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Effects of allantoin on cognitive function and hippocampal neurogenesis

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ABSTRACT

Allantoin is contained in *Nelumbo nucifera* (lotus) and a well-known cosmetic ingredient reported to have anti-oxidative and anti-inflammatory activities. In the present study, we investigated whether allantoin affects cognitive function in mice. The subchronic administration of allantoin (1, 3 or 10 mg/kg, for 7 days) significantly increased the latency time measured during the passive avoidance task in scopolamine-induced cholinergic blockade and normal naïve mice. Allantoin treatment (3 or 10 mg/kg, for 7 days) also increased the expression levels of phosphorylated phosphatidylinositide 3-kinase (PI3K), phosphorylated protein kinase B (Akt) and phosphorylated glycogen synthase kinase-3β (GSK-3β). Doublecortin and 5-bromo-2-deoxyuridine immunostaining revealed that allantoin significantly increased the neuronal cell proliferation of immature neurons in the hippocampal dentate gyrus region. In conclusion, allantoin has memory-enhancing effects, and these effects may be partly mediated by the PI3K-Akt-GSK-3β signal pathway. These findings suggest that allantoin has therapeutic potential for the cognitive dysfunctions observed in Alzheimer's disease.

1. Introduction

As the elderly population increases, the numbers of patients with Alzheimer's disease (AD) also increases. AD is one of neurodegenerative disorders as the main part of dementia (Drever et al., 2008). Currently, the primary treatment in AD therapy consists of an acetylcholinesterase inhibitor, such as donepezil, galantamine or rivastigmine (Isik et al., 2012). Even though monoclonal antibodies against the amyloid-β (Aβ) protein fail to improve cognitive function in AD patients (Imbimbo, 2009), the Aβ protein is still one of targets in AD therapy because it causes oxidative stress and inflammation in the brain (Chan et al., 2012). Other studies have reported that Aβ protein-induced cognitive deficits can be rescued by regulating oxidative stress and by inflammatory mediators (Zhang et al., 2013). Therefore, any compound that has anti-oxidative or anti-inflammatory effects would be considered a potential candidate for AD therapy.

Nelumbo nucifera (lotus) is widely cultivated in Asia, north Australia, Egypt and the Caspian sea, and its rhizome has long been used as a food source (Huang et al., 2011). The rhizome of *N. nucifera* has also been used to treat diarrhea, hemorrhage and constipation (Talukder and Nessa, 1998). Recently, it has been reported that *N. nucifera* rhizome extract improves learning and memory behavior in a rodent model (Yang et al., 2008). However, it is unclear which compound(s) is responsible for this cognitive activity. We aimed to isolate the active compounds responsible for improving these cognitive functions using activity-guided fractionation and identified allantoin. Allantoin can be isolated not only from *N. nucifera* rhizome but also from many other plant species, such as sugar beet and leguminous plants (Todd et al., 2006). Allantoin is a purine-derived compound that is known to be safe and non-toxic (Bordoni et al., 2005). Furthermore, allantoin is a well-known cosmetic ingredient that has anti-oxidative and anti-inflammatory activities (Doi et al., 2009; Huang et al., 2011; Jin et al., 2012). If allantoin can improve cognitive performance, then it would be considered a potential treatment for the cognitive dysfunctions observed in AD patients because of its anti-inflammatory and anti-oxidative effects and its non-toxicity (Bordoni et al., 2005).

Abbreviations: Aβ, amyloid-β; AChE, acetylcholinesterase; AD, Alzheimer's disease; ANOVA, analysis of variance; BrdU, 5-bromo-2-deoxyuridine; ChAT, choline acetyltransferase; DCX, doublecortin; DG, dentate gyrus; GSK-3β, glycogen synthase kinase-3β; LTD, long-term depression; LTP, long-term potentiation; PI3K, phosphatidylinositide 3-kinase; SGZ, subgranular zone.

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Currently, no studies investigating whether allantoin has cognitive-enhancing or cognitive-ameliorating activity have been reported.

In this study, we investigated the effect of allantoin on memory enhancement using the passive avoidance task in mice. To uncover the mechanism regulating of allantoin-mediated memory enhancement, we used Western blotting to measure the expression levels of signal molecules, including phosphatidylinositide 3-kinase (PI3K) and its downstream signal molecules in hippocampal tissue. In addition, we investigated the effects of allantoin on neurogenesis in the dentate gyrus (DG) region of hippocampus.

2. Materials and methods

2.1. Animals

Male ICR mice weighing 25–27 g (6 weeks old) were purchased from the Orient Co., Ltd., which is a branch of Charles River Laboratories (Gyeonggi, Korea). The animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care-international (AAALAC-i) and provided with food and water ad libitum. The animal facility was maintained at a constant temperature (23 ± 1 °C) and humidity (60 ± 10%) under a 12 h light/dark cycle (light on between 07:30 and 19:30 h). All of the behavioral tasks were conducted between 10:00 h and 16:00 h. Animal treatment and care were conducted in accordance with the Animal Care and Use Guidelines, and the experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) (approval No. KHP-2011-07-03). The number of animals used in each group for behavioral tests was described in Table 1.

2.2. Materials

Allantoin, donepezil hydrochloride monohydrate, (-)-scopolamine hydrobromide, piracetam, 5-bromo-2-deoxyuridine (BrdU) and Tween 80 were purchased from Sigma–Aldrich (St. Louis, MO). Zoletil 50® was obtained from Virbac (06516 Carros, France). A complete protease inhibitor cocktail and the PhosSTOP phosphatase inhibitor cocktail were purchased from Roche (Palo Alto, CA). Goat polyclonal anti-doublecortin (DCX), rabbit polyclonal anti-protein kinase B (Akt) and rabbit polyclonal anti-glycogen synthase kinase-3β (GSK-3β) antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Rabbit polyclonal anti-phosphorylated Akt and rabbit polyclonal anti-phosphorylated GSK-3β (pGSK-3β) at Ser 9 antibodies were purchased from Cell Signaling Technology (Cell Signaling, MA). Rat anti-BrdU antibody was purchased from Abcam (Cambridge, UK). An avidin–biotin peroxidase complex (ABC) kit was purchased from Vector Laboratories (Burlingame, CA). All other chemicals for immunostaining and Western blotting were of the highest grade available and were obtained from Ducksan Co. (Seoul, Korea) or Sigma–Aldrich (St. Louis, MO).

Table 1
Number of animals used in each group.

Experiment	Group	Number of animals
Passive avoidance test (Normal model)	Control	9
	Allantoin (1)	8
	Allantoin (3)	10
	Allantoin (10)	8
	Piracetam (200)	8
Passive avoidance test (Scopolamine model)	Control	10
	Vehicle + Scopolamine (1)	8
	Allantoin (1) + Scopolamine (1)	8
	Allantoin (3) + Scopolamine (1)	10
	Allantoin (10) + Scopolamine (1)	9
	Donepezil (5) + Scopolamine (1)	10
Open field test	Control	8
	Allantoin (1)	8
	Allantoin (3)	8
	Allantoin (10)	8

The number in parenthesis indicates the dose administered (mg/kg).

2.3. Drug administration

Allantoin was dissolved in 10% Tween 80 solution. Donepezil hydrochloride monohydrate or (-)-scopolamine hydrobromide was dissolved in 0.9% saline solution. All drugs for oral administration via oral gavage were dissolved as mg of drug/kg/5 ml of each vehicle solution. As shown in Fig. 1A, for memory ameliorating study, mice were administered vehicle solution (an equal volume of the 10% Tween 80 solution), allantoin (1, 3 or 10 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) at the same time (10:00–12:00 a.m) and same place for 7 days. For memory enhancing study, mice were administered vehicle solution, allantoin (1, 3 or 10 mg/kg, p.o.) or piracetam (200 mg/kg, i.p.). The final administration of allantoin, donepezil or piracetam was performed 1 h before an acquisition trial in the passive avoidance task. In a separate experiment, mice were administered allantoin (1, 3 or 10 mg/kg, p.o.) for 7 days and sacrificed 1 h after the final dose administration for Western blotting. To investigate the effect of allantoin-induced neuronal cell proliferation, BrdU (50 mg/kg, i.p.) was dissolved in 50 mM phosphate-buffered saline (PBS) and was administered on the 6th day (3 times, at 2 h intervals) after the allantoin administration (Fig. 1B).

2.4. Passive avoidance task

Passive avoidance tasks were conducted in two identical light and dark Plexiglas square boxes (20 cm × 20 cm × 20 cm, respectively) which were separated by a guillotine door (5 cm × 5 cm), as described elsewhere (Kim et al., 2008). The light compartment contained a 50 W bulb, and its floor was composed of 2 mm steel rods spaced 1 cm apart. The floor of the non-illuminated, dark compartment also consisted of 2 mm steel rods spaced 1 cm apart. The animals underwent two separate trials: an acquisition trial and a retention trial 24 h later. For the acquisition trial, mice were initially placed in the illuminated compartment, and the door between the two compartments was opened 10 s later. When the mice entered the dark compartment, the door automatically closed, and an electrical foot shock (0.5 mA) was delivered through the stainless steel rods for 3 s. If a mouse did not enter the dark compartment within 60 s, the mouse was gently forced to enter the dark compartment. The other procedure was the same as described above. In this case, training trial was assigned a latency of 60 s (ceiling score). One hour before the acquisition trial, the final administration of allantoin, donepezil or vehicle solution was administered to the test mice. To induce memory impairment, mice were treated with scopolamine (1 mg/kg, i.p.) or 0.9% saline 30 min before the acquisition trial. Twenty-four hours after the acquisition trial, a retention trial was conducted by placing each mouse in the light compartment. The time taken for a mouse to enter the dark compartment after opening the door was defined as the latency for the acquisition and retention trials. The latency prior to entering the dark compartment was recorded for up to 300 s.

To investigate the memory enhancing effect, allantoin (1, 3 or 10 mg/kg) or piracetam (200 mg/kg, i.p.) was administered 1 h before the acquisition trial without scopolamine. When the mice entered the dark compartment, the door automatically closed, and an electrical foot shock (0.25 mA) was delivered through the stainless steel rods for 3 s. Latencies were recorded for up to 600 s to avoid ceiling effects, and the other procedures were the same as those previously described.

2.5. Open field test

The open field test was carried out in cleared black Plexiglas boxes (45 cm × 45 cm × 45 cm) equipped with the video-based Ethovision System (Noldus, Wageningen, The Netherlands), as previously described (Lee et al., 2013b). To evaluate horizontal locomotor activity, mice were placed in the center of the apparatus, and the locomotor behaviors were recorded for 30 min using the video-tracking system. The horizontal locomotor activity was expressed as the total ambulatory distance. Mice were administered allantoin (1, 3, or 10 mg/kg, p.o.) for 7 days. Each box was cleaned with 70% ethanol between each subject.

2.6. Tissue preparation

Mice were anesthetized with Zoletil 50® (10 mg/kg, i.m.) at 7 days after the administration of allantoin and were perfused transcardially with 50 mM PBS (pH 7.4) followed by ice-cold 4% paraformaldehyde for prefixation. The brains were removed, postfixed in phosphate buffer (50 mM, pH 7.4) containing 4% paraformaldehyde overnight, immersed in a 30% sucrose solution (in 50 mM PBS), and stored at 4 °C until sectioned. Frozen brains were coronally sectioned at a thickness of 30 μm using a cryostat, and the sections were stored in a storage solution at 4 °C.

2.7. Immunohistochemistry

Every tenth section from −1.50 mm posterior to the bregma was selected to obtain anatomically matched section according to the Atlas of mouse brain (Paxinos and Franklin, 2004). For BrdU immunohistochemistry as described elsewhere (Kim et al., 2010), free-floating sections were rinsed extensively with PBS, and the sections were incubated for 30 min in HCl (1 N) at 37 °C to denature DNA. Thereafter, the sections were neutralized for 20 min in borate buffer (0.1 M, pH