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Agmatine Attenuates Oxidative Stress In Alcohol-Related Neurodevelopmental Disorder In Prenatal Ethanol Exposure Rat Model

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Abstract:

The present study investigates the potential of agmatine, a neuromodulator with antioxidant properties, as a therapeutic intervention for addressing behavioural abnormalities in offspring with prenatal ethanol exposure. Though underlying pathology remains to be investigated, existing literature examined involvement oxidative stress resulted through several pathways in prenatal ethanol exposure. In the present study female SD rats were administered intragastrically with 0 or 6 g/kg ethanol (20 % wt/vol) from gestation days 9 to 20 to induce ADHD-like behavioural deficits in offspring. After weaning, male offspring were assigned to treatment groups and received agmatine intraperitoneally from postnatal day 21 to 35. The results showed that prenatally ethanol exposed offspring possessed hyperactivity, anxiety and depression like behaviour. Treatment with agmatine (40 and 80 mg/kg) and its modulators L-arginine (60 mg/kg), Arcaine (30 mg/kg) and Aminoguanidine (50 mg/kg) effectively reduced the hyperactivity in adult offspring and also shows anxiolytic and antidepressant action in EPM and FST. Additionally, prenatal ethanol exposure resulted in impaired attention which was reverse by chronic treatment agmatine and modulators. Also, offspring showed cognitive impaired with agmatine suggests that agmatine may play a crucial role in mitigating the neurodevelopmental consequences of prenatal alcohol exposure. Overall, our findings highlight the promising potential of agmatine as a novel pharmacological approach for addressing attention deficits associated with prenatal ethanol exposure and underscore the need for further research in this area.

Keywords: Fetal alcohol spectrum disorder (FASD), Alcohol-related neurodevelopmental disorder (ARND), Attention-deficit/hyperactivity disorder (ADHD), Agmatine.

Introduction:

The most prevalent form of fetal alcohol spectrum disorder (FASD) is alcohol-related neurodevelopmental disorder (ARND), which involves major CNS abnormalities (Clarren et al. n.d.; Riley and McGee 2005; Stratton, Howe, and Battaglia 1996). Because alcohol has neurotoxic effects on developing brain, ethanol exposure during pregnancy shows diverse brain abnormalities in children. Patients diagnosed with ARND have cognitive as well as behavioural deficits as manifested in attention-deficit/hyperactivity disorder (ADHD) and neurodevelopmental disorders (Bhatara, Loudenberg, and Ellis 2006; Fryer et al. 2007; Hausknecht et al. 2005; O'Malley and Nanson 2002). Clinical reports suggest growing incidence of ADHD in children with in utero ethanol exposure, indicating that comorbid conditions exist within some individuals in FASD (Rojas-Mayorquín, Padilla-Velarde, and Ortuño-Sahagún 2016).

Though underlying pathology remains to be investigated, existing literature examined involvement of several pathways in prenatal ethanol exposure including altered neurotransmission, hypothalamic–pituitary–adrenal (HPA) axis, or expression of neurotropic factors and inflammatory mediators (Atalar, Uzbay, and Karakaş 2016; Barron et al. 2012; Boschen and Klintsova 2017; GABRIEL et al. 2006; Sharma et al. 2021; Yu et al. 2020). The previous experiments in animals with prenatal ethanol exposure proposed cognitive impairment associated with hippocampal abnormality (Dudek et al. 2014). Of relevance to these findings, we reported that prenatal ethanol exposure result in cognitive impairment and behavioural alteration in offspring which could be associated with altered BDNF expression in and proinflammatory cytokines in hippocampus (Aglawe et al. 2021). Furthermore, adult with ADHD demonstrated decreased executive control which is linked with impairment in source and prospective memory, indicating ADHD is associated with cognitive impairment which can adversely affect execution of everyday task (Butzbach et al. 2019).

Prenatal ethanol exposure is known to induce significantly increase in oxidative stress, responsible for behavioural abnormalities in offspring. There are growing number of studies indicates that oxidative damage modulates physiological brain signalling. The recent metanalysis study by Joseph et al. also reported significant neuronal damage in ADHD associated with increased oxidative stress (Joseph et al. 2015). Furthermore, in-vitro and in-vivo animal models have undercover many possible mechanisms by which prenatal alcohol exposure induces neurotoxicity, of which oxidative stress is an attractive mechanism. As the brain possess high amount of oxygen relative to its mass thus, high substrate available for the process of oxidation compared to low antioxidant activity. Moreover, developing brain in more susceptible to oxidative stress induce neurotoxicity compared with adult due to low antioxidant defence. The prenatal ethanol exposure has been associated with development of anxiety and depression, which mainly accompanied by imbalance in oxidative state (Brocardo et al. 2012). Several literatures indicated increased in oxidative and nitrative stress in ADHD associated with environmental, lifestyle, genetic and nutritional factor (Lopresti 2015; Sezen et al. 2016). Thus, making oxidative and nitrative stress a crucial target in prenatal ethanol exposed ADHD-like neurological abnormalities.

Agmatine is a biogenic amine, synthesised from L-arginine by arginine decarboxylase, found to be an endogenous ligand for imidazoline receptor. It fulfils almost criteria for neurotransmitter. This neuromodulator also binds with high affinity to α2-adrenergic, imidazoline (I1) receptor and blocks NMDA glutamate receptors (Reis and Regunathan 2000; Yang and Reis 1999). Agmatine has been associated with protective and therapeutic roles in several mental illnesses including anxiety, depression, psychosis, etc. (Aglawe et al. 2014; Bence et al. 2003; Dixit et al. 2018; Feng, Yan, and Yan 2005; Lavinsky, Arteni, and Netto 2003; Lee et al. 2009; Liu and Bergin 2009; Neis et al. 2015; Satriano et al. 2001; Taksande et al. 2014, 2017, 2009).

Agmatine has found to attenuate symptoms associated with addiction as well as their withdrawal including nicotine, morphine and ethanol withdrawal symptoms (Aricioglu, Means, and Regunathan 2004; Chimthanawala et al. 2020; Kotagale et al. 2014, 2015, 2018; Su et al. 2009; Taksande et al. 2010, 2019; Yananlı et al. 2007). The molecule efficiently reduced self-administration associated with ethanol (Taksande et al. 2019). Additionally, treatment with agmatine in ethanol addiction and withdrawal period has been found to improve performance in numerous behavioral tasks (Chimthanawala et al. 2020; Uzbay et al. 2000). Furthermore, our previous study also explored the involvement of agmatine in cognitive impairment in offspring associated with gestation ethanol exposure (Aglawe et al. 2021). All of these activities could be of interest in treating neurodevelopmental complications associated with prenatal ethanol exposure.

Thus, we hypothesize the pharmacological importance of agmatine in ameliorating cognitive and behavioral deficits associated with prenatal ethanol exposure induced ADHD. In the present study, we used a different behavioral paradigms to investigate whether rats with prenatal ethanol exposure show impairments in attention similar to those observed in children with ADHD and FASD. Further, we tried to examine the neurochemical impact of agmatine administration in male offspring.

2. Methodology:

2.1 Animals

The adult male and female Sprague–Dawley rats were used and paired housed with same sex in a temperature (25 °C) and light (12/12-h light/dark cycle) controlled facility. All animals had free access to food and water. All animal experiments were reviewed and approved by Institutional Animal Care (IAEC) of Smt. Kishoritai Bhoyar College of Pharmacy, Kamptee, Nagpur, India (853/1AEC/20-21/23) and were consistent with Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India (CPCSEA) guidelines. All efforts were taken to reduce the suffering of animals used for the study, while maintaining number for statistically valid analysis.

2.2 Drugs

Ethanol (Merck Chemicals; Mumbai, India) was administered through intragastric intubation. Agmatine Sulphate, L-arginine (Agmatine precursor), Aminoguanidine (diamine oxidase inhibitor), Arcaine (Agmatinase Inhibitor) (Sigma-Aldrich Co.; USA) were dissolved in saline (0.9%) and administered by intraperitoneal (i.p.) route. Behavioural experiments were performed between 9:00 AM and 1:00 PM. Doses and timings of injections for behavioural testing were employed on the basis of previous experiments (Taksande et al. 2010, 2013)

2.3. Prenatal treatment

Animals were housed under standard facility and female were paired and mated with male SD rats in cages overnight. The conformation of copulation was carried out through vaginal plug examination and presence of anestrus phase were regarded as gestation day (GD) 0. In order to mimic behavioural and cognitive deficits associated with ADHD, the ethanol was administered via intragastric intubation between GD9-20. Pregnant rats were administered with 0 or 6 g/kg ethanol (20% [wt/vol]) diluted in saline daily in two divided doses (6 h apart), except on weekends a single daily dose of 0 or 4 g/kg ethanol was administered (Hausknecht et al. 2005). The dams in control group were administered with the equal amount of sucrose solution (30% [wt/vol]) diluted in saline as isocaloric substitute for ethanol. After the delivery, the pups were weaned with mother for 3 weeks. In present experiment, the animal model differs from Aglawe et al., 2021, in which they administered ethanol in liquid modified diet (Aglawe et al. 2021). As the unit for analysis was litter, thus single male litter from pre-treated damps were considered for further analysis so as to avoid litter effect. The offspring were separated from mother on post-natal day 22 and the male offspring were assigned to different treatment (n=7 per group). Agmatine and modulators were administered intraperitoneally from PND 21 to PND 35 and different paradigm were performed using adult offspring.

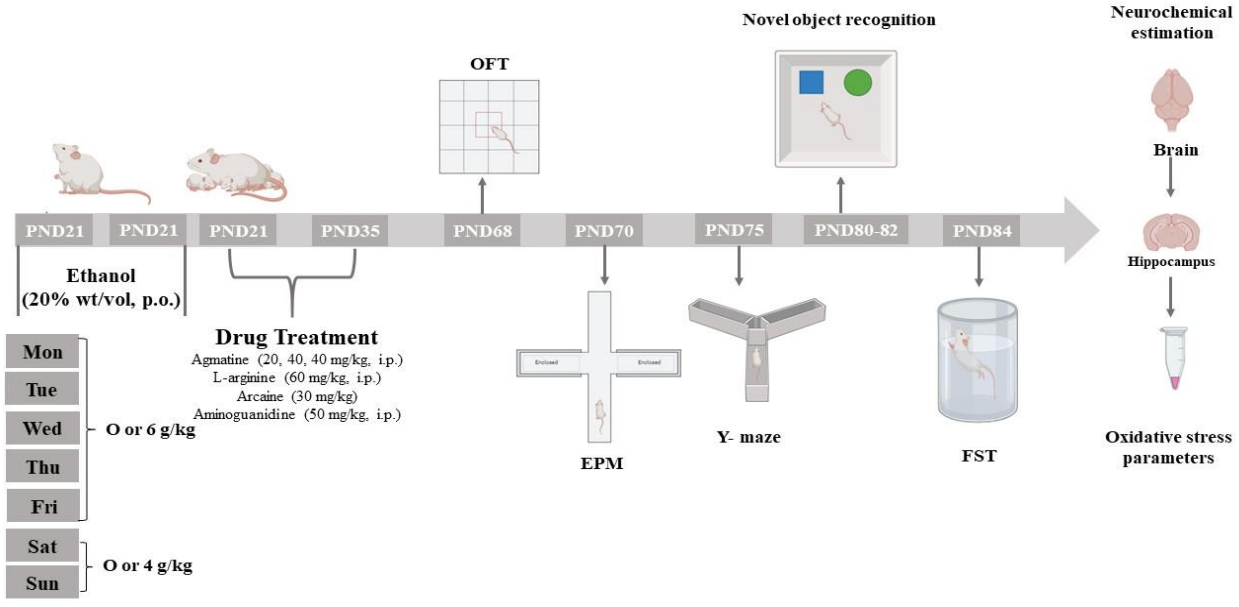

Figure 1. Schematic representation of experimental design

2.4. Blood alcohol concentration (BEC)

For all ethanol administer rats, 40 µl blood sample was collected from tail vein 2hr after the second dose of ethanol on GD20 and stored with perchloric acid (3%) at 4^0C. BEC were determined by alcohol dehydrogenase assay and expressed as mg/dl of serum. Briefly blood sample was incubated with alcohol dehydrogenase and β-nicotinamide adenine dinucleotide (β-NAD) in Tris-Cl buffer for 40 min at room temperature. The reduction of β-NAD to β-NADH were recorded at 340nm (Zapata and Shippenberg 2006).

2.5. Behavioural analysis

2.5.1. Locomotor activity:

On PND 68, the animals were subjected to the open field test, to examine the effect of agmatine administration on prenatal stress induced altered locomotion and exploration behaviour. The apparatus consisted of white box (80 x 80 x 60), the base divided in 16 squares (20 x 20). The experiment was performed by placing the animals individually on the center of box and were allowed to explore freely. The animal behaviour was recorded for 10 min trial period and the total number of ambulation, rearing and grooming were counted manually. Between the testing the box was cleaned with 75% of ethanol and dried before testing next animals.

2.5.2. Elevated plus maze:

The anxiety-like behaviour of the animals was evaluated using the elevated plus-maze (PND 70). The apparatus consisted of black Plexiglas, comprising two open arms measuring 50 cm × 10 cm and two closed arms measuring 50 cm × 10 cm × 50 cm with an open roof, arranged such that the two open arms were positioned opposite each other. Initially, each rat was placed in the center, facing one of the open arms. Subsequently, the total number of entries into both closed and open arms, along with the cumulative time spent, was automatically calculated using a video camera system over a 5-minute period (Walf and Frye 2007).

2.5.3. Y maze:

The Y-maze test was carried out to evaluate attention on PND75. The apparatus consisted of Plexiglas with three arm maze measured 10 cm in width, 50 cm in length, and 20 cm in height. Rats were place in the start arm of the Y-maze and allowed to move freely for a duration of 8 minutes. The percentage of spontaneous alternation and the total arm entries of animals were recorded and analysis using software. The calculation for spontaneous alternation was performed using the formula:

Spontaneous alternation = ((number of alternations/total arm entries) - 2) × 100.

2.5.4. Novel object recognition test:

The novel object recognition test has been used to evaluate memory and learning based on the exploration time and recognition index (Alkam et al. 2010). The test consisted of three phases performed on consecutive days. On first day (habituation trial) the rat were place in central arena of open field apparatus (50 X 50 X 40 cm) for 5 min session. On the second day, in acquisition trial animals were allow to explore a floor-fixed two identical wooden object (A and B) placed at symmetrical position. During, the session the total duration of exploration was recorded. On the third day (PND82) during retention trial, one of the objects from acquisition trial was replaced with a novel object and the exploration time (sec) was recoded for 5 min session. The two objects were differed in shape but identical in colour and size. After the test the recognition index was calculated for retention trial using the formula (TN×100) / (TF + TN), where TN and TF are the time spent near novel and familiar object, respectively. The inclusion criteria for exploration were sniffing (from distance of 3cm) and touching the object with nose and/or forepaws. The apparatus and the objects were meticulously cleaned with 75% alcohol between the trials to avoid olfaction cues.

2.5.5. Forced swim test:

Immobility behaviour was assessed in FST using cylindrical Plexiglas containers (45 cm high × 25 cm diameter) filled with tap water to 30 cm and maintained at room temperature during testing (25 ± 1.0 °C) (Yankelevitch-Yahav et al. 2015). Test consisted of 2 sessions; an initial 15-min 'pre-test' followed by 5-min 'test' after 24 h. Briefly, animal is individually forced to swim in a cylindrical glass tank and the duration of immobility was monitored (PND84). The rat was considered to be immobile when it floats without struggling and making only those movements necessary to keep its head above the water. An increase in the duration of immobility time reflects depression-like phenotype. The total duration of immobility during the test session

recorded by trained and skilled observer blind to experimental protocol was considered for determining the effects on depression-like behaviour.

2.6. Biochemical analysis

After behavioral assessment, animals were humanely euthanized using an overdose of pentobarbital sodium. The brains were surgically extracted, rinsed with isotonic saline, and 10% (w/v) hippocampal homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate were centrifuged at 10,000 ×g at 4 °C for 15 minutes, the resultant supernatant were utilized for the determination of biochemical analysis.

2.6.1. Lipid peroxidation

The thiobarbituric acid (TBA) reaction was used to estimate lipid peroxidation after acute prenatal exposure. Briefly, the brain supernatant was mixed with 2 volumes of trichloroacetic acid (TCA) and was reacted with 0.67% w/v TBA in a boiling water bath. The pink color pigment obtained after MDA formation was measured at 532 nm using a spectrophotometer (Wills 1966).

2.6.2. Reduced glutathione (GSH)

The reduced GSH concentration in the sample was determined by method described by Ellman and colleagues. Briefly, 1 ml of tissue supernatant was digested and precipitated using 4% sulfosalicylic acid. After centrifugation at 1200 rpm for 15min the supernatant was reacted with 5,5-dithiobis 2-nitrobenzoic acid (DTNB) in 0.1 M phosphate buffer (pH 8). The yellow product so formed was read at 412nm using a UV-VIS spectrophotometer. The GSH was calculated using a molar extinction coefficient (1.36 l04 M cm-1)(Ellman 1959).

2.6.3. Nitrate/nitrite

The nitrite levels in the supernatant was used as an indicator of the nitric oxide generation. An equal volume of brain supernatant and Griess reagent were mixed and incubated at 37°C for 15 minutes. The absorbance was recorded at 540 nm using a spectrophotometer and the nitrite concentration was calculated using the standard curve of sodium nitrite (Green et al. 1982).

2.7. Statistical Analysis

All values are stated as mean ± SEM. One-way analysis of variance (ANOVA) with post hoc Sidak's comparison was used to define statistical significance in OFT, EPM, Y maze, SPT and for biochemical analysis. Two-way ANOVA followed by post hoc Sidak's comparison test were used to analyses the results of NOR. Values of $p < 0.05$ were considered statistically significant for all the tests.

3. Results

3.1. Gestation data

We analysed the dams weight on GD 8 and 21 and pups weight on PND1, 7 and 21. The pregnant dams administered with ethanol with defined schedule did not show any significant alteration in the body weight of animals. There was no alterations in litter size of ethanol treated dams compared with control damps. However, the ethanol exposed offspring shows significant reduction in body weight with the time (PND7 and PND21).

Table 1. Weight of the animals during the ethanol administration protocol.

Treatment	Pregnant dams		Offspring		
	GD8	GD21	PND1	PND7	PND21
Saline Control	270.28±0.76	338.76±3.53	6.2±0.16	14.34±0.15	55.06±0.89
PF	272.91±1.46	344.16±1.46	6.08±0.13	14.74±0.18	50.22±0.92
EtOH	272.12±0.72	340.58±2.70	5.94±0.09	13.44±0.25$^{*\#}$	48.7±0.46$^{**\#\#}$

Each values represents mean ± SEM (n=7). *p < 0.05, ** P < 0.01 vs saline control, # P < 0.05, ## P < 0.01 vs PF animals.

3.2. Blood ethanol concentration

The intra-gastric intubation of pregnant rats with 0 or 6 g/kg ethanol in two divided doses (6 h apart) between GD9-20 resulted in moderate increase in BEC after first dose i. e. 177 mg/dl whereas the mean BEC levels after 2nd dose was found to be 298.11 mg/dl (±14.84 S.E.M.) for the ethanol group.

3.3. Agmatine attenuates the hyperactivity associated with prenatal ethanol exposure in adult offspring

Fig 2. Shows the effect of agmatine (20, 40, and 80 mg /kg) and its modulators L-arginine (60 mg/kg), Arcaine (30 mg/kg), and Aminoguanidine (50 mg/kg) administration on locomotor activity using an open field test in ethanol exposed offspring. Prenatal ethanol exposure significantly alters the locomotor activity of offspring evident with increase in the number of ambulation and distance travelled as compared with control animals [$t=4.815$, $df=45$; $F(7, 48) = 4.309$, $p < 0.001$ and $F(7, 48) = 4.487$, $p < 0.0$]. As shown in Fig. 2. (A and B), post hoc Sidak's multiple comparison test demonstrated that administration of agmatine (20, 40, 80 mg/kg, i.p.) significantly reduced ambulation count and distance travelled in ethanol exposed animals as compared to ethanol treated rats (tAgmatine 20 treatment= 3.818, $p < 0.05$; tAgmatine 40 treatment= 4.269, $p < 0.01$; tAgmatine 80 treatment= 3.961, $p < 0.01$ and tAgmatine 40 treatment= 3.997, $p < 0.01$; tAgmatine 80 treatment= 4.263, $p < 0.01$). Similarly, *post hoc* Sidak's multiple comparison test revealed that administration of L-arginine (60 mg/kg, i.p.) ($p< 0.01$), aminoguanidine (50 mg/kg, i.p.) ($p< 0.05$) and arcaine (30 mg/kg, i.p.) ($p< 0.05$) significantly reduced ambulation count as well as distance travelled as compared to ethanol treated rats (tL-arginine treatment= 3.344, $p < 0.05$; tAminoguanidine treatment= 3.913, $p < 0.01$; tArcain treatment= 3.605, $p < 0.05$ and tL-arginine treatment= 3.520, $p < 0.05$; tAminoguanidine treatment= 4.227, $p < 0.01$; tArcain treatment= 3.482, $p < 0.05$). There was no significant alterations in rearing behavior were observed with prenatal ethanol exposure.

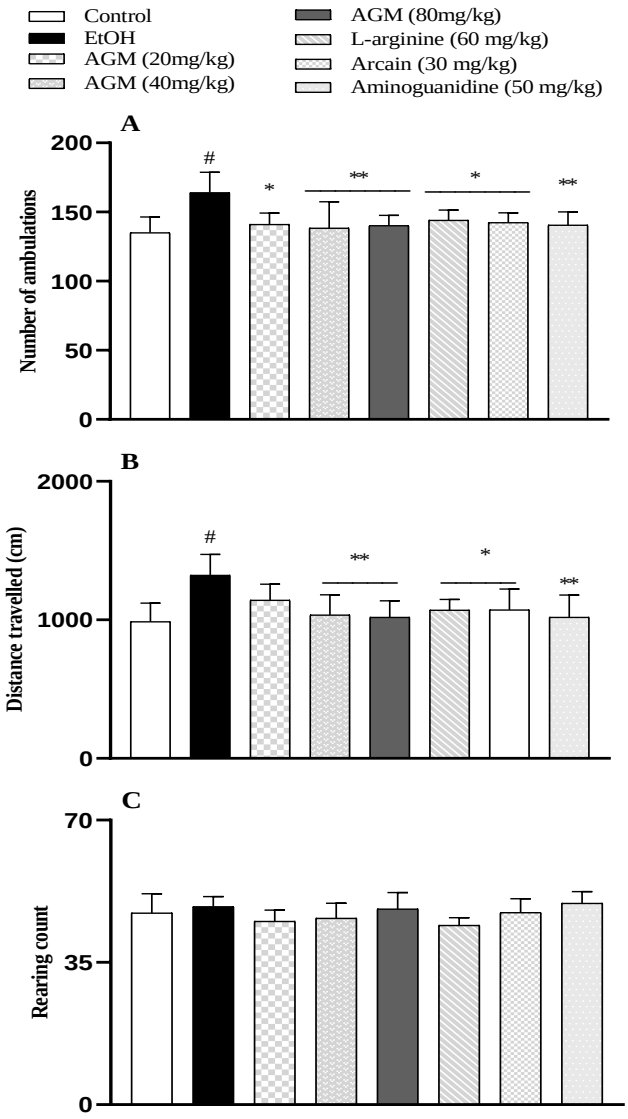

Figure 2. Effect of agmatine (20, 40 and 80 mg/kg, i.p.) and agmatinergic modulators L-arginine (60 mg/kg), aminoguanidine (50 mg/kg) and arcaine (30 mg/kg) on ambulation (A), distance travelled (B) and rearing (C) in prenatal ethanol treated offspring. Each data point represents mean OFT indices ± SEM (n = 7). # $P < 0.001$ vs. control rats, *$p < 0.05$, **$p < 0.01$ vs. EtOH offspring (One way ANOVA followed by *post hoc* Sidak multiple comparison test).

3.4. Agmatine modulates anxiety like behaviour associated with prenatal ethanol exposure in adult offspring

As shown in figure 3, One-way ANOVA followed by post hoc Sidak multiple comparison test demonstrated that prenatal ethanol exposed offspring showed a significant reduction in number of entries in open arm ($p<0.001$) and time spent in open arm ($p<0.001$) as compared with control group [$t = 4.414$, $df = 48$; $F (7, 48) = 23.35$ and $t = 5.045$, $df = 48$; $F (7, 48) = 13.10$]. Treatment of Agmatine (40 and 80 mg/kg) group showed significant improvement in number of entries ($t_{Agmatine\ 40\ treatment} = 3.653$, $p < 0.05$ and $t_{Agmatine\ 80\ treatment} = 10.20$, $p < 0.001$), and time spend in open arm ($t_{Agmatine\ 40\ treatment} = 3.734$, $p < 0.01$ and $t_{Agmatine\ 80\ treatment} = 8.212$, $p < 0.001$), as compared to prenatal ethanol exposed offspring group. Additionally, post hoc analysis also revealed treatment with Aminoguanidine (50 mg/kg) and arcaine (30 mg/kg) increases number of open arm entries ($t_{Aminoguanidine\ treatment} = 7.988$, $p < 0.001$; $t_{Arcain\ treatment} = 4.690$, $p < 0.01$) and time spent ($t_{Aminoguanidine\ treatment} = 4.306$, $p < 0.01$; $t_{Arcain\ treatment} = 3.95$, $p < 0.01$) in open arm as compared to prenatally ethanol exposed offspring. No significant changes were observed in the time spent ($P > 0.9999$) in open arm with L-arginine treatment whereas L-arginine effectively increases the % open arm entries ($t = 3.384$, $p<0.05$) in ethanol exposed offspring. No significant changes were observed in the total entries by agmatine and its modulators.

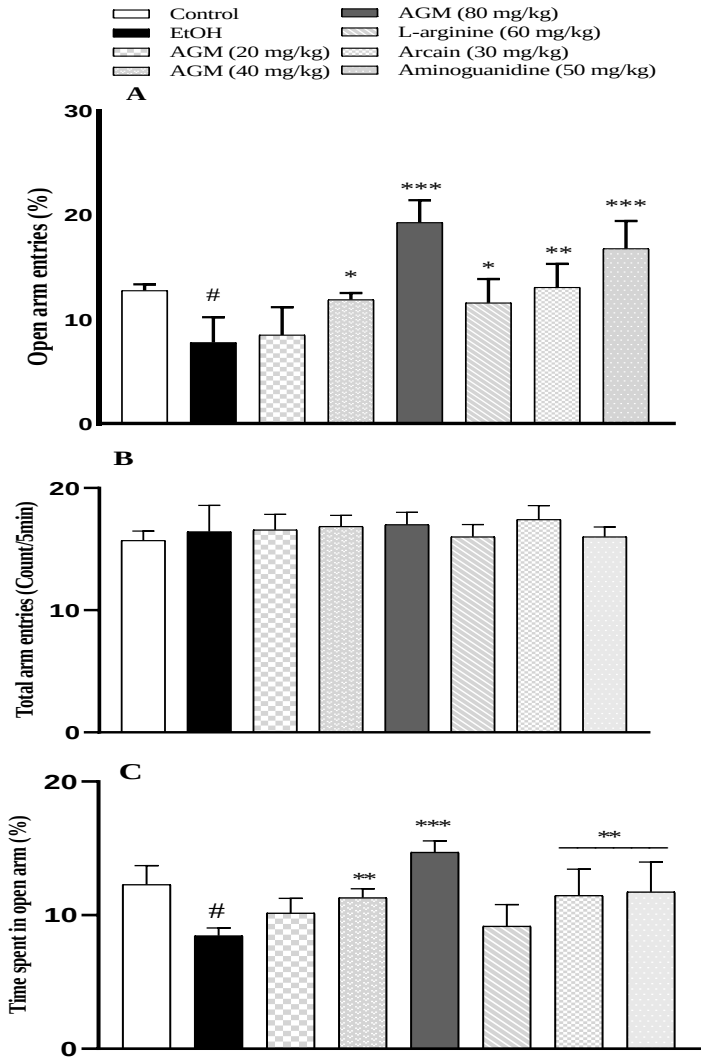

Figure 3. Effect of agmatine (20, 40 and 80 mg/kg, i.p.) and agmatinergic modulators L-arginine (60 mg/kg), aminoguanidine (50 mg/kg) and arcaine (30 mg/kg) % open arm entries (A), total arm entries (B) and time spent in open arm (C) in prenatal ethanol treated offspring. Each data point represents mean OFT indices ± SEM (n = 7). # $P < 0.001$ vs. control rats, *$p < 0.05$, **$p < 0.01$, *** $P < 0.001$ vs. EtOH offspring (One way ANOVA followed by *post hoc* Sidak multiple comparison test).

3.5. Agmatine modulates Depression-like behaviour associated with prenatal ethanol exposure in adult offspring

As shown in figure 4, One-way ANOVA followed by post hoc Sidak's multiple comparison test demonstrated that prenatal ethanol exposed offspring showed increased immobility duration as compared with control group [$F (7, 48) = 58.23$ and $t = 22.69$, $df = 48$]. Treatment of Agmatine (40 and 80 mg/kg) group showed significant reduction in immobility time as compared to prenatal ethanol exposed offspring ($t_{Agmatine\ 40\ treatment} = 4.589$, $p < 0.05$ and $t_{Agmatine\ 80\ treatment} = 6.781$, $p < 0.001$). Additionally, post hoc analysis also revealed treatment with L-arginine (60 mg/kg) and Aminoguanidine (50 mg/kg) reduced the immobility behavior in prenatally ethanol exposed offspring ($t_{L\text{-}arginine\ treatment} = 5.287$, $p < 0.05$; $t_{Aminoguanidine\ treatment} = 7.004$, $p < 0.001$). No significant changes were observed in the immobility time with low dose of agmatine (20 mg/kg) and Arcaine (30 mg/kg) in ethanol exposed offspring.

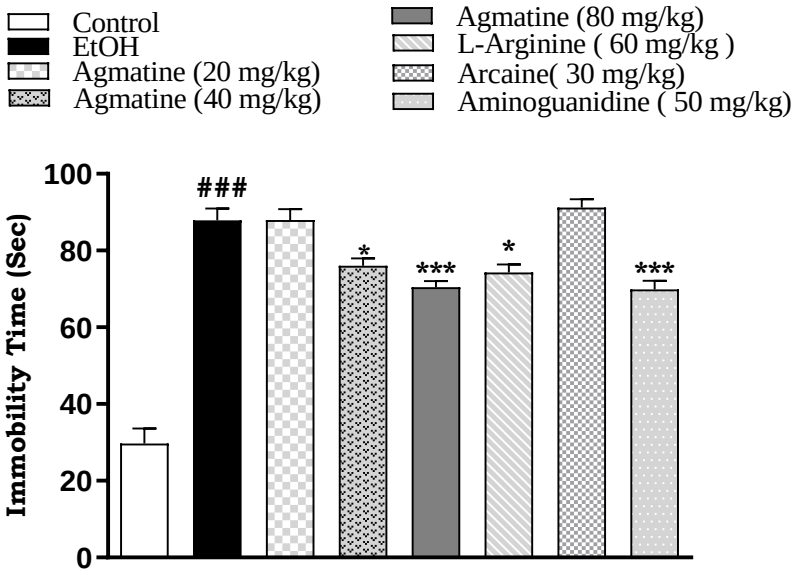

Figure 4. Effect of agmatine (20, 40 and 80 mg/kg, i.p.) and agmatinergic modulators L-arginine (60 mg/kg), aminoguanidine (50 mg/kg) and arcaine (30 mg/kg) on immobility time in prenatal ethanol treated offspring. Each data point represents mean immobility (sec) ± SEM (n = 7). # P < 0.001 vs. control rats, *p < 0.05, *** P < 0.001 vs. EtOH offspring (One way ANOVA followed by *post hoc* Sidak multiple comparison test).

3.6. Agmatine mitigates memory impairment associated with prenatal ethanol exposed adult offspring

As shown in figure 5, Two-way ANOVA followed by post hoc Sidak's multiple comparison test demonstrated significant impairment in memory in prenatal ethanol exposed offspring as compared with control group as shown in figure 5 A [$F_{Interaction}$ (7, 42) = 44.31 , $F_{Treatment}$ (7, 42) = 3.535, F_{Object} (1, 6) = 341.1, p < 0.001]. Post hoc analysis showed no significant alteration in exploration time spent by the different group with both the object from acquisition trial (Fig. 5. B). However, during retention trial control animals spent more time with the novel object as compared with the familiar object from acquisition trial indicating intact novelty, learning and memory in control group (t = 10.53, df = 42, p < 0.001). Whereas, offspring exposed to ethanol during prenatal period spent more time with familiar object as compared with novel object indicating cognitive impairment (t = 23.37, df = 42, p < 0.001). Treatment of Agmatine (40 and 80 mg/kg) group showed significant improvement in memory as the animals spent more time with novel object as compared to prenatal ethanol exposed offspring ($t_{Agmatine\ 40\ treatment}$= 3.631, p < 0.05 and $t_{Agmatine\ 80\ treatment}$= 6.650, p < 0.001). The post hoc analysis also revealed treatment with L-arginine (60 mg/kg), Arcaine (30 mg/kg) and Aminoguanidine (50 mg/kg) reduced the memory impairment in prenatally ethanol exposed offspring ($t_{L\text{-}arginine\ treatment}$= 5.549, p < 0.001; $t_{Arcain\ treatment}$= 6.065, p < 0.001 and $t_{Aminoguanidine\ treatment}$= 5.628, p < 0.001). No significant changes were observed object exploration time with low dose of agmatine (20 mg/kg) in ethanol exposed offspring (fig 5. C). Additionally, figure 5. D, showed that prenatal ethanol-exposed offspring exhibited a reduced recognition index (%) as compared to control animals [F (7, 48) = 50.30, t = 13.83, df =48). However, one-way ANOVA followed by post hoc analysis revealed that treatment with agmatine (40 and 80 mg/kg) showed significant improvement in the recognition index in ethanol exposed offspring ($t_{Agmatine\ 40\ treatment}$= 9.586, p < 0.001 and $t_{Agmatine\ 80\ treatment}$= 12.64, p < 0.001). Similarly, administration of modulators L-arginine (60 mg/kg), Arcaine (30 mg/kg) and Aminoguanidine (50 mg/kg) significantly improved the recognition index as compared with prenatal ethanol exposed offspring ($t_{L\text{-}arginine\ treatment}$= 10.85, $t_{Arcain\ treatment}$= 11.13 and $t_{Aminoguanidine\ treatment}$= 10.82, p < 0.001). This suggests that the agmatine treatment effectively reversed the impairment in object recognition associated with prenatal ethanol exposure in the adult offspring.

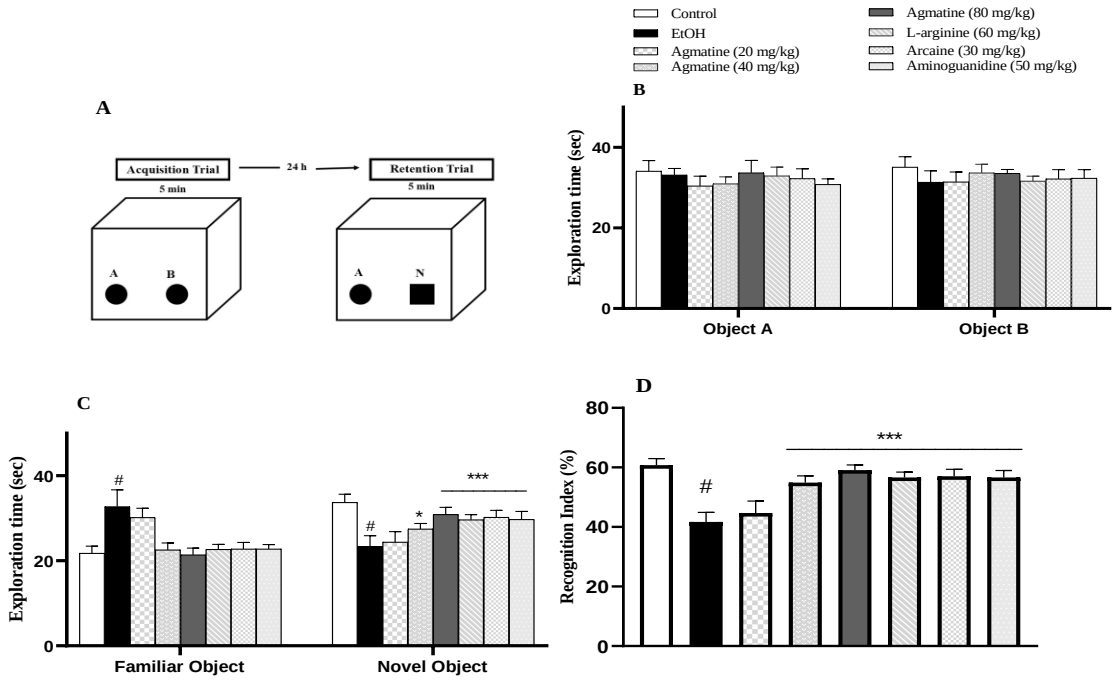

Figure 5. Effect of agmatine (20, 40 and 80 mg/kg, i.p.) and agmatinergic modulators L-arginine (60 mg/kg), aminoguanidine (50 mg/kg) and arcaine (30 mg/kg) on memory impairment in prenatal ethanol treated offspring. Each data point represents mean ± SEM (n = 7). # $P < 0.001$ vs. control rats, *$p < 0.05$, **$P < 0.01$, *** $P < 0.001$ vs. EtOH offspring (One way ANOVA followed by *post hoc* Sidak multiple comparison test).

3.7. Agmatine improves attention in prenatal ethanol exposed adult offspring

One-way ANOVA post hoc Sidak's comparison test suggest altered attention in the prenatal ethanol exposed adult offspring as demonstrated with reduction in % alteration as the offspring revisits the same arm of Y-maze suggesting changes in attentional focus or cognitive processing as compared to control animals which seeks novelty behaviour by visiting the alternate arms of maze [$F(7, 56) = 6.273$, $t = 4.235$, $p < 0.01$]. Administration of agmatine (40 and 80 mg/kg) shows significant improvement in attention indicated with increase % alteration in ethanol exposed adult offspring ($t_{\text{Agmatine 40 treatment}} = 4.239$, $p < 0.05$ and $t_{\text{Agmatine 80 treatment}} = 4.975$, $p < 0.001$). Similarly, administration of modulators L-arginine (60 mg/kg), Arcaine (30 mg/kg) and Aminoguanidine (50 mg/kg) significantly improved % alteration indicating improved attention as compared with prenatal ethanol exposed offspring ($t_{\text{L-arginine treatment}} = 3.506$, $p < 0.05$; $t_{\text{Arcaine treatment}} = 5.222$, $p < 0.001$ and $t_{\text{Aminoguanidine treatment}} = 3.887$, $p < 0.01$). Additionally, figure 6 showed the elevated total arm entries with prenatal ethanol exposed offspring as compared with control animals [$F(7, 56) = 9.287$, $t = 6.520$, $p < 0.001$]. Treatment with agmatine ($t_{\text{Agmatine 40 treatment}} = 5.565$ and $t_{\text{Agmatine 80 treatment}} = 5.108$, $p < 0.001$) and modulators ($t_{\text{L-arginine treatment}} = 5.191$, $p < 0.001$; $t_{\text{Aminoguanidine treatment}} = 5.524$, $p < 0.001$) reduced the hyperactivity associated with prenatal ethanol exposure.

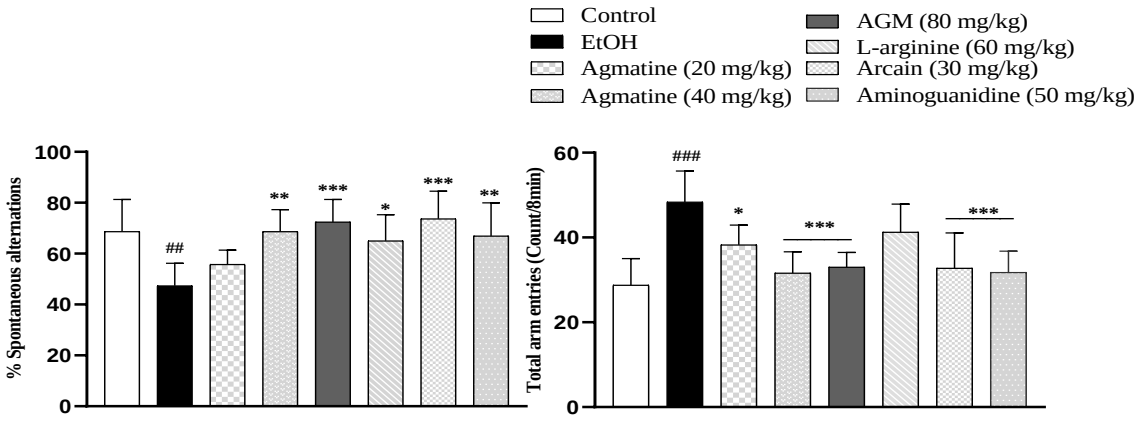

Figure 6. Effect of agmatine (20, 40 and 80 mg/kg, i.p.) and agmatinergic modulators L-arginine (60 mg/kg), aminoguanidine (50 mg/kg) and arcaine (30 mg/kg) on impaired attention in prenatal ethanol treated offspring. Each data point represents mean ± SEM (n = 7). ## P < 0.01, ### P < 0.001 vs. control rats, *p < 0.05, **P < 0.01, *** P < 0.001 vs. EtOH offspring (One way ANOVA followed by *post hoc* Sidak multiple comparison test).

3.8. Agmatine mitigates oxidative stress parameters in prenatal ethanol exposed adult offspring

One-way ANOVA followed by post hoc Sidak's multiple comparison test showed that prenatal exposure to ethanol led to a notable increase in oxidative stress, as evidenced by elevated levels of MDA ($p < 0.001$), NO ($p < 0.001$), coupled with a reduction in the antioxidant defenses, such as glutathione (GSH) ($p < 0.001$). However, treatment with agmatine (40 and 80 mg/kg) resulted in a significant improvement in antioxidant status and reduction in lipid peroxidation and NO observed in ethanol-exposed offspring ($p < 0.01$ and $p < 0.001$). These findings suggest that the agmatine administration effectively mitigated oxidative stress induced by prenatal ethanol exposure, highlighting its potential therapeutic efficacy in ameliorating oxidative damage and restoring antioxidant defense mechanisms. Administration of agmatine modulators shows similar result, l-arginine, aminoguanidine and arcaine reduced the lipid peroxidation as well as NO levels and elevates the GSH levels in ethanol treated animals. However, treatment with low dose of agmatine (20 mg/kg) did not produced any significant alterations in oxidative stress and antioxidant levels.

Table 2. Effect of Agmatine and modulators on oxidative stress parameters

Parameters	**MDA (nM/mg of brain tissue)**	**Reduced glutathiOne (GSH) (mM/mg of brain tissue)**	**Nitrate/nitrite (mM/mg of brain tissue)**
Control	0.01136 ± 0.0001769	0.08665 ± 0.001314	99.40 ± 2.225
EtOH	0.02446 ± 0.0003577$^{\#}$	0.04903 ±0.001364$^{\#}$	134.4 ± 1.820$^{\#}$
Agmatine (20 mg/kg)	0.02410 ± 0.0006803	0.04968±0.002365	120.9 ± 0.7959
Agmatine (40 mg/kg)	0.02267 ± 0.0005161**	0.07336±0.002672*	112.9 ± 1.032**
Agmatine (80 mg/kg)	0.02077 ± 0.0003461***	0.07116±0.002747***	101.4 ± 2.989***
L-arginine (60 mg/kg)	0.01936 ± 0.0001893***	0.06802±0.001132***	113.4 ± 1.327***
Aminoguanidine (50 mg/kg)	0.02036 ± 0.0001323***	0.06753±0.001026***	109.3± 2.246**
Arcaine (30 mg/kg)	0.01036 ± 0.0002093	0.05299±0.001647*	118 ± 0.3252*

Each data point represents mean ± SEM (n = 7). # P < 0.001 vs. control rats, *p < 0.05, **P < 0.01, *** P < 0.001 vs. EtOH offspring (One way ANOVA followed by *post hoc* Sidak multiple comparison test).

4. Discussion

Prenatal ethanol exposure has long been associated with a spectrum of neurodevelopmental disorders, including ADHD-like behaviours in offspring (Choi et al. 2012; Han et al. 2015; Schneider, Moore, and Adkins 2011). The mechanism underlying this association has been extensively studied, with emerging evidence highlighting oxidative stress as a significant contributor in the pathogenesis (Corona 2020; Joseph et al. 2015; Lopresti 2015). Oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) production and antioxidant defense mechanisms, can disrupt neuronal function and development, ultimately leading to behavioural abnormalities observed in offspring prenatally exposed to ethanol.

The present study corroborates previous findings by demonstrating that prenatal ethanol exposure induces ADHD-like behaviour in offspring, and further elucidates the role of oxidative stress in mediating these effects. By subjecting pregnant dams to ethanol during critical periods of fetal neurodevelopment, the offspring exhibited hyperactivity and impaired attention, core symptoms reminiscent of ADHD.

Agmatine, a neuromodulator with antioxidant properties, presents a promising way for ameliorating the adverse effects associated with prenatal ethanol exposure. In the present study chronic agmatine administration effectively mitigated hyperactivity, anxiety, and depression associated with prenatal ethanol exposure, suggesting its therapeutic potential in managing comorbid symptoms associated with ADHD. The data form previous studies also suggest agmatine can effectively manage the comorbid symptoms including anxiety and depression associated with numerous neurological disorders (Freitas et al. 2014; Kale et al. 2020; Neis et al. 2016; Rahangdale et al. 2021; Taksande et al. 2009, 2013). It also mitigates the behaviour of animals during addition and withdrawal phase (Chimthanawala et al. 2020). These results were consistent with our previous study which indicating the effectiveness of agmatine in managing the behavioural alterations associated with prenatal ethanol exposure (Aglawe et al. 2021).

Although memory impairment is not typically considered a primary symptom of ADHD, studies highlight the cognitive deficits associated with the disorder (Kofler et al. 2011; Rhodes et al. 2012). Declarative memory, particularly encoding of information, has been reported to be impaired in individuals with ADHD (Skodzik, Holling, and Pedersen 2017). This impairment aligns with findings from a MRI data, which revealed decreased hippocampal size in children with ADHD compared to controls, suggesting a potential neurobiological basis for memory deficits in this population (Hoogman et al. 2017). In our study, we observed a positive effect of agmatine on memory function. Agmatine demonstrated improvements in memory tasks, specifically in object recognition. Moreover, the ability of agmatine to alleviate impaired memory and reduce oxidative stress levels highlights its multifaceted neuroprotective properties.

The observed improvement in attention, as evidenced by enhanced performance in the Y maze task, among offspring with prenatal ethanol exposure following agmatine administration underscores the potential of agmatine as a therapeutic intervention for mitigating the neurodevelopmental consequences of prenatal alcohol exposure. This finding suggests that agmatine may exert beneficial effects on attentional processes, possibly through its neuroprotective and neuromodulatory properties, offering promise for addressing attention deficits associated with prenatal ethanol exposure and neurodevelopmental disorders such as ADHD. The observed improvements in behavioural outcomes following agmatine treatment may be attributed to its antioxidative and neuroprotective effects. Agmatine acts as a scavenger of ROS, thereby attenuating oxidative stress-induced damage to neuronal structures and preserving cognitive function (El-Sayed et al. 2019; Freitas et al. 2014; Song et al. 2014).

Notably, the findings regarding the efficacy of agmatine in ameliorating ADHD-like behaviours underscore the importance of exploring alternative treatment strategies that target underlying neurobiological mechanisms. Traditional pharmacotherapies for ADHD often focus on symptomatic relief and may be associated with adverse side effects. In contrast, agmatine presents a novel approach by addressing oxidative stress, potentially offering a safer and more holistic therapeutic option. While the current study provides compelling evidence supporting the therapeutic potential of agmatine in mitigating prenatal ethanol-induced ADHD-like behaviours, further research is warranted to elucidate its precise mechanisms of action and optimize treatment protocols.

In conclusion, prenatal ethanol exposure induces ADHD-like behaviour in offspring through oxidative stress-mediated mechanisms, underscoring the detrimental effects of maternal alcohol consumption on fetal neurodevelopment. However, treatment with agmatine emerges as a promising therapeutic strategy for attenuating ADHD-related symptoms by counteracting oxidative stress. This study contributes to our understanding of the complex interplay between prenatal insults, oxidative stress, and neurobehavioral outcomes, offering insights into novel therapeutic approaches for mitigating neurodevelopmental disorders.

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