Ascorbic acid (AA), also called vitamin C, is a key micronutrient for normal metabolic function, although its action on central nervous system (CNS) has also been increasingly reported, given its ability to potentiate gamma-aminobutyric acid (GABA)ergic neurotransmission (1). In this context, Bigelow et al. suggested that ascorbate release is mediated by the GABA receptor and located synaptically in rats (2). On the other hand, GABA(A) receptors exist in the intra-ventral tegmental area exert a pivotal action on narcotic drug-induced AA release modulation in the nucleus accumbens of freely moving rats (3,4). In this context, Naseer and coworkers indicated that AA (0.5 mM) exerts its mediated neuroprotective effect through GABA(B) receptor and protein kinase A-alpha expression modulation in the prenatal rat brain (5). However, the AA effect is concentration-dependent. Research findings by Grigor’ev and Neokesar’inskii (1986) found that at higher doses (10^{-6} M) AA markedly inhibits, and at lower doses (10^{-7} M) highly stimulates 3H-GABA binding capacity in rat brain cortical synaptosomes (6).

Diazepam (DZP), a benzodiazepines group’ drug, is often prescribed for treating several health conditions, such as anxiety, withdrawal syndromes (e.g., alcohol, benzodiazepine), muscle spasms, seizures, sleeping disturbance, and restless legs syndrome (7). However, DZP...
has some side effects, such as sleepiness, memory loss, coordination trouble, occasionally excitement or agitation (8, 9). It additionally has some serious side effects, including suicide, decreased breathing, and an increased seizures risk whether frequently used in epileptic patients (7, 10). Moreover, long-term use may lead to tolerance, dependence, and withdrawal symptoms (e.g., cognitive problems) on dose reduction (7). Therefore, it should be avoided during pregnancy or breastfeeding. DZP is known to act by increasing the GABA effect (9).

A paper published by Pavlovic and his coworkers suggested that AA (10, 100, 100 µg/mL) reduces the DZP-induced apoptosis in rat thymocytes by restoring the cellular content of glutathione which may be helpful in preventing DZP-induced immunosuppression (11). In a similar fashion, Suke and colleagues found that AA (100 mg/kg, p.o.) has ability to reverse cognitive impairment and antioxidants-induced brain oxidative stress (OS), following analysis of the malondialdehyde (MDA) levels, protein carbonyl, and reduced glutathione (GSH) activity in Wistar rats (12). In addition, research findings indicated that AA (100 mg/kg, p.o.) can alter Pb-induced reduction of Purkinje cells and reduce the synaptophysin, glutamic acid decarboxylase 67, and axonal myelin basic protein expression in rat brain (13).

Thus, in light of the previous discussion, the present study aims at evaluating the modulatory effects of AA on the DZP-mediated anxiolytic effect in mice. Additionally, molecular docking study has been performed to confirm the possible involvement of GABA receptor(s) in this modulatory action of AA.

Materials and Methods

Animals

Two-month-old Swiss (Mus musculus) albino mice (24–30 g) of either sex provided by the Animal House department of Jahangirnagar, Dhaka University, Bangladesh were used throughout this investigation. Animals had water and food ad libitum and were kept under standard laboratory conditions (dark/light 12 h-cycle, at 26±2 °C). Experiments were conducted from 08:00 am to 1:00 pm. This study received approval from the Ethics Committee in Animal Experimentation of BSMRSTU, Bangladesh (#PHR-BSMRSTU-T01/2019); all experiments were done according to relevant guidelines and regulations.

Drugs and treatment groups

AA and FLU were from Sigma-Aldrich (St. Louis, MO, USA) whereas DZP was obtained from the Acme Laboratories Limited., Bangladesh. Forty mice were randomly divided into 8 groups (5 mice each). Group I was used as control (NC) and received distilled water (10 mL/kg). Groups II, III, and IV received DZP (2 mg/kg), FLU (2.5 mg/kg), and AA (25 mg/kg), respectively. Co-treatment groups V, VI, VII, and VIII received, respectively, DZP+FLU, DZP+AA, FLU+AA and DZP+FLU+AA. All treatments were intraperitoneally (i.p.) given.

Experimental procedures

Each of the following tests was performed after 30 min of each treatment.

Open-field test

This study was performed according to the procedure outlined by Archer (14). Briefly, after 30 min of treatment, spontaneous locomotor activity, grooming, and rearing were monitored during 5 min. After each test, the equipment floor was washed with soap and water, cleaned with 70% ethanol, and dried.

Hole cross test

This experiment was accomplished following a procedure described by Subhan et al. (15). After 3 min of open-field test, each mouse was placed on one side of the hole-board instrument. Then, the spontaneous movement of each mouse from one chamber to another through the hole was observed for 5 min. After each test, equipment floor was washed and cleaned accordingly.

Swing test

Swing test was executed according to Islam et al. (16). After 3 min of the hole cross test, each mouse was placed on one side of the swing box and the spontaneous mice movement from the one to the other side was observed for 5 min. After each test, swing box floor was cleaned with 70% ethanol and dried.

Light-dark test

This study was done following the Crawley (17) protocol. Briefly, after 3 min of swing test, the time spent (sec) in the dark and light portion of each animal was counted by means of a stopwatch during 5 min. After each test, the equipment floor was washed and cleaned accordingly.

Molecular docking study

Molecular docking study with AA was performed on 3 therapeutically important proteins: GABA(A1), GABA(B1), and GABA(Aρ). Homology models of these essential proteins were constructed using Modeler 9.19 and taking reported models from a protein data bank (PDB) as models (PDB ID’s are given in Table 1) (18). The ligand AA was prepared using Autodock tools and proteins binding sites predicted through the ProBiS server (19). Initial docking grid of 40 × 40 × 40, 0.8 Å was generated by closing the active binding site, and the prepared ligand was inserted into the predicted selected proteins’ binding pocket using AutodockVina (20).

Statistical analysis

Data are shown as mean ± SEM values. Analysis of

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Binding affinity (Kcal/mol)</th>
<th>Interacting amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA(A1)</td>
<td>-4.6</td>
<td>Ala37, Val38, Gly39, Ala66, Trp67, Arg68, Thr127, Tyr126, Arg169</td>
</tr>
<tr>
<td>GABA(B1)</td>
<td>-4.7</td>
<td>Glu172, Gly173, Val175, Tyr176, Gly177, Val178, Asn179, Lys180, Tyr191</td>
</tr>
<tr>
<td>GABA(Aρ)</td>
<td>-5.1</td>
<td>Asp102, Val103, Gln104, Arg125, Tyr127, Lys232, Ser236, Leu237, Lys238</td>
</tr>
</tbody>
</table>
Table 2. Open-field test of the ascorbic acid and/or diazepam in Swiss albino mice.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose (i.p.), mg/kg</th>
<th>NFC</th>
<th>Grooming</th>
<th>Rearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>10</td>
<td>109.25±2.51</td>
<td>6.75±2.72</td>
<td>28.00±1.05</td>
</tr>
<tr>
<td>DZP</td>
<td>2</td>
<td>84.25±3.02</td>
<td>8.50±1.45</td>
<td>15.25±1.91</td>
</tr>
<tr>
<td>FLU</td>
<td>2.5</td>
<td>128.25±1.91d</td>
<td>2.27±0.58</td>
<td>36.09±1.41e</td>
</tr>
<tr>
<td>AA</td>
<td>25</td>
<td>118.00±2.25v</td>
<td>4.50±1.67</td>
<td>27.55±1.91e</td>
</tr>
<tr>
<td>DZP+FLU</td>
<td>2+2.5</td>
<td>150.09±2.21d</td>
<td>4.27±0.58</td>
<td>34.03±1.08e</td>
</tr>
<tr>
<td>DZP+AA</td>
<td>2+25</td>
<td>142.25±3.57d</td>
<td>5.75±1.28</td>
<td>32.25±1.97e</td>
</tr>
<tr>
<td>FLU+AA</td>
<td>2.5+25</td>
<td>152.75±2.72d</td>
<td>3.25±0.58</td>
<td>38.75±1.97e</td>
</tr>
<tr>
<td>DZP + FLU + AA</td>
<td>2+2.5+25</td>
<td>148.00±1.91d</td>
<td>4.58±0.78</td>
<td>36.09±1.41e</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=5); ANOVA followed by t-Student–Neuman–Keuls post-hoc test; p<0.05 when compared NC, DZP.
1 mL/kg; AA, ascorbic acid; DZP, diazepam; FLU, flumazenil; NC, control, distilled water; NFC: Number of field cross.

Table 3. Hole cross, swing, and light-dark tests of the ascorbic acid and/or diazepam in Swiss albino mice.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose (i.p.), mL/kg</th>
<th>NHC</th>
<th>NS</th>
<th>Light-dark test</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1</td>
<td>30.50±1.80</td>
<td>17.00±2.71</td>
<td>RID (Sec)</td>
</tr>
<tr>
<td>DZP</td>
<td>2</td>
<td>13.75±1.72</td>
<td>9.75±1.28</td>
<td>256.75±8.82</td>
</tr>
<tr>
<td>FLU</td>
<td>2.5</td>
<td>42.25±1.72</td>
<td>24.00±2.88</td>
<td>175.00±2.58</td>
</tr>
<tr>
<td>AA</td>
<td>25</td>
<td>38.75±1.28</td>
<td>20.50±2.85</td>
<td>189.25±9.11</td>
</tr>
<tr>
<td>DZP + FLU</td>
<td>2+2.5</td>
<td>35.25±0.58</td>
<td>32.00±0.78</td>
<td>201.75±1.44</td>
</tr>
<tr>
<td>DZP + AA</td>
<td>2+25</td>
<td>33.50±1.80</td>
<td>28.00±4.88</td>
<td>225.00±2.62</td>
</tr>
<tr>
<td>FLU + AA</td>
<td>2.5+25</td>
<td>42.00±1.72</td>
<td>36.75±1.28</td>
<td>162.25±2.58</td>
</tr>
<tr>
<td>DZP + FLU + AA</td>
<td>2+2.5+25</td>
<td>38.25±1.80</td>
<td>34.75±1.28</td>
<td>178.75±1.78</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=5); ANOVA followed by t-Student–Neuman–Keuls post-hoc test; p<0.05 when compared NC, DZP.
1 mL/kg; AA, ascorbic acid; DZP, diazepam; FLU, flumazenil; NC, control, distilled water; NFC: Number of field cross; NS: Number of hole cross; RID, swing; RID (Sec), residence in dark; RIL (Sec), Residence in light.

variance (ANOVA) was used, followed by t-Student–Neuman–Keuls's as post-hoc test. GraphPad Prism software (version 6.0) was used for all statistical analysis, with an alpha set at 0.05.

Results

Open-field test

In this study, DZP (at 2 mg/kg, i.p.) reduced the number of field cross (NFC), grooming, and rearing parameters when compared to the NC group (Table 2). On the other hand, AA (25 mg/kg, i.p.) and FLU (2.5 mg/kg, i.p.) significantly (p<0.05) increased NFC and rearing number when compared to NC and DZP groups; however, grooming numbers were reduced in these two groups. Reduced NFC and rearing number, accompanied by an increase in grooming number were observed in AA when compared to FLU-treated group. In addition, it was found that FLU and/or AA co-treated groups with DZP significantly increased the NFC and rearing number, whereas the grooming number markedly decreased (p<0.05) when compared to NC and DZP groups. Moreover, data pertaining to group VIII (DZP+FLU+AA) revealed that NFC, grooming, and rearing numbers range between the other co-treated groups (Group V to VII).

Hole-board, swing, and light-dark tests

Listed in Table 3 are the results related to the effect of AA and standard drugs in mice behavior in hole-board, swing, and light-dark tests. Our findings revealed that animals treated with DZP had a reduced number of hole cross (NHC), swing (NS), and residence in light (RIL), while increased the residence in dark (RID) compared to the NC group. However, mice treated with FLU evidenced a pronounced increase in NHC, NS, and RID, and reduction in RID values when compared with AA-treated group. Results from the open-field test showed that co-treatment groups display similar results. Thus, animals treated with FLU and/or AA combined with DZP (2 mg/kg, i.p.) showed an increase in NHC, NS, and RID, and a decrease in RID value as compared to DZP-treated group. However, all values related to the hole-board, swing, and light-dark tests of Group VIII (DZP+FLU+AA) were found among the other co-treated groups (Group V to VII).

In silico study

Results obtained from molecular docking study show that AA possesses good-to-moderate affinity towards all target proteins used, with binding affinity ranging from −4.6 to −5.1 Kcal/mol (Table 1). The interactions between the ligand and the amino acid residues around the binding proteins sites were also addressed (Figure 1). Red lines mean the hydrophobic interactions, whereas green ones refer to the hydrogen bonds. AA showed best interaction with GABA(Aρ₁) (−5.1 Kcal/mol) and GABA(B1) (−4.7 Kcal/mol).

Discussion

Recent findings by Badshah and coworkers indicated that AA (1 mg/kg, p.o.) exerts anti-depressant effect in mice, possibly by GABA(B) receptors inhibition (21).
Ascorbic acid antagonizes the sedative effect of diazepam.

In addition, AA (100 mg/kg, p.o.) was found to reverse the ethanol-induced increased GABA(B1) receptor and neuronal apoptosis expression through changes in Bax/Bcl-2 ratio, cytochrome C release, and caspase-3 and -9 activation (22). In a similar fashion, Ullah and colleagues showed that AA (250 mg/kg, i.p.) exerts anti-convulsive effect in rats through GABAB1R/CaMKII/CREB pathway activation, thus suggesting a feasible therapeutic action in epilepsy (23). Our in silico findings suggest that AA has interaction capacity with GABA(B1) receptor. Moreover, there was evidence that AA (0.1–3 μM) antagonizes these receptors. Thus, based our experimental and molecular docking results, it can be suggested that AA may antagonize the DZP’s action in Swiss albino mice, possibly through GABA(Ap) and GABA(B1) receptors inhibition.

**Conflict of interests**

None declared.

**References**

Ascorbic acid antagonizes the sedative effect of diazepam.