Neuroprotective effect of morin against unpredictable chronic mild stress induced oxidative stress and behavioural deficits in Wistar rats

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ABSTRACT
Morin is a bioflavonoid widely found in fruits and vegetables, which possesses the protective activity to various neurological diseases. An earlier report showed that the administration of morin had antidepressant-like effect against acute stress conditions. Acute stress induces only transient changes in the brain while the prolonged stress increases vulnerability to mental disorders. To better understand its anti depressive activity, we explored the neuroprotective role of morin against unpredictable chronic mild stress (UCMS) induced rats. Male Wistar rats were randomized and grouped into control, UCMS exposed (different stressors) UCMS and morin (30 & 60 mg/kg) co-treated and morin alone treated (60 mg/kg). Morris water maze test (MWMT) and sucrose preference test (SPT) were employed to study the effect of morin on learning, memory and anhedonia of rats. In addition, the levels of thiobarbituric acid (TBARS) and reduced glutathione (GSH) and the activities of super oxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were estimated. Behavior studies indicated that morin treatment significantly increased the memory and sucrose intake as shown by MWMT and SPT respectively. Moreover, it exhibited the antioxidant activity by reducing the amount of lipid peroxidation products (TBARS) and GSH and activities of enzymatic antioxidants in UCMS rats. Our experiment suggested that the antidepressant-like actions of morin could be due to its potent antioxidant property.

INTRODUCTION
Depression is a devastating neuronal disability, represented by anhedonia, feelings of guilt or fatigue, sadness, disrupted sleep or loss of appetite and poor concentration, affecting about 350 million people globally (WHO, 2016). Various factors shown to be involved in the etiology and progression of depression were alteration in environmental factors, sedentary lifestyle, socioeconomic status, nutrition and eating habits (Patel, 2001). These circumstances are linked with stressful events in life. UCMS exposure affected the hypothalamic pituitary adrenal axis (Slavich and Irwin, 2014) thereby increasing corticosterone levels, which in turn triggers oxidative stress that ultimately leads to memory deficits (McEwen, 2008).

Moreover UCMS rats have been reported to develop anhedonia, anxiety, alcohol preference, changes in sleep pattern and decrease in memory (Willner, 2005).
Table 1: UCMS schedule

<table>
<thead>
<tr>
<th>Stressors</th>
<th>Days of UCMS experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social crowding (ten animals/cage for 24 h)</td>
<td>1,15,27</td>
</tr>
<tr>
<td>Swimming at room temperature for 20 min</td>
<td>2,16,25,36</td>
</tr>
<tr>
<td>24 h Food deprivation</td>
<td>3,19,37</td>
</tr>
<tr>
<td>Swimming in cold water at 4°C for 5 min</td>
<td>10,20,31</td>
</tr>
<tr>
<td>Kept in Tilting cage at 30° for 24 h</td>
<td>4,13,26,41</td>
</tr>
<tr>
<td>Cage-tilting 45° for 24 h</td>
<td>5,21,29,40</td>
</tr>
<tr>
<td>Wet bedding for 24 h</td>
<td>6,17,38</td>
</tr>
<tr>
<td>Sawdust empty for 24 h</td>
<td>8,18,30,39</td>
</tr>
<tr>
<td>Reversal of day and night</td>
<td>9,24,32</td>
</tr>
<tr>
<td>Isolation for 24 h</td>
<td>7,14,28,35,42</td>
</tr>
<tr>
<td>Exposure to constant light for 24 h</td>
<td>12,22,34</td>
</tr>
<tr>
<td>Water deprivation for 24 h</td>
<td>11,23,33</td>
</tr>
</tbody>
</table>

Tricyclic antidepressants, serotonin and serotonin-norepinephrine reuptake inhibitors are being currently used drugs for treating depression. Their treatment needs about 14-28 days to attain curative effect and have stern side effects like sleep disturbance, sexual impairment and heart problems (Bostwick, 2010). About 60% of patients are responding to these drugs and the remaining patients display remission. Therefore, there is an urgent requirement for more potent and rapid acting antidepressant agents with no or fewer side effects (Pochwat et al., 2014). Since ancient times, plant extracts and their active compounds have gained attention in the prevention and treatment of physical and mental illnesses.

Figure 1: Experimental protocol. SPT, sucrose preference test; MWMT, Morris water maze test.

Morin, a bio-flavonoid, ubiquitously exist in the Moraceae plants family (eg. white mulberry) and found in almond, sweet chestnut, guava and apple. It is present abundantly in red wine. It offered neuroprotective effect against lipo-polysaccharide-induced neuroinflammation (Zhang et al., 2010), schizophrenia-like behaviors (Ben-Azu et al., 2018), streptozotocin-induced oxidant-antioxidant imbal-

ance and cognitive impairments (Ola et al., 2014), 1-methyl,4-phenyl,1,2,3,6-tetrahydropyridine induced Parkinson’s disease, sleep deprivation-induced neuro behavioural impairments (Zhang et al., 2010) and amyloid beta-induced Alzheimer’s disease (Lemkul and Bevan, 2012). (Olonode et al., 2017) suggested that morin hydrate attenuated the acute stress-induced anxiety, depression, oxidative stress and hyperglycemia in mice. Acute stress stimulates transitory alteration in the brain, but the prolonged stress enhances susceptibility to mental disorders (Alkadhi, 2013). Hence, we investigated the neuroprotective efficacy of morin in unpredictable chronic mild stress rats, the established rat model of depression, which is not reported till now.

Figure 2: Consumption of sucrose solution in control and experimental groups. UCMS - unpredictable chronic mild stress; Mor-Morin.

MATERIALS AND METHODS

Animals
Male Albino Wistar rats ranging from 200–225g
were obtained and maintained at standard conditions with food and water ad libitum at Central Animal House, Rajah Muthiah Medical College & Hospital, Annamalai University. The experiment protocols were followed according to guidelines of the Institutional Animal Ethics Committee (Reg. No. 160/1999/CPCSEA, Proposal No. AU-IAEC/1212/4/18).

Chemicals
Morin, 5, 5-dithiobis-(2-nitrobenzoic acid), trichloroacetic acetic acid (TCA), thiobarbituric acid (TBA) and reduced glutathione were purchased from Sigma-Aldrich, Bangalore, India and used in this study. Other chemicals used in this experiment were of in analytical grade.

Experimental design

Chronic mild stress (UCMS) protocol and morin treatment

After the acclimatization phase of one week, thirty rats were separated into five groups of six animals. Group I control rats were kept without disturbance for 42 days under standard conditions. Group II rats subjected to UCMS for 6 weeks (Table 1) (Lucca et al., 2009; Yang et al., 2017) Group III and IV rats were subjected to UCMS and oral administration of morin (30 and 60 mg/kg) (Ola et al., 2014) for 42 days. Group V animals were orally treated with morin (60 mg/kg) for 42 days (Figure 1).

Sucrose preference test
SPT was performed as indicted by (Stepanichev et al., 2016) 48 h before the end of UCMS, one bottle filled with pure drinking water placed left side of the cages and another with 5% sucrose solution was presented in right side of the cages. The bottle positions were changed to prevent the place preference. After adaptation period, during the SPT, animals were housed individually and allowed to access freely with a bottle containing 200 ml of 5% sucrose and another of 200 ml of tap water. After 24 h, the following formula was used to measure

$$SP = \frac{\text{sucrose solution intake}}{\text{sucrose solution intake} + \text{water intake}} \times 100$$

Morris water maze test

The MWM apparatus consisted of circular tank containing 1/3rd water with an escape platform located about a centimeter below the surface of water (Burda et al., 2011). Several visual signs were marked in the walls and the animals placed in the same place for each trial. This test was conducted as initial training and probe test. During the training period, the animal was placed on the hidden platform for one min to recognize and remember the environment. Then the rat was permitted to swim freely till they reached the hidden platform. The unsuccessful animals were assisted to reach the target by viewer. Four trials per day with the interval of 15 min for 4 days continuously were given for each rat and the placing position was changed for each and every trial. The probe test (Day 5) was conducted after the removal of platform. The time
taken to reach the place of removed platform is represented as escape latency and the number of times the rat crossed that place was also noted.

Figure 5: Activities of SOD in control and experimental groups. UCMS - unpredictable chronic mild stress; Mor-Morin.

Tissue collection

The rats were sacrificed, hippocampus, amygdale and cerebral cortex were quickly removed, washed thoroughly in saline solution, frozen and stored at −80°C.

Estimation of TBARS

To 0.2 ml phenyl methosulphate, brain tissues were kept at 37 °C in water bath shaker for 1 h. Then 0.4 ml of 0.67% TBA and 0.4 ml of 5% trichloroacetic acid were added and centrifuged (3000 × g) for 15 min. Then the supernatant was separated, boiled for 10 min, cooled and read at 532 nm (Bhattacharya et al., 2001). The rate of lipid peroxidation was represented as nmol of TBARS formed/g tissue.

Assay of SOD

SOD activity was assessed by (Oberley, 1998) method and expressed as units/min/mg protein. To the sodium carbonate buffer, 0.025 mm nitro blue tetrazolium, 0.1 mm xanthine, and 0.1 mm ethylenediamine-tetraacetic acid, xanthine oxidase and brain supernatant were added. Optical density differences were recorded spectrophotometrically at 560 nm. Protein content was determined by the (Lowry et al., 1951).

Assay of catalase

Examination of catalase activity was done according to the (Aebi, 1984) method and expressed as nmol of H₂O₂ decayed/min/mg protein. To the brain tissue extracts, 10mM H₂O₂, Tris–HCl buffer, 5mM EDTA and double distilled water were added. The H₂O₂ decomposition was measured at 240 nm by spectrophotometer.

Assay of GPx

The reaction mixture contains Tris–HCl buffer with EDTA, distilled water, glutathione reductase solution (10 U/ml), 0.1 M GSH, 2 mm NADPH, 7 mm H₂O₂ and brain extract. The amount of GPx required to oxidize 1 μmol of NADPH per min (Yamamoto and Takahashi, 1993) was measured.

Estimation of GSH

Tissue extracts was centrifuged (16,000 × g) at room temperature for 15 min. To the supernatant, ice-cold 1 M phosphate buffer containing 0.1 mm 5, 5-dithiobis [2-nitrobenzoic acid] were added and the absorbance was measured at 412 nm (Jollow et al., 1974).

Data analysis

Data (n = 6) were expressed as mean ± Standard Error (SEM). The statistical significance was calculated by one-way analysis of variance (ANOVA) using Duncan’s Multiple Range Test (DMRT). Groups not sharing a common alphabet (p< 0.05) are differ significantly with each other.

RESULTS AND DISCUSSION

UCMS exposure produces depressive-like phenotypes including chronic behavioral alterations, which were validated by sucrose preference, Morris water maze, open field, forced swimming and tail suspension test. The anti depressive action of morin on UCMS induced anhedonia was observed by the percentage of sucrose preference in SPT (Figure 2). Exposure to UCMS exhibited a significant diminish in the preference to the sucrose solutions in rats as compared to the non-stressed rats. Morin (30 & 60 mg/kg) treatment to UCMS rats dose dependently attenuated the impairment of sucrose preference as compared to UCMS alone exposed animals. The neuroprotective effect of morin on UCMS provoked memory and cognitive dysfunctions was assessed by
the MWM test (Figure 3). UCMS exposure showed declined learning process as measured by enhanced latency to reach the hidden platform and the place of platform on days 3 & 4 and 5 respectively. It also decreased number of time passing the place of removed platform in probe test as compared with control group. Morin (30 and 60 mg/kg) administration to UCMS rats significantly improved the learning progress as shown by significant reduction in latency to reach the hidden platform and the place of removed platform on days 3 & 4 and 5 respectively. It also decreased number of time passing the place of removed platform in probe test as compared with control group. Morin (30 and 60 mg/kg) administration to UCMS rats significantly improved the learning progress as shown by significant reduction in latency to reach the hidden platform and the place of removed platform and increase in the number of times passing the removed platform in probe test as compared to UCMS exposed rats.

Morin administration to non-stressed rat showed no significant alterations in anhedonia, memory and cognitive function as compared with control rats. SPT is used to identify the anhedonia, the irresponsible to rewards like a low consumption of sucrose solution representing depression (Papp et al., 1991), whereas MWMT is used to observe the hippocampal function indicating memory status of animals which is affected during stress exposure. Morin exhibited its anti-depressant effect in acute stress (Olonode et al., 2018) and ketamine-induced schizophrenia model (Ben-Azu et al., 2018) of mice was further supported by other behavioural tests of our present findings.

In the present study, exposure to UCMS significantly enhanced the levels of TBARS (Figure 4), a reliable marker of lipid membrane peroxidation and diminished GSH levels (Figure 8), SOD (Figure 5), catalase (Figure 6) and GPx (Figure 7) activities than unstressed rats. Administration of exogenous antioxidants like polyphenols, flavonoids and terpenoids may offer an effective defense against free radicals in central nervous system (Bharathi et al., 2018; Rather et al., 2018; Essa et al., 2015) and exhibited antidepressant effects in various experimental models (Sairam et al., 2002; Vanzella et al., 2012). Morin exhibited the antioxidant activity by diminishing the levels of ROS and increasing the amount of GSH and SOD, catalase and GPx activities in the brain of ifosfamide (Çelik et al., 2020) doxorubicin (Kuzu et al., 2018), ketamine (Ben-Azu et al., 2018), lead acetate (Thangarajan et al., 2018) and gentamyin (Jonnalagadda et al., 2013) treated rats, which corroborate with our present results.
CONCLUSIONS

As to conclude, the present study suggested that morin attenuated UCMS exposed neurotoxicity by reducing ROS production, improving antioxidants and reversing the behavioural impairments. This might be due to its anti depressive property which is not explored in chronic mild stress condition till now, in addition to the already established antioxidant, anti amyloidogenic, anti inflammatory, and neurotrophic factor-enhancing effects. However, further research is desirable to shed light on molecular mechanisms underlying the neuroprotective effect of morin.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

REFERENCES


WHO 2016. WHO Depression.


