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Original Article

Effects of raw and sulfur-fumigated *Rhizoma Dioscoreae* on rat hippocampal neurotransmitter concentrations

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Abstract: Objective: *Rhizoma Dioscoreae* (*Dioscoreae polystachya*, Chinese yam or Shan Yao) extract (RDE) is an herbal preparation with possible immunoregulatory, pro-digestive, anti-hyperglycemic, anti-aging, and nootropic properties, effects that may depend in part on regulation of neurotransmitters such as dopamine (DA), 5-hydroxytriptamine (5-HT), norepinephrine (NE), and acetylcholine (ACh). Sulfur-fumigation is used to preserve herbal preparations but may also influence therapeutic efficacy. We assessed the impacts of *Rhizoma Dioscoreae* sulfur fumigation on RDE-induced changes in hippocampal neurotransmitter concentrations. Methods: Rats received either vehicle, raw RDE, or sulfur-fumigated RDE for 90 days by intragastric administration, and hippocampal concentrations of DA, 5-HT, NE, and ACh were measured by reverse phase HPLC or colorimetric assay. Results: Compared to the Control group, raw (sulfur-free) RDE significantly enhanced hippocampal NE, DA, and ACh concentrations ($P < 0.05$). Sulfur-fumigated RDE slightly augmented the effect on ACh and markedly enhanced that on NE but partially mitigated the effect on DA. Neither raw nor sulfur-fumigated RDE altered hippocampal 5-HT. Conclusion: *Rhizoma Dioscoreae* extract effectively enhances the synthesis of ACh and several monoamine neurotransmitters in rat hippocampus. Study indicates that sulfur likely greatly enhance the body's external stress capacity by increasing the concentration of neurotransmitter NE in hippocampus.

Keywords: *Rhizoma Dioscoreae,* sulfur fumigation, rat, neurotransmitter

The hippocampus is critical for mammalian learning and memory. Cognitive function is directly impacted by brain levels of the neuromodulatory transmitters dopamine (DA), norepinephrine (NE), 5-hydroxytryptamine (5-HT), and acetylcholine (ACh) [1]. Thus, treatments that regulate the synthesis, release, or uptake of these transmitters may have cognitive-enhancing (nootropic) effects. NE enhances the excitability of afferent nerves, thereby enhancing the effects of other excitatory inputs. In addition, NE triggers vasoconstriction, thereby elevating blood pressure, promotes catabolism, and accelerates the breakdown of hepatic glycogen to elevate blood sugar. Dysfunction of the NE system is implicated in psychiatric disorders, bradycardia, and bronchial asthma [2]. DA is also implicated in numerous psychiatric and neurodegenerative disorders. For instance, the symptoms of senile dementia and Parkinson's disease are caused primarily by DA deficiency in specific brain regions [3]. 5-HT is primarily an inhibitory neurotransmitter with multiple functions in cognition, endocrine regulation, and gastrointestinal function regulation [4]. Thus, agents able to enhance the hippocampal concentrations of these transmitters may have broad beneficial effects against age-related cognitive decline and dementia. Finally, acetylcholine (ACh) also plays a crucial role in learning and memory. Decreased ACh is associated with accelerating brain cell aging, impairing memory, and even senile dementia [5]. Chang reported increased hippocampal ACh during memory tasks [6], and elevation of neural ACh content can significantly improve the symptoms of Alzheimer's disease [7]. Conversely, ACh content tends to decrease with age, so maintaining ACh content can effectively prevent memory loss and improve cognitive function.

Rhizoma Dioscoreae (RD), the dry rhizome of *Dioscorea polystachya*, has long been used in

Traditional Chinese Medicine (TCM) to promote digestion, lower blood glucose, increase immunity, invigorate the body, and delaying aging. Studies have also shown that RD can promote the release of catecholamines (CAs) and increase sympathicoadrenal system activity [8]. Thus, the active components of RD extracts (RDEs) are promising candidate therapeutics against age- and disease-related cognitive decline.

Sulfur fumigation has been used for nearly 100 years in RD processing. Unlike the raw form, sulfur-fumigated RD has no acidic smell and possesses a bright white appearance. In addition to bleaching, sulfur fumigation sterilizes RD and prevents spoilage, greatly increasing shelf-life. However, sulfur fumigation may leave a residue of SO_2, a powerful reducing agent that may impact the chemical composition and pharmacological actions of RD. The investigators of this study examined the potential of sulfur fumigation to alter the pharmacological properties of RD by comparing the effects of raw and sulfur-fumigated RD on NE, DA, 5-HT, and ACh concentrations in rat hippocampus.

Materials and apparatus

Animals

Thirty female specific pathogen-free (SPF) Sprague-Dawley (SD) rats weighing 160-180 g were purchased from Hunan Slack Jingda Laboratory Animal Co., Ltd. (Certificate of Conformity: SCXK (Xiang) No. 2013-0004) and maintained on Grade B fed (Hunan Slack Jingda Laboratory Animal Co., Ltd., Certificate of Conformity: SCXK (Xiang) No. 2014-0002). All animal care and experimental procedures conformed with the guidelines of the National Institutes of Health and were approved by the National Institute of Mental Health Animal Care and Use Committee.

Experimental drugs

Rhizoma Dioscoreae and sulfur were purchased from Zhangshu Tianqi Pharmaceutical Co., Ltd. *Rhizoma Dioscoreae* was identified as the dried rhizome of *Dioscorea polystachya* (formerly *Dioscorea opposita* Thunb) by Dr. Jinlong Chen, Nanchang University.

Reagents

Monoamine standards were prepared by mixing 0.01 mo1/L aqueous perchloric acid, 0.01% L-cysteine, and 0.50 mmol/L EDTA-2Na with various DA, NE, and 5-HT concentrations. Hippocampal tissue was mixed with 0.60 mo1/L acqueous perchloric acid, 0.01% L-cysteine, and 0.50 mmol/L EDTA-2Na for lysis and neurotransmitter measurements. Prior to HPLC, perchloric acid was precipitated by adding 1.20 mo1/L K_2HP0_4 and 2.00 mmo1/L EDTA-2Na.

Acetylcholine was measured using a kit was provided by Nanjing Jiancheng Bioengineering Institute (batch number: 20151009). DA (batch number 100070-201507), NE (batch number 100169-201404), and 5-HT (batch number 111656-200401) standards were provided by the National Institutes for Food and Drug Control.

Main experimental apparatus

Acetylcholine colorimetric assays were conducted on a DNM-9602 microplate reader (Beijing Pulang New Technology Co., Ltd.). Monoamines were measured by HPLC using an Agilent high performance liquid chromatograph system (Agilent, USA). All supernatants were prepared using a TCL-16C high speed centrifuge (Shanghai Anting Scientific Instrument Factory). All chemicals were weighed using a JA2003 electronic balance (Shanghai Liangping Instrumentation Co., Ltd.).

Methods

Preparation of sample materials

Preparation of saturated fumigated Rhizoma Dioscoreae: Fresh *Rhizoma Dioscoreae* was washed, peeled with a bamboo knife, and cut into sections. Sections were evenly placed in a fumigation chamber together with a crucible containing an appropriate amount of sulfur. The sulfur was ignited and the fumigator door closed when an obvious blue flame appeared. The door was then sealed with transparent plastic. After fumigation for 12 h, the exhaust fan was started and the air cleared for 1 h, followed by 3 additional 12-h fumigation steps as described above. The fumigated RD was then removed, cut into 2-3 mm slices, and placed in an oven for low temperature drying at 60°C.

Preparation of raw (sulfur-free) Rhizoma Dioscoreae: Fresh *Rhizoma Dioscoreae* sections were placed in a drying oven for 1 h at 105°C, followed by low-temperature drying at 60°C.

Table 1. Hippocampal acetylcholine content ($\bar{x} \pm s$)

Treatment group	Number of animals	ACh (μg/mg)
Control	10	1.124 ± 0.188
Sulfur-free RDE	10	1.422 ± 0.313*
Sulfur-fumigated RDE	9	1.461 ± 0.316*

ACh: acetylcholine; RDE: *Rhizoma Dioscoreae* extract $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the Control group.

Table 2. Neurotransmitter recovery rates from rat hippocampus (n = 3)

Transmitter	Sample content μg/L	Standard content μg/L	Recovered quantity (μg)	Recovery rate (%)
NE	74.8	47.5	117.261	95.88
	74.8	95	160.451	94.49
	74.8	142.5	208.748	96.06
DA	41.5	11.5	53.016	100.03
	41.5	23	62.758	97.30
	41.5	46	84.415	96.47
5-HT	1.47	1.95	3.278	96.11
	1.47	3.9	5.165	96.18
	1.47	7.8	8.765	94.55

Table 3. Linear ranges for HPLC measurements of NE, DA, and 5-HT

	Linear equation	Range ($\times 10^{-4}$ μg)	Correlation coefficient R^2
NE	$Y = 0.0002X + 4 \times 10^{-5}$	9.5-47.5	0.9999
DA	$Y = 0.0001X + 0.0003$	11.5-57.5	0.9991
5-HT	$Y = 2 \times 10^{-5}X + 10^{-5}$	1.95-9.75	0.9998

X = transmitter concentration.

Sections were then removed, cut into 2-3 mm slices, and subjected at low-temperature drying at 60°C until semi-dry. Dried samples were then crushed into powder as described.

Preparation of concentrated aqueous extract of Rhizoma Dioscoreae: Forty grams of sulfur-free or sulfur-fumigated *Rhizoma Dioscoreae* powder was added to 8 volumes of distilled water, cold soaked for 30 min, boiled for 1 h, filtered through gauze, and concentrated to a volume of 20 mL (2 g/mL) [9].

Animal treatment

Thirty SD rats were adapted to the feeding protocol for 1 week, then randomly divided into three groups of ten: a sulfur-fumigated RDE group (20 g/kg), sulfur-free RDE group (20 g/kg), and blank Control group. Treatment-group animals received intragastric administration at 20 mL per kg body weight RDE once a day for 90 days while the Control group received equivolume distilled water over the same period. Five rats per cage received water *ad libitum*.

Specimen collection

The rats were sacrificed by cervical dislocation. The hippocampus were removed, placed into 2-mL cryopreservation tubes, and flash-frozen in liquid nitrogen for future analysis.

Assay of neurotransmitter

Assay of ACh in rat hippocampus: Frozen hippocampus were thawed, carefully weighed, and homogenized in a 5 mL glass homogenizer. Colorimetric reactions were performed in strict accordance with the kit manufacturer's instructions. After the reaction, 200-μL samples were pipetted into 96-well plates and the optical density (OD) at 550 nm measured on a microplate reader (DNM-9602 microplate reader, Beijing Pulang New Technology Co., Ltd.). The ACh concentration was determined by comparing sample OD values against a standard curve.

Assay of monoamine neurotransmitter in rat hippocampus: Frozen hippocampus were thawed and placed into a 5 mL glass homogenizer with 10 volumes per unit weight lysis solution. Lysates were centrifuged at 8000 rpm for 15 min and the supernatants stored at -20°C. For monoamine transmitter measurements, supernatant samples were placed on ice to thaw. The perchloric acid was precipitated by adding 1:1 (v:v) 1.20 mo1/L K_2HP0_4 with 2.00 mmo1/L EDTA-2Na. Samples were placed in an ice bath for 10 min and then centrifuged at 8000 rpm for 15 min to remove precipitate crystals. The resulting supernatant was passed through a 0.22-μm Millipore filter before chromatographic measurements. Chromatographic separation was achieved using a Hypersil ODS_2 C_{18} column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of 8:1:1 aqueous buffer (0.5 mmo1/L 1-sodium heptanesulfonate, 0.5 mmo1/L Na_2EDTA, 50 mmol/L citric acid, 5 mmo1/L tri-

Table 4. Hippocampal monoamine concentrations (μg/g, $\bar{x}$ ± s, n = 10)

Treatment Group	NE	DA	5-HT
Control	3.195 ± 0.170	1.462 ± 0.155	0.0569 ± 0.0147
Sulfur-free RDE	3.58 ± 0.597*	1.623 ± 0.089*	0.0677 ± 0.0277
Sulfur-fumigated RDE	3.830 ± 0.340**,#	1.537 ± 0.102	0.0549 ± 0.0119

RDE: *Rhizoma Dioscoreae* extract *P < 0.05 and **P < 0.01 vs. Control group; #P < 0.05 vs. Sulfur-free RDE group.

ethylamine, and 50 mmo1/L sodium acetate):methanol:acetonitrile, and the pH was adjusted to 3.8. Flow velocity was 1.0 mL/min and the injection volume was 10 μL. Transmitters were detected using an excitation wavelength of 280 nm and transmission wavelength of 330 nm.

Preparation of standard solution: Briefly, 3.9 mg 5-HT standard was dissolved in 10 mL of lysis solution. A 1 mL volume was pipetted into a 10 mL volumetric flask, to which 1.9 mg NE and 2.3 mg DA standard were added. The solution was mixed, diluted fortyfold, and stored at 4°C.

Statistical methods

Microsoft Excel and SPSS19.0 software were used for statistical calculations. Data are expressed as mean ± standard deviation ($\bar{x}$ ± s). One-way ANOVA and Dunnett's multiple comparison test were used to assess the differences among treatments using the Statistical Package for the Social Sciences 19.0 software. A $p < 0.05$ is regarded as significant and $p < 0.01$ as very significant.

Experimental results

Changes in hippocampal ACh concentration

After 90 days of feeding, sulfur-free RDE and sulfur-fumigated RDE group rats exhibited significantly elevated hippocampal ACh concentrations compared to the Control group, with no difference between sulfur-free and sulfur-fumigated RDE groups ($p > 0.05$).

Changes in hippocampal monoamine concentrations

Precision investigation: The mixed standard solution was injected six times under the above chromatographic conditions (Section 2.4) to investigate measurement precision. Retention time fluctuation of the 6 chromatographic peaks was less than 0.03 min, and the relative SD (RSD) of peak area was only 0.58%-1.75%, sufficient precision for the changes induced by RDE (**Tables 1-4**).

Sample adding standard recovery rate: Three samples of hippocampal homogenate with known transmitter concentrations (high, middle, and low) were analyzed under these same chromatographic conditions (Section 2.4) to assess recovery rates (**Table 2**). Recovery rates using reverse phase HPLC detection ranged from 93% to 105%, with a mean of 96.34% and RSD = 1.704%, confirming the accuracy and reliability of this multiple CA measurement method.

Linear relationship: The peak chromatographic areas were strongly correlated with neurotransmitter standard concentrations (**Table 3**), confirming the accuracy of tissue concentration estimation.

Results of monoamine neurotransmitter detection: Hippocampal NE, DA, and 5-HT concentrations were determined in each group according to the methods described in Section 2.4. **Figure 1A** and **1B** present typical standard and sample chromatographs, and **Table 4** summarizes the results for all groups (Control, raw/Sulfur-free RDE, and Sulfur-fumigated RDE groups).

Sulfur-free RDE significantly enhanced hippocampal NE and DA ($P < 0.05$). Sulfur-fumigated RDE enhanced NE in rat hippocampus ($P < 0.01$) to a level significantly higher than that measured in the Sulfur-free RDE group ($P < 0.05$). However, Sulfur-fumigated RDE induced a smaller increase in DA. Neither extract altered 5-HT in rat hippocampus.

Evaluation and discussion

Colorimetric assays and reverse phase HPLC were utilized to detect changes in the NE, DA, 5-HT, and ACh contents of rat hippocampus following prolonged ingestion of either unprocessed (sulfur-free) or sulfur-fumigated *Rhizoma Dioscoreae* extract. After 90 days of continuous administration, sulfur-free RDE significantly elevated NE, DA, and ACh in rat hippocampus, while sulfur-fumigated RDE induced a similar elevation in ACh but a significantly great-

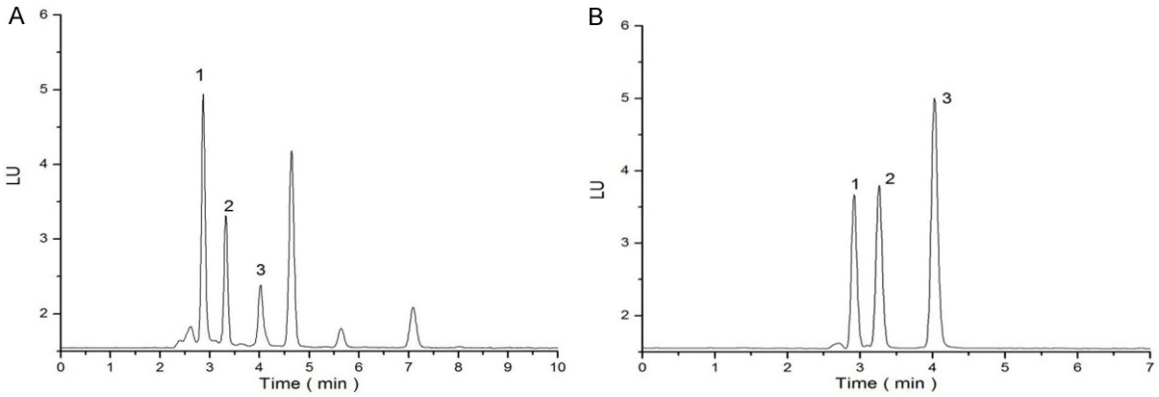

Figure 1. Sample chromatograms of (A) standards and (B) tissue extracts. 1: NE; 2: DA; 3: 5-HT.

er increase in NE. Further, sulfur fumigation partially reduced the effect on DA. Thus, sulfur fumigation does influence the pharmacological properties of RDE, likely due to the effects of residual SO_2.

Rhizoma Dioscoreae can significantly increase ACh in cerebral tissues, likely by reducing AChE activity, suggesting that RDE may enhance cholinergic transmission. Given the inverse relationship between brain cholinergic transmission and cognitive function, RDE may serve to improve or maintain cognition in neurodegenerative diseases by augmenting hippocampal ACh. The monoamine neurotransmitters NE, DA, and 5-HT are also implicated in behavioral regulation, learning, and memory [11], and dysregulation of each of these systems is involved in neuropsychiatric diseases. For instance, NE plays an important role in memory retention, while promotion of DA release improves memory, and the 5-HT system regulates emotion, motivation, and appetitive behaviors, which are strongly associated with learning efficacy [12]. During aging, the metabolism of intracerebral monoamine neurotransmitters may be disrupted, resulting in decreased NE, DA, and 5-HT contents with concomitant effects on memory, emotion, and behavior [13]. Indeed, senile dementia and Parkinson's disease are associated with functional impairments in these transmitter systems [14]. Further, disturbances in monoamine neurotransmitter metabolism contribute to depression [15, 16], and most current antidepressant medications aim to increase brain concentrations of NE, DA, and (or) 5-HT by inhibiting uptake [17].

This experiment suggests that *Rhizoma Dioscoreae* can substantially increase NE, ACh, and DA contents in rat hippocampus, which may in turn improve the symptoms of depression as well as cognitive decline associated with aging and neurodegenerative disease. Sulfur-fumigated RDE can significantly increase the content of NE, probably because sulfur, as a warm medicinal material, has the efficacy of supplementing fire of vital gate and invigorating yang. Thus, internal administration can enhance the external stress resistance and improve the cognitive ability. As a result, sulfur-fumigated RDE also has this efficacy. However, sulfur fumigation may affect other properties, such as the efficacy for promoting DA release. Further studies are needed to assess the full spectrum of these effects on RDE pharmacology.

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Disclosure of conflict of interest

None.

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