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Effect of Prenatal Forced-Swim Stress and Morphine Co-Administration on Pentylentetrazol-Induced Epileptic Behaviors in Infant and Prepubertal Rats

ABSTRACT: *Prenatal exposure to stress and morphine has complicated effects on epileptic seizure. Many reports have shown an interaction between morphine- and stress-induced behavioral changes in adult rats. In the present study, effect of prenatal forced-swim stress and morphine co-administration on pentylentetrazole (PTZ)-induced epileptic behaviors was investigated in rat offspring to address effect of the interaction between morphine and stress. Pregnant rats were divided to four groups of control-saline, control-morphine, stressed-saline and stressed-morphine. In the stressed group, the rats were placed in 25˚C water on 17–19 days of pregnancy. In the morphine/saline group, the rats received morphine/saline on the same days. In the morphine/saline-stressed group, they were exposed to stress and received morphine/saline simultaneously. On postnatal day 15 (P15), blood samples were collected to determine corticosterone (COS) level. On P15 and P25, PTZ was injected to the rest of pups to induce seizure. Then, epileptic behaviors of each rat were individually observed. Latency of tonic–colonic seizures decreased in control-morphine and stressed-saline groups while increasing in stressed-morphine rats compared to control-saline group on P15. Duration of tonic–colonic seizures significantly increased in control-morphine and stressed-saline rats compared to stressed-morphine and control-saline rats on P15, but not P25. COS levels increased in stressed-saline group but decreased in control-morphine group compared to control-saline rats. Body weight was significantly higher in morphine groups than saline treated rats. Prenatal exposure to forced-swim stress potentiated PTZ-induced seizure in the offspring rats. Co-administration of morphine attenuated effect of stress on body weight, COS levels, and epileptic behaviors.* © *2014 Wiley Periodicals, Inc.* Dev Psychobiol 56: 1179–1186, 2014.

Keywords: *forced-swim stress; PTZ; tonic–colonic seizure; body weight; corticosterone*

INTRODUCTION

Many reports have shown that stress during gestation can induce early and long-lasting effects on neurobehavioral development in rats. Previous works have demonstrated that prenatal restraint and predator stresses potentiate epileptic behaviors and increase susceptibility to seizures in offspring rats (Ahmadzadeh,

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Saboory, Roshan-Milani, & Pilehvarian, 2011a; Saboory, Ahmadzadeh, & Roshan-Milani, 2011; Sadaghiani & Saboory, 2010). Morphine abuse during gestational period also produces long-term alterations in CNS, alters density of hypothalamic opioid receptors and affects seizure susceptibility of the exposed animals (Vathy, 2001). Complex effect of morphine on seizure has been reported in terms of type and experimental condition. According to previous reports, effect of morphine on seizure activity appears to be biphasic, potentiates seizures at dose of 2 mg/kg or above and inhibits seizures at lower doses in several seizure models (Ahmad & Pleuvry, 1995; Massotti & Gale, 1989; Shafaroodi et al., 2004). With respect to prenatal morphine exposure, it has been reported that maternal morphine shows different effects on seizure threshold and severity of rat progeny in a sex- and age-dependent manner (Schindler, Veliskova, Slamberova, & Vathy, 2000; Schindler, Slamberova, & Vathy, 2001; Vathy, 2001; Vathy, Veliskova, & Moshe, 1998). On the other hand, other researchers' investigations have represented an interaction between morphine- and stress-induced behavioral changes in adult rats. Restraint stress as well as acute or chronic morphine injections increase immobility in the swim test and these effects can be blocked by the nonspecific opiate antagonist, naloxone (Schindler et al., 2001; Vathy, 2001). Thus, it has been suggested that stress (Amir, Brown, & Amit, 1980) and morphine can induce immobility (Schindler et al., 2001; Vathy, 2001) in a forced-swim test, probably through the endogenous opioid system. Whether exposure to morphine during fetal development affects stress responses or not is not well known.

The observation that the highest concentrations of opioid receptors are present in several brain areas including the limbic system, thalamus, striatum, hypothalamus, midbrain, and spinal cord suggests that physiological mechanisms other than analgesia and pain perception could be affected by opiate exposure. As far as stress is concerned, previous studies have suggested that the neural systems (NE and opioids) mediating stress responses are modified by prenatal exposure to opiates (Vathy, 2002). It is known that males and females respond differently to a variety of stressors and activity of the hypothalamo-pituitary-adrenal (HPA) axis is highly sexually dimorphic. It has been also reported that prenatal morphine exposure induces adrenal atrophy and hypoactivity in newborns, indicating long-lasting effects on the HPA axis (Deloof, Montel, & Chatelain, 1994; Lesage et al., 1998). Moreover, according to previous studies, many stressed humans have used or abused opiates/alcohol to cope with stressful situations (Brady & Sonne, 1999). Population of opiate-addicted individuals is much higher in stressful than standard communities (Jacobson, Riesch, Temkin, Kedrowski, & Kluba, 2011). Therefore, it was hypothesized in this study that prenatal exposure to stress affects epileptic behaviors in the adult offspring due to opioid mediating stress responses, as shown in the studies above. This study aimed to investigate effect of prenatal forced-swim stress, morphine administration and their interaction on body weight, corticosterone (COS) blood level and PTZ-induced epileptic behaviors in rats at different time points.

MATERIALS AND METHODS

This research was an experimental trial. Male and female Wistar rats (200–250 g) were obtained from the animal facility at Urmia University of Medical Sciences, Urmia, Iran. They were 8 weeks old on delivery. The rats were housed in groups of four per cage and kept in standard conditions as follows: standard 12 hr light/dark cycle, $22 \pm 2^{\circ}C$ and food and water ad libitum. All the experimental protocols and procedures were complied according to guidelines of 1975 declaration of Helsinki as reflected in the guidelines of Medical Ethics Committee, Ministry of Health, I.R. Iran. Also, Regional Medical Ethics Committee in West Azarbyjan province, I.R. Iran, approved this study. When they were 12 weeks old, all the female rats were mated with a sexually experienced male of the same genotype. Each female was paired with one male at 8 am and checked for plugs at 3 pm. The pregnant rats were immediately moved to new cages in which they were four rats per cage for the entire gestation period. They were divided to four groups ($n = 21$, in each group): control-saline, control-morphine, stressed-saline, and stressed-morphine. The stressed groups were exposed to forced-swim stressor on gestation days 17, 18, and 19 (GD17, GD18, and GD19, respectively). The morphine group was treated with 10, 12, and 15 mg/kg morphine sulfate (Temad, Tehran, Iran) intraperitoneally (IP) on GD17, GD18 and GD19, respectively. The doses for prenatal morphine exposure were chosen according to the previously reported studies (Laborie et al., 2005; Lesage et al., 1998; Rimanoczy, Slamberova, Riley, & Vathy, 2003). The saline groups received 1 ml saline IP on the same days. The control rats were transported to the experimental room on GD17, GD18, and GD19 and handled similar to the stressed rats but were not exposed to stress. This gestational age as "late-gestational period" was chosen because of its importance in developing opioid system (McDowell & Kitchen, 1987), HPA axis and nervous system (Weinstock, 2001). Some neuronal systems present at birth in humans continue to develop in rats in the perinatal period or for several days/weeks after parturition (Weinstock, 2001). Prenatal stress, particularly during third weeks of pregnancy, plays an important role in increasing seizure vulnerability in rat offspring (Sadaghiani & Saboory, 2010).

Forced-Swim Stress

The pregnant rats were forced to swim individually for 30 min in a plastic cylinder (50 cm in height and 30 cm in diameter) filled up to 30 cm with 25 ± .5°C clear and fresh water (Jezek et al., 2010). Temperature of the water was controlled by an automatic temperature controller (Campden Instruments, Ltd., Loughborough, UK). Period of each stress session was 30 min once per day between 9 and 11 am. Afterwards, the rats were dried by paper towels and returned to their home cages (Badowska-Szalewska, Spodnik, Klejbor, & Morys, 2010). All the animals survived the experience and no additional follow-up care was required. Depth of the water was chosen 30 cm to prevent the rats from standing up on their feet and tails.

Sample Collection

After parturition, the pups in each litter were counted and weighed at 9 am on the first postnatal day (P1). The pups in each group were mixed and equally divided in the dams in case their birth date was the same. Each dam along with her pups was maintained in the individual cage (Sadaghiani & Saboory, 2010). On P15, 12 pups ($n = 6$, for each sex) in all the experimental groups were decapitated under halothane anesthesia at 0830 hr to collect trunk blood samples. Blood was collected in 1.5-ml EDTA-coated micro-centrifuge tubes, was kept on ice and was later centrifuged for 15 min at 9,000 rpm at 3°C. Its plasma was 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, Ellsworth, MI).

Behavioral Assessment

On P15, the pups ($n = 6$, for each sex) were first weighed and then injected with PTZ (45 mg/kg, IP) in all the experimental groups. Because of an extremely close margin between PTZ lethal and convulsive doses, PTZ was used at convulsive dose of 40–50 mg/kg, which was very close to its subconvulsive dose (Szyndler et al., 2010). Following the injection, behavior of each rat was observed and documented at least every 15 min for 120 min and seizure rating was done using a previously defined scale (Becker, Grecksch, Thiemann, & Hollt, 2000; Gholami & Saboory, 2012), in which 0 = no response, 1 = ear and facial twitching, 2 = myoclonic jerks without rearing, 3 = myoclonic jerks with rearing, 4 = turning over onto one side with tonic–clonic seizures, and 5 = turning onto back with generalized tonic–clonic convulsions. Other monitored parameters were first behavioral change and duration of tonic–clonic convulsion. The animals were monitored for fatal effect of PTZ 24 hr after the injection. The same protocol was carried out on P25 for the remaining rats ($n = 6$, for each sex) in all the experimental groups. In rats, the occurrence of seizure increases during the second and third postnatal week(s) in response to convulsant agents (Hablitz & Heinemann, 1987; Moshe & Albala, 1983; Veliskova & Velisek, 1992) and decreases between postnatal days 30–35, just prior to puberty (Moshe et al., 1995).

Statistical Analysis

Normally distributed data related to body weight and COS blood levels were analyzed using parametric techniques. Data related to body weight were analyzed using a four-factor general linear model (morphine × stress × sex × age). Values of body weight at different ages (P6, P15, and P25) were treated as repeated measures for each animal. For the comparison of body weight between experimental groups at each age (P6, P15, or P25), one-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test, when required. To analyze the data related to COS, two-way ANOVA was performed for two factors of stress and morphine (these data were available just on P15). The data related to epileptic behaviors that were not normally distributed were analyzed using Mann–Whitney *U*-test and/or Kruskal–Wallis one-way ANOVA. Also, post-hoc analyses were done using Dunn's test. Results were expressed as mean ± S. E. of the mean. Differences were considered statistically significant at $p < .05$.

RESULTS

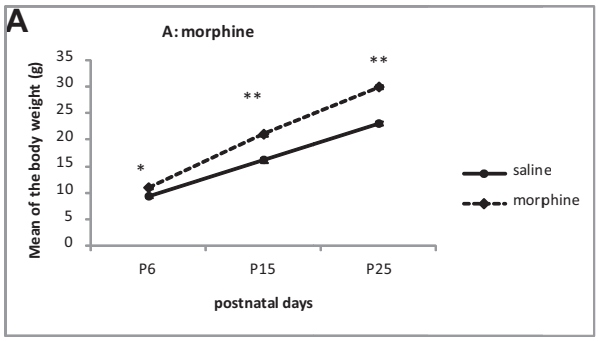
First, data of both sexes were separately analyzed. There was no significant difference between male and female pups in terms of body weight, epileptic behaviors, and COS. Therefore, data of both sexes were mixed and analyzed together.

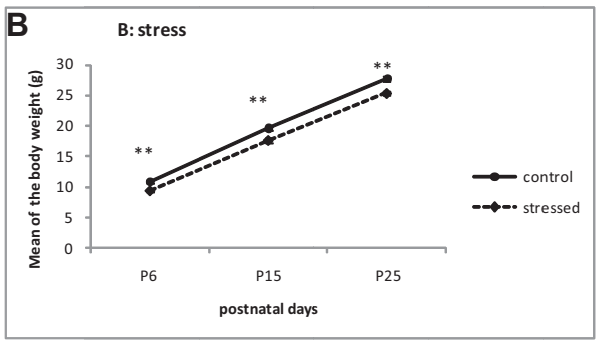
Effects of Prenatal Morphine and Stress Exposure on Body Weight

Results of general linear model (GLM) repeated measures analysis indicated that effect of morphine was significant ($F(1,40) = 250.1$; $p < .001$), effect of stress was significant ($F(1,40) = 47.03$; $p < .001$) and effect of sex was non-significant ($F(1,40) = .019$; $p = .891$). Interaction of morphine × sex was non-significant; but, interaction of stress × sex was significant ($F(1,40) = 6.02$, $p = .019$). Also, there was a significant interaction between stress and morphine (stress × morphine, $F(1,40) = 13.29$, $p = .001$). Therefore, subgroup analysis was performed. In this regard, mean of body weight was separately compared on P6, P15, and P25. Body weight significantly decreased in stressed pups compared to their respective control group on P6, P15, and P25 ($p < .001$). It was significantly higher in morphine-treated rats than saline-treated ones ($p < .001$) and in stressed-morphine rats than stressed-saline group ($p < .001$) on P6, P15, and P25 (Fig. 1).

Effects of Prenatal Morphine and Stress Exposure on COS Blood Levels

Data related to COS were analyzed by two-way ANOVA for two factors of stress and morphine. Effect





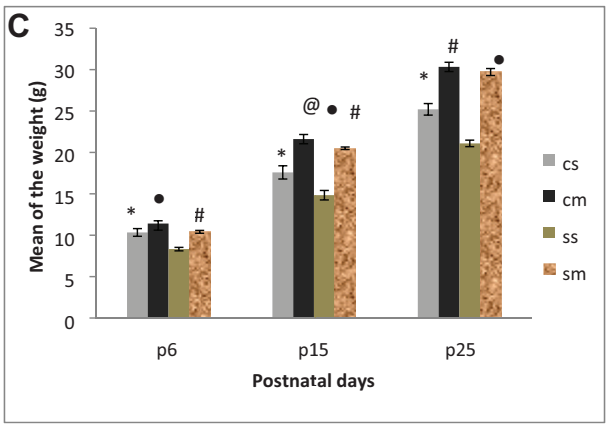


FIGURE 1 Effect of prenatal exposure to forced-swim stress and co-administration of either saline or morphine on body weight. Panel A and B illustrate the effect of morphine and stress on body weight, respectively (GLM repeated measure, each point indicates $n = 12$, * indicates $p < 0.05$ and ** indicates $p < 0.01$). Panel C demonstrates the effect of any combination of the intervention on body weight; each bar represent $n = 6$. * Indicates $p < .001$ ss versus cs; # indicates $p < .001$ ss versus sm; • indicates $p < .001$, cm versus ss at p6. * Indicates $p < .001$, cs versus cm; @ indicates $p < .001$, cm versus ss; • indicates $p = .005$, sm versus cs, and # indicates $p < .001$, sm versus ss at p15. * Indicates $p < .001$, cm, ss and sm versus cs; # indicates $p < .001$, cm versus ss and • indicates $p < .001$, sm versus ss at p25; control-saline (cs), control-morphine (cm), stress–saline (ss), stress–morphine (sm).

of stress was significant ($F(1,44) = 13.9$, $p = .001$, size effect = .24) and effect of morphine was also significant ($F(1,44) = 37.2$, $p < .001$, size effect = .46). But, interaction of morphine × stress was non-significant ($F(1,44) = .43$, $p = .51$, size effect = .01). COS blood level increased in stress–saline group and decreased in control-morphine and stress–morphine groups compared to control-saline rats (Fig. 2).

Effects of Prenatal Morphine and Stress Exposure on Duration and Latency of Tonic–Clonic Seizure

Duration of tonic–clonic seizure significantly increased in control-morphine and stressed-saline groups compared to control-saline group on P15 ($p = .026$, Kruskal–Wallis). There was an insignificant increase of seizure duration in stressed pups on P25. None of the rats in stressed-morphine group showed tonic–clonic seizure on P15 (Fig. 3).

Latency of tonic–clonic seizure in control-morphine and stressed-saline groups decreased on both P15 and P25. These changes were significant on P15 ($p = .017$, Kruskal–Wallis); but, it was not significant on P25 while co-administration of morphine with stress (stressed-morphine group) increased tonic–clonic latency (Fig. 4).

Effects of Prenatal Morphine and Stress Exposure on Mortality Rate

The pups were also monitored for fatal effect of PTZ 24 hr after the injection. There was no significant difference between groups on P15 and P25 in terms of mortality rate 24 hr after PTZ injection.

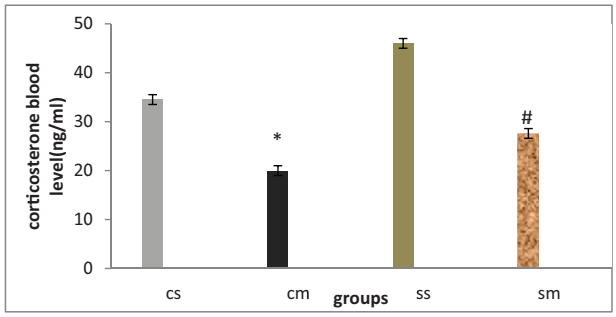


FIGURE 2 Effect of prenatal exposure to forced-swim stress and co-administration of either saline or morphine on corticosterone blood level (ng/ml) at p15 in rats * indicates $p < .001$, control-morphine (cm) versus other groups; # indicates $p = .002$, stress–morphine (sm) versus stress–saline (ss).

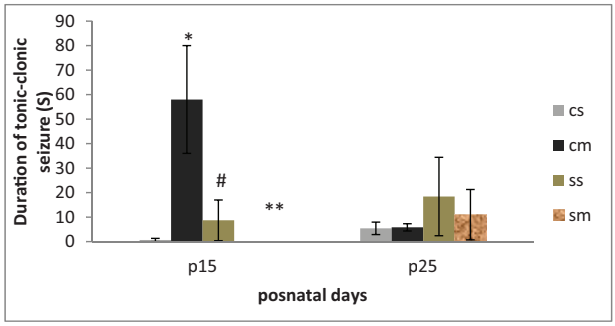


FIGURE 3 Effect of prenatal exposure to forced-swim stress and co-administration of either saline or morphine on duration of PTZ-induced tonic–clonic seizure (s) in infant and prepubertal rats. * Indicates $p = .007$, cm versus cs; # indicates $p < .02$, ss versus cm, and ** indicates $p < .001$, sm versus of all groups at p15; control-saline (cs), control-morphine (cm), stress–saline (ss), stress–morphine (sm).

DISCUSSION

In the present study, COS blood levels, body weights, and PTZ-induced epileptic behaviors were investigated in rats, prenatally exposed to forced-swim stress, morphine or both. The main findings were that prenatal stress (PS) increased COS blood levels while exposure to morphine decreased it. Duration of tonic–colonic seizures significantly increased while latency decreased in control-morphine and stressed-saline rats compared to stressed-morphine and control-saline groups. Weight of the rats increased in the morphine group while decreasing in the stressed group. Body weight variation in the stressed-morphine group was in the middle. This findings suggested that morphine attenuated stress-induced effects on epileptic behaviors and COS levels,

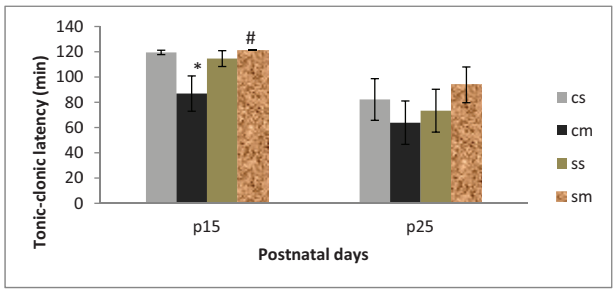


FIGURE 4 Effect of prenatal exposure to forced-swim stress and co-administration of either saline or morphine on latency of tonic–clonic (min) in rats * indicates $p = .02$, cm versus cs, and # indicates $p = .01$, sm versus cm, at p15; control-saline (cs), control-morphine (cm), stress–saline (ss), stress–morphine (sm).

which might explain why people use morphine in stressful societies to cope with stressful situations.

Previous studies have shown impact of sex on the dynamic interplay among stress, morphine exposure and convulsive responsivity (Schindler et al., 2000; Vathy, 2001). In the present study, one male and one female pup from each litter were assigned to each experimental group and the data of both sexes were separately analyzed to investigate sex-dependent differences. In spite of some differences between male and female rats in experimental groups, statistical analysis revealed no significant changes in most parameters, probably due to small sample size ($n = 12$ for each group, 6 male/6 female). Sex-dependent influence of prenatal stress on epileptic behavior could differ depending on the applied models of epilepsy/stress and experimental/animal conditions.

Effects of Prenatal Morphine and Stress Exposure on Pups' Body Weight

Several lines of studies have reported that prenatal exposure to morphine and stress leads to low birth weight (LBW). Maternal stress affects growth and organ development of fetuses, targeting some organs more than others, strikingly attenuates Glucose transporter 1 expression and diminishes fetal plasma glucose, growth hormone, and adrenocorticotropic hormone levels. It is documented that opioid receptors are also involved in physiological control of food and water intake. However, effects of morphine on food intake and body weight have been controversial (Gosnell & Krahn, 1993). With respect to maternal morphine, effect of prenatal morphine exposure on body weight is also controversial and both decreased (Kaltenbach, Berghella, & Finnegan, 1998; Naeye, Blanc, Leblanc, & Khatamee, 1973) or increased (Chiang, Hung, Lee, Yan, & Ho, 2010) changes have been reported. Chiang et al. (2010) reported that, although birth weight of the rats prenatally exposed to morphine did not alter, there was a significant increase in body weight of the offspring on day 7 (Chiang et al., 2010). The mechanisms by which prenatal exposure to stress and morphine affects pup growth remain largely unknown. However, it has been postulated that overexposure to catabolic effects of maternal glucocorticoids in utero could underlie such an alteration (Seckl, 2004). Long-lasting effects of stress and morphine have been related to disturbance in the function of HPA axis and alteration of its feedback regulation (Avishai-Eliner, Brunson, Sandman, & Baram, 2002; Slamberova, Rimanoczy, Riley, & Vathy, 2004), which influences fetal growth and development (Seckl, 2004). In the present study, body weight of the pups increased in the

control-morphine and stressed-morphine compared to control-saline and stressed-saline groups, which was consistent with the above-mentioned study. It seems that morphine suppresses effect of stress on decreasing body weight.

Effects of Prenatal Morphine and Stress Exposure on COS Blood Level

According to previous studies, maternal stress is associated with raised plasma levels of ACTH and COS, and may increase brain developmental delays and abnormalities (Van den Bergh, Mulder, Mennes, & Glover, 2005) and influence hippocampal synaptic plasticity (Alfarez, Wiegert, Joels, & Krugers, 2002). As already mentioned, prenatal stress-induced disturbance in the function of HPA axis and alteration of its feedback regulation causes higher basal secretion of corticotrophin releasing factor (CRF). CRF and glucocorticoids show pro-convulsant effects in pups and are known to decrease seizure threshold and cause alterations in fetal central nervous systems (Barrot et al., 2001; Kapoor, Dunn, Kostaki, Andrews, & Matthews, 2006; Li et al., 2009). The present findings were consistent with these data. As outlined in Figure 2, blood COS levels increased in both stressed groups compared with control rats. Therefore excitatory effect of prenatal stress on infant rats could be probably due, at least in part, to greater glucocorticoid production. Meanwhile, it has been also shown that prenatal morphine exposure suppresses restraint stress-induced ACTH levels in both diestrus and proestrus females and prenatal morphine exposure alters HPA axis and regulates stress response and sensitivity of negative feedback that are affected by fluctuation of ovarian (Slamberova et al., 2004). Chronic exposure of male rats to morphine also markedly increases concentration of corticosteroid binding globulin in blood. This issue, in turn, appears to greatly reduce the amount of COS available to intracellular receptors (Nock, Cicero, & Wich, 1998). The present results were also consistent with these findings. COS blood level increased in stressed-saline group and decreased in control-morphine and stressed-morphine groups compared to control-saline rats. These data indicated that morphine decreased COS blood levels and might suppress excitatory effects of stress on COS blood level, as illustrated in Figure 2.

Effect of Interaction Between Gestational Morphine Exposure and Stress on PTZ-Induced Seizure

PS potentiates seizure in different animal models. To confirm this point, it has been reported that prenatal restraint and predator stresses potentiate pilocarpine-induced seizure in rats (Ahmadzadeh, Saboory, Roshan-Milani, & Pilehvarian, 2011b; Saboory et al., 2011; Sadaghiani & Saboory, 2010). On the other hand, opioid system is one of the endogenous modulatory mechanisms that affect seizure susceptibility (Saboory et al., 2007). In this study, morphine administration decreased latency of tonic–clonic seizure and onset time of seizure. Morphine also potentiated PTZ-induced tonic–clonic seizure at P15 but not at P25. These results were consistent with those of previous studies, which showed that effect of morphine on seizure susceptibility was age-dependent (Gholami & Saboory, 2012; Schwartzkroin, 1994; Vathy et al., 1998). Therefore, the present data confirmed that prenatal exposure to morphine or stress alone potentiated PTZ-induced seizure age-dependently while co-administration of morphine and stress had an opposite effect, which indicated that morphine might suppress excitatory effect of stress on PTZ-induced seizure. It is possible that prenatal morphine exposure may modify capacity of endogenous stress systems to effectively respond to exogenous stressors (Vathy, 2002). It seems that HPA axis, endogenous norepinephrine and opioid systems of the developing fetus are most sensitive to maternal opiate abuse (Vathy, 2002), play a significant role in synaptic plasticity and are implicated in seizure susceptibility (Albertson, Joy, & Stark, 1984; Cowan, Geller, & Adler, 1979; Frenk, 1983). Thus, prenatal morphine exposure could alter development of adaptive responses to environmental stimuli by altering the stress-sensitive brain circuitry.

In conclusion, this study indicated that prenatal exposure of rats to stress, morphine and stress–morphine age-dependently changed their susceptibility to PTZ-induced seizure during infancy and prepubertal period. These data also suggested that co-administration of morphine suppressed stress-induced effects on body weight, COS blood levels and epileptic behavior and may modify capacity of endogenous stress systems to effectively respond to exogenous stressors.

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.

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