



Protective Effect of Hemin Against Experimental Chronic Fatigue Syndrome in Mice: Possible Role of Neurotransmitters

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Abstract

Chronic fatigue syndrome (CFS) is a disorder characterized by persistent and relapsing fatigue along with long-lasting and debilitating fatigue, myalgia, cognitive impairment, and many other common symptoms. The present study was conducted to explore the protective effect of hemin on CFS in experimental mice. Male albino mice were subjected to stress-induced CFS in a forced swimming test apparatus for 21 days. After animals had been subjected to the forced swimming test, hemin (5 and 10 mg/kg; *i.p.*) and hemin (10 mg/kg) + tin(IV) protoporphyrin (SnPP), a hemoxygenase-1 (HO-1) enzyme inhibitor, were administered daily for 21 days. Various behavioral tests (immobility period, locomotor activity, grip strength, and anxiety) and estimations of biochemical parameters (lipid peroxidation, nitrite, and GSH), mitochondrial complex dysfunctions (complexes I and II), and neurotransmitters (dopamine, serotonin, and norepinephrine and their metabolites) were subsequently assessed. Animals exposed to 10 min of forced swimming session for 21 days showed a fatigue-like behavior (as increase in immobility period, decreased grip strength, and anxiety) and biochemical alteration observed by increased oxidative stress, mitochondrial dysfunction, and neurotransmitter level alteration. Treatment with hemin (5 and 10 mg/kg) for 21 days significantly improved the decreased immobility period, increased locomotor activity, and improved anxiety-like behavior, oxidative defense, mitochondrial complex dysfunction, and neurotransmitter level in the brain. Further, these observations were reversed by SnPP, suggesting that the antifatigue effect of hemin is HO-1 dependent. The present study highlights the protective role of hemin against experimental CFS-induced behavioral, biochemical, and neurotransmitter alterations.

Keywords Forced swimming · Chronic fatigue syndrome · Anxiety · Hemin · Oxidative stress · Neurotransmitters

Introduction

Chronic fatigue syndrome (CFS) (also known as myalgic encephalomyelitis) is a disease of unknown etiology with estimated prevalence of 0.1–0.5% (Cortes Rivera et al. 2019). It is characterized by persistent or relapsing debilitating fatigue for at least 6 months and a combination of symptoms that result in substantial reduction in occupational, personal, social, and educational status (Cortes Rivera et al. 2019; Lim et al.

2020). CFS is associated with a wide of spectrum symptoms such as arthralgias, muscle pain, headaches, sleep disorders, or intolerance to physical exertion (Lim et al. 2020). To date, there is no single therapy for chronic fatigue and related complications. Substantial progress has been made in the last few decades in understanding the pathophysiology of CFS in animal models, and different theories have been put forward such as immunological weakness, CNS dysfunction, disturbance of the hypothalamic–pituitary–adrenal (HPA) axis, brain neurotransmitters imbalance, mitochondrial dysfunctions, and oxidative stress (Singh et al. 2002; Surapaneni et al. 2012). Prevailing treatment strategies are largely directed towards limiting the symptoms rather than treating its root cause. Along with energy production, mitochondria are involved in various physiological processes including the production of reactive oxygen species (ROS), regulation of cellular levels of substrates (amino acids, enzyme cofactors), apoptosis, metal (Fe–S cluster and heme) metabolism, calcium homeostasis, and neurotransmitter synthesis. Thus, damage to

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mitochondria can have extensive repercussions, resulting in oxidative stress, which has been well implicated in the pathophysiology of CFS (Nunnari and Suomalainen 2012; Filler et al. 2014).

Many stress-inducing pathological conditions are involved in the induction of HO-1 enzymes which show antioxidant activities (Son et al. 2013) by breaking the pro-oxidant heme group and generate equimolecular quantities of carbon monoxide (CO), iron, and biliverdin. The induction of HO-1 is primarily regulated at the transcriptional level, secondary to nuclear translocation of nuclear factor erythroid 2-related factor (Nrf2) from the cytoplasm. Nuclear factor-erythroid 2-related factor 2/antioxidant responsive element (Nrf2/ARE) is one of the most important defense mechanisms of the body's cells against oxidative damage (Ragy et al. 2016).

Hemin, a HO-1 substrate, provides a positive countervailing effect against oxidative stress in various CNS disorders (Khan et al. 2015; Ragy et al. 2016). Hemin is a highly potent and selective HO-1 inducer, and its effect may be attributed to its catalytic activity as well as to the antioxidant and anti-inflammatory properties of its breakdown products, i.e., bilirubin and CO (Ragy et al. 2016). The role of the HO-1 pathway in CFS and related problems has not been explored yet. Thus, modulation of the HO-1 system was explored using a suitable pharmacological modulator like tin(IV) protoporphyrin (SnPP), a synthetic heme analog that selectively inhibits HO-1.

In rodents, for induction of a chronic stress-like illness, the commonly used model is the forced swimming test (Singh et al. 2002). The forced swimming test produces a CFS-like condition as evidenced by a significant decrease in locomotor activity and an increase in an anxiety-like state, as well as immobility, which is suggestive of physical exhaustion and depression. An increase in the immobility period and reduced locomotor activity in rodents is also suggestive of increased fatigue, which is a core symptom of CFS (Dhir and Kulkarni 2008; Surapaneni et al. 2012). The present study was undertaken to explore the potential role of hemin to counteract the effects of forced swimming-induced alterations in behavioral, biochemical, mitochondrial, and neurotransmitter levels in the mouse brain.

Material and Methods

Drugs and Chemicals

Tin(IV) protoporphyrin (SnPP) (Frontier Scientific, Inc., Newark, DE, USA) and hemin (Hi-Media, New Delhi, India) were used. Unless stated, all other chemicals and biochemical reagents used in this study were of the highest analytical grade.

Experimental Animals

Male albino mice (2–3 months of age), weighing 20–30 g (Laca strain) procured from the Central Animal House Facility of ISF College of Pharmacy, Moga, India, were used in the study. The animals were kept in polyacrylic cages in groups of three in a controlled atmosphere (room temperature 25 ± 1 °C and relative humidity of 60%) with a 12-h light/dark reverse cycle. The animals were maintained on a commercial food diet in the form of dry pellets and water ad libitum. All the behavioral parameters were analyzed between 9:00 and 17:00 h. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) with approval no. IAEC/CPCSEA/M14/P246. All the experiments carried out in accordance with guidelines for the use and care of experimental animals. All the experiments for a given treatment were performed using age-matched animals to avoid variability between experimental groups.

Treatment Schedule

The experimental protocol included six groups with six animals in each group (total 36 animals). The groups were scheduled as follows:

Group I: Vehicle-treated group: saline + 0.1 N NaOH; *i.p.*

Group II: CFS (10-min forced swimming test session daily for 21 days).

Group III: CFS + hemin (5 mg/kg; *i.p.*).

Group IV: CFS + hemin (10 mg/kg; *i.p.*).

Group V: CFS + tin(IV) protoporphyrin (SnPP) (40 μ M/kg; *i.p.*). Tin(IV) protoporphyrin (SnPP) is a synthetic heme analog that selectively inhibits HO-1. CFS animals were treated with tin(IV) protoporphyrin (SnPP) to explore the effect of tin(IV) protoporphyrin (SnPP) alone.

Group VI: CFS + SnPP (40 μ M/kg; *i.p.*) + hemin (10 mg/kg; *i.p.*).

Hemin and tin(IV) protoporphyrin (SnPP) solutions were prepared by dissolving in 0.1 N NaOH and diluted with phosphate-buffered saline (pH adjusted to 7.4). All drugs or vehicles were administered once daily for 21 days by the *i.p.* route after swimming in a constant volume of 1 ml/100 g of body weight, and doses were selected from previous literature and our lab studies (Khan et al. 2015; Ragy et al. 2016). On the 1st, 7th, 14th, and 21st days, behavioral parameters, including immobility period, locomotor activity, rotarod (for determination of grip strength), elevated plus maze, and mirror chamber apparatus (to evaluate anxiolytic and anxiogenic effect), were assessed. On day 22, all animals were sacrificed, and the brain was separated to estimate biochemical parameters, mitochondrial function (complexes I and II), and neurotransmitter (dopamine (DA), serotonin (5-HT), and norepinephrine (NA) and their metabolites) levels.

Behavioral Assessment

Forced Swimming Test (Measurement of Immobility Period)

The forced swimming test is used to measure the immobility period (lack of activity aside from small movements needed to keep the body floating was measured) in mice as per the procedure described by Surapaneni et al. (2012). The individual animal was forced to swim for 10 min daily for 21 days in a glass jar (25 × 12 × 25 cm) containing water at room temperature (22 °C ± 3 °C). The water depth was calibrated to 15 cm throughout the experiment. After 5 min of initial period of vigorous activity, each animal assumed a typical immobile posture. The duration of immobility was measured up to 300 s. When the mice stopped the struggling movement of their limbs to keep their heads above water, they were considered immobile. The prolonged immobility time caused by continued forced swimming was considered to be a condition similar to CFS (Surapaneni et al. 2012; Mitra et al. 2017; Sarvaiya and Goswami 2016).

Assessment of Gross Behavioral Activity (Locomotor Activity)

The gross locomotor activity was recorded for a period of 10 min using an actophotometer (IMCORP, Ambala) on the 1st, 7th, 14th, and 21st days, with a view to assess the impact of the forced swimming test session on motor activity. Each mouse was kept in the actophotometer for a 1-min habituation period prior to the actual 10-min recording. Each animal was examined in a square (30-cm) closed platform with infrared light-sensitive photocells using a digital actophotometer. Locomotor activity was indicated by the total photobeam counts for 10 min/animal (Kumar et al. 2012; Mitra et al. 2017).

Grip Strength Measurement

A digital grip forced meter (DFIS series, Chatillon, Greensboro, NC, USA) was used to test the grip strength of the fore limbs. The mice were placed in such a position to grab the grid with the fore limbs and were gently pulled so that grip strength could be recorded (in kgf) (Khan et al. 2015).

Measurement of Anxiety Using Elevated Plus Maze

Anxiety was evaluated by using an elevated plus maze apparatus. The test takes advantage of the natural tendency of mice to explore novel environments. The mouse is given the choice of spending time in open, unprotected maze arms or enclosed, protected arms, all elevated approximately 50 cm above the floor. Mice tend to avoid the open areas, especially when they are anxious, favoring darker, more enclosed spaces. The apparatus was placed 50 cm above the ground on a platform on

the closed side of the apparatus. It is made of two open arms (16 × 5 cm) and two enclosed arms (16 × 5 × 12 cm) extended from a central platform (5 × 5 cm). Mice were placed individually in the apparatus, facing either of the open arms, at the central platform. The following parameters were observed during a 5-min session: (a) time spent by each animal in the closed arm and the open arm and (b) number of entries in the closed arm. An anxiogenic result was observed with an increase in the number of entries and duration in the closed arm (Dhir and Kulkarni 2008; Mitra et al. 2017).

Mirror Chamber

The mirror chamber is another specific and a quantitatively/qualitatively different measure of anxiety. The mirror chamber is designed to detect anxiolytic agents. It is based on the principle that when faced with a mirror image, many species show an approach–avoidance conflict behavior. The mirrored cube (30 × 30 × 30 cm) consists of 5 pieces of mirror glass. The mirrored surfaces are in such a position that these face the interior of the cube. The container box (40 × 40 × 30.5 cm) had opaque black walls and a white bottom. Placing of the mirrored cube into the center of the container resulted in forming a 5-cm corridor which surrounded the mirrored chamber completely. A sixth mirror was placed on the container wall positioned in such a way that it faced the single open side of the mirrored chamber. The intensity of light in the corridor surrounding the mirrored chamber was 200 lx, in contrast to luminance of 100 lx within the minor compartment. Mice, while exposed to the chamber of mirrors, were evaluated only once to avoid habituation issues. Mice were positioned at a single, fixed starting point at the same corner of the corridor and allowed to move freely around the corridor and into the chamber of mirrors. During 5-min sessions, the number of entries and time spent in mirrored chamber by mice were observed and recorded. The criterion for entry into the chamber was all four feet being placed on the floor panel of the mirrored chamber (Mitra et al. 2017).

Dissection and Homogenization

On the 22nd day, after behavioral quantification as described above, animals were randomly divided into two groups: one for biochemical estimations and the other for neurochemical estimations. The animals were sacrificed by decapitation immediately, and the brains were dissected out. A 10% (w/v) whole-brain homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) and centrifuged at 10,000×g for 15 min. Aliquots of the supernatant were separated and used for biochemical estimations.

Measurement of Oxidative Stress Parameters

Measurement of Lipid Peroxidation

The quantification of the malondialdehyde (MDA), an end product of lipid peroxidation, was performed in the mouse brain homogenate according to the method described by 1966 (Wills 1966). The homogenate and Tris–HCl were mixed in equal volumes and incubated for 2 h at 37 °C. After incubation, ice-cold 10% trichloroacetic acid (TCA) was added and centrifuged at 1200×*g* for 10 min. Afterwards, 1 ml of 0.67% thiobarbituric acid (TBA) was added to 1 ml of supernatant and tubes were placed in a water bath for 10 min at 60 °C. The mixture was then allowed to cool, and the optical density was recorded at 532 nm using a Shimadzu spectrophotometer (Wills 1966).

Estimation of Nitrite

The quantification of nitrite to determine NO production in the striatal supernatant was performed using Griess reagent (0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid) described by Green et al. (1982). The striatal supernatant and Griess reagent were mixed in equal volumes and incubated for 10 min in the dark at room temperature. Finally, the absorbance was recorded at 540 nm using Shimadzu spectrophotometer (Green et al. 1982).

Estimation of Glutathione Levels

The quantification of the antioxidant enzyme glutathione in brain supernatant was done according to method described by Ellman and Lysko (1979). To 1 ml of supernatant, 1 ml of 4% sulfosalicylic acid was added and placed at 4 °C for 1 h. The mixture was centrifuged at 1200×*g* for 15 min, and then, phosphate buffer (0.1 mmol/l, pH 8, 2.7 ml) and 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) (2 ml) were added to 1 ml of clear supernatant. Finally, the absorbance of the yellow-colored mixture was recorded at 412 nm using a Shimadzu spectrophotometer (Ellman and Lysko 1979).

Protein Estimation

Protein levels were quantified in brain samples using Folin phenol reagent as per the method described by Lowry et al. (1951). Of the brain supernatant, 0.2 ml was taken, and 0.8 ml of distilled water was added to make up the final volume of 1 ml. To this, 4.5 ml of reagent I with a composition of 2% Na₂CO₃ in 0.1 N NaOH, 1% KNaC₄H₄O₆·4H₂O, and 0.5% CuSO₄·5H₂O was added and incubated for 10 min. In the next step, 0.5 ml of reagent II with a composition of one part Folin's phenol [2 N]:one part water was added and again incubated for the next 30 min. The mixture developed a light

green color, and the absorbance was recorded at 650 nm using a Shimadzu spectrophotometer. The amount of protein present in the sample was calculated from the standard graph.

Mitochondrial Complex Estimation

Isolation of Mouse Brain Mitochondria

Mouse brain mitochondria were isolated as per the description given by Berman and Hastings (1999). An isolation buffer was used for the homogenization of brain regions, and then, homogenates were centrifuged at 13,000×*g* for 5 min at 41 °C. Pellets were suspended again in the isolation buffer with ethylene glycol tetra-acetic acid (EGTA) and rotated again at 13,000×*g* for 5 min. The resulting supernatant was taken in tubes and filled with the isolation buffer with EGTA and again rotated at 13,000×*g* for 10 min. Pellets consisting of pure mitochondria were suspended again in isolation buffer without EGTA.

Complex I (NADH Dehydrogenase Activity)

Complex I enzyme activity was measured spectrophotometrically as per the description given by King and Howard (1967). This method involves the reduction of cytochrome-c preceded by catalytic oxidation of nicotinamide adenine dinucleotide (NADH) to NAD⁺. The reaction mixture was made of 0.2 mol/l glycylglycine buffer, pH 8.5; 6 mmol/l NADH in 2 mmol/l glycylglycine buffer; and 10.5 mmol/l cytochrome C. The reaction was started by adding the requisite quantity of solubilized mitochondrial sample, and absorbance change was measured at 550 nm for 2 min.

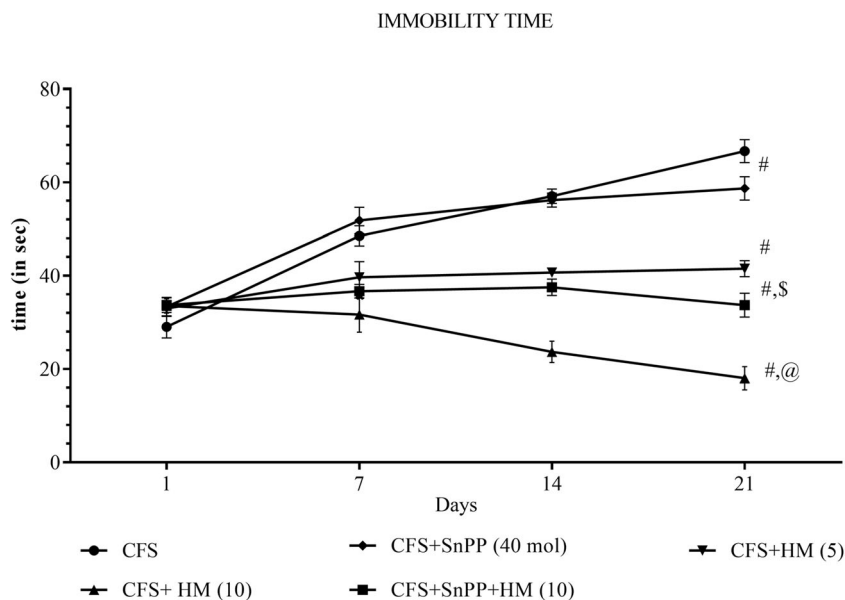
Complex II (SDH Activity)

Succinate dehydrogenase (SDH) was recorded spectrophotometrically according to the procedure described by King (1967). The process involves the oxidation of succinate by potassium ferricyanide, which is an artificial electron acceptor. The reaction mixture consisted of 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. The reaction was started by adding of the striatal mitochondrial sample, and the absorbance change was followed for 2 min at 420 nm.

Neurotransmitter Estimation

Catecholamines (DA, 5-HT, and NE) and their metabolites, i.e., 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA), and homovanillic acid (HVA) levels, were determined by HPLC with the help of an electrochemical detector as per the method given by Patel et al. (2005) and Jamwal et al. (2015). A Waters (Milford,

Fig. 1 Hemin, HO-1 dependently reduces immobility period prolonged by CFS in mice. [#]*p* < 0.001 v/s CFS, [@]*p* < 0.001 v/s HM (5), [§]*p* < 0.001 v/s HM (10). CFS chronic fatigue stress, HM hemin (5 and 10 mg/kg), v/s versus



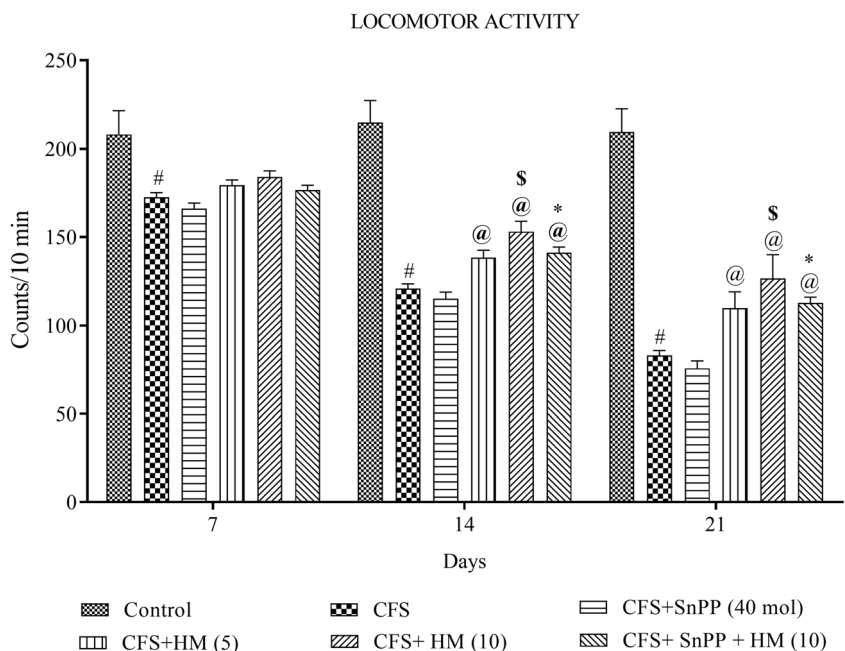
MA, USA) standard system containing a high-pressure isocratic pump, a 20- μ l manual injector valve, a C₁₈ reverse phase column, and a electrochemical detector was used in the study. The mobile phase consisted of sodium citrate buffer (pH 4.5)–acetonitrile (87:13 v/v). Electrochemical conditions used in the study were + 0.75 V; sensitivity ranged from 5 to 50 nA. Separation was done at a flow rate of 0.8 ml/min, and samples (20 μ l) were injected manually. Frozen brain samples were thawed and homogenized in homogenizing solution having 0.2 M per chloric acid on the day of the experiment, followed by samples being centrifuged at 12,000 \times g for 5 min. Before injecting supernatant in the HPLC sample injector, it was filtered through 0.22- μ m nylon filters. Data were

collected and analyzed with the assistance of the Breeze software. Concentrations of neurotransmitters and their metabolites were calculated from the standard curve.

Statistical Analysis

Values are expressed with means \pm SD. The behavioral assessment data were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test for multiple comparisons. For biochemical parameters, one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was used for comparison. *p* < 0.05 was considered statistically significant.

Fig. 2 Hemin HO-1 dependently attenuates locomotor activity alteration induced by CFS in mice. [#]*p* < 0.001 v/s control, [@]*p* < 0.001 v/s CFS, [§]*p* < 0.001 v/s HM (5), ^{*}*p* < 0.001 v/s HM (10). CFS chronic fatigue stress, HM hemin (5 and 10 mg/kg), v/s versus



Results

Hemin HO-1 Dependently Reduces Immobility Period Prolonged by CFS in Mice

The immobility period in the CFS group was significantly increased as compared with the vehicle-treated group (Fig. 1). Hemin (5 and 10 mg/kg; *i.p.*) treatment for 21 days significantly ($p < 0.05$) reduced the increased immobility period as compared with the CFS-alone group. The inhibitor of HO-1 enzyme, SnPP (40 $\mu\text{M}/\text{kg}$), pretreated with hemin (10 mg/kg) significantly reversed hemin protective effect as compared with the CFS alone-treated group.

Hemin HO-1 Dependently Attenuates Locomotor Activity Alteration Induced by CFS in Mice

Locomotor activity was recorded in order to detect the association of forced swimming activity on motor activity. Interestingly, locomotor activity in the CFS group was significantly decreased as compared with the vehicle control group (Fig. 2). Hemin (5 and 10 mg/kg; *i.p.*) treatment for 21 days significantly ($p < 0.05$) reduced the impairment in locomotor activity at the 14th and 21st days, whereas SnPP (40 $\mu\text{M}/\text{kg}$) (inhibitor of HO-1 enzyme) pretreated with hemin (10 mg/kg) significantly reversed its protective effect as compared with the CFS alone-treated group.

Hemin HO-1 Dependently Attenuates Grip Strength Alteration Induced by CFS in Mice

The grip strength of the CFS group decreased significantly as compared with the vehicle control group due to the chronic stress-induced impaired grip strength (Fig. 3). Hemin (5 and 10 mg/kg; *i.p.*) treatment for 21 days significantly reduced the

alteration in grip strength on the 14th and 21st days ($p < 0.05$). The inhibitor of HO-1 enzyme SnPP (40 $\mu\text{M}/\text{kg}$) pretreated with hemin (10 mg/kg) significantly reversed its protective effect as compared with the hemin-alone group.

Hemin HO-1 Dependently Reduces Anxiety-Like Behavior Induced by CFS in Mice

The CFS-related anxiety behavior was measured using the elevated plus maze test. The CFS group showed a significant ($p < 0.001$) increase in time spent and the number of entries into the closed arm and a significant decrease in time spent in the open arm ($p < 0.001$) as compared with the vehicle-treated group (Table 1). Hemin (5 and 10 mg/kg; *i.p.*) treatment for 21 days significantly improved the anxiety-like behavior on the 14th and 21st days ($p < 0.05$). The inhibitor of HO-1 enzyme SnPP (40 $\mu\text{M}/\text{kg}$) pretreated with hemin (10 mg/kg) significantly blunted its protective effect ($p < 0.05$) as compared with the hemin alone-treated group.

Hemin HO-1 Dependently Reduces Anxiety (Mirror Chamber)-Like Behavior Induced by CFS in Mice

The CFS-induced anxiety behavior was measured using the mirror chamber test. The total time spent and number of entries into mirror chamber were significantly decreased ($p < 0.001$) in the CFS group as compared with the vehicle control group (Table 2). Hemin (5 and 10 mg/kg; *i.p.*) treatment for 21 days significantly improved time spent and number of entries on the 14th and 21st days ($p < 0.05$). The inhibitor of HO-1 enzyme SnPP (40 $\mu\text{M}/\text{kg}$) pretreated with hemin (10 mg/kg) significantly blunted its protective effect as compared with hemin alone (10 mg/kg; *i.p.*).

Fig. 3 Hemin HO-1 dependently attenuates grip strength alteration induced by CFS in mice.

$p < 0.001$ v/s control, @ $p < 0.001$ v/s CFS, \$ $p < 0.001$ v/s HM (5), * $p < 0.001$ v/s HM (10). CFS chronic fatigue stress, HM hemin (5 and 10 mg/kg), v/s versus

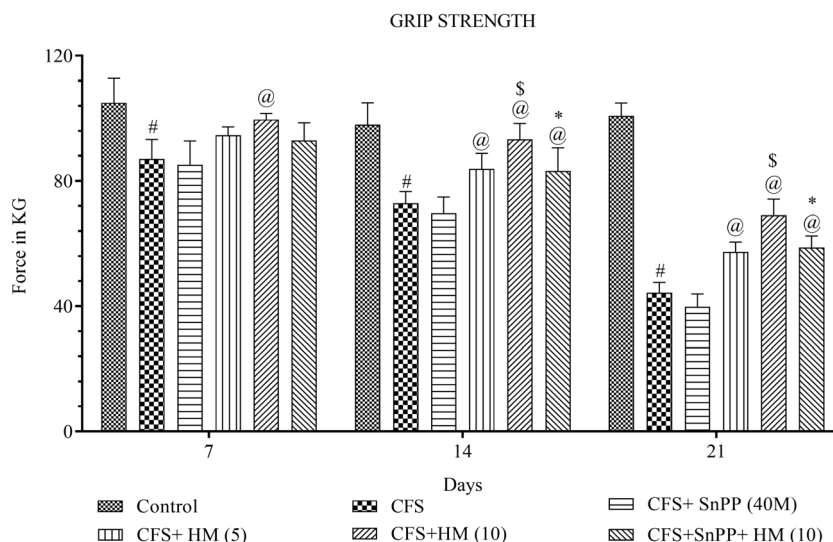


Table 1 Effect of hemin on anxiety-like behavior in elevated plus maze. Data expressed as mean \pm S.D. # $p < 0.001$ v/s control, [®] $p < 0.001$ v/s CFS, [§] $p < 0.001$ v/s HM (5), ^{*} $p < 0.001$ v/s HM (10). CFS: chronic fatigue stress, HM (5): hemin (5 mg/kg), HM (10): hemin (10 mg/kg)

Treatment group	Day 7			Day 14			Day 21		
	Number of entries in closed arm	Time spent (in s)		Number of entries in closed arm	Time spent (in s)		Number of entries in closed arm	Time spent (in s)	
		Open arm	Closed arm		Open arm	Closed arm		Open arm	Closed arm
Control	12.1 \pm 0.3	198.3 \pm 4.6	57.5 \pm 0.8	11.5 \pm 0.3	191.7 \pm 5.3	69.2 \pm 1.8	11.8 \pm 0.3	193.3 \pm 4.9	50.7 \pm 1.1
CFS	5.8 \pm 0.3 [#]	16.6 \pm 0.3 [#]	69.7 \pm 1.1 [#]	5.5 \pm 0.1 [#]	11.8 \pm 0.3 [#]	88 \pm 0.8 [#]	3.6 \pm 0.2 [#]	6 \pm 0.3 [#]	130.3 \pm 4.1 [#]
CFS + SnPP (40 μ M)	4.8 \pm 0.1 [®]	49.17 \pm 1.3 [®]	65.8 \pm 4.1 [®]	4.8 \pm 0.2 [®]	40 \pm 1.1 [®]	79.8 \pm 4.4 [®]	3 \pm 0.2 [®]	21.5 \pm 1.3 [®]	100.2 \pm 4.8 [®]
CFS + HM (5)	9.5 \pm 0.5 [®]	57.8 \pm 0.8 [®]	97.3 \pm 0.6 [®]	16.8 \pm 0.4 [®]	75.5 \pm 1.5 [®]	82.1 \pm 1.2 [®]	14.2 \pm 0.3 [®]	75.8 \pm 2.5 [®]	53.3 \pm 1.4 [®]
CFS + HM (10)	15.1 \pm 1 ^{®,\$}	80.6 \pm 1.1 ^{®,\$}	74 \pm 0.8 ^{®,\$}	14.5 \pm 0.8 ^{®,\$}	94.6 \pm 0.6 ^{®,\$}	49.2 \pm 1.5 ^{®,\$}	15 \pm 1 ^{®,\$}	128.3 \pm 3.8 ^{®,\$}	20 \pm 1.1 ^{®,\$}
CFS + SnPP + HM (10)	12 \pm 0.3 ^{®,*}	54.5 \pm 0.7 ^{®,*}	82.3 \pm 1.2 ^{®,*}	10 \pm 0.3 ^{®,*}	70.5 \pm 1.2 ^{®,*}	54.2 \pm 1.2 ^{®,*}	11.5 \pm 0.3 ^{®,*}	91.5 \pm 1.3 ^{®,*}	27.5 \pm 1.2 ^{®,*}

Hemin HO-1 Dependently Reduces CFS-Induced Oxidative Stress (Lipid Peroxidation, Nitrite, and Reduced Glutathione) Levels in the Mouse Brain

The 10-min test of forced swimming sessions for 21 days significantly ($p < 0.001$) increased lipid peroxidation and nitrite concentration and depleted glutathione enzyme activity in the brains of CFS group animals as compared with the vehicle control group. Hemin (5 and 10 mg/kg; *i.p.*) treatment for 21 days significantly reduced alteration in lipid peroxidation (Fig. 4), nitrite concentration (Fig. 5), and levels of antioxidant enzyme glutathione (Fig. 6) as compared with the CFS alone-treated group. Inhibitor of HO-1 enzyme SnPP (40 μ M/kg) pretreatment with hemin (10 mg/kg) significantly blunted its protective effect.

Hemin HO-1 Dependently Reduces CFS-Altered Mitochondrial Enzyme Complexes (Complexes I and II) in the Mouse Brain

The 10-min forced swimming sessions for 21 days significantly impaired the mitochondrial enzyme complexes (I and II) as compared with the vehicle control animals. Hemin (5 and 10 mg/kg; *i.p.*) administration for 21 days significantly ($p < 0.001$) reduced the mitochondrial dysfunction as compared with the CFS group (Fig. 7). Inhibitor of HO-1 enzyme SnPP (40 μ M/kg) pretreatment with hemin (10 mg/kg) significantly blunted its protective effect.

Hemin HO-1 Dependently Reduces CFS-Altered Brain Neurotransmitters (NE, DA, 5-HT) and Their Metabolite Levels in the Mouse Brain

The 10-min sessions of forced swimming for 21 days resulted in a significant ($p < 0.001$) decrease in levels of catecholamines (NE, DA, and 5-HT) in the brain (Fig. 8) and increased levels of DOPAC and HVA metabolites (Fig. 9) as compared with the vehicle control group. Hemin (5 and 10 mg/kg; *i.p.*) treatment for 21 days significantly ($p < 0.001$) reduced the alteration in levels of NE, DA, 5-HT, and their metabolites as compared with the CFS group. Inhibitor of HO-1 enzyme SnPP (40 μ M/kg) pretreatment with hemin (10 mg/kg) significantly blunted its protective effect.

Discussion

Hemin administration significantly decreased the immobility period, improvement in locomotor activity, anxiety-like behavior, oxidative defense, mitochondrial complex dysfunction, and neurotransmitter levels in the brain. The core finding of the present study revealed that HO-1 enzyme induction by hemin attenuated various behavioral, biochemical, and

Table 2 Effect of hemin on anxiety-like behavior in mirror chamber. Data expressed as mean \pm S.D. # p < 0.001 v/s control, @ p < 0.001 v/s CFS, \$ p < 0.001 v/s HM (5), * p < 0.001 v/s HM (10). CFS: chronic fatigue stress, HM: hemin (5 and 10 mg/kg)

Treatment groups	Day 7		Day 14		Day 21	
	Number of entries	Time spent (in s)	Number of entries	Time spent (in s)	Number of entries	Time spent (in s)
Control	12.3 \pm 0.3	156.6 \pm 2.9	12.5 \pm 0.3	146.6 \pm 4.6	12.5 \pm 0.3	150 \pm 4.9
CFS	10.1 \pm 0.1 [#]	74.7 \pm 0.7 [#]	7.8 \pm 0.1 [#]	39.5 \pm 1.1 [#]	4.1 \pm 0.1 [#]	12.5 \pm 0.7 [#]
CFS + SnPP (40 μ M)	12.6 \pm 0.2 [@]	86.7 \pm 1 [@]	7.5 \pm 0.1 [@]	53.3 \pm 0.6 [@]	3.5 \pm 0.1 [@]	21.2 \pm 0.9 [@]
CFS + HM (5)	7.1 \pm 0.1 [@]	39.1 \pm 0.9 [@]	10.1 \pm 0.1 [@]	62.5 \pm 1.5 [@]	11.3 \pm 0.3 [@]	93 \pm 1.2 [@]
CFS + HM (10)	6.1 \pm 0.2 ^{@,\$}	70 \pm 1.1 ^{@,\$}	8.3 \pm 0.1 ^{@,\$}	93.8 \pm 0.5 ^{@,\$}	13.5 \pm 0.3 ^{@,\$}	109.8 \pm 2.1 ^{@,\$}
CFS + SnPP + HM (10)	4.5 \pm 0.1 ^{@,*}	51 \pm 1.2 ^{@,*}	7.8 \pm 0.1 ^{@,*}	71.1 \pm 1.1 ^{@,*}	11.1 \pm 0.1 ^{@,*}	93.6 \pm 1.2 ^{@,*}

neurochemical alterations in forced swimming–induced chronic fatigue mice. These results are consistent with previous findings that exercise-induced rodent models of chronic fatigue are associated with inflammation, fatigue, mitochondrial deficits, oxidative stress, and neurotransmitter imbalance (Singh et al. 2002; Dhir and Kulkarni 2008; Sarvaiya and Goswami 2016; Mitra et al. 2017). Chronic exposure to forced swimming activity daily for 21 days produced a fatigue-like state with a steady increase in anxiety-like behavior and impaired activity levels. These behavioral alterations are characteristic features of fatigue syndrome which might appear due to increasing oxidative stress and mitochondrial dysfunction (Kennedy et al. 2005). Excessive generation of free radicals in CFS patients, which results in oxidation of lipids and proteins, may be related to a variety of altered biological processes. It has been well reported that the levels of endogenous antioxidants such as glutathione, superoxide dismutase, catalase, GSH peroxidase, and GSH reductase were significantly

decreased, while ROS and reactive nitrogen species (RNS) (malondialdehyde (MDA), conjugated dienes, hydroperoxides, and nitric oxide) were significantly increased in plasma/serum samples of CFS patients (Filler et al. 2014). The results are similar to previous reports and proved that CFS was associated with oxidative damage in the brain (Dhir and Kulkarni 2008; Surapaneni et al. 2012). Hemin treatment significantly reversed the behavioral changes (immobility period, locomotor activity, anxiety) and oxidative stress possibly by activation of the HO-1 enzyme.

Further, the study found a significant decline in the mitochondrial enzyme complex (I and II) activity in the mouse brain. Mitochondrial membrane-bound enzymes play a vital role in neuronal energy metabolism as a part of respiratory chain and in tricarboxylic acid (TCA) cycle. These results are in accordance with a previous report stating that mitochondrial enzyme activities are critical for mitochondrial function,

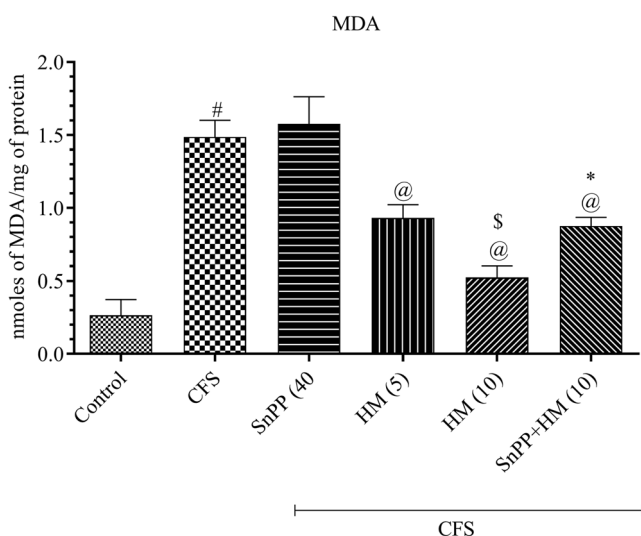


Fig. 4 Hemin HO-1 dependently reduces CFS-induced oxidative stress (lipid peroxidation) levels in the mouse brain. Data expressed as mean \pm S.D. # p < 0.001 v/s control, @ p < 0.001 v/s CFS, \$ p < 0.001 v/s HM (5), * p < 0.001 v/s HM (10). CFS chronic fatigue stress, HM hemin (5 and 10 mg/kg), v/s versus

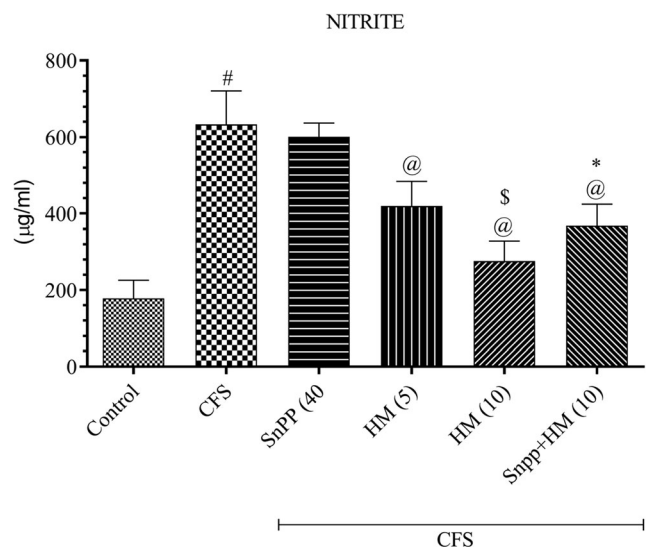


Fig. 5 Hemin HO-1 dependently reduces CFS-induced oxidative stress (nitrite) levels in the mouse brain. Data expressed as mean \pm S.D. # p < 0.001 v/s control, @ p < 0.001 v/s CFS, \$ p < 0.001 v/s HM (5), * p < 0.001 v/s HM (10). CFS chronic fatigue stress, HM hemin (5 and 10 mg/kg), v/s versus

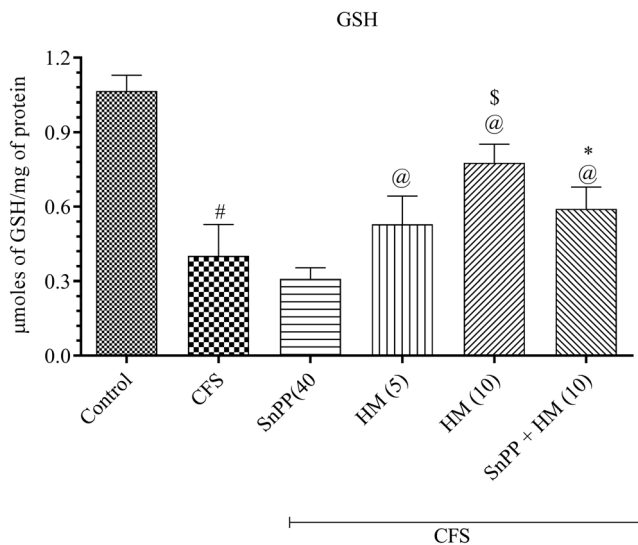


Fig. 6 Hemin HO-1 dependently reduces CFS-induced oxidative stress (reduced glutathione) levels in the mouse brain. Data expressed as mean ± S.D. #*p* < 0.001 v/s control, @*p* < 0.001 v/s CFS, §*p* < 0.001 v/s HM (5), **p* < 0.001 v/s HM (10). CFS chronic fatigue stress, HM hemin (5 and 10 mg/kg), v/s versus

decreased during high-intensity continuous or intermittent frequency exercise (Belzung et al. 2001). Treatment with hemin (5 and 10 mg/kg) significantly restored the mitochondrial enzyme complex (I and II) activity, suggesting the protective role of hemin against mitochondrial dysfunction. Multiple neurotransmitters, including endogenous opioid, dopaminergic, serotonergic, and noradrenergic systems, also play an important role in the pathophysiology of CFS (Pizzigallo et al. 1999; Afari and Buchwald 2003). In the present study, chronic

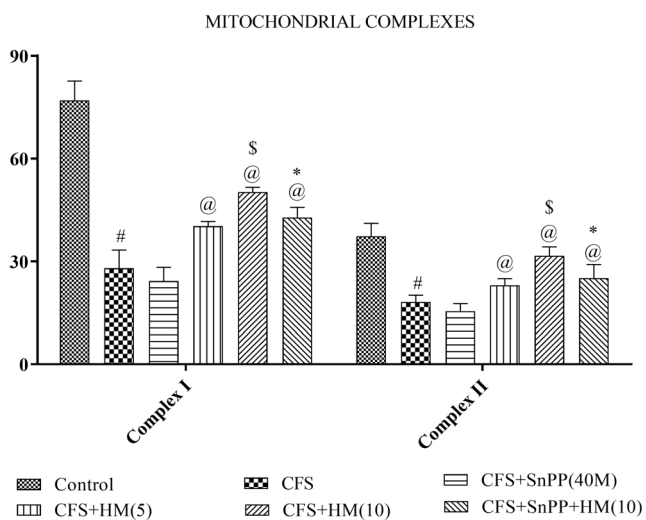


Fig. 7 Hemin HO-1 dependently reduces CFS-altered mitochondrial enzyme complexes (complexes I and II) in the mouse brain. Data expressed as mean ± S.D. Data analyzed by one-way ANOVA followed by Tukey’s post hoc test. #*p* < 0.001 v/s control, @*p* < 0.001 v/s CFS, §*p* < 0.001 v/s HM (5), **p* < 0.001 v/s HM (10). CFS chronic fatigue stress, HM hemin (5 and 10 mg/kg), v/s versus

fatigue stress significantly altered the level of catecholamines (NE, DA, and 5-HT) and their metabolites (DOPAC, HVA, and 5-HIAA) in the mouse brain. These results are consistent with previous studies reporting a similar decline in the level of neurotransmitters in CFS (Belzung et al. 2001; Dhir and Kulkarni 2008). The decreased levels of 5-HT and its metabolites 5-HIAA were reported in CFS patients (Pizzigallo et al. 1999). Treatment with hemin significantly prevented the alteration in levels of neurotransmitters and their metabolites, whereas SnPP significantly inhibited the protective effect of hemin. Therefore, it can be concluded that induction of HO-1 enzyme by hemin plays an important role in mediating the protective effect against CFS besides other possible effects due to antioxidant and subsequent maintenance of neurotransmitter homeostasis by hemin.

Pharmacological activation of HO-1 has been shown to provide neuroprotection against behavioral, oxidative stress, mitochondrial, and neurotransmitter alterations. SnPP is a tin protoporphyrin IX, a powerful inhibitor of HO that maintains the HO level by a double action by inhibiting the HO enzyme activity and increasing the synthesis of HO enzyme protein (Jang et al. 2007). In the present study, when SnPP was injected along with a higher dose of hemin, it significantly reversed the protective effect of hemin and so confirmed the role of HO-1 in the protective effect of hemin. Hemin is an essential component of hemoglobin (Hb) and the iron protoporphyrin IX complex, which is essential for a number of proteins. Hemin is the prosthetic group for a large number of proteins that play a vital role in oxygen delivery, mitochondrial function, and signal transduction, including hemoglobin, cytochromes, prostaglandin endoperoxide, and nitrous oxide synthases, catalase, and peroxidases. Hemin induced HO-1 in several in vitro and in vivo studies and represent a protective system potentially active in the brain against brain oxidative injury, by producing the vasoactive molecule CO and the potent antioxidant bilirubin (Alam and Cook 2003; Eftekharzadeh et al. 2010; Kravets et al. 2004; Schenck and Zimmerman 2004). HO-1 has been well established to provide neuroprotection through the generation of biliverdin, free iron, and CO (Kaizaki et al. 2006; Zhang et al. 2008). It has been documented that even a small quantity of bilirubin has the ability to protect cells from 10,000-fold higher concentrations of the oxidant hydrogen peroxide (Liu et al. 2006). Iron inhibits lipid peroxidation, contributes to protection against oxidative stress, and increases the synthesis of ferritin (Morse and Choi 2002). In stress conditions, release of HO-1 is beneficial, but in chronic stress or conditions, there is decrease in HO-1, due to which hemin is used as an inducer of HO-1. In the present study, the possible explanation for the observed effect of hemin may be attributed to its antioxidant activity. Recently, our lab proved the neuroprotective role of HO-1/glycogen synthase kinase-3b modulators against 3-nitropropionic acid-induced neurotoxicity in rats (Khan et al. 2015).

Fig. 8 Hemin HO-1 dependently reduces CFS-altered brain neurotransmitters (NE, DA, 5-HT) and their metabolite levels in the mouse brain. Data expressed as mean \pm S.D. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. # $p < 0.001$ v/s control, @ $p < 0.001$ v/s CFS, \$ $p < 0.001$ v/s HM (5), * $p < 0.001$ v/s HM (10). CFS chronic fatigue stress, HM hemin (5 and 10 mg/kg), v/s versus

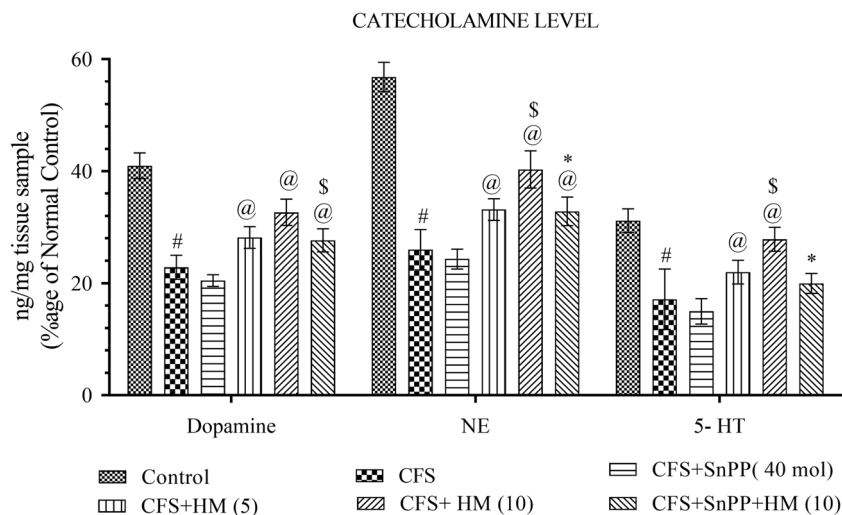
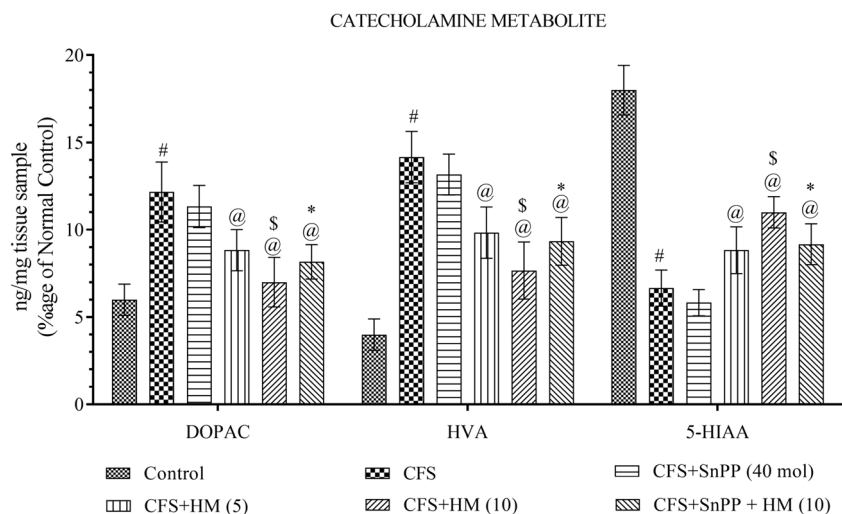


Fig. 9 Hemin HO-1 dependently reduces on CFS-altered brain neurotransmitter metabolite levels. Data expressed as mean \pm S.D. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. # $p < 0.001$ v/s control, @ $p < 0.001$ v/s CFS, \$ $p < 0.001$ v/s HM (5), * $p < 0.001$ v/s HM (10). CFS chronic fatigue stress, HM hemin (5 and 10 mg/kg), v/s versus



Conclusions

Salient findings of the study revealed that hemin protects against the behavioral, oxidative stress, and mitochondrial dysfunction and neurotransmitter imbalance in FST-induced CFS. The outcomes of the present study suggest that hemin is beneficial and therefore might emerge as a therapy for treatment for CFS-like symptoms.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Statement The protocol of the study was approved by the Institutional Animal Ethics Committee (IAEC) Approval No. IAEC/CPCSEA/M14/P246 and was carried out in accordance with guidelines for the use and care of experimental animals. All the experiments for a given treatment were performed using age-matched animals to avoid variability between experimental groups.

Abbreviations CFS, chronic fatigue stress; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; FST, forced swimming test; GSH, reduced glutathione; HO-1, heme oxygenase; HVA, homovanillic acid; LPO, lipid peroxidation; NE, norepinephrine; SnPP, tin protoporphyrin; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid

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