Schisandra N-butanol extract improves synaptic morphology and plasticity in ovarectomized mice★

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Abstract

Preliminary work by our research team revealed that Schisandra, a renowned traditional Chinese medicine, causes learning and memory improvements in ovariectomized mice. This activity was attributed to active ingredients extracted with N-butyl alcohol, named Schisandra N-butanol extract. In this study, ovariectomized mice were pretreated with Schisandra N-butanol extract given by intragastric administration. This treatment led to the enhancement of learning, and an increase in hippocampal CA1 synaptic, surface and postsynaptic density. A decrease in the average size of the synaptic active zone was also observed. These experimental findings showing that Schisandra N-butanol extract improved synaptic morphology indicate an underlying mechanism by which the ability of learning is enhanced in ovariectomized mice.

Key Words

Schisandra N-butanol extract; ovariectomy; mice; behavioral learning; hippocampal CA1; synaptic morphology; synaptic density; neural regeneration

Abbreviations

OVX, ovariectomy; EB, estradiol benzoate; PSD, postsynaptic density

INTRODUCTION

Schisandra, a traditional Chinese sedative and tranquilizer, is the dried fruit of *Schisandra chinensis* (Turcz.) Baill or *Schisandra sphenanthera* Rehd. etWils. Schisandra was ordinarily used clinically as a single medicine or as a compound with other herbs for the treatment of neurasthenia, palpitations and insomnia[1]. The active ingredients of Schisandra consist of lignans, polysaccharides and triterpene acids[2]. Schisandra obtained with ethanol extraction gives access to lignan active ingredients, including schizandrin, gomisin A, deoxyschizandrin, r-schizandrin, and pseudo-r-schizandrin (gomisin N)[3]. It is reported that Schisandra lignans play a role in regulating the central nervous system, eg, improving human brain activity, enhancing working efficiency and promoting the quality of work[4].

Our preliminary studies showed that Schisandra ethanol extract could ameliorate learning and memory in ovariectomized (OVX) mice[5]. To further understand the active ingredients of Schisandra ethanol extract, Schisandra was further extracted in differing solvents to obtain N-butanol extract, ethyl acetate extract and chloroform extract from the ethanol extract. It was found that only Schisandra N-butanol extract caused improvement of behavioral learning in OVX mice, while ethyl acetate extract and chloroform extracts showed little effect[6]. Therefore, we speculate that Schisandra N-butanol extract may contain several active lignans, which may underlie the pharmacologic function seen in preliminary studies *i.e.* ameliorating learning and memory in OVX mice. Nevertheless, the mechanisms underlying Schisandra action in the central nervous system, especially the influence on cellular morphology, remain

poorly understood in OVX mice. Synaptic structural plasticity underlies learning and memory[7]. Therefore, this study aimed to observe effects of Schisandra N-butanol extract on behavioral learning and synaptic ultrastructure in OVX mice.

RESULTS

Quantitative analysis of experimental animals

A total of 62 mice were randomly divided into five groups: sham group (n = 14); OVX group (n = 14); estradiol benzoate (EB) group (n = 12; OVX + EB); low concentration (LW) group (n = 10; OVX + 1.0 g crude Schisandra N-butanol extract/mL); and high concentration (HW) group (n = 12; OVX + 1.5 g crude Schisandra N-butanol extract/mL). Bilateral OVX was performed in all mice except the sham group. Each mouse in the EB group was injected intraperitoneally with 20 µg EB per day; both LW and HW groups were orally administered Schisandra N-butanol extract (1.0 g and 1.5 g crude drug/mL, respectively) every morning and afternoon (total volume at 0.4 mL per day). Both the sham and OVX groups were given distilled water in equivalent volumes. No animals died during the experimental procedure; therefore, all were incorporated into the final analysis.

Schisandra N-butanol extract enhanced behavioral learning in OVX mice

The results of Y maze testing are shown in Table 1.

Table 1 Effects of Schisandra N-butanol extract on the rate of correct responses (%) in ovariectomized mice

Time (day)	Sham group (n = 10)	Ovariectomized group (n = 10)	Estradiol benzoate group (n = 12)	LW group (n = 10)	HW group (n = 12)
1	53.0±17.8	51.0±11.7	58.0±12.0	51.0±12.7	53.0±11.4
2	65.0±13.7	54.0±9.1[b]	65.0±13.5[c]	68.5±13.8[c]	65.5±13.2[c]
3	82.0±11.8	71.5±11.3[a]	78.0±10.4	73.0±16.5	83.0±12.1[c]
4	93.0±14.8	73.5±8.5[b]	91.0±10.8[d]	89.5±8.3[c]	90.5±6.9[d]
5	96.5±3.4	82.0±8.6[a]	94.0±6.5	95.0±5.3[d]	94.5±4.4[d]
6	96.5±4.1	93.0±7.9	99.0±2.2[c]	96.0±5.2	96.5±4.1
7	98.0±3.5	95.0±5.3	98.0±3.5	96.0±6.6	98.0±2.6
8	99.5±1.6	93.0±7.5[a]	99.0±2.2[c]	99.0±2.1[c]	98.0±4.2[c]

Rate of correct responses refers to the percentage of correct responses in total trials per day. Criterion of learning completion is the rate of correct responses ≥ 90%. Data were expressed as mean ± SD and evaluated by Student's *t*-test (two samples) between two groups.

[a]$P < 0.05$, [b]$P < 0.01$, *vs.* sham group; [c]$P < 0.05$, [d]$P < 0.01$, *vs.* ovariectomized group. LW group: ovariectomy + 1.0 g crude Schisandra N-butanol extract/mL; HW group: ovariectomy + 1.5 g crude Schisandra N-butanol extract/mL.

Higher rates of correct responses were observed in the EB, LW and HW groups, compared with OVX group.

Moreover, mice in the EB and HW groups reached learning standard on day 4 ($P < 0.01$, $P < 0.05$), whereas mice in the LW group were up to standard on day 5 ($P < 0.05$). These findings show that the rate of correct responses was significantly reduced in OVX mice, but significantly increased after Schisandra N-butanol extract treatment, especially at a concentration of 1.5 g crude drug/mL. Rates to reach learning standard were analyzed during 8 days of behavioral training. In the OVX group, the rate was significantly lower than that in the sham group (60% *vs.* 100%; $P < 0.05$). The EB group and HW group showed rates of 90% and 100%, respectively. These rates were significantly higher than that in OVX group ($P < 0.05$). A trend towards significance appeared in the LW group when compared with the OVX group, with a rate of 80% ($P > 0.05$). The OVX and LW groups failed to pass the standard during 8-day behavioral training. By contrast, the sham, EB and HW groups all achieved the standard. Our results demonstrated that the rate of reaching learning standard was reduced in OVX mice but increased after treatment with Schisandra N-butanol extract in 1.5 g crude drug/mL.

Schisandra N-butanol extract improved Gray type I synapses in the hippocampal CA1 region of OVX mice

Gray divided cerebral cortex synapses into type I and type II based on interface characteristics. Type I synapses have an asymmetrical interface, that is a larger postsynaptic membrane than presynaptic one. Also, the synaptic cleft broadens and the length of the active zone is extended in Gray type I synapses. As for type II synapses, they are symmetrical, with similar presynaptic to postsynaptic membrane thickness accompanied by narrow synaptic cleft and short active zone. Gray type I synapses exhibited excitability and contributed to excitatory synaptic transmission, which was different to Gray type II inhibitory synapses[8]. In this paper, we focused on Gray type I synapses and analyzed their ultrastructural morphology.

As shown in Table 2, surface density, synaptic density and postsynaptic density (PSD) thickness were all decreased in hippocampal CA1 synapses of OVX mice at 6 weeks post-operation, while the average size of the synaptic active zone was significantly increased ($P < 0.01$). Both the synaptic interface curvature and the ratio of perforated synapses were slightly decreased, but this decline was not significant ($P > 0.05$). The indexes of surface and synaptic density, the average size of synaptic active zone, PSD thickness and interface curvature at 10 weeks post-OVX, were not significantly changed when compared with those at 6 weeks post-OVX ($P > 0.05$). The ratio of perforated synapses declined further, but still without significance ($P > 0.05$). Our results suggest that synaptic structure reached

stability in mice over 6 weeks after OVX.

The surface density, synaptic density and PSD thickness of hippocampal CA1 synapses in the HW group were all significantly increased with Schisandra N-butanol extract treatment, compared with that in OVX group at 10 weeks. The average size of the synaptic active zone was significantly decreased ($P < 0.01$ or $P < 0.05$), but both the ratio of perforated synapses and synaptic interface curvature increased without significance ($P > 0.05$). In particular, we observed more presynaptic vesicles in the HW group. Our results demonstrate that Schisandra N-butanol extract can improve surface density, synaptic density and PSD thickness of hippocampal CA1 synapses and reduce the average size of the synaptic active zone in OVX mice (Table 2, Figure 1).

Table 2　Effects of Schisandra N-butanol extract on hippocampal CA1 synaptic ultrastructure in ovariectomized mice

Item	Sham group	OVX group (6 weeks)
Surface density ($\mu m^2/\mu m^3$)	0.176±0.045	0.146±0.051[a]
Synaptic density (μm^{-3})	2.651±0.677	1.675±0.591[a]
Average size of synaptic active zone (μm^2)	0.071±0.015	0.092±0.033[a]
Postsynaptic density thickness (μm)	0.078±0.023	0.067±0.019[a]
Synaptic interface curvature	1.031±0.062	1.026±0.042
Ratio of perforated synapses (%)	4.78	1.91

| Item	OVX group (10 weeks)	HW group
Surface density ($\mu m^2/\mu m^3$)	0.149±0.038	0.186±0.046[b]
Synaptic density (μm^{-3})	1.876±0.549	2.630±0.880[b]
Average size of synaptic active zone (μm^2)	0.082±0.015	0.074±0.021[c]
Postsynaptic density thickness (μm)	0.068±0.017	0.070±0.014[b]
Synaptic interface curvature	1.029±0.05	1.031±0.055
Ratio of perforated synapses (%)	0.71	2.48

Ratios of perforated synapses were expressed with average value and evaluated by the chi-square test. Other indexes were expressed as mean ± SD and evaluated by Student's *t*-test (two samples).

[a] $P < 0.01$, *vs*. sham group; [b] $P < 0.01$, [c] $P < 0.05$, *vs*. OVX group at 10 weeks. A total of 30 electromicrographs were obtained for each group. OVX: Ovariectomy; HW group: ovariectomy + 1.5 g Schisandra N-butanol crude extract/mL.

DISCUSSION

Hippocampus-dependent learning and memory can be impaired by OVX[9]. Indeed, our results found behavioral learning in OVX mice was attenuated in the Y maze test. Administration with estrogen or 1.5 g crude Schisandra N-butanol extract/mL improved learning of OVX mice. To study morphological plasticity, the population of synapses and their changing number were investigated.

It has been reported that hippocampal CA1 dendritic spine density was reduced in OVX rodents[10-11]. In this study, surface density, PSD thickness, the ratio of perforated synapses and synaptic density of hippocampal CA1 synapses were decreased in OVX mice over 6 weeks, while the average size of the synaptic active zone area was increased. All the above findings are consistent with our behavioral results, suggesting that the disorder of behavioral learning is, in part, attributable to reduced morphological plasticity in hippocampal CA1 synapses of OVX mice.
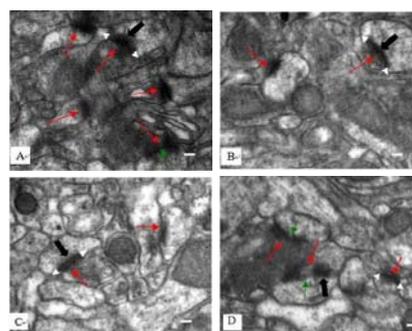
Figure 1　Synaptic morphometry and synaptic number in the hippocampal CA1 area of mice (transmission electron micrograph, × 40 000; scale bars: 100 nm).

The synapse is indicated by a long red arrow; the synaptic active zone by two white arrowheads. The black arrow shows postsynaptic density thickness and the dashed green arrow shows perforated synapses.

In the OVX group at 6 weeks (B), the number of synapses and the thickness of postsynaptic density decreased, but the length of synaptic active zone was increased. Meanwhile, both the number of perforated synapses and synaptic interface curvature were reduced compared with that in the sham group (A), but no differences were seen when compared with the OVX group at 10 weeks (C).

In the HW group (D), opposite changes in the number of synapses, thickness of postsynaptic density and the length of synaptic active zone were observed compared with that in the OVX group at 10 weeks (C). Meanwhile, the number of perforated synapses and synaptic curvature increased.

OVX: Ovariectomy; HW group: ovariectomy + 1.5 g crude Schisandra N-butanol extract/mL.

Synaptic density reflects the number of synapses within a three-dimensional space, while surface density indicates the total area of the synaptic active zone. It is reported that both the number of dendrites and synaptic density in the hippocampus fluctuate with the change of estrogen during the menstrual cycle, with enhancement of estrogen levels leading to the formation of new hippocampal synapses and dendrites[12]. The density of hippocampal CA1 synapses was reduced following OVX, while replacement therapy restored the number of synapses with estrogen treatment[13]. Results of this study found that synaptic density was reduced in OVX mice, and this change stabilized 6 weeks post-surgery. Nevertheless, synaptic density was increased with

treatment of Schisandra N-butanol extract in 1.5 g crude drug/mL, suggesting this extract seems to promote the formation of new synapses.

Intriguingly, we found that hippocampal CA1 synaptic density declined in OVX mice. This decline was accompanied by an increase in the average size of the synaptic active zone, which is determined by the ratio of surface density to synaptic density. Both surface and synaptic density were significantly increased with the treatment of Schisandra N-butanol extract, while the average size of the synaptic active zone was decreased. Among all the parameters of synaptic ultrastructure, PSD is the easier to change[14]. It is reported that PSD thickness of hippocampal CA1 synapses was reduced in OVX mice, but increased with estrogen therapy[15]. Our study also found that PSD thickness of hippocampal CA1 synapses was significantly thinner in OVX mice, and it was remarkably increased with Schisandra N-butanol extract treatment. For the ratio of perforated synapses and synaptic curvature, our study observed no significant impact of low estrogen level by OVX, suggesting that the effect of estrogen on PSD may be mainly at the synaptic thickness level, and the compensatory function of surviving synapses are not associated with the change in synaptic curvature. Presently, we also observed that there were no significant changes in synaptic perforation and synaptic curvature with Schisandra N-butanol extract treatment in OVX mice.

In conclusion, our research indicates that Schisandra N-butanol extract can improve learning and memory impairment in OVX mice. Administration of Schisandra N-butanol extract restored synaptic ultrastructure, enhancing hippocampal CA1 surface, synaptic density and PSD thickness, and decreasing the average size of the active synaptic zone in OVX mice. We suggest that the mechanism by which Schisandra N-butanol extract enhances learning and memory in OVX mice could be associated with improvement of synaptic ultrastructural morphology.

MATERIALS AND METHODS

Design
A randomized, controlled, animal experiment.

Time and setting
All procedures were performed in Physiology Laboratory, School of Life Science, South China Normal University, China from February 2009 to March 2010.

Materials
Animals
Sixty-two 2-month-old healthy female mice, weighing 24 ± 2 g, were provided by the Experimental Animal

Center, Guangzhou University of Chinese Medicine, China (license No. SCXK (Yue) 2008-0020). All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, published by the Ministry of Science and Technology of China.

Drug preparation
Dried Schisandra fruit (Guangzhou Pharmacy, identified by Guangdong Pharmaceutical University, China) was crushed into small particles (diameter 0.5 mm), defatted with two-fold volume of petroleum ether impregnation for 24 hours, and the filtrate was recycled. Residues were soaked (five times) with two-fold volumes of 90% ethanol at room temperature for 24 hours. Extract filtrates were combined, filtered and vacuum concentrated, giving Schisandra ethanol extract. Subsequently, Schisandra extract was dissolved into three-fold volumes of distilled water, filtered, and then extracted with chloroform, ethyl acetate and N-butanol in turn. This process was repeated six times. Filtrate combination and vacuum concentration yielded Schisandra N-butanol extract.

Methods
Establishment of OVX model
Mice were anesthetized with 0.7% sodium pentobarbital *via* intraperitoneal injection, and then both ovaries were removed. In the sham group, only adipose tissue around the ovary was removed. Two weeks later, we confirmed the success of the model according to absence of periodic changes in vaginal cells following the performance of vaginal smear examination for 7 days.

Drug administration
Mice were administered relevant drugs 6 weeks following OVX. Both LW and HW groups were given Schisandra N-butanol extract (1.0 g and 1.5 g crude drug/mL, respectively) orally every morning and afternoon in a total volume at 0.4 mL per day. Both sham and OVX groups were given distilled water in an equivalent volume. EB group were injected intraperitoneally with 20 μg/kg EB per day. Drug administration was continuous over 28 days.

Y maze behavioral test
Behavioral learning was determined in a Model MG-2 maze (Shijiazhuang Sanxing Teaching Equipment Factory, Shijiazhuang, China) using the fixed time stochastic restless testing method[16]. This testing began from day 21 of drug administration and lasted for eight continuous days.

Synaptic ultrastructural morphology observation
We observed hippocampal CA1 synaptic ultrastructural morphology at the periods of 6 and 10 weeks after OVX.

The latter time point also corresponded to the end of behavioral learning. In brief, four mice were randomly selected from the sham and OVX groups at 6 weeks after OVX. Four mice were also randomly selected from the OVX and HW groups at the end of behavioral learning (10 weeks). Since the LW group exhibited weaker improvement in Y maze behavioral learning, compared with the HW group, we chose the HW group for ultrastructural morphological observation.

Mice were anesthetized by intraperitoneal injection of 0.7% pentobarbital sodium (35−40 mg/kg), which was followed by perfusion and fixation with 4% paraformaldehyde + 1% glutaraldehyde *via* the carotid artery. Then, brains were removed, embedded in EPON812, sectioned into 70−80 nm per micrograph with a LKB ultramicrotome (LKB Corporation, Switzerland), and stained with uranyl acetate followed by lead citrate, then observed and photographed using a Hitachi H-7500 microscope (Hitachi High-Technologies Corporation, Tokyo, Japan). Six to eight sheets of each sample were selected randomly from electron micrographs. Main parameters of Gray type I synapses were measured with Image-Pro Plus 6.0 software (Media Cybernetics Inc., Silver Spring, MD, USA)[16].

Statistical analysis

With the application of SPSS 13.0 software (SPSS Inc, Chicago, IL, USA), rate of reaching learning standard and ratio of perforated synapses were expressed as percentage and evaluated using the chi-square test. Other data were expressed as mean ± SD and evaluated using the Student's *t*-test (two-samples). Statistical significance was considered at $P < 0.05$.

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(Edited by Gao HY, Wang RT/Yang Y/Wang L)