg) and RP extract (75, 150, and 300 mg/kg) on antagonism of reserpine-induced symptoms in mice. Data were expressed as percent of positive symptoms of individual groups. Statistical differences were determined using χ^2 test. * $P<.05$ vs. sham, $^{\#}P<.05$, $^{\#\#}P<.01$ vs. CIR.

especially ptosis, were more severe in CIR mice than in sham mice, and RP extract (300 mg/kg) was as potent as clomipramine in the antagonism of any of the three symptoms (ptosis, akinesia, and mortality).

3.3. Effects of clomipramine and radix puerariae extract on norepinephrine, dopamine, serotonin, and their metabolites in mice brain tissues

The contents of monoamines in the hippocampus, striatum, and frontal cortex after short-term (2 days) treatment are shown in Tables 2–4. Compared with 5-HT system, NE and DA systems showed more significant differences between CIR and drugs-treated mice. For NE, there were significant differences between the groups in the hippocampus and striatum [$F(5,66)=3.53$, $P=.007$, and $F(5,66)=24.54$, $P<.001$, respectively], and both RP extract and clomipramine significantly increased NE levels in the brain regions above. For DA, there was no significant difference between the groups in the striatum [$F(5,66)=2.18$, $P=.067$], but there was a significant difference between the groups in the hippocampus [$F(5,66)=3.23$, $P=.011$] where RP extract (150 mg/kg and 300 mg/kg) and clomipramine (17.5 mg/kg) significantly increased DA levels. For MHPG, although a great significant difference between the groups in the hippocampus [$F(5,66)=5.61$, $P<.001$] was observed, only clomipramine showed the significant effect of increasing MHPG ($P<.001$). For DOPAC, there was a great significant difference between the groups in the hippocampus [$F(5,66)=8.56$, $P<.001$], where CIR decreased the level of DOPAC ($P<.01$) and RP extract (75 and 300 mg/kg) and clomipramine (17.5 mg/kg) significantly increased its level. No significant dif-

Table 2
Effects of short-term (2 days) administration of clomipramine (Clom, 17.5 mg/kg) and RP extract (75, 150, and 300 mg/kg) on NE, DA, 5-HT, MHPG, DOPAC, and 5-HIAA levels in the hippocampus of the mice

Group	Dose (mg/kg)	Hippocampus (ng/g)					
		NE	DA	5-HT	MHPG	DOPAC	5-HIAA
Sham		601 ± 31	215 ± 24	540 ± 136	13770 ± 2054	617 ± 96	1138 ± 182
CIR		525 ± 64[a]	190 ± 25[b]	509 ± 86	12031 ± 1876	499 ± 72**	1170 ± 143
CIR + RP	75	631 ± 104[#]	229 ± 44	578 ± 153	12926 ± 2751	598 ± 118[#]	1238 ± 246
	150	665 ± 120[##]	248 ± 89[#]	555 ± 143	12641 ± 2444	585 ± 106	1196 ± 200
	300	673 ± 157[##]	245 ± 38[#]	580 ± 70	13823 ± 1475	626 ± 96[##]	1294 ± 119
CIR + Clom	17.5	629 ± 68[#]	258 ± 43[##]	517 ± 65	16188 ± 2167[##]	755 ± 96[##]	1211 ± 90

Values were mean ± S.E.M. expressed as nanograms per gram of tissue of 12 animals in each group. Data analysis was performed using Dunnett's test.
[a] $P=.109$ vs. sham.
[b] $P=.113$ vs. sham.
** $P<.01$ vs. sham.
[#] $P<.05$ vs. CIR.
[##] $P<.01$ vs. CIR.

Table 3
Effects of short-term (2 days) administration of clomipramine (Clom, 17.5 mg/kg) and RP extract (75, 150, and 300 mg/kg) on NE, DA, 5-HT, MHPG, DOPAC, and 5-HIAA levels in the striatum of the mice

Group	Dose (mg/kg)	Striatum (ng/g)					
		NE	DA	5-HT	MHPG	DOPAC	5-HIAA
Sham		303 ± 52	14896 ± 2591	558 ± 72	12540 ± 3293	960 ± 203	967 ± 234
CIR		225 ± 93 *	13033 ± 1397	547 ± 93	12529 ± 1553	818 ± 75	936 ± 101
CIR + RP	75	296 ± 68	13209 ± 3629	537 ± 95	11120 ± 4596	825 ± 233	907 ± 255
	150	316 ± 72$^{\#}$	13866 ± 1884	504 ± 75	11249 ± 2487	933 ± 187	939 ± 185
	300	520 ± 113$^{\#\#}$	13602 ± 1045	527 ± 72	8619 ± 2195$^{\#}$	968 ± 236	963 ± 249
CIR + Clom	17.5	453 ± 56$^{\#\#}$	15676 ± 2962$^{\#}$	482 ± 101	11285 ± 2346	960 ± 139	888 ± 159

Values were mean ± S.E.M. expressed as nanograms per gram of tissue of 12 animals in each group. Data analysis was performed using Dunnett's test.
* $P < .05$ vs. sham.
$^{\#}$ $P < .05$ vs. CIR.
$^{\#\#}$ $P < .01$ vs. CIR.

ference between the groups in either 5-HT or 5-HIAA was observed.

4. Discussion

As mentioned earlier, RP extract markedly shortened the increased immobility time induced by CIR in the FST and TST, which are behavioral paradigms that are predictive of antidepressant activity in rodents (Porsolt et al., 1977; Steru et al., 1985), and produced no overt behavioral change or motor dysfunction in the open-field test, indicating a specific antidepressant activity. In the present study, we analyzed the effects of the tricyclic antidepressant, clomipramine (a relative selective serotonin reuptake inhibitor, SSRI), and the RP extract on brain monoamine concentrations and on the antagonism of reserpine-induced symptoms in CIR mice to get further insight into the mode of antidepressant action of the RP extract.

It is well known that reserpine irreversibly inhibits the vesicular uptake of catecholamines, ultimately resulting in the depletion of catecholamine stores, which can induce syndromes, such as ptosis and akinesia (Kandel, 1991), and it has been used in animal depression models to evaluate antidepressants activity. Further investigations reveal that such activity spectra of the drugs on the reserpine-induced symptoms may be attributed to their different effects on each brain monoaminergic system, as it is reported that ptosis antagonism is obtained by the stimulation of α-adrenergic or serotonergic receptors, akinesia antagonism by stimulation of dopaminergic receptors, and hypothermia (or mortality) antagonism by stimulation of β-adrenergic receptor (Bourin et al., 1983). Our data showed that the syndromes induced by reserpine in CIR mice were more severe than that observed in sham mice, indicating the possibility of disturbance in monoamine systems induced by CIR, while RP extract (300 mg/kg) was as potent as clomipramine (17.5 mg/kg) in antagonism of any of three symptoms, probably by the mechanism of receptors. These results supported the potential benefits of RP extract for poststroke depression and suggested a prospective relationship existing between the NE, DA, and/or 5-HT systems and the antidepressant effects of RP extract.

The major finding of this study was that both clomipramine and RP extract mainly affected concentrations of NE and DA, but not 5-HT in the hippocampus and striatum. The downtrend of NE level (P=.109 vs. sham mice) in the hippocampus and the significant decreased NE level observed in the striatum of CIR mice were in good correlation with our previous results. (Previous dynamic evaluations showed that the durations of immobility in FST and TST of CIR mice were significantly longer on days 3–6 than on other days postsurgery, and the neurochemical changes were most significant on days 3–4 postsurgery. This is why we performed the present

Table 4
Effects of short-term (2 days) administration of clomipramine (Clom, 17.5 mg/kg) and RP extract (75, 150, and 300 mg/kg) on NE, DA, 5-HT, MHPG, DOPAC, and 5-HIAA levels in the frontal cortex of the mice

Group	Dose (mg/kg)	Frontal cortex (ng/g)					
		NE	DA	5-HT	MHPG	DOPAC	5-HIAA
SHAM		248 ± 46	559 ± 24	391 ± 65	5714 ± 219	347 ± 97	446 ± 64
CIR		249 ± 44	559 ± 90	367 ± 96	5704 ± 379	326 ± 96	435 ± 59
CIR + RP	75	218 ± 36	521 ± 70	384 ± 82	5746 ± 759	317 ± 79	455 ± 76
	150	217 ± 54	527 ± 98	433 ± 97	5483 ± 561	315 ± 79	470 ± 64
	300	218 ± 71	568 ± 96	411 ± 82	5617 ± 428	319 ± 59	472 ± 61
CIR + Clom	17.5	232 ± 31	567 ± 59	408 ± 89	5540 ± 862	318 ± 80	443 ± 64

Values were mean ± S.E.M. expressed as nanograms per gram of tissue of 12 animals in each group. Data analysis was performed using Dunnett's test.

experiments on day 3 postsurgery.) Interestingly, RP extract produced similar changes in central NE concentrations as the tricyclic antidepressant, suggesting a similar mode of action, although for a long time, the most widely accepted theories of the biological basis of depression involve the dysfunction of either the NE or 5-HT neurotransmitter system. 5-HT has received far more attention than NE in recent years because of the availability of SSRIs. Scientists believe that the development of new compounds that have a selective action on the noradrenergic system has encouraged reconsideration of the role of NE in depression (Stuart, 1997). It was reported recently that the changes caused by some antidepressants in the brain were based partly either on the subsensitivity of presynaptic α_2-adrenergic receptors (Sacchetti et al., 2000), which control the release of NE from central neurons (Langer, 1981), or on the regulation of central β-adrenergic receptor (Sulser et al., 1978). Judging from the facts that reserpine led to the depletion of catecholamines (including NE) and RP extract increased the level of NE, the antidepressant effects of RP extract may be partly due to the influence on the function of adrenergic receptors and/or on the synthesis and metabolism of NE. The speculation needs to be validated by further experiments using antagonism of ptosis or hypothermia with noradrenergic activity.

Similarly, a significant decreased concentration of DOPAC in the hippocampus and the downtrend of DA level (P=.113 vs. sham mice) in the striatum were observed in CIR mice. At the same time, clomipramine and the RP extract increased DA and DOPAC levels, which suggested that the drugs influenced the synthesis and metabolism of DA to keep normal dopaminergic function, and the DA system was also involved in the antidepressant effects. Serra et al. (1979) proposed the involvement of dopaminergic presynaptic receptors in the action of antidepressants. Moreover, it has been reported that amineptine, which blocks DA reuptake, had antidepressive activity (Simoni et al., 1986). Thus, the importance of DA system in the pathophysiology and treatment of poststroke depression should not be ignored. Of course, further elaborate experiments including the antagonism of some reserpine-induced symptoms with dopaminergic activity should be performed to speculate about the mechanism of the possible antidepressant-like effect of RP extract.

Data showed that the antidepressant effect of RP extract was comparable to that of clomipramine. RP extract contains flavonoids, coumarins, and especially isoflavones, such as puerarin, daidzein, daidzin, and daidzin-4′,7-diglucoside(Cao et al., 1999), which were safe and of great benefit to the vascular diseases. Considering the undesirable side effects of clomipramine, which limited its clinical usage, RP extract seemed to be a promising antidepressant for stroke patients.

Based on the present study, it was speculated that NE and DA systems were the targets of the treatment for poststroke depression. In addition, no obvious changes were found in the frontal cortex either in CIR or in drugs-treated mice, and it was probably due to the limitation of short-term effects of drugs. Considering that clinical antidepressant effects often appear after chronic treatment, long-term effects of RP extract should be evaluated and further in vivo studies should focus on the receptors and signal systems to elucidate the mechanism of the possible antidepressant-like effect of RP extract.

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