Evaluation of the anti-stress effect of olanzapine against cold restraint stress in rats

1.1 Introduction

There is compelling evidence that stress is implicated in the development of mental illness (Herbert, 1997). The hypothalamic-pituitary-adrenal (HPA) axis is the key regulator of the stress response. Both hyper- and hypo-activation of the HPA axis have been linked to presentations of neuropsychiatric disorder (Bradley and Dinan, 2010). The hypothalamic-pituitary-adrenal (HPA) axis dysfunction may play a key role in triggering depression and stress ulcers (Zhang et al., 2012). An earlier study, shown that re-strain stress-induced catalepsy behaviour plasma corticosterone and serotonin level in rats (Bhattacharya and Parmar, 1985). Both typical and atypical antipsychotics are prescribed drugs for neuropsychiatric disorders, but atypical drugs are mostly preferred over typical antipsychotics because of fewer extrapyramidal side effects (He et al., 2009). A previous study found increased blood serotonin levels in schizophrenic patients (erythematosus Rheumatoid, 1981). Olanzapine is an atypical antipsychotic used in psychological disorders. The previous study has found olanzapine to have a significant modulating effect on anxiety and memory. This acts by inhibiting dopaminergic and serotonergic hyperactivity. In stress, there is a modulation of serotonergic and monoaminergic systems. So the aim of the present study is to evaluate the effect of olanzapine on cold restraint stress in rats. To achieve the hypothesis, we have measured plasma corticosterone as a marker of HPA axis activity. Further, we have measured dopamine and norepinephrine for neurochemical correlation with stress.

1.2 Material and methods

1.2.1 Animals

Charles Foster strain rats were procured from the central animal house, BHU. They were assigned into five groups as required for treatment as the control, sham (stress control), doses of 0.1mg, 1mg and 10mg/kg body weights of OLZ to be administered. All the animals were housed and treated following the Principles of laboratory animal care (National Research Council US Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011 guidelines). The experimental procedures were prior approved by the Institutional animal ethical committee, Banaras Hindu University (Ref No. Dean/10-11/148).

1.2.2 Drugs and chemicals

OLZ was procured from Sigma Aldrich Ltd. (St. Louis, MO, USA). All other chemicals and reagents of HPLC were of analytical grade and procured from local suppliers.

1.2.3 Drug treatment

Animals were orally pretreated with 0.5% carboxymethylcellulose (CMC) suspensions of either olanzapine (OLZ) for 21 days with fixed doses of 0.1, 1.0 and 10.0 mg/kg for three different treatment groups. Control groups were administered with just 0.5% CMC. Dose ranges were selected based upon related antipsychotic doses (Llorente-Berzal et al., 2012).

1.2.4 Cold Restraint Stress (CRS)

CRS model was used to induce stress in the animals. On the 21st day of the dosing schedule, the animals were subjected to stress after 18hrs of prior fasting. They were immobilized in a metallic cage using cotton to prevent any gaps and then placed in the refrigerator at a cold temperature of 2^{o}C for 2hrs. Since there were chances of death due to continuous exposure, the door of the refrigerator was opened every 15 minutes to cuddle the animals with a slight hand touch. After the intended duration of exposure, they were freed, decapitated on the same day to collect blood and brain samples, which were stored at -80^{o}c until further experimentation (Krishnamurthy et al., 2013).

1.2.5 Evaluation of Catalepsy behaviour

This behavioural parameter is screened to evaluate the extrapyramidal side effects of antipsychotic drugs (Pozzi et al., 2003). Since different doses of drugs are used, the presence of catalepsy behaviour indicates if the drug in the doses used is effective in the absence of extrapyramidal side effects. Catalepsy is defined as the acceptance and retention of abnormal posture and was measured employing the bar test. The bar test was carried by gently removing rats from their home cage and placing their forepaws on a horizontal bar, fixed at the height of 10 cm above the working surface. The length of time during which the animal retained in this position was recorded by measuring the time from the placement of the rat until removal of one of its forepaws (mean of three consecutive trials; cut-off time =60s). All the groups were tested on the 21st day.

1.2.6 Evaluation of Ulcer Index

After decapitation, the stomach was cut through greater curvature, and the ulcer index was calculated by following standard protocol by a blind observer using a dissecting microscope (Frederick et al.,1978; Gianluca et al., 2011; Carnevale et al., 2011; Saheed et al., 2015). The ulcers were counted and categorized based on severity and size in the gastric mucosa as follows: 0: almost normal mucosa, 1: redness or vascular congestion or a limited number of petechiae, 2: mucosal erosions or a lot of petechiae or small ulcers ($\leq$1 mm 2), 3: 1 to 3 large ulcers or severe lesions ($\geq$1 mm^2), 4: more than 3 large ulcers or very severe lesions 5: perforating ulcer or mucosa full of lesions.

The Ulcer Index (U.I) was calculated by

$$U.I. = [\text{Ulcerated area/total stomach area}] \times 100.$$

This ulcer index is the function of the severity of stress. The drug's ability to mitigate stress can be observed by the reduction in ulcer index.

1.2.7 Estimation of plasma corticosterone and norepinephrine by HPLC

The levels of plasma corticosterone were measured by HPLC using a UV detector While, the levels of plasma norepinephrine (NE) were estimated by HPLC connected with an electrochemical detector (ECD) (Krishnamurthy et al., 2013).

1.2.8 Estimation of Serotonin, Dopamine and their Metabolites by HPLC

The brains were removed after decapitation and microdissected as soon as possible on glass plates over ice into five regions: the prefrontal cortex (PFC), hippocampus (HIP), amygdala (AMY) and hypothalamus (Krishnamurthy et al., 2013). The levels of 5-HT, DA and their metabolites were estimated using HPLC/ECD (Krishnamurthy et al., 2013). In brief, the brain tissue samples were homogenized in 0.17 M perchloric acid by a Polytron homogenizer. Homogenates were then centrifuged at 33,000g (BiofugeStratos, Heaureas, Germany) at 4°C. Twenty microliters of supernatant were injected via HPLC Mobile phase consisted of methanol: water (70:30) pump (Model 515, isocratic pump, Waters, Milford, MA, USA) into a column (Spherisorb, RP C18, 5 lm particle size, 4.6 mm i.d. 9250 mm at 30°C) connected to an ECD (Model 2465, Waters, Milford, MA, USA) at a potential of 0.8V with a glassy carbon working electrode Vs. Ag/AgCl reference electrode. Mobile phase consisted of 32mM citric acid, 12.5mM disodium hydrogen orthophosphate, 1.4mM sodium octyl sulfonate, 0.05mM EDTA and 16% (v/v) methanol (pH 4.2) at a flow rate of 1.2ml/min. Quantification was done by comparing the peak heights of the samples to the corresponding standard curve. Two ranges of standard curves, i.e., 10-100 and 100-1,000ng/ml, were used depending upon the abundance of monoamines in respective brain regions. A constant amount (25ng/ml) of DHBA was added to the tissue samples to calculate recovery. The protein content was estimated and the neurotransmitters were quantified in terms of a fixed weight of protein (Krishnamurthy et al., 2013).

1.2.9 Statistics for Data Analysis

The plasma CORT, NE and brain monoamines data were analyzed by using the Graph pad prism

Version 5 software. Statistical analysis of data was done using one-way ANOVA with Newman–Keuls Post- hoc analysis.

1.3 Results

1.3.1 Effect of OLZ (0.1, 1.0 and 10mg/kg) on CRS-induced alteration on catalepsy behaviour

Escape latency is considered to measure the extrapyramidal effect of antipsychotics. Here, escape latency is measured as latency to escape from the abnormal posture. The effect of olanzapine (0.1, 1.0 and 10mg/kg) on CRS-induced alteration escape latency is represented in **fig.1.1.** Statistical analysis (one-way ANOVA followed by post hoc) revealed that there was no significant difference observed after cold re-stress and olanzapine at the dose of 1.0 and 10mg/kg [$F(4, 20) = 0.25$, $p>0.05$].

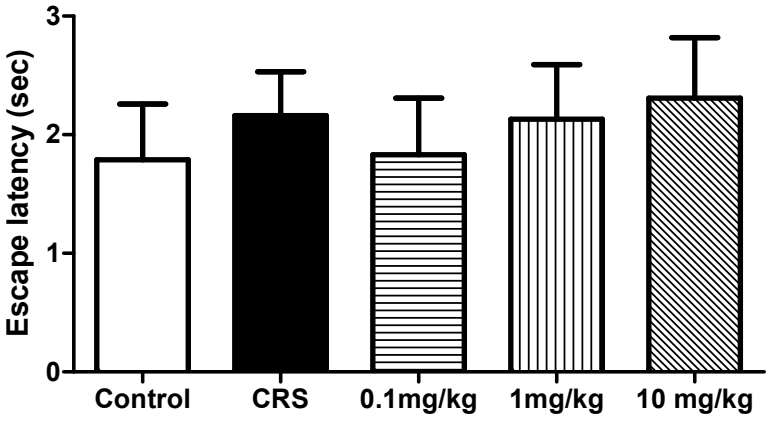

Fig 1.1 The effect of repeated treatment of OLZ (0.1, 1.0 and 10mg/kg) on stress-induced changes in catalepsy behaviour of rats in the bar test. All the values are Mean ± SEM with n=5 [One-way ANOVA followed by Student Newman-keuls test].

1.3.2 Effect of OLZ (0.1, 1.0 and 10mg/kg) on CRS-induced alteration on ulcer index

Ulcer index is a commonly observed parameter after stress. Statistical analysis (one-way ANOVA followed by post hoc) revealed that there was a significant difference among the groups [$F(4, 20) = 899$, $p<0.05$] represented in **fig-1.2.** CRS significantly increased the ulcer index and treatment with olanzapine at the dose of 1.0 and 10 mg/kg significantly attenuated the CRS-induced increase in ulcer index.

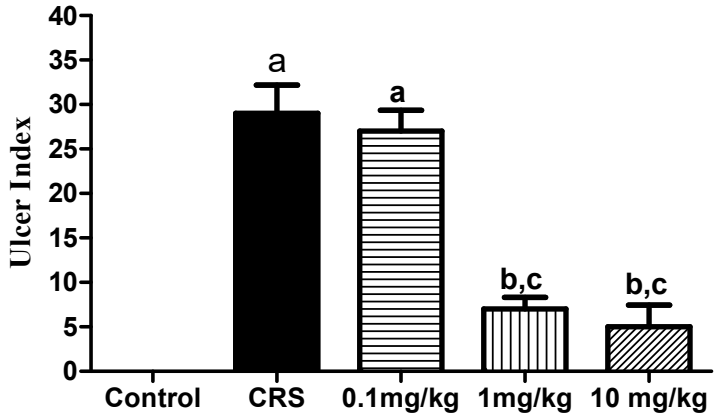

Fig 1.2 effect of repeated treatment of OLZ (0.1, 1.0 and 10mg/kg) on stress-induced changes in gastric ulcer. All values are Mean ± SEM with n=5. $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to CRS and $^{c}P<0.05$ compared to OLZ (0.1 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

1.3.3 Effect of OLZ (0.1, 1.0 and 10mg/kg) on CRS-induced alteration on plasma corticosterone

The effect of olanzapine (0.1, 1.0 and 10mg/kg) on CRS-induced increased in the plasma corticosterone level is depicted in Fig-1.3. Statistical analysis by one-way ANOVA showed that there was a significant difference in plasma corticosterone [F (4, 20) = 1412; P<0.05] level among groups. Post-hoc analysis revealed that CRS caused a significant increase in the level of corticosterone in plasma compared to control animals. Treatment with olanzapine at the doses 1 mg and 10 mg/kg significantly altered the CRS-induced increased in the plasma corticosterone level.

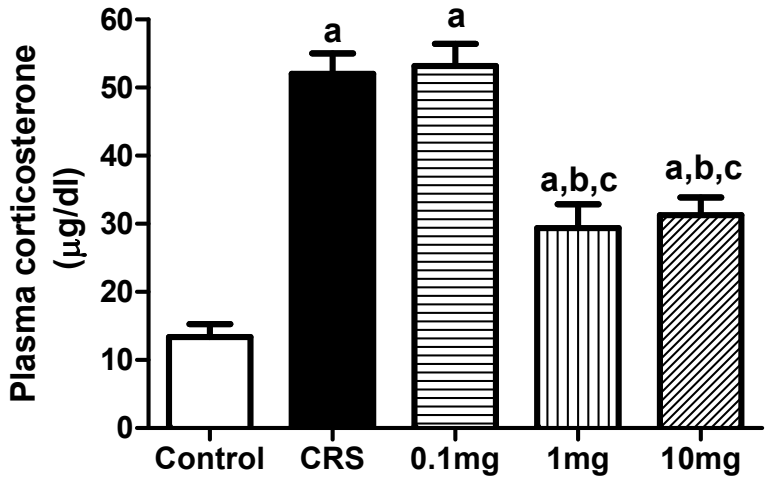

Fig 1.3 The effect of repeated treatment of OLZ (0.1, 1.0 and 10mg/kg) on stress-induced changes in plasma corticosterone. All the values are Mean ± SEM with n=5. ^{a}P<0.05 compared to control, ^{b}P<0.05 compared to CRS and ^{c}P<0.05 compared to OLZ (0.1 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

1.3.4 Effect of OLZ (0.1, 1.0 and 10mg/kg) on CRS-induced alteration on plasma norepinephrine

The effect of olanzapine (0.1, 1.0 and 10mg/kg) on CRS-induced increased in the plasma norepinephrine level is depicted in **Fig-1.4**. Statistical analysis by one-way ANOVA showed that there was a significant difference in plasma nor-epinephrine level [F (4, 20) = 1412; P<0.05] level among groups. Post-hoc analysis revealed that CRS caused a significant increase in the level of nor-epinephrine in plasma compared to control animals. Treatment with olanzapine at the dose 1 mg and 10 mg dose significantly altered the CRS-induced increased in the plasma nor-epinephrine level.

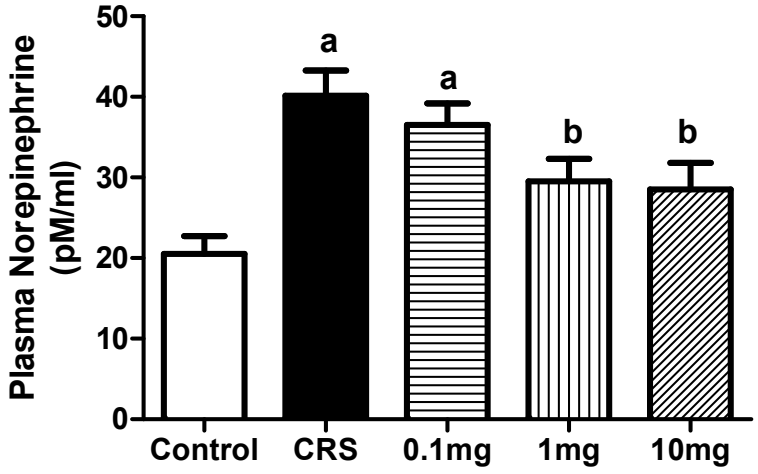

Fig 1.4 The effect of repeated treatment of OLZ (0.1, 1.0 and 10mg/kg) on stress-induced changes in plasma nor-epinephrine. All the values are Mean ± SEM with n=5. [a]P<0.05 compared to control, [b]P<0.05 compared to CRS [One-way ANOVA followed by Student Newman-keuls test].

1.3.5 Effect of OLZ (0.1, 1.0 and 10mg/kg) on CRS-induced alteration on serotonin and its metabolite in PFC, HIP and AMY

5HT: Repeated treatment with OLZ (0.1, 1.0 and 10 mg/kg) on 5-HT levels among brain regions is shown in **Fig-1.5**. One-way ANOVA shows that, there was significant differences among groups in the 5-HT levels in PFC [F (4, 20) = 5.145, p>0.05, HIP [F (4, 20) = 0.76, p<0.05] and AMY [F (4, 30) = 10.305, p<0.05]. Post-hoc analysis by Newman–Keuls showed that Stress significantly increased 5-HT levels in all regions compared to control. Repeated doses of OLZ also showed a decline in levels of 5-HT at the dose of 1 and 10 mg/kg in PFC, HIP and AMY except for 0.1mg/kg dose.

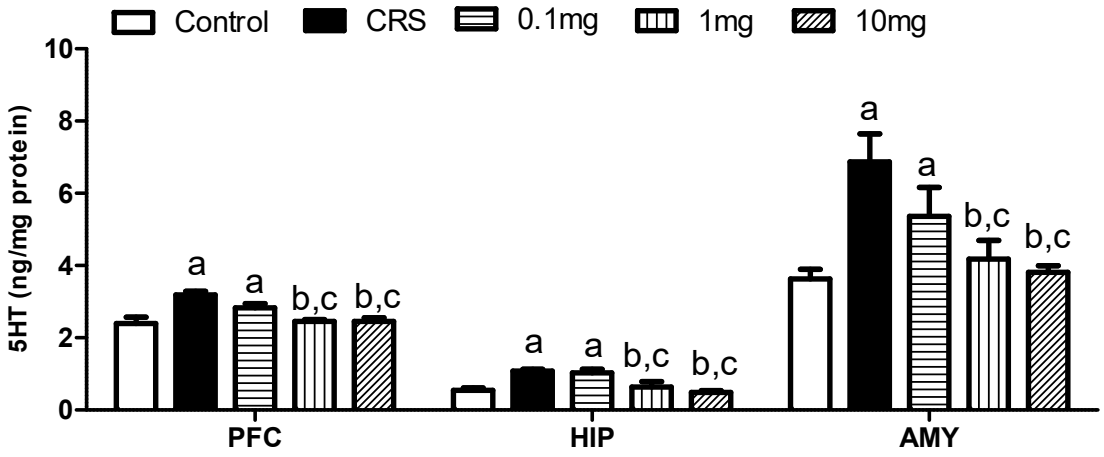

Fig 1.5 The effect of repeated treatment of OLZ (0.1, 1.0 and 10mg/kg) on brain 5HT levels in regions like PFC, HIP, AMY. All values are Mean ± SEM with n=5. [a]p<0.05 compared to control, [b]p<0.05 compared to Stress and [c]p<0.05 compared to OLZ (0.1 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

5HIAA: Fig-1.6 shows 5-HIAA levels in different brain regions in cold restraint rats after repeated treatment with OLZ. One-way ANOVA analysis indicates significant differences in the 5-HIAA levels PFC [F (4, 20) = 5.329, $p<0.05$]; HIP [F (4, 20) = 3.337, $p>0.05$] and AMY [F (4, 20) = 3.9 $p<0.05$]. Post-hoc analysis showed that Stress decreased 5-HIAA levels in all the brain regions. However, repeated OLZ treatment (OLZ 1 and 10mg/kg) increased the 5HIAA in PFC, HIP and AMY.

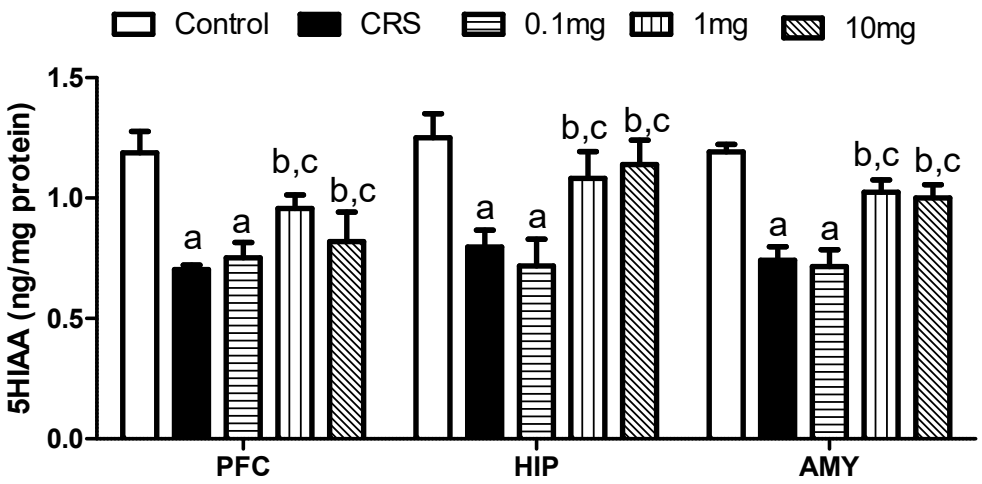

Fig 1.6 The effect of repeated treatment of OLZ (0.1, 1.0 and 10mg/kg) on brain 5HIAA levels in regions like PFC, HIP, AMY. All values are Mean ± SEM with n=5. All values are Mean ± SEM with n=5. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to Stress and $^{c}p<0.05$ compared to OLZ (0.1 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

5-HIAA/5-HT: Fig-1.7. represents the effect of repeated treatment of OLZ on 5-HIAA/5-HT ratios in different brain regions. On analysis with one-way ANOVA there is significant interaction of treatment between groups; PFC [$F(4, 24) = 6.4$, $p<0.05$], HIP [$F(4, 20) = 3.09$, $p<0.05$] AMY [$F(4, 24) = 9.14$, $p<0.05$] and HYP [$F(4, 24) = 8.637$, $p<0.05$]. Further, Post-hoc analysis showed that the stress decreased the 5-HIAA/5-HT ratios in all the brain regions. This decrease in 5-HIAA/5-HT was reversed by OLZ administration in varying doses in different brain regions. OLZ mitigated the CRS-induced decline in 5-HIAA/5-HT in the entire brain region at doses of 1 and 10mg/kg.

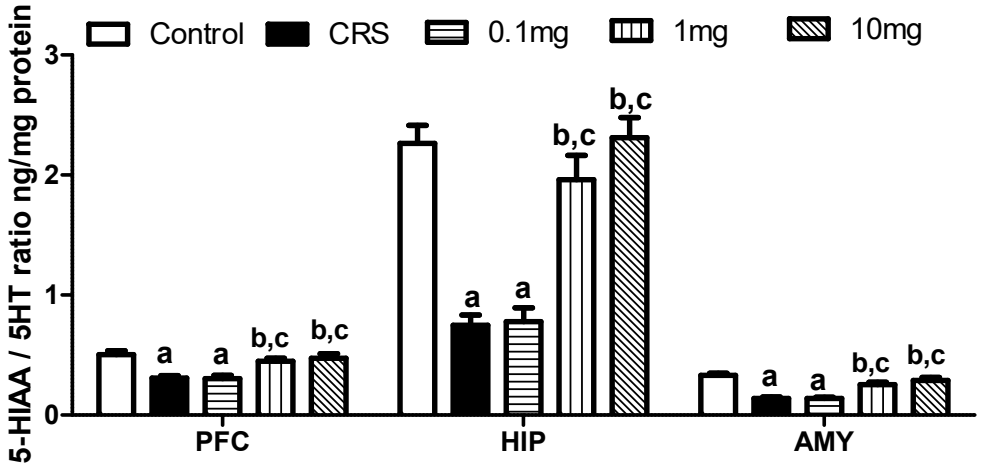

Fig 1.7 The effect of repeated treatment of OLZ (0.1, 1.0 and 10mg/kg) on brain 5-HIAA/5-HT levels in regions like PFC, HIP, AMY. All values are Mean ± SEM with n=5. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to Stress and $^{c}p<0.05$ compared to OLZ (0.1 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

1.3.6. Effect of OLZ (0.1, 1.0 and 10mg/kg) on CRS-induced alteration on dopamine level in PFC, HIP and AMY

The effect of repeated treatment of OLZ (0.1, 1.0 and 10 mg/kg) on DA levels in different brain regions in stress is depicted in **Fig-1.8**. Analysis of data by One-way ANOVA showed that there was significant differences in the DA levels in PFC [F (4, 20) = 8.9, $p<0.05$], HIP [F (4, 20) = 20, $p>0.05$], AMY [F (4, 20) = 1.272, $p>0.05$]. *Post-hoc* analysis showed that Stress significantly increased the DA levels in all the brain regions. Repeated OLZ; 1 mg/kg and 10 mg/kg treatment mitigated stress-induced increase in DA levels in PFC, HIF and AMY.

Fig 1.8 The effect of repeated treatment of OLZ (0.1, 1.0 and 10mg/kg) on brain DA levels in regions like PFC, HIP, AMY. All values are Mean ± SEM with n=5. [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to Stress and [c]$p<0.05$ compared to OLZ (0.1 mg/kg) [One-way ANOVA followed by Student Newman-keuls test].

1.4 Discussion

Olanzapine shows a significant anti-stress effect in terms of ulcer index, plasma corticosterone, and plasma norepinephrine and brain monoamines.

PTSD is a mood disorder that aggravates under stressful conditions. The drugs effective in the treatment of PTSD should possess anti-stress effects. The first set of the study was taken up to check the efficacy of these antipsychotic drugs in stress. An acute stress model involving exposure of animals to a single but severe Cold Restraint stress session was used (Krishnamurthy et al., 2011). The maximal therapeutic effect of the antipsychotic drugs is seen only after one to three weeks of repeated administration (Ofer et al., 2006). So, in order to have an optimal therapeutic effect, drug dose was repeatedly administered for four weeks before subjecting animals to acute stress in the first set of three experiments. In the second set of studies stress restress (SRS) model was used to induce PTSD in animals. PTSD is a chronic disease and the SRS model has scope for dosing along with multiple stress sessions for 28 days. So, the chosen antipsychotic drugs were administered after the first stress session and were continued until the final stress session on the 28^{th} day. So, to achieve the maximal therapeutic response of antipsychotics drugs, repeated treatment was given for three to four weeks in both sets of experiments.

In this study, the CRS significantly enhanced the gastric ulcers and this was alleviated by OLZ at 1 and 10 mg/kg doses. The plasma corticosterone was significantly enhanced by CRS and was mitigated by repeated treatment with OLZ-1 and 10mg/kg. The levels of plasma norepinephrine were also enhanced by CRS, but OLZ-1 and 10mg/kg decreased the enhanced levels of plasma norepinephrine. The drug also showed no catalepsy behaviour in all of the doses tested. In the brain, the levels of brain monoamines showed significant variations due to stress. The 5HT levels were enhanced due to stress in all the brain regions PFC, HIP and AMY. The treatment with OLZ mitigated the enhanced levels of 5HT at doses 1 and

10mg/kg. The 5HIAA levels were decreased in the three brain regions selected. This was mitigated at 1 and 10mg/kg doses by OLZ. The 5HT turnover was decreased in PFC, AMY and HIP and OLZ treatment showed an enhancement in the levels. Similar to 5HT, DA levels were increased in PFC, HIP and AMY, which was alleviated by OLZ at a dose of 1 and 10 mg/kg doses.