Malic Acid Improves Behavioral, Biochemical, and Molecular Disturbances in the Hypothalamus of Stressed Rats

Khaled M. M. Koriem1,*, Hatem A. K. Tharwat2

1Department of Medical Physiology, Medical Research and Clinical Studies Institute, National Research Centre, Dokki, 12622 Giza, Egypt
2Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt
*Correspondence: kkoriem@yahoo.com (Khaled M. M. Koriem)
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Abstract

**Background:** Stress can lead to emotional and mental symptoms such as anxiety, sadness, panic attacks, and depression. Malic acid was chosen due to its ability to improve antioxidant activity and improve liver damage. This study evaluated malic acid anti-depressant activity in the hypothalamus of stressed rats. **Methods:** Thirty-six male albino rats were divided into 2 equal groups; normal and chronic mild stress (CMS) rats. Normal rats were divided into 3 equal groups; control, malic acid, and venlafaxine drug groups; normal rats were administered orally with 1 mL of saline solution, 250 mg/kg of malic acid, and 20 mg/kg of venlafaxine drug, respectively. CMS rats were divided into 3 equal groups; CMS, CMS + malic acid, and CMS + venlafaxine drug. CMS rats were administered orally with 1 mL of saline solution, 250 mg/kg of malic acid, and 20 mg/kg of venlafaxine drug, respectively. All the above-mentioned treatments were administered once a day by oral gavage for 6 weeks. **Results:** The obtained results revealed that the animal behavioral stress response tests such as forced swimming test, tail suspension test, sucrose preference test, and open-field test (center square entries test, center square duration test, and distance travelled test), noradrenaline, dopamine, serotonin, γ-aminobutyric acid, nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity, oxidant index, conjugated dienes, catalase, glutathione peroxidase, and superoxide dismutase, malondialdehyde, interleukin-6, tumor necrosis factor-α, interleukin-10, interleukin-1β, sodium/potassium-ATPase activity, and histamine-N-methyl transferase (Hnmt) and tyrosine hydroxylase (TH) enzymes in the hypothalamus of stressed rats, were returned to approaching the normal state in the stressed group after treating with malic acid for 6 weeks. **Conclusions:** Malic acid ameliorated stress-related symptoms and it inhibited superoxide anion and neuro-inflammation in the hypothalamus of stressed rats.

Keywords: stress; antioxidants; neurotransmitters; inflammation; histology

1. Introduction

Changes in physiology, the nervous system, hormones, and behavior are all a result of stress. Continuous excessive stress leads to obsessive brain damage and serious neurological dysfunction [1]. Human memory and the brain are impacted by stress.

By interfering with biological hormones and proteins within the human body, it changes the brain and memory and thus leads to human neurological dysfunction [2].

Prolonged stress throughout adolescence increases the risk of developing neuropsychiatric disorders because it alters neuronal structure and behavior in all major brain regions [3]. The hypothalamus was selected for this study because it is a more precise target for stress or depression [4] due to its involvement in two main axes: (1) the hypothalamic-pituitary-adrenal (HPA) axis, which is a major factor in the neural/hormonal cycle that reacts to both internal and external stressors. Human mental and physical states are completely under the control of the HPA axis, and dysregulation of this axis has been linked to a number of mental and physical diseases [5]. (2) Stress or depression also inhibits the hypothalamic-pituitary thyroid (HPT) axis. Many interactions exist between the aminergic systems that play a role in stress or depression and the HPA- and HPT-axis [6]. Emotional and mental symptoms like anxiety or irritation, melancholy, panic attacks, and depression can be brought on by stress. Stress on an ongoing internal and external level leads to depression. Over 350 million people worldwide suffer from depression [7]. Depression is characterized by a decrease in brain neurotransmitter levels, an increase in the hypothalamic-pituitary-adrenal axis, elevated inflammatory markers, and changes in the gut flora [8].

The potential processes underlying the link between stress and depression have been the subject of numerous recent investigations. These research [9] demonstrated links between stress and depression, including those involving the immune system, the microbiota, hormones, and brain abnormalities. In the USA, depression affects adolescents and accounts for one-third of adolescent fatalities [10].

There are many animal models that mimic human depression, but the chronic mild stress (CMS) model is the most widely used model that replicates the main symptoms of human depression [11]. In this model, rats are exposed to multiple stressors, which alters their behavior and biochemistry by altering the chemistry, plasticity, expression, and function of their neurons, neuroreceptors, and neurotrophin [12].
Depression is a chronic illness that lasts for years; as a result, it requires affordable and secure treatment. Given its high antioxidant activity, malic acid was selected for this investigation from natural herbs, which are a viable source in this regard [13]. In this regard, malic acid enhanced brain neural connection function, reduced inflammation, improved amino acid metabolism, energy metabolism and neurotransmitter metabolism, and decreased dopaminergic degeneration [14]. Malic acid is used to treat fibromyalgia [15], where it has a neuroprotective role against the loss of glutamic acid decarboxylase activity, depletion of γ-aminobutyric acid level, increased propidium iodide uptake, and increased protein level [16]. Malic acid is a naturally occurring acid with the chemical formula C4H6O5 that is produced by numerous species [17,18]. Malates are the names for the salts and esters of malic acid [19]. Malic acid is a prominent component of several fruits, including blueberries, cherries, blue, apricots, blackberries, plums, grapes, pears, mirabelles, peaches, and quince. Malic acid is also a minor component of citrus [20]. A new kind of phytopharmaceutical with cardioactive properties is malic acid [21]. Malic acid inactivates catalase and ascorbate peroxidase by acting as an antioxidant. In the plant, it binds to cadmium and lowers the concentration and toxicity of the metal [22]. Malic acid can increase antioxidant activity and slow down ageing [23]. Malic acid has a clear therapeutic effect on treating liver damage, and as a result, it protects the liver [24]. Malic acid is used to treat consequences of diabetes mellitus (type 2), such as xerostomia [25]. Malic acid is a crucial component of clinical nanomedicine since it is utilized to diagnose and treat diseases [26].

This study aims to study the anti-depressant activity of malic acid through modifying animal behavior, neurotransmitters, oxidative stress, inflammation, sodium/potassium-ATPase activity, and histamine-N-methyl transferase (Hmnt) and tyrosine hydroxylase (TH) enzymes in the hypothalamus of CMS rats.

2. Materials and Methods

2.1 Materials

DL-malic acid (99% pure malic acid, 6915-15-7, Sisco Research Laboratories (SRL) Pvt. Ltd., New Delhi, India). Ethylene glycol-bis(2-amino-ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), nicotinamide-adenine dinucleotide phosphate (NADPH), potassium phosphate monobasic (KH2PO4), lubrol, and lucigenin (9, 9'-bis[N-methyl acridinium nitrate] were purchased from Sigma-Aldrich Co (Merck Group, St. Louis, MO, USA). Venlafaxine (93413-69-5, a standard anti-depressant drug used for depression treatment) was obtained from the International Drug Agency for Pharmaceutical Industry, Cairo, Egypt. All kits reagents used for biochemical analysis were purchased from Bio-diagnostics Company (Birmingham, UK), by a local Egyptian branch.

2.2 Animals

The animal house of the National Research Centre, Dokki, Giza, Egypt provided this study by thirty-six male albino rats of Sprague Dawley strain (130 ± 10 g, 12 weeks old). The experimental plastic cages held the animals. They were fed commercial rat food and drank tap water. The study was carried out following receipt of the approval sheet (approval number 12041126) from the National Research Centre of Egypt’s ethics committee. The research used laboratory animals with the appropriate handling and care (NIH publication no. 85:23, revised 1985).

2.3 Study Protocol

Thirty-six male albino rats were divided into 6 equal groups (6 rats/group) as follows: Control group: normal rats were administered orally with 1 mL of saline solution. Malic acid-treated group: normal rats were administered orally with 250 mg/kg of malic acid [27] dissolved in 1 mL distilled water. This dose increases the motor activity and excitatory processes in the sensory motor brain areas. This dose increases carbohydrate reserves and decreases oxygen consumption of the brain tissues [27]. Venlafaxine drug-treated group: normal rats were administered orally with 20 mg/kg of the venlafaxine drug [28] dissolved in 1 mL distilled water. CMS group: CMS animals were administered orally with 1 mL of saline solution. CMS + malic acid (250 mg/kg)-treated group: CMS rats were administered orally with malic acid (250 mg/kg of malic acid dissolved in 1 mL water). CMS + Venlafaxine drug (20 mg/kg)-treated group: CMS rats were administered orally with 20 mg/kg of Venlafaxine drug dissolved in 1 mL distilled water.

During 6 weeks, oral gavage was used to provide all of the aforementioned therapies once daily. All rats were observed throughout the entire study for any aberrant symptoms, such as rat hair loss, skin patches, convulsions, and a reduction of normal locomotor activity, as well as any deaths.

2.4 Chronic Mild Stress Induction

The authors have already conducted this stress animal model [29–31]. According to a predetermined plan, the normal rats were exposed to 1 or 2 stressors everyday as follows: The first day involved forced swimming in hot water (45 °C for 5 minutes); the second day cage tilting and wet bedding; the third day animal shaking for 10 minutes; the fourth day nipping the tail for 1 minute; the fifth day involved forced swimming in cool (4 °C for 5 minutes); the sixth day food deprivation for 24 hours and overnight illumination; the seventh day water deprivation for 24 hours and all the above aforementioned stressors were applied once a week for 6 weeks, after which time all rats underwent the following tests: (1) sucrose preference test, (2) open-field test (which included the distance travelled test, the center square entries test, and the center square duration test), (3) tail suspension test, and (4) forced swimming test.
Fig. 1. Chronic mild stress (CMS) induction. Summarize the program for chronic mild stress induction as follows: The first day involved forced swimming in hot water (45°C for 5 minutes); the second day cage tilting and wet bedding; the third day animal shaking for 10 minutes; the fourth day nipping the tail for 1 minute; the fifth day involved forced swimming in cool (4°C for 5 minutes); the sixth day food deprivation for 24 hours and overnight illumination; the seventh day water deprivation for 24 hours and all the above aforementioned stressors were applied once a week for 6 weeks.

Fig. 1 could serve as a summary of the induction of mild chronic stress in rats.

2.5 Hypothalamus Tissue Preparation

The rats were inhaled a solution of diethyl ether as an anesthesia after the final dose of each treatment. The rats’ heads were detached, and then they were dissected. The tissue from the brain’s hypothalamus was taken and soaked in saline solution. This procedure followed Palkovits’ [32] instructions for isolating the hypothalamus. The entire hypothalamic tissue, which has 3 regions (the supraoptic, tuberal, and mammillary regions) was examined in the study.

The filter papers were used to dry this tissue. Two pieces of hypothalamic tissue were separated, and the first piece was dissolved in 2.5 mL of Tris buffer solution. Thereafter, for 10 minutes at room temperature, hypothalamic tissue was homogenized in an automated homogenizer. The supernatant, which was used to calculate the biochemical parameters, was removed from the tissue by centrifuging it for 15 minutes at –4°C and 7000 rpm. The second portion of the tissue from the hypothalamus was used for histological analysis. The hypothalamic tissue was sufficient to conduct biochemical, molecular, and histological examinations despite the fact that the hypothalamus is a small tissue.
Table 1. Effect of malic acid on physiological measures of CMS rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Malic acid</th>
<th>Venlafaxine drug</th>
<th>CMS rats</th>
<th>CMS rats + Malic acid</th>
<th>CMS rats + Venlafaxine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body weight (g)</td>
<td>165 ± 6.24</td>
<td>167 ± 6.13 (1.21%)</td>
<td>164 ± 6.52 (–0.61%)</td>
<td>128 ± 5.09a (–22.42%)</td>
<td>163 ± 6.24b (27.34%)</td>
<td>162 ± 6.24b (26.56%)</td>
</tr>
<tr>
<td>Food consumption (g/day)</td>
<td>11.6 ± 1.2</td>
<td>11.4 ± 1.4 (–1.72%)</td>
<td>11.5 ± 1.6 (–0.86%)</td>
<td>7.2 ± 1.3a (–37.93%)</td>
<td>11.3 ± 1.6b (56.94%)</td>
<td>11.2 ± 1.5b (55.56%)</td>
</tr>
<tr>
<td>Water intake (mL/day)</td>
<td>12.4 ± 1.7</td>
<td>12.6 ± 1.5 (1.62%)</td>
<td>12.3 ± 1.2 (–0.81%)</td>
<td>7.8 ± 1.3a (–37.09%)</td>
<td>12.2 ± 1.7b (56.41%)</td>
<td>12.1 ± 1.7b (53.85%)</td>
</tr>
<tr>
<td>Liver weight (g/100 g bw)</td>
<td>2.7 ± 0.08</td>
<td>2.6 ± 0.06 (–3.70%)</td>
<td>2.5 ± 0.09 (–7.41%)</td>
<td>1.8 ± 0.05a (–33.33%)</td>
<td>2.4 ± 0.08b (33.33%)</td>
<td>2.3 ± 0.08b (27.78%)</td>
</tr>
<tr>
<td>Kidney weight (g/100 g bw)</td>
<td>0.37 ± 0.03</td>
<td>0.37 ± 0.05 (1.67%)</td>
<td>0.35 ± 0.04 (–2.78%)</td>
<td>0.21 ± 0.02a (–41.67%)</td>
<td>0.33 ± 0.06b (57.14%)</td>
<td>0.34 ± 0.05b (61.90%)</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>0.56 ± 0.07</td>
<td>0.57 ± 0.08 (1.79%)</td>
<td>0.58 ± 0.06 (3.57%)</td>
<td>0.39 ± 0.04a (–30.36%)</td>
<td>0.53 ± 0.09b (35.90%)</td>
<td>0.54 ± 0.06b (38.46%)</td>
</tr>
<tr>
<td>Pancreas weight (g/100 g bw)</td>
<td>0.27 ± 0.03</td>
<td>0.27 ± 0.02 (8.00%)</td>
<td>0.24 ± 0.04 (–4.00%)</td>
<td>0.16 ± 0.02a (–36.00%)</td>
<td>0.23 ± 0.03b (43.75%)</td>
<td>0.22 ± 0.03b (37.50%)</td>
</tr>
<tr>
<td>Spleen weight (g/100 g bw)</td>
<td>0.34 ± 0.06</td>
<td>0.33 ± 0.04 (–2.94%)</td>
<td>0.36 ± 0.05 (5.88%)</td>
<td>0.21 ± 0.03a (–38.24%)</td>
<td>0.32 ± 0.05b (52.38%)</td>
<td>0.31 ± 0.07b (47.62%)</td>
</tr>
<tr>
<td>Heart weight (g/100 g bw)</td>
<td>0.36 ± 0.04</td>
<td>0.35 ± 0.06 (–2.78%)</td>
<td>0.38 ± 0.05 (5.56%)</td>
<td>0.23 ± 0.03a (–36.11%)</td>
<td>0.34 ± 0.03b (47.83%)</td>
<td>0.33 ± 0.03b (43.49%)</td>
</tr>
<tr>
<td>Adrenal gland weight (mg/100 g bw)</td>
<td>5.6 ± 0.19</td>
<td>5.4 ± 0.16 (–3.57%)</td>
<td>5.5 ± 0.17 (–1.79%)</td>
<td>3.6 ± 0.13a (–35.71%)</td>
<td>5.3 ± 0.19b (47.22%)</td>
<td>5.2 ± 0.19b (44.44%)</td>
</tr>
<tr>
<td>Urinary volume (mL/100 g/8 h)</td>
<td>0.96 ± 0.19</td>
<td>0.94 ± 0.16 (–2.08%)</td>
<td>0.97 ± 0.18 (1.04%)</td>
<td>0.64 ± 0.13a (–33.33%)</td>
<td>0.93 ± 0.17b (45.31%)</td>
<td>0.95 ± 0.18b (48.43%)</td>
</tr>
<tr>
<td>Fecal pellet count</td>
<td>38 ± 4.07</td>
<td>37 ± 3.86 (–2.63%)</td>
<td>36 ± 4.20 (–5.26%)</td>
<td>25 ± 3.14a (–34.21%)</td>
<td>35 ± 4.35b (40.00%)</td>
<td>34 ± 4.26b (36.00%)</td>
</tr>
</tbody>
</table>

Number of animals = 6 rats/group. Data are represented as mean ± SEM. CMS, Chronic mild stress. a Highly significant change compared to control. b Highly significant change compared to CMS rats. (%): Percentage of change compared to control or CMS rats.
Table 2. Effect of malic acid on behavioral tests of CMS rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Malic acid</th>
<th>Venlafaxine drug</th>
<th>CMS rats</th>
<th>CMS rats + Malic acid</th>
<th>CMS rats + Venlafaxine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose preference test (%)</td>
<td>89.2 ± 1.7</td>
<td>88.3 ± 1.5 (–1.01%)</td>
<td>87.9 ± 1.6 (–1.46%)</td>
<td>48.8 ± 1.5a (–45.29%)</td>
<td>86.3 ± 1.4b (76.84%)</td>
<td>89.7 ± 1.2b (83.81%)</td>
</tr>
<tr>
<td>Distance traveled test (cm)</td>
<td>226.7 ± 90.1</td>
<td>220.0 ± 74.1 (–2.96%)</td>
<td>223.7 ± 80.5 (–1.32%)</td>
<td>70.0 ± 16.3a (–69.12%)</td>
<td>220.6 ± 78.0b (215.14%)</td>
<td>224.7 ± 44.3b (221.0%)</td>
</tr>
<tr>
<td>Center square entries test (/5 minutes)</td>
<td>1.40 ± 1.8</td>
<td>1.50 ± 0.97 (7.14%)</td>
<td>1.46 ± 1.6 (4.29%)</td>
<td>0.20 ± 0.10a (–85.71%)</td>
<td>1.36 ± 0.15b (580.0%)</td>
<td>1.39 ± 0.72b (595.0%)</td>
</tr>
<tr>
<td>Tail suspension test (seconds)</td>
<td>3.60 ± 1.3</td>
<td>3.43 ± 1.4 (–4.72%)</td>
<td>3.54 ± 1.7 (–1.67%)</td>
<td>7.18 ± 11.9a (99.44%)</td>
<td>3.62 ± 1.1b (–49.85%)</td>
<td>3.64 ± 0.9b (–49.30%)</td>
</tr>
<tr>
<td>Forcswimming test (seconds)</td>
<td>83.7 ± 3.6</td>
<td>83.4 ± 3.9 (–0.36%)</td>
<td>82.9 ± 3.2 (–0.96%)</td>
<td>1170 ± 40a (64%)</td>
<td>3230 ± 50b (176.0%)</td>
<td>3220 ± 70b (175.2%)</td>
</tr>
</tbody>
</table>

Number of animals = 6 rats/group. Data are represented as mean ± SEM. CMS, Chronic mild stress. a Highly significant change compared to control. b Highly significant change compared to CMS rats. (%): Percentage of change compared to control or CMS rats.

Table 3. Effect of malic acid on antioxidants levels in hypothalamus of CMS rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Malic acid</th>
<th>Venlafaxine drug</th>
<th>CMS rats</th>
<th>CMS rats + Malic acid</th>
<th>CMS rats + Venlafaxine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (U/g tissue)</td>
<td>3250 ± 60</td>
<td>3240 ± 50 (–0.31%)</td>
<td>3245 ± 55 (–0.15%)</td>
<td>1170 ± 40a (–64.0%)</td>
<td>3230 ± 50b (176.0%)</td>
<td>3220 ± 70b (175.2%)</td>
</tr>
<tr>
<td>Glutathione peroxidase (U/g tissue)</td>
<td>785 ± 19</td>
<td>780 ± 24 (–0.64%)</td>
<td>785 ± 21 (0.0%)</td>
<td>365 ± 18a (–53.50%)</td>
<td>775 ± 16b (112.32%)</td>
<td>770 ± 21b (110.96%)</td>
</tr>
<tr>
<td>Catalase (µmol H₂O₂/min/mg tissue)</td>
<td>0.18 ± 0.05</td>
<td>0.19 ± 0.04 (5.56%)</td>
<td>0.17 ± 0.06 (0.0%)</td>
<td>0.09 ± 0.03a (–50.0%)</td>
<td>0.17 ± 0.06b (88.89%)</td>
<td>0.16 ± 0.05b (77.78%)</td>
</tr>
<tr>
<td>Malondialdehyde (µmol/g tissue)</td>
<td>8.52 ± 0.60</td>
<td>8.49 ± 0.72 (–0.35%)</td>
<td>8.50 ± 0.69 (–0.23%)</td>
<td>19.38 ± 5.4a (127.46%)</td>
<td>9.54 ± 0.83b (–50.77%)</td>
<td>9.58 ± 0.90b (–50.56%)</td>
</tr>
<tr>
<td>NADPH oxidase activity (mg/mg protein × 10⁵)</td>
<td>12.3 ± 1.18</td>
<td>12.5 ± 1.54 (1.62%)</td>
<td>12.4 ± 1.37 (0.81%)</td>
<td>7.6 ± 1.31a (–38.21%)</td>
<td>11.6 ± 1.25b (52.63%)</td>
<td>11.9 ± 1.26b (56.57%)</td>
</tr>
<tr>
<td>Conjugated dienes (µmol/g tissue)</td>
<td>1.45 ± 0.19</td>
<td>1.42 ± 0.17 (–2.06%)</td>
<td>1.43 ± 0.15 (–1.37%)</td>
<td>2.13 ± 0.14a (46.89%)</td>
<td>1.46 ± 0.18b (–31.46%)</td>
<td>1.47 ± 0.17b (–30.98%)</td>
</tr>
<tr>
<td>Oxidative index (A₂₃₃/A₂₁₅ ratio)</td>
<td>0.49 ± 0.03</td>
<td>0.47 ± 0.05 (–4.08%)</td>
<td>0.50 ± 0.03 (2.04%)</td>
<td>0.82 ± 0.04a (67.34%)</td>
<td>0.53 ± 0.04b (–35.36%)</td>
<td>0.51 ± 0.03b (–37.80%)</td>
</tr>
</tbody>
</table>

Number of animals = 6 rats/group. Data are represented as mean ± SEM. CMS, Chronic mild stress; NADPH, nicotinamide-adenine dinucleotide phosphate. a Highly significant change compared to control. b Highly significant change compared to CMS rats. (%) : Percentage of change compared to control or CMS rats.
2.6 Behavioral and Biochemical Tests

2.6.1 Behavioral Tests in a Rat Model by Using Automated Software

2.6.1.1 Sucrose Preference Test. The Strekalova and Steinbusch [33] technique was used for this test. In this technique, the rats were trained to absorb sucrose by being housed in cages with two sucrose bottles (1% w/v) for a period of 72 hours. One bottle was then swapped out for one that contained tap water. To determine the sucrose preference, the amount of water and sucrose that were consumed were both measured.

2.6.1.2 Forced Swimming Test. This test was located using the Zhang et al. [34] technique. We used a plastic cylinder that was 25 cm in diameter and 50 cm high, and we filled it with water that was 23–25 °C to a specific height (≈ 45 cm). For 5 minutes, every single rat was submerged in this cylinder. The rat was taken out of the water and allowed to dry. Each and every rat was returned to its cage. Each rat’s immobility period was determined by measuring how long it spent floating in the water without making any effort to maintain its head above the surface.

2.6.1.3 Tail Suspension Test. The results of this test were calculated using the techniques proposed by Beloviova et al. [35] and Castagné et al. [36]. The animals were suspended independently from their tails for 5 minutes, 58 cm from the ground. After the struggle phase, the rat became immobile and immobile time of each rat was detected.

2.6.1.4 Open Field Test. According to Zhang et al. [34] technique, the open field test was conducted using the distance travelled test, center square entries test, and center square duration test. Twenty-five squares made up an extra-large cage with dimensions of 75 cm × 75 cm × 40 cm. Each animal was evaluated separately by being left in the center for 5 minutes so that it could learn its surroundings. Each session included calculations for the number of crossings, rearing, and central square entrances times.

2.6.2 Biochemical Tests

2.6.2.1 Hypothalamus Antioxidants Determination. The activity of superoxide dismutase (SOD) was measured using the Suttle [37] method. The Pagalia and Valentine [38] method was used to measure the activity of glutathione peroxidase (GPx). Catalase (CAT) activity was calculated using the Aebi [39] technique. Malondialdehyde was measured as a sign of lipid peroxidation using the Ohkawa et al. [40] method. Following the instructions in the kit booklets, all of the aforementioned antioxidants were detected using spectrophotometry.

To detect conjugated dienes (CD), the method of Kogure et al. [41] was used. Hypothalamus homogenate was added to a solution containing 1 mL of 10 mmol/L phosphate buffer (pH 7.4) and 1% Lubrol (0.01 mg of protein). Using a spectrophotometer, conjugated dienes were measured using the absorbance ratio A<sub>233</sub>/A<sub>215</sub> (oxidative index) [42,43].

Superoxide radical (O<sub>2</sub><sup>−</sup>) production in NADPH oxidase activity was examined using a chemiluminescence assay to measure NADPH oxidase activity [44]. Hypothalamus homogenate (250 μL) and phosphate buffer (50 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 1 mmol/L EGTA, 150 mmol/L sucrose, pH 7.4) were the solutions to which NADPH (0.1 mmol/L) was added. The aforementioned solution was supplemented with lucigenin (5 mmol/L). NADPH oxidase activity was determined using a multimode microplate fluorometer at 30 °C/5 seconds for a period of 10 minutes [45].

2.6.2.2 Hypothalamus Neurotransmitters Determination. The Kitagawa [46] method was used to measure the serotonin level. The Guo et al. [47] method was used to determine the dopamine level. The norepinephrine level was determined using the Kapoor and Chalmers [48] technique. To measure the level of γ-aminobutyric acid (GABA), the Sciotti et al. [49] method was used. For all of the aforementioned neurotransmitters, enzyme-linked immunosorbent assay (ELISA) kits (20386, SinoGeneClon, Hangzhou, China) were utilized, and the kit guidelines were followed.

2.6.2.3 Hypothalamus Inflammatory Markers Determination. Tumor necrosis factor-α (TNF-α) was detected using the Matalka et al. [50] method. The Interleukin-1β (IL-1β) level was measured using the DeCicco et al. [51] method. Both Interleukin-6 (IL-6) and Interleukin-10 were calculated using the Stelmasiak et al. [52] method. All of the aforementioned inflammatory indicators were detected using ELISA kits in accordance with the instructions provided in the kit booklets.

2.6.2.4 Hypothalamus Sodium/Potassium-ATPase Determination. The primary solution was made up of the following ingredients: NaCl (80 mM), Tris HCl (50 mM, pH 7.4), MgCl<sub>2</sub> (5 mM), KCl (20 mM), and ATP disodium salt (3 mM). Sodium/potassium-ATPase activity was then determined. To start the reaction, 50 mL of hypothalamus homogenate was added to the aforementioned solution, which was then incubated at 37 °C for 10 minutes. Trichloroacetic acid (50 mL) was then added to terminate the reaction. After that, the solution was centrifuged for 15 minutes (at 3000 rpm). Then, the supernatant (1 mL) was collected and added to the mixture of ascorbic acid, trichloroacetic acid, and ammonium molybdate (250 mL of each ingredient). A spectrophotometer was used to measure the acquired color at 680 nm [53].

2.6.2.5 Detection of Hypothalamus Histamine-N-methyl transferase (Hmtt) and Tyrosine Hydroxylase (TH) Enzymes Expression by Using Real Time PCR (4385610, Hercules, CA, USA). RNA was extracted from the hypothalamus using an Invitrogen® PureLink® RNA Mini Kit (b12183018A, Invitrogen™, ThermoFisher Scientific
Company, Waltham, MA, USA). This study’s primary methodology is the use of the RNA-to-cDNA Kit to convert cDNA to RNA (Applied Biosystems kit reagents, Biorad Company, Hercules, CA, USA). Tyrosine hydroxylase is activated by the primer (Rn Th 1 SG), Histamine N-methyl transferase is activated by the primer (Rn Hnmt 1 SG), and Glyceraldehyde 3-phosphate dehydrogenase is activated by the primer (Rn GAPDH 1 SG) (GAPDH). The Qiagen Corporation (Qiagen, Hilden Town, Germany) was used to purchase all of the primers indicated above. The mRNA for the GAPDH gene was identified using 2-CT, and the fold change from the control is expressed [54].

2.7 Histopathological Investigation

Hypothalamus specimens were preserved in 10% formalin. It was a routine task to embed hypothalamic tissue in paraffin blocks. Five meters of partitioning separated these blocks. Hematoxylin and eosin was used to stain all of the blocks before they were examined under a light microscope.

2.8 Statistical Analysis

The results were presented as mean standard error mean in tables (SEM). In this study, the normal distribution or the Gaussian distribution was used. The SPSS 13 application (IBM Corp., Chicago, IL, USA) was used to run a one-way Analysis of Variance (ANOVA) test. p-values ≤ 0.05 were considered significant when using the fisher least significant difference (FLSD) test in post-hoc analysis among all treatment groups.

3. Results

3.1 Physiological Measures Results

Table 1 exhibits that there is a significant decrease in total body weight, food consumption, water intake, organ (liver, kidney, brain, pancreas, spleen, heart, adrenal gland) weight, and urine and fecal output in CMS rats with percentage of change equal to –22.42%, –37.93%, –37.09%, –33.33%, –41.67%, –30.36%, –36.00%, –38.24%, –36.11%, –35.71%, –33.33%, and –34.21% compared to the control group. On the contrary, malic acid and venlafaxine drug oral administration to CMS rats returned the total body weight, food consumption, water intake, organ (liver, kidney, brain, pancreas, spleen, heart, adrenal gland) weight, and urine and fecal output to approach the control values with percentage of change equal to 27.43% and 26.56%, 5.94% and 55.56%, 56.41% and 53.85%, 33.33% and 27.78%, 57.14% and 61.90%, 35.90% and 38.46%, 43.75% and 37.50%, 52.38% and 47.62%, 47.83% and 43.49%, 47.22% and 44.44%, 45.31% and 48.43%, and 40.00% and 36.00%, respectively compared to CMS rats.

3.2 Behavioral Results

Table 2 reveals malic acid effect on behavioral tests in CMS rats. It is clear that CMS caused a highly significant decrease (p ≤ 0.01) in the sucrose preference test, distance travelled test, and center square entries test with percentages of change equal to –45.29%, –69.12%, and –85.71% compared to the control group. A highly significant increase (p ≤ 0.01) was observed in the center square duration test, tail suspension test, and forced swimming test with percentages of change equal to 99.44%, 182.98%, and 60.57% compared to the control group. On the other hand, malic acid and venlafaxine drug oral administration to CMS rats pushed the above-mentioned behavioral tests (sucrose preference test, distance travelled test, center square entries test, center square duration test, tail suspension test, and forced swimming test) to be near the control values with percentages of change equal to 76.84% and 83.81%, 215.14% and 221.0%, 580.0% and 595.0%, –49.85% and –49.30%, 64.28% and –62.79%, and –39.46% and –37.38% of these tests compared to CMS rats. Moreover, the oral administration of malic acid or venlafaxine drug to normal rats without behavioral change in all tests applied through the study period.

3.3 Antioxidants Results

Table 3 shows malic acid effect on antioxidant levels in the hypothalamus of CMS rats. It can be concluded from the table that CMS induced a highly significant decrease (p ≤ 0.01) in superoxide dismutase, glutathione peroxidase, catalase activities, and NADPH oxidase activity with percentages of change equal to –64.0%, –53.50%, –50.0%, and –38.21% compared to the control group. A highly significant increase (p ≤ 0.01) was recorded in malondialdehyde, conjugated dienes, and oxidative index with percentages of change equal to 127.46, 46.89, and 67.34%, respectively, compared to the control group. Furthermore, oral administration of malic acid or venlafaxine to CMS rats pushed antioxidant tests to approach the control levels with a percentage of change equal to 176.0% and 175.2%, 112.32% and 110.96%, 88.89% and 77.78%, 52.63% and 56.57%, –50.77% and –50.56%, –31.46% and –30.98%, –35.36% and –35.24% for superoxide dismutase, glutathione peroxidase, catalase activities, NADPH oxidase activity, malondialdehyde, conjugated dienes, and oxidative index, compared to CMS rats. In the contrary, malic acid or venlafaxine drug oral administration in normal rats did not show any change in any of the antioxidants used in this study.

3.4 Neurotransmitters Results

Fig. 2 exhibits malic acid effect on neurotransmitter levels in the hypothalamus of CMS rats. It is obvious that CMS caused a highly significant decrease (p ≤ 0.01) in serotonin, dopamine, norepinephrine, and γ-aminobutyric acid levels with percentages of change equal to –54.13, –47.14%, –47.22%, and –47.06% compared to the control group. On the other hand, malic acid or venlafax-
Fig. 2. Effect of malic acid on neurotransmitters levels in hypothalamus of chronic mild stress (CMS) rats. Fig. 2 explains hypothalamus neurotransmitters. (A) Serotonin (ng/g tissue). (B) Dopamine (ng/g tissue). (C) Norepinephrine (ng/g tissue). (D) Gamma amino butyric acid (µmol/g tissue). Number of animals = 6 rats/group. Data are represented as mean ± SEM. a Highly significant change compared to control. b Highly significant change compared to CMS rats. Cont., Control; Mal., Malic acid; Venla., Venlafaxine drug; CMS, Chronic mild stress; CMS rats + Mal., CMS rats + Malic acid; CMS rats + Venla., CMS rats + Venlafaxine drug.

The oral administration of malic acid or venlafaxine drug to normal rats without change in all neurotransmitters in this research. with percentages of change equal to 116.7% and 115.3%, 88.51% and 88.17%, 87.36% and 85.26%, and 74.07% and 66.67% compared to CMS rats.

Fig. 3. Effect of malic acid on inflammatory markers in hypothalamus of CMS rats. Fig. 3 shows hypothalamus inflammatory markers. (A) Tumor necrosis factor-α (ng/g tissue). (B) Interleukin-1β (ng/g tissue). (C) Interleukin-6 (pg/g tissue). (D) Interleukin-10 (pg/g tissue). Number of animals = 6 rats/group. Data are represented as mean ± SEM. a Highly significant change compared to control. b Highly significant change compared to CMS rats. Cont., Control; Mal., Malic acid; Venla., Venlafaxine drug; CMS, Chronic mild stress; CMS rats + Mal., CMS rats + Malic acid; CMS rats + Venla., CMS rats + Venlafaxine drug.
3.5 Inflammatory Markers Results

Fig. 3 shows malic acid effect on inflammatory markers in the hypothalamus of CMS rats. It can be estimated that depression induced a highly significant increase \((p \leq 0.01)\) in tumor necrosis factor-\(\alpha\), interleukin-1, and interleukin-6 levels with percentages of change equal to 82.05%, 101.53%, and 95.97% compared to control levels. A highly significant decrease \((p \leq 0.01)\) was found in interleukin-10 level with percentages of change equal to \(-51.92\%\) compared to the control group. Furthermore, oral administration of malic acid or venlafaxine drug to CMS rats pushed these inflammatory markers to approach the control levels with percentages of change equal to \(-46.83\%\), \(-46.47\%\), \(52.67\%\), \(-51.90\%\), \(-47.57\%\), \(-46.72\%\), and \(103.42\%\) and \(93.71\%\), respectively, compared to CMS rats. Furthermore, the oral administration of malic acid or venlafaxine drug to normal rats without change in all inflammatory markers in the experimental study.

3.6 Sodium/Potassium-ATPase Results

Fig. 4 exhibits the effect of malic acid on sodium/potassium-ATPase activity in the hypothalamus of CMS group. It is obvious from this figure that CMS induced a highly significant decrease \((p \leq 0.01)\) in sodium/potassium-ATPase activity with a percentage of change equal to \(-39.69\%\) compared to the control group. On the other hand, oral administration of malic acid or venlafaxine to CMS rats pushed the above-mentioned sodium/potassium-ATPase activity to near the control values with a percentage of change equal to 65.09% and 61.82% compared to CMS rats. Moreover, oral administration of malic acid or venlafaxine drug to normal rats without change in sodium/potassium-ATPase activity during the study period.

3.7 Histamine-N-methyl transferase (Hnmt) and Tyrosine Hydroxylase (TH) Enzymes Results

Fig. 5 reveals malic acid effect on the fold change of Hnmt and TH enzyme expressions in the hypothalamus of CMS rats. The data indicate that CMS caused a highly significant decrease \((p \leq 0.01)\) in Hnmt and TH enzymes, with percentages of change equal to \(-41.58\%\) and \(-23.08\%\), respectively, compared to the control group. Moreover, malic acid or venlafaxine drug oral administration to CMS rats pushed the above-mentioned Hnmt and TH enzymes to approach the control values with a percentage of change equal to 66.10% and 62.71% compared to CMS rats. Furthermore, the oral administration of malic acid or venlafaxine drug to normal rats without change in Hnmt and TH enzymes during the study period.

3.8 Histology Results

Fig. 6 reveals hypothalamus tissue in the control group, showing the control neurons with large nuclei (pale-stained neurons) (black arrows) (Fig. 6A). Hypothalamus
sections of the CMS rats showed small cells that were missing the normal large cells and their normal nuclei. These small cells appear as rings and these neurons contain newly dark dead neurons (black arrows) (Fig. 6B). CMS rats treated with malic acid (250 mg/kg bw) or venlafaxine drug (20 mg/kg bw) showed large normal neurons with normal nuclei that looked like control group (Fig. 6C).

4. Discussion

Stress causes depression by increasing brain superoxide anion and neuro-inflammation due to stimulation of the hypothalamus-pituitary-adrenal axis [55]. The pathogenesis and symptoms of human depression are comparable to those shown in the CMS animal model [56]. In comparison to the control group, the study found a reduction in food intake, water consumption, urine production, and organ weights. CMS significantly affects the animal’s desire for food and water, which in turn causes the animal’s total and organ weights to decrease. This decrease in animal appetite is brought on by the CMS rats’ lower levels of insulin and leptin, and these findings concur with those of Aluko and Umukoro [57], Flak et al. [58], and Wang et al. [59]. CMS caused a decline in the sucrose preference test, travel distance test, and center square entries test, but a rise in the center square duration test, tail suspension test, and forced swimming test. In tests using inescapable cases like the tail suspension test and the forced swimming test to force the rat to confront depressing and challenging circumstances, CMS is associated with stress exposure in rats. These studies assessed the animal’s management strategy in these circumstances. At the conclusion of each rat’s struggling phase throughout these tests, the immobility time is recorded [57]. In the forced swimming test and the tail suspension test, CMS rats showed an increase in the immobility period. These findings explain the animal’s management strategy in an unavoidable circumstance that closely resembles evident depressive symptoms, and they are corroborated by earlier investigations [34,60].

CMS makes people respond more anxiously, which has an impact on their automatic behavior. By introducing the rat to a novel habitat and observing the animal’s adaptability, risk assessment-correlated behavior, and exploratory activities like the open field test, anxiety in rats was studied [35]. In the open field test, CMS rats display a decrease in the number of rearing, crossed lines, and central square entries. These findings, which related to anxiety and a poorer risk assessment behavior, are corroborated by other studies [34,59]. Anhedonia simulates the loss of pleasure that is a hallmark of depressive patients’ symptoms [61]. In depressive individuals, anhedonia is associated with indoleamin 2,3-dioxygenase I expression. The sucrose preference test can be used to measure hedonic behavior in rats. CMS rats show a decrease in this test, and the degree of the decline depends on the length of CMS exposure. These results are consistent with those of Zhang et al. [34] and Filho et al. [62].

The decrease of distance travelled test in CMS rats is due to 2 reasons; (1) the decrease of hypothalamus neurotransmitters serotonin, dopamine, norepinephrine, and γ-aminobutyric acid in CMS rats compared to that in control rats [63]; where rats after 5 weeks of chronic unpredictable stress had depressive-like behaviors such as decreased total travel distance and decreased open field test [64,65] and (2) depressive-like behaviors in CMS rats change the concentration of mood-related hormones, and cause immune/endocrine dysfunction [66].

CMS caused also a decrease in hypothalamus neurotransmitters, where CMS is characterized by disturbances of both psychological and physiological conditions [67], chronic mild stress decreased 5-hydroxytryptamine levels [68]. Malic acid oral administration to CMS rats amended the behavioral disturbances and hypothalamus neurotrans-
mitters in CMS rats. This observation is related to the antidepressant activity of malic acid, which, in accordance with many previous studies such as Tanasiević et al. [69], stated that malic acid treated xerostomia, which led to depression. Also, Ferreira et al. [70] showed that malic acid treated patients with fibromyalgia, and the acid reduces the patient’s pain and its depressive symptoms. Moreover, von Eggelkraut-Gottanka et al. [71] revealed that malic acid plays a key role in the treatment of mild/moderate depressive disorders. Furthermore, Lian et al. [72] reported that malic acid has an antidepressant effect.

CMS induced oxidative stress in the hypothalamus of the CMS group, where CMS increased hydrogen peroxide production and neuroinflammation [73]. Malic acid oral administration to CMS rats jumped the antioxidant enzyme activities in the hypothalamus of those rats. This effect is related to the antioxidant activity of malic acid. Many previous studies agree with these results. As Quiroga et al. [74] stated, malic acid has antioxidant activity. Also, Yan et al. [75] found that malic acid improves the antioxidants in pigs. Moreover, Mousavi et al. [22] revealed that malic acid increases catalase, ascorbate peroxidase, and many antioxidants but it decreases cadmium toxicity inside the plant. Furthermore, Calvo et al. [76] and Taher et al. [77] proved that malic acid has antioxidant, antihypertensive, and hypoglycemic activities.

CMS increased the inflammatory markers in the hypothalamus of CMS rats, where CMS induce neuroinflammation [73]. The oral administration of malic acid to CMS rats decreased these markers in the hypothalamus. This observation is related to the anti-inflammatory activity of malic acid, where Tang et al. [78] stated that malic acid reduced serum levels of tumor necrosis factor-α and platelet aggregation, and consequently, malic acid has anti-inflammatory and antiplatelet aggregation properties. Also, Barragán-Zarate et al. [79] revealed that malic acid exhibits antioxidant and/or anti-inflammatory activity. Moreover, Sahbaz et al. [80] reported that malic acid exerts anti-inflammatory activity. Furthermore, Ahiabarro-Ortega et al. [81] showed that malic acid inhibits neuro-inflammation, free radicals in the brain, microbes, and tyrosinase in the neurons.

CMS induced a decrease in sodium/potassium-ATPase activity in the hypothalamus of CMS rats, where CMS induced oxidative stress that caused the depletion of antioxidant enzymes. The imbalance in the oxidative state caused a deficiency in sodium/potassium-ATPase activity [82]. The oral administration of malic acid to CMS rats amended sodium/potassium-ATPase activity in the hypothalamus of CMS rats. This observation is related to the finding that malic acid increases oxygen consumption and decreases glucose secretion [83]. Also, malic acid increases the K+ accumulation inside the cell, which activates the plasma membrane H+-ATPase and phosphoenolpyruvate carboxylase cycle [84] and consequently restores sodium and potassium-ATPase activity in the hypothalamus of CMS rats. Finally, sodium/potassium-ATPase activity was elevated secondary to the alleviation of oxidative stress by malic acid [22].

CMS caused a decrease in Hnmt and TH enzyme overexpression in the hypothalamus of CMS rats. Brain histamine is a neurotransmitter and regulates many physiological functions, and histamine depletion causes many neurological disorders such as sleeping disorders, depression, and Parkinson’s disease. Hnmt is a histamine-metabolising enzyme expressed in the brain. The deficiency causes the depletion in the concentration of catecholamines because it is an important rate-limiting enzyme in their biosynthesis. Hnmt is an essential enzyme for the elimination of histamine [85,86]. The TH and Hnmt genes were inhibited in CMS rats. The decrease of Hnmt and TH enzymes will perturb the synthesis of neurotransmitters and enhance neuroinflammation. The oral administration of malic acid to CMS rats restored Hnmt and TH enzymes in the hypothalamus of CMS rats to levels approaching the control levels due to malic acid’s inhibition of neuro-inflammation and hydrogen peroxide production in the neurons [22,78,80].

Histological investigation showed acid cytoprotective on the hypothalamus of CMS rats, and these results are related to the fact that malic acid inhibits neuro-inflammation and hydrogen peroxide production in the neurons. Such an observation is supported by Martin-Nizard et al. [87], who revealed that malic acid inhibits low-density lipoprotein-induced cellular toxicity. Also, Bhattacharya and Tulsawani [88] showed that malic acid has maximum cytoprotection against the toxicity of potassium cyanide.

Exposing animals to many stressors causes CMS rats. CMS was associated with disturbances of animal behaviors such as decreasing sucrose preference testing, distance travelled testing, and center square entry testing but increasing center square duration testing, tail suspension testing, and forced swimming tests. Depression decreased hypothalamus antioxidants such as catalase and NADPH oxidase activities but increased malondialdehyde, conjugated dienes, and the oxidative index. It also decreased hypothalamic neurotransmitter levels. Moreover, it increased hypothalamic inflammatory marker levels. Furthermore, it decreased hypothalamic sodium/potassium-ATPase activity. Finally, it decreased hypothalamic Hnmt and TH enzymes in CMS rats compared to the control group. Malic acid or venlafaxine drug oral administration to CMS rats pushed the above-mentioned behavioral tests, antioxidants, neurotransmitters, inflammatory markers, sodium/potassium-ATPase activity, and Hnmt and TH enzymes in the hypothalamus of depressed rats to approach the control values.

Malic acid’s neuroprotective mechanism depends on a rise in adenosine triphosphate (ATP) levels and a fall in propidium iodide (PI) nucleic acid levels. Malic acid’s neuroprotective function stops the degenerative degeneration of the neurons as well as the loss of mitochondrial O2 consumption. Malic acid thus participates in anaerobic phosphorylation [89]. However, the neuroprotective effect of
venlafaxine drug is dependent on changes in calcium homeostasis and neurotransmitter levels in the neurons of CMS rats [90].

Malic acid is a potent antioxidant, yet it has no effect on all the parameters tested in the normal rats. This effect is due to the acid’s preservation properties, which allow food goods to be preserved for months without experiencing any change in the composition of the contents. Malic acid is listed as a food additive with the E number (E296). The European Union [91], the United States [92], Australia, and New Zealand [93] all utilize it as a food additive for years.

The limitation of the study includes small sample size was used. Also, all other study factors, including internal and external variables, are under control. A small sample size and thorough control of the experimental circumstances also make it simple to determine the neuroprotective impact of malic acid on CMS rats. Consequently, there is a better and accurate result obtained from this study but this result is difficult to apply in depressive human subjects due to external, psychological, and internal variables, as well as, small sample size used in the study.

5. Conclusions

CMS modified animal behaviors, as well as, neurotransmitters, antioxidants, inflammatory markers, sodium/potassium-ATPase activity, and Hnmt and TH enzymes in the hypothalamus of CMS rats. Malic acid was given orally to CMS rats to treat these CMS-related symptoms. This result is associated with malic acid’s ability to reduce neuro-inflammation and the generation of hydrogen peroxide in the neurons. The clinical study that will be conducted as the second stage of this research will examine the impact of malic acid on depressive patients, and the results will be positive if malic acid may reduce depressive-like symptoms in those individuals.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

KMMK selected the topic and created the research plan. The experiments for the study were conducted by KMMK and HAKT. KMMK carried out the study’s statistical analysis and collected the outcome data. The initial and final forms of the work were written by KMMK. The article’s final form was authorized by KMMK and HAKT. Both authors contributed to editorial changes in the manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study and all experimental methods were authorized by the National Research Centre Institutional animal care and use committee; the approval code was 12041126. This study was conducted at the National Research Centre, which adhered to strict guidelines for the housing of animals and all other experimental study procedures, particularly the protocol for the care and use of animals.

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Conflict of Interest

The authors declare no conflict of interest.

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