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OLANZAPINE COMBINED WITH ETHANOL MODULATES THE ACTIVITY OF MITOCHONDRIAL ENZYMES AND ACETYLCHOLINESTERASE IN METHYLPHENIDATE INDUCED MANIA

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ABSTRACT

Background/Aim: Mood disorders (unipolar and bipolar disorders) are currently the 4th leading contributor to the global burden of disease (DALYs) in 2000. The present study was aimed to evaluate the effect of olanzapine (OLZ) with ethanol (EtOH) on methylphenidate (MPD) induced mania in Swiss albino mice. **Materials and Methods:** The effect of EtOH on OLZ treated MPD induced manic mice was analyzed by Krebs cycle enzymes activities on isocitrate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, α-ketoglutarate dehydrogenase and acetylcholinesterase. **Results:** MPD induced mania was manifested by decreased the activity of Krebs cycle enzymes and AChE in brain was observed. Our result shows that OLZ controlled the activity of Krebs cycle enzymes and AChE in brain to treated MPD administrated mice. EtOH rather than being treated alone, when combined with OLZ significantly worsen the activity of Krebs cycle enzymes and AChE in MPD administrated mice. **Conclusion:** Based on the Krebs cycle enzymes and AChE in brain studies of our results, it can be concluded that EtOH attenuates the OLZ treatment and thereby reduced Krebs cycle enzymes and enhanced AChE activity in MPD induced manic mice. Further studies are needed to discover the mechanisms responsible for these findings.

KEYWORDS: Manic disorder, Methylphenidate, Olanzapine, Ethanol, Krebs cycle enzymes, AChE.

INTRODUCTION

In most of study reported methylphenidate (MPD) is psychomotor stimulant which produces hyperactivity and induced mania in rodents[1,2] and it has been shown to influence oxidative stress.[3,4] It is well known that those alterations could lead to activation of apoptotic pathways.[5] In fact, initial activation of caspases can be from plasma membrane upon ligation of death receptor or from mitochondrial damage.[6] MPD has also been shown to alter energy metabolism, including alteration of creatine kinase, an enzyme important for normal energy homeostasis,[7] enzymes of Krebs cycle, such as citrate synthase and isocitrate dehydrogenase,[4] and in mitochondrial respiratory chain enzymes[8] were reported. In addition, the changes caused by MPD can be blockade in dopamine transporter and by increase of dopamine levels in the brain areas, which are also activated by abused drugs.[9,10] In fact, the excess in dopamine could lead to reactive oxygen species (ROS) production, which in turn directs to mitochondrial dysfunction and decrease in ATP production.[11-14] Thus, in this study we have employed MPD as mania inducer and evaluated mitochondrial changes occur in our experimental period. Several agents used in acute and prophylactic mania treatment including lithium, valproate, olanzapine (OLZ), quetiapine, and risperidone, which often can set the stage for non-adherence. Present study we used OLZ for mania treatment.

OLZ is an atypical thienobenzodiazepine-class antipsychotic with an affinity for dopamine binding sites D1 and D4, as well as serotonin 5-HT,[15,16] muscarin (subtypes 1-5), adrenergic (alfa1) and histamine (H1) binding sites. The drug possesses weak D2 receptor blockade properties and its serotonin receptor 5-HT 2A blockage is about 8 times more intense than that of dopamine receptor D2.[15] Clinical assay suggests that an OLZ decrease both positive and negative in symptoms of schizophrenia and it is associated with a low incidence of extra-pyramidal side effects.[17] Ji[18] demonstrated that OLZ acts by alters the enzymatic activity of complex I of the mitochondrial respiratory chain in some brain areas, such as the cerebral cortex and hippocampus, in addition to inhibiting subunits of complex V or adenosine triphosphate (ATP) synthase. Keck[19] have described the efficacy of OLZ in treating a broad spectrum of symptoms such as agitation, aggression, depression and suicidality. OLZ drug normalizing mood disturbances in the BPD and they may improve cognitive functions.[20-22]

Ethanol (EtOH) elicits behavioral effects in MPD induced manic like behavior in mice by altering the neurotransmitter systems[23] and also alter the transduction of cellular signal generated by different neurotransmitter systems.[24] Enhanced generation of ROS, which consequently leads to an increase in oxidative damage to lipids, proteins, DNA and decrease in the cellular antioxidant defense mechanisms upon exposure to EtOH.[25,26] In addition, EtOH impairs normal brain function and plasticity, by both acute and chronic EtOH exposure.[27,28] Acetylcholinesterase (AChE) hydrolyzes the neurotransmitter acetylcholine (ACh) at the synaptic cleft of cholinergic synapses and neuromuscular junctions.[29] In addition, its role in cholinergic transmission, AChE is associated with brain development, learning, memory and neuronal damage.[30-32] As far our knowledge, there are no scientific evidance on EtOH and OLZ combination on MPD treated manic mice. While, manic patients treating with antipsychotic drug even they consuming EtOH leads to changes in mitochondrial. Thus present study was designed to investigate the effect of EtOH and OLZ on MPD induced manic mice mitochondrial changes.

MATERIALS AND METHODS

Animals: Healthy adult male Swiss Albino mice (*Mus musculus*) 8-10 weeks old, bred and reared in the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University were used for the experiment. Weight matched animals (25-30g) were selected and housed in polypropylene cages layered with husk and kept in a semi-natural light/dark condition (12h light/12h dark). The animals were allowed free access to water and standard pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd. Bangalore, India). Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 160/1999/CPCSEA, Proposal number: 1027) and animals were carried in accordance with the "Guide for the care and use of laboratory animals" and "Committee for the purpose of control and supervision on experimental animals".

Preparation of samples: Methylphenidate (Sigma - Aldrich, India) were dissolved in physiologic saline (0.9% NaCl), at a dose of 2.0 mg/kg b.w. i.p[33] was injected intraperitoneally for 21 days. Olanzapine (6 mg/kg b.w)[34] were dissolved in two drops of 10% lactic acid that was then brought up to volume with distilled water and acidic (pH=5-6) with 1 M NaOH and administered via oral gavages. Ethanol (2.0 g/kg b.w)[35,36] were prepared by 96% ethanol dissolved in distilled water and administered via oral gavage. All chemicals were used analytical grade obtained from E. Merck and Himedia, Mumbai, India. All the drugs were administrated in the range of 5-ml/kg /body weight.

Experimental design: In this experiment, saline (2 mg/kg/b.w i.p) injected mice recorded as control group and methylphenidate (2 mg/kg b.w i.p) injected mice recorded as mania mice. Control and methylphenidate treated mice were divided into eight group, each comprising of six mice totaling to forty eight mice; Group 1: Control (saline 2 mg/kg/b.w); Group 2: Control + OLZ; Group 3: Control + EtOH; Group 4: Control + OLZ + EtOH; Group 5: MPD (Mania); Group 6: MPD + OLZ; Group 7: MPD + EtOH; Group 8: MPD + OLZ + EtOH; the mice were sacrificed by with cervical dislocation; immediately, mice brain tissues were collected and stored at −80°C until used for the biochemical analysis.

Isolation of mitochondria: The animals were sacrificed by cervical dislocation immediately after the behavioral analysis (OFT, FST); datas were published[23] in mice brain tissues were stored at −80°C until used for the biochemical analysis. The mice brain tissues were thawed, weighed, and then mitochondrial fraction of the brain tissue was isolated by the standard method of Takasawa.[37] The brain tissue was put into ice-cold 50 mM Tris–HCl buffer; pH 7.4 containing 0.25M sucrose and homogenized. The homogenates were centrifuged at 700×g for 20 min and then the supernatants obtained were centrifuged at 9,000×g for 15 min. Then, the pellets were washed with 10 mM Tris–HCl buffer (pH 7.8) containing 0.25 M sucrose and finally resuspended in the same buffer.

Activity of Krebs cycle enzymes

Assay of isocitrate dehydrogenase: The activity of isocitrate dehydrogenase (ICDH) in the brain mitochondrial fraction was assayed by the method of King.[38] The incubation mixture contained 0.4 ml of Tris-HCl buffer, 0.2 ml of substrate, 0.2 ml of manganese chloride, 0.2 ml of nicotinamide adenine dinucleotide phosphate (NADP+) and 0.2 ml of mitochondrial fraction. The NADP+ was replaced by 0.2 ml of saline in tubes labeled as control. A suitable aliquot of enzyme preparation was added and mixed well. The tubes were then incubated at 37°C for 60 min. At the end of the incubation period, 1.0 ml of the coloring reagent and 0.5 ml of EDTA were added. The contents of the tubes were mixed well and allowed to stand at room temperature for 20 min and 10 ml of 0.4 N NaOH was added and the color intensity was read at 420 nm.

Assay of succinate dehydrogenase: The activity of succinate dehydrogenase (SDH) in the brain mitochondrial fraction was assayed by the method of Slater and Borner.[39] The reaction mixture contained 1.0 ml of phosphate buffer, 0.1 ml of EDTA, 0.1 ml of sodium cyanide, 0.1 ml of bovine serum albumin, 0.3 ml of sodium succinate, 0.2 ml of potassium ferricyanide, and made up to 2.8 ml with distilled water. The reaction was initiated by the addition of 0.2 ml of mitochondrial fraction. The change in optical density was recorded at 15s intervals for 5 min at 420 nm.

Assay of malate dehydrogenase: The activity of malate dehydrogenase (MDH) in the brain mitochondrial fraction was assayed by the method of Mehler.[40] The reaction mixture contained 0.75 ml of phosphate buffer, 0.15 ml of reduced nicotinamide adenine dinucleotide (NADH), and 0.75 ml of oxaloacetate. The reaction was done at 25°C and was started by the addition of 0.2 ml of mitochondrial fraction. The control tubes contained all reagents except NADH. The change in optical density at 340 nm was measured for 2 min at an interval of 15s in a Systronics UV-visible spectrophotometer.

Assay of α-ketoglutarate dehydrogenase: The activity of α-ketoglutarate dehydrogenase (α-KGDH) in the brain mitochondrial fraction was assayed by the method of Reed and Mukherjee.[41] The incubation mixture contained 0.1 ml of phosphate buffer, 0.1 ml of thiamine pyrophosphate, 0.1 ml of magnesium sulfate, 0.1 ml potassium α-ketoglutarate, 0.1 ml of potassium ferricyanide and distilled water to a final volume of 1.4 ml. A suitable aliquot of the mitochondrial fraction was added in test, while it was replaced by distilled water in the control. The mixture was then incubated at 30°C for 30 min. At the end of this period, the reaction was terminated by the addition of 1.0 ml of 10% TCA. The mitochondrial fraction was added to the control after TCA was added. The mitochondrial fraction was centrifuged. To this, 1.0 ml of supernatant, 1.0 ml of 10% TCA, 1.5 ml of distilled water, 1.0 ml of 4% duponol, and 0.5 ml of ferric ammonium sulfate–duponol reagent were added. Then, the tubes were allowed to stand at room temperature for 30 min. The color intensity was measured at 540 nm in a spectrophotometer. ATP concentration in the brain mitochondrial fraction was measured by the method of Williams and Coorkey.[42]

Assay of acetylcholinesterase activity: The assays for mouse brain AChE activity were carried out according to the method of Ellman.[43] To 3.0 ml of phosphate buffer (pH 8.0), 0.1 ml of tissue homogenates were added separately and stirred. Then 100 µl of 0.01M DTNB (5-5-dithiobis-2-nitrobenzoic acid) was added to each tube and the initial color was measured spectrophotometrically at 412 nm. Brain homogenates was added and then to start the reaction, 20 µl of acetyl thiocholine iodide (75mM) was added to each tube as substrate and the reaction allowed continuing for 15 min at room temperature. Changes in absorbance were measured at 412 nm.

Estimation of protein in the brain mitochondrial fraction: Protein content in the mitochondrial fraction was estimated by the method of Lowry.[44]

Statistical analysis: All quantitative measurements were expressed as mean ± SD for control and experimental animals. The data were analyzed using two way analysis of variance (ANOVA) on SPSS/PC (statistical package for social sciences, personal computer) and the group means were compared by post hoc comparisons performed using the Tukey HSD test. The results were considered statistically significant if the P value is less than 0.05.

RESULTS

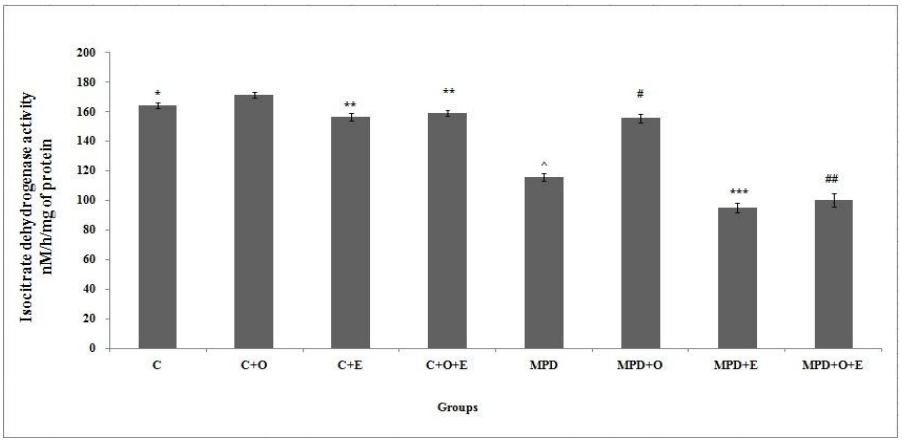

Figure 1: Effects of OLZ and EtOH (alone, or in combination) treated with MPD induced manic and control mice (C) groups on Isocitrate dehydrogenase (ICDH). Group C: Control (saline 2 mg/kg/b.w); Group C + O: Control + OLZ; Group C + E: Control + EtOH; Group C + O + E: Control + OLZ + EtOH; Group MPD: Depression (MPD alone); Group MPD + O: MPD + OLZ; Group MPD + E: MPD + EtOH; Group MPD + O + E: MPD + OLZ + EtOH. Values are expressed as mean ± S.D with n=6 in each group; two-way ANOVA followed by post hoc comparisons with the average on Tukey HSD. *P<0.05 as compared to control (saline) group. #P<0.05 as compared to control (saline) group. **P<0.05 as compared to MPD administrated group. ##P<0.05 as compared to MPD administrated group.

Effects of MPD treatment on OLZ and EtOH on the activity of ICDH: Figure 1 shows the effect of control with EtOH and combined OLZ with EtOH treated control group significantly decreased **ICDH** (Fig. 1; $F(2,15) = 0.574$, $P= 0.575$) when compared to the control group. MPD administrated mice significantly decreased

ICDH (Fig. 1; F(1,10) = 0.074, P= 0.792) when compared with the control group. MPD treated with OLZ significantly increased **ICDH** (Fig. 1; F(1,10) = 0.053, P= 0.823) when compared with MPD treated alone group. Reduced **ICDH** (Fig. 1; F(2,15) = 0.931, P= 0.416) were observed in EtOH alone and combined OLZ with EtOH treated groups of MPD administered mice when compared to MPD alone treated group.

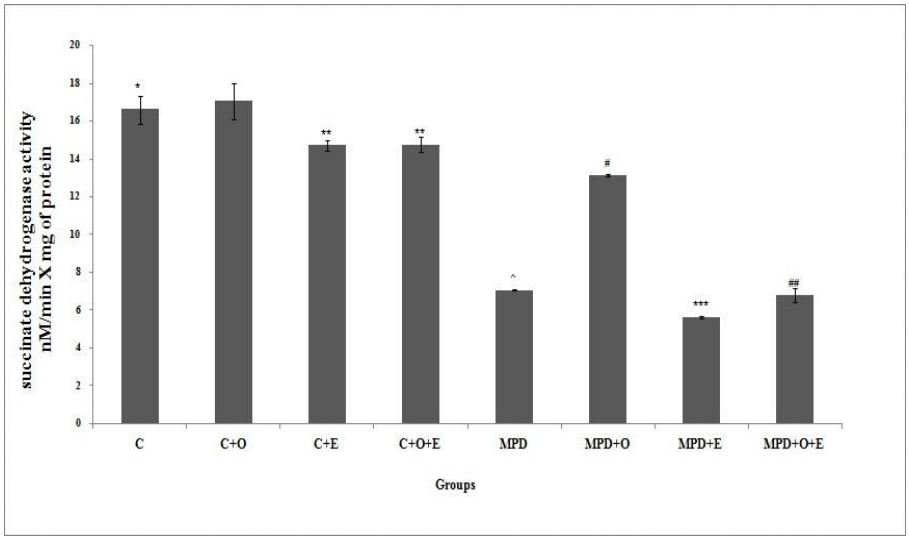

Figure 2: Effects of OLZ and EtOH (alone, or in combination) treated with MPD induced manic and control mice (C) groups on succinate dehydrogenase (SDH). Group C: Control (saline 2 mg/kg/b.w); Group C + O: Control + OLZ; Group C + E: Control + EtOH; Group C + O + E: Control + OLZ + EtOH; Group MPD: Depression (MPD alone); Group MPD + O: MPD + OLZ; Group MPD + E: MPD + EtOH; Group MPD + O + E: MPD + OLZ + EtOH. Values are expressed as mean ± S.D with n=6 in each group; two-way ANOVA followed by post hoc comparisons with the average on Tukey HSD. *P<0.05 as compared to control (saline) group. #P<0.05 as compared to control (saline) group. **P<0.05 as compared to MPD administrated group. ##P<0.05 as compared to MPD administrated group.

Effects of MPD treatment on OLZ and EtOH on the activity of SDH: Figure 2 depicts the activity of control with EtOH and combined OLZ with EtOH treated control group significantly decreased **SDH** (Fig. 2; F(2,15) = 3.667, P= 0.051) when compared to the control group. MPD administrated mice significantly decreased **SDH** (Fig. 2; F(1, 10) = 17.352, P= 0.002) when compared with the control group. MPD treated with OLZ significantly increased **SDH** (Fig. 2; F(1,10) = 2.894, P= 0.120) when compared with MPD treated alone group. Reduced **SDH** (Fig. 2; F(2,15) = 4.798, P= 0.025) were observed in EtOH alone and combined OLZ with EtOH treated groups of MPD administered mice when compared to MPD alone treated group.

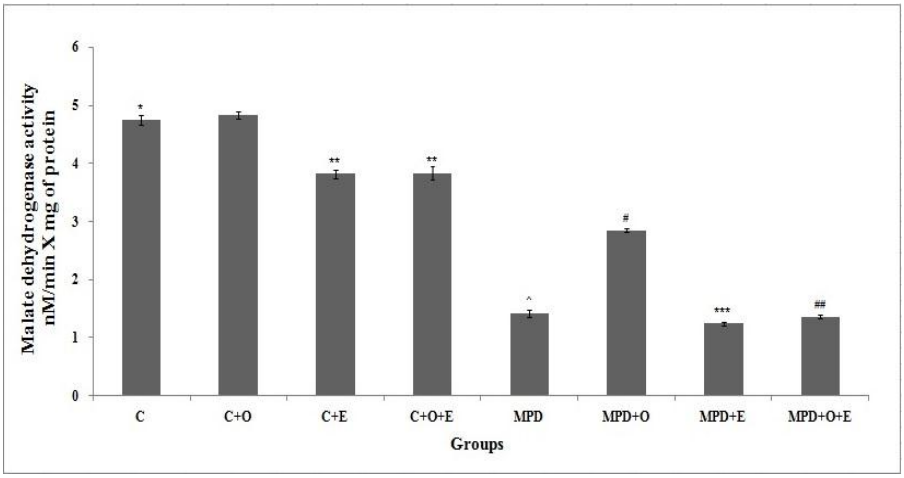

Figure 3: Effects of OLZ and EtOH (alone, or in combination) treated with MPD induced manic and control mice (C) groups on malate dehydrogenase (MDH). Group C: Control (saline 2 mg/kg/b.w); Group C + O: Control + OLZ; Group C + E: Control + EtOH; Group C + O + E: Control + OLZ + EtOH; Group MPD: Depression (MPD alone); Group MPD + O: MPD + OLZ; Group MPD + E: MPD + EtOH; Group MPD + O + E: MPD + OLZ + EtOH. Values are expressed as mean ± S.D with n=6 in each group; two-way ANOVA followed by post hoc comparisons with

the average on Tukey HSD. *P<0.05 as compared to control (saline) group. #P<0.05 as compared to control (saline) group. **P<0.05 as compared to MPD administrated group. ##P<0.05 as compared to MPD administrated group.

Effects of MPD treatment on OLZ and EtOH on the activity of MDH: Figure 3 shows the effect of control with EtOH and combined OLZ with EtOH treated control group significantly decreased **MDH** (Fig. 3; F(2,15) = 0.375, P= 0.694) when compared to the control group. MPD administrated mice significantly decreased **MDH** (Fig. 3; F(1,10) = 0.144, P= 0.712) when compared with the control group. MPD treated with OLZ significantly increased **MDH** (Fig. 3; F(1,10) = 0.863, P= 0.375) when compared with MPD treated alone group. Reduced **MDH** (Fig. 3; F(2,15) = 0.750, P= 0.489) were observed in EtOH alone and combined OLZ with EtOH treated groups of MPD administered mice when compared to MPD alone treated group.

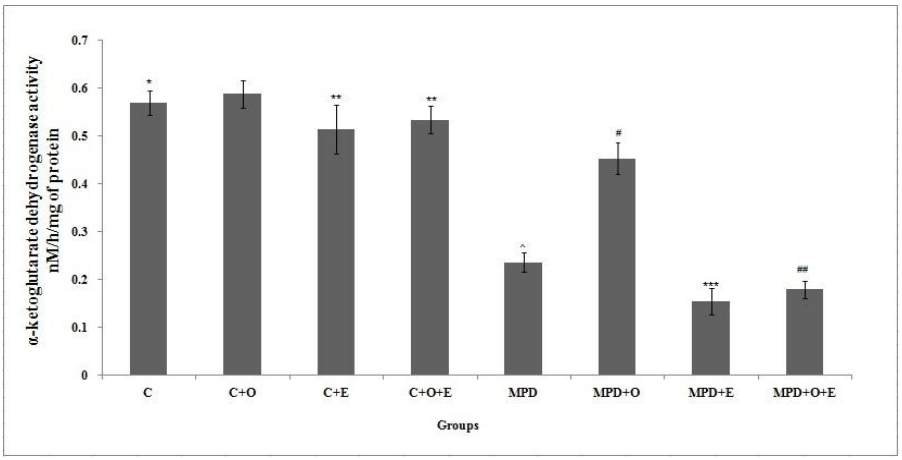

Figure 4: Effects of OLZ and EtOH (alone, or in combination) treated with MPD induced manic and control mice (C) groups on α-ketoglutarate dehydrogenase (α-KGDH). Group C: Control (saline 2 mg/kg/b.w); Group C + O: Control + OLZ; Group C + E: Control + EtOH; Group C + O + E: Control + OLZ + EtOH; Group MPD: Depression (MPD alone); Group MPD + O: MPD + OLZ; Group MPD + E: MPD + EtOH; Group MPD + O + E: MPD + OLZ + EtOH. Values are expressed as mean ± S.D with n=3 in each group; *P<0.05 as compared to control (saline) group. **P<0.05 as compared to control (saline) group. #P<0.05 as compared to MPD administrated group. ##P<0.05 as compared to MPD administrated group.

Effects of MPD treatment on OLZ and EtOH on the activity of α-KGDH: The activity of α-KGDH in the control with EtOH and combined OLZ with EtOH treated control group significantly decreased **α-KGDH** (Fig. 4; F(2,15) = 2.688, P= 0.101) when compared to the control group. MPD administrated mice significantly decreased **α-KGDH** (Fig. 4; F(1,10) = 0.263, P= 0.619) when compared with the control group. MPD treated with OLZ significantly increased **α-KGDH** (Fig. 4; F(1,10) = 2.250, P= 0.165) when compared with MPD treated alone group. Reduced **α-KGDH** (Fig. 4; F(2,15) = 0.913, P= 0.422) were observed in EtOH alone and combined OLZ with EtOH treated groups of MPD administered mice when compared to MPD alone treated group.

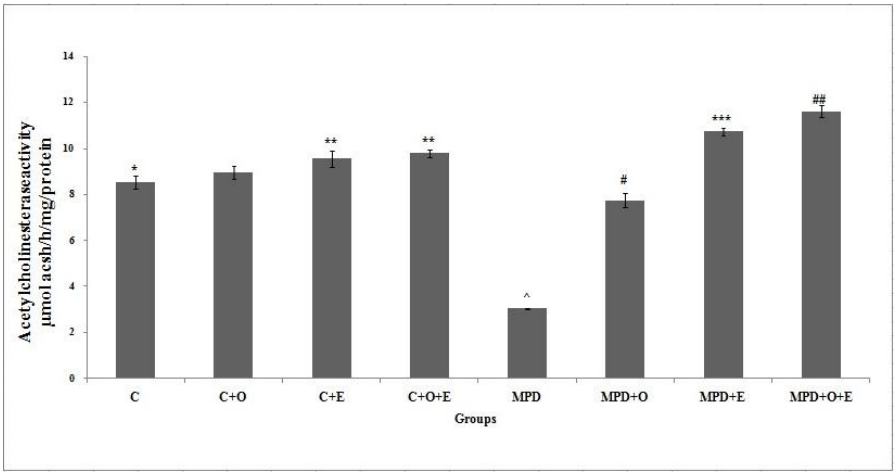

Figure 5: Effects of OLZ and EtOH (alone, or in combination) treated with MPD induced manic and control mice (C) groups on acetylcholinesterase (AChE). Group C: Control (saline 2 mg/kg/b.w); Group C + O: Control + OLZ; Group C + E: Control + EtOH; Group C + O + E: Control + OLZ + EtOH; Group MPD: Depression (MPD

alone); Group MPD + O: MPD + OLZ; Group MPD + E: MPD + EtOH; Group MPD + O + E: MPD + OLZ + EtOH. Values are expressed as mean ± S.D with n=3 in each group; *P<0.05 as compared to control (saline) group. **P<0.05 as compared to control (saline) group. #P<0.05 as compared to MPD administrated group. ##P<0.05 as compared to MPD administrated group.

Effects of MPD treatment on OLZ and EtOH on the activity of AChE: The activity of AChE in the control with EtOH and combined OLZ with EtOH treated control group significantly increased AChE (Fig. 5; F(2,15) = 1.851, P= 0.191) when compared to the control group. MPD administrated mice significantly decreased AChE (Fig. 5; F(1,10) = 19.063, P= 0.001) when compared with the control group. MPD treated with OLZ significantly increased AChE (Fig. 5; F(1,10) = 7.810, P= 0.019) when compared with MPD treated alone group. Enhanced AChE (Fig. 5; F(2,15) = 4.487, P= 0.030) were observed in EtOH alone and combined OLZ with EtOH treated groups of MPD administered mice when compared to MPD alone treated group.

DISCUSSION

Several researches have been focused on MPD effects in the central nervous system during childhood and adolescence exposure despite to alterations in the dopaminergic circuits function,[45,46] gene expression[47,48] and others molecular changes related to neuronal metabolism.[49] Previously we reported, behavioral and neurochemical activities of ethanol on olanzapine treated methylphenidate induced mania like behaviours in swiss albino mice.[23] However, changes in the Krebs cycle enzymes have been little studied. In our present study we demonstrated the effect of the activity of Krebs cycle enzymes and acetylcholinesterase (AChE) in methylphenidate (MPD) induced mania mice treated olanzapine (OLZ) with ethanol (EtOH). The ICDH activity was reduced in the brain of adult rats after treatment with MPD.[4] Previously reported administration of MPD decreased SDH enzyme in brain of young rats.[8] The activity of α-KGDH, MDH and AchE enzymes activities were significantly decreased in the brain of MPD mice.[50] Based on this previous studies we reported MPD treated mice reduced ICDH, SDH, α-KGDH, MDH and AChE activity the mice brain.

Most antipsychotic administration before achieving clinical effects; the mechanisms involved in this delay are not known, but their therapeutic efficacy is probably mediated by long-term molecular adaptations. In this context, several studies demonstrated that mitochondrial respiratory chain enzymes are activated in the brain of adult rats after administration of paroxetine, nortriptyline, and venlafaxine.[51] Aripiprazole antipsychotic increased the mitochondrial enzyme in the rat brain.[52,50] Studies also demonstrated that citrate synthase, SDH, MDH and creatine kinase are increased by administration of paroxetine in brain of adults' rats.[53,54] Based on the hypothesis that previous antipsychotic studies, in our study reported OLZ increased mitochondrial enzyme such as ICDH, SDH, MDH and α-KGDH. In addition, the procognitive effects of OLZ have been associated with their ability to elevate extracellular acetylcholine concentrations in medical regions. Thus, OLZ, markedly increase AChE in the rat[55,56] through a still unidentified mechanism. Additionally, It was previous proposed that OLZ might increase AChE release by blocking muscarinic autoreceptors.[57] Accordingly, the present study reported enhanced AChE activity of mice brain.

Previous study reported the attenuation of ICDH activity is the intermediary events in EtOH induced cellular damage to that in control cells.[26] EtOH ingestion recorded reductions in activity of SDH.[58] Observations on MDH background demonstrate significant increases in EtOH tolerance with reductions in MDH activity. This observation strongly suggests the operation of the malate/ pyruvate cycling in adaptation to alcohol exposure.[59] To test this hypothesis, reduction in MDH activity influence adult tolerance to alcohol.[60] Protective action of α-KGDH reduced EtOH toxicity is realized through stimulating both alcohol dehydrogenase and antioxidant activities.[61] Based on that report in our study showed increased EtOH were reduced α-KGDH activity. Information about the effects of AChE on EtOH induced apoptosis is scarce. Mice treated with acute dose of EtOH experience an apoptotic cascade resulting in increased levels of AChE.[62] We hypothesize that EtOH decreased ICDH, SDH, α-KGDH, MDH and increased AChE activity in the mice brain. Finding of the present study supports the previous reports, EtOH alone and its combined with OLZ were significantly reduced ICDH, SDH, α-KGDH, MDH and increased AChE activity in treated MPD induced manic mice. Therefore, based on the present results observed in mitochondrial enzymes and acetylcholinesterase activities, MPD induced mania under the study period shown decreased ICDH, SDH, α-KGDH, MDH and AChE activity of mice and OLZ treatment enhanced ICDH, SDH, α-KGDH, MDH and AChE activity and reverse the activities of MPD induced manic mice. An interestingly finding of the present study of that EtOH attenuates OLZ treatment and its suppress MPD activity of ICDH, SDH, α-KGDH, MDH and stimulant AChE in treated EtOH alone and combined EtOH with OLZ. Further studies we have been needed to explore of endocrinological and mRNA expression involved are also affected by those drugs used in this study.

CONCLUSION

In conclusion, our data postulated that EtOH impairs the OLZ treatment and suppress the mitochondrial enzymes and stimulant acetylcholinesterase activities in brain tissue and also confirm that EtOH worsen the condition of the mania even in the regular intake of mood stabilizer. This study gives a clear indication about the

effect of inducer and its impact on the physiology and biochemistry of the animal models of mania and forms the base for further investigation of neurophysiological changes in the brain with reference to EtOH stimulation.

ACKNOWLEDGEMENTS
-Nil-.

CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

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