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SHORT COMMUNICATION

Chronic Intraperitoneal and Oral Treatments with Hesperidin Induce Central Nervous System Effects in Mice

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Hesperidin (HN) is a flavanone glycoside abundantly found in citrus fruits. This flavonoid mediated central nervous system activity following intraperitoneal (i.p.) acute treatment. The responses of mice after the chronic i.p. (4 and 30 mg/kg/day) or the oral intake administration of this drug (20, 50 and 100 mg/kg/day) were studied by using the holeboard, the plus-maze and the locomotor activity tests. Hesperidin, chronically administered by the i.p. route, exerted a decrease in the locomotor and exploratory activities, thus evidencing a depressant activity. In turn, the chronic oral intake of this flavonoid induced anxiolytic-like effects. These varied responses could be attributed to the different routes of administration that could lead to the production of diverse active metabolites. **Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: hesperidin; central nervous system; depressant action; anxiolytic activity; intraperitoneal treatment; oral intake

INTRODUCTION

Flavanones are the main type of flavonoids present in citrus fruits and juices. They rarely occur in other food in appreciable quantities. Hesperidin (HN, hesperetin-7-rutinoside) is a flavanone glycoside found abundantly in citrus fruits such as lemons and oranges (Ross and Kasum, 2002). In human and animal studies it contributes to the reduction of cholesterol (Park *et al*., 2001), blood pressure (Yamamoto *et al*., 2008) and bone density loss (Chiba *et al*., 2003). It was also reported to provide health benefits, including antioxidant, antiinflammatory and anticarcinogenic properties (Nielsen *et al*., 2006).

We have previously reported the presence of HN in the roots and rhizomes of *Valeriana officinalis* and *V. wallichii* and, for the first time, described its remarkable activity on the central nervous system (CNS) (Marder *et al*., 2003). The depressant and antinociceptive activities in mice after acute intraperitoneal (i.p.) administration of HN have been reported extensively. Also, the possible beneficial use of the association of HN with benzodiazepines, not only to improve sedation but also in the management of pain, has been suggested, with the additional advantage of the known lack of toxicity and side effects of HN (Fernández *et al*., 2005; Loscalzo *et al*., 2008).

We have also demonstrated that HN's depressant action in mice could be partially blocked by naltrexone, a non-selective opioid receptor antagonist and by norbinaltorphimine, a potent and highly selective κ-opioid receptor antagonist. These results indicate that the depressant effect of HN is mediated, at least partially, by an opioid mechanism of action (Loscalzo *et al*., 2008). Subsequently, it has been suggested that the participation of adenosine receptors might be involved in the sedative action of HN (Guzmán-Gutiérrez and Navarrete, 2009).

Notwithstanding the evidence for HN mediated CNS activity on acute treatments there is no information on its action following chronic administration. The present study was designed to examine the efficacy of HN chronically administered by the i.p. route or after its oral intake, thus closely mimicking the oral route conditions in humans.

MATERIALS AND METHODS

Animals. Adult male Swiss mice weighing 25–30 g were obtained from the Central Animal House of the School of Pharmacy and Biochemistry, University of Buenos Aires. Mice were housed in groups of four or five in a controlled environment (20–23 °C), with free access to food and liquid (water, a solution of diazepam (DZ) in water, a solution of carboxymethyl cellulose (CMC) 0.05%, or HN in CMC 0.05%); and maintained on a 12 h/12 h day/night cycle, light on at 06:00 a.m. Housing, handling, and experimental procedures complied with the recommendations set forth by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985) and CICUAL (Institutional Committee for the Care and Use of Laboratory Animals, University of Buenos Aires, Argentina). All efforts were taken in order to

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minimize animal suffering. The number of animals used was the minimum number consistent with obtaining significant data. The animals were randomly assigned to any treatment groups and were used only once. The behavioral experiments were performed between 10:00 a.m. and 2:00 p.m. in an arrangement of 24 h apart between behavioral tests.

Drug solutions and experimental procedures. Hesperidin was obtained from Exquim S.A. (Zoster S.A., Spain) and DZ was obtained from Roche Diagnostics (Argentina, purity 99.9%).

For the treatment developed by the intraperitoneally (i.p.) route, HN and DZ were dissolved by using the sequential addition of dimethylsulphoxide, a solution of 0.25% Tween 80 and saline; up to final concentrations of 5%, 20% and 75%, respectively. Mice were administered with a single i.p. injection with vehicle or a solution of HN (4 and 30 mg/kg) or DZ (0.3, 1 and 10 mg/kg) 20 min before the beginning of the test in the acute treatments. For the chronic treatment mice received daily either an i.p. injection of vehicle or the drug for 10 days, at 10:00 a.m. The day of the assay mice were injected 20 min prior to testing. The volume of i.p. injections was 0.15 mL/30 g of the body weight.

In the oral study six different groups of mice were treated in parallel with: water, CMC 0.05%, DZ in water, HN in CMC 0.05% (0.1 mg/mL; 0.25 mg/mL and 0.5 mg/mL) as their sole source of drinking fluid for 14 days before performing the tests.

The volume of liquid consumed daily by mice was approximately 200 mL/kg, and no significant differences were found among all the experimental groups. The concentration of HN and DZ in the liquid was determined from the average daily liquid consumption (× mL/mouse/day), and the average body weight per mouse (g/mouse) to achieve the desired doses. Thus, the dose of HN ingested per day was 20 mg/kg, 50 mg/kg or 100 mg/kg. The concentration of the solution of DZ was 0.1 mg/mL, so the dose of DZ was 20 mg/kg per day.

Body weight, food intake and liquid consumption were determined every 2 days and did not differ among the groups (data not shown). The drug solutions were replaced every 2 days.

Behavioral studies. *Holeboard assay.* This assay was conducted in a walled black Plexiglass arena with a floor of 60 cm × 60 cm and 30 cm high walls, with four centred and equally spaced holes in the floor, 2 cm in diameter and illuminated by a dim indirect light. Each hole housed an infrared emitting diode and an infrared detector oriented along a diameter and perfectly aligned. Each of these pairs forms a hole exploration sensor, as previously described (Fernández *et al*., 2006). Mice were placed in the center of the holeboard and allowed to freely explore the apparatus for 5 min. The number of holes explored and the duration of the explorations were measured automatically and shown in real time to the observer. At the end of the experiment all the information was stored in a file for post-experiment study. The number of mouse "rearings" was detected visually and recorded by the observer, who was blinded to the drug treatments.

Elevated plus-maze test. The elevated plus-maze setup consisted of a maze of two open arms, 25 × 5 cm, crossed by two closed arms of the same dimensions, with free access to all arms from the crossing point. The closed arms had walls 15 cm high all around. The maze was suspended 50 cm from the room floor. Mice were placed on the central part of the cross facing an open arm. The number of entries and the time spent going into open arms were counted during 5 min under dim red light. The total exploratory activity (number of entries in both arms) was also determined (Lister, 1987). The observer recording the plus-maze parameters was blinded to the drug treatments.

Locomotor activity test. The spontaneous locomotion activity was measured in a box made of Plexiglass, with a floor of 30 cm by 15 cm and 15 cm high walls with 15 infrared emitting diodes and 15 infrared detectors arranged in perfectly aligned pairs, which form movement sensors, as previously described (Fernández *et al*., 2006). The sensor interruptions measure the animal activity along a single axis. The data were shown in real time to the operator and at the end of the experiment all the information was automatically stored in a file for post-experiment study. The locomotor activity was expressed as total light beam counts per 5 min.

Statistical analyses. The effects of the compounds in mice after the oral intake of the drugs were analysed by one-way analysis of variance (ANOVA) and *post hoc* comparisons between treatments and vehicle were made using the Dunnett multiple comparison test (GraphPad Prism program version 5.0). For the i.p. administration, acute and chronic treatments were analysed by two-way ANOVA followed by Sidak's multiple comparison test (SPSS software version 14.0). Significance levels were set at $p < 0.05$.

RESULTS AND DISCUSSION

The data presented in this work confirm the depressant action of HN after its acute i.p. administration in mice previously reported (Marder *et al*., 2003; Fernández *et al*., 2006; Loscalzo *et al*., 2008). A comparison of the acute and chronic i.p. treatments with HN in mice is shown in Fig. 1. The acute administration of DZ, the reference drug, produced a decrease in the parameters measured in the holeboard and locomotor activity assays at 10 mg/kg, according to the sedative effect of this benzodiazepine. The injection of 10 mg/kg of DZ for 10 days displayed tolerance to the sedative effect. The chronic treatment with 1 mg/kg and 10 mg/kg of DZ produced an increase in the number of rearings elicited by mice (Fig. 1C), evidencing the remnant anxiolytic effect of this drug. The depressant action of the acute and chronic treatments with 4 mg/kg and 30 mg/kg of HN was evidenced by the reduction in all the parameters measured in the holeboard and locomotor activity tests. The chronic i.p. administration of 4 mg/kg of HN did not develop tolerance, and additionally this treatment improved the depressant action (Fig. 1C). The rather high dosage of 30 mg/kg of HN chronically administered developed tolerance only to the locomotor effect (Fig. 1D).

Route of administration is one of the most important factors affecting the results of *in vivo* effects. The choice of administration route should depend upon the

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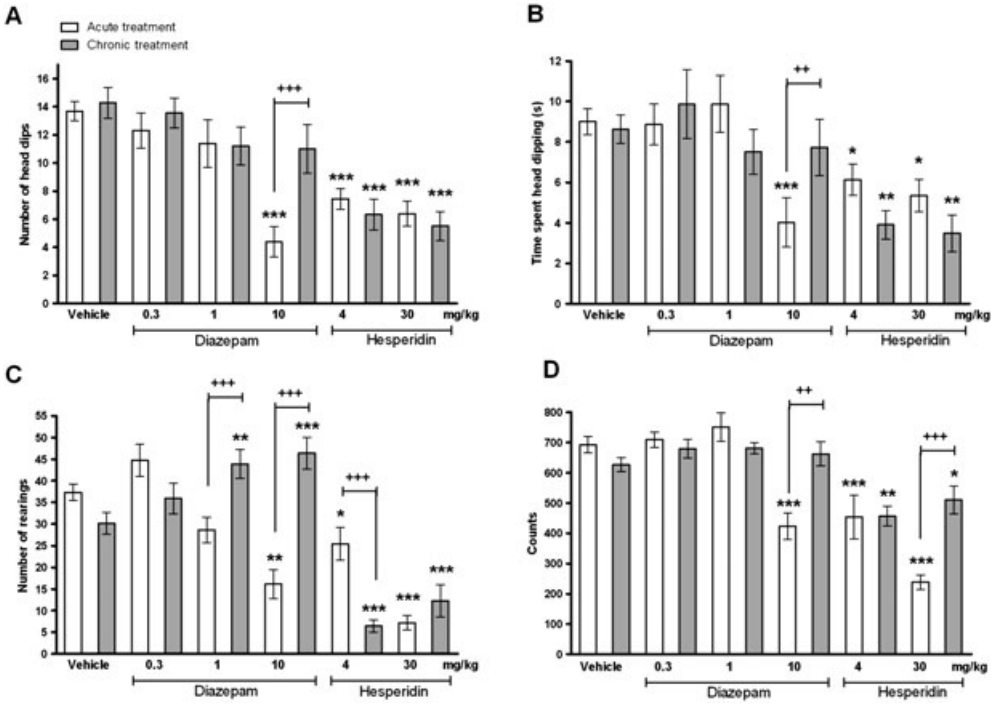

Figure 1. Effects of acute and chronic intraperitoneal administration of hesperidin and diazepam in the holeboard and locomotor activity tests in mice. Results are expressed as mean ± SEM of (A) number of holes explores, (B) time spent head dipping, (C) number of rearings measured in the holeboard test and (D) spontaneous locomotor activity counts: registered in 5-min sessions. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ denote significantly different from the corresponding vehicle; $^{+++}p < 0.001$, $^{++}p < 0.01$ denote significant difference between acute and chronic treatment, Sidak's multiple comparison test after two-way ANOVA (n = 6–30 mice/group).

purpose of the assay and the nature of the test substance. We have found that up to 300 mg/kg of HN tested 1 h and 4 h after its acute administration by the oral route produced no effects in the holeboard and locomotor activity tests (data not shown). Hesperidin is proposed to have limited bioavailability due to the rutinoside moiety attached to the flavonoid. There is also much controversy as to whether natural flavonoid glycosides can be absorbed by the gastrointestinal tract, or whether they are hydrolysed in the small intestine prior to absorption. Thus, absorption and metabolism of flavonoids are complex processes that determine its bioavailability, which until now remains unclear (Londoño-Londoño *et al.*, 2010). Several studies have reported that dietary HN was converted to conjugated metabolites, such as hesperetin glucuronides and sulphoglucuronides during absorption and metabolism (Matsumoto *et al.*, 2004). Circulating glucuronides, sulphates and O-methylated forms are believed to be those most likely to exert bioactivity and express beneficial effects in humans and animals (Spencer *et al.*, 2004).

To determine the effect of the regular intake of HN, this drug and DZ were administered for 14 days prior to behavioural testing in mice, thus closely mimicking the oral route conditions in humans. The effect of the chronic ingestion of HN and DZ in the holeboard, plus-maze and locomotor activity assays are shown in Fig. 2. Twenty mg/kg/day of DZ for 14 days increased the number of holes explored in the holeboard test, according to its anxiolytic effects ($p < 0.05$, Fig. 2A). Fifty and 100 mg/kg/day of HN produced a significant increase in the number of rearings in the holeboard test ($p < 0.05$ and $p < 0.001$, respectively, Fig. 2A), providing evidence for an increase in vertical exploratory activity. An increase in locomotor/exploratory activity of mice was also shown in the plus-maze test, as evidenced by the number of closed arms and total arms explored (Fig. 2B), and by the number of counts measured in the locomotor activity test: only for the dose of 50 mg/kg ($p < 0.05$, Fig. 2C). Additionally, the chronic oral administration of 100 mg/kg/day of HN presented an anxiolytic-like effect, indicated by a reduction of fear to enter the open arms, and the time spent in these arms, of the elevated plus-maze test.

No significant differences in all the parameters measured in these tests were found between mice that ingested water and CMC 0.05%.

The present work demonstrated that HN, administered by the i.p. route in mice in acute and chronic treatments, produced depressant action on the locomotor and exploratory activities. In turn, the chronic oral intake of this flavonoid induced anxiolytic-like effects. These varied responses could be attributed to the different routes of administration that could lead to the production of diverse active metabolites.

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Conflict of Interest

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

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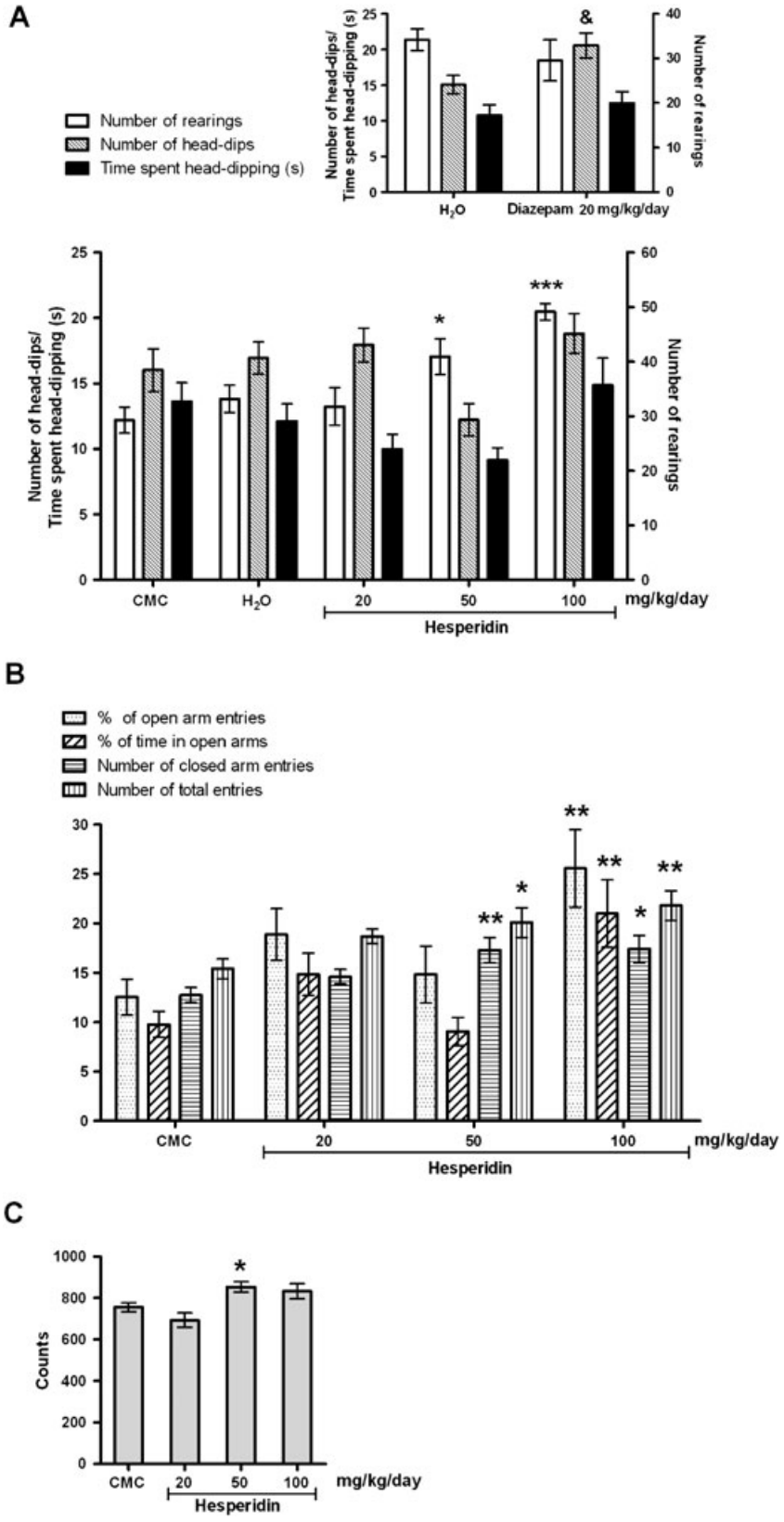

Figure 2. Effects of the chronic intake of hesperidin and diazepam in the holeboard, plus-maze and locomotor activity tests in mice. Results are expressed as mean ± SEM of (A) the holeboard parameters, (B) the plus-maze data and (C) spontaneous locomotor activity counts: registered in 5-min sessions. $***p < 0.001$, $**p < 0.01$, $*p < 0.05$ denote significantly different from the corresponding vehicle; Dunnett's multiple comparison test after ANOVA (n = 6–17 mice/group).

REFERENCES

Chiba H, Uehara M, Wu J, *et al*. 2003. Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. *J Nutr* **133**: 1892–1897.

Fernández SP, Wasowski C, Paladini AC, Marder M. 2005. Synergistic interaction between hesperidin, a natural flavonoid, and diazepam. *Eur J Pharmacol* **512**: 189–198.

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Fernández SP, Wasowski C, Paladini AC, Marder M. 2006. Central nervous system depressant action of flavonoid glycosides. *Eur J Pharmacol* **539**: 168–176.

Guzmán-Gutiérrez SL, Navarrete A. 2009. Pharmacological exploration of the sedative mechanism of hesperidin identified as the active principle of *Citrus sinensis* flowers. *Planta Med* **75**: 295–301.

Lister RG. 1987. The use of a plus maze to measure anxiety in the mouse. *Psychopharmacology (Berlin)* **92**: 180–185.

Londoño-Londoño J, Lima VR, Jaramillo C, Creczynski-Pasa T. 2010. Hesperidin and hesperetin membrane interaction: understanding the role of 7-O-glycoside moiety in flavonoids. *Arch Biochem Biophys* **499**: 6–16.

Loscalzo LM, Wasowski C, Paladini AC, Marder M. 2008. Opioid receptors are involved in the sedative and antinociceptive affects of hesperidin as well as in its potentiation with benzodiazepines. *Eur J Pharmacol* **580**: 306–13.

Marder M, Viola H, Wasowski C, Fernández S, Medina JH, Paladini AC. 2003. 6-Methylapigenin and hesperidin: new valeriana flavonoids with activity on the CNS. *Pharmacol Biochem Behav* **75**: 737–745.

Matsumoto H, Ikoma Y, Sugiura M, Yano M, Hasegawa Y. 2004. Identification and quantification of the conjugated metabolites derived from orally administered hesperidin in rat plasma. *J Agric Food Chem* **52**: 6653–6659.

Nielsen IL, Chee WS, Poulsen L, *et al*. 2006. Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. *J Nutr* **136**: 404–408.

Park YB, Do KM, Bok SH, Lee MK, Jeong TS, Choi MS. 2001. Interactive effect of hesperidin and vitamin E supplements on cholesterol metabolism in high cholesterol-fed rats. *Int J Vitam Nutr Res* **71**: 36–44.

Ross JA, Kasum CM. 2002. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* **22**: 19–34.

Spencer JP, Abd-el-Mohsen MM, Rice-Evans C. 2004. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. *Arch Biochem Biophys* **423**:148–161.

Yamamoto M, Suzuki A, Hase T. 2008. Short-term effects of glucosyl hesperidin and hesperetin on blood pressure and vascular endothelial function in spontaneously hypertensive rats. *J Nutr Sci Vitaminol (Tokyo)* ***54****: 95–98.*

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