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Interactive effects of prenatal exposure to restraint stress and alcohol on pentylenetetrazol-induced seizure behaviors in rat offspring

Paria Hashemi [a,d], Shiva Roshan-Milani [b,c,*], Ehsan Saboory [a,b], Loghman Ebrahimi [a], Maryam Soltanineghad [a]

[a] Department of Physiology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[b] Neurophysiology Research Center, Urmia University of Medical Sciences, Urmia, Iran
[c] Cellular and Molecular Research Center, Urmia University of Medical Sciences, Urmia, Iran
[d] Physiology Research Center, Department of Physiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Prenatal exposure to stress or alcohol increases vulnerability of brain regions involved in neurobehavioral development and programs susceptibility to seizure. To examine how prenatal alcohol interferes with stress-sensitive seizures, corticosterone (COS) blood levels and pentylenetetrazol (PTZ)-induced seizure behaviors were investigated in rat pups, prenatally exposed to stress, alcohol, or both. Pregnant rats were exposed to stress and saline/alcohol on 17, 18, and 19 days of pregnancy and divided into four groups of control–saline (CS), control–alcohol (CA), restraint stress–saline (RS), and restraint stress–alcohol (RA). In CS/CA groups, rats received saline/alcohol (20%, 2 g/kg, intraperitoneally [i.p.]). In RS/RA groups, rats were exposed to restraint stress by being held immobile in a Plexiglas® tube (twice/day, 1 h/session), and received saline/alcohol, simultaneously. After parturition, on postnatal days 6 and 15 (P6 & P15), blood samples were collected from the pups to determine COS level. On P15 and P25, PTZ (45 mg/kg) was injected into the rest of the pups and seizure behaviors were then recorded. COS levels increased in pups of the RS group but not in pups of the RA group. Both focal and tonic-clonic seizures were prevalent and severe in pups of the RS group, whereas only focal seizures were prominent in pups of the CA group. However, pups prenatally exposed to co-administration of alcohol and stress, unexpectedly, did not show additive epileptic effects. The failure of pups prenatally exposed to alcohol to show progressive or facilitatory epileptic responses to stressors, indicates decreased plasticity and adaptability, which may negatively affect HPA-axis performance or hippocampal structure/function.

Introduction

Environmental factors during the prenatal period are believed to play a causal role in brain development and its subsequent functions, as extensive brain growth and differentiation take place in this period (Vestergaard et al., 2005). Several lines of studies have indicated that stress during gestation can induce early and long-lasting effects on neurobehavioral development. Previously, we and others showed that prenatal stresses potentiate epileptic behaviors and increase susceptibility to seizures in rat offspring (Ahmadzadeh, Saboory, Roshan-Milani, & Pilehvarian, 2011; Edwards, Dortok, Tam, Won, & Burnham, 2002; Hashemi, Ebrahimi, Saboory, & Roshan-Milani, 2013; Moriyama et al., 2013; Tavassoli et al., 2013). In addition, several lines of evidence have indicated that ethanol abuse during gestation is also associated with a broad spectrum of abnormalities in offspring, including persistent CNS damage and a pattern of mental and physical defects (Margret et al., 2006; Riley et al., 2003), known as fetal alcohol syndrome (FAS), which is also related to a higher susceptibility to convulsions (Bonthius, Pantazis, et al., 2001; Bonthius, Woodhouse, Bonthius, Taggard, & Lothman, 2001; O'Malley & Barr, 1998; Paintner, Williams, & Burd, 2012; Stokkeland, Ebrahim, Hultcrantz, & Ekbom, 2013; Sun et al., 2009). The hippocampal formation, a region of the brain involved in the processes of learning and epilepsy, is quite susceptible to the effects of ethanol, both pre- and postnatally (Berger, 1984). Clinical studies have reported that children

* Corresponding author. Neurophysiology Research Center, Urmia University of Medical Sciences, Urmia, 5756115111, Iran. Fax: +98 044 1278 0801.
E-mail addresses: shiva_muk@yahoo.com, shivamilani@umsu.ac.ir (S. Roshan-Milani).

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suffering from FAS are seizure-prone, and epilepsy is a major sign of neurologic dysfunction in these children (Bonthius, Pantazis, et al., 2001; O'Malley & Barr, 1998; Paintner et al., 2012; Stokkeland et al., 2013; Sun et al., 2009). Such clinical reports about increased susceptibility to seizure mediated by prenatal ethanol exposure have also been supported by studies in rodents (Berman, Beare, Church, & Abel, 1992; Bonthius, Woodhouse, et al., 2001; Riljak, Maresova, Jandova, Bortelova, & Pokorny, 2012). However, some controversial issues can be found in the literature, which may be related to the kind of study (Abel, Berman, & Church, 1993). Ethanol can interact with nearly all the identified neurotransmitters; however, it has critically opposite effects on excitatory and inhibitory amino acid neurotransmissions, which result in the mediation of its behavioral effects. Prenatal exposure to ethanol induces hyperdifferentiation of glutamatergic neurons, which may underlie the ethanol-induced hyper-excitability phenotype and seizure susceptibility (Kim et al., 2010). It should be noted that the effects of prenatal ethanol exposure on seizure susceptibility are dose- and age-dependent (Kim, Dalal, Pinel, & Weinberg, 1994; Ng, Hauser, Brust, & Susser, 1988; Riljak et al., 2012).

Previous studies have indicated an interaction between alcohol- and stress-induced behavioral changes in animals. Both prenatal alcohol and prenatal stress exposures are known to alter morphology, function, and neuronal development of the hippocampus, the seizure-prone temporal lobe structure, in rat offspring (Berman & Hannigan, 2000; Mychasiuk, Gibb, & Kolb, 2012; Suenaga, Yukie, Gao, & Nakahara, 2012). Moreover, the hypothalamic–pituitary–adrenal (HPA) axis is a major component of the stress system, which is also influenced by prenatal alcohol exposure (Weinberg, Sliwowska, Lan, & Hellemans, 2008). The HPA axis can be altered by an initial stressor delivered weeks or even months earlier (Lesage et al., 2002), as well as by alcohol, even once discontinued (Allen, Lee, Koob, & Rivier, 2011; Logrip et al., 2013). Intake of alcohol during gestation is known to have marked effects on behavioral and HPA responsiveness to stressors (Sliwowska et al., 2010; Weinberg et al., 2008). Previous results suggest an important role of brain catecholamines in modulating the short- and long-term consequences of alcohol exposure on the activity of the HPA axis in adult and adolescent rats (Allen et al., 2011; Lee, Craddock, & Rivier, 2011).

In humans, a clear association between stress and drinking behavior has yet to be established, and mechanisms leading to the development of alcoholism in stressed humans are still unknown. Alcohol is most likely to be used in response to stress, since individuals believe that alcohol will help them to reduce their stress. Some evidence also links excessive drinking to the anticipation of a major stress or even to the duration of stress. Although alcohol consumption is less prevalent among pregnant women as compared to non-pregnant women, it can create a host of clinical challenges in them when encountered (DeVido, Bogunovic, & Weiss, 2015). As alcohol abuse and alcoholism are a source of substantial stress for pregnant women, and because stress and alcohol may mutually exacerbate each other's effects, it is worthy to study combined effects of prenatal stress and alcohol and their possible interaction on offspring.

This study was performed because the interactive effects of early environmental teratogens such as alcohol and stressors are not well known. In addition, no studies have examined the modulation of epileptic behavior and seizure susceptibility by prenatal stress, in prenatally alcohol-exposed animals. We hypothesized that, in accordance with the previous data, prenatal alcohol exposure would potentiate epileptic behavior, and that prenatal stress acting on a sensitized HPA axis would be additive and have greater epileptic effects on prenatally alcohol-exposed animals than on the control pups. This study, therefore, aimed to investigate the interactive effects of prenatal restraint stress and alcohol administration on body weight, corticosterone (COS) blood level, and PTZ-induced epileptic behaviors in rats at different time points.

Materials and methods

In these experiments, 10-week-old female Wistar rats (200–250 g) were obtained from the animal facility, Urmia University of Medical Sciences, Urmia, Iran. The rats were housed in groups of three per cage under a 12-h light/dark cycle (lights on from 7:00 AM to 7:00 PM), at 22 ± 2 °C, with free access to food and water. All the experimental protocols and procedures were followed according to the guidelines of the 1975 Declaration of Helsinki, as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, Iran. In addition, this study was approved by the Regional Medical Ethics Committee in West Azerbaijan Province, Iran. All the female rats were mated at 12 weeks with sexually experienced males of the same genotype. Each female was paired with one male at 8:00 AM and checked for plugs at 3:00 PM. If a plug was present, the female rat was immediately moved to a new cage, where three pregnant rats were kept for the entire gestation period. If no plug was observed, the animal was returned to her home cage for a new mating chance. The pregnant rats were divided into four groups (n = 18, in each group), including control–saline (CS), control–alcohol (CA), restraint stress–saline (RS), and restraint stress–alcohol (RA). The pregnant rats were housed three per cage, all from the same group, for the entire gestation period. The pregnant rats of the restraint stress–saline group received 2 mL saline intraperitoneally (i.p.) and were then exposed to the restraint stressor on gestation days 17, 18, and 19 (E17, E18, and E19, respectively). The pregnant rats of the restraint stress–alcohol group were treated with ethanol solution i.p., immediately prior to the stress induction and then similarly exposed to the stress. The pregnant rats of the control–saline group received saline similar to those in the restraint stress–saline group. They were then transported to the experimental room on the same gestational days and handled similarly to the stressed rats; however, they were not exposed to stress. The pregnant rats of the control–alcohol group were treated with ethanol similar to those in the restraint stress–alcohol group but were not exposed to stress. These gestational days (E17, E18, and E19) were chosen as "late-gestational period" because of their importance in developing the HPA axis and alterations induced by gestational stress (Weinstock, 2001). Prenatal stress, particularly during the 3rd week of pregnancy, plays an important role in increasing seizure vulnerability in rat offspring (Sadaghiani & Saboory, 2010).

Prenatal ethanol exposure

Dams from the alcohol groups (CA & RA) were injected with 2 g/kg ethanol solution (20%, i.p.) on E17, E18, and E19, which likely resulted in a blood alcohol concentration (BAC) of roughly 200 mg/dL (Nation, Burkey, & Grover, 1993; Rinker et al., 2011; Roma, Chen, Barr, & Riley, 2007), indicating a severe exposure condition (White et al., 2011). Immediately after alcohol injection, pregnant dams from the restraint stress–alcohol (RA) group were exposed to stress, according to the restraint–stress procedure. Pregnant dams from the control–alcohol (CA) group were transported to the experimental room on the same gestational days and handled similarly to the stressed rats; however, they were not exposed to stress.

Prenatal restraint–stress procedure

For restraint–stressed rats, stress involved being transported from the home cage to the experimental room and the placement of

the pregnant females in a restraint chamber (a transparent, plastic, cylindrical chamber, 6 cm in diameter and 16 cm in length) under normal room conditions. The animals were restrained for 120 min (twice daily, 60 min per session, from 8:00 to 9:00 AM and from 3:00 to 4:00 PM) for three consecutive days. This procedure causes alterations in regulation of the HPA axis in the offspring (Sadaghiani & Saboory, 2010; Weinstock, 2001).

Body weight measurement

After parturition, the pups in each litter were counted and weighed at 9:00 AM on the first postnatal day (P1). The pups in each group were mixed and equally divided among the dams if their birth dates were the same. Reallocating the pups among litters after birth created equal-sized litters across conditions. Each dam along with her pups was maintained in the individual cage. The weight of each pup was recorded again at 9:00 AM on P6 and P15 (n = 12, 1 male and 1 female pup of 6 different dams/group).

Sample collection

On P6 and P15, 12 pups of each experimental group ($n = 12$, 1 male and 1 female pup of 6 different dams/group) were decapitated under halothane anesthesia at 8:30 AM to collect trunk blood samples. Blood was collected in 1.5-mL EDTA-coated micro-centrifuge tubes, kept on ice, and later centrifuged for 15 min at 9000 rpm at 3 °C. The blood plasma was also transferred to clean 1.5-mL micro-centrifuge tubes and stored frozen at −80 °C until COS levels were determined. This hormone was measured using a commercial ELISA kit (Cayman, Ann Arbor, MI, USA) and the values were expressed in ng/mL. Moreover, COS levels of dams were measured using the same procedure. Moreover, 6 dams of each experimental group were decapitated on P6 and P15 using the same procedure. The pups were reallocated among 6 remaining dams of each experimental group if their birth dates were the same and used later for behavioral experiments.

Behavioral assessment

On P15, the pups ($n = 12$, 1 male and 1 female pup of each dam/group) were first weighed and then injected with PTZ (45 mg/kg, i.p.). Due to an extremely close margin between PTZ lethal and convulsive doses, PTZ was used at the convulsive dose of 45 mg/kg, which was very close to its sub-convulsive dose (Szyndler et al., 2010). Following the injection, behavior of each rat was observed and recorded for 120 min with a digital camera. The animals were monitored for epileptic behaviors including number and duration of focal and tonic-clonic seizures, percentage of focal and tonic-clonic seizures, and the fatal effect of PTZ (up to 24 h after the injection). The same protocol was carried out on P25 for the remaining pups ($n = 12$, 1 male and 1 female pup of 6 different dams/group) in all the experimental groups.

Statistical analysis

The results were expressed as mean ± SEM. Normally distributed data (weight and COS) were analyzed using parametric techniques. The data related to body weight were analyzed using repeated-measure analysis of variance (ANOVA) followed by a multiple comparison for all pair-wise evaluations. Data related to COS blood levels were analyzed for two factors of stress and alcohol using two-way ANOVA. For the comparison of COS blood levels within experimental groups at each age (P6 or P15), baseline ANOVA was performed, followed by Tukey's *post hoc* test, when required. The data related to epileptic behaviors, which were not normally distributed, were analyzed by Kruskal–Wallis one-way ANOVA followed by the Mann–Whitney *U* test when appropriate. The data related to mortality rate and percentage of focal and tonic-clonic seizures were analyzed using Fisher's exact tests. The results with $p < 0.05$ were considered significant.

Results

First, the data of pups for both sexes were separately analyzed ($n = 6$ for each sex/group, in all the experiments). As already mentioned, 1 male and 1 female pup were randomly selected from 6 different dams in each group. According to our analysis, there was no significant difference between male and female pups in terms of body weight, COS levels, and epileptic behaviors. Therefore, data of both sexes were mixed and analyzed together ($n = 12$ pups for each group, in all the experiments).

Effects of prenatal exposure to alcohol and restraint stress on body weight in rat pups

Significant differences were detected among the experimental groups regarding pups' body weight using a repeated-measures ANOVA test, at different time points. The main finding was that in the restraint–saline (RS) pups, mean body weight of the offspring significantly decreased in comparison to the control–saline (CS) pups on P1 ($p < 0.05$), P6 ($p < 0.05$), and P15 ($p < 0.001$). In the control-alcohol (CA) pups, mean body weight of the offspring on P1 and P6 did not significantly differ from that of the CS pups, but significantly decreased compared to the CS pups on P15 ($p < 0.01$). Mean body weight of the restraint–alcohol (RA) pups did not significantly differ from those of the RS and the CA pups at any of the time points tested, but significantly decreased compared to the CS pups on P1 ($p < 0.05$) and P15 ($p < 0.001$). A summary of results is provided in Fig. 1.

Effects of prenatal exposure to alcohol and restraint stress on COS blood levels in rat dams and pups

The results of a two-way ANOVA indicated a significant interaction between stress and alcohol subjects on pups' COS blood

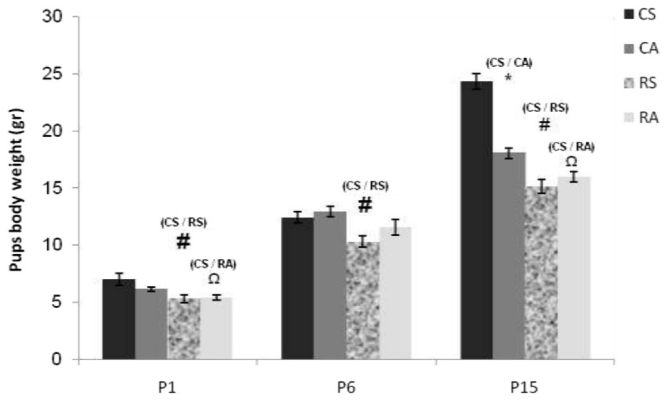

Fig. 1. Comparison of pups' body weight among experimental groups at different time points. Significant differences were detected among experimental groups on P1, P6, and P15 (repeated-measures ANOVA). For clarity and in order to show the most relevant results, not all significant changes are shown. # represents significantly different means of CS compared to RS (P1 & P6: $p < 0.05$; P15: $p < 0.001$). Ω represents significantly different means of CS compared to RA (P1: $p < 0.05$; P15: $p < 0.001$). * represents significantly different means of CS compared to CA (P15: $p < 0.01$). CS: control–saline; CA: control–alcohol; RS: restraint stress–saline; RA: restraint stress–alcohol; P1: postnatal day 1; P6: postnatal day 6; P15: postnatal day 15.

levels (Fig. 2A and B). Accordingly, at P6, the effect of alcohol was significant ($F[1,20] = 11.49$, $p = 0.003$). The effect of stress was not significant. However, interaction of alcohol × stress was significant ($F[1,20] = 17.69$, $p < 0.001$). At P15, alcohol and stress had no significant impact; however, interaction of alcohol × stress was significant ($F[1,20] = 8.97$, $p = 0.007$). For the comparison of COS blood levels within experimental groups at each age (P6 or P15), baseline ANOVA was also performed (Fig. 2C). Accordingly, COS blood levels significantly increased in pups of the RS group compared to those of the CS group on both P6 ($p = 0.004$) and P15 ($p = 0.02$). COS blood levels also increased in pups of the CA group compared to CS on both P6 and P15; however, such increases were not significant. Unexpectedly, COS levels significantly decreased in pups of the RA group compared to those of the RS group, on both P6 ($p = 0.001$) and P15 ($p = 0.03$), and reached approximately the same COS blood level in the control group at both time points.

In the dams, two-way ANOVA indicated a significant interaction between stress and alcohol subjects on COS blood levels, at P6 (Fig. 3). Accordingly, the effect of alcohol was significant ($F[1,20] = 22.94$, $p < 0.001$) and the effect of stress was not significant. However, interaction of alcohol × stress was significant ($F[1,20] = 8.27$, $p = 0.009$). At P15, the effects of alcohol, stress, and their interaction on the dams' COS blood levels did not show any significant changes.

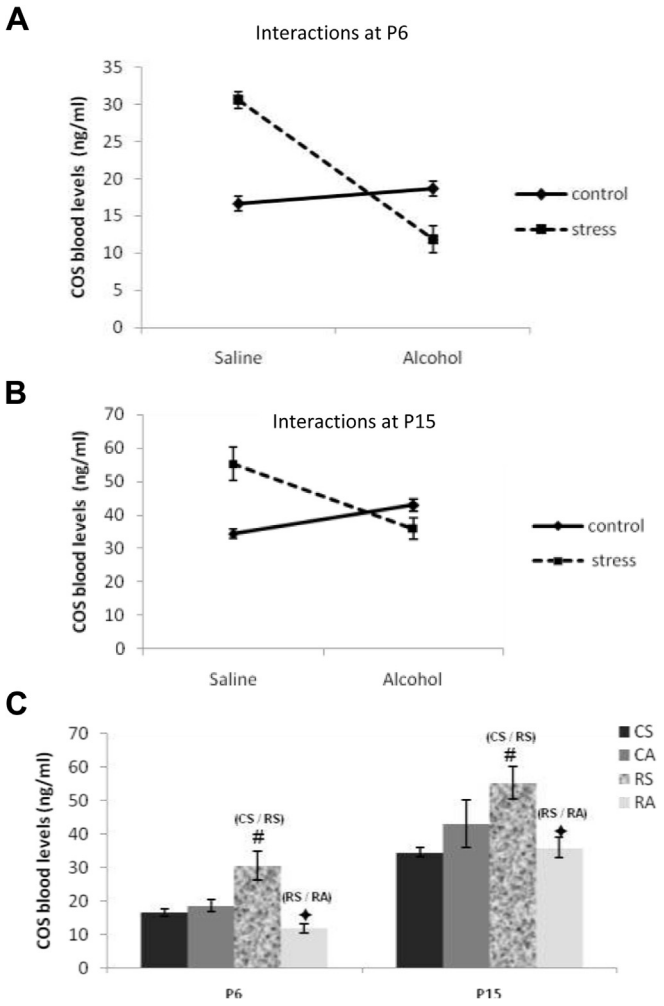

Fig. 2. Effects of prenatal exposure to restraint stress and co-administration of either saline or alcohol on COS blood levels (ng/mL) at P6 and P15 in rat pups. Panels A and B illustrate the effect of interaction of restraint stress × alcohol on COS blood levels at P6 and P15, respectively (two-way ANOVA, each bar indicates $n = 12$). At P6, effect of alcohol was significant ($p = 0.003$), effect of stress was not significant and interaction of alcohol × stress was significant ($p < 0.001$). At P15, the effects of alcohol and stress were not significant, but interaction of alcohol × stress was significant ($p = 0.007$). Panel C illustrates comparison of COS blood levels within experimental groups at each age (baseline ANOVA). For clarity and in order to show the most relevant results, not all significant changes are shown. # represents significantly different means of CS compared to RS (P6: $p = 0.004$; P15: $p = 0.02$). ✦ represents significantly different means of RS compared to RA (P6: $p = 0.001$; P15: $p = 0.03$). CS: control-saline; CA: control-alcohol; RS: restraint stress-saline; RA: restraint stress-alcohol; P6: postnatal day 6; P15: postnatal day 15.

Effects of prenatal exposure to alcohol and restraint stress on epileptic behaviors in rat pups

In order to compare prenatal alcohol- and prenatal stress-induced effects on epileptic behaviors and their interactive effects, statistical analyses were performed on non-normally distributed epileptic data using a Kruskal–Wallis ANOVA followed by multiple comparisons of all pair-wise evaluations. A summary of these effects on PTZ-induced seizure behaviors is provided in Table 1. Epileptic behaviors were markedly affected by prenatal stress, prenatal alcohol, and their interaction in this study. Statistical analysis showed significant differences among experimental groups and indicated significant interaction between main effects. In this respect, seizure durations, frequencies, and seizure percentages were significantly different, depending on type of intervention in each time point (all $p < 0.05$). Focal attacks were more frequently and intensively observed in pups of the CA group on P15, as the number and duration of these attacks significantly increased compared to the CS pups at this time point (both $p = 0.01$). However, no focal attacks were observed in the CA pups on P25. In addition, the number and duration of both focal and tonic-clonic seizures increased in pups of the RS group compared to those of the CS group on both P15 and P25 (all were significant except the focal seizures in the youngest age group). In spite of these pro-epileptic effects of prenatal exposure to alcohol and stress, pups prenatally exposed to both alcohol and stress in the RA group did not show additive and synergistic epileptic effects. As presented in Table 1, the occurrence, frequency, and intensity of the most epileptic behaviors (except for a minority in the youngest age group), unexpectedly decreased in pups of the RA group in

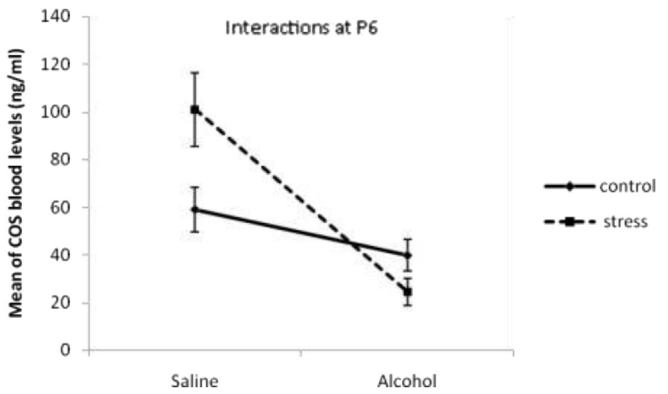

Fig. 3. Effects of restraint stress and co-administration of either saline or alcohol in gestational period on COS blood levels (ng/mL) at P6 in rat dams. The figure illustrates the effect of interaction of restraint stress × alcohol on COS blood levels at P6 in rat dams (two-way ANOVA, each bar indicates $n = 12$). Effect of alcohol was significant ($p < 0.001$), effect of stress was not significant and interaction of alcohol × stress was significant ($p = 0.009$). CS: control–saline; CA: control–alcohol; RS: restraint stress–saline; RA: restraint stress–alcohol; P6: postnatal day 6.

comparison to pups of the RS group, and reached approximately the same levels as the control pups, especially on P25.

Fisher's exact test was performed for statistical analysis of percentage of focal and tonic-clonic attacks and mortality rate. Statistical analysis showed a significant interaction between main effects. Accordingly, percentage of occurrence of the focal seizure (percentage of pups that showed at least one focal attack in each group) significantly increased from 8.3% (1 of 12 pups) in pups of the CS and RS groups to 58.3% (7 of 12 pups) in pups of the CA group ($p = 0.01$) on P15. Percentage of focal seizures also increased in pups of the RS group (66.6%, 8 of 12 pups) compared to pups of the CS group (33.3%, 4 of 12 pups); however, percentage of focal seizures decreased significantly in pups of the RA group (25%, 3 of 12 pups) on P25 ($p < 0.05$). None of the rats in the CA group showed focal seizures on P25. In addition, percentage of occurrence of the tonic-clonic seizure (percentage of pups that showed at least one tonic-clonic attack in each group) significantly increased in pups of the RS group (66.6%, 8 of 12 pups) compared to pups of the CS group (8.3%, 1 of 12 pups). However, it was significantly decreased in pups of the RA group (16.6%, 2 of 12 pups) on P25 ($p < 0.05$). None of the pups in the CA group showed tonic-clonic seizures on P15 and P25. The pups were also monitored for fatal effects of PTZ during attacks up to 24 h after the injection. On P25, the mortality rate was 8.3% (1 of 12 pups) during attacks in the RS pups, whereas no mortality was observed on P15 and P25 in other groups. Statistical analysis using Fisher's exact test showed no significant changes in mortality rate among experimental groups (Table 1).

Discussion

In the present study, body weights, COS blood levels, and PTZ-induced epileptic behaviors have been investigated in rats that were prenatally exposed to restraint stress, alcohol, or both. The main findings were that the body weight significantly decreased in pups prenatally exposed to stress (on P1, P6, and P15) and alcohol (on P15), while their COS blood levels increased, compared to the control group. However, pups prenatally exposed to the co-administration of alcohol and stress did not show additive effects. In respect to epileptic behaviors, focal seizures were more prevalent and severe in pups prenatally exposed to alcohol (on P15). Moreover, both focal and tonic-clonic seizures were more prevalent and severe in pups that were prenatally exposed to stress (on P15 and P25). Again, however, pups prenatally exposed to the co-administration of alcohol and stress did not show additive effects in the most epileptic behaviors.

In the present study, dams from the alcohol groups (CA & RA) were injected with 2 g/kg ethanol solution (20%, i.p.), which likely results in a BAC of 200 mg/dL, indicating a severe exposure condition, according to the previous reports. Previous studies have shown that 1.5–2 g/kg ethanol injection results in a BAC of 150–200 mg/dL (Nation et al., 1993; Rinker et al., 2011; Roma et al., 2007). The National Institute on Alcohol Abuse and Alcoholism (2004) has designated a BAC level of 80 mg/dL or higher as an indicator of heavy drinking (White et al., 2011). Although alcohol consumption is less prevalent among pregnant women, when encountered, it could frequently exacerbate stress during pregnancy. The combination of alcohol and stress during gestation may mutually exacerbate each other's effects and create a host of clinical challenges, especially for offspring. Therefore, the purpose of the present study was to investigate the combined effects of these prenatal manipulations on offspring susceptibility to seizure induction.

Previous studies have reported that maternal stress influences growth and organ development of fetuses and alters endocrine function of the feto-placental unit, growth hormone, and corticosterone and corticotropin hormone levels in rats (Lesage et al., 2002; Mairesse et al., 2007). It has been suggested that overexposure to catabolic effects of maternal glucocorticoids *in utero* could underlie such an alteration (Mairesse et al., 2007; Seckl, 2004). Our findings are consistent with the results obtained in these studies. As shown in Figs. 1 and 2, the pups' body weight decreased whereas blood COS levels increased in pups of the RS group compared to the CS rats on both P6 and P15. Therefore, early and long-lasting effects of prenatal stress on fetal growth and development are at least in part due to altered maternal and/or fetal glucocorticoid exposure. It is documented that prenatal exposure to alcohol is also involved in fetal and postnatal growth (Aros et al., 2011; Day et al., 1989), involving growth hormone and insulin-like growth factor axis (Aros et al., 2011; Gundogan et al., 2008; Halmesmaki, Valimaki, Karonen, & Ylikorkala, 1989). In the present study, there was a significant decrease in the body weight of offspring in CA compared to CS rats on P15, which is in agreement with the above-mentioned studies. According to our data (Fig. 2C), an increase in the COS blood level was also observed in pups prenatally exposed to alcohol on both P6

Table 1
Summary of the effect of prenatal alcohol and stress exposure on PTZ-induced seizure behaviors on P15 and P25 in rat pups.

Epileptic behaviors	Age	CS	CA	RS	RA	*p* value
Duration of immobility (min)	P15	$7.37 \pm 1.10^{\#}$	5.33 ± 0.43	3.95 ± 0.4	4.91 ± 0.43	$p = 0.008$ (CS/RS)$^{\#}$
	P25	$6.08 \pm 0.79^{*}$	12.66 ± 2.07	6.04 ± 0.85	7.75 ± 1.21	$p = 0.01$ (CS/CA)*
Duration of focal seizures (min)	P15	$0.03 \pm 0.03^{*}$	0.38 ± 0.12	0.16 ± 0.16	0.07 ± 0.15	$p = 0.01$ (CS/CA)*
	P25	$0.1 \pm 0.04^{\#}$	0	$0.56 \pm 0.13^{\blacklozenge}$	0.17 ± 0.02	$p = 0.01$ (CS/RS)$^{\#}$ & (RS/RA)$^{\blacklozenge}$
Number of focal seizures (min)	P15	$0.08 \pm 0.08^{*}$	1.08 ± 0.43	0.25 ± 0.25	0.41 ± 0.14	$p = 0.01$ (CS/CA)*
	P25	$0.33 \pm 0.14^{\#}$	0	$1.16 \pm 0.24^{\blacklozenge}$	0.41 ± 0.19	$p < 0.02$ (CS/RS)$^{\#}$ & (RS/RA)$^{\blacklozenge}$
Duration of tonic-clonic seizures (min)	P15	$0.01 \pm 0.01^{\#}$	0	2.55 ± 0.88	3.16 ± 1.68	$p = 0.01$ (CS/RS)$^{\#}$
	P25	$0.09 \pm 0.04^{\#}$	0	$1.56 \pm 0.56^{\blacklozenge}$	0.08 ± 0.04	$p < 0.05$ (CS/RS)$^{\#}$ & (RS/RA)$^{\blacklozenge}$
Number of tonic-clonic seizures (min)	P15	$0.08 \pm 0.08^{\#}$	0	2.66 ± 0.94	1.33 ± 0.85	$p = 0.01$ (CS/RS)$^{\#}$
	P25	$0.33 \pm 0.14^{*}$	0	$1 \pm 0.36^{\blacklozenge}$	0.16 ± 0.11	$p < 0.05$ (CS/CA)* & (RS/RA)$^{\blacklozenge}$
Percentage of focal seizures (%)	P15	8.3%*	58.3%	8.3%	41.6%	$p = 0.01$ (CS/CA)*
	P25	33.3%*	0	66.6%$^{\blacklozenge}$	25%	$p < 0.05$ (CS/CA)* & (RS/RA)$^{\blacklozenge}$
Percentage of tonic-clonic seizures (%)	P15	33.3%*	0	41.6%	25%	$p < 0.05$ (CS/CA)*
	P25	8.3%$^{\#}$	0	66.6%$^{\blacklozenge}$	16.6%	$p < 0.05$ (CS/RS)$^{\#}$ & (RS/RA)$^{\blacklozenge}$
Mortality rate during attacks (%)	P15	0	0	0	0	
	P25	0	0	8.3%	0	
Mortality rate during 24 h (%)	P15	0	0	0	0	
	P25	0	0	0	0	

Each value represents the mean ± SEM in 12 pups (0 value was assigned for pups in which no related seizure behavior was observed and applied in mean). CS: control-saline; CA: control-alcohol; RS: restraint stress-saline; RA: restraint stress-alcohol; P15: postnatal day 15; P25: postnatal day 25. * represents significantly different means of CS compared to CA. $^{\#}$ represents significantly different means of CS compared to RS. $^{\blacklozenge}$ represents significantly different means of RS compared to RA.

and P15; however, such increases were not significant. These data together indicate that increased levels of COS may be involved in prenatal alcohol- and stress-induced effects on body weight. Long-term effects of stress and alcohol may be related to disturbance in the function of the HPA axis and alteration of its feedback regulation, which influences fetal growth, development, and endocrine function (Mairesse et al., 2007; Seckl, 2004). However, the mechanisms by which prenatal exposure to stress and alcohol affect pup growth remain largely unknown. Unexpectedly, the effect of prenatal alcohol in combination with stress on the body weight and COS levels were not additive based on our data, as the combined effects of these prenatal manipulations did not produce greater effects than either treatment by itself. One explanation for this finding is that prenatal exposure to alcohol exerts long-lasting effects on brain stress circuits and produces changes in the responsiveness of the HPA axis to stress, which is in agreement with the results of other studies (Hellemans, Sliwowska, Verma, & Weinberg, 2010; Logrip et al., 2013).

With respect to epileptic behaviors, in the present study, the COS blood levels and the number, duration, and occurrence of the focal and tonic-clonic seizures significantly increased in pups of the RS group compared to the control pups, which in general indicates potentiation of seizure behaviors in the stressed pups. These results are consistent with those of previous studies that showed the effect of stress on seizure susceptibility to be at least in part due to involvement, activities, and engagement of the HPA system (Edwards et al., 2002; Maguire & Salpekar, 2013; Reddy & Rogawski, 2002; Rosen et al., 1994; Sadaghiani & Saboory, 2010; Yang, Zou, Wang, & Ding, 2011). The significantly elevated COS levels in pups of the RS group on P6 and P15 clearly indicate that they were exposed to stress during gestation. Prenatal stress also inhibits feedback regulation of the HPA axis, resulting in higher basal secretion of corticotropin-releasing factor and associated adrenal corticosteroid production in the offspring, even days later (Sadaghiani & Saboory, 2010; Viltart et al., 2006). COS levels also increased in dams of the RS group compared to CS on P6, but not on P15. A feedback regulation of the HPA axis may underlie some small changes in COS levels in the dams on P15. Prenatal alcohol administration also potentiated PTZ-induced focal, but not tonic-clonic seizures, in pups in the CA group compared to those in the CS group on P15. Moreover, neither focal nor tonic-clonic attacks were observed on P25 in the CA group. This observation suggests a pro-epileptic effect of prenatal alcohol on 15-day-old rats and an anti-epileptic effect on 25-day-old rats. This finding is consistent with the result of the study by Riljak et al. (2012), who, using electrophysiological recording techniques, found that ethanol intake during pregnancy differentially influenced the seizure susceptibility of 18- and 25-day-old rats, suggesting age-dependent effects of prenatal ethanol on the seizure susceptibility of rat offspring. Developmental differences may therefore account for such differences. Controversial findings have been obtained regarding the impact of prenatal alcohol on seizure, depending on duration, pattern, and intensity of alcohol consumption or exposure (Berman et al., 1992; Kim et al., 1994; Riljak et al., 2012; Stokkeland et al., 2013; Sun et al., 2009; Vestergaard et al., 2005). Differently designed animal experiments have also indicated that maternal alcohol shows different effects on seizure threshold and severity in rat offspring, in a dose- and age-dependent manner (Kim et al., 1994; Riljak et al., 2012). Prenatal exposure to alcohol influences seizure susceptibility by acting on excitatory/inhibitory brain systems (Riljak et al., 2012), involving glutamate receptor systems (Chandler, Newsom, Sumners, & Crews, 1993) and inhibiting neurogenesis (Nixon & Crews, 2002) and oxidative stress (Crews & Nixon, 2009).

In spite of the pro-epileptic effects of stress in both younger and older pups and the pro-epileptic effects of alcohol in younger pups, the combined effects of gestational alcohol exposure with maternal stress unexpectedly did not result in greater effects than either manipulation by itself, on both the offspring COS levels and their susceptibility to seizure, which indicated that alcohol might suppress excitatory effects of stress on seizure susceptibility through an HPA-dependent mechanism/s. As mentioned previously, the number, duration, and incidence of the tonic-clonic seizures significantly increased in the RS group compared to the CS group; however, exposure to both restraint stress and alcohol in the RA group caused seizure parameters to decline to approximately the same levels as the control levels (except for a minority in the youngest age group). It is possible that prenatal alcohol exposure may modify the capacity of endogenous stress systems to effectively respond to exogenous stressors. It seems that the HPA axis, endogenous norepinephrine, and neuroendocrine systems of the developing fetus are most sensitive to maternal alcohol abuse (Hellemans et al., 2010), which alters vulnerability to stress and neurobehavioral disorders, including seizure (Hellemans et al., 2010; Witt, 2010). Thus, prenatal alcohol exposure could have altered the development of adaptive responses to environmental stimuli by altering the stress-sensitive brain circuitry. Alcohol may influence, and possibly facilitate, the ability of the body to maintain or restore homeostasis and to organize appropriate behavioral reactions in response to stressors, by modifying the activity of the HPA axis (Allen et al., 2011; Zou et al., 2014). Supporting this, Sliwowska et al. (2010) showed that rats prenatally exposed to ethanol failed to maintain an appropriate (i.e., suppressive) hippocampal neurogenic response to stressors and showed an inability to maintain homeostasis. Therefore, the failure of pups prenatally exposed to alcohol to show a progressive or facilitatory epileptic response to stressors could also indicate decreased plasticity and adaptability, which may negatively affect the HPA axis performance or the hippocampal structure/function.

In conclusion, this study documented that prenatal exposure of rats to stress and alcohol, alone or in combination with each other, age-dependently changed susceptibility to PTZ-induced seizure during critical periods in postnatal life. These data also suggested that the co-administration of alcohol and stress during gestation suppressed stress-induced effects on body weight, COS blood levels, and seizure behavior, and might alter the development of adaptive responses to environmental stimuli by altering the stress-sensitive brain circuitry.

Ethical approval

We confirm that we have read the journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflict of interest statement

The authors have no conflicts of interest to declare regarding the study described in this article and the preparation of the article.

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