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Effects of prenatal exposure to alcohol on activity, anxiety, motor coordination, and memory in young adult Wistar rats

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Abstract

The objective of the present study was to examine the effects of prenatal exposure to ethanol on motor performance, emotionality, learning and memory in young-adult, male Wistar rats. Alcohol was delivered to the pregnant dams intragastrically, throughout gestation days (GD) 7–20, at the dose of 6 g/kg/day resulting in the peak blood alcohol concentration (BAC) of 350 mg/dl as assessed on GD 20. Isocaloric intubation and untreated control groups were included. Alcohol exposed rats were not impaired in the rotarod/accelerod tests. Their behavior in the open field and plus maze suggested increased neophobia. Hyperactivity was not observed. In the spatial-navigation task in the water maze, by the middle of the training, fetal alcohol rats showed a tendency towards a slower place acquisition compared to controls, but statistical analysis of the data did not yield between-group differences significant. Towards the end of the training, all rats reached a similar performance level. No detectable between-group differences were noted either in memory retention after a delay, in reversal learning, or in working memory task. Our findings demonstrate that the adverse behavioral effects of a binge-like alcohol administration during half of the first and throughout the second trimester equivalent are difficult to be disclosed in young-adult male Wistar rats. The possible reasons of the lack of significant behavioral deficits in the fetal-alcohol rats observed in the present study are discussed.

Keywords: Prenatal alcohol; Motor coordination; Locomotor activity; Emotionality; Spatial learning; Reference and working memory; Rat

Ethanol readily crosses the placental barrier producing approximately equal maternal and fetal blood alcohol concentrations (BACs) (Waltman and Iniquez, 1972). The fetus is limited in its ability to metabolize alcohol due to a low activity of hepatic alcohol dehydrogenase (ADH), the major metabolizing enzyme for alcohol. Therefore, the elimination of alcohol from the fetus is through passive diffusion of alcohol across placenta followed by maternal elimination. In addition, the rate of alcohol elimination from amniotic fluid is approximately half that from maternal blood, resulting in relatively high alcohol concentrations in amniotic fluid when alcohol levels are low or eliminated from maternal blood. Thus, amniotic fluid may act as a reservoir for alcohol, and the fetus can be actually exposed to it for a longer period than predicted on the basis of maternal alcohol concentration (Brien et al., 1983).

Ethanol was shown to be a potent teratogen inducing a massive wave of apoptosis in the developing brain (Goodlett et al., 2005; Ikondomidou et al., 2000; Light et al., 2002; Olney et al., 2002a). In line with this, increased neuronal losses were reported by several authors within different brain regions of juvenile rats perinatally (prenatally and/or postnatally) exposed to alcohol (Bonthius and West, 1990; Goodlett et al., 1997; Livy et al., 2003; Mihalick et al., 2001; Miki et al., 2003; Pierce and West, 1987; Tran and Kelly, 2003). According to some authors, these neuronal deficits were permanent, extending into adulthood (Barnes and Walker, 1981; Bonthius and West, 1991). The apoptotic potential of ethanol is among other factors attributed to the induction of oxidative stress and activation of caspase-3 effecter proteases (Henderson et al., 1995; Marino et al., 2004; Olney et al., 2002b). The developing brain, which has only a fraction of the antioxidant enzyme activity of the

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adult brain, is thought to be more susceptible to the neurotoxic effects of oxidative stress than the adult brain (Henderson et al., 1999). In addition, certain regions of the brain, such as the cerebellum and the hippocampus, show especially low levels of endogenous Vitamin E, an important biochemical antioxidant. These regions seem to be particularly vulnerable to oxidative stress and were shown to suffer the most from alcohol insult (Barnes and Walker, 1981; Maier and West, 2001; Pierce et al., 1999). Whereas the cerebellum plays an important role in control over the organism's motor performance, the hippocampus is known to be crucial for the episodic memory. It is not surprising then that motor and cognitive deficits are the most common consequences of alcohol intoxication.

Both in humans and in rodents, one of the most characteristic effects of perinatal alcohol intoxication is locomotor hyperactivity. Locomotor hyperactivity has been consistently reported in children and in preweaning and juvenile rats after perinatal exposure to ethanol (Abel, 1982; Abel and Reddy, 1997; Bond and Di Giusto, 1977; Leonard, 1988; Mattson et al., 2001; Tran et al., 2000). However, studies on adult rats (2 months and older), brought controversial results. Some authors reported increased locomotor activity in prenatally alcohol-exposed rats at ages varying between 2 and 18 months (Osborn et al., 1980; Abel and Berman, 1994). In many other studies, however, hyperactivity has not been observed in adult fetal-alcohol rats (Abel and Berman, 1994; Bond and Di Gusto, 1977; Randall and Hannigan, 1999; Westergren et al., 1996).

It was also shown that preweaning rats prenatally exposed to alcohol were worse than the control animals in their performance on rotating drum, and fell off an inclined plane at a less steep angle, suggesting alcohol-induced decrease in muscle strength and sensorimotor coordination (Abel and Dintcheff, 1978). On the other hand, however, sensorimotor functions in adult subjects exposed to fetal-alcohol intoxication have not been investigated as much.

In addition to its effects on the motor functions, fetal-alcohol was also reported by some authors to result in elevated anxiety (Ogilvie and Rivier, 1997; Osborn et al., 1998; Weinberg et al., 1996) and cognitive deficits including attention and learning impairments. It has been postulated that cognitive deficits may be observed even in the absence of a full-blown fetal alcohol syndrome (Girard et al., 2000). Learning and memory impairments have been demonstrated especially in spatial tasks sensitive to hippocampal damage such as spatial navigation in the Morris Water Maze (MWM) (Blanchard et al., 1987; Cronise et al., 2001; Gianoulakis, 1990; Girard et al., 2000; Goodlett and Peterson, 1995; Goodlett and Johnson, 1997; Hamilton et al., 2003; Hannigan et al., 1993; Johnson and Goodlett, 2002; Kim et al., 1997; Matthews and Simson, 1998; Pauli et al., 1995; Tomlinson et al., 1998; Westergren et al., 1996), food-rewarded spatial navigation in the radial arm maze (Neese et al., 2004; Reyes et al., 1989) and place acquisition as well as conditional alternation in T-maze (Lee and Rabe, 1999; Nagahara and Handa, 1997; Thomas et al., 1996; Zimmerberg et al., 1991). Most of these studies were investigating effects of perinatal alcohol intoxication on place learning dependent on spatial reference memory. Relatively few studies examined alcohol-dependent changes in the spatial working memory capacity (Girard et al., 2000; Nagahara and Handa, 1997; Neese et al., 2004; Reyes et al., 1989; Thomson et al., 1996; Zimmerberg et al., 1991). Experimental results concerning behavioral effects of alcohol have not always been consistent. Generally the more pronounced deficits in learning and memory tasks were noted in juveniles as compared to adult subjects (Blanchard et al., 1987; Cronise et al., 2001; Gianoulakis, 1990; Girard et al., 2000; Goodlett and Peterson, 1995; Goodlett and Johnson, 1997, Nagahara and Handa, 1997; Pauli et al., 1995; Thomson et al., 1996). Conversely, adult animals perinatally exposed to ethanol were reported by some authors to show none or very small cognitive deficits, especially when alcohol was administered prenatally during the 2nd trimester equivalent (Abel, 1979; Clausing et al., 1995; Cronise et al., 2001; Minetti et al., 1996).

The present study is revisiting the still open issue of endurance of behavioral effects of fetal alcohol insult with ethanol administered in a binge-like manner throughout 2/3 of the gestation period at a dose producing high BAC. In previous studies on the behavioral effects of prenatal ethanol intoxication in rats usually only one or two aspects of behavior have been examined. Conversely, in the present study, a wide battery of tasks was applied to the same group of either fetal-alcohol or control young-adult male Wistar rats to thoroughly investigate all aspects of behavior that have earlier been reported by some authors to be susceptible to alcohol effects. Applied behavioral tasks included tests for emotionality, as well as for motor and cognitive performance. A disadvantage of the application of more than one behavioral task to the same experimental subject is an uncontrolled effect of previous experience on the subsequent performance. However, complete counterbalancing of different conditions would be very difficult with the large number of the tasks applied. On the other hand, the potential interference arising from a previous experience may be overlooked if cross-sectional analysis is more important than longitudinal comparison of the data, with all the animals subjected to the same tasks at the same time. In addition, in the present study, order and timing of the tasks were adjusted to minimize the possible proactive effects.

1. Material and methods

1.1. Subjects

Large number of 3-months old, naive, male and female Wistar rats, purchased from the Hýfzýsýhha Serum-Production Facility (Ankara—Turkey), were used for breeding in the present study. The rats were housed in a secluded room with an ambient temperature of 22 °C and under 12 h/12 h light/dark cycle commencing at 07:00 h. Female rats were individually housed in transparent Plexiglas cages. For mating, a male rat, picked at random, was placed into a female's cage. Rats were allowed to mate nightly until a vaginal plug was observed on the following morning. The presence of a vaginal plug was evidence of a successful fertilization and this day was marked as gestational day 0 (GD 0). The pregnant dams (22) and then

the offspring (initially 231) were monitored with regard to body weight gain. The day of birth was referred to as postnatal day 0 (P 0). At birth, the number of pups in each litter was counted. Until weaning, pups remained with their natural mothers. Cross-fostering was considered unnecessary because postnatal maternal influences induced by prenatal treatment with alcohol at the dose applied in the present study were reported as not having significant adverse effect on the offspring development or behavior (Hanningan, personal communication). Weaning occurred at P 30, after which the offspring were group-housed by litter and sex in transparent Plexiglas cages (46 × 24 × 20 cm) until one week before testing. Behavioral testing was started at 80–85 and completed at 142–147 days of age. Female rats were not taken into experiments to avoid fluctuations in mnemonic capacity related to estrus cycle and changing estrogen levels. To limit the effects attributable to contributions from individual litters the rats from each treatment group were intermixed between litters. We expected animal losses to be more likely among fetal alcohol rats than among control animals, therefore, from the beginning, the size of the alcohol group (9 subjects) was kept larger than that of control groups (7 subjects/group). However, in the course of the experiments, we lost three animals from the control groups and none from the alcohol group, such that the final numbers of subjects were 9 in the alcohol group and, respectively, 6 and 5 in control groups.

1.2. Treatment

On GD 7, pregnant dams were assigned (counterbalanced for initial body weight) to one of three treatment groups: Alcohol Group (A), Intubated Control Group (IC), and Nonintubated Control Group (C). Starting from the GD 7 throughout GD 20, dams from group A were daily administered 6 g alcohol/kg body weight, with ad libitum access to laboratory chow and water. Animals in IC group, a control for possible intubation-induced stress effects, received the same volume of fluid with sucrose substituted isocalorically for ethanol. Animals in C group received ad libitum access to laboratory chow and water with no additional treatment. The alcohol/sucrose solution was delivered by intragastric intubations using stainless curved feeding needle (18 ga, 3 in, Stoelting Co., USA). The alcohol/sucrose solution administration protocol was strictly timed. Daily portion of alcohol/isocaloric sucrose solution was divided into two equal doses given to animals 1 h apart, at 10:00 a.m. and 11:00 a.m. Food was removed from all dams at 08:00 a.m. to allow chyme to clear from the stomach and to facilitate the absorption of the alcohol. The food was replaced approximately 4–5 h after the second intubation. The water was removed from all dams' cages prior to the first intubation and was replaced immediately following the second intubation. Binge-like regime of alcohol administration was chosen as producing higher BACs (Pierce and West, 1986; West et al., 1981) and thus being more damaging as compared to the liquid diet.

Ethyl alcohol (96.5% v/v, Merck) was used in the study. The alcohol was prepared daily as a 20% (weight/volume) solution mixed with distilled water and stored at room temperature. Blood alcohol concentration was assessed on GD 20, in a different set of pregnant dams (4) by using spectrophotometric enzymatic procedure (Dudek and Abbott, 1984).

1.3. Behavioral testing

Male pups from all three groups were tested for motor activity, anxiety, sensorimotor coordination and muscle strength, and eventually for learning and memory in a series of behavioral tasks which included open field (first 3 days), plus maze (1 day), rotarod/accelerod (4 days), and Morris water maze (30 days).

1.3.1. Open field

The open-field activity monitoring system (MAY 9908 model, Commat Ltd, Turkey) comprised of eight Plexiglas cages (42 × 42 × 30 cm) equipped with a total of 30 infrared photocells located at 2.5 cm intervals, 2 and 8 cm above the floor on the rear sides of each activity cage. Interruptions of photocell beams by the animal were detected by a computer system and the animal's location was calculated by the software at 0.1 s sensitivity. Animals' vertical (rearing) and horizontal (ambulation) movements were automatically recorded over 30 min in 5 min intervals.

1.3.2. Plus maze

The plus maze (Commat Ltd, TR), specifically designed to measure anxiety was constructed of polyester and consisted of a central platform (10 × 10 cm), two open arms (50 × 10 cm) and two closed arms (50 × 10 cm) with black Plexiglas walls extending 30 cm high and no ceiling. The arms were arranged in a cross shape with the two open arms facing each other and two closed arms facing each other. The maze was positioned 45 cm above the floor. On the testing session, each animal was placed in the center of the maze facing an open arm. Rats were allowed to explore the maze for 5 min. During this time, the number of entries with all four paws to the closed and open arms, the total time spent in closed and open arms separately, and total time spent on the central platform were recorded by the experimenter.

1.3.3. Rotarod/accelerod

A rotarod/accelerod apparatus ((Rotamex V-EE/85, Columbus, OH, USA) was used for measuring muscle strength and sensorimotor coordination as previously described by Uzbay and Kayaalp (1995). The apparatus contained a cylinder 6.5 cm in diameter and was rotated with a pre-selected speed. Four animals were tested simultaneously. The test was repeated over four consecutive days, each day under different conditions. On the first shaping day, the speed of rotation was stable and set to 20 revolutions per minute (rpm). Animals remained on the rod until they fell down or after 10 min elapsed. When falling off the rod, rats came into contact with a metal grid beneath them kept under a mild electrical voltage. No measurements were taken on this day. On the second day of testing, the conditions remained the same but the time the animals spent on the rotarod before falling down was measured. On the third day of testing, the speed of the rod was linearly increased from 0 to 80 rpm within 10 min. On the fourth last day, the speed of the rod was

accelerated from 0 to 80 rpm within 4 min. The time rats remained on the rod was automatically measured in seconds by built-in timer of the apparatus.

1.3.4. Circular water maze (Morris type)

The tank was 150 cm in diameter and 60 cm high. It was filled to the depth of 45 cm with water maintained at 23 °C (±1) by an automatic heater. Yellow nontoxic watercolor paint was added to make the water opaque. A movable platform (10×10 cm) made of transparent Plexiglas was located in the center of one of the quadrants. In its raised position, the top of the platform was 1 cm below the surface of the water so that the rat could easily climb on it to escape from the water. In its lowered position, the top of the platform was 30 cm below the water surface and not available to the rat. The tank was divided into four quadrants (NE, NW, SE, and SW) by two imaginary perpendicular lines crossing in the center of the tank. A computerized video tracking system (EthoVision Image Analysis 3.1, Noldus Information Technology, Holland) was used to record data. A camera was mounted to the ceiling above the pool and was connected to a microprocessor. The experimental room was furnished with several extra-maze cues immobile throughout the entire experimental period. Indirect illumination was provided by diffused light coming from the four sides of the room.

The water maze is used to monitor spatial learning and memory in small rodents (Morris, 1984). Training and testing in the MWM comprised of 6 consecutive stages: Shaping Training; Place Learning (reference memory test); Probe Trials/Extinction Training; Reversal Training; Memory Retention Test after a 10 day rest period; and Repeated Acquisition Training (working memory test).

1.3.5. Shaping training

This training was meant to reduce the possible confounding non-mnemonic factors arising from being introduced to a novel stressful situation. On the first day in the pool, each rat was trained to swim in the water and climb on the escape platform. The raised platform was placed 30 cm from the edge of the pool in a random position. Dark curtains were drawn around the tank to eliminate the distal cues that were used for subsequent place learning. Animals were released into the pool four times from different starting points: first in the vicinity of the platform, then from a gradually increased distance from the platform. Each time an animal swam in the water until it found the platform. If an animal failed to find the platform within 60 s it was gently guided to it by the experimenter.

1.3.6. Place learning

During the place learning, the platform was placed in the center of NE quadrant where it remained throughout this stage of experiment. Rats were given four daily trials for 6 consecutive days. Each rat was released into the water facing the pool wall at one of the four starting points (N, S, E, W) which were used in a pseudorandom order so that each position was used only once during the daily experimental session. The trial terminated when the rat found the platform and was allowed to remain there for 10 s, or in 60 s, whichever came first. If the animal did not find platform within 60 s, the experimenter guided the animal to the platform. Afterwards the rat was returned to its cage for a 5 min. inter-trial interval. During platform trials, two measures of performance were recorded: *Escape latency* was the time between leaving the start location and climbing on the escape platform; *Swim distance* (path length) was the distance (cm) swum from the start location to the escape platform.

1.3.7. Probe trials/extinction training

On the completion of 6-day place learning, for 3 consecutive days, animals received 45 s probe trials. During probe trials, the platform was in its lowered position. On the computer screen, an imaginary 40 cm diameter annulus (annulus 40) was drawn around the place where originally platform was located. The total time an animal spent in: (a) platform quadrant (NE); (b) the opposite quadrant (SW); and (c) the annulus 40 was recorded.

1.3.8. Reversal training and memory retention test after 10-day rest period

During reversal training, the position of the escape platform was changed from the NE to the opposite SW quadrant. The training lasted for three days with 4 trials/day, and 5 min inter-trial intervals. Similarly to the original training, here too, animals were released to the pool from four semi-randomly varied start positions. Ten days after the completion of reversal training, animals received a single 45 s probe trial.

1.3.9. Repeated acquisition training: working memory test

This procedure was adopted after Vann and Aggleton (2003). During this experiment, 12 platform positions, which varied in their distance from the pool perimeter, were used along with 8 different start positions designated by the compass points (N, E, S, W, NE, NW, SE, SW). The entire training lasted 16 days. Daily sessions constituted of 4 trials. The location of the platform remained constant across the four trials of a given day but varied between days. The animal was released into the pool, facing the wall, from one of the eight start points. The same starting point was used for the first two trials of each session but was then varied for the remaining two trials. Each swim was finished when the animal either located the hidden platform or after 120 s had elapsed. If the animal had not found the platform at the end of 120 s, it was guided there by the experimenter and remained on the platform for 10 s. For the first 12 days, all inter-trial intervals were of equal duration, approximately 15 s. On days 13–16, the delay between the first and second trial was increased to 30 min. During this time, the animal was returned to the home cage. After the second trial the inter-trial interval was 15 s as before. Throughout the training, distal visuospatial cues were stable.

All procedures in the present study are in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (Washington, D.C. USA, 1996) and the Declaration of Helsinki. All efforts were made to minimize animal suffering and to keep the number of animals used as low as possible.

1.4. Statistical analysis

From all measures group means±SEM were calculated. The data were analyzed with treatment [A ($n=9$), IC ($n=6$), and C ($n=5$)] as independent factor, and sessions, trials, or blocks of trials as repeated measures. The Tukey test was used for post hoc analysis of the data. Additionally, the data analysis has been verified with LSD (Least Significant Difference) test provided by the SPSS statistical package used in the present study. . The criterion of statistical significance was $p=0.05$, two-tailed. Due the lack of significant differences between C and IC groups on all the tasks expect working memory test with 30 min delay where A and IC groups were significantly better than C group, all the statistical analyses were re-done with pooled C and IC data to improve the power of test.

2. Results

2.1. Body weights

During the last two weeks of pregnancy (GD 6–20), there was a constant increase in the dams' body weight in all three groups. A two-way repeated measures ANOVA (treatment *x* days) applied to the dams' body weight data yielded significant main effect of day ($F_{(14,266)}=206.18$, $p<0.001$), insignificant main group effect, but significant group *x* day interaction ($F_{(28,266)}=6.66$, $p<0.001$]. One-way ANOVAs performed for each gestation day independently revealed significant between-group differences on the last four days only, with lower body weight of dams from A group as compared to controls ($F_{(2,21)}=3.4$, $p=0.054$; $F_{(2,21)}=5.7$, $p=0.010$; $F_{(2,21)}=6.3$, $p=0.008$, and $F_{(2,21)}=9.0$, $p=0.002$, respectively]. The difference between C and IC groups was insignificant.

The mean body weight at births for the pups from A group was 4.9 g as compared to 5.9 g and 5.8 g in IC and C group, respectively. This difference in the pups' body weight on P 0 between fetal-alcohol and control animals was yielded significant ($p<0.001$) and it still persisted on P 5 but disappeared on P 10. No difference has been observed between groups in the litter size.

2.2. Blood alcohol concentrations

The mean maternal blood alcohol concentrations estimated 2 and 3 h after the second intubation on the gestation day 20, were 334.45±18 and 349.65±48.4 mg/dl, respectively.

2.3. Behavioral testing

2.3.1. Open field test

In all three groups, the highest ambulation (measured by the total covered distance) was noted on the first day of testing, during the first five min of exposure to the open field (Fig. 1A).

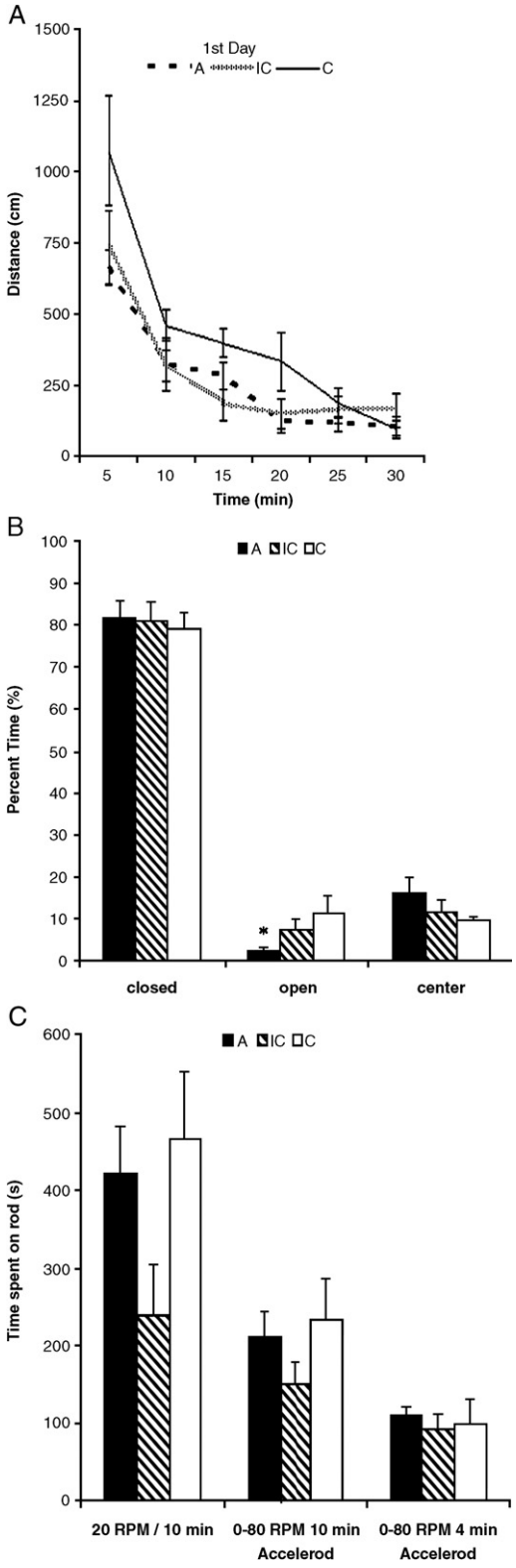

Fig. 1. Measures of animals' motor performance and emotionality. A. Mean distance (±SEM) covered by rats from each group in the consecutive 5-min intervals of the total 30-min testing period during the first day in the open field. B. Mean percent of time (+SEM) spent in each of three compartments of the plus maze: closed arms, open arms, and center, in A, IC, and C group, respectively. C. Group mean times (+SEM) spent on a rotating rod until falling off under three different conditions: rotarod 20 rpm; accelerod 0–80 rpm in 10 min; and accelerod 0–80 rpm in 4 min. Error bars denote SEM. Asterisk indicates significant difference at $p\leq 0.5$.

One-way ANOVA performed for the first 5 min of the first OF session revealed marginal significance of the group effect ($F_{(2,19)}=3.17$, $p=0.06$). Between group comparisons yielded marginally significant difference between A and C group only ($p=0.06$) with lower locomotor activity in fetal-alcohol group as compared to controls. Two-way repeated measures ANOVA for groups and all six 5-min intervals of the first OF session yielded significant effect of interval ($F_{(5,85)}=59.11$, $p=0.001$) confirming a general decrease in overall locomotor activity in the course of the session as a result of habituation to the novel environment. The main effect of group and group x interval interaction were significant at the level $p=0.06$ ($F_{(2,17)}=3.33$) and $p=0.041$ ($F_{(10,85)}=2.02$), respectively. Repeated measure ANOVA for the total distance covered within each of the 3 consecutive days in the OF yielded significant main effect of day ($F_{(2,34)}=10.57$, $p=0.001$) confirming decrease in the locomotor activity throughout the whole testing period. Additionally, there was a significant group x day interaction ($F_{(4,34)}=4.74$, $p=0.004$) due to a relatively low ambulatory activity in A group on the first day and relatively high activity in this group on the third day, as compared to controls from the group C. The main effect of group was not yielded significant. For the vertical movements none of the comparisons was significant.

2.3.2. Plus maze

As revealed by one-way ANOVAs, pups exposed prenatally to alcohol spent significantly less time in the open arms ($F_{(2,19)}=3.55$, $p=0.051$), spending relatively more time on the central platform as compared to the control rats (Fig. 1B). The mean numbers entries to closed and open arms were as follow: A=3.0, IC=3.2, C=2.2 and A=1.0, IC=1.7, C=1.8, respectively.

2.3.3. Rotarod/accelerod

Under none of the three conditions tested on the rotarod/accelerod (rotation speed fixed at 20 rpm, rotation speed increasing from 0 to 80 rpm in 10 min, and rotation speed increasing from 0 to 80 rpm in 4 min) were any significant between-group differences detected (Fig. 1C).

2.3.4. Place learning in the MWM

In the place acquisition task, mean escape latency and mean swim distance were calculated for each animal on each of 6 testing days, and the means were taken into analysis. Two-way repeated measures ANOVA (group x day) performed for escape latency yielded a significant day effect showing a general decrease in overall latency throughout the training period in the MWM ($F_{(5,85)}=40.65$, $p=0.001$) (Fig. 2A). None of the other comparisons showed significant differences. The same analysis

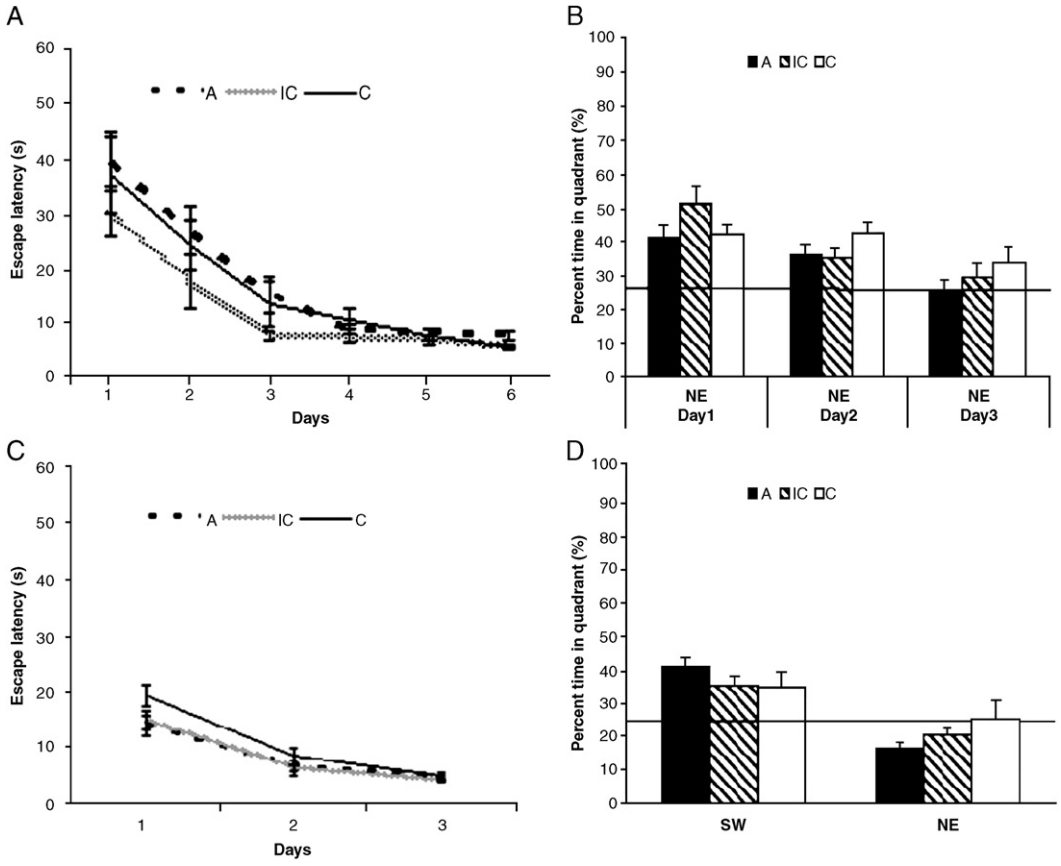

Fig. 2. Measures of place learning and retention of spatial reference memory in the MWM. A. Mean escape latency (±SEM) to locate invisible platform calculated for each training day and each treatment group independently. B. Mean percent of time (+SEM) spent in the platform quadrant (NE) on each of the three consecutive 45 s probe trials in each treatment group independently. Line at 25% represents chance level. C. Mean escape latency (±SEM) to locate invisible platform calculated for three consecutive reversal training days in each treatment group. D. Mean percent of time (+SEM) spent in the new platform quadrant (SW) and in the opposite NE quadrant on the probe trial carried out 10 days after the completion of reversal training. Error bars denote SEM.

performed for the distance measure revealed a significant effect of both, the day ($F_{(5,85)}=39.46$, $p=0.001$) and the group ($F_{(2,17)}=3.71$ $p=0.046$). Subsequent post hoc comparisons using the Tukey test confirmed significant difference between A and IC groups ($p=0.037$) with A vs C and IC vs C differences remaining insignificant. Analysis of the simple effects, however, did not revealed significant between-group differences on any of the days ($p>0.1$).

A statistical analysis performed on the data from A group versus the pooled data from C and IC groups confirmed a significant between-group difference in the swim distance ($F_{(1,18)}=5.9$, $p=0.026$), and lack of such a difference in the overall escape latency. Comparison of simple effects yielded marginally significant between-group differences in the distance measure on days 2 and 3 ($F_{(1,19)}=3.40$, $p=0.082$, $F_{(1,19)}=2.92$ $p=0.1$, respectively) and a significant difference at $p<0.05$ level only on the day 6 ($F_{(1,19)}=5.1$, $p=0.036$). On the other hand, however, two-factorial ANOVA with repeated measures confined to the last three days of training in the MWM (days 4–6) revealed neither a significant main group effect nor a significant group *x* day interaction.

2.3.5. Probe trials/extinction training

On the first probe trial, regardless of the treatment, animals' performance was well above the chance level, with 40–50% of the time spent in the platform quadrant (see Fig. 2B). Over the three daily probe trials (extinction training), in all three groups, time spent in the platform quadrant decreased approaching the chance level. Two-way repeated measures ANOVA (group *x* day) revealed insignificant group effect and insignificant group *x* day interaction for all three measures applied: time in the platform quadrant, time in annulus 40, and the ratio of the time spent in the platform quadrant to the time spent in the opposite quadrant. The main effect of day was significant ($F_{(2,32)}=16.57$, $p=0.001$) showing the extinction of the preference for the platform quadrant in all rats.

2.3.6. Reversal training and memory retention test after 10 days rest period

One-way ANOVA applied to the data from the first day of reversal training yielded significant between-group differences neither in the escape latency nor in the swim distance. Repeated measures ANOVA for all 3 days of reversal training when applied to the latency measure revealed a significant main effect of day ($F_{(2,34)}=57.44$; $p=0.001$) showing a general decrease in overall latency throughout the training period (Fig. 2C). None of the other comparisons reached significance. When the same analysis was applied to the distance measure, a significant day effect ($F_{(2,34)}=49.53$, $p=0.001$), marginally significant group effect ($F_{(2,17)}=3.22$, $p=0.065$), and insignificant group *x* day interaction were revealed. Subsequent comparisons using the Tukey test confirmed marginally significant difference between two control groups ($p=0.065$) with the slower rate of acquisition of a new platform position in group C. The differences between fetal-alcohol group and either of the control groups were insignificant.

Ten days after the completion of the reversal training, in all groups subjected to a probe trial with removed platform, the mean time spent in the new platform quadrant was above the chance level (25%) (Fig. 2D). A one-way ANOVA yielded no significant between-group differences.

2.3.7. Repeated acquisition training

The first 12 days of the repeated acquisition training was a variant of a delayed matching-to-position working-memory task with a short 15 s delay between the sample (trial 1) and test (trial 2) as well as the remaining two trials. The 12 days of acquisition were blocked in groups of three (Fig. 3A) and repeated measures ANOVA was performed for the escape latencies and swim distances on the test trials using group and block factors. None of the main effects or interactions was significant.

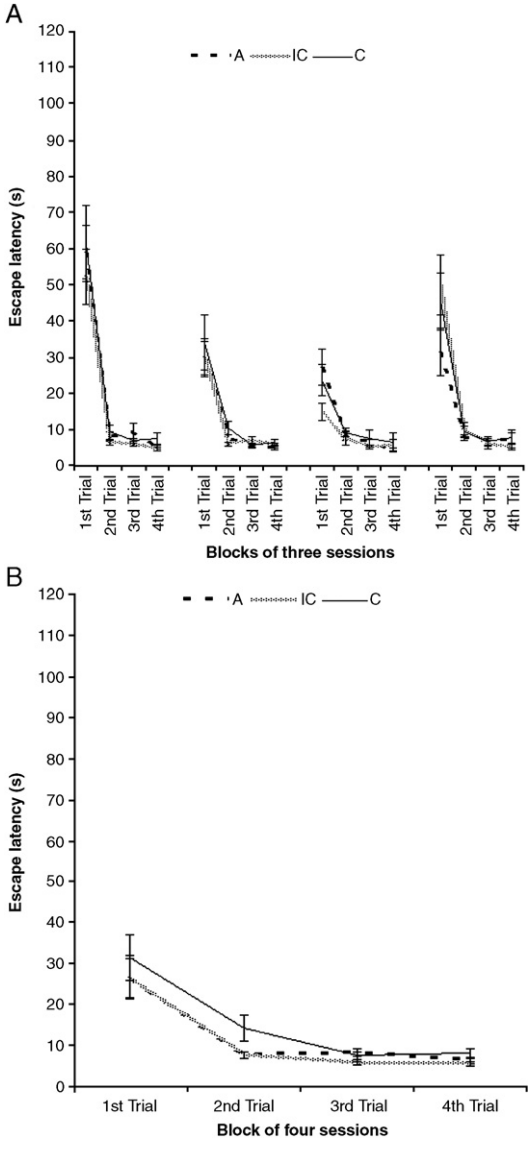

Fig. 3. Performance on the spatial working memory task. A. The mean escape latency (±SEM) calculated for the four consecutive trials in four blocks of three sessions each, during repeated acquisition training. B. The mean escape latency (±SEM) calculated for the four consecutive trials in a block of four sessions during repeated acquisition training with 30-min interval between first (sample) and second (test) trial.

The next stage (days 13–16) involved a 30 min delay between the sample and test trial, with the remaining two trials run as before. To determine the effect of delay on performance, the last block of sessions with 15 s delay (days 10–12) was compared with the subsequent block of sessions with longer 30 min delay. Variable delay was between trials 1 and 2, with the remainder of the trials being run as normal, therefore only trials 1 and 2 were included in the analysis. Repeated measures ANOVA for escape latency and swim distance revealed a significant effect of trial ($F_{(1,134)}=107.35$, $p<0.001$ and $F_{(1,134)}=106.11$ $p<0.001$, respectively), confirming shortening the latency and the distance on the second trial as compared with the first trial (Fig. 3A and B). The main effect of delay was also significant ($F_{(1,134)}=5.22$, $p=0.024$ and $F_{(1,134)}=4.5$ $p=0.036$, respectively) with longer latency/distance after longer delay, but the main effect of group was yielded insignificant ($p>0.1$). However, one-way ANOVA performed on the data from the 30 min test trials yielded significant between-group differences with shorter escape latencies and swim distances in A and IC groups as compared to the C group ($p<0.05$).

For all the behavioral tests, the outcomes of the analyses run on the pooled data and by the LSD post hoc test confirmed the results yielded by the analyses with C and IC groups when treated independently and when the more conservative Tukey HSD post hoc test was applied.

3. Discussion

The results of this study demonstrate that the behavioral effects of prenatal exposure to a fairly high dose of ethanol were very mild when tested in young-adult male offspring. There was noted an increased neophobia. However, no hyperactivity or sensorimotor disorder and/or muscle weakness was observed. Fetal-alcohol rats, when tested as young adults, showed a tendency towards initially slightly slower place acquisition in the MWM, but in the second half of this training both alcohol and control rats obtained similar level of performance. The latter result together with the lack of a negative effect of prenatal ethanol on the strength of place preference, reversal learning, memory retention after a delay, and eventually working-memory argues against significant cognitive deficits in the fetal-alcohol rats.

In the present study, retardation of the dams' weight gain was confined to the last three days of gestation only. Nevertheless, birth weight of pups from the alcohol group was significantly lower than that of control offspring. The between-group differences in the body weight disappeared, however, within the ten postnatal days. These observations are consistent with the reports by Tran et al. (2000) showing that offspring of rats subjected to chronic alcohol exposure during gestation tend to be significantly lighter at birth but the difference rapidly dissipates with maturation.

As assessed by the open field and rotarod/accelerod tasks, neither hyperactivity nor sensorimotor deficits were observed in fetal-alcohol pups when tested as young adults. Hyperactivity is the most frequently reported behavioral problem in children with fetal alcohol effects (after Driscoll et al., 1990). Furthermore, hyperactivity has been commonly observed in juvenile rats regardless of the dose and period of alcohol administration (Abel, 1982; Abbel and Reddy, 1997; Bond and Di Giusto, 1977; Tran et al., 2000) and it has been linked by some authors to a deficit in response inhibition (Abel, 1982; Westergren et al., 1996). Lack of hyperactivity in adult rats that were prenatally exposed to alcohol found in the present study is consistent with earlier reports by Bond and Di Giusto (1977). In the latter study, offspring born from dams receiving a liquid diet with 35% ethanol concentration throughout the whole gestation, displayed significantly greater ambulation in the open-field at 28 and at 56 days of age, but not at 112 days of age. Randall and Hannigan (1999) also did not report increased locomotor activity in offspring at PN 90–150 after binge-like alcohol administration throughout GD 8-20. According to the outcome of Bond's (1981) analysis of the update literature regarding prenatal alcohol effect on the animal locomotor activity, an increase in activity as compared to controls was observed in rats receiving prenatally alcohol doses of 6 or more g/kg/day only when tested prior to 70 days of age. On the other hand, locomotor hyperactivity has been reported in the adult rats after neonatal alcohol administration (Kelly et al., 1987; Tran et al., 2000). Thus, it possible that in rat, early neonatal period corresponding to the human third trimester, represents the time window of the highest vulnerability for this dysfunction.

Perinatal alcohol-related sensorimotor deficits also seem to attenuate with maturation. Purkinje cells of the cerebellum having functional relation to the neuroanatomical circuit for motor coordination and gait have been demonstrated by other authors to suffer from prenatal alcohol intoxication (West et al., 1990; Maier and West, 2001). In the current study, however, rats prenatally treated with alcohol and tested as young-adults were unimpaired in sensorimotor coordination and did not show muscle weakness. Such dysfunction was earlier reported by Abel (1982) in rats tested prior to weaning and also in rats raised in isolation and in impoverished environment (Hannigan et al., 1993). It is also possible that cerebellum is more vulnerable to growth restrictions and neuronal depletion induced by alcohol exposure during the neonatal brain growth spurt. As shown by Goodlett and associates (1991), administration of ethanol as 10.2% (v/v) solution confined to two daily feedings on PN 4–9 produced significant reductions in the whole brain and cerebellar weight in alcohol-exposed rats. In these rats, a significant impairment in the acquisition of the rotarod task was still observed at approximately 405 days of age, as compared to controls.

On the plus maze task, alcohol-treated rats spent significantly less time on the open arms than the control animals. The latter observation is in line with a lower ambulation score in this group as compared to controls during the first 5 min in the open field and may suggest an increased level of anxiety in alcohol pretreated rats. Osborn et al. (1998) showed that pre-exposure to an open field reduced subsequent exploratory responses in the plus maze more in the fetal alcohol than in control rats, with females more affected than males. In the latter experiments, however, animals were placed in an OF for 5 min before a single 5-min exposure to the plus maze, while in the present

study, the open field testing was carried out over 3 consecutive days, 30 min each day, giving a chance for habituation of the potential fear responses to the novel environment. In the present study, elevated anxiety in the fetal alcohol rats as assessed by their plus maze behavior is consistent with reports by several authors (Ogilvie and Rivier, 1997; Osborn et al., 1998; Weinberg et al., 1996) that animals exposed to alcohol *in utero* are typically hyperresponsive to stressors in adulthood as indicated by increased activation of the hypothalamic–pituitary–adrenal axis. It is also consistent with the report that prenatal exposure to alcohol decreases sensitivity to $GABA_A$ receptor's allosteric modulators such as endogenous neurosteroid, allopregnanolone, which is thought to act as an endogenous anti-anxiety agent in novel or stressful situations (Zimmerberg et al., 1995).

In the place learning, by the middle of the training, swim distances were longer in alcohol rats as compared to controls but the differences appeared to be insignificant ($p>0.05$) and towards the end of the training all rats reached similar and almost asymptotic performance levels with mean escape latency oscillating around 10 s, and mean escape distance of around 200 cm. On the remaining cognitive tasks including repeated probe trials, reversal learning, memory retention after a delay, and spatial working-memory test, fetal alcohol rats were as good as or even better than control subjects. Our results argue against substantial learning and memory deficit in male Wistar rats after exposure to a relatively high dose of alcohol (BAC 350 mg/dl) over the last two weeks of pregnancy (approximately second trimester equivalent). The present results indicate that alike hyperactivity, and sensorimotor and gait anomalies, learning and memory deficits too are difficult to be disclosed after maturation. Lack of a serious learning and memory deficit in young-adult rats prenatally exposed to ethanol observed in the present study is in line with findings by other authors. Abel (1979) with similar alcohol regime applied to the pregnant dams also did not observe significant differences between alcohol-exposed group and pair-fed controls either in avoidance or in MWM task. In the avoidance task, fetal-alcohol adult males performed even slightly better than control animals. Cronise et al. (2001) reported that fetal-alcohol rats demonstrated longer latencies during place learning in the MWM when juveniles, however, this deficit was found to be transient because no differences were observed among adults. Lee and Rabe (1999) testing young-adult fetal-alcohol rats in T-maze place discrimination, and Hannigan et al. (1993) testing rats in the MWM, all reported no change in the acquisition and only some impairment during the reversal training. Matthews and Simson (1998) observed longer escape latencies in fetal-alcohol rats (no distance and/or swim speed information given) on test days with invisible platform after few days of training with visible platform but only when testing took place 3 days after the initial training. Westergren et al. (1996) observed only a tendency towards a lower acquisition of place learning in the MWM in rats prenatally exposed to ethanol (the difference was at $p=0.06$ level). Kim et al. (1997) also reported longer escape latencies (no distance and/or swim speed information given) in fetal alcohol group but animals were tested at 16–17 month of age.

It is difficult, however, to compare the results of different studies since the severity of the behavioral deficits may involve the interaction of several factors, such as the amount of consumed alcohol, duration and pattern of alcohol consumption, peak BAC, degree of difficulty of the employed behavioral task, and eventually age at testing, gender, and even strain of tested animals. Sellin and Laakso (1987) reported for instance that ethanol was more effective in reducing motor performance in Long-Evans rats than in Wistar rats. In addition, ethanol produced a greater reduction in the population spikes recorded from hippocampal slices (*in vitro*) of Long-Evans rats compared to Wistar rats. These data suggest that differences in ethanol sensitivity may exist between species and even standard laboratory rat strains. Differential sensitivity to perinatal alcohol-induced restriction of brain weight was reported in inbred strains of Sprague–Dawley rats (MR and M520) and between Long-sleep and Short-sleep mice, selectively bred for differences in initial sensitivity to the hypnotic effects of acute alcohol administration (Goodlett et al., 1989). In the present study, the lack of adverse effects of the prenatal alcohol intoxication on rats' behavior may be partially related to a lower sensitivity of Wistar rats compared to Sprague–Dawley rats, the latter used in many of the studies reporting detrimental effects of perinatal alcohol on behavior and brain morphology. These differences not necessarily must be associated with the differences in blood alcohol concentrations and thus ADH activity (Goodlett et al., 1989). There are discrepancies among the findings related to sex differences in susceptibility to perinatal alcohol intoxication. Some authors reported females as more impaired on spatial memory tasks (Kelly et al., 1988; Kelly et al., 1987; Lee and Rabe, 1999; Minetti et al., 1996; Neese et al., 2004; West et al., 1984a,b), whereas others found male rats more deficient compared with females (Blanchard et al., 1987; Goodlett and Peterson, 1995; Johnson and Goodlett, 2002; Zimmerberg et al., 1991).

It has been also postulated that alcohol effects may precipitated with aging. They may be present in juveniles, attenuate in young-adults and re-appear in old-age, but this hypothesis has not been documented yet. Regardless of the rat strain, gender and age of testing, binge-like alcohol exposure during the neonatal brain growth spurt (P 4–9) seems to cause more enduring deficits in spatial learning (Girard et al., 2000; Johnson and Goodlett, 2002; Pauli et al., 1995; Tomlinson et al., 1998), which may indicate that brain structures implemented in spatial memory during this time window of development show greater vulnerability to teratogenic effects of ethanol.

In the context of the above discussion on the potential factors affecting severity and endurance of fetal alcohol effects, the results of the present study may suggest higher resistance in our laboratory strain of Wistar rats to the detrimental effects of the prenatal alcohol exposure. Our previous results suggest that among juvenile rats of both sexes exposed to the same alcohol regime, juvenile females appeared to be more affected by fetal alcohol than the juvenile males but the endurance of this effect is still to be examined (Dursun and

Jakubowska-Dogru, 2006). It is also possible that an overall behavioral deficit in alcohol-exposed Wistar rats from our laboratory strain would be greater after neonatal ethanol administration.

Attenuation of alcohol-induced behavioral deficits with maturation may also take place due to some kind of morphological and/or functional regeneration in the still developing nervous system. Morphological studies by Davies and Smith (1981) based on qualitative and quantitative evaluation of Golgi-impregnated hippocampal pyramidal cells revealed a marked reduction in the extent of basilar dendrites in alcohol-exposed animals at early postnatal stages only. Miki et al. (2003) showed that initially a significantly reduced number of neurons in the hilus region of hippocampus in rat pups exposed to high dose alcohol at PN 10–15 later returned to normal. Ferrer et al. (1988) reported reduced hippocampal dendritic spine densities in young (PN 15) but not adult (PN 90) prenatally alcohol exposed rats. However, there is still very little known about the mechanisms of a potential recovery of the central nervous system from the damaging effects of the perinatal alcohol insult. Elucidation of these mechanisms would an interesting subject for further investigations.

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