List of Publications


Hasan S, Bilal N, Fatima S, Suhail N, Anwar K, Sharma S, Banu N. Multivitamin-mineral supplement is more efficacious than vitamins (E+C) in the prevention of chronic unpredictable stress induced oxidative damage in mice. (Communicated)

Suhail N, Bilal N, Hasan S, Ahmad A, Banu N. Enhancement of carcinogenic and genotoxic potential 7-12-dimethylbenz (a) anthracene (DMBA) following exposure to chronic unpredictable stress. (Communicated)

Suhail N, Bilal N, Hasan S, Ahmad A, Banu N. Augmentation of DMBA-TPA induced hepatotoxicity and nephrotoxicity in Swiss albino mice by prior exposure to chronic unpredictable stress (CUS). (Communicated)
Bilal N, Suhail N, Ashraf GM, Hasan S, Khan HY, Banu N. Exacerbation of N-nitrosodiethylamine induced hepatotoxicity and DNA damage in mice exposed to chronic unpredictable stress. (Communicated)
Multivitamin–Mineral and Vitamins (E+C) Supplementation Modulate Chronic Unpredictable Stress-Induced Oxidative Damage in Brain and Heart of Mice

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Abstract Brain is a target of stress along with the immune, metabolic, and cardiovascular systems of the body. In the present work, the preventive roles of a multivitamin–mineral supplement and vitamins (E+C) in chronic unpredictable stress (CUS)-induced oxidative damage were studied in the brain and heart of Swiss albino mice. Thirty-two mice were randomized to one of the following groups: control+vehicle, CUS+vehicle, CUS+multivitamin–mineral, and CUS+vitamins (E+C). CUS was applied for 4 weeks, and multivitamin–mineral and vitamins (E+C) were administered orally for the same period. CUS led to a negative impact on all the biochemical parameters analyzed. Elevation in malondialdehyde and reduction in glutathione levels were found. The activities of superoxide dismutase, catalase, glutathione S-transferase, and glutathione reductase were decreased. Treatment with multivitamin–mineral and vitamins (E+C) brought these parameters to near normal levels. Multivitamin–mineral was found more restitutive than combined vitamins (E+C) doses. The present study hypothesizes that supplementation with a multivitamin–mineral may prove more effective than vitamin treatment alone in the alleviation of oxidative damage in brain and heart during periods of chronic stress.

Keywords Chronic unpredictable stress · Multivitamin–mineral · Vitamin E · Vitamin C

Abbreviations

CUS Chronic unpredictable stress
GR Glutathione reductase
GSH Reduced glutathione
GST Glutathione S-transferase

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LPO  Lipid peroxidation
MDA  Malondialdehyde
MM  Multivitamin–mineral
ROS  Reactive oxygen species

Introduction

The brain is the key to interpreting and responding to potentially stressful events and the behavioral and physiological responses that are produced. It is especially vulnerable to free radical-induced damage because of its high oxygen consumption, abundant lipid content, and a limited amount of antioxidant capacity. Repeated and unpredictable stress situations through activation of hypothalamic–pituitary–adrenal axis and increased glucocorticoid release can enhance reactive oxygen species (ROS) generation in central nervous system cells, leading to oxidative stress [1].

Psychosocial factors are known to play an important role in the etiology and progression of coronary heart diseases as well. The brain, through stress-induced activation of certain regions, leads to a response from hormones or the sympathetic nervous system that ultimately affects the heart function. Chronic mild stress results in marked increase in plasma corticosterone levels which produces an aggravated atherogenic lipid profile accelerating atherosclerosis [2]. A recent study has shown that chronic mild unpredictable stress induces physiological and morphological changes that may contribute to the development of atherosclerosis by mechanisms related to deficiency in nitric oxide production and dyslipidemia [3]. Oxidative stress again plays a significant role in the pathogenesis of cardiovascular diseases (CVD). Oxidation of low density lipoprotein particles is a key step in atherogenesis. Cardiomyocytes and vasculature of the heart can be severely damaged by oxidative stress [4].

Exogenous supplementation of antioxidants exerts protective effects in various pathological states in which free radicals are involved. Vitamins E and C are effective antioxidants of major importance for protection against diseases and degenerative processes caused by oxidative stress [5]. Vitamin E is the only well-recognized, lipid-soluble, chain-breaking antioxidant that plays an important role in protecting lipid-rich structure like brain from free radical damage. It has been shown to be effective in reducing exercise-induced oxidative stress in rats [6]. Antioxidant vitamins either through diet, supplementation, or both prevent oxidative stress-associated coronary heart diseases. Vitamins E, C, and other antioxidants are known to reduce CVD by trapping organic free radicals, by deactivating excited oxygen molecule or both, to prevent tissue damage. Antioxidant supplementation has been shown to inhibit atherosclerosis progression in animal models. Some prospective studies showed a direct inverse association between dietary antioxidant and the development of CVD incidents [7].

Nowadays, there is a considerable interest in multivitamin–mineral (MM) supplements for prophylactic and disease mitigating purposes. They are a good insurance for protection against diseases, like heart disease, depression, cataract, immune function, cancer, particularly for those with inadequate diets. One such dietary supplement was found to show remarkable extension of physical function in old mice. It prevented cognitive declines and protected mice from radiation [8]. In a recent epidemiologic study, telomere length which is a marker of biological aging was found to be enhanced in women on routine multivitamin supplement use [9]. MM also improves cognitive and mood effects in healthy children [10]. Supplementation with MM has been shown to be inversely associated with risk of myocardial infarction in men and women [11].
The present study investigates and compares the effect of treatment with a multivitamin–mineral supplement and vitamins (E+C) on selected biochemical parameters in mice brain and heart during the course of chronic unpredictable stress-induced oxidative damage.

Materials and Methods

Chemicals

MM supplement (Galaxy 490 mg) was purchased from Roots Life Sciences Pvt. Ltd. India; vitamin E (Evion 200 mg) was obtained from Merck, India. Vitamin C (Celin 500 mg) was purchased from GlaxoSmithKline Pharmaceuticals Limited, India. NADPH; oxidized and reduced glutathione; 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman’s reagent); 1-chloro-2,4-dinitrobenzene (CDNB); and Pyrogallol were acquired from SRL India. All other chemicals were of analytical grade.

Experimental Protocol

Thirty-two healthy male Swiss albino mice each weighing approximately 40 g and 5–6-week old were acclimatized for 1 week to mice feed (Ashirwad industries, Chandigarh, India) with food and water available ad libitum and alternating light and dark cycles of 12 h. Mice were divided into four groups with eight mice in each group. Group I consisted of the non-stressed control mice. Control mice were manipulated everyday for 10 min in the home cage to control for nonspecific handling effects. Group II mice (stress alone) were exposed to 4 weeks of CUS (Table 1), previously described by Oritz et al. [12]. Specific details of the CUS procedure are as follows: For restraint stress, mice were placed individually in body-sized wire mesh cages attached to wooden boards, with no movement allowed. Wet bedding was carried out by filling 300-ml tap water in home cage. Forced swim and cold forced swim were accomplished by placing the mice in a cylindrical tank (50-cm height×20-cm diameter) filled with water to a 20-cm depth at 25°C or 4°C, respectively. Crowding was done by placing an iron divider in the cage to provide minimum space for housing. Lastly, illumination was attained by placing an illuminated tube light on the cages overnight. After each stressor, animals were kept in a recovery room for 1–2 h, following which they were placed in clean cages with fresh bedding and returned to the housing facility. Group III mice were given oral dose of MM supplement (200 mg/kg body weight (bw)/day, p.o.) followed by chronic unpredictable stress

<table>
<thead>
<tr>
<th>Day</th>
<th>Stress type and schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000 hours, restraint, 3 h</td>
</tr>
<tr>
<td>2</td>
<td>1100 hours, wet bedding (25°C), 2 h</td>
</tr>
<tr>
<td>3</td>
<td>1500 hours, forced swim (25°C), 30 min</td>
</tr>
<tr>
<td>4</td>
<td>1300 hours, crowding, 2 h</td>
</tr>
<tr>
<td>5</td>
<td>1900 hours, lights on, overnight</td>
</tr>
<tr>
<td>6</td>
<td>0900 hours, cold forced swim (4°C), 15 min; 2200 hours, crowding, overnight</td>
</tr>
<tr>
<td>7</td>
<td>1000 hours, restraint, 2 h; 1900 hours, food deprivation, overnight</td>
</tr>
</tbody>
</table>
stressor as in group II. The MM supplement contained lycopene 6% (2,000 μg), α-lipoic acid (50 mg), beta-carotene 10% (10 mg), citrus bioflavonoid (50 mg), lutein (250 μg), chromium picolinate (150 μg), selenium dioxide (70 μg), vitamin C (60 mg), vitamin E (15 mg), vitamin D (400 IU), vitamin K (10 μg), vitamin B1 (1.5 mg), vitamin B2 (1.7 mg), niacin (20 mg), vitamin B6 (3 mg), folic acid (1.5 mg), vitamin B12 (5 μg), biotin (300 μg), pantothentic acid (10 mg), calcium (20 mg), phosphorus (48 mg), iodine (150 μg), magnesium oxide (10 mg), zinc (15 mg), copper (2 mg), chloride (72 mg), potassium (80 mg), silicon dioxide (2 mg), carbonyl iron (15 mg), boron (150 μg), nickel (5 μg), and vanadyl sulfate (10 μg). Group IV mice were given oral dose of vitamins (E+C) (100 mg/kg body weight/day, p.o., each) followed by chronic unpredictable stressor as in group II.

MM and vitamin C were dissolved in drinking water (100 μl/mouse) while vitamin E was given as such. Groups of control and CUS were given vehicle (drinking water, 100 μl/mouse) orally for the same duration. To avoid product oxidation, the supplementations were prepared fresh. MM and vitamin (E+C) doses were made comparable by taking 100 mg/kg bw/day each of vitamins E and C, making a total of 200 mg/kg bw/day of vitamins supplementation, and an equal dose of MM (200 mg/kg bw/day) was given. Every 7 days, the weight of the animals was measured to adjust the treatment doses.

All the experimental protocols adhered to the guidelines of the Institutional Ethical Committee of the university.

After 4 weeks of CUS paradigm, animals from all the groups were sacrificed by cervical decapitation. Brain and heart samples were collected for biochemical studies and were rinsed with normal saline and homogenized in 0.1 M phosphate buffer pH 7.4 (10% w/v) followed by centrifugation at 10,000×g (at 4°C for 15 min) to remove cellular debris, and the supernatant was used for further studies.

Biochemical Estimations

Lipid Peroxidation The levels of malondialdehyde (MDA) formed were assessed by measuring the concentration of thiobarbituric acid reactive substances [13].

Total Reduced Glutathione Levels of reduced glutathione (GSH) were measured using sulfosalicylic acid and DTNB by the method of Jollow et al. [14].

SOD Activity SOD activity was monitored by the inhibition of auto-oxidation of Pyrogallol at 420 nm [15].

Glutathione S-Transferase Activity Glutathione S-transferase (GST) activity was measured using CDNB by the method of Habig [16].

CAT Activity Catalase was assayed by following the rate of decomposition of H₂O₂ at 240 nm [17].

Glutathione Reductase Activity Glutathione reductase (GR) was assayed by measuring the oxidation of NADPH at 340 nm by the method of Carlberg and Mannervik [18].

Protein Estimation Protein content was measured by the method of Lowry et al. using BSA as standard [19].
Statistical Analysis

Data were expressed as group mean ± SEM of six values and analyzed by one-way ANOVA for differences among controls and treatment groups. *P* values less than 0.05 were considered statistically significant.

Results

Effect of Treatment with MM and Vitamins (E+C) on CUS-Induced Decline in Antioxidant Enzyme Activities

Tables 2 and 3 illustrate the effect of supplementation with MM (200 mg/kg bw/day, p.o.) and vitamins (E+C) (100 mg/kg bw/day, p.o., each) on SOD, CAT, GST, and GR activities in the brains and hearts, respectively, of control+vehicle, CUS+vehicle, CUS+MM, and CUS+(E+C) groups.

### Table 2 Effect of Chronic Unpredictable Stress (CUS) and Treatment with Multivitamin–Mineral Supplement (MM, 200 mg/kg bw/day) and Vitamins (E+C) (100 mg/kg bw/day, each) on Enzymatic Antioxidant Parameters of Brain

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>GST (U/mg protein)</th>
<th>GR (U/mg protein × 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control+vehicle</td>
<td>234.6±7.32</td>
<td>98.77±4.13</td>
<td>70.13±2.12</td>
<td>5.38±0.14</td>
</tr>
<tr>
<td>CUS+vehicle</td>
<td>135.33±5.51</td>
<td>48.93±3.26</td>
<td>19.99±2.33</td>
<td>0.30±0.08</td>
</tr>
<tr>
<td>CUS+MM</td>
<td>203.22±7.90</td>
<td>83.67±4.80</td>
<td>64.32±1.98</td>
<td>4.78±0.21</td>
</tr>
<tr>
<td>CUS+(E+C)</td>
<td>180.54±8.10</td>
<td>71.43±4.60</td>
<td>51.67±2.60</td>
<td>3.45±0.23</td>
</tr>
</tbody>
</table>

Data represent mean with their SEM of eight mice

* *P* <0.001, as compared to controls
  b *P* <0.001, as compared to stressed mice
  c *P* <0.05, CUS+MM as compared to CUS+(E+C) group

### Table 3 Effect of Chronic Unpredictable Stress (CUS) and Treatment with Multivitamin–Mineral Supplement (MM, 200 mg/kg bw/day) and Vitamins (E+C) (100 mg/kg bw/day, each) on Enzymatic Antioxidant Parameters of Heart

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>GST (U/mg protein)</th>
<th>GR (U/mg protein × 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control+vehicle</td>
<td>178.86±6.15</td>
<td>112.11±3.62</td>
<td>30.34±2.29</td>
<td>3.37±0.16</td>
</tr>
<tr>
<td>CUS+vehicle</td>
<td>132.65±4.35</td>
<td>78.13±3.94</td>
<td>13.93±1.58</td>
<td>1.48±0.19</td>
</tr>
<tr>
<td>CUS+MM</td>
<td>203.22±6.34</td>
<td>107.17±3.10</td>
<td>27.64±2.16</td>
<td>2.73±0.17</td>
</tr>
<tr>
<td>CUS+(E+C)</td>
<td>180.54±5.47</td>
<td>94.87±3.50</td>
<td>20.06±1.43</td>
<td>2.28±0.20</td>
</tr>
</tbody>
</table>

Data represent mean with their SEM of eight mice

* *P* <0.001, as compared to controls
  b *P* <0.001, as compared to stressed mice
  c *P* <0.05, CUS+MM as compared to CUS+(E+C) group
CUS+vitamins (E+C) mice. Results indicate that exposure to 4 weeks of CUS significantly decreased all antioxidant enzyme activities ($P<0.001$), as compared to controls, while MM and vitamins (E+C) treatment to stressed mice significantly ($P<0.001$) upturned the enzyme activities. However, recovery in the CUS+MM group was significantly higher ($P<0.05$) than in the CUS+vitamins (E+C) group.

Effect of Treatment with MM and Vitamins (E+C) on CUS-Induced Decline in Non-enzymatic Antioxidant Levels

CUS alone produced a significant decrease in brain and heart GSH levels ($P<0.001$; Figs. 1 and 2, respectively). Administration of MM and vitamins (E+C) to the stressed mice for the same period resulted in a significant improvement of these levels ($P<0.001$). GSH levels in the CUS+MM group was significantly higher ($P<0.05$) than in the CUS+vitamins (E+C) group, thus proving the former being more effective than vitamins (E+C) in restoring GSH levels toward their control values.

Effect of Treatment with MM and Vitamins (E+C) on Lipid Peroxidation

The significantly increased production of MDA by CUS and its recovery in stressed animals receiving MM and vitamins (E+C) in brain and heart are shown (Figs. 3 and 4, respectively). As depicted, CUS caused an excessive accumulation of the aldehydic product of lipid peroxidation, MDA ($P<0.001$). MM and vitamins (E+C) significantly decreased MDA
levels in stressed animals ($P<0.001$). However, the decline in MDA levels in the CUS+MM group was significantly more ($P<0.05$) when compared to the CUS+vitamins (E+C) group, thus proving MM to be a better antioxidant for the prevention of lipid peroxidation (LPO) in comparison to vitamins (E+C).

MM proved more restitutive than vitamins (E+C) in the prevention of CUS-induced oxidative damage. Besides, no side effects were observed by either.

**Discussion**

In the present work, the mice were exposed to “unpredictable” stressors of mild to moderate intensity everyday for a period of 4 weeks. LPO was significantly increased in the stressed groups of both brain and heart samples as compared to the controls. The increased ROS production during stress may have led to an enhanced LPO by propagating the initial attack on lipid membrane. A similar enhancement of lipid peroxidation on CUS exposure has been
reported earlier in rat brain and heart by Nayantara et al. [20]. Further studies on brain cerebellum, striatum, and brain cortex showed elevated levels of MDA after CUS treatment. Similar increase was also detected in the sub-mitochondrial particles of cortex with chronic mild stress but not in hippocampus and prefrontal cortex [21–23].

GSH is a multifunctional intracellular non-enzymatic thiol antioxidant and is an essential compound for maintaining cell integrity because of its reducing properties and participation in the cell metabolism. Depletion in brain and heart GSH was observed in our results which are in line with other studies where GSH was found to be decreased in brain cortex [24]. Whereas to our knowledge, there have been no studies showing the effect of CUS on enzymatic and non-enzymatic antioxidant parameters in the heart tissue.

Antioxidant enzymes are the primary defense mechanisms that protect biological macromolecules from oxidative damage. Our investigations showed a decline in the activities of both GR and GST in the stressed groups of brain and heart samples. In a study by Kamper et al. [25], chronic mild stress treatment caused a decline in GR activities in the brain of female rats. Other major antioxidant enzymes include SOD and CAT. We found reductions in the activities of SOD and CAT in both the organs after CUS exposure. Lucca et al. [21] reported decreased SOD activity in prefrontal, hippocampus, striatum and cortex in stressed rats. Reduced CAT activity has been reported with oxidative stress in brain regions [26].

As a novel result of the current study, 4 weeks of MM and vitamins (E+C) supplementation prior to the stress exposure brought about an improvement in the antioxidant status of brain and heart.

In the present study, MM and vitamins (E+C) were found to cause a significant decline in LPO accompanied by a significant increase in the activities/levels of SOD, CAT, GST, GR, and GSH in both brain and heart samples. However, MM proved more effective than combined doses of vitamins (E+C) in alleviating the disturbed oxidative status. We therefore hypothesize that taking a single supplement of the MM is more beneficial than taking combined vitamins E and C doses, as the former is also fortified with several vitamins and trace elements. Thus, different antioxidants when combined together act synergistically to improve the overall antioxidative potential of an MM supplement as compared to individual vitamins alone. The study discussed here highlights the way to adopt optimal nutrition and emphasizes the need to opt for a complete and safe multivitamin–mineral supplement instead of relying on intake of separate micronutrients. Further investigations regarding the role of specific nutrients and their interactions remain to be explored which will thereby lead to the development of new and more effective supplements.

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References