EFFECTS OF PRENATAL ALCOHOL EXPOSURE ON ACTIVITY, ANXIETY AND LEARNING IN YOUNG ADULT WISTAR RATS

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ABSTRACT

EFFECTS OF PRENATAL ALCOHOL EXPOSURE ON ACTIVITY, ANXIETY AND LEARNING IN YOUNG ADULT WISTAR RATS

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The objective of the present study was to examine the effects of prenatal exposure to alcohol on sensorimotor coordination, emotionality, learning and memory in young adult Wistar rats. Most of the recent reports concerning behavioral effects of fetal alcohol exposure refer to the juvenile period of life and very few studies investigated different aspects of behavior simultaneously in the same subjects. In the current study, alcohol was delivered to the pregnant dams by intragastric infusions, throughout gestation days (GD) 7-20, at the dose of 6g /kg maternal body weight /day. This dose resulted in relatively high peak blood alcohol concentration (340 mg/dl) as assessed on GD 20. A pair-fed isocaloric and untreated control groups were included. Prenatal alcohol administration retarded dams' weight gain significantly, and had an adverse effect on pups' weight at birth but not in adulthood. No between-group differences were observed in the litter size and in the pups' mortality. The adult brain weight was neither affected. Pups were subjected to a series of behavioural tests as young adults (at 2.5 months of age). In adulthood, rats prenatally treated with alcohol were not impaired in sensorimotor coordination and/or did not show muscle weakness as assessed by rotarod/accelerod tests. Their behavior in the open field and plus maze suggested alcohol-induced increase in

anxiety level and some decrease in behavioral flexibility, but hyperactivity was not observed. In cognitive tasks, alcohol treated rats showed slightly slower rate of initial place learning in the water maze. However, memory retention tested after 1 and 10-day delay, reversal learning, rate of extinction of place preference, as well as working memory capacity appeared to be the same in alcohol exposed and control rats. The possible reasons of this negative result are discussed.

Key words: Prenatal Alcohol, Rat, Spatial Learning and Memory, Locomotor Activity, Emotionality

ÖZ

DOĞUM ÖNCESİ DÖNEMİNDE ALKOLE MARUZ BIRAKILAN WISTAR SIÇANLARDA AKTİVİTE, KAYGI VE ÖĞRENME ÜZERİNDEKİ ETKİLERİN İNCELENMESİ

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Bu çalışma, Wistar sıçanlarında doğum öncesi alkole maruz bırakılmanın sensorimotor koordinasyonu, duygu hassasiyeti, öğrenme ve hafızaya etkisini incelemeyi amaçlamaktadır. Fötal dönemde alkole maruz bırakılmanın davranışa etkileri üzerine günümüze kadar yapılan çalışmların çoğu ergenlik öncesi döneme yöneliktir ve çok az sayıda araştırma aynı deneklerde davranışın farklı yönlerini aynı zamanda çalışmaktadır. Bu çalışmada, sıçanlara hamileliklerinin 7-20 günleri arasında 6g/ kg vücut ağırlığı/ gün dozunda alkol besleme iğnesi yoluyla doğrudan mideye verildi. Bu dozla gebeliğin 20. gününde kanda oldukça yüksek alkol konsantrasyonuna (340 mg/ dl) ulaşıldığı gözlendi. Çalışma, alkolün veriliş yönteminden kaynaklanan, stres etkisine maruz bırakılan ve bırakılmayan kontrol gruplarını da içermektedir. Doğum öncesi dönemde alkole maruz bırakılma, hamile sıçanların kilo almasını anlamlı ölçüde geciktirdi ve yavruların doğum ağırlıklarını olumsuz yönde etkilemekle birlikte ergenlik ağırlıklarını etkilemedi. Bir batında doğurulan yavru sayısında ve yavru ölümünde gruplararası farklar görülmedi. Ergin bireylerin beyin büyüklüğünün de alkolden etkilenmeği görüldü. Yavrular 2.5 aylık

iken genç erginler olarak bir dizi davranış testine tutuldu. Doğum öncesi alkole maruz bırakılan sıçanlarda, rotarod/ accelerod testleriyle ölçüldüğü üzere, ergenlikte sensorimotor koordinasyon bozukluğu ve/veya kas güçsüzlüğü görülmemiştir. Açık alanda ve "+" labirentdeki davranışları, alkole bağlı olarak anksiyete seviyesinde bir yükselme ve davranış esnekliğinde biraz düşmeyi işaret etmektedir. Bununla beraber, hiperaktivite gözlemlenmemiştir. Kognitif testlerde alkole maruz kalan sıçanlar, su labirentinde "ilk yer öğrenme"de çok az olmakla birlikte daha yavaş bir hız gösterdiler. Bununla birlikte, 1 ve 10 gün gecikme ile test edilen hafıza güçlülüğü, platformun yerinin değiştirilmesinden kaynaklanan yeni yer öğrenme, yer tercihinin ortadan kalkma hızı ve kısa süreli hafıza kapasitesi alkole maruz bırakılan ve kontrol sıçanlarda hemen hemen aynıydı. Bu negatif sonuçların muhtemel nedenleri tartışıldı.

Anahtar Kelimeler: Doğum öncesi dönemde alkol, Sıçan, Mekansal öğrenme ve hafıza, Lokomotor aktivite, Duygu hassasiyeti

To My Family

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LIST OF ABBREVIATIONS

A	Alcohol Group
ACh	Acetylcholine
AChE	Acetylcholine Esterase
ADH	Alcohol Dehydrogenase
ANOVA	Analysis of Varience
ARBD	Alcohol Related Birth Defect
ARND	Alcohol Related Neurodevelopmental Disorder
BAC	Blood Alcohol Concentration
BACs	Blood Alcohol Concentrations
BDNF	Brain Derived Neurotrophic Factor
C	Nonintubated Control Group
CA1	Cornu Ammonis
CA3	Cornu Ammonis
ChAT	Choline Acetyl Transferase
CNS	Central Nervous System
E	East
EDC	Ethanol Derived Calories
EPSPs	Excitatory Postsynaptic Potentials
FAE	Fetal Alcohol Effect
FAS	Fetal Alcohol Syndrome
$GABA_A$	Gamma-Amino-Butyric Acid
GD	Gestational Day
HAS	High Alcohol Sensitivity
IC	Intubated Control Group
LAS	Low Alcohol Sensitivity
min	minute
MWM	Morris Water Maze
N	North

NBM	Nucleus Basalis Magnocellularis
NE	North-East
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartate
NW	North-West
p75	Low Affinity NGF Receptor
PN	Postnatal Day
rpm	Revolutions Per Minute
s	second
S	South
SE	South-East
SEM	Standard Error of Means
SPSS	Statistical Package for the Social Sciences
SW	South-West
Trk	High Affinity Tyrosine Kinase Family Receptor
W	West

CHAPTER 1

INTRODUCTION

1.1. Fetal Alcohol Syndrome

Alcohol, the leading known human teratogen, affects a variety of organ systems in both humans and laboratory animals. In 1968, Lemoine *et al.,* and in 1973, Jones *et al.,* and Jones and Smith described a set of specific human birth defects related to maternal alcohol consumption during pregnancy as Fetal Alcohol Syndrome (FAS). FAS is characterized by pre- and postnatal growth retardation, craniofacial defects and central nervous system (CNS) dysfunctions. Today, FAS is considered the most common nonhereditary cause of mental retardation.

FAS generally describes the most severe condition resulting from prenatal alcohol exposure. The term fetal alcohol effects (FAE) has been used to describe cases in which children exposed to alcohol prenatally do not meet all three of the diagnostic criteria of FAS. More recently, three terms have been developed to characterize children who were affected by alcohol prenatally but not meet all criteria for FAS. The term "partial FAS" refers to children with confirmed prenatal alcohol exposure and characteristic facial anomalies without full FAS. The term "alcohol-related birth defects" (ARBD) is used for children who have physical malformations or physiological abnormalities. Finally, the term "alcohol-related neurodevelopmental disorder" (ARND) describes children with either physical CNS abnormalities (i.e., smaller head size or structural brain abnormalities) or with behavioral and/or cognitive abnormalities, such as deficits in memory, language skills or learning abilities (Stratton *et al.,* 1996). Children exposed to prenatal alcohol abuse require special treatment. Therefore consumption of alcohol during pregnancy is an issue of widespread public concern.

1.2. Teratogenic Effects of Alcohol

Alcohol is lipid soluble and thus rapidly absorbed from the stomach and gastrointestinal tract following ingestion, and is evenly distributed throughout the fluids and tissues of the body and brain. It also 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 lack 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 and then 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).

The severity of the deficits may involve the interaction of several risk factors, such as the amount of consumed alcohol, duration and pattern of alcohol consumption, the timing of alcohol consumption relative to critical windows of vulnerability, or the inherent differential vulnerability among the various brain regions to alcohol-induced brain injury. The dose/duration of alcohol exposure has been identified as an important factor in alcohol teratogenesis. Human epidemiological studies have reported a dose dependency (Ernhart *et al.,* 1989), and animal studies have definitively confirmed a dose-response curve in the teratogenic effects of prenatal alcohol exposure (Chernoff, 1977). The minimum dose required to produce deficits or the amount of alcohol that can be "safely" consumed during pregnancy have not been established. It was demonstrated, however, that more critical than the absolute amount and duration of alcohol exposure is the blood alcohol concentration (BAC) achieved. A smaller daily dose of alcohol can be more damaging than a larger daily dose, if it is consumed in a binge-like pattern that produces relatively higher BACs.

Indeed, binge drinking was shown to be more harmful to the fetus than the same amount of alcohol consumed at a steady rate over an extended period of time (Pierce and West, 1986a). Also in inter-species comparisons of teratogenic alcohol effects, BAC rather than the absolute dose of alcohol provides a more meaningful estimate of compound bioavailability due to the inter-species differences in alcohol metabolism.

As mentioned earlier, the type and severity of alcohol-induced morphological anomalies and functional disorders may also be related to the period of alcohol exposure. The critical periods of alcohol exposure overlap with periods of greatest development and/or maturation of organ systems. For example, alcohol exposure in the first trimester is more often associated with organ and musculoskeletal anomalies while exposure in the second and third trimesters is linked to growth, intellectual and behavioral deficits (Aronson and Olegard, 1987). During the first three months of gestation in humans, the development of the facial and skull bones occurs. Thus, alcohol exposure occurring in the first trimester can result in the characteristic facial abnormalities observed in children with FAS. Conversely, alcohol exposure in the second or third trimester is more often associated with growth retardation and neurological defects because fetal growth and brain development occur more rapidly during those gestational stages. If maternal alcohol consumption occurs during all three trimesters, the fetus is exposed to alcohol during the critical periods for the development of facial characteristics, growth patterns and CNS function, and thus may develop full FAS.

1.3. Animal Models of Human FAE /FAS

Studies on developmental and neurobehavioral effects of fetal alcohol exposure are important for designing successful therapies of alcohol related dysfunctions in humans. Legal and ethical constraints on research with humans create the need for animal models to determine the effects of perinatal alcohol exposure.

Among different animal species, rodents and particularly rats have been most widely used in modeling fetal alcohol effects mainly because of the ease of handling, short gestation period and relatively low cost to purchase, house and feed (Keane and Leonard, 1989). The mechanisms of alcohol metabolism were shown to be similar in humans and rats, with the exception that rats as small endotherms have faster metabolic rate than man and, therefore, metabolize alcohol more quickly.

To date human and animal literature regarding neurobehavioral effects of perinatal alcohol exposure shows a great deal of inconsistence. The general categories of CNS dysfunction seem to be similar both in humans and rodents. They include deficits in cognitive performance, attentional deficits, hyperactivity, and motor disorders.

Numerous factors (independent variables) appear to contribute to the behavioral specificity of the neurotoxic effects of in utero alcohol exposure, including: alcohol dosage, duration of gestational exposure, pattern and route of administration, gender, species, and/or age of assessment. The combination of these factors makes the effects of prenatal alcohol exposure somewhat variable. Among above mentioned factors, the protocol of alcohol exposure seems to be of great importance.

1.3.1. Alcohol Exposure Protocols in Animals

Animals do not normally consume enough alcohol voluntarily to maintain chronically high blood alcohol concentrations (BAC) during pregnancy. As a result, many other ways of administering alcohol to the developing fetus have been introduced in addition to initially commonly used liquid diet. They include injections, inhalation, and intraoral / intragastric gavage or intubation. No method is ideal and each has its advantages and disadvantages.

As mentioned before, the primary method employed for administering alcohol to a pregnant animal has been by a liquid diet serving as the animal's sole source of nutrition. Alcohol is added to this diet either at a low concentration usually equivalent ~18% ethanol derived calories (EDC) or at a higher concentration usually equilavent ~35% ethanol derived calories (EDC). These alcohol concentrations result in daily alcohol intake of ~12 and ~18 g/kg/day respectively. This method generally includes two control groups. The first is pair-fed to either 18 or 35% alcohol group and receives a similar liquid diet with a carbohydrate i.e. sucrose, substituted for the alcohol (Berman and Hannigan, 2000; Driscoll *et al.,* 1990). This procedure equates the total daily caloric intake across groups and therefore, serves as a control for reduced caloric intake which is typical in the alcohol treated groups, and which might result in malnutrition that per se could affect fetus development. The second control group has continuous access to standard laboratory chow and water. If the alcohol group differs from both control groups, and the two control groups do not differ from each other, the effect may be attributed to alcohol intake. Alcohol administration with a liquid diet is more natural (Uzbay and Kayaalp, 1995), however, a basic disadvantage of this procedure is that there is a great individual variation in the consumption of alcohol-containing solutions, and thus variation in the blood alcohol concentrations among pregnant dams.

Another frequently employed method involves direct intraoral or intragastric intubation of dams with alcohol solutions, producing doses typically between 2-6 g/kg/day. In this procedure, alcohol is mixed with a vehicle and administered directly to the stomach via a feeding needle. To increase the portion of the day with elevated BACs, the absolute daily dose has occasionally been divided into two administrations. This method also includes two controls. One control is pair-fed to alcohol group and receives the same volume of fluid as the alcohol group via intubation, except that carbohydrate is substituted isocalorically for alcohol. The other control group has continuous access to standard laboratory chow and water (Berman and Hannigan, 2000; Driscoll *et al.,* 1990).

Intragastric intubation allows for more strict control of dosage and timing of alcohol administration. By administering alcohol via intragastric gavage, experimenter is able to administer the same amount of alcohol at approximately the same time to all animals, and is able to produce relatively high BAC over a sustained period of time, thus circumventing the problem of varying and fluctuating BACs due to individual differences in amount and pattern of alcohol consumption when provided with the liquid diet. The disadvantage of this method may be stress created by intubation. However, isocalloric control also controls the potential effect of stress related with animal manipulation during intubation. It might even be possible that, compared to the experimental group, control pair-fed animals are more stressed by the procedure since alcohol-treated subjects may be slightly sedated especially at time of second intubation.

Peak BAC appears more critical than the alcohol dose in determining the degree of severity of brain damage and behavioral deficits in rats prenatally exposed to alcohol (Pierce and West, 1986a,b; Kelly *et al.,* 1987; Bonthius and West, 1988, 1990; Bonthius *et al.,* 1988; West *et al.,* 1989). On the other hand, peak BAC has been shown to depend on the pattern of alcohol exposure. West *et al.,* 1989, carried out a series of experiments in which various doses of alcohol (6.6 to 9.8 g/kg in a milk formula) delivered during postnatal days 4-10 (a period of brain growth spurt in rat, equivalent to part of the human third trimester) were condensed into fewer and fewer hours each day. Condensing the dose produced higher BACs (345.8 +/- 15.6 mg/dl on postnatal day 6) for a given dose and produced more severe microencephaly, greater neuronal loss, behavioral hyperactivity and impaired spatial navigation. These data suggest that patterns of alcohol consumption that produce high BACs, such as binge drinking, may be especially harmful to the brain of the developing fetus. Pierce and West (1986b) showed that BACs above 425 mg/dl were lethal, while BAC threshold for producing microencephaly was between 140 and 197 mg/dl with female rats more vulnerable to adverse alcohol effects than male.

In the light of these results, an animal model of FAS allowing a strict control over the time and dose of alcohol administration and thus resulting in a predictable and sufficiently high BAC is better suited for the studies on the effects of prenatal and/or neonatal alcohol exposure on the nervous system development and behavior. Such model is provided by binge-like alcohol delivery via intra-oral or intra-gastric intubation.

1.3.2. Critical Period of Exposure to Alcohol in Animals

The animal models typically entail alcohol exposure during either the prenatal or early postnatal periods, which are roughly equivalent to the first two and third human trimesters respectively (Bayer *et al.,* 1993).

The fetal development of humans and rats follow similar stages, but differ in the relative timing of birth. For humans, the major brain growth spurt occurs during the third trimester of gestation and growth then continues for about two years postnatally (West, 1987). In contrast, the major brain growth spurt in the rat occurs during the first 10-14 days of postnatal life (West *et al.,* 1989). Due to this difference, both prenatal and postnatal alcohol exposure in rats have been used to address different types of research questions.

1.4. Effects of Chronic Exposure to Alcohol in Adult Life on Brain Morphology and Behavior

In adult rats, chronic alcohol intake was reported to produce damage to the basal forebrain cholinergic system, which is known to be involved in learning and memory (O'Keef and Nadel, 1978; Aggleton *et al.,* 1986, 1992; Connor and Thal, 1989; Connor *et al.,* 1991; Dunnet *et al.,* 1987; Etherington *et al.,* 1987; Hagan *et al.,* 1988; Markowska *et al.,* 1989, 1990).

Arendt *et al.,* (1988) reported that in adult rats, after alcohol intake (20% v/v) for 12 weeks, in the target areas of the basal forebrain cholinergic system: neocortex and hippocampus, there was observed reduction of choline acetyl transferase (ChAT) and acetylcholine esterase (AChE) activity to 74% and 81% of the control values respectively. It was accompanied by the reduction in the content of acetylcholine (ACh) down to 56% of control value in these brain areas. At the same time, substantial reduction (to 83% of control value) in the number of acetylcholine esterase (AChE) positive neurons was noted in the nucleus basalis magnocellularis (NBM). Lukoyanov *et al.,* (1999) also reported 18% cell loss in CA1 and 19% cell loss in CA3 hippocampal regions in the rats consuming alcohol at the average dose of 7.5 g/kg/day between 2 and 15 months of age.

Parallel to morphological damage, chronic alcohol consumption by adult subjects was reported to result in the impairment of memory and learning as measured by different tasks. Interestingly, it was shown that alcohol does not impair or impairs less the acquisition of nonspatial memory tasks and yet produces deficits in reference and working spatial memory, memory dependent on the integrity of the basal forebrain cholinergic system (Arendt, 1994; Fadda and Rossetti, 1998; Hodges *et al.,* 1991; Markwiese *et al.,* 1998).

1.5. Effects of Perinatal Exposure to Alcohol on the Brain Development

The developing brain is even more vulnerable to the deteriorating effects of alcohol. Alcohol exposure during brain development may be producing neuron attrition in multiple ways, including inhibition of protein synthesis, alterations in lipid solubility, and thus disruption of membrane integrity or disruption of cytoskeletal elements. The animal models typically entail alcohol exposure during either the prenatal or early postnatal period, which are roughly equivalent to the first two and third human trimesters respectively (Bayer *et al.,* 1993). It has been demonstrated that prenatal or early postnatal (neonatal) exposure to alcohol leads to

microencephaly with significant growth deficits in the cerebrum including basal forebrain, cerebellum, and brain stem of rats of either sex.

Morphological, neurochemical and electrophysiological studies suggest that among forebrain structures the hippocampal formation is one of the brain regions most sensitive to the teratological consequences of prenatal exposure to alcohol.

1.5.1. Morphological Studies

Prenatal alcohol-induced changes in the hippocampus morphology include permanent loss of pyramidal neurons, decrease in the dendritic spine density on the pyramidal cells, alterations in synaptic circuitry, and decreased morphological plasticity in this structure. Barnes and Walker (1981) reported for instance that administration of 35% EDC liquid diet through GD 10-21 to Long Evans dams resulted in 20% reduction in hippocampal pyramidal cells as assessed on PN 60, interestingly, with no change in the dentate gyrus.

West *et al.,* (1987); Bonthius and West (1991) observed brain weight deficits (microencephaly) and neuronal losses within both cerebellum and hippocampus. However, these deficits were still recorded in the adult brain (PN 90) only after condensed administration of alcohol to the pups during brain growth spurt on PN 4 through PN 9. In this study, in the hippocampus, only the CA1 pyramidal cells were significantly reduced in number and only in the group receiving the most condensed alcohol treatment producing highest BAC (around 400 mg/dl). In the cerebellum, the severity of Purkinje cell and granule cell losses varied among lobules, and Purkinje cell vulnerability appeared to depend on the maturational state of the neuron at the time of the alcohol exposure, with, interestingly, more mature Purkinje cells being more vulnerable.

Several authors (Kelly *et al.,* 1987; Goodlett *et al.,* 1991) observed significant reductions in the whole brain and cerebellar weight after neonatal (PN 4-10) alcohol intoxication (6.6 g/kg/day).

However, cerebellar and hippocampal damage did not always occure together. Pierce *et al.,* (1989) as well as Maier and West, (2001) reported cerebellar damage with unaffected cell densities in hippocampal formation after respectively early postnatal (PN 4-10), and prenatal (GD 1-20) exposures to alcohol applied at the doses varying between to 2.25 –6.5 g/kg/day, with brains examined on the PN 10. Such results may indicate varying vulnerability of different brain structures to alcohol abuse, with cerebellum more susceptible to deleterious alcohol effects as compared to hippocampus, showing degeneration already after a single alcohol dose delivered intragastrically on postnatal day (PN) 4 (Light *et al.,* 2002). Some studies, however, point towards the possibility that neural plasticity may be effective in promoting recovery in hippocampus by replacing lost neurons by either newborn cells or by abnormal cell migration, or both. (Ferrer *et al.,* 1988; Miki *et al.,* 2003).

Nevertheless, in the light of the latter findings, the issue of critical periods for alcohol deleterious effects is of special importance. Literature regarding critical periods for the effects of alcohol exposure on brain development in rats bears, however, contradictory results. Miller (1995) compared the effects of prenatal (GD 6-21) and neonatal exposure (PN 4-12) to alcohol resulting in intermediate BACs (144 and 231 mg/dl respectively). Histological examination of the rats' brains carried out on the PN 30-35 revealed reduced neuron numbers in hippocampal CA1 area only after prenatal alcohol treatment. Conversely, in similar study, Livy *et al.,* (2003) reported the largest decrease in the brain weight/ body weight ratio (as compared to pair-fed controls) and significant reduction in the neuron numbers in the CA1 and CA3 regions of hippocampus only in PN 4-9 group with prenatal alcohol exposure remaining ineffective.

Disparate findings regarding critical periods for perinatal alcohol effects may arise from the fact that apart from using different rat strains investigators employed

different methods of alcohol administration, which may not be equivalent in producing central nervous system dysfunctions. During prenatal exposures to alcohol either liquid diet or intragastric intubation were applied. These methods even when total daily dose of alcohol is similar produce different BACs, which actually determine the severity of alcohol effects. On the other hand, neonatal alcohol exposure has commonly been accomplished via artificial rearing (Light *et al.,* 1998) allowing for strict control of alcohol dose and time of alcohol administration and thus BACs, but blood alcohol levels obtained with this method are usually much higher than with the liquid diet procedure. Interestingly, in the study BACs were high, consistent, and comparable across both pre- and postnatal periods, at the behavioral level, no differences in the pre- or postnatal short-term exposure groups were detected, this argues against critical period suggesting rather that the adverse effects of alcohol may accumulate during whole development (Kim *et al.,* 1997). On the other hand, linear regression analysis between peak blood alcohol concentration (BAC) and percent reduction in cerebellar Purkinje cells covering different postnatal days: PN 4, PN 5, PN 6, PN 7, and PN 8 or beyond (+) indicated that PN 4-6 period constitutes the most vulnerable window for alcohol-induced loss of Purkinje cells (Pierce *et al.,* 1999).

It has been demonstrated that even when no significant reduction in cell numbers was observed intrauterine exposure to alcohol produced a dramatic change in the topography of hippocampal mossy fibres. An aberrant distal infrapyramidal mossy fibre terminal band was present at mid-temporal levels (where it does not normally occur). This anomaly in fiber distribution endured until adulthood and was detected on PN 60. Rats exposed to alcohol after birth showed even greater aberrations in the mossy fibre terminal field than rats exposed to alcohol in utero (West *et al.,* 1984; West and Pierce, 1984; West *et al.,* 1981).

1.5.2. Molecular Studies

Alcohol-induced changes in the hippocampus have been described also at the molecular level. Prenatal exposure to alcohol was reported to decrease sensitivity of the adult rat (PN 70-90) hippocampus (CA1 area) to NMDA (Morriset *et al.*, 1989), producing also alterations in the expression of hippocampal $GABA_A$ receptor and its pharmacological properties (Iqbal *et al.*, 2004). Alterations in receptor functions may affect signals transmission in the hippocampus and contribute to hippocampal-related behavioural deficits observed after perinatal alcohol exposure.

It has been proposed by some researchers that neurodegeneration reported after perinatal alcohol intoxication can be partially related to alcohol-induced interruption of neurotropic support leading among other effects to activation of the mitochondrial pathway of apoptosis. In line with this notion it has been reported that chronic but even acute (single intragastric alcohol infusion on the GD 15) prenatal administration of alcohol leads to decreased expression and decreased brain levels of neurotrophins such as NGF and BDNF (Tapia-Arancibia *et al.*, 2001; Angelucci *et al.,* 1997, 1999; Climent *et al.,* 2002). Chronic alcohol intake during gestation and/or lactation was also shown to decrease expression of p75, low affinity NGF receptor (Seabold *et al.,* 1998), and increase the ratio of truncated to full-length TrkB receptors in the developing cerebral cortex (Climent *et al.,* 2002). These changes are accompanied by reduction in neurotrophin-activated extra- and intracellular signal transduction pathways leading to increased loss and/or dysfuction of cholinergic neurons known to be dependent on neurotrophin support. Reduction in the number of cholinergic neurons in the basal forebrain gives rise to the cholinergic deafferentation of the hippocampus and cortical mantle.

Some of the studies demonstrated direct relation between chronic adult or perinatal alcohol intake and expression of cholinegic phenotype by the basal forebrain cholinergic neurons. It was shown that degenerative changes in the basal forebrain are paralleled by the concomitant reduction of presynaptic cholinergic markers

including activity of ChAT, as well as content and release of acetylcholine in the neocortex and hippocampus (Arendt, 1994; Arendt *et al.,* 1988).

1.5.3. Electrophysiological Studies

Adverse effects of alcohol intake on the central nervous system morphology inevitably result in functional deficits and anomalies in affected neuronal groups. In line with this prenatal alcohol exposure was reported to change the hippocampal electrophysiology. It was shown that prenatal alcohol exposure caused augmentation of paired-pulse facilitation representing one type of short-term hippocampal plasticity (Hablitz, 1986; Tan *et al.,* 1990). This effect can be still observed in adult rats (PN 60-90) and can be explained by alcohol-induced decrease in recurrent inhibition due to reduced numbers of inhibitory GABAergic interneurons, changed pattern or number of connections between principal pyramidal cells and interneurons, or altered GABAergic transmission. It was also postulated that prenatal exposure to moderate alcohol levels can produce a long-lasting deficit in synaptic enhancement in neural pathway believed to be critical in certain forms of learning and memory. It was manifested by decreased amplitude of field EPSPs and population spikes (Sutherland *et al.,* 1997; Krahl *et al.,* 1999). Cortese *et al.,* (1997) examined the effects of prenatal alcohol exposure on hippocampal theta activity in adult rats. The 5 g/kg male group demonstrated a significantly different theta score than controls, indicating either an increase in type I (movement-associated) theta and/or a decrease in type II (information-processing) theta activity. These results are consistent with prior reports that prenatal alcohol exposure alters hippocampal function.

1.6. Effects of Perinatal Exposure to Alcohol on Behavior

1.6.1. Effects on Locomotor Activity, Exploration and Anxiety

Behavioral dysfunctions observed in juvenile and/or adult subjects are, perhaps, one of the most sensitive indicators of the effects of perinatal alcohol exposure on the developing fetus. As mentioned earlier, they include motor dysfunctions, hyperactivity, increased distractibility, deficits in behavioural inhibition and behavioural flexibility, and eventually impairments in learning and memory although correlation between potential alcohol-related behavioural deficits, threshold alcohol doses, time windows of vulnerability during brain development, and eventually underlying mechanisms of neuroteratogenity are not quite clear. Their elucidation requires further investigations. In addition, literature related to behavioural effects of perinatal alcohol exposure brings many inconsistent results. The discrepancies in the effects of the fetal alcohol on behaviour have the same reasons as discrepancies in the effects of perinatal exposure to alcohol on brain morphology and functional characteristics of neural assemblies described earlier. Below, there is presented a short review of the related literature.

The most often mentioned motor dysfunction linked to prenatal intoxication with alcohol is locomotor hyperactivity. Locomotor hyperactivity is typical to FAS in man and was observed in animal models of FAS. In animal studies, however, locomotor activity is usually examined in the open field wherein, animals' ambulation is affected by contradictory tendencies such as tendency to explore a new environment and anxiety or even fear arising from the contact with the new environment. Therefore, reliable interpretation of the open field results is possible only when changes in activity scores are carefully analysed across the few consecutive sessions in the open field, and when activity data is complemented by defecation scores and/or description of animal behaviour in a task such as plus maze especially designed to measure anxiety level. Most of the studies are lacking such

careful comparisons. Nevertheless, when intermediate dose of alcohol was administered through the liquid diet to pregnant dams during the whole gestation period, increased locomotor activity was observed at PN 28 and 56 but not at PN 112 (Bond and Di Giusto, 1977). In this study, no differences between treatment groups were noted in defecation. In the line with the latter finding, increased activity in the open filed and increased frequency of inter-trial responses in the shuttle box avoidance task was reported in rats exposed to alcohol through part of gestation (GD 10-14) and tested at PN 63-65 (Osborn *et al.,* 1980). Conversely, Randal and Hannigan (1999), despite of binge-like alcohol administration (always producing higher peak maternal BAC) throughout the longer period of gestation (GD 8-20) did not observed between- group differences in locomotor activity when testing at PN 90-150. On the other hand, however, when high to moderate alcohol doses (6,6-4,5 g/kg) were applied to neonatal pups (equivalent of human third trimester) hyperactivity was observed not only in juvenile but also in adult rats (PN 90) (Kelly *et al.,* 1987; Tran *et al.,* 2000). Commonly, higher activity in alcohol-exposed rats was observed in juvenile subjects regardless of the dose and period of alcohol administration (Melcer *et al.,* 1994, Abel and Reddy, 1997). Bond (1985,1986 a,b) in series of elegant experiments examined modulatory effects of agonists and antagonists of three principal neurotransmitters: dopamine, serotonine, and acetylcholine, on hyperactivity in pups born from dams receiving liquid diet containing 35% ethanol-derived calories through GD 6-19. In these studies, offspring prenatally exposed to alcohol were hyperactive compared to controls at 16 and 22 days, but not at 10 or 28 days. The time courses of the effects on activity of d-amphetamine (dopamine agonist), α-methyl-paratyrosine (catecholamine synthesis inhibitor), and parachlorophenylalanine (inhibitor serotonine synthesis) were the same regardless of the treatment received during gestation. On the contrary, effects of scopolamine (ACh receptor blocker) depended on prenatal treatment. Scopolamine appeared to have no effect on activity in any group at 10 days. At 16 days it reduced activity in the alcohol treated offspring but had no effect on the controls. At 22 days, it led to a dose-related increase in activity in controls but had no effect on the already high levels of activity in the alcohol treated pups.

At 28 days, scopolamine increased activity in all three groups. On the basis of these results it was postulated that hyperactivity associated with fetal alcohol exposure is likely to arise from alterations in the ontogeny of cholinergic/inhibitory system and that this system becomes functional in control pups before 22 days, but in pups exposed to alcohol prenatally development is delayed by a number of days.

As mentioned earlier, activity when measured in the open field apparatus may be confounded by changed levels of anxiety, which should additionally be controlled by concomitant testing in, for instance, the elevated plus maze. There is, however, very little available data regarding potential effect of perinatal alcohol exposure on the animals' behaviour in the plus maze.

Alcohol-induced hyperactivity manifested itself not only in the open field or activity box but also in other experimental situations and behavioral tasks, wherein, depending on whether it was compatible (active avoidance) or incompatible (passive avoidance) with the task requirements, led to improvement or impairment in the animals' performance. In a series of experiments, Riley *et al.,* (1979a) showed increased nose-poking and head-dipping behaviours in juvenile rats born from dams receiving different doses of alcohol throughout gestation (8-35% EDC in the liquid diet). Impairment in the passive avoidance task and in the conditioned taste aversion to a lithium chloride solution were also observed in both sexes of juvenile rats (Riley *et al.,* 1979b; Lochry and Riley, 1980; Abel, 1982; Barron and Riley, 1990), with females apparently more susceptible to perinatal alcohol-induced deficit in response inhibition. These deficits do not appear to persist into adulthood (Abel, 1982). Prenatally exposed to alcohol pups were also deficient in spontaneous alternation and in reversal learning in a T-maze shock-escape paradigm showing tendency for perseveration of previously executed responses (Riley *et al.,* 1979c). The results are interpreted in terms of an alcohol-induced delay in the development of a central inhibitory system.

1.6.2. Effects of Perinatal Exposure to Alcohol on Learning and Memory

So far, the attempts to elucidate the effects of perinatal alcohol exposure on learning abilities and memory have been undertaken by several different research groups with alcohol effects being examined under different experimetal pradigmes in juvenile and adult offspring of both sexes and of different strains. However, many contradictions exist in the body of related literature. These discrepancies in the experimental results regarding behavioral effects of perinatal alcohol intake arise from the same reasons as earlier discribed disparate findings related to the effects of fetal alcohol exposure on brain morphology. As mentioned before, disparity of the experimental results may be explained by the fact that behavioral outcome of perinatal exposure to alcohol depends on multiple factors such as: applied dose and duration of alcohol administration (and created by them peak BAC), period of exposure, age, gender, and even strain of tested animals, and eventually cognitive demands and degree of difficulty of the employed behavioral task.

As it was shown earlier, neuroanatomical and physiological studies demonstrated that perinatal alcohol alters neurobiology of specific brain regions with cerebellum and hippocampus beeing most affected. It is not surprising then, that, as it has been demonstrated by numerous neurobehavioral studies, animals exposed prenatally to alcohol were impaired in many of the same spatial learning and memory tasks sensitive to hippocampal damage, including place discrimination as well as spontaneous and conditional alternation in T-maze (Thomas *et al.,* 1996; Nagahara and Handa, 1997; Zimmerberg *et al.,* 1991; Lee and Rabe, 1999), place learning in the Morris Water Maze (MWM) (Blanchard *et al.,* 1987; Goodlett and Peterson 1995; Pauli *et al.,* 1995; Westergren *et al.,* 1996; Gianoulakis, 1990; Kim *et al.,* 1997 ;Tomlinson *et al.,* 1998; Girard *et al.,* 2000) and food-rewarded spatial navigation in the radial arm maze (Reyes *et al.,* 1989; Neese *et al.,* 2004). Similarly like after the hippocampal damage, here too performance on nonspatial tasks seemed to be less affected (Kim *et al.,* 1997, Means *et al.,* 1986). Apart from the fact that hippocampus is thought to be much less implemented in forms of learning

that do not contain spatial components, it has been postulated that nonspatial tasks are easier than the spatial ones, thus reducing the chance that moderate doses of alcohol will have an effect. In line with this notion it has been demonstrated that perinatal exposure to alcohol had deteriorating effect on animal performance in some nonspatial tasks having a higher level of difficulty. Thus, the impairments caused by perinatal alcohol can be tighed in the first place to the cognitive demands of the task (i.e. the spatial demands) but also to the level of task difficulty.

Animal literature regarding deleterious effects of perinatal exposure to alcohol is consistent with similar human literature. Children prenatally exposed to alcohol were reported to suffer from serious cognitive deficits and behavioral problems as well as from alcohol-related changes in brain structure. Brain imaging studies have identified structural changes in various brain regions of these children, including the basal ganglia, corpus callosum, cerebellum, and hippocampus, that may account for the cognitive deficits (Mattson *et al.,* 2001). The alcohol-exposed children were impaired on the free recall task but not on the recognition memory task (Mattson and Riley, 1999), and additionally they were shown to be impaired at place learning but not cued-navigation in a virtual Morris water task (Hamilton *et al.,* 2003).

Not always, however, perinatal exposure to alcohol was reported to have adverse effect on animal cognitive functions. Similarly as in the case of locomotor hyperactivity, severity of alcohol-induced cognitive impairments depend on the age of testing and the lack of any effect was recorded more often when rats were tested as adults than when they were tested as juveniles. Abel (1979), for instance, observed deficits neither in active two-way avoidance task nor in the MWM task in 5 months old Long Evans rats exposed throughout the whole gestation to a daily dose of alcohol of 4 or 6 g/kg maternal body weight, delivered via intragastric intubation and resulting in maternal peak BAC of 150 and 262 mg/%, respectively. Minetti *et al.,* (1996) did not observe significant differences between experimental and control groups in the MWM in both juvenile and adult Wistar offspring (PN 45 and 90), but this time dams were subjected to acute exposure to alcohol in the form

of two interperitoneal injections (2.9 g/kg each) on GD 8. Hannigan *et al.,* (1993) also did not observed any impairment in the initial acquisition of place discrimination in the classical MWM. Some impairment was observed only during reversal learning when platform was shifted to a new position after 4 days of training on the original task. Similarly, Lee and Rabe (1999), when testing adult (PN 80) Long Evans rats prenatally exposed to alcohol training in T-maze place discrimination, reported no change in the task acquisition, and only some deficit during reversal training. Impairment in the MWM performance in juvenile rats prenatally exposed to alcohol reported by Blanchard *et al.,* (1987), was confined to longer swim distance, both during initial and reversal training, indicating altered search pattern in these animals rather than deficit in learning per se. Cronise *et al.,* (2001) testing in their experiments different age groups (PN 23-24, and PN 120-121), observed impairment in the place learning in the MWM only in juvenile rats despite of prolonged alcohol exposure covering whole gestation and eight postnatal days and binge-like alcohol administration via intragastric intubation demonstrated to produce high peak BAC. In other studies, when alcohol was administered with liquid diet (35% EDC) during the last week of gestation, offspring from all three age groups: PN 38-44 (juvenile), PN 82-89 (young adult) and PN 173-180 (adult) were shown to be impaired in food rewarded alternation in T-maze with 30s and 60s delay between consecutive runs. However, only juvenile rats were impaired on this task with the shortest 10s delay (Nagahara and Handa, 1997). Conversely, Gianoulakis (1990) and Westergren *et al.,* (1996) found that rats born from dams receiving alcohol containing liquid diet (35% EDC) throughout the whole or at least second half of the gestation period were impaired in their performance in the MWM in adulthood (at 3 and 6 months of age respectively). In general however, it can be concluded that deteriorating behavioral effects of perinatal alcohol exposure are more pronounced in the young age. There is some evidence that they may re-appear again in elderly subjects. The transient character of alcohol-induced cognitive deficits raises the question whether perinatal exposure to alcohol produces developmental delay rather than developmental dysfunction. Dissipation of alcohol effects with maturation would argue for the first possibility. On the other hand,

persistence of alcohol effects in some cases would argue for the second possibility. If the latter is true, dissipation of alcohol effects in adult subjects may be related with some regeneration occurring in the central nervous system (some evidence of such regeneration was presented earlier) or compensation at the behavioral level by employing new strategies. This issue requires further investigations.

Several research groups attempted to determine time windows of the highest vulnerability to deteriorating behavioral effects of perinatal exposure to alcohol by administering alcohol during different periods of gestation and lactation. Neese *et al.,* (2004) when testing adult offspring of both sexes born from dams receiving alcohol containing liquid diet (35% EDC) during selected periods of gestation (GD 1-7, GD 8-14, or GD 15-21) found largest deficit in the spatial memory as assessed in the radial maze, after exposure to alcohol during last week of gestation (GD 15-21). Conversely, when the spatial memory was assessed in the classical MWM after binge-like alcohol administration via intragastric intubation through gestation days 1-10, 11-22, postnatal days 2-10, or all three intervals, only juveniles (PN 19-31) born from dams receiving alcohol throughout the whole pre- and postnatal period demonstrated impaired performance, however, this deficit was found to be transient because no differences were observed among adults (PN 90-103) (Cronise *et al.,* 2001). On the other hand, when memory deficit was assessed after different exposures during early postnatal period (PN 4-6, PN 7-9, PN 4-9), male juvenile offspring (26 days of age at the beginning of the training) were shown to be affected after exposure covering PN 7-9 when tested in MWM, but required longer exposure covering postnatal days from 4 through 9 to be impaired in conditional alternation in T-maze (Goodlett and Peterson, 1995; Thomas *et al.,* 1996). MWM in its classical version is the test for spatial reference memory, while conditional alternation (alike spontaneous alternation) in the T-maze, as well as radial arm maze are tests for spatial working memory. The latter results may suggest that spatial working memory is more vulnerable to fetal alcohol effects than spatial reference memory, whereas spatial reference memory is more vulnerable to neonatal alcohol effects than the spatial working memory.

In some studies, however, it has been demonstrated that even an acute exposure to alcohol during the brain growth spurt (early postnatal period thought to be an equivalent of the human third trimester) is able to produce significant cognitive impairment as assessed by the place learning in the MWM (Pauli *et al.,* 1995). In these experiments, pups were exposed to 7.5 g/kg body weight of alcohol administered as a 10% solution via an intragastric cannula over an 8 h period either on the 5th (PN 5) or the 10th (PN 10) postnatal day of age. Both the PN 5 and PN 10 alcohol treated groups when tested at PN 41-54 had significant deficits in their spatial learning in the MWM as compared with the control groups with no significant difference in the degree of impairment between the PN 5 and PN 10.

Animal gender is another factor that may influence behavioral effects of perinatal exposure to alcohol. There is, however, small number of works studying both males and females especially as adults. Adult females are not readily taken into experiments on learning and memory to avoid fluctuations in mnemonic capacity related to estrus cycle and changing estrogen levels. From the available literature it is seen that when both males and females were tested as juveniles after prenatal exposure to alcohol, no differences between sexes were observed in the acquisition and reversal of place discrimination in the MWM (Blanchard *et al.,* 1987; Cronise *et al.,* 2001). Juvenile males, however, were reported, to be more susceptible to neonatal alcohol intoxication than juvenile female as assessed by their performance in the MWM (Goodlett and Peterson, 1995). When tested as young adults (at PN 80), after prenatal exposure to alcohol throughout the whole gestation (35% EDC in the liquid diet), females were shown to be more impaired than males in the reversal (but not in the acquisition) of T-maze place discrimination carried out under water deprivation (Lee and Rabe, 1999) indicating towards faster recovery in males. The available results regarding differences between sexes in the susceptibility to deleterious effects of fetal alcohol exposure when tested in the spatial working memory tasks are contradictory. Zimmerberg *et al.,* (1991) found adult male offspring more impaired on the working memory task in the T-maze after fetal exposure to alcohol (through whole gestation) via the liquid diet (35% EDC).

Conversely, Neese *et al.,* (2004) reported lower performance in the adult female offspring exposed to alcohol via liquid diet (35% EDC) through GD 15-21 when tested in the radial arm maze. These disparate results may arise from the differences in the applied tasks. Alcohol exposed female rats might have had altered lateralization of responses as compared to male subjects what would have differentially affected their performance in the T-maze alternation task.

In summary, from the literature related to the neurobehavioral effects of perinatal alcohol exposure it is vivid that alcohol intoxication during brain development is affecting a variety of behaviors such as motor activity, exploration, anxiety, learning and memory although experimental results not always are consistent and correlation between effects of perinatal alcohol on different aspects of behavior is difficult due to the examination of these effects by different research groups under varying experimental protocols, in animals of different strain, age, and gender. The issue of endurance of the deleterious behavioural effects of perinatal alcohol exposure into adulthood is also still open. In the present study, effects of prenatal exposure to the relatively high dose of alcohol on different types of behavior were examined in the same group of rats tested as young adults.

1.7. Aim of the Study

The aim of the present study was to examine the possible effects of prenatal exposure to alcohol on sensorimotor coordination, emotionality, learning and memory in the same group of young adult Wistar rats, applying intragastric method of alcohol administration.

CHAPTER 2

MATERIALS AND METHODS

2.1. Subjects

Large number of 2.5-3 months old, naive, male and female Wistar rats, obtained from the Hıfzısıhha Serum-Production Facility (Ankara), were used for breeding in the present study. Breeding, alcohol delivery to the pregnant dams, rising the pups, and three first behavioral test (Open Field, Rotarod/ Accelerod, and Plus maze) were done in the Psychopharmacology Research Unit of Gülhane Military Medical Academy (GMMA), Ankara. Memory testing took place at the Department of Biological Sciences, Middle East Technical University (METU). Throughout the experiments, rats were kept in Psychopharmacology Research Unit in GMMA and then in METU, with controlled temperature (22 ± 1 °C), under 12 h/12 h light/dark cycle (lights on at 07:00 h, lights off at 19:00 h), and with free access to food (laboratory chow) and water. Only male offspring was taken to the behavioral tests. Females were not used as subjects to avoid potential confound arising from fluctuations in learning that may correspond to estrus-related changes in brain plasticity (Warren and Juraska, 1997). Tests were carried out in the light phase of the light/dark cycle.

2.2. Breeding

Female rats were individually housed in Plexiglas cages with wood shaves. One week prior to mating, male and female cages were put next to each other. For mating, a male rat, picked at random, was placed into a female's cage. Rats were mated nightly until a vaginal plug was observed on the following morning (Figure 2.1).

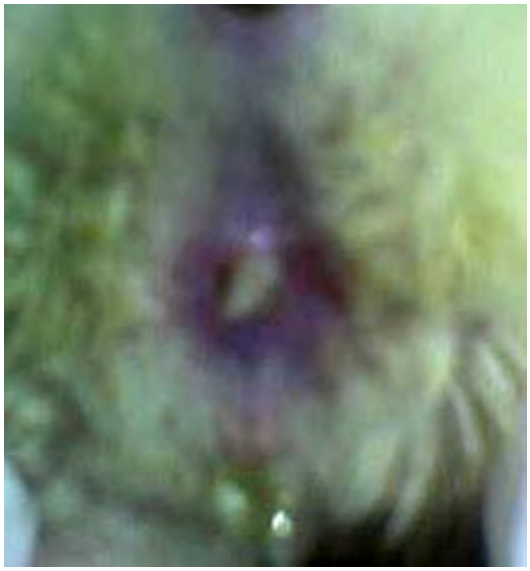

Figure 2.1. The appearance of a vaginal plug.

The presence of a vaginal plug was used as evidence of successful fertilization and this day was marked as Gestational Day (GD) 0. At this time, the male was removed and the female left undisturbed in its home cage with free access to laboratory chow and water until treatment started. Each of the pregnant dams was weighed between GD 6 to GD 20.

2.3. Diet

Alcohol was delivered to pregnant dams between 7-20 Gestational Day (GD), inclusive, to omit sever developmental defects that could have occurred if high dose of alcohol is delivered during the first week of gestation, the period of organogenesis. Alcohol can be delivered either with a liquid diet or by intragastric intubation. Alcohol administration with a liquid diet is more natural, however, great individual variation in the daily diet consumption has been observed. This gave rise to a variation in the dose of alcohol delivered to individual dams. For this reason, alcohol was administered via intragastric intubation.

Figure 2.2. The moment of intragastric intubation.

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, dams in the alcohol-treated group 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 as the alcohol group, except that sucrose was substituted isocalorically for alcohol. Animals in C group received ad libitum access to laboratory chow and water with no additional treatment. The alcohol administration protocol was strictly timed so that the dams received the alcohol at the same time each day. Alcohol and isocaloric sucrose solution was divided into two equal doses given to animals one 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 facilitate the absorption of the alcohol, and it 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. The alcohol was prepared daily as a 20 %

(weight/volume) solution mixed with distillated water and stored at room temperature. Alcohol was delivered by intragastric gavage using stainless curved feeding needle directly into stomach of the dam (Needle, Curved, 18ga, 3 in, Stoelting Co. USA). All treatments for Alcohol Group and Intubated Control Group were completed on GD 20.

2.4. Pups

At birth, the number of pups in each litter was counted. The day of birth referred to as postnatal day 0 (PN 0). Body weight of pups was recorded on PN 0, 5, and 10. They remain with their natural mothers until weaning. 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). Pups weaned at 30-35 days of age. At that time, male and female pups were removed from the litters and housed separately in groups of 4 per cage. The average age of the animals when training began was 82 days (ranging from 80 to 85), whereas the animals' average age at the last test day was 145 days (ranging from 142 to 147).

Figure 2.3. The new born pup.

2.5. Apparatus

Noncognitive variables can influence performance on the cognitive tasks. Although a test may accurately reflect memory, it inevitably reflects many other psychological processes such as sensorimotor coordination, emotionality or sensory perception. For more reliable interpretation of the results obtained in cognitive tasks, the other aspects of the behavior must be also tested.

2.5.1. The Open Field

The open-field (Figure 2.4) is a simple apparatus that can be used to measure motor activity, but also to assess exploratory drive, and/or anxiety. Animals' motor activity was measured by open-field activity monitoring system (MAY 9908 model, Activity Monitoring System, Commat, TR). This system comprised of eight Plexiglas cages (42 × 42 × 30 cm) equipped with infrared photocells. Fifteen photocell emitter and detector pairs were located 2 cm above the floor at intervals of 2.5 cm on both counter sides of each activity cage, and another 15 photocell pairs were located 8 cm above the floor. Interruptions of photocell beams were detected by a computer system and place of animal was calculated by the software at 0.1 s sensitivity. If a calculated place was changed completely, then it presented the ambulatory activity. Other behaviors that caused interruptions of beams, but not a change in place, are represented by the horizontal activity (i.e. grooming), and vertical activity (i.e. rearing).

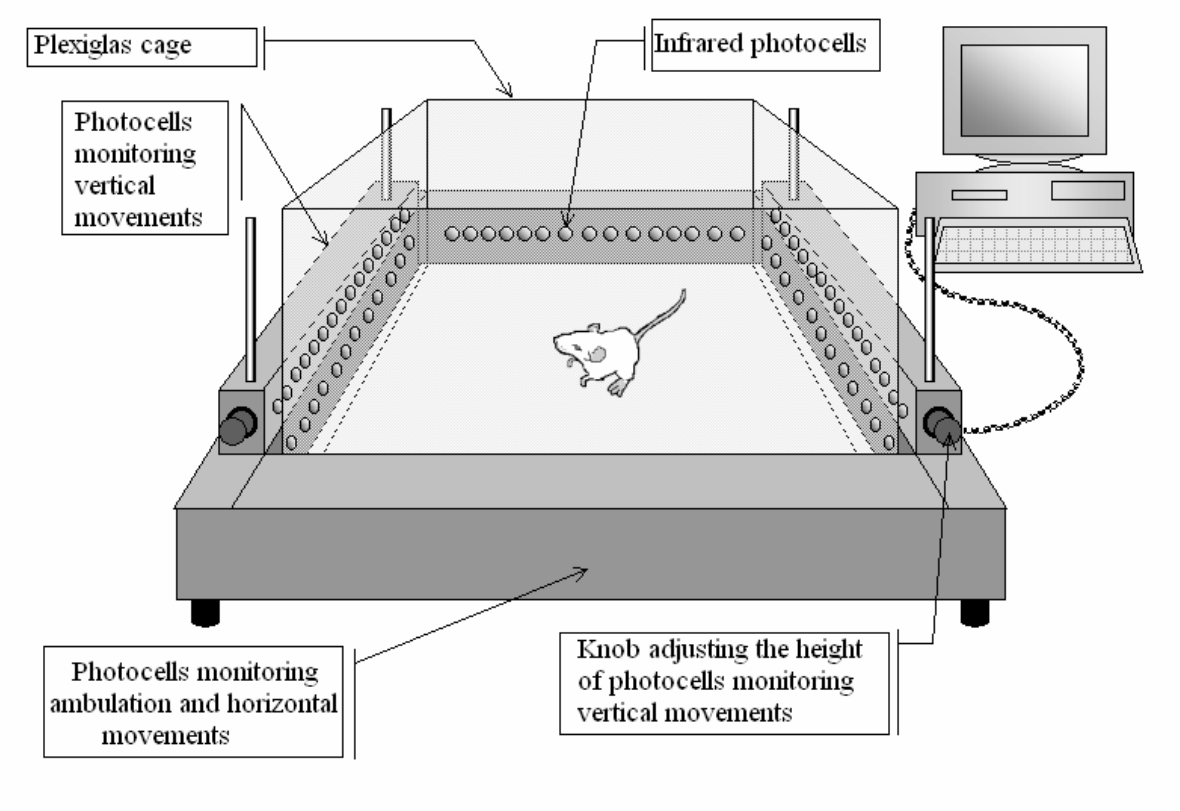

Figure 2.4. Open Field Apparatus (Adopted from Uzbay, 2004).

2.5.2. Plus Maze

The plus maze (Commat, TR, Figure 2.5) is specifically designed to measure anxiety. It is constructed of polyester and consisted of a central platform (10 × 10), two open arms (50 × 10) and two closed arms (50 × 10) with black Plexiglas walls extending 30 cm high and no ceiling. The arms are arranged in a plus shape with the two open arms facing each other and two closed arms facing each other. The maze is positioned 45 cm above the testing room floor.

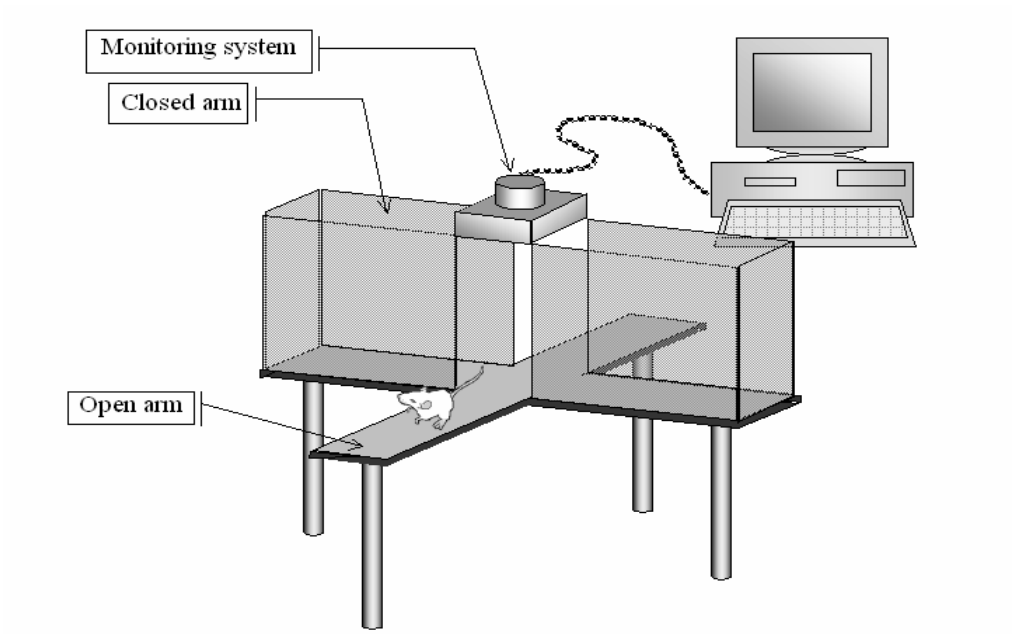

Figure 2.5. Plus Maze Apparatus (Adopted from Uzbay, 2004).

2.5.3. Rotarod / Accelerod

Rotarod/accelerod apparatus (Columbus Instruments, USA; Figure 2.6) is used to assess animals' sensorimotor coordination. The size of the rotating cylinder is 6.5 cm diameter. The speed of rotation is stable (rotarod) or gradually increased (accelerod). Four animals can be tested simultaneously.

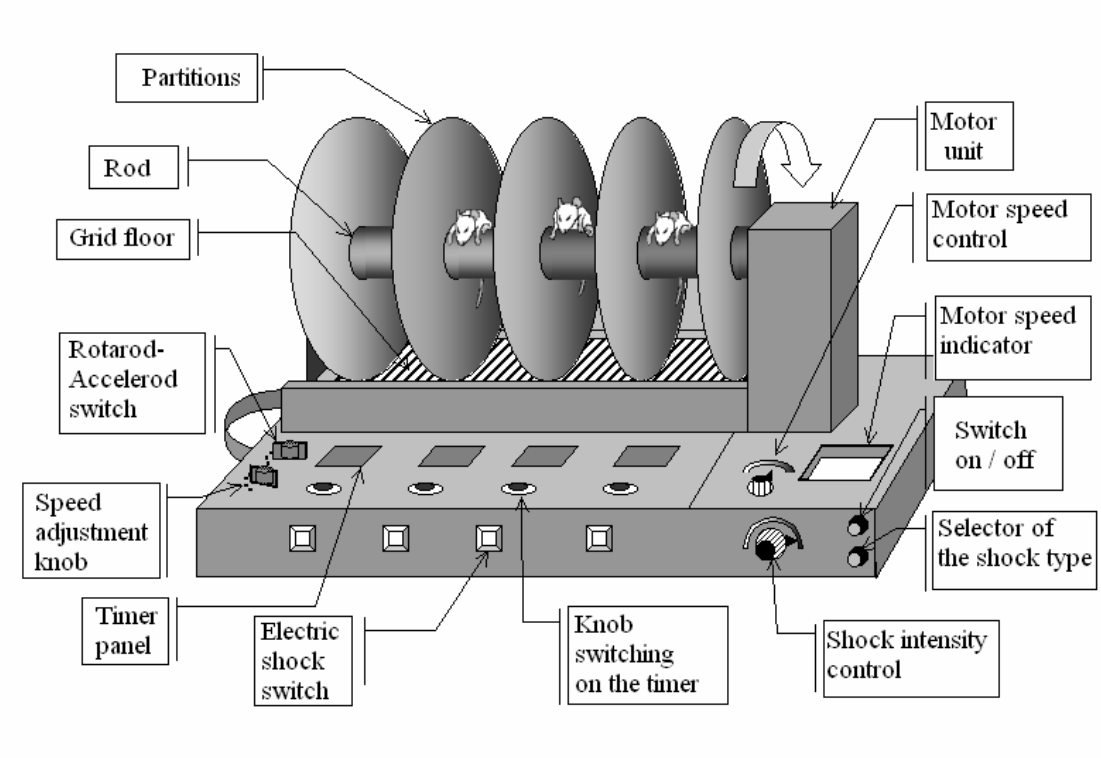

Figure 2.6.Rotarod / Accelerod Apparatus (Adopted from Uzbay, 2004).

2.5.4. Morris Water Maze

Figure 2.7. Morris Water Maze Apparatus.

Morris Water Maze (MWM) is commonly used to test spatial learning and memory in rodents. It is a circular tank, 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. Nontoxic watercolor paint was added to make water opaque. Computerized video tracking system (EthoVision System by Noldus Information Technology, Holland) was used to track the animal in the pool and to record data. On the computer screen, the pool was divided into four quadrants by two imaginary perpendicular lines crossing in the center of the pool. The quadrants were marked by the four compass points (N, S, E, and W) and were called North-East (NE), North-West (NW), South-East (SE), and South-West (SW). A movable platform (11 ×11) made of transparent Plexiglas and thus invisible to the animals, was located in the center of one of the quadrants. The top of the platform was 2 cm below the surface of the water such that the animal could climb on it in order to escape from the water. A camera was

mounted to the ceiling above the pool and was connected to a microprocessor. 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 sides of the room.

2.6. Procedure

2.6.1. Experimental Design and Behavioral Training

Tests	Days
Handling	**5**
Open Field	**3**
Rotarod / Accelerod	**4**
Plus Maze	**1**
Rats moved form GMMA to METU: Habituation Period	**10**
MWM Shaping	**1**
Acquisition Training	**6**
Probe Trial/Extinction Training	**3**
Reversal Training	**3**
Probe Trial	**1**
Repeated Acquisition Training	**16**

Figure 2.8. Time schedule of the experiments.

2.6.2. Handling

For five consecutive days prior to the experiments, rats were daily handled 30 s each so that the rats get use to the experimenter.

2.6.3. Open Field Test

Open field represents a novel environment that is distinctly different from any environment rats have previously encountered, with the diameters vastly greater than the boundaries of their usual living quarters. In such novel and strange environment, rats, on one hand, may show anxiety manifested by an increased freezing and increased defecations, but on the other hand may show exploratory tendency manifested by increased locomotion. According to some authors (Denenberg, 1968) the meaning of the activity score changes from the first day of testing to the second and third day. In order to make a meaningful interpretation of the activity scores, minimum three days of testing are required. Each day, at the same time and in the same order, animals were placed into the apparatus and their activity was automatically recorded over 30 min in 5 minutes intervals.

The measures recorded were:
1. Horizontal activity score (or ambulation) as an index of exploratory tendency but at the same time of emotional reactivity;
2. Vertical activity (number of rearing incidents) as an index of exploratory behavior.

2.6.4. Plus Maze Test

On the following day, in order to measure the level of anxiety, each animal was placed in the center of the plus maze facing an open arm. Rats were allowed to explore the maze for a 5 min.

During this test five measures were recorded:
1. Number of entries to the closed arms;
2. Number of entries to the open arms;
3. Total time spent in closed arms;

4. Total time spent in open arms;
5. Time spent on the central platform;

2.6.5. Rotarod / Accelerod Test

On the completion of the open field testing, the animals were subjected to the tests on the rotarod/accelerod to evaluate their sensorimotor coordination. During this test, rats were placed on a rotating rod. Animals must have been continuously walking forward to avoid falling off the rod. This test was repeated over four consecutive days, each day under different conditions. On the first day of testing (referred to as 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 10 min elapsed. When falling of the rod, rats got into touch with a metal grid beneath kept under the mild electrical voltage. This short training taught rats to make en effort to stay on the rod as long as possible. On this very day no measures were taken. On the second day of testing the conditions remained the same but the time animals spent on the rotarod before falling down was measured. On the third day of testing, the speed of the rod was accelerated from 0 to 80 rpm within 10 min. On the fourth day, the speed of the rod was accelerated from 0 to 80 rpm within 4 min. The time rats remained on the rod was recorded.

2.6.6. Morris Water Maze Task

Ten days after plus maze test, memory testing in the Morris Water Maze began.

2.6.6.1. Reference (long term) Memory Tests in a Classical MWM

In the MWM, the rats use long term memory to learn the position of a hidden platform in reference to the visuo-spatial distal cues belonging to the room.

2.6.6.1.1. Shaping Training

On the first day of experiments, response shaping took place. During shaping training, a curtain was drawn round the pool to eliminate the distal cues. Animals were released into the pool four times from different start points: first in the vicinity of the platform, then from gradually longer distance from the platform. Each time animal swam in the water until it found the platform or for 60 s, then it was gently guided to the platform by experimenter. The purpose of this procedure was to habituate the rats to the water and to teach them to escape from the water by climbing onto the platform.

2.6.6.1.2. Place Learning (Acquisition Training)

During the place learning curtains were removed. The platform was placed in the center of North-East quadrant where it remained throughout the experiment. Rats were given four trials daily, 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) that were used in a pseudorandom order such that each position was used once during the daily experimental session. On each trial, the starting positions were the same for all animals. The rat was allowed a maximum of 60 s to find the hidden platform. The trial finished when the animal found the platform and was allowed to remain there for 15 s. If the animal did not find platform within 60 s, the experimenter guided the animal to the platform where it remained for 15 s. Afterwards the rat was returned to its cage for 5 min. inter-trial interval. Throughout the experiment, the distal and

proximal visuospatial cues stayed the same. The experimenter remained near the computerized recording system for the duration of the trial except for when introducing the animal into or removing it from the pool (Morris, 1984).

The Noldus EthoVision video-tracking system was automatically recording following measures:

1. Swim path trajectory;
2. Escape latency: the time between leaving the start location and climbing on the escape platform;
3. Swim distance (path length): the distance swum, in centimeters, from the start location to the escape platform;
4. Mean swim velocity;

2.6.6.1.3. Probe Trials / Extinction Training

The probe trial is used to assess the strength of the acquired response and, indirectly, to assess degree of learning. Repeated probe trials allow measure the rate of response extinction.

On the completion of 6 day place learning, for 3 consecutive days, animals received 45 s probe trials. On a probe trial, the platform was removed from the pool. 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 were recorded. Additionally, annulus crossing were counted for NE quadrant.

2.6.6.2. Reversal Training and Memory Retention Test after 10 Days Rest Period

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

2.6.6.3. Working Memory Test: Repeated Acquisition in the MWM ("Delayed Matching-to-Place")

This procedure was adopted after Vann *et al.,* 2003. During this experiment, 12 platform positions, which varied in their distance from the pool perimeter, were used along with 8 different (N, E, S, W, NE, NW, SE, SW) start positions designated by the compass points. The entire training lasted 16 days. Daily session 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 start 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 30 s. For the first 12 days, all intertrial 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 intertrial interval was 15 s as before. Throughout the training, distal visuo-spatial cues were stable. Testing was done at the same time of

day, beginning at 09:00 a.m. After the last daily trial, the rats were towel dried and the wood shaves were changed in cages.

All procedures involving animals were carried out according to the rules in the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health (USA).

2.6.7. Blood Alcohol Concentration (BAC)

Blood alcohol concentration was determined by the method of Dudek and Abbott (1984) with slight modifications. Blood samples were taken, 2 and 3 hours after the second alcohol intubation, via a nick to the tip of the tail of alcohol treated dams on GD 20. Then, 190 µl of 0.53 N Perchloric Acid and 10 µl of blood was placed into each eppendorf (centrifuge) tube and tubes were kept on ice until centrifugation. After that, acid was neutralized with 200 µl of 0.3 M potassium carbonate. Then samples were centrifuged at 5000 rpm for 15 minutes. Final supernatant was taken and kept on -20 ^{0}C until assay. Alcohol in supernatants was determined by enzymatically with alcohol dehydrogenase (ADH). The reaction medium contained 800 µl of 500 mM Tris pH 8.8 containing 1.875 mM NAD^{+}, 100 µl ADH (89.25 unit/ml) and 100 µl of supernatant. The reaction tubes were vortexed and incubated one hour at room temperature. At the same time standard alcohol solutions ranging between 50 to 500 mg/dl were used in order to draw calibration curve and then optical density was read at 340 nm against blank solutions containing no alcohol (Shimadzu 1601 UV/Vis Double-beam Spectrophotometer, Kyoto, Japan).
Blood alcohol concentrations were determined according to equation 1.

Blood alcohol concentration (mg/dl) =ODsample/Slope of calibration curve (eq.1)

As it was shown by other authors (Abel, 1979; Tran *et al.,* 2000) BAC reaches maximum level 3 hr after intragastric intubation, with higher alcohol levels in pregnant as compared to nonpregnant female rats.

2.6.8. Brain Weights

At the completion of behavioral testing, half of rats were killed with an overdose of anesthetic (ketamine plus xylazine). Brains were removed and weighted. Remaining rats were deeply anaesthetized (10 mg/kg body weight xylazine and 80 mg/kg body weight ketamine) and perfused intracardially with room temperature saline followed by 4% formaldehyde solution in 0.1M phosphate buffer (pH 7.2 at 4 °C). Brains are stored at room temperature in 10% formaldehyde waiting for histological examination.

2.7. Data Analyses

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. Tukey test was used for Post Hoc analysis of the data. The statistical packages Minitab and SPSS were used.

CHAPTER 3

RESULTS

3.1. Changes in the Dams' Body Weights between GD 6 – 20

The mean body weight ± SEM was calculated for each day between 6 and 20 GD. Figure 3.1 shows that compared to control groups, in A group, body weight remained lower throughout gestation. Two-way repeated measure ANOVA (treatment x days) confirmed significant effect of treatment ($F_{(2,329)}= 32.94$, $p<0.001$) and days ($F_{(14,329)}= 17.13$, $p<0.001$).Post hoc tests, however, yielded significant difference in the body weight between A and control groups only during last three gestation days ($F_{(2:20)}= 5.6$, $p<0.01$; $F_{(2:20)}= 6.053$, $p<0.01$; $F_{(2:20)}= 9.008$, $p<0.002$, respectively).

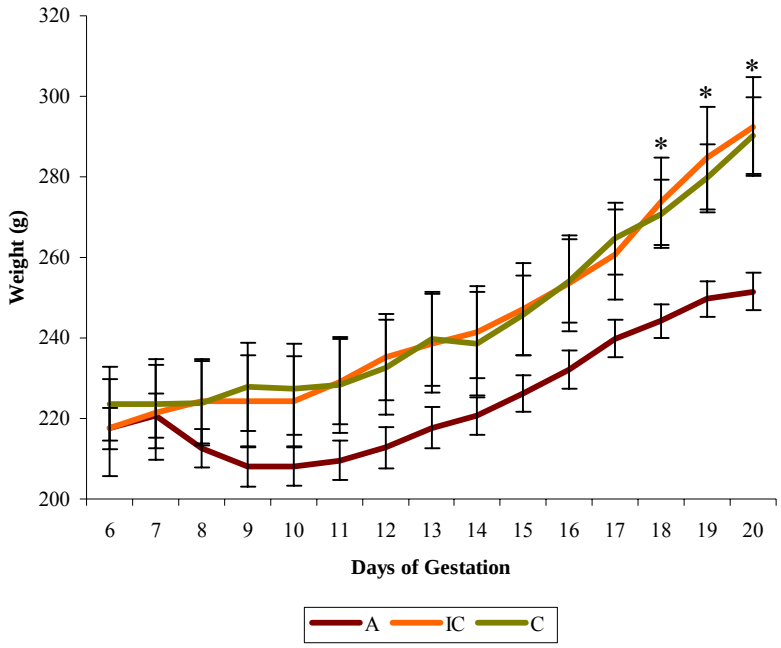

Figure 3.1. Comparison of body weight gain for three groups of dams, A, IC, and C, between 6 and 20 gestation day.

3.2. Pups Weight at PN 0, PN 5, and PN 10

Figure 3.2 shows that at birth, and still at PN 5, the average body weight of pups in A group was lower as compared to control animals. At PN 0, mean body weights ± SEM were as follows: 4.9 ± 0.07 in A group, 5.9 ± 0.09 in IC group, and 5.8 ± in C group. One-way ANOVA done separately for PN 0 and PN 5 revealed significant difference between A and control groups ($F_{(2,192)} = 47.827$, $p<0.001$ and $F_{(2,183)} = 26.284$, $p<0.001$). On PN 10, significant difference in the pups' body weight was revealed only between A and IC group ($F_{(2,151)} = 5.627$, $p<0.004$).

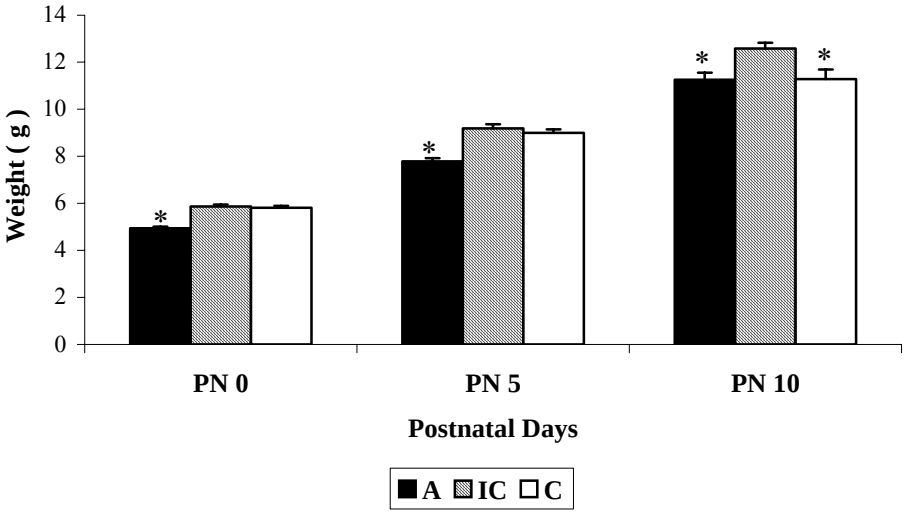

Figure 3.2. The mean body weight ± SEM for all male and female pups born under each of three treatments, A, IC, and C, were calculated for postnatal days 0, 5, and 10.

3.3. Litter Size on PN 0

No between-group difference has been observed in the litter size. The mean number of pups ± SEM in A, IC, and C groups was 12.2 ± 0.49, 12.25 ± 0.85, and 12.33 ± 1.20, respectively.

3.4. Pups' Survival Rate

Percentage of pups that survived on PN 30 in each litter has been calculated. Mean values ± SEM for A, IC, and C groups are as follow: 64 ± 7.6, 62 ± 7.2, and 69 ± 24, respectively, with the greatest variation in control group. No significant between group difference in the pups' survival rate has been revealed.

3.5. Results of Behavioral Tests

3.5.1. Open Field Test

As seen from Figure 3.3 in the open field, in all three groups, the highest ambulation score was noted during the first five min on the first testing day. Then habituation to the novel environment obviously took place, what was reflected by the overall decrease in locomotor activity. Decline in the locomotor activity, both within the first testing session, as well as across days, seemed to be the steepest in the control group. On the contrary, in A group, both, increase in the locomotor activity after being introduced to a novel environment, and then, decline in locomotor activity due to habituation were relatively mild, such that ambulation recorded in A group was lower than in C group during the first 5 min of the first testing session but higher during the third testing session. This could indicate towards alcohol-brought decrease in behavioral flexibility in this group. However repeated measure ANOVA yielded significant effect of time (5 min interval, and session) only. The effect of group was insignificant.

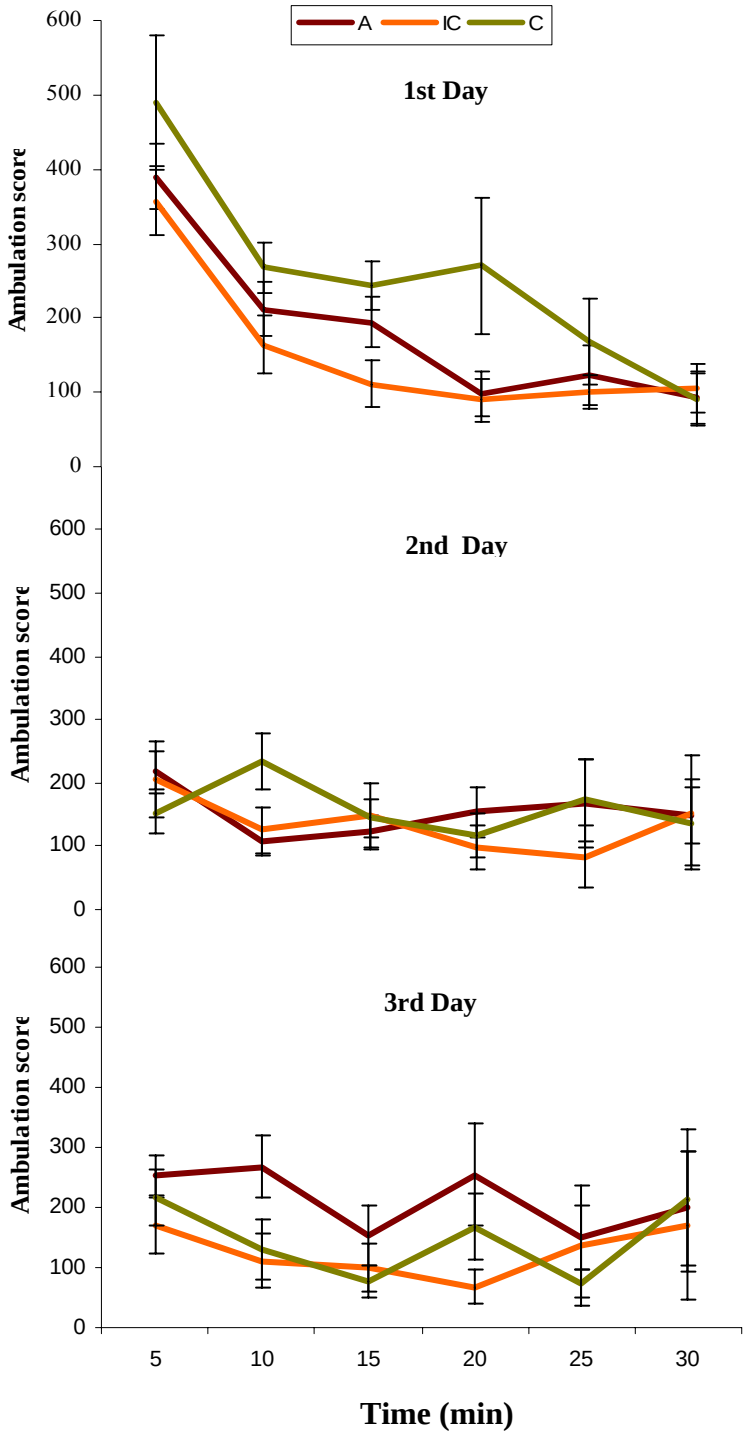

Figure 3.3. Spontaneous open field locomotor activity showed as mean (± SEM) ambulation score calculated for the 5-min intervals of the total 30-min testing period, on three consecutive testing sessions in each treatment group.

As seen from the Figure 3.4 the distance score confirmed observations done when ambulation has been measured.

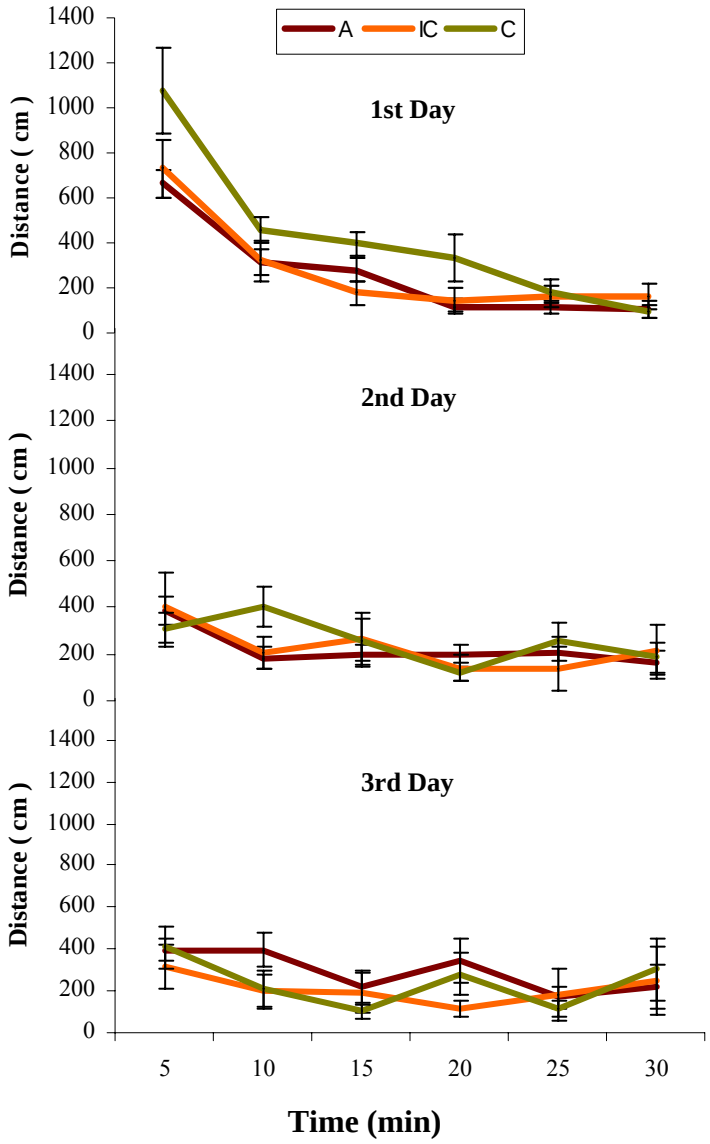

Figure 3.4. Mean (± SEM) distance in the open field calculated for the 5-min intervals of the total 30-min testing period on three consecutive testing sessions, and for each treatment group independently.

Within- and across sessions changes in the frequency of rearing incidents (vertical movements) were parallel to the changes in ambulation, and again the fluctuation in

the vertical motility were the least pronounced in the animals in utero exposed to alcohol (Figure 3.5)

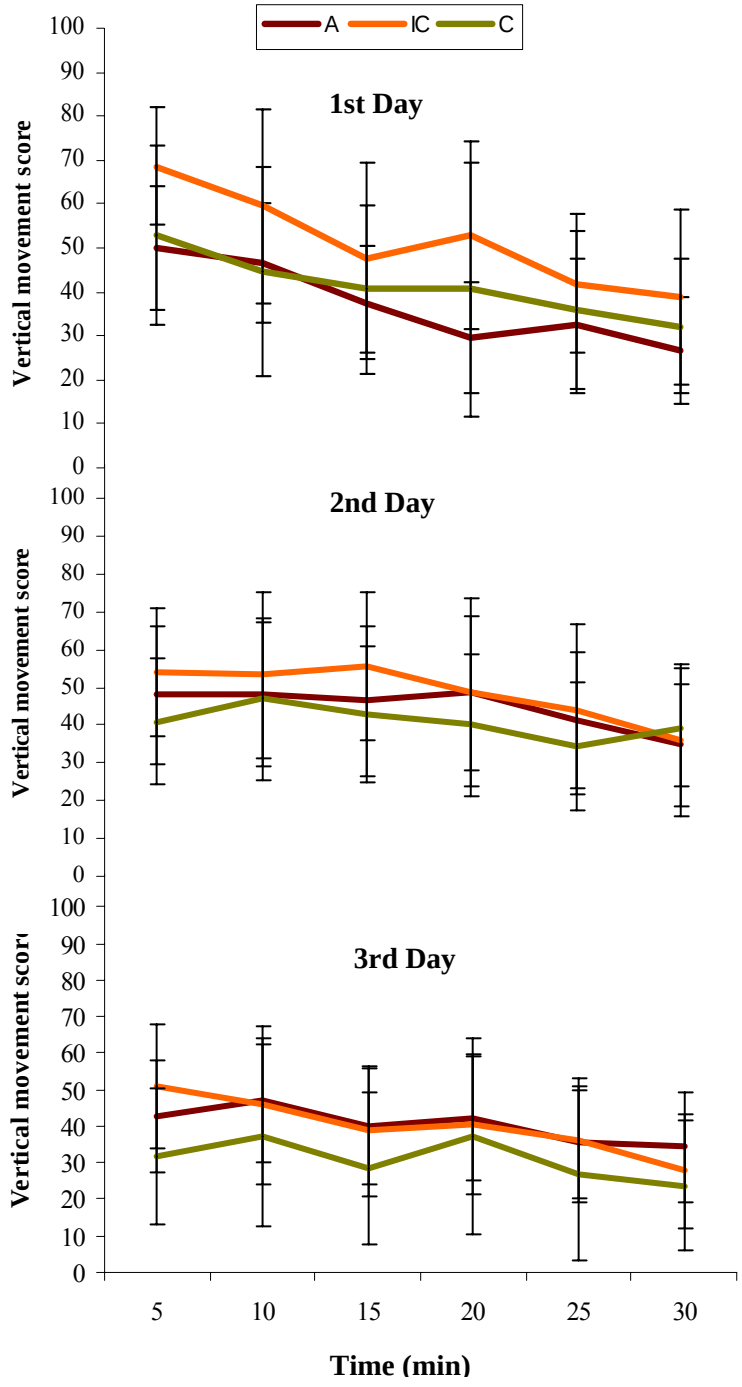

Figure 3.5. Mean (± SEM) group vertical movements (rearing) recorded on three consecutive testing sessions for the 5 min intervals, and for each treatment group independently.

3.5.2. Plus Maze Test

As seen from the Figure 3.6 pups exposed prenatally to alcohol spent less time in the open arms, and spent relatively more time on the central platform. One way ANOVA carried out for each compartment independently, yielded significant group effect only for the open arms ($F_{(2, 20)} = 4.187$, $p<0.03$). This observation is in line with lower ambulation score in A group as compared to control during the first 5 min in the open field. It could indicate increased level of anxiety in this group.

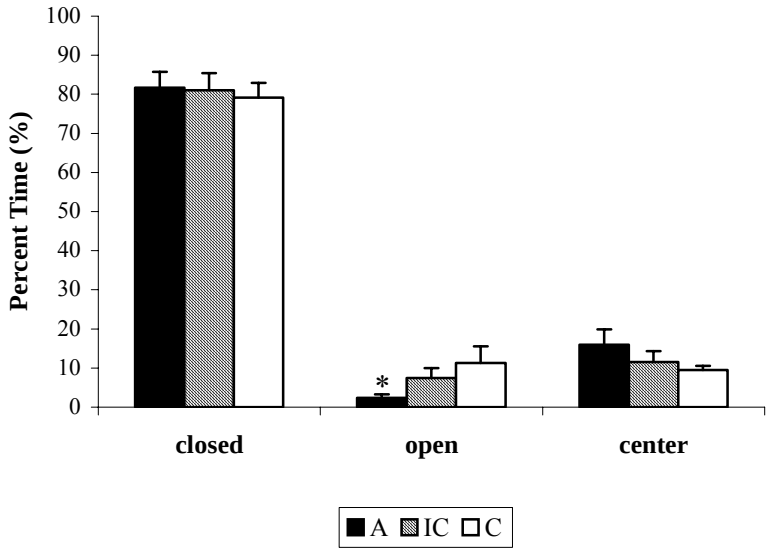

Figure 3.6. Percentage of time spent in each of three compartments of the plus maze: closed arms, open arms, and center.

3.5.3. Sensorimotor Performance on the Rotarod/Accelerod

Under three conditions tested on the rotarod/ accelerod the lowest performance was shown by IC group. Sensorimotor coordination and muscle strength in A group were same as in C group. However, one-way ANOVA applied independently to each testing condition yielded group effect insignificant. Results were given in Figure 3.7.

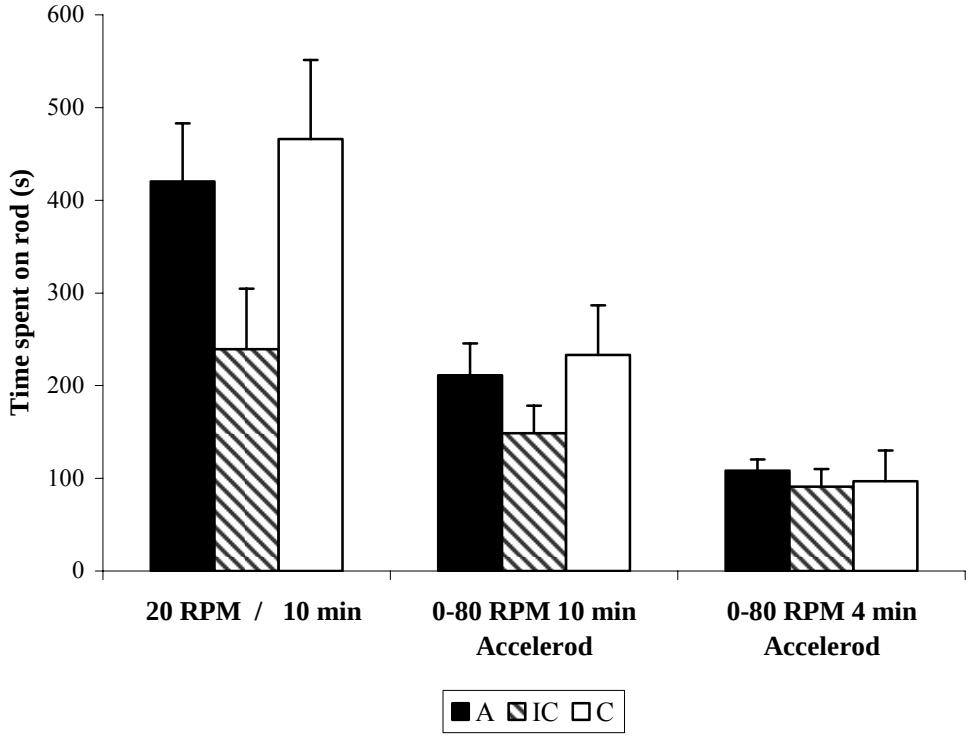

Figure 3.7. Mean time (s) ± SEM spent on a rotating rod until falling off under three different conditions: (a) when the speed of rotation is stable and fixed at 20 rpm, (b) when the speed of rotation gradually increases from 0 to 80 rpm in 10 min, and (c) when rotation speed increases within the same range 0-80 rpm in 4 min.

3.5.4. Classical MWM Training

In the water maze, by the fourth training day all three groups of rats, regardless of the treatment, reached the asymptotic performance with mean escape latency oscillating around 10s (Figure 3.8.). The rate of reaching this level of performance was the fastest in IC group and the slowest in A group.

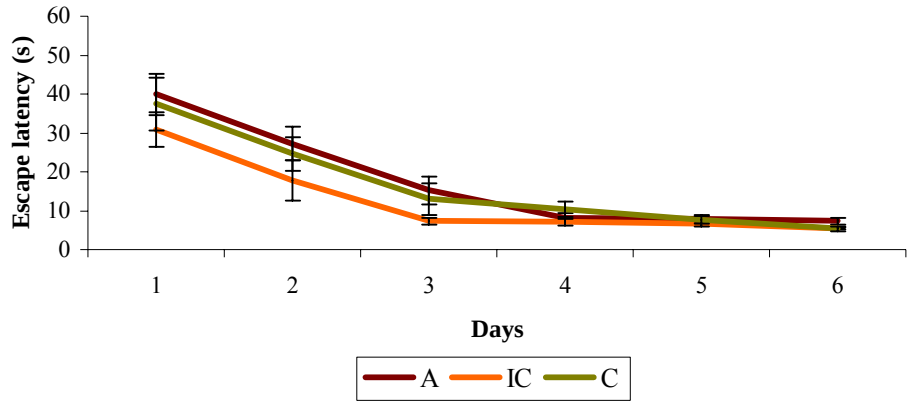

Figure 3.8. Mean escape latency ± SEM to locate invisible platform in the water maze calculated for each training day and each treatment group independently.

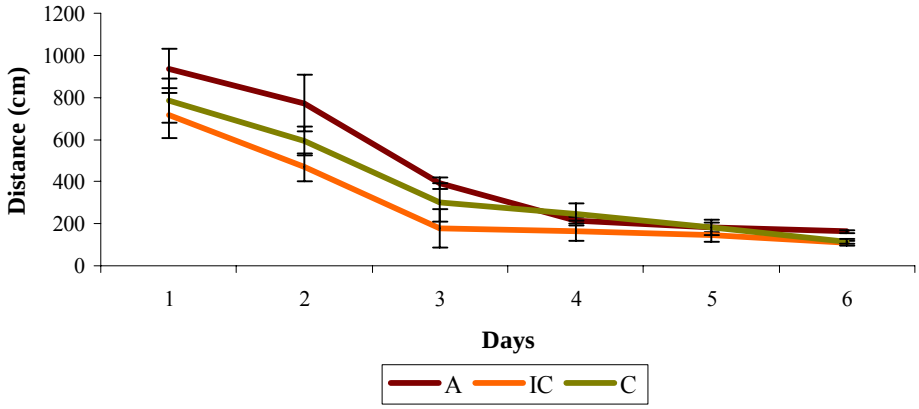

Figure 3.9. Mean distance (path length) ± SEM covered by rats from each group on consecutive training days to reach the invisible platform in the water maze.

Two-way ANOVA (treatment x days) for repeated measures yielded main effect of group and day significant ($F_{(2,479)} = 6.52$, $p<0.002$, and $F_{(5, 479)} = 58.41$, $p<0.0001$ respectively). The path length measure replicated the results obtained when the swim time (latency) was measured.

As seen from the Figure 3.10, there was no substantial difference in the swimming speed between alcohol and control groups throughout the training in the water maze.

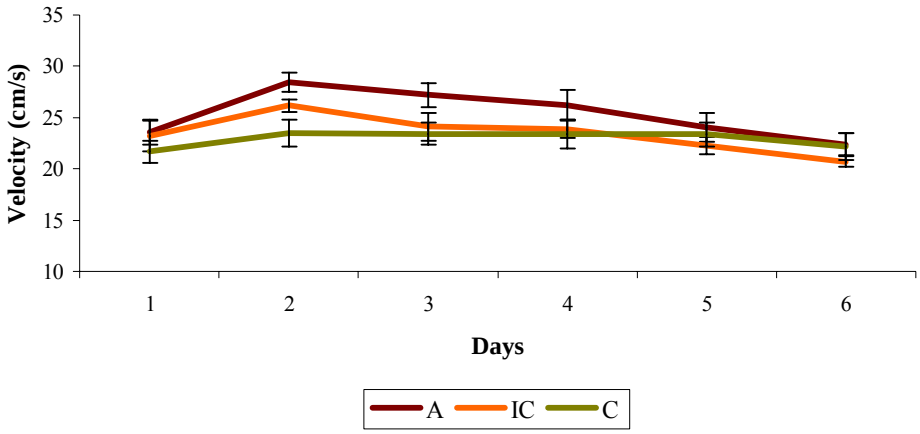

Figure 3.10. Mean velocity of swimming in the water maze calculated for each treatment group and each training day independently. Error bars denote SEM.

3.5.5. Probe Trial

Animal performance in the water maze on the three daily probe trials (platform removed) was assessed by the percentage of time spent in the platform quadrant as compared with the total 45 s in the pool (Figure 3.11), time spent in the platform quadrant versus time spent in the opposite quadrant (Figure 3.12), ratio of these two time measures (Figure 3.13), and time in annulus 40 (Figure 3.14).

As seen from Figure 3.11 regardless of the treatment, animals' performance on the first probe trial (carried out on the day after the completion of acquisition training in the water maze), was well above the chance level, with 40-50 % of the time spent in the platform quadrant. Over two consecutive days (extinction training), in A and IC groups, time spent in the platform quadrant declined to the chance level. In C group, even on the third probe trial, time in the platform quadrant remained relatively high

suggesting slowest extinction rate in this group. Nevertheless, two–way repeated measure ANOVA, yielded significant neither the main group effect nor group x day interaction.

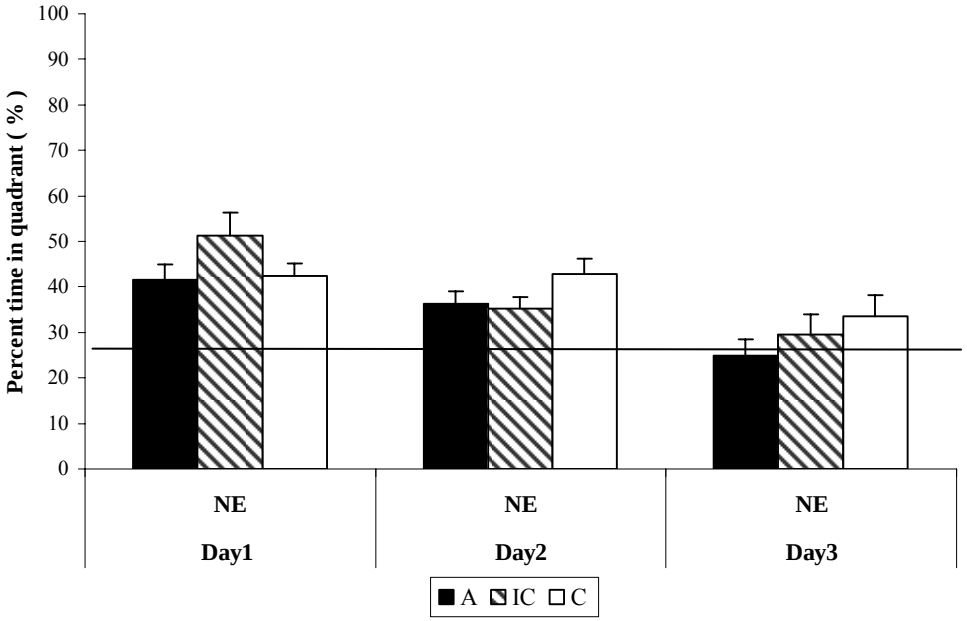

Figure 3.11. Percentage of time spent in the platform quadrant on the three consecutive 45-s probe trials in each treatment group independently. Line at 25% represents chance level. Error bars denote SEM.

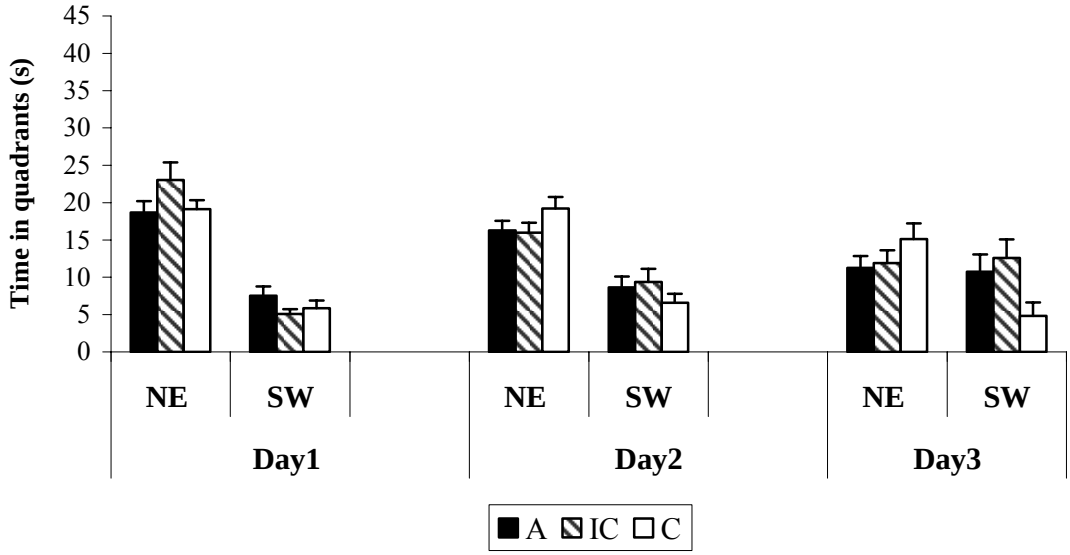

Figure 3.12. Performance on the three consecutive probe trials of the water maze task (platform removed) as assessed by the time spent in the original platform quadrant (NE) and in the opposite quadrant (SW).

High preference for the original platform quadrant in control animals even on the third probe trial (third extinction day) is best illustrated by the ratio of the total time spent in the platform quadrant (NE quadrant) to the total time spent in the opposite quadrant (SW quadrant) (Figure 3.13). In the C group, on the last, third probe trial, preference for the NE quadrant was, as an average, 5 times higher than this for the opposite SW quadrant, however, due to a great individual variation in this group this effect did not yield significant result.

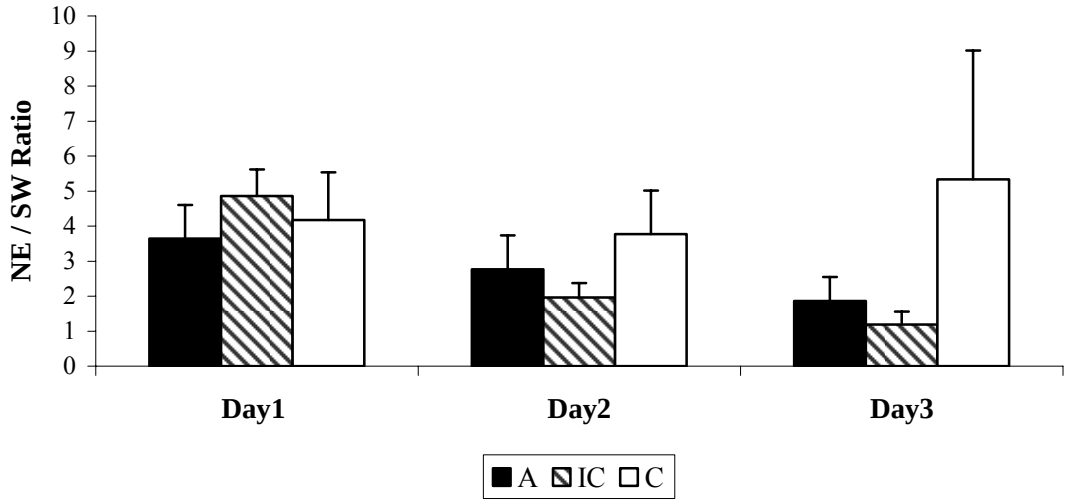

Figure 3.13. Ratio of the total time spent in the platform quadrant (NE) to the total time spent in the opposite quadrant (SW) for each daily probe trial and each treatment group independently.

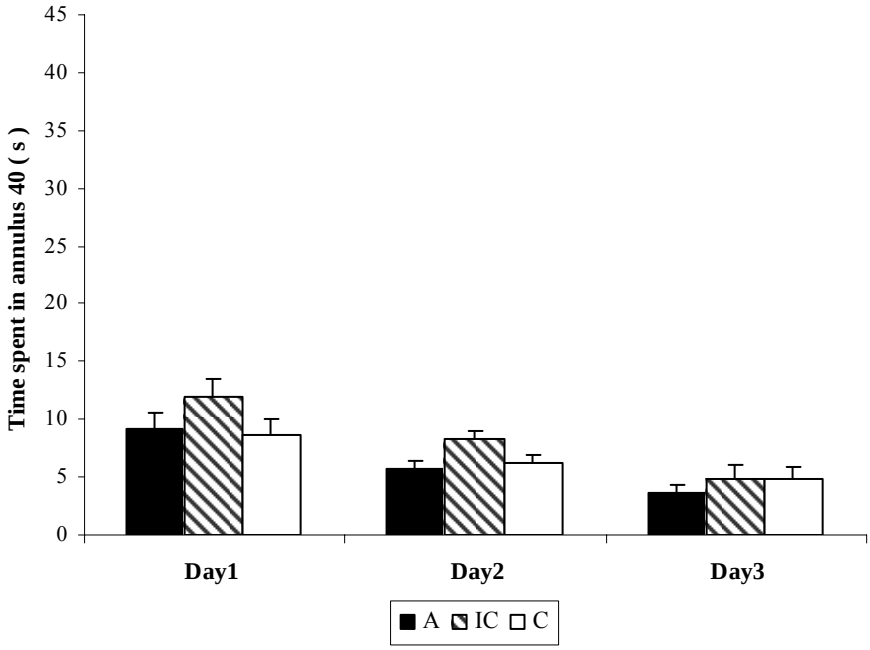

Figure 3.14. Time in annulus 40 ± SEM calculated for three consecutive probe trials and for each treatment group independently.

No significant between-group difference was observed in the time spent in annulus 40.

3.5.6. Reversal Training

During three-day acquisition training with invisible platform moved to the opposite SW quadrant, rate of learning was slightly better in A and IC as compared with control group (Figure 3.15), as suggested by significant main effect of group ($F_{(2,239)} = 2.84$, $p<0.06$).

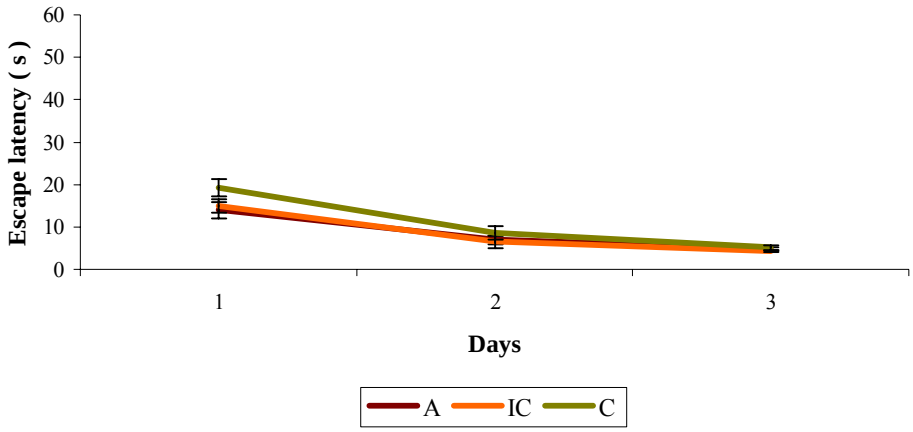

Figure 3.15. Mean escape latency ± SEM to locate invisible platform in the water maze calculated for three consecutive reversal training days in each treatment group.

3.5.7. Probe Trial Carried Out 10 Days after the Reversal Training

As seen from the Figure 3.16 in all three groups, 10 days after the completion of the reversal training the time spent in the new platform quadrant was above the chance level (25%). A one-way between subjects ANOVA revealed no significant difference among the groups.

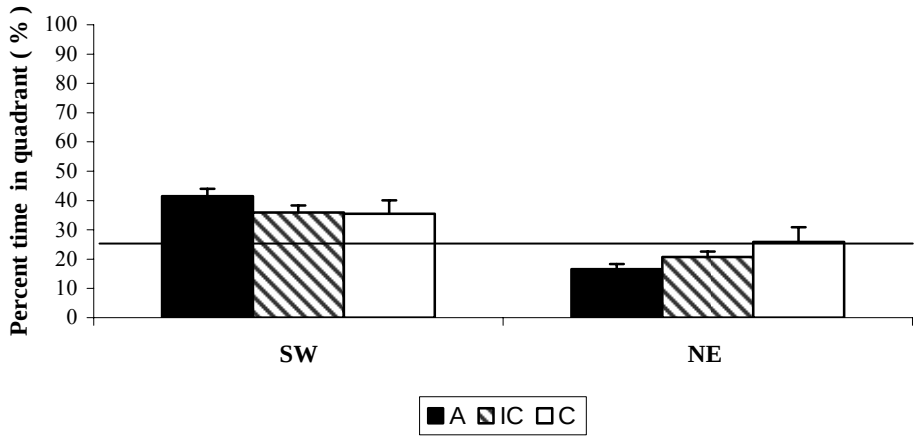

Figure 3.16. Percentage of the total time spent in the new platform quadrant (SW) and in the opposite quadrant (NE) on the probe trial carried out 10 days after the completion of the reversal training, for each treatment group. Line at 25% level represents chance level. Error bars denote SEM.

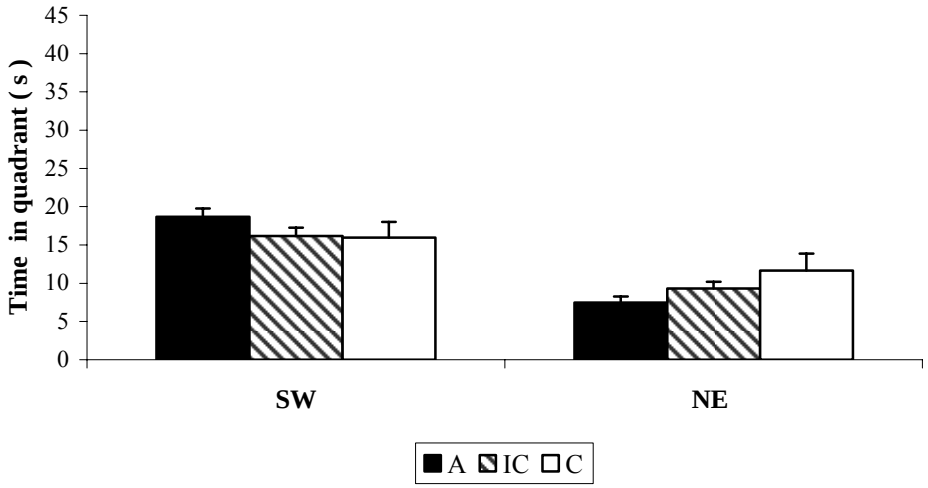

Figure 3.17. Total time spent in the new platform quadrant (SW) and in the opposite NE quadrant on the probe trial carried out 10 days after the completion of reversal training. Error bars denote SEM.

There was also no significant difference between groups in the absolute time spent in the platform quadrant (Figure 3.17), however, preference for the opposite NE quadrant was lower in A group as compared with control ones, and this difference was marginally significant ($F_{(2,19)} = 2.88$, $p<0.08$).

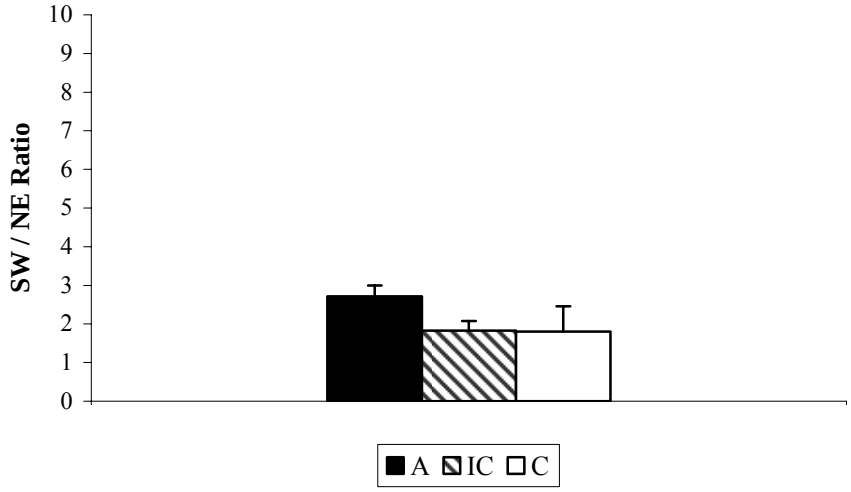

Figure 3.18. Ratio of the total time spent in the new platform quadrant (SW) to the total time spent in the opposite quadrant (NE) on the probe trial carried out 10 days after the completion of reversal training. Error bars denote SEM.

The ratio of the total time spent in the platform quadrant to the total time spent in the opposite quadrant was also higher in the A group as compared with control animals, with the no significance of the between group difference at $p<0.15$ ($F_{(2, 19)} = 2.128$).

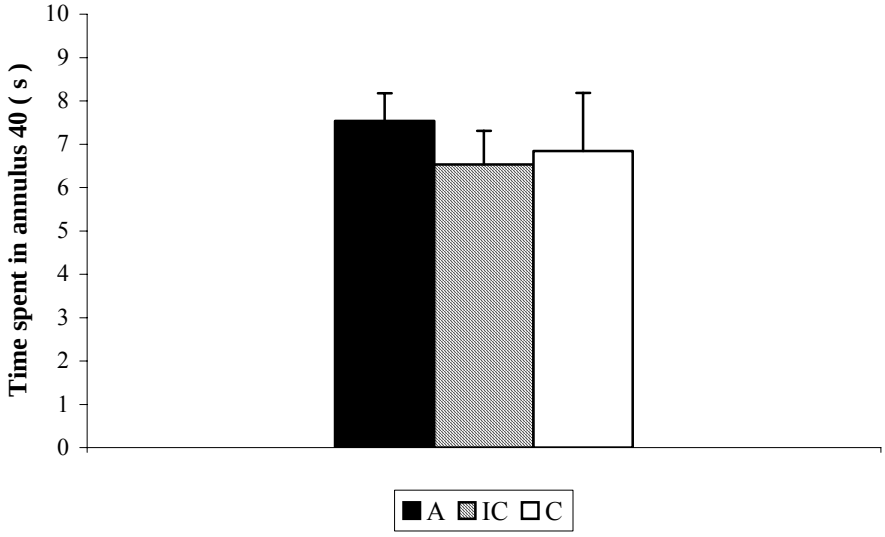

Figure 3.19. Time in annulus 40 ± SEM calculated for the probe trial carried out 10 days after the completion of reversal training.

No significant between group difference was found for the time in annulus 40.

In summary, pups exposed prenatally to alcohol showed slower rate of place learning in the classical MWM. However, during reversal learning, and on probe trials assessing memory retention and habit strength, rats from A group showed performance that was as good as or even slightly better than that of control subjects.

3.5.8. Repeated Acquisition Training

The first 12 days of the repeated acquisition training involved the standard working memory task in the water maze, with a 15 s inter-trial interval 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, and analyses were performed using group, block and trial factors. Three factorial ANOVA for repeated measures revealed significant effect of session block showing a general decrease in overall latency over the training period ($F_{(3,959)} = 12.76$, $p<0.001$) (Figure 3.20). There was also significant effect of trial ($F_{(3,959)} = 196.34$, $p<0.001$) showing some improvement by all groups over the trial (Figure 3.20). The main effect of the group as well as group x trial and group x session interactions were, however, insignificant Changes in the path length (distance) paralleled the changes in the swim time (latency) (Figure 3.21).

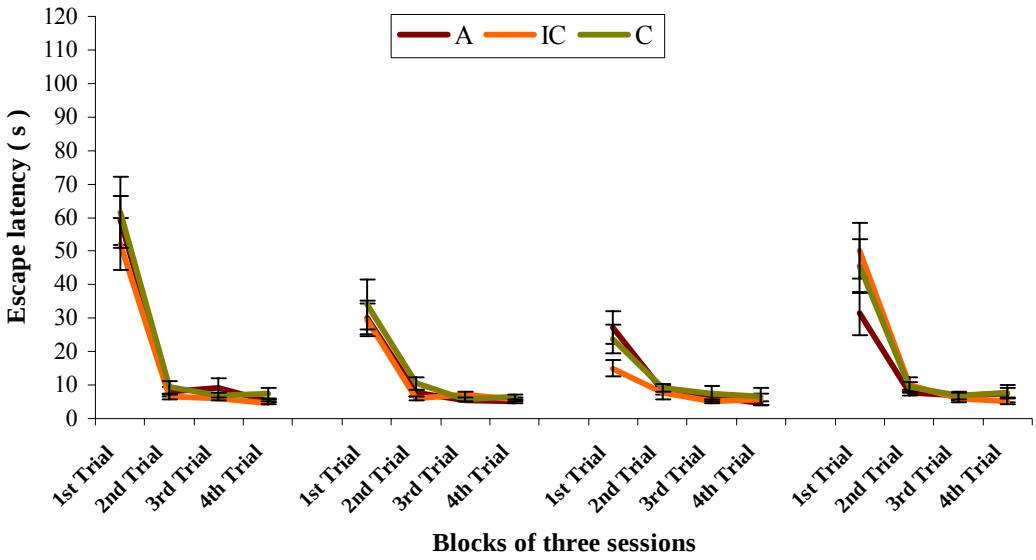

Figure 3.20. Performance on the repeated acquisition training (platform position varying every day) with 15-s inter-trial interval. The mean escape latency in (s) was calculated for the four consecutive trials in four blocks of three sessions each. Error bars represent SEM.

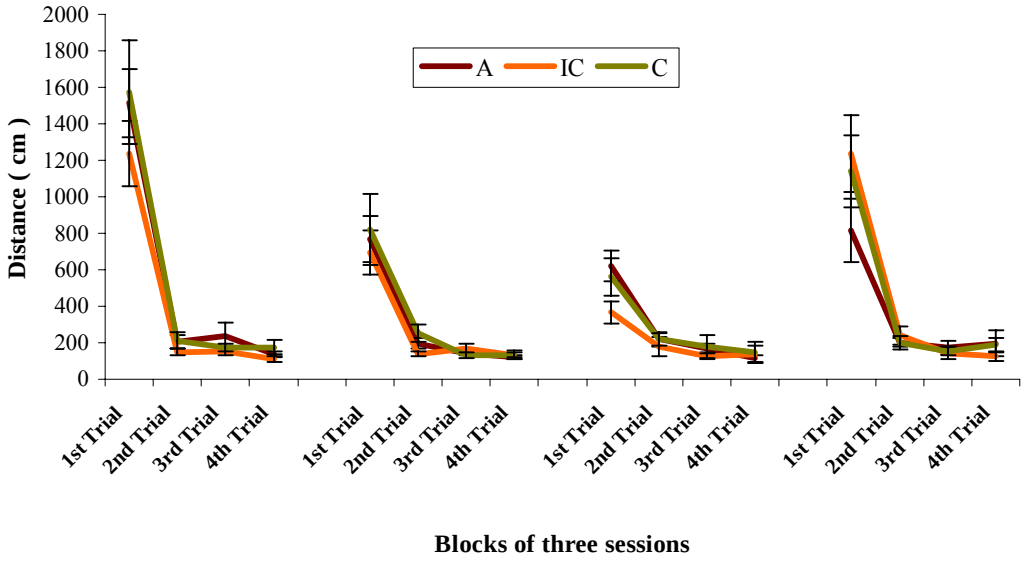

Figure 3.21. The mean distance (s) to platform calculated for the four consecutive trials in four blocks of three sessions each, during repeated acquisition training Error bars represent SEM.

The next stage (four consecutive training days) involved 30 min delay between trial 1 (sample) and trial 2 (working memory test), with the remaining two trials run as before (Figure 3.22 and 3.23). With both latency and distance taken into analysis trial effect was highly significant ($F_{(3,319)} = 38.29$, $p<0.001$, and $F_{(3,319)} = 38.8$, $p<0.001$ respectively). Conversely, group effect and interactions were insignificant.

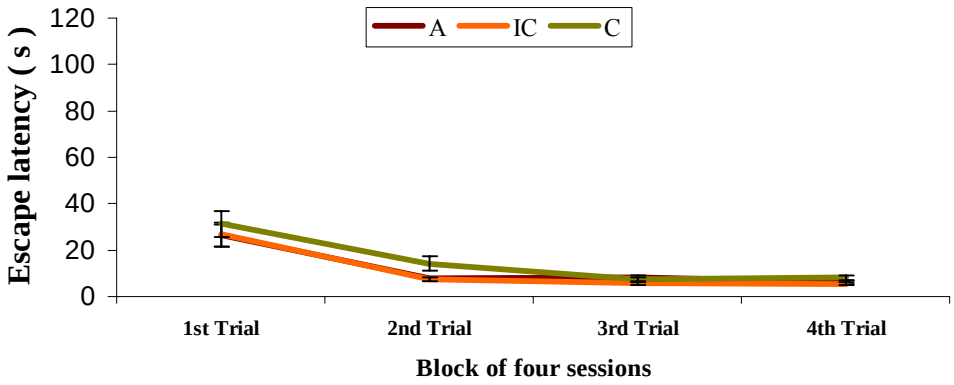

Figure 3.22. The mean escape latency in (s) calculated for the four consecutive trials in a block of four sessions during repeated acquisition training with 30-min interval between first and second trial. Error bars represent SEM.

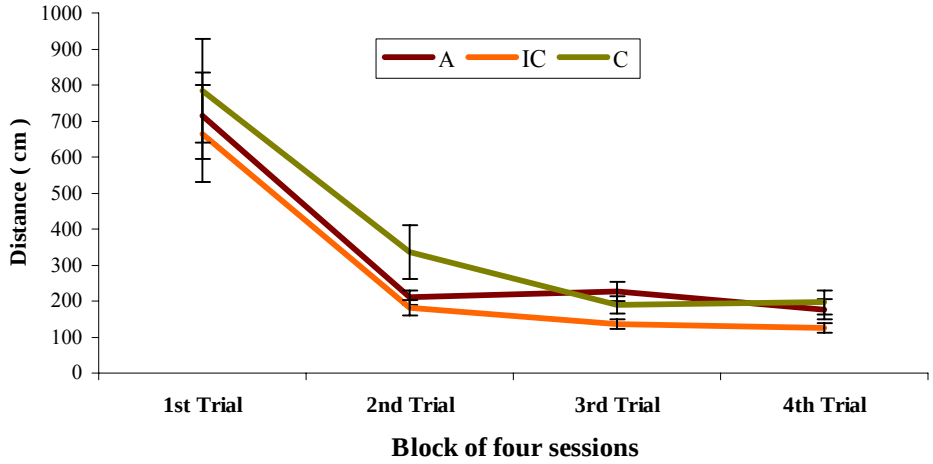

Figure 3.23. The mean distance moved (path length in cm) calculated for the four consecutive trials in a block of four sessions during repeated acquisition training with 30-min interval between first and second trial. Error bars represent SEM.

3.6. Brain Weights

A one-way between subjects ANOVA revealed no significant difference in the brain weight among the groups (Figure 3.24).

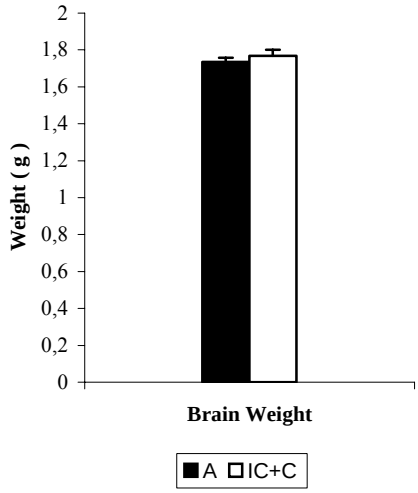

Figure 3.24. Mean brain weight (± SEM) at the completion of experiments.

3.7. Blood Alcohol Concentration

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

CHAPTER 4

DISCUSSION

Poor learning scores are commonly attributed to the children with fetal alcohol effects. In children, prenatal alcohol was related to deficits in spatial memory and integration, verbal memory and integration, flexible problem solving, and attention (after Driscoll *et al.,* 1990). Learning deficits on numerous tasks, especially those containing spatial components such as place discrimination as well as spontaneous and conditional alternation in T-maze, spatial navigation in radial arm maze and MWM, were described also in rats exposed perinatally to alcohol. Learning and memory impairments usually were manifested by juvenile subjects (Blanchard *et al.,* 1987; Girard *et al.,* 2000; Goodlett and Peterson, 1995; Pauli *et al.,* 1995; Thomas *et al.,* 1996), however, greater or smaller deficits were also reported in young adult rats (60-95 days of age) in both, spatial working memory (Nagahara and Handa, 1997; Neese *et al.,* 2004; Reyes *et al.,* 1989; Zimmerberg *et al.,* 1991) and spatial reference memory, the latter commonly assessed on the basis of animals' performance in the MWM. Some authors reported impairment in the task acquisition (Gianoulakis, 1990; Kim *et al.,* 1997; Tomlinson *et al.,* 1998), some others observed deficits only in the strength and/or retention of the acquired place preference on the probe trials carried out with removed platform (Matthews and Simson, 1998), or impairment during reversal training carried out with platform moved to another location, but with the initial learning unaffected (Hannigan *et al.,* 1993; Lee and Rabe, 1999). Westergren *et al.,* (1996) observed impaired performance in the MWM even in six month old rats exposed prenatally to alcohol delivered to the pregnant dams with a liquid diet through gestation days 11-20. In contrast to these results, in the present study, alcohol-exposed rats were not different from controls on any of the spatial reference memory tasks applied and including initial acquisition training in the MWM, test of the strength of acquired place preference on the probe trial, extinction of the place preference, reversal

learning, and finally, retention of the acquired new place preference over the ten day period. Alcohol-treated rats did not showed impairment in the repeated acquisition task (a task for working spatial memory) with 30 min delay between sample and test swim either. Our results are consistent with finding by Cronise *et al.,* (2001), reporting impairment of spatial navigation in the MWM only in juvenile rats (19-31 days of age) and only in the group exposed to alcohol throughout the whole gestation and 2-10 postnatal days. This deficit was found to be transient because no differences were observed among adults (90-103 days of age). Similarly, Girard *et al.,* (2000) when examining spatial working memory in the modified MWM task, reported impaired performance in alcohol-treated group (exposed to alcohol at PN 5-18) when tested as juveniles (PN 35) and only with the longest 2 hr delay between sample and test swim.

In the present study, alcohol was delivered to the pregnant dams in a binge-like manner by daily intragastric infusions, over the period between 7 and 20 gestation days. Offspring rats were thus subjected to a treatment resulting in high BAC (around 340 mg/dl) during the period of gestation overlapping with the period of formation of the stratum piramidale in the hippocampal CA1 and CA3 areas occurring on GDs 16-20 (Altman and Bayer, 1990). Under these conditions, at least some damage to the hippocampal formation could be expected in alcohol treated rats.

In several studies by other authors even lower perinatal BACs as compared to the one recorded in the present study, were shown to cause adverse morphological and functional alterations in the hippocampus. Mostly, the alcohol-induced adverse effects were, however, observed in juvenile subjects (Barnes and Walker, 1981; Hablitz, 1986; Krahl *et al.,* 1999; Miller, 1995; Morriset *et al.,* 1989; West *et al.,* 1989) but sometimes they persisted into adulthood (Krahl *et al.,* 1999, Morriset *et al.,* 1989).

There are, however, some reports about lack of significant cell loss in hippocampus after prenatal exposure to alcohol in contrast to neonatal exposure. Livy *et al.,* (2003) reported that alcohol intoxication during first two trimester equivalent did not affect cell numbers in either hippocampal CA1 or hippocampal CA3 areas. Cell loss was observed only after neonatal alcohol administration, but in this study maternal blood alcohol levels were lower than in the present study, on average varying between 268-314 mg/dl. Conversely, West *et al.,* (1984) and Pierce *et al.,* (1989) reported microencephaly not accompanied by changes in the hippocampal morphology even after neonatal alcohol administration during PN 4-10 at the dos 6.6 g/kg/day that resulted in a very high BAC of 480 mg/dl. Also Maier and West (2001) after alcohol treatment via intragastric intubation throughout GDs 1-20 at the doses 2.25, 4.5, and 6.5 g/kg/day observed reduction in the total number of cells in cerebellum but not in hippocampal CA1 and CA3 areas.

On the other hand, not always alterations in hippocampal morphology were reported to be accompanied by behavioral impairment. Lukoyanov *et al.,* (1999) found that when adult rats were continuously exposed to alcohol at the average dose of 7.5 g/kg (producing BAC 130 mg/kg) between 2 and 15 months of age, 18% cell loss in CA1 and 19% cell loss in CA3 hippocampal regions in the alcohol consuming adult rats was observed. The brain damage was aggravated by alcohol withdrawal. However, no deficits were observed either in place learning or spatial working memory in the MWM.

Fact that adverse effects of fetal alcohol exposure tend to be more pronounced in immature than adult animals would indicate alcohol-induced developmental delay rather than alcohol-induced permanent developmental dysfunction. It may be the case that a drug applied prenatally will retard the development of the nervous system. In this situation, animals exposed to this drug in utero but tested for its effects in adulthood may not differ from untreated controls. Indeed, qualitative and quantitative evaluation of Golgi-impregnated hippocampal pyramidal cells revealed a marked reduction in the extent of basilar dendrites in alcohol-exposed animals

what suggested a delay in neuronal development (Davies and Smith, 1981). Morphological evidence for a delay of neuronal maturation in fetal alcohol exposure was also presented by Hammer Jr and Scheibel (1981).

Alternatively, it has been suggested by some authors that this apparent age-dependence of perinatal alcohol exposure on learning abilities may be caused by "recovery" from impairments during maturation due to some regeneration process. In line with this hypothesis are findings by Miki *et al.,* (2003) that exposure of rat pups to high dose alcohol at PN 10-15, initially significantly reduced numbers of neurons in the hilus region of hippocampus, but it appeared that they can be later replaced either by newborn cells or by abnormal cell migration. On the other hand, Ferrer *et al.,* (1988) reported reduced hippocampal dendritic spine densities in young (PN 15) but not adult (PN 90) prenatal alcohol exposed rats, suggesting the possibility that that neural plasticity was effective in promoting recovery in hippocampus during normal postnatal development.

The absence of an acquisition deficit in our study may also be due to the fact that the place discrimination in the MWM was an easy task for the alcohol-exposed rats to learn, and that delays between sample and test swims in the working memory task were too short. Some authors (Riley, 1990) proposed that prenatal exposure to alcohol may have long-lasting adverse effects on animals' behavior which, however, might only occur under challenging or stressful circumstances. It has also been hypothesized that animals exposed prenatally to alcohol develop compensatory strategies as adults that, however, are not adequate when animals are tested under challenging conditions. In line with this suggestion is the report by Girard *et al.,* (2000) who found impairment in the working memory in rats exposed to alcohol during PN 15-18 only at 2 hr delay.

Effect of fetal alcohol exposure may be further modified by the maternal and offspring response to alcohol as determined by genetic factors. In the studies reporting cognitive deficits when similar doses of alcohol were administered usually

Spraque Dawley and Long Evans rats were used. A careful analysis of the update literature regarding prenatal alcohol effect on memory and learning abilities could maybe help to answer the question whether rats belonging to the Wistar strain, the strain used in the present study, are more resistant to the alcohol effects than the other strains.

In the present study, rats prenatally exposed to alcohol were not impaired also on the motor tasks. Although the Purkinje cells of the cerebellum having functional relation to the neuroanatomical circuit for motor coordination and gait were demonstrated to suffer from perinatal alcohol intake, in the current study, rats prenatally treated with alcohol and tested as adults were not impaired in sensorimotor coordination and/or did not show muscle weakness as assessed by rotarod/accelerod testing. Such dysfunction was reported by Abel *et al.,* (1979) 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 restriction and neuronal depletion induced by alcohol exposure during the brain growth spurt of neonatal rats. As shown by Goodlett and co-workers (1991), pups given alcohol as 10.2% (v/v) solution in two of the 12 daily feedings (resulting in peak BACs of 361 mg/dl) on PN 4-9 had significant reductions in whole brain and cerebellar weight, and at the same time, showed significant impairment in the acquisition of the rotarod task at approximately 405 days of age, as compared to controls).

In the present study, young adult rats receiving fetal alcohol treatment did not display hyperactivity either. This observation is consistent with earlier findings by Bond and Di Giusto, (1977) that offspring born from dams receiving throughout the whole gestation liquid diet containing 35% EDC, displayed significantly greater activity (ambulation) in the open-field at 28 and at 56 days of age, but not at 112 days of age. Also Randal and Hannigan (1999), after binge-like alcohol administration throughout GD 8-20 did not report increased locomotor activity in offspring at PN 90–150. According to the outcome of Bond's (1981) analysis of the

update literature regarding prenatal alcohol effect on the animal locomotor activity, increase in activity as compared to controls was observed in rats receiving prenatally alcohol dose of 6 or more g/kg per day and tested prior to 70 days of age.

Hyperactivity is the most frequently reported behavioural problem in children with fetal alcohol effects (after Driscoll *et al.,* 1990) and, as it was mentioned earlier, it has been commonly observed in juvenile rats regardless of the dose and period of alcohol administration. In adult rats, locomotor hyperactivity has been reported after neonatal alcohol administration (Kelly *et al.,* 1987; Tran *et al.,* 2000). Maybe, thus third trimester equivalent represents the time window of the highest vulnerability for this dysfunction.

Children of women who consume high amounts of alcohol during their pregnancies vary greatly in physical and behavioral outcomes. Although many factors, such as dose and timing of exposure, undoubtedly contribute to this variation, one important determinant may be genetic differences in the response to alcohol. Thomas *et al.,* (1998) examined activity levels in high alcohol sensitivity (HAS) and low alcohol sensitivity (LAS) rats following neonatal alcohol exposure (6 g/kg/day from PN 4 through 7 and 3 g/kg/day on PN 8 and 9). In this study, activity level was measured in automated activity monitors for 30 min daily on PN 18 through PN 21. Neonatal alcohol exposure produced overactivity in HAS rats, relative to their controls, but the same alcohol treatment had no effect on the LAS rats. Importantly, there were no differences in blood alcohol concentrations (around 420 mg/dl) observed between the two lines during the treatment period. These findings suggest that behavioral teratogenic effects of alcohol may be affected by genetic makeup of the subjects. However, analysis of the rat data on different activity measures after perinatal alcohol exposure presented in a rewiev by Bond (1981) and together with some more recent reports gives an impression that Spraque Dawley rats are more susceptible than Wistar rats to the stimulating effect of perinatal alcohol on animals' activity, although the age factor seems to be predominating.

In the present study, although the mean level of activity (both ambulation and rearing) recorded on the three consecutive days in the open field did not distinctly differ in alcohol-treated and control subjects, rats prenatally exposed to alcohol showed different pattern of activity changes across the sessions, with slightly lower activity on the first session and slightly higher activity on the third session, as compared to controls. The fact that activity of the alcohol treated rats on the first day in the open-field was lower but on the third day of testing higher than that of control rats (borderline significance of this effect was revealed) could indicate towards alcohol-induced decrease in the behavioural flexibility manifested as deficit in habituation (extinction of orienting response) to a novel environment. The latter result is similar to the finding reported by Westergren *et al.,* (1996) that rats exposed to alcohol through GD 11-22, when tested as young adults in the resident maze and activity chamber, on the first day of testing showed lower activity as compared to controls, conversely, on the second day their activity increased while that of control animals decreased. This pattern of activity changes can reflect decrease in behavioural flexibility manifested by alterations in habituation to a novel test situation, or increased neophobia.

Against decreased behavioural flexibility argues the fact that alcohol treated rats were as good as control groups in extinction of place preference for the platform quadrant during three consecutive daily probe trials with removed platform, and in reversal learning. On the other hand, for increased neophobia are arguing results obtained in the plus maze, a task designed to measure the level of anxiety. In the presents study, alcohol treated rats spent significantly less time on open arms of the plus maze. This observation is in line with reports by other authors (Ogilvie and Rivier, 1997; Weinberg *et al.,* 1996) that animals exposed to alcohol in utero are typically hyperresponsive to stressors in adulthood as indicated by increased adrenocortical activation. It is also consistent with the report of decreased sensitivity in rats prenatally exposed to alcohol to $GABA_A$ receptor's allosteric modulators such as endogenous neurosteroid, allopregnanolone, which is believed

to act as an endogenous anti-anxiety agent in novel or stressful situations (Zimmerberg *et al.,* 1995).

In the present study, lack of an adverse alcohol effect on behavior was observed despite of significant retardation of dams' weight gain throughout pregnancy, adversely affecting the pups' weight at birth and in the early postnatal period. However, the between-group differences in the body weight disappeared at time of testing. These observations are consistent with the reports by other authors showing that offspring of rats subjected to chronic alcohol exposure during gestation tend to be significantly lighter at birth and remain lighter than age-matched control animals during the first few weeks of postnatal life but the difference dissipates with maturation (Abel, 1979; Barnes and Walker,1981; Kim *et al.,* 1997; Mihalick *et al.,* 2001; Tran *et al.,* 2000; Westergren *et al.,*1996).

The adult brain weight was not affected either. We did not examine the brain weights at birth, so we do not know whether, microencephaly was present during the neonatal and/or juvenile period in the alcohol exposed offspring, in the current study. If, however, fetal alcohol exposure produced neonatal microencephaly, similarly as in the case of the total body weight, the catch-up growth in the brain weight took place.

CHAPTER 5

CONCLUSION

In the present study, prenatal exposure to alcohol at the dose of 6 g/kg/day delivered to the pregnant dams via intragastic infusions through GDs 7-20 slightly increased neophobia in alcohol treated offspring when tested as young adults. Otherwise, it had no adverse effect either on the motor performance or on learning and memory in these rats. The results of this study confirm that the effect of prenatal alcohol intoxication on behavior is age-dependent, and if there is a function recovery during maturation, it refers equally to both motor and cognitive aspects of behavior. Elucidation of the mechanisms of recovery from deteriorating behavioural effects of perinatal alcohol exposure in the adulthood requires further investigations.

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APPENDIX A

ANOVA TABLES FOR DAMS' BODY WEIGHT, PUPS WEIGHT

Changes in the Dams' Body Weights between GD 6 – 20

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	14	113415,3	118258,9	8447,1	17,13	0,000
Treatment	2	32484,5	32484,5	16242,3	32,94	0,000
Day*Treatment	28	7647,6	7647,6	273,1	0,55	0,969
Error	285	140538,8	140538,8	493,1		
Total	329	294086,1				

Gestational Day 18	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3567,433	2	1783,717	5,600	,013
Within Groups	5733,233	18	318,513		
Total	9300,667	20			

Gestational Day 19	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4980,376	2	2490,188	6,053	,010
Within Groups	7405,433	18	411,413		
Total	12385,810	20			

Gestational Day 20	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7221,943	2	3610,971	9,008	,002
Within Groups	7215,200	18	400,844		
Total	14437,143	20			

Pups Weight at PN 0, PN 5, and PN 10

PN 0	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
A	75	4,941	,606	7,001E-02	4,802	5,081	3,7	6,7
IC	59	5,858	,680	8,847E-02	5,681	6,035	4,5	7,3
C	59	5,812	,570	7,420E-02	5,663	5,960	3,4	6,9
Total	193	5,488	,755	5,436E-02	5,380	5,595	3,4	7,3
PN 5	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
A	69	7,788	1,131	,136	7,517	8,060	4,9	9,7
IC	57	9,189	1,342	,178	8,833	9,546	6,7	12,2
C	58	9,000	1,098	,144	8,711	9,289	6,9	11,4
Total	184	8,604	1,345	9,917E-02	8,409	8,800	4,9	12,2
PN 10	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
A	61	11,246	2,357	,302	10,642	11,850	6,3	16,3
IC	49	12,582	1,744	,249	12,081	13,083	6,5	15,5
C	42	11,279	2,658	,410	10,450	12,107	7,2	14,8
Total	152	11,686	2,341	,190	11,310	12,061	6,3	16,3

Postnatal Day 0	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	36,663	2	18,331	47,824	,000
Within Groups	72,828	190	,383		
Total	109,490	192			
Postnatal Day 5	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	74,532	2	37,266	26,284	,000
Within Groups	256,624	181	1,418		
Total	331,157	183			
Postnatal Day 10	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	58,092	2	29,046	5,627	,004
Within Groups	769,136	149	5,162		
Total	827,228	151			

APPENDIX B

ANOVA TABLES FOR OPEN FIELD TEST

Ambulation score

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatmen	2	23112	23112	11556	0,79	0,460
Day	2	518550	556009	278004	18,97	0,000
Treatmen*Day	4	68205	68205	17051	1,16	0,338
Error	51	747511	747511	14657		
Total	59	1357378				

Vertical movement score

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatmen	2	2260	2260	1130	0,52	0,596
Day	2	1918	2245	1122	0,52	0,598
Treatmen*Day	4	513	513	128	0,06	0,993
Error	51	110281	110281	2162		
Total	59	114971				

APPENDIX C

ANOVA TABLES FOR PLUS MAZE TEST

Closed Arm	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
A	10	81,670	12,868	4,069	72,465	90,875	53,7	99,3
IC	6	81,017	10,764	4,394	69,721	92,312	63,7	92,7
C	5	79,120	8,356	3,737	68,745	89,495	67,3	90,7
Total	21	80,876	10,887	2,376	75,920	85,832	53,7	99,3
Open Arm	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
A	10	2,3670	2,8138	,8898	,3541	4,3799	,00	8,67
IC	6	7,4450	6,1354	2,5048	1,0062	13,8838	,00	13,67
C	5	11,3320	9,4420	4,2226	-,3918	23,0558	,00	25,33
Total	21	5,9524	6,7183	1,4660	2,8943	9,0105	,00	25,33
Center	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
A	10	15,9670	12,3532	3,9064	7,1301	24,8039	,67	44,00
IC	6	11,5550	6,7400	2,7516	4,4818	18,6282	6,67	24,67
C	5	9,5320	2,2550	1,0085	6,7320	12,3320	7,33	13,33
Total	21	13,1743	9,4365	2,0592	8,8788	17,4697	,67	44,00

Closed Arm	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21,841	2	10,920	,084	,920
Within Groups	2348,897	18	130,494		
Total	2370,738	20			
Open Arm	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	286,619	2	143,309	4,187	,032
Within Groups	616,083	18	34,227		
Total	902,701	20			
Center	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	160,056	2	80,028	,889	,428
Within Groups	1620,888	18	90,049		
Total	1780,945	20			

APPENDIX D

ANOVA TABLES FOR ROTAROD/ ACCELEROD TEST

20 RPM 10 min	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
A	10	420,3000	198,5341	62,7820	278,2773	562,3227	164	600
IC	6	239,5000	159,7883	65,2333	71,8125	407,1875	81	464
C	5	466,0000	190,8023	85,3294	229,0877	702,9123	191	600
Total	21	379,5238	199,9604	43,6349	288,5029	470,5447	8	600
0-80 RPM 10 min	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
A	10	211,3000	108,2405	34,2287	133,8694	288,7306	15	335
IC	6	148,8333	72,4111	29,5617	72,8425	224,8241	37	225
C	5	233,2000	119,3868	53,3914	84,9618	381,4382	107	405
Total	21	198,6667	102,7455	22,4209	151,8975	245,4358	15	405
0-80 RPM 4 min	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
A	10	108,2000	39,0635	12,3530	80,2557	136,1443	25	163
IC	6	91,0000	46,9297	19,1590	41,7503	140,2497	8	149
C	5	97,0000	73,6716	32,9469	5,5247	188,4753	15	163
Total	21	100,6190	48,8113	10,6515	78,4004	122,8377	8	163

20 RPM/ 10 min	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	171657,638	2	85828,819	2,460	,114
Within Groups	628025,600	18	34890,311		
Total	799683,238	20			
0-80 RPM/ 10 min	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22458,933	2	11229,467	1,071	,363
Within Groups	188673,733	18	10481,874		
Total	211132,667	20			
0-80 RPM/ 4min	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1195,352	2	597,676	,232	,796
Within Groups	46455,600	18	2580,867		
Total	47650,952	20			

APPENDIX E

ANOVA FOR CLASSICAL MWM TRAINING

Latency

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatmen	2	2351,3	2351,3	1175,6	6,52	0,002
Day	5	57717,0	52636,8	10527,4	58,41	0,000
Trial	3	6855,6	6136,2	2045,4	11,35	0,000
Treatmen*Day	10	1336,7	1336,7	133,7	0,74	0,685
Treatmen*Trial	6	1557,9	1557,9	259,7	1,44	0,198
Day*Trial	15	5746,9	4709,9	314,0	1,74	0,041
Treatmen*Day*Trial	30	2408,5	2408,5	80,3	0,45	0,996
Error	408	73537,7	73537,7	180,2		
Total	479	151511,7				

APPENDIX F

ANOVA FOR REVERSAL TRAINING

Latency

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatmen	2	268,42	268,42	134,21	2,84	0,061
Day	2	5224,26	5347,87	2673,93	56,59	0,000
Trial	3	8000,38	7693,07	2564,36	54,27	0,000
Treatmen*Day	4	171,70	171,70	42,92	0,91	0,460
Treatmen*Trial	6	24,81	24,81	4,13	0,09	0,997
Day*Trial	6	13441,11	13191,66	2198,61	46,53	0,000
Treatmen*Day*Trial	12	209,28	209,28	17,44	0,37	0,973
Error	204	9638,54	9638,54	47,25		
Total	239	3 6978,48				

APPENDIX G

ANOVA TABLES FOR REPEATED ACQUISITION TRAINING

15 sec Delay

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	2	745,2	745,2	372,6	1,32	0,268
Trials	3	173697,8	166530,3	55510,1	196,34	0,000
block of three sessions	3	11207,2	10774,3	3591,4	12,70	0,000
Treatment*block of three sessions	6	1699,6	1699,6	283,3	1,00	0,423
Treatment*Trials	6	421,5	421,5	70,2	0,25	0,960
Error	939	265480,1	265480,1	282,7		
Total	959	453251,5				

30 min Delay

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	2	745,5	745,5	372,7	1,81	0,165
Trial	3	24289,2	23600,3	7866,8	38,29	0,000
Treatment*Trial	6	418,0	418,0	69,7	0,34	0,916
Error	308	63282,4	63282,4	205,5		
Total	319	88735,1				