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EVALUATE NEUROPROTECTIVE EFFECT OF HESPERIDIN USING CHRONIC UNPREDICTABLE STRESS MODEL

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ABSTRACT

Current study was investigate the effect of hesperidin on unpredictable chronic stress induce behavioral and biochemical alterations in mice. Chronic unpredictable stressors can produce a situation similar to clinical depression, and such animal models can be used for the preclinical evaluation of antidepressants. Many findings have shown that the levels of proinflammatory cytokines (e.g., TNF-α) and oxidative stress (increased lipid peroxidation, decreased glutathione levels, and endogenous antioxidant enzyme activities) are increased in patients with depression. Mice were subjected to different stress paradigms daily for a period of 45 days to induce depressive like behavior such as memory acquisition, and retention. Chronic treatment with hesperidin significantly reversed the unpredictable chronic stress-induced behavioral (improve memory function), biochemical changes (decreased glutathione levels, superoxide dismutase), and inflammation surge (serum TNF-α IL 1β) in stressed mice. The study revealed that hesperidin exerted effects in behavioral despair paradigm in chronically stressed mice, specifically by modulating central oxidative stress and inflammation.

KEYWORDS: Hesperidin; TNF-α; IL 1 β; Unpredictable chronic stress; Antioxidants.

INTRODUCTION

Cognitive impairment is a common and usual co morbidity associated with prolonged stress (Radley JJ *et al.*, 2004). Chronic stress is influence cognitive performance in various psychiatric patients and also increases corticosterone secretion, which involves deterioration of hypothalamic-pituitary-adrenocortical (HPA) axis.[1,2] Corticosterone secretions were also triggers oxidative stress that ultimately leads to memory deficits.[3] These physiological responses of stress depend on the intensity and duration of the stressor and on how an organism perceives and reacts to the noxious stimulus. Therefore, chronic unpredictable stress (CUS) model has been standardized to study the development and progress of stress and related problems.[4] Degeneration of cholinergic neurons is one of the major hallmarks in the brain of cognitive deficit patient.[5] Along with this, study report also suggest that neuronal functions are altered by generation of reactive oxygen species which leads to oxidative stress; a prominent feature in the pathogenesis of cognitive dysfunction.[6] Various antioxidants have been tried for their effectiveness in reducing deleterious effects on neurons due to oxidative stress.[7] Dietary and medicinal phyto-antioxidants are being used as an adjuvant therapy to limit their side effects and increase their effectiveness. Hesperidin is a potent antioxidant[8], anti inflammatory agent[9], hepatoprotective.[10] There is increasing evidence that supports that potent antioxidants and anti inflammatory agents can provide protection against neurodegenerative changes associated with stress conditions. In light of these reports, the present study aims to investigate the protective effect of hesperidin against chronic unpredictable stress induced cognitive deficits and oxidative damage in mice.

MATERIALS AND METHODS

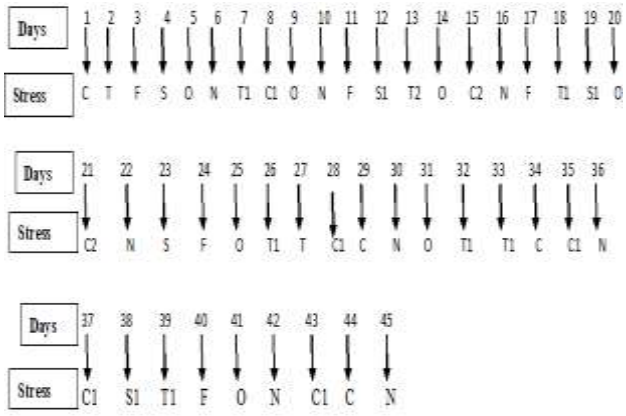
Animals: Three-month-old male Swiss albino mice (20–30 g) bred at Central Animal House (CAH), Sicra, Hyderabad, were used. They were housed (six mice per cage) under standard (25±2°C, 60–70% humidity) laboratory conditions, maintained on a 12-h natural day–night cycle, with free access to standard food and water. Animals were acclimatized to laboratory conditions before the test. The experimental protocol was approved by Institutional Animals Ethics Committee (253/IAEC/SICRA/PhD/2017) and animal care was taken as per the guidelines of CPCSEA (1821/PO/RE/S/15/CPCSEA).

Drugs

Hesperidin was purchased from Yarrow chem. pvt. Ltd (Mumbai, Maharastra, India www.yarrowpharma.com). Hesperidin was administered daily for 40 days by oral gavage.

Experimental procedure for neuroprotective effect of hesperidin on chronic unpredictable stress (CUS) model

Mice were exposed to a random pattern of mild stressors[11] daily for 45 days. The order of various stressors used in the present study is depicted below.



C — Cold swim (8 G, 5 min); T — Tail pinch (1 min); F — Food and water deprivation (24 h); S — Swimming at room temperature (24± 2 G, 20 min); O — Overnight illumination; N — No stress; T1 — Tail pinch (1.5 min); C1 — Cold swim (10 G, 5 min); S1 — Swimming at room temperature (24±2 G, 15 min); T2 — Tail pinch (2 min); C2 — Cold swi m (6 G, 5 min).

Drug treatment

Randomly divided into eight experimental groups (n=6-8). First and second group was named as normal and control (CUS) group respectively. Hesperidin (0.01, 0.1 and 1 mg/kg, p.o.) were treated as group 3-5 respectively. Hesperidin was prepared in 1% of Sodium CMC and administered orally on the basis of body weight (1 ml/100 g).

Solutions were made fresh at the beginning of each day of the drug treatment. Drugs were administered daily 30 minutes before CUS procedure (described in material and methods) for 40 days. The entire study was conducted in multiple phases.

Twenty four hour after the last treatment, all the animals were euthanized by cervical dislocation and the brain was dissected out from the cranial cavity. The brain was washed in 0.9% NaCl solution and kept in an ice cold PBS (pH 7.4) in a petriplate and was minced into small pieces. It was further homogenized immediately in Teflon homogenizer under the cold condition and cold centrifuged at 4°C to obtain 10% w/v brain tissue homogenate was subjected for estimation of total protein, reduced glutathione (GSH)[12], superoxide dismutase (SOD)[13], inflammatory mediators such as TNF α, IL 1β[14] and acetyl choline esterase (AchE)[15] and also performed behavioral pattern of mice.

Behavioral assessments

Elevated plus maze paradigm

The elevated plus maze (EPM) consists of two opposite black open arms, crossed with two closed walls of the same dimensions of 12 cm height. The arms were connected with a central square of dimensions 5x5 cm. The entire maze was elevated to a height of 25 cm from the floor. Acquisition and retention of memory processes were assessed as previously described.[16] Acquisition of memory was tested on day 20 of CUS procedure. Animal was placed individually at one end of the open arm facing away from the central square. The time taken by the animal to move from the open arm to the closed arm was recorded as the initial transfer latency (ITL). Animal was allowed to explore the maze for 20 sec after recording the ITL and then returned to the home cage. If the animal could not enter closed arm within 90 sec, it was guided to the closed arm and ITL was given as 90 sec. Retention of memory was assessed on day 37 as first retention transfer latency (1st RTL) and on day 38 as the second retention transfer latency (2nd RTL) respectively, upto 40th day.

Morris water-maze test

Morris water-maze apparatus (MWM) is most commonly used model to test spatial memory.[17] The MWM procedure is based on the principle that animal dislikes swimming and hence when placed in a large pool of water its tendency is to escape it by searching for a platform. MWM consists of large circular pool (90 cm in diameter, 40 cm in height). The tank was divided into four equal quadrants. A submerged platform (10 cm in diameter and 26 cm high), painted white was placed in the middle of the target quadrant of this pool, 1 cm, below surface of water. The position of platform was kept unaltered throughout the training session. The tank was located in a large room where there were several brightly colored cues external to the maze; these were visible from the pool and could be used by the mice for spatial orientation. The position of the cues remained unchanged throughout the study. The water maze task was carried out for four consecutive days from day 37-40. The mice received daily four consecutive training trials, with each trial having a ceiling time of 120 sec. For each trial, individual mouse was gently put into the water at one of four starting positions, the sequence of which being selected randomly and allowed 120 sec to locate submerged platform. Then, it was allowed to stay on the platform for 20 sec. If animal failed to find the platform within 120 sec, it was guided gently onto platform and allowed to remain there for 20 sec. Acquisition trial - Each mouse was subjected to four trials on each day (starting from day 37-40). A rest period of 1 hour was allowed in between each trial. Four trials per day were repeated for four consecutive days. Starting position on each day to conduct four acquisition trials was changed as described below and acquisition trials. Q4 was maintained as target quadrant in all.

Mean escape latency time (ELT) calculated for each day during acquisition trials was used as an index of acquisition.

Retrieval trial - On day 39, the platform was removed. Animal was placed in water maze and allowed to explore the maze for 120 sec. Mean time spent in the target quadrant, i.e. Q4 in search of missing platform provided an index of retrieval. Care was taken that relative location of water maze with respect to prominent visual clues was not disturbed during the total duration of study.

Y-maze task

Y-maze task is frequently used in monitoring spatial learning. Animals were allowed to learn alternation between arms based on their memory of the previously visited arms. The experimental apparatus consisted of a white-painted Y-maze that is made from acryl. Each arm of the Y-maze was 30 cm long, 14 cm high, and 8 cm wide, and it is positioned at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 5 min session. Spontaneous alteration behavior was defined as the consecutive entry into all three arms in overlapping triplet sets. During each trial, spontaneous alternations were recorded.. The percentage (%) of spontaneous alternation behavior was determined by dividing the total number of alternations by the total number of arm entries, subtracting 2, and then multiplying by 100 according to the following equation: % alternation = [(number of alternations)/(total number of arm entries-2)] × 100. One hour before the test, mice were orally administrated with vehicle. Acquisition trial - Each mouse was subjected to four trials on each day (starting from day 37-40). Hesperidin (0.01, 0.1 and 1 mg/kg), and 30 min later, the mice were injected with vehicle .The Y-maze arms were thoroughly cleaned in between tests to remove residual odors.

STATISTICAL ANALYSIS

All data are expressed as the means ± SEM. Statistical differences among the experimental groups were tested by using a one way analysis of variance (ANOVA) and Dunnet test was employed for multiple comparisons. P-values less than 0.05 were accepted as significant.

RESULTS

Effect of hesperidin on initial transfer latency in elevated plus maze test

Initial transfer latency was significantly increased in chronically stressed mice as compared to control mice (12±2.1 sec to 32±1.2sec; p<0.001***). Treatment with hesperidin significantly and dose dependently decreased initial transfer latency to 18.2±2.8 sec; p<0.05*when compared to CUS group (Fig.1).

Effect of hesperidin on swimming time in the target quadrant of Morris water maze

Time to reach target quadrant was significantly decreased in chronically stressed mice as compared to control mice (92.9±1.2 sec to 39.5±5.2 sec; p<0.01***) (Table 1; Fig. 1). Treatment with hesperidin significantly and dose dependently decreased time reach to target quadrant to 69.5±0.8 sec; p<0.05*) when compared to CUS group (Fig. 2).

Effect of hesperidin on % of alteration on 40th day on Y-maze task

Percentage of alteration was significantly decreased in chronically stressed mice as compared to control mice (75.7±2.1% to 44.6±0.2%; p<0.01**) (Table 1; Fig. 1). Treatment with hesperidin significantly, dose dependently decreased percentage of alteration in mice. Hesperidin (1 mg/kg) administration to unstressed mice show significant decreased percentage of alteration latency to 65.3±0.8%; p<0.05* (Fig. 3).

Effect of hesperidin on brain biochemical parameters against CUS rats

Brain protein content was significantly decreased in CUS group (30.2±0.5µg/mg to 14.6±0.7µg/mg; p<0.01**) compare to normal control**.** Treatment with hesperidin significantly and dose dependently increased protein content 12.6±2.1 µg/mg to 22.1±1.5 µg/mg; p<0.01**) when compared to CUS group. Brain SOD levels was significantly decreased in CUS group (61.2±0.4 U/mg to 23.2±1.2 U/mg; p<0.001***) compare to normal control. Hesperidin treatment mice brain SOD activity was significantly increase to 47.9±1.9 (p<0.01), when compared to CUS group Brain GSH activity was significantly decreased in CUS group (0.09±0.01µ.mol to 14.6±0.7 µ.mol; p<0.01**) compare to normal control. Hesperidin treatment mice brain GSH activity was significantly increase to 0.069±0.01µ.mol; p<0.05*, when compared to CUS group. Brain AchE activity was significantly increased in CUS group (0.4±0.02 µ.mol to 2.92±0.03µ.mol; p<0.001***) compare to normal control. Hesperidin treatment mice brain GSH activity was significantly increase to 0.92±0.02 µ.mol; p<0.05*, when compared to CUS group (Table 1).

Effect of hesperidin on brain TNF alfa and IL 1 beta in CUS rats

Brain inflammatory mediator levels such as TNF α (1800±5.9 pg/gr tissue to 16400±12.4 pg/gr tissue; p<0.001***) and IL 1β (2550±21.5 pg/gr tissue to 35000±23.6 pg/gr tissue; p<0.001***) was significantly increased in CUS group compare to normal control. Hesperidin treated mice brain TNF α levels significantly decreased to 5604±9.5 pg/gr tissue; p<0.001***) and IL 1β (5450±35.7 pg/gr tissue; p<0.001***) compare to CUS group (Fig 4).

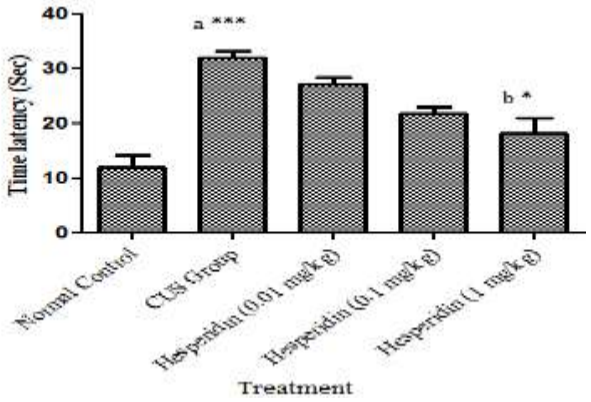


Fig. 1: Effect of hesperidin on transfer latency on plus maze.

All values are expressed in Mean± SEM. CUS group significantly increase in transfer latency (**p <0.01), compared with normal control. Treatment with Hesperidin (1 mg/kg bd. wt.) significantly decreased (*p <0.05) compared to CUS group.

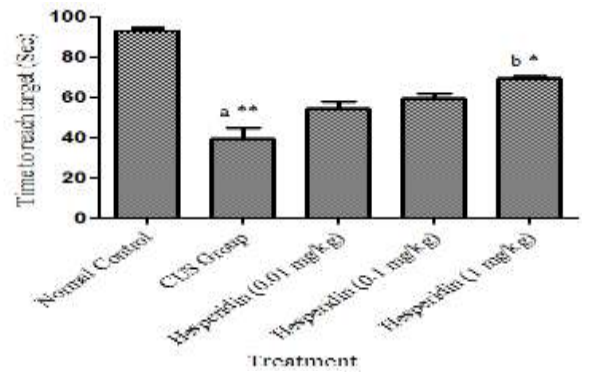


Fig. 2: Effect of Hesperidin on swimming time in the target quadrant on Morris water maze.

All values are expressed in Mean±SEM. CUS group significantly decrease in Swimming time in the target quadrant (**p <0.01), compared with normal control. Treatment with Hesperidin (1 mg/kg bd. wt.) significantly increase (*p <0.05 compared to CUS group.

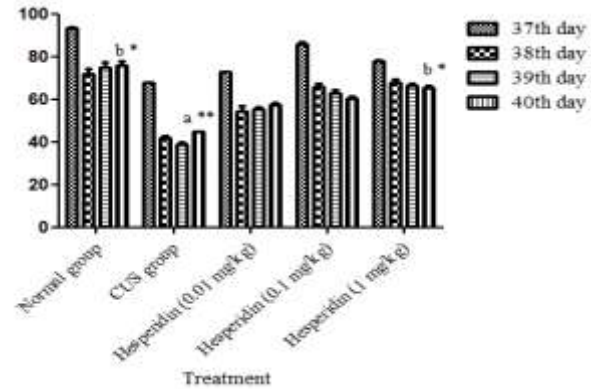


Fig. 3: Effect of Hesperidin on memory enhancing effects of hesperidin on Y-maze task.

All values are expressed in Mean±SEM. CUS group significantly decrease in % Alternation (**p <0.01), compared with normal control. Treatment with Hesperidin (1 mg/kg bd. wt.) significantly increase (*p <0.05 compared to CUS group.

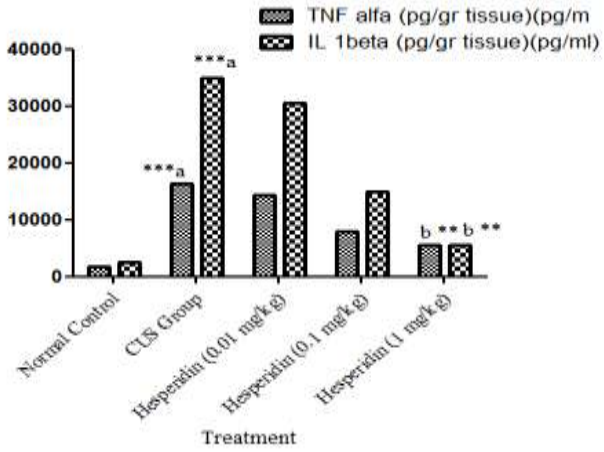


Fig. 4: Effect of hesperidin on brain TNF alfa and IL 1 beta in CUS rats.

All values are expressed in Mean±SEM. a denotes significant difference between Normal Control vs CUS group. b denotes significant difference between treatment vs CUS group.

Table 1: Effect of Hesperidin on brain biochemical parameters against CUS rats.

Treatment	Protein content [µg/mg tissue]	GSH (µmole of GSH/mg pr.)	SOD (units/mg pr)	µmole of acetylthiocholine iodide hydrolyzed/ min/mg pr.
Normal Control	30.2±0.5	0.09±0.01	61.2±0.4	0.4±0.02
CUS Group	14.6±0.7**a	0.03±0.01***a	23.2±1.2***a	2.92±0.03***a
Hesperidin (0.01 mg/kg)	17.6±0.8	0.05±0.01	29.4±1.2	1.51±0.04
Hesperidin (0.1 mg/kg)	19.2±0.6	0.06±0.01	34.6±0.9	1.32±0.01
Hesperidin (1 mg/kg)	25.3±0.5**b	0.069±0.01**b	47.9±1.9**b	0.92±0.02**b

All values are expressed in Mean±SEM. CUS group significantly decrease in % Alternation (**p <0.01), compared with normal control. Treatment with Hesperidin (1 mg/kg bd. wt.) significantly increase (*p <0.05 compared to CUS group.

DISCUSSION

Complex relationship between stressful situations, mind and body's reaction to stress, and the onset of cognitive disturbances.[18] Chronic administration of various uncontrollable stresses, a procedure known as chronic unpredictable stress, is generally thought to be the most reliable and valuable experimental model to study stress pathology.[19] Chronic unpredictable stress (CUS) has been shown to influence brain regions which play a critical role in spatial navigation and memory.[20] Thus in the present study, hesperidin has been tried as a drug strategy against chronic unpredictable stress induced oxidative damage and cognitive deficits in mice. In the present study, memory performance was evaluated by Morris water maze (MWM), Y Maze (YM) as well as elevated plus maze (EPM). Though elevated plus maze test is primarily used for anxiety, it can also be employed as an experimental model for evaluation of long term memory in rodents.[21] In the present study, chronic unpredictable stress resulted in a significant impairment of cognitive performance in MWM, YM and EPM tests as compared to normal mice. These results are consistent with the previous finding.[22]

Hesperidin treatment for 40 days significantly improved cognitive performance in MWM, YM and EPM indicating its therapeutic potential against chronic stress induced memory impairment. The results are in accordance with previous studies by[23] which showed a significant decrease in cognitive function CUS mice. Further, hesperidin in a dose dependent manner significantly restored the cognitive function in chronic unpredictable stress mice. Hippocampus has been well known to play a key role in spatial learning and memory.[24] Since hippocampus has abundant inputs from the basal forebrain cholinergic system and thus acetylcholine (ACh) plays a crucial role in learning and memory.[25] Acetylcholine is degraded by the enzyme acetylcholinesterase, terminating the physiological action of the neurotransmitter. Cognitive dysfunction affects cholinergic system resulting in increased activity of acetylcholinesterase.[26] Stress has been well documented to induce alterations in acetylcholinesterase enzyme activity.[27] In the present study, CUS caused a significant increase in acetylcholinesterase activity leading to memory deficits, but later was significantly attenuated by chronic hesperidin treatment, implicating its role in cholinergic transmission processes.

CONFLICT OF INTEREST: No.

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