Ginsenoside Rb1 ameliorates cisplatin-induced learning and memory impairments
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ABSTRACT
Background: Ginsenoside Rb1 (Rb1), a dominant component from the extract of Panax ginseng root, exhibits neuroprotective functions in many neurological diseases. This study was intended to investigate whether Rb1 can attenuate cisplatin-induced memory impairments and explore the potential mechanisms.
Methods: Cisplatin was injected intraperitoneally with a dose of 5 mg/kg/wk, and Rb1 was administered in drinking water at the dose of 2 mg/kg/d to rats for 5 consecutive wk. The novel objects recognition task and Morris water maze were used to detect the memory of rats. Nissl staining was used to examine the neuron numbers in the hippocampus. The activities of superoxide dismutase, glutathione peroxidase, cholineacetyltransferase, acetylcholinesterase, and the levels of malondialdehyde, reactive oxygen species, acetylcholine, tumor necrosis factor-α, interleukin-1β, and interleukin-10 were measured by ELISA to assay the oxidative stress, cholinergic function, and neuroinflammation in the hippocampus.
Results: Rb1 administration effectively ameliorates the memory impairments caused by cisplatin in both novel objects recognition task and Morris water maze task. Rb1 also attenuates the neuronal loss induced by cisplatin in the different regions (CA1, CA3, and dentate gyrus) of the hippocampus. Meanwhile, Rb1 is able to rescue the cholinergic neuron function, inhibit the oxidative stress and neuroinflammation in cisplatin-induced rat brain.
Conclusion: Rb1 rescues the cisplatin-induced memory impairment via restoring the neuronal loss by reducing oxidative stress and neuroinflammation and recovering the cholinergic neuron functions.

1. Introduction
Chemotherapy is an effective treatment for many types of cancer; however, several side effects of chemotherapy have also been reported. Among them, postchemotherapy cognitive impairment (PCCI) is the most important. It encompasses a wide range of neurological disorders, including the impairments in cognition, clarity of thought, attention, executive functioning, and information processing speed [1]. Approximately 20–30% of patients who undergo chemotherapy suffer from different level of PCCI [2]. In the USA, up to 3.9 million individuals with cancer and cancer treatments are living with persistent cognitive problems. Cognitive deficits that occur from cancer or its treatment vary and may be subtle or dramatic, temporary or permanent, and stable or progressive [3]. However, the underlying mechanisms of PCCI are still elusive, and no effective treatments are currently available.
Cisplatin, cis-diaminedichloroplatinum(II), is a drug used for treating various types of tumors by injecting into a vein [4]. Cisplatin could reportedly induce apoptosis in cancer cells by cross linking DNA with the purine bases, impairing the repair mechanisms of DNA and inducing DNA damage. Although it is very effective in cancer therapy, the highly toxic effects of cisplatin, especially neurotoxicity, have been widely reported including the PCCI. In mice and rats, administration of cisplatin can induce severe cognitive malformations via different mechanisms [5], such as elevation of reactive oxygen species (ROS) [6], induction of neuronal loss by promoting apoptosis and inhibiting neurogenesis [7], and stimulation of neuroinflammation [8]. Therefore,
treatment with antioxidants and anti-inflammation drugs may restore the inflammatory factors and endogenous antioxidants to physiological levels and thereby prevent the neuronal loss and memory function induced by cisplatin.

Ginseng, also known as Korean or Asian ginseng, is the root of *Panax ginseng* Meyer. The benefits of ginseng in traditional oriental medicine have been widely recognized for over a thousand years. The major bioactive components in ginseng are ginsenosides, and their neuroprotective effect on different neurologic diseases has been studied [9,10]. Numerous studies have proven that ginsenosides can ameliorate learning/memory deficits in different brain diseases and during senescence in humans and animals [11,12]. There are many important components in ginsenosides; Rg1 and Rb1 are two of them with slightly varying molecular structures. According to previous studies, the beneficial effects of ginseng root on learning and memory are often attributed to Rb1 [13]. Previous studies showed that Rb1 stimulated neurite outgrowth [14] and promoted synaptic vesicle release by enhancing the phosphorylation of synapsins through a cyclic adenosine monophosphate-dependent protein kinase pathway [15]. Additionally, Rb1 was suggested to prevent from either ischemia [16] or neurodegeneration induced by glutamate in hippocampal neurons [17]. However, whether Rb1 is also protective to the memory impairments induced by cisplatin is not known.

In this study, we assessed whether ginsenoside Rb1 could protect against the cognitive deficit induced by cisplatin. We found that oral administration of Rb1 effectively attenuated the memory impairments caused by cisplatin. Rb1 also rescued the neuronal loss and cholinergic dysfunction. Finally, we reported that Rb1 reduces the oxidative stress and neuroinflammation in the hippocampus.

2. Materials and methods

2.1. Animals and drugs

Wistar rats (male, 250 ± 20 g) were purchased from the Henan Provincial Laboratory Animal Center (Zhengzhou, Henan). They were housed on a 12-h light/dark cycle (light on at 7:00 AM) for 1 wk before use with food and water *ad libitum*. The experiment protocols were approved by Care and Use of Laboratory Animals of HeNan Province. In total, 40 rats were divided randomly into four groups: control (*n* = 10); cisplatin (Cisp, *n* = 10); cisplatin plus Rb1 (Cisp + Rb1, *n* = 10); and Rb1 (Rb1, *n* = 10). The control group rats were subjected with 0.2-ml normal saline once per wk via intraperitoneal (i.p.) injection. The Cisp group rats received 5 mg/kg/wk i.p. cisplatin. The Cisp + Rb1 group rats were administrated cisplatin (5 mg/kg/wk, i.p.) plus Rb1 (2 mg/kg/d in drinking water). The Rb1 group rats received Rb1 (2 mg/kg/d in drinking water). The effective doses of cisplatin and Rb1 were chosen based on literatures [18–20] (Fig. 1).

Ginsenoside Rb1 and cisplatin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Some commercial kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiang Su, China), including superoxide dismutase (SOD) assay kit, malondialdehyde (MDA) assay kit, glutathione peroxidase (GSH-Px) assay kit, acetylcholine (ACH) assay kit, cholineacetyltransferase (ChAT) assay kit, acetylcholinesterase (AChE) assay kit, tumor necrosis factor-α (TNF-α) assay kit, interleukin-1β assay kit (IL-1β), and interleukin-10 assay kit (IL-10).

2.2. Novel object recognition task

The novel object recognition task (NORT) was performed, as described previously [21]. The equipment comprises a Plexiglas box (45 cm × 45 cm × 50 cm) with identical objects located in the opposite corners. In the habituation period, rats were put into the center of the empty box for 5 min without objects. The training session was performed 24 h after the habituation. Two identical objects (familiar objects, A and A’) were placed in opposite corners, and the rats were allowed to explore for 5 min. The latency to approach each object was recorded. The testing phase was carried out after a 24-h retention interval, and rats were returned to the testing environment for 5 min where the familiar object (A’) was replaced by a novel object (B), which differed from the familiar object in shape and texture. Measures of interaction were recorded as the amount of time that the animal spent with its head and nose oriented toward and within 2 cm of the object. For both training and testing phases, the exploration was operationally defined as an active event in which the rats were facing, sniffing, or pawing at the object. During the whole task, all objects were cleaned with 70% ethanol between each session. The object preference value was calculated by the formula: $T_{\text{novel}}/(T_{\text{novel}} + T_{\text{familiar}}) \times 100$, $T_{\text{familiar}}/(T_{\text{novel}} + T_{\text{familiar}}) \times 100$. The discrimination ratio was calculated as the exploring novel object time compared with the familiar object relative to the total time, as per the following formula: $(T_{\text{novel}} - T_{\text{familiar}})/T_{\text{total}}$ [22,23].

2.3. Morris water maze

Morris water maze was used to assess the spatial learning and memory of rats. The maze was a circular (180 cm in diameter) water tank filled with water (20–21°C) up to a height of 40 cm. Briefly, rats were first trained to find a hidden platform in the target quadrant that was submerged in 1–2 cm of water by using a stationary array of cues outside the pool. Acquisition training in the hidden platform was conducted for 5 d consecutively including 20 trials. In each trial, the rat was placed into the pool at one of the four possible locations (randomly ordered). All rats were given 60 s free time to find the hidden platform, and the time spent to reach the escape platform was defined as escape latency. If the rat did not find

**Fig. 1.** Time-line of experimental procedures of the whole study. After 7 d adaption, Wistar rats were administered ginsenoside Rb1 (2 mg/kg/d) in drinking water for 5 wk. Cisplatin (0.2 mL, 5 mg/kg) or saline (0.9%, 0.2 mL) was injected intraperitoneally on Day 7, Day 14, Day 21, Day 28, and Day 35. Next, the learning and memory abilities of rats were assessed by the novel object recognition task and Morris water maze. After the behavioral tasks, the rats were killed on Day 45. Five rats were fixed for Nissl staining, and the other rats were prepared for making hippocampal samples to detect the cholinergic function and biochemical biomarkers. i.p., intraperitoneal; MWM, Morris water maze; NORT, novel object recognition task; p.o., per os.
the platform within 60 s, it was gently guided by the observer to the platform for 30 s. After rest for 1 d, a 60-s probe test was conducted in which the platform was removed. Both during the training and test period, the swimming pathways, percentage of time spent in the target quadrant, platform area crossings, and swimming speed were recorded and calculated using a computer software (Smart V3.0, Panlab Harvard Apparatus) [24–26].

2.4. Nissl staining

We used Nissl staining to detect the neuronal population of hippocampus. Five rats in each group were anesthetized and fixed by perfusion of 4% paraformaldehyde phosphate-buffered solution. After the perfusion, the brains were harvested and post-fixed for 24 h. The hippocampal tissues were cut into 25 μm slices using a vibratome (VT1000s, Leica, Germany). The slices were placed in Nissl staining fluid for 5–10 min. The cell numbers of hippocampal CA1, CA3, and dentate gyrus (DG) regions were counted in the six consecutive sections using a grid-installed light microscopy (BX51; Olympus, Tokyo, Japan). Three visual fields of the hippocampus (CA1, CA3, and DG areas) were found in each slice at low magnification and were used as statistical areas for counting neurons with nuclei. The average of six sections for every region was taken as the final value for that region [27].

2.5. Preparation of hippocampal samples

The hippocampi were homogenized according to the method described in previous studies, with minor modifications [28,29]. The rats were killed following measurement of spatial memory. Next, the right hippocampi were separated and homogenized with 10 volumes of ice-cold 50mM Tris-HCl, pH 7.4, 2mM EDTA. The homogenates were centrifuged at 4,000 ×g for 10 min at 4°C, and the resulting supernatants were stored at −80°C for assaying the cholinergic function and biochemical assays, including AChE, ChAT, ACh, TNF-α, IL-1β, IL-10, SOD, MDA, GSH-Px, and ROS levels.

2.6. Analysis of cholinergic function

The activity of ChAT and AChE and the level of ACh in the hippocampal supernatant were measured using ChAT assay kit, AChE assay kit, and ACh assay kit, respectively, according to the manufacturer’s instructions.

2.7. Assays of oxidative damage

The activities of SOD and GSH-Px as well as the level of MDA were assayed according to the manufacturer’s instructions. The ROS content was measured by the products of 2’,7’-dichlorofluorescein (DCFH)-DA, as described previously. After the transfer into cells, DCFH-DA is cleaved to form DCFH, which in turn is transformed into highly fluorescent DCF upon reaction with ROS. DCF was quantified in each sample using a fluorescence monitor [30].

2.8. Detection of inflammatory cytokines

The inflammatory cytokine levels in the hippocampus were assessed using TNF-α, IL-1β, and IL-10 assay kits according to the manufacturer’s instructions. The concentrations of these inflammatory cytokines were calculated according to the optical density determined spectrophotometrically using a micro ELISA reader.
2.9. Statistical analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The differences between the groups were tested by ANOVA, followed by Fisher’s least significant difference post hoc test. For a single comparison, the significance of differences was determined by the t test. A value of \( p < 0.05 \) is considered to be statistically significant.

3. Results

3.1. Effect of ginsenoside Rb1 on cisplatin-induced object recognition memory impairment

After the completion of different treatment, rates were subjected to a series of behavioral tasks to assess their cognitive function. In the NORT test, as shown in Fig. 2, we found that control rats spent more time exploring the novel object than the familiar object \( (t_{18} = 6.12, p < 0.01; \text{Fig. 2A}) \). In contrast, cisplatin-treated rats could not distinguish between the novel and familiar objects \( (t_{18} = 1.08, p > 0.05) \). Rb1 treatment blocked cisplatin-induced memory impairment without affecting normal recognition function \( (\text{Cisp}, t_{18} = 3.86, p < 0.05; \text{Rb1}, t_{18} = 5.72, p < 0.01) \). The recognition index was significantly decreased in cisplatin-treated group, and this was restored by ginsenoside Rb1 \( [F(3,36) = 6.792, p < 0.01; \text{Fig. 2B}] \). All rats spent a similar amount of time on object exploration during training and test sessions, indicating that the significant differences of each group were not due to the alternation of exploratory behavior on objects \( (\text{Figs. 2C and 2D}) \). These results suggest that ginsenoside Rb1 improves cisplatin-induced memory impairment.

3.2. Effect of Rb1 on cisplatin-induced spatial memory deficit

We also detected the spatial cognitive abilities of rats using Morris water maze. We found that the cisplatin group rats spent more time to find the hidden platform from the 4th d compared with the control in the training period; however, the Cisp + Rb1 group rats showed learning latency similar to that of control rats \( (\text{Fig. 3A}) \). During the probe test, the cisplatin group rats showed poor memory retention, spent less time in the target quadrant \( [F(1,18) = 10.66, p < 0.01; \text{Fig. 3B}] \), and less crossing times of the platform’s previous location \( [F(1,18) = 6.14, p < 0.05; \text{Fig. 3C}] \) as compared with the controls. Similarly, the learning and memory impairment induced by cisplatin was significantly arrested by ginsenoside Rb1 \( [F(1,18) = 6.14, p < 0.05] \). The rats could remember the previous platform location and exhibit more exploring time in target quadrant \( [F(1,18) = 10.24, p < 0.05] \). Throughout the tasks, the swimming speed of rats among each group showed no significant difference, indicating that the effect of drugs is on learning and memory rather than swimming ability \( (\text{Fig. 3D}) \).

3.3. Ginsenoside Rb1 promotes neuronal survival in the hippocampus

As we know, hippocampus is an important structure for learning and memory [31], and selective hippocampal lesions can induce place and cue memory impairment [32]. In this study, the behavioral tests showed most obvious cognition impairment in mice, and we thought these changes were related with hippocampus. The effect of cisplatin or/and ginsenoside Rb1 on the neuronal population of hippocampus was detected via Nissl staining. A large
number of neuron cell bodies were seen at the CA1, CA3, and DG areas in hippocampi, distributed with big and round nuclei. The results of statistics revealed a significant reduction of hippocampal neuronal population in the cisplatin-treated group \( F(3,12) = 4.46, p < 0.05 \). The least significant difference post hoc test revealed a considerable reduction in the hippocampal neuronal population of cisplatin animals \( p < 0.05 \). Ginsenoside Rb1 treatment increased the survival of neurons in the hippocampus of rats \( p < 0.05 \), whereas ginsenoside Rb1 single treatment showed no significant effect compared with the controls (Fig. 4).

3.4. Ginsenoside Rb1 rescues cholinergic dysfunction induced by cisplatin

Cholinergic dysfunction is a primary cause related to cognitive degeneration; therefore, we measured the ACh level and AChE and ChAT activities in the hippocampi of rats. From Fig. 5, we found that cisplatin caused a significant decrease in ACh levels (control, 18.68 ± 1.12 μg/mg; cisplatin, 10.69 ± 1.54 μg/mg, \( p < 0.01 \)), increased AChE activity (control, 0.84 ± 0.05 U/mg; cisplatin, 1.192 ± 0.09 U/mg, \( p < 0.01 \)), and a dramatic reduction in ChAT activity (control, 276.70 ± 14.71 U/mg; cisplatin, 209.26 ± 20.68 U/mg, \( p < 0.01 \)) in the hippocampus compared with the controls. These results verified that cisplatin impairs cholinergic function. The treatment of ginsenoside Rb1 could rescue these cholinergic abnormalities, as indicated by elevated ACh levels (16.57 ± 1.55 μg/mg, \( p < 0.01 \)), increased ChAT activity (269.42 ± 18.46 U/mg, \( p < 0.05 \)), and reduced AChE activity (1.04 ± 0.04 U/mg, \( p < 0.01 \)). Meanwhile, administration of ginsenoside Rb1 alone did not alter cholinergic dysfunction significantly. These data support the behavioral test results and suggest that ginsenoside Rb1 may exert its neuroprotection by rescuing cholinergic function.

3.5. Ginsenoside Rb1 reduces oxidative stress in hippocampus

It is reported that cisplatin can interrupt the cholinergic neurotransmitter through trigger peroxidation and can cause memory impairment [33,34]; therefore, we measured the activity of SOD and GSH-Px as well as the levels of MDA and ROS in the brains of rats (Fig. 6). We found cisplatin treatment decreased the
activities of SOD (control, 12.23 ± 0.41 U/mg; Cisp, 8.12 ± 1.12 U/mg, p < 0.01) and GSH-Px (control, 6.58 ± 0.31 U/mg; Cisp, 3.88 ± 0.25 U/mg, p < 0.01) compared with the treatment for control group, whereas ginsenoside Rb1 significantly increased the activities of SOD (10.22 ± 0.42 U/mg) and GSH-Px (4.91 ± 0.29 U/mg) compared with the Cisp group (p < 0.05). MDA and ROS levels significantly increased in the brain tissue of the cisplatin group rats compared with the control group rats (p < 0.01); ginsenoside Rb1 administration ameliorated this increase (p < 0.05 and p < 0.01).

3.6. Ginsenoside Rb1 attenuates the cisplatin-induced inflammatory response

At last, we detected the changes in the hippocampus interleukin levels using ELISA kits. The results showed that cisplatin significantly enhanced the levels of TNF-α and IL-1β (p < 0.01), implying stronger inflammatory response, which was prevented by Rb1 treatment (p < 0.01 and p < 0.05; Figs. 7A and 7B). In contrast, the anti-inflammatory IL-10 level in the cisplatin group showed a decline compared with that in the control group (p < 0.01; Fig. 7C), and Rb1 partly arrested the effect of cisplatin. We did not find significant difference in all inflammatory factor levels between the rats treated with Ginsenoside Rb1 alone and the controls, neither.

4. Discussion

As a platinum-based compound, cisplatin was widely used as an anticancer chemotherapeutic agent and was reported to elicit a lot of side-effects, including hepatotoxicity [35], nephrotoxicity [36], ototoxicity [37], neurotoxicity [38], and nausea/vomiting [39]. Among them, neurotoxicity is particularly important. In this study, we first evaluated the learning and memory abilities by NORT and Morris water maze task. We reported that cisplatin-treated rats displayed lower discrimination ratio in the novel object recognition and longer swimming latency, and shorter duration and less crossing times in the Morris water maze task, suggesting impairment in the learning and memory induced by cisplatin. These findings are consistent with those of previous reports [18,40,41]. Meanwhile, we also observed that upon the treatment of cisplatin, the neuron numbers were reduced in the different subregions of hippocampus. In accordance to our finding, cisplatin was found to cause a dramatic decrement in the number of DCX-positive neurons in the DG region of the adult rat hippocampus when compared with vehicle-treated ones [42]. Furthermore, the hypernitration and LMO4 down-regulation play important roles in cisplatin-induced neuronal apoptosis [43]. Thus, the loss of neurons upon cisplatin treatment may be due to the reduction of neurogenesis and promotion of neuron apoptosis. In our study, we also demonstrated that application of Rb1 rescued the memory impairments both in the novel object recognition and Morris water maze. Simultaneously, the neuron numbers were restored after the Rb1 treatment, suggesting neuroprotection of Rb1. As reported before, Rb1 was shown to inhibit cell death caused by kainic acid in both CA1 and CA3 regions of rat hippocampus and protected hippocampal neurons from ischemia [44]. Rb1 injection also increases brain-derived neurotrophic factor level, reduces caspase-3...
activity, and promotes neurogenesis in the hippocampus [45]. Then, the application of Rb1 could both enhance the neurogenesis and protect against neuron apoptosis, which is effective in neuroprotection upon cisplatin insult.

The neurotoxic effects of cisplatin have been widely studied, and it is suggested that the overproduction of ROS [6], high levels of Pt-DNA binding and the following neuronal apoptosis are the main reasons [46]. It is known that ROS is mainly generated from the mitochondria, and exposure to cisplatin results in the increment of intracellular ROS in normal cells. For example, an in vivo study has shown that cisplatin exposure induces an increase in the activity of SOD and MDA, and also levels of H$_2$O$_2$, and a reduction in glutathione (GSH) and activity of GSH reductase in the cochlear [47]. Another in vitro study found that cisplatin could initiate ROS response via a mitochondria-dependent pathway by directly affecting the mitochondrial DNA. This impairs the synthesis of electron transport chain proteins and accelerates the nuclear DNA impairments, which leads to the enhancement of cytotoxic effect [48]. Thus, application of molecules with antioxidant property will be beneficial to protect the neurotoxicity induced by cisplatin. Here, we also detected the oxidative stress level on cisplatin administration. A reduction in the activities of SOD and GSH-Px and elevation in the levels of MDA and ROS were found after the cisplatin treatment. This indicated that the overproduction of oxidative free radicals and inhibition of anti-oxidative enzymes in the hippocampus were induced by cisplatin. However, treatment of ginsenoside Rb1 significantly reduced the oxidative stress level as demonstrated by restoring the activities of SOD and GSH-Px, suppressing the MDA and ROS. In many previous studies, Rb1 had been shown to protect against oxidative insult in numerous neurological disorders [49]. The possible mechanism by which Rb1 protects against the oxidative insult is via Nrf2 pathway activation and G1PI3K/Akt pathway in ER-dependent manner and heme oxygenase-1 pathway [50,51].

In the current study, we also reported that Rb1 alleviates the cisplatin-induced neuroinflammation by reducing the levels of TNF-α and IL-1β and restoring the level of IL-10. In some previous studies, cisplatin was found to stimulate the generation of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and transforming growth factor-β1 [52,53]. TNF-α is a prototypical inflammatory cytokine; it activates other cytokines [54] to mediate the cisplatin-induced renal injury [55]. In a mice obesity model induced by high-fat diet, Rb1 supplement decreased the levels of TNF-α, IL-6, and/or IL-1β [56]. Additionally, the reduction of TNF-α expression by Rb1 could inhibit the severity of inflammatory response in collagen-induced arthritis mice [57]. It has also been shown that IL-10 acts as the anti-inflammatory cytokine to protect against acute renal injury induced by cisplatin [58,59]. And Rb1 treatment attenuated carbon tetrachloride-induced liver fibrosis by elevating IL-10 level in rats [60]. Thus, the Rb1 application could rebalance the inflammation and anti-inflammation factors in the hippocampus and then exert its neuroprotective effects. Furthermore, Rb1 administration here decreased the dysfunction of cholinergic neurons by restoring the ACh level, ChAT activity, and reducing the AChE activity. The physiological cholinergic signaling could modulate peripheral cytokine production by activation of the cholinergic anti-inflammatory pathway. Thus, the rescuing
cholinergic function also participates in the neuroprotection of Rb1 on cisplatin-induced neuroinflammation. Conclusively, we investigated the neuroprotective effects of ginsenoside Rb1 on cisplatin-treated rats. We demonstrated that Rb1 markedly improved the cognitive decline and neuronal loss by inhibition of oxidative stress, rescuing the cholinergic function, and suppression of neuroinflammation in the hippocampus. Taken together, we concluded that Rb1 might be used as a potent neuroprotector against cisplatin-induced learning and memory impairments.

Conflicts of interest

The authors have declared that there is no conflict of interest.

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