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Research Article

The Contribution of Administration Techniques Related to Pseudo-Stress in the Preventive Effectiveness of Hesperidin Against Neurobehavioral and Immunological Disorders Induced by an Air Jet Stress in Wistar Rats

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ABSTRACT
Aggressors from outside the body appear to alter its equilibrium, usually associated with an adaptive response against an aggressor that remains unknown to the nervous system as it works as a sufficiently intensified stimulus, capable of activating pain centers and evoking memorial trauma. This study was conducted on male Wistar rats, a model of choice for the study of anxiety has shown the importance of non-invasive administration techniques for the treatment by hesperidin, including the oral administration technique. Compared with this administration technique, a month of hesperidin injections (40 mg/kg) did not show enough preventive ability against significant immune and behavioral alterations induced by 2 h of air jet in the cage of the animal identified by the leukocyte formula and elevated T-maze test. This study suggests that unlike oral administration, the handling and pain associated with repeated intra-peritoneal injections cause the generation of a pseudo-stress expressed by a significant increase of the obtained plasma levels of the adrenocorticotropin hormone (ACTH). These immune-endocrine mediations triggered after such a negative contact with the animal (injections) may have side effects on the efficiency of hesperidin by slowing the anxiolytic properties in addition to the less obvious impact of white cells due to the immune resistance to glucocorticoids.

Key words: Administration techniques; hesperidin; anxiety-like behavior; immunological disorders; ACTH; air jet stress

INTRODUCTION

Aggressors from outside the body appear to alter its equilibrium, usually associated with an adaptive response against an aggressor that remains unknown to the nervous system as it works as a sufficiently intensified stimulus, capable of activating pain centers and evoking memorial trauma[1,2].These stressors can be a result of environmental perturbations or social interactions. Regardless of the type of stressor, prolonged exposure to stress can have profound, long-lasting effects on physiology and behavior [3]. These changes are mediated by specific neuroendocrine mechanisms, including the activation of the hypothalamo-pituitary-adrenal (HPA) axis. Increased HPA activity in response to stress, and the resultant release of adrenal glucocorticoids, can exert substantial effects on physiology, including brain remodeling and immune modulation [3].

Such stimuli may be similar to the treatment of laboratory animals, for instance with natural antioxidants such as hesperidin. Although whether taken orally or by an intra-peritoneal injection, hesperidin represents an important way to reduce metabolic disorders and the abundance of free radicals caused by the action mode of stress hormones and cytokines on the oxidation of target cells [4-6]. Therefore, the handling and infection processes are likely to compromise the quality of the results obtained by blood numbering and behavioral tests aimed at demonstrating the effectiveness of hesperidin using any experimental protocol due to the negligence of choosing an administration technique that minimizes the anxiety state of the animal that is the subject of this study.

We studied this effect by using two different administration techniques (intra-peritoneal injection and oral administration) with the same hesperidin dose to treat leukocyte and behavioral alterations caused by psychogenic stress (the air jet stress) in Wistar.

MATERIALS AND METHODS

Animals and housing

Thirty-two Wistar rats with initial weights between 230-250 g were purchased from the Pasteur Institute (Algiers, Algeria) to carry out this protocol where they were housed in translucent cages and acclimated to the conditions including a constant temperature of (25 ± 1 °C) with a

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Table 1. Details of materials used

Name	Hesperidin
Source	Sigma, St. Louis, MO, USA
Synonym	Hesperetin 7-rhamnoglucoside, Hesperitin-7-rutinoside
Empirical Formula	$C_{28}H_{34}O_{15}$
Molecular Weight	610.56
Biochem/physiol Actions	Flavonoid phytochemical extracted from citrus species. It is the rhamnoglucoside (rutinoside) of hesperetin

Table 2. Plasma levels of ACTH in rats treated with the hesperidin (40mg/kg) by oral and intra-peritoneal routes.

Groups	ACTH (pg/ml)
T	$1,93\pm0,107^{C}$
S	$3,51\pm0,301$
IH	$2,92\pm0,06^{b}$
GH	$2,46\pm0,354^{C\,;\,\alpha}$

light/dark cycle of 12 h/12 h (lights on at 7:30 am). The rats had access to standard food and drinking water presented in ad libitum bottles. The study protocol was carried out according to the NIH revised Guidelines for the Care and Use of Laboratory Animals (no. 80–23, 1996).

The treatment and experimental groups

Thirty-two rats were divided into four groups (n = 8). The T group is the intact group. The GH group underwent a week of training receiving 2 ml of 5% sugar solution directly from the syringe. The animals adapted quickly to this procedure, which allowed the transition to oral administration of hesperidin (Sigma, St. Louis, MO, USA) at 40 mg/kg. The same dose is given to the IH group, but via intra-peritoneal injection (i.p.). The antioxidant treatment duration took one month before the application of the air jet stress, which occurred simultaneously in the S group. Recent advances in the field of psychological trauma suggest exploring methods to prevent the onset of anxiety disorders up to 30 days before their apparitions take place[7].

Air jet stress was chosen due to its recommendation by numerous scientific studies [8-14], and it is an emotional stressor consisting of creating a 2-h constant air pressure of 1 bar using a compressor equipped with a gauge in the rat cage through a side port. After the air jet stress session, the behavior of the four groups of animals was tested in elevated T-maze tests. The rats were then sacrificed by decapitation under mild anesthetic diethyl ether and the blood collection was carried out in ethylenediaminetetraacetic acid (EDTA) tubes to determine the total white blood cell count. The serum prepared immediately by centrifugation at 3,000×g for 15 min. The supernatant is used for the measurement of the adrenocorticotropin hormone (ACTH).

The elevated T-maze

This test is applied to determine the anxiety disorders in rodents according to their escape behavior[15]. The device is prepared as a result of the obstruction of one closed arm of the elevated plus-maze test with a heavy cardboard barrier (12 cm × 40 cm) [16]. Every rat was placed in the distal end of one of the two open arms and the time (latency) it takes to escape to the closed arms was measured in three consecutive sessions (escape 1, escape 2 and escape 3) at 30 second intervals. A maximum of 300 seconds was devoted for all sessions, indicating the end of the session if the animal does not display escape behavior. After each session, the rat was returned to its cage and the device was wiped with 70% ethanol.

White blood cell analysis

Total white blood cell count (WBC) was measured using a fully automated blood cell counter (PCE-210 model 2009, Japan).

ACTH

To measure the plasma levels of the ACTH hormone, ELISA tests were performed as directed by the manufacturers. The kit for ACTH assay was obtained from Phoenix Pharmaceuticals Inc. Burlingame, USA.

Statistical analysis

All data are expressed as the mean ± SEM (standard error of the mean). All groups showed normal distributions, so a parametric statistical method; the one-way analysis of variance (ANOVA), followed by the post-hoc Dunnett's test when necessary, was used for multiple comparisons. The value of $p<0.05$ was considered as significantly different. Data were analyzed using MINITAB (Minitab ® 15.1.1.0., Minitab Inc., USA).

RESULTS

Anxious behavior in the elevated T-maze test

Rats exposed to air jet stress have shown a significantly low escape latency compared with the T group rats ($p <0.001$), but no significant difference was noted during the three escape sessions between the S and IH groups. This is not the case with rats administered hesperidin orally for escape attempts 1, 2 and 3 ($p <0.001$). These results explain the difference in the GH group compared with the IH group during the three sessions of the test ($p <0.01$) for escape attempt 1 and ($p <0.001$) for escape attempts 2 and 3 (Fig. 1).

White blood cell count

The results of WBC formula show a highly significant decrease in rats exposed to air jet stress ($p <0.001$, Fig. 2) compared with the intact group T. between the GH and IH groups, there is a significant difference ($p <0.05$).

Plasma level of ACTH

The results are expressed as the mean ± SEM. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ vs. S; $^{\alpha}P <0.05$ IH vs. GH.

The results after two hours of air jet stress show a significant increase in the plasma levels of ACTH compared with groups T, GH ($p <0.001$) and IH ($p<0.01$). However there is significant difference between the GH group and IH group ($p<0.05$).

DISCUSSION

Hesperidin (HN, hesperetin-7-rutinoside) is a flavanone glycoside abundant in the fruits of the citrus family such as oranges and lemons [17]. Experimental applications of this substance on animals and humans have many beneficial effects including antioxidant and anti-inflammatory

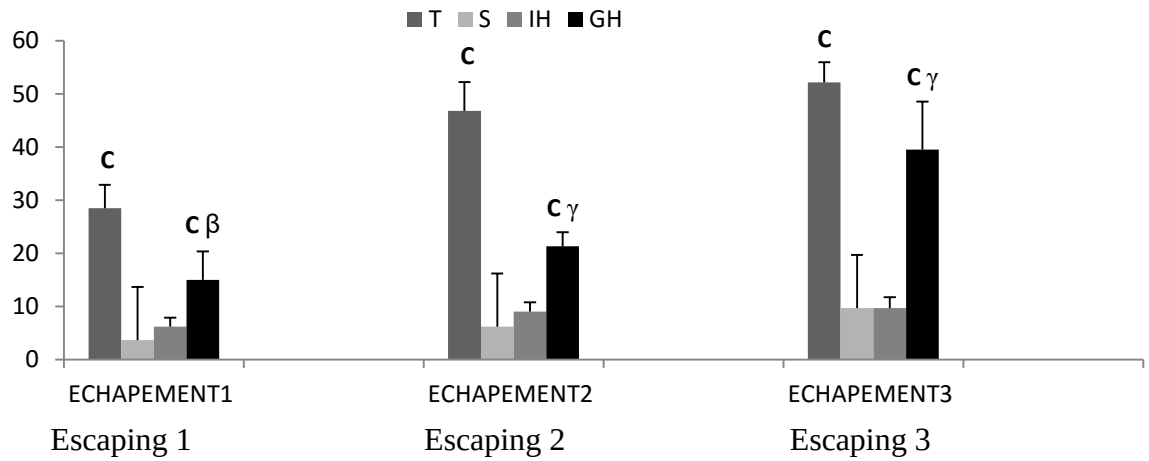

Figure 1: The parameters in the elevated T-maze test among rats pretreated with hesperidin by oral and intra-peritoneal routes and exposed for 2 h to air jet stress. The results are expressed as the mean ± SEM. $^{c}P < 0.001$ vs. S; $^{\beta}P < 0.01$ and $^{\gamma}P < 0.001$ IH vs. GH.

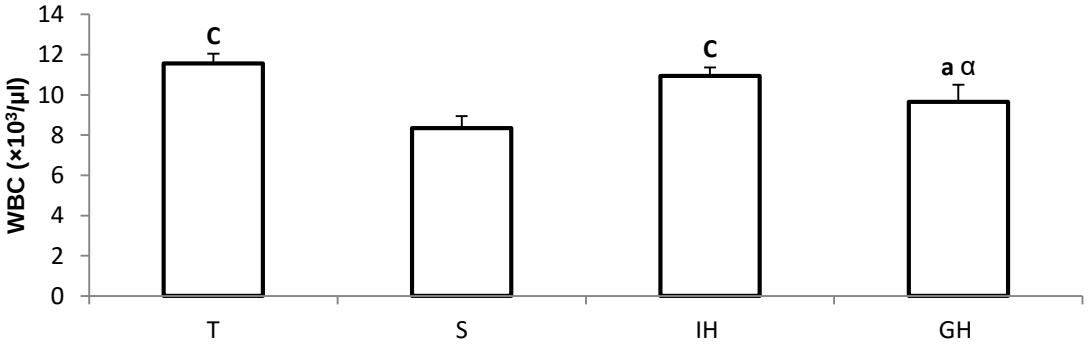

Figure 2: The white blood immune cell count of rats pretreated with hesperidin by oral and intra-peritoneal routes and exposed for 2 h to air jet stress. The results are expressed as the mean ± SEM. $^{a}P < 0.05$ and $^{c}P < 0.001$ vs. S; $^{\alpha}P < 0.05$ IH vs. GH.

effects, in addition to a remarkable ameliorative capacity of the central nervous activity and the hematological system[18-19]. Very soluble in water, hesperidin could be administered orally and intra-peritoneally (i.p.). In many recent studies, such as the research of Wasowski, who demonstrated that acute and chronic i.p. administration depress the locomotor and exploratory activities within mice, unlike oral route that remain intact and induce an anxiolytic effect [4].

The same finding was obtained in this study after one month of hesperidin pretreatment in Wistar rats with an average of dose of 40 mg/kg. Compared with the oral administration, the injected group had less protection against behavioral alterations in response to the air jet stress as reflected in the three sessions of the elevated T-maze test, we found an escaping behavior in rats injected with hesperidin similar to that of the stressed group, with a learning latency between sessions, which revealed a slight improvement in the time spent in the open arm from one session to the next. Stress caused by negative interactions with the experimenter, will damage the learning and cognition abilities of the rat; thereby undermining the usefulness of the animals in biomedical research and limiting the external validity of the conclusions drawn from the data [20-22]. In contrast, positive contact between animals and humans, such as the administration of hesperidin directly from the syringe after habituation training of the procedure for one week with a 5% sweet solution, can reduce the stress response associated with experimental practices. This was previously reported in the results of the elevated T-maze tests, which indicates that this group responds better to the antioxidant. These observations suggest that animal interactions with humans during the oral administration of a natural antioxidant imitate the positive social interactions of the species (e.g. tickling, substituting the tactile stimulation, caressing and substituting the tactile stimuli received during social grooming) could be used as alternative rewards that replace the aversive effects of the injections [23-24].

Behavioral changes elucidated after the rats were exposed to the air jet stress showed a particular immune distribution; white blood cell levels were depressed in associated with a significant increase in ACTH level. In fact, scientific studies have shown that the exposure of rodents to important social challenges, in this case to a 2-h episode of jet air causes the high level of oxidative damage that can probably disrupt the balance between proliferation and blood cell apoptosis [25-31]. The exact mechanism is not clear; however, researchers suspect the mediation of glucocorticoid-related stress. Pretreatment with hesperidin

could partly prevent these immune alterations triggered by sending a jet of air in to the cages of treated rats, as hesperidin has important antioxidant properties and acts through several physiological systems[1, 6, 32-35] and directly affects the stress axis (HPA). According to Cai et al, hesperidin inhibits the expression of the mRNA of corticotrophin-releasing factor (CRF) in the hypothalamus and regulates the increase of ACTH hormone and the GR glucocorticoid receptor protein [36].

The main issue our results raise is why, unlike the outcomes of the behavioral tests, did the injected hesperidin seem to better restore the values of white cells from damage from the oral air jet stress?

Some studies suggest there is a specific window in which the development of specific response may be altered by stress. During a primary response, exposure to stress just before or during the 24 h following vaccination would be a critical period. Stress occurring later would have little or no effect [37-40]. From this perspective, we argue that the handling coupled to the injection procedure repeated for one month is a pseudo-chronic stressor that prevents an immune deviation when exposed to the air jet stress such as the one observed in the group administered hesperidin orally and wherein the lack of aversive contact with the rats makes them immunologically naive to the stressful session of the air jet following treatment. Neglecting such an immune-resistance process by researchers will likely decrease the quality of collected immunity data by overestimating the immune-pharmacological effectiveness of any natural product, including hesperidin, due to non-consideration of the anxiety impact of the treatment technique on the psychological status of the experimental model. Despite this, the negative effects of pain, stress and distress and their influence on study outcome are either not reported or underreported in published scientific papers [41]. What is clear, however, are that handling effects can significantly alter an animal's immune status□either enhancing or compromising□and could have important methodological implications [38].

We have finally demonstrated that hesperidin, administered either by intra-peritoneal injection or oral administration, maintains a preventive efficiency against neurobehavioral and immunological changes that may be induced during psychological stress, as is the case of air jet stress. However, this work supports the consideration of non-invasive techniques such as oral administration that provides the animal with a positive contact link with the experimenter and avoids the physical aggressiveness of handling and the pain of repeated narcosis. As we demonstrated will compromise the data of behavioral tests during treatment with hesperidin by triggering a corticotropin response of ACTH level. In addition to air jet stress, it amplifies the animal's anxiety and thus delays its beneficial anxiolytic effect and also impacts the immune system. These disturbances may compromise the clarity of the behavioral and immunological results of research focused on the benefits of natural antioxidants including hesperidin. Consequently, we support, in an experimental protocol, the distinction between the control group receiving the vehicle (placebo) and the intact group to isolate the effect of the drug on the active group compared with the administration effect itself.

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