ORIGINAL ARTICLE

What effects can be expected of prenatal ethanol exposure in pregnant mice and their offspring?

Que efeitos podem ser esperados da exposição pré-natal ao etanol em camundongas prenhes e sua descendência?

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ABSTRACT

Objective: To investigate the effects of chronic alcohol consumption in pregnant mice and their offspring. **Methods:** Twenty eight female C57BL/6J pregnant mice were distributed in two weight-matched groups. One group received a high protein *ad libitum* liquid diet containing 27.5% of ethanol-derived calories, from gestation day 5 to 19. The control group received the same volume of diet containing isocaloric amounts of maltose-dextrin. On postnatal day 6 the pups were counted and weighed at variable intervals up to the 60th day of life. On postnatal day 60, the males of the two groups (control and ethanol) were randomly assigned into 4 subgroups which were injected subcutaneously either with neurotoxin 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine or vehicle control. Seven days after the injection the subjects were weighed, sacrificed, and their brains were removed and processed for immunohistochemistry and neuronal counting by stereological methods. **Results:** The number of pups from the ethanol diet mothers was significantly smaller compared with the control group (3.54 ± 0.45 and 6.5 ± 0.42 respectively; $p < 0.01$), in addition of increased neonatal mortality and teratogeny, like gastroschisis. Decreased number of pups was observed among the male offspring of the ethanol diet mothers (1.54 ± 0.31 and 2.87 ± 0.48; $p < 0.05$). The brains of the ethanol diet group that received either the toxin or solvent showed a significantly decreased number of dopaminergic neurons in the pars compacta of substantia nigra as related to the control group that received the solvent. An increased number of reactive astrocytes was observed in the striatum of subjects of the alcohol/diet group injected with the toxin. **Conclusions:** Data showed that gestational alcoholism has an important role in teratogeny as well as modifying the nigrostriatal dopaminergic system of the mice offspring.

Keywords: Pregnancy, animal; Ethanol/adverse effects; Gastroschisis; Neurotoxins; Solvents; Mice: Controlled clinical trials

RESUMO

Objetivo: Investigar os efeitos do consumo crônico de álcool em camundongas durante a prenhez e na sua prole. **Métodos:** Vinte e oito camundongas da linhagem C57BL/6J prenhes foram divididas em dois grupos com pesos equivalentes. Um grupo recebeu dieta líquida rica em proteínas *ad libitum* contendo 27,5% das calorias derivadas do etanol, do 5º ao 19º dia de gestação. O grupo controle recebeu o mesmo volume de dieta contendo quantidades isocalóricas de dextrino-maltose. No 6º dia pós-natal os filhotes foram contados e pesados, em intervalos variáveis, até o 60º dia de vida. No 60º dia de vida, os camundongos do sexo masculino dos dois grupos foram randomicamente divididos em quatro subgrupos, tendo recebido injeções subcutâneas de uma neurotoxina, o 1-metil 4-fenil 1,2,3,6-tetraidropiridina ou o solvente. Sete dias depois da injeção, os animais foram pesados e sacrificados, seus cérebros removidos e processados por imunoistoquímica e feita contagem neuronal por método estereológico. **Resultados:** O número de filhotes do grupo de mães com dieta contendo etanol foi significativamente menor quando comparado ao grupo controle ($3,54 \pm 0,45$ e $6,5 \pm 0,42$, respectivamente; $p < 0,01$), além de ter se encontrado aumento da mortalidade neonatal e teratogenicidade, como gastrosquise. Encontrou-se número diminuído de filhotes do sexo masculino no grupo dieta etanol em relação ao controle ($1,54 \pm 0,31$ e $2,87 \pm 0,48$, respectivamente; $p < 0,05$). Os cérebros do grupo dieta/etanol que receberam toxina ou solvente mostraram uma significativa diminuição do número de neurônios dopaminérgicos na pars compacta da substância negra em relação ao grupo controle que recebeu solvente. Foi observado um aumento do número de astrócitos reativos no corpo estriado dos animais do grupo dieta/etanol que receberam injeção de toxina. **Conclusões:** Os dados apresentados mostraram que o alcoolismo gestacional em camundongas tem um importante papel na teratogenicidade da prole e também modifica o sistema nigroestriatal dopaminérgico desses animais.

Descritores: Prenhez; Etanol/efeitos adversos; Gastrosquise; Neurotoxinas; Solventes; Camundongas; Ensaios clínicos controlados

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INTRODUCTION

Much is known about the potential consequences of prenatal alcohol exposure through data developed in animals and man. Ethanol consumed during gestation is potentially teratogenic to several species of animals and human beings and may experimentally cause malformations in central nervous system of the offspring in animals, and in human beings. There are specific brain areas that are more affected by prenatal ethanol than others[1].

Fetal alcohol syndrome (FAS) is identified by a series of signs and symptoms, like intra and extrauterine growth restriction, mental retardation and two or more features of facial dysmorfim. Behavioural deficits may also accompany the picture. Little attempt has been made to compare the human and animal literature with respect to qualitative and quantitative similarities and differences. To this end, a comparison was made between the effects reported in humans following moderate levels of alcohol exposure and the neurobehavioral effects detected using animal models. A good deal of congruence was found with respect to qualitative endpoints. General functional categories, such as deficits in learning, inhibition, attention, regulatory behaviors, and motor performance were reported to be affected in both animals and children. Moreover, ethanol can cause neurotoxic effects in the fetal and offspring CNS, as initially described since 1968[1-2]. When clinical manifestations are not complete, without the signs of facial dismorfism, the syndrome is called "Fetal Alcohol Effects" (EAF). The most notable features of fetal alcohol syndrome involve the face and eyes, and include microcephaly, short palpebral fissures, an underdeveloped philtrum and a thin upper lip. Evidence of intrauterine or postnatal growth retardation, mental retardation or other neurologic abnormalities, and at least two of the typical facial features are necessary to make the diagnosis. Newborns with the syndrome may be irritable, with hypotonia, severe tremors and withdrawal symptoms. Mild mental retardation, the most common and serious deficit, and a variety of other anomalies may accompany fetal alcohol syndrome. Sensory deficits include optic nerve hypoplasia, poor visual acuity, hearing loss, and receptive and expressive language delays. Atrial and ventricular septal defects, as well as renal hypoplasia, bladder diverticula and other genitourinary tract abnormalities, may occur[3].

The disease has an overall estimated incidence of 9.7 cases for every 10,000 live births[4]. An epidemiologic survey in São Paulo, in 1997, showed that there is an approximate incidence of 1 per 1000 live borns[5]. Quantitatively, although the dose required to produce an effect differs across species, the resultant circulating blood alcohol levels are quite similar. In addition, while compelling data are limited, the magnitude of the observed effects are generally dose-related for humans and animals[6].

Glial cells and their interactions with neurons play vital roles during the ontogeny of the nervous system and in the adult brain. Clinical and experimental evidence indicate that in utero alcohol exposure induces structural and functional abnormalities in gliogenesis and in glial-neuronal interactions, suggesting a potential role of glial cells on ethanol-induced developmental brain abnormalities. In vivo studies have shown ethanol-associated alterations in the migration of neurons and radial glial as well as in astrogliogenesis and myelin development. In astrocytes in primary culture, ethanol has been found to 1. impair cell growth and differentiation, 2. decrease the levels of glialfibrillary acidic protein or GFAP (an astrocyte marker) and its gene expression and 3. interfere with the stimulatory effect of trophic factors affecting their release and receptor expression. Evidence also suggests that ethanol affects intracellular protein trafficking, which may mediate some effects of ethanol on astroglial cells. These findings suggest that glial cells are target of ethanol toxicity during brain development and may underlie the neurodevelopmental abnormalities observed after in utero alcohol exposure and in FAS[7].

OBJECTIVES

To investigate the effects of chronic consumption of an alcoholic diet by female mice during pregnancy on their offspring, in the number of pups, and in the morfologic changes that occur in the dopamine neurons of the nigrostriatal system of their offspring, marked by the tirosine hydroxilase immunoreactivity and quantified by specific stereologic method.

METHODS

Pathogen free female C57BL6J isogenic mice 10 weeks of age (20-25 g) obtained from the Central Animal Colony of the Biomedical Sciences Institute (University of São Paulo) were used in this study. For breeding, each nulliparous female mouse was caged with an individual stud male in the evening. The following morning, females were examined for vaginal plugs. The presence of a plug was considered indicative of conception and that day was designated as embryonic day (E) 0. Pregnant females were divided into two weight-matched groups: an ethanol consuming dams (n=16) which were given liquid diet ad libitum

containing 27.5% ethanol-derived calories (5,28% v/v; alcohol diet; BioServ, N.J.) and pair-fed dams (n=12) which received the same volume of liquid diet containing isocaloric amounts of maltose-dextrin substituted for ethanol (control diet; BioServ, N.J.). Thus, four female mice of the control diet group had two pair-fed dams of the ethanol-diet group.

Administration of the diet began on E5 and continued until E19. The ethanol was gradually introduced as follows: a mixture of 1/3 ethanol and 2/3 control diets in the first day, 2/3 ethanol and 1/3 control diets in the second day and 3/3 ethanol diet in the third day. On E18 the ethanol was inversely withdrawn as introduced with the onset of gestation.

Diets were replenished between 8:00 and 9:00 a.m. with 30 to 50 ml of diet, everyday. All dams were housed individually under constant conditions of temperature, humidity and lighting (12 h light-dark cycle). Pair-fed dams were fed the same volume of liquid diet as that consumed ad libitum by the weight-matched ethanol-fed dams on the respective embryonic day.

The volume of the liquid diet consumed by each mouse from both groups was recorded daily, and the body weight of the dams was verified every second day. The length of gestation was carefully recorded in both groups.

The neonates were examined and their number counted on postnatal day 6 (P6). The neonates were not manipulated before P6 to avoid maternal aggression against them, which could interfere with the number of live offspring. The body weight of the offspring was regularly recorded from P6 to P60.

On P60, 18 male mice were randomly separated from the offspring, 8 belonging to control diet group, and 10 to the alcohol diet group. The mice were kept in controlled conditions of temperature and humidity, and day/night cycle, receiving tap water and pellets. Either neurotoxin 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) (40 mg/kg dissolved in 1ml of saline) or the solvent was administered subcutaneously[8]. All animals were sacrificed 7 days after MPTP injection, and submitted to sectioning of brains with a cryostat (Leica, Germany) and processed for immunochemistry. Control experiments were done with other series of sections, to demonstrate the specificity of the immunostaining. Then, stereological analysis of the estimated number of dopaminegic neurons and astrocytes in substantia nigra was performed with the optical fractionator[9-11].

STATISTICAL ANALYSIS

The study of the results was performed on the Statview software (version 4.0) of MacIntosh, at the Laboratory of Neurotrophic Factors and Neuronal Plasticity, Department of Anatomy, Biomedical Sciences Institute, University of São Paulo. According to the nature of the variables, we used the *t* test, parametric, to compare the variations in number of offspring in mice of control and alcohol groups. In all tests we used the level of 5% (alfa=0,05) to reject the hypothesis of null, so that values of $p<0,05$ were considered significant.

RESULTS

The consumption of hiperproteic liquid diet by all mice utilized in the present study (control and ethanol groups) is summarized in figure 1.

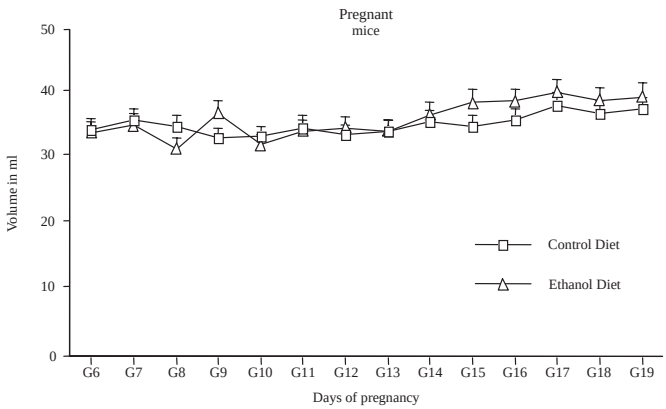

Figure 1. Means and standard deviations of daily consumption of the control and ethanol diets, of all pregnant mice. N = 12 , group N = 16 diet control and diet ethanol, $p<0,05$, according to test *t* unpaired.

Consumption of diet with or without ethanol by the pregnant mice was very similar throughout gestation. On the 19th day of pregnancy, control group mice consumed 8.18% more diet than on the 6th day, and ethanol group mice consumed 13.78% more diet than on the 6th day. However, no difference was observed in the volume of liquid diet consumed by both groups.

Table 1 shows the number of pups alive on the 6th day of life for both groups.

Table 1. Number of newborn mice in the two groups. Mean and standard deviation

Group	Number of newborn mice
Control diet	6,50 ± 0,42
Ethanol diet	3,54 ± 0,45*

*p < 0,01

As can be seen in table 1 there was a significantly diminished number of offspring of the ethanol diet mothers.

Table 2 shows the number of pups according to sex.

Table 2. Number of newborn mice according to sex. Mean and standard deviation

	Number of newborn mice	
Group	**Male**	**Female**
Control diet	2,87 ± 0,48	2,87 ± 0,35
Ethanol diet	1,54 ± 0,31*	2,00 ± 0,40

*p<0,05

As can be seen in table 2, the ethanol diet mothers had a significantly diminished number of male offspring alive on the 6th day of life.

There were no significant differences among the gestational age of both groups as well as the body mass of the pregnant mice or the effectiveness of gestation which was 75% for the two groups.

Figure 2 shows that from the 18th post natal day, pups of the ethanol group started gaining more weight than the ones from the control group, but on the 46th and the 60th day after birth no difference in the mean weight was observed in any group.

Analysis of the marked dopaminergic cells in offspring substantia nigra of the diet group showed a significant decrease of those cellular profiles in

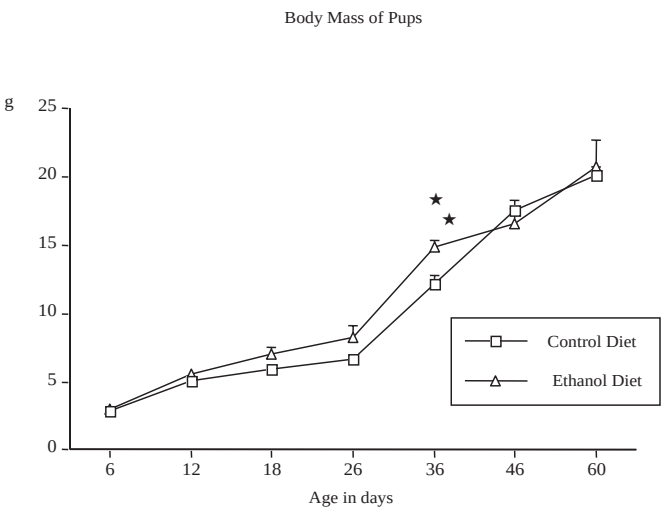

Figure 2. Means and standard deviations of the body masses of the newborn pups of groups control diet and ethanol diet, obtained on 6th, 12th, 18th, 26th, 36th, 46th and 60th days of post natal life. p<0,01 according to t test, unoaired.

comparison with the control group. However, the remaining dopaminergic neurons after ethanol administration showed an increased cell volume in the adult offspring, when compared to the control offspring.

Also, the dopaminergic terminals marked in the offspring striatum showed decreased immunoreactivity in animals treated with MPTP, but MPTP did not provoke additional decrease in the number of dopaminergic neurons in substantia nigra of the adult offspring that received ethanol during pregnancy. On the other hand, astrocytes marked by the immunoreactivity of GFAP had decreased size, but an increased number in substantia nigra (figure 3).

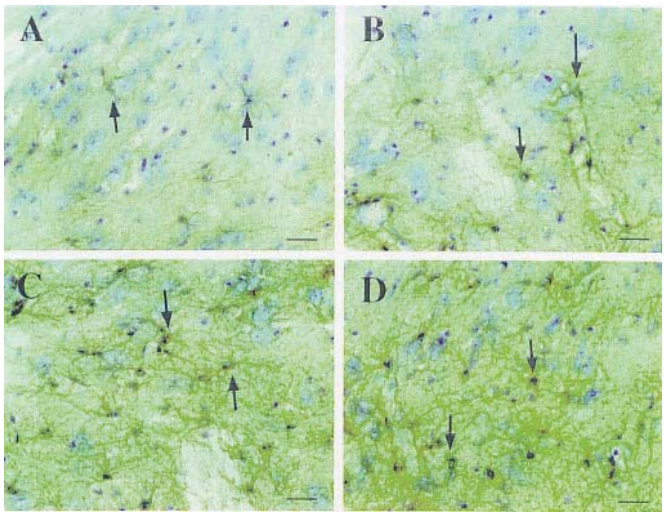

Figure 3. Astrocytes marked by the immunoreactivity of GFAP.

Among the malformations that were found, it is interesting to emphasize the presence of a stillborn with gastroschisis (figure 4).

Figure 4. The presence of a stillborn with gastroschisis.

DISCUSSION

There are several studies published in the literature that pointed out the effects of ethanol in offspring of alcoholized mice, in order to make clear the association between alcoholism during pregnancy and the teratogenic defects found in the fetus[12-15]. Female mice of C57BL/6J strain were chosen in this study because of small size, low cost and easy handling. The

comparison between findings in offspring of humans and mice exposed to ethanol in utero are fairly congruent, confirming the choice for this species in laboratory studies of the FAS. Other species of animals were used to study the effects of ethanol administered during pregnancy like golden hamsters used to observe the voluntary consumption of ethanol during pregnancy and lactation[16]. Cartwright and Smith[17] utilized chick embryos, administering one single dosis of ethanol to show the fenotipic malformations of the teratogeny of ethanol. Furthermore, ethanol has been described to be teratogenic to fish, dogs, pigs and monkeys[18-21].

In this study, concentration of ethanol in the diet corresponded to 27.5% of the calories. The concentration of ethanol utilized in this study was chosen due to the fact that the majority of researchers used between 10% and 35% of the caloric content in the form of ethanol[1,22]. This is also the concentration under which gestational alcoholism showed the teratogenic effects of ethanol in the offspring of rodents[22].

The alcoholic diet was introduced gradually from the 5th day of pregnancy on in order to assure the continuity of pregnancy, with less ocurrence of reabsorbed embryos or interruption of pregnancy. It is interesting to stress that ethanol can be given orally ad libitum[23], as in the present study, via intragastric gavage[13], or with an intraperitoneal injection[24]. Some researchers used inhalatory administration of ethanol in their experimental design[25].

In the present study, the consumption of the liquid diet by the female mice was equivalent in both alcohol and control groups throughout pregnancy. The mice of both groups showed equivalent weight gain during pregnancy, remarkably the ones who ended gestation with live neonates. The weights of the ethanol and control groups of mice was very similar by the end of gestation, reinforcing the fact that the nutritional factor did not interfere with the results. Ethanol did not modify the continuity of pregnancy, and when the female mice were mated with males they were receiving diet with no alcohol at all.

In the studied sample, the number of animals was adequate, allowing the distribution in two groups: control and experiment. Although the possibility of reabsorbed embryos or miscarriage was always present due to the alcoholization of the mice, the effectiveness of the pregancies in both groups was 75%, according to what is described in the literature[14,22]. The same can be said about the time of gestation, that in both groups was 19 to 20 days, with no statistical differences.

The offspring was counted only on 6th day after birth, to avoid mothers's agression against the pups. The decreased number of pups of the alcoholized group of mice is in accordance with what has already been found[26]. In this paper the authors used a liquid diet based on concentrated fatty acids and safflower oil, instead of the hiperproteic diet which was used in the present study.

The decreased number of male pups of the ethanol group, in comparison with the control group, was an experimental finding of the present study not yet published in the literature.

Some of the pups of the alcohol group had weights below average, with high mortality rate up to the 7th postnatal day, a finding that has already been described[27].

It is possible that the decreased weight of some pups could be compensated by the increased weight of other members of the offspring. Besides, it is possible that the decreased number of offspring of the ethanol group caused an increased offer of milk for the surviving pups by their mothers, who in turn may have contributed to the increased weight gain in the immediate postmilking period, observed in the present study. However, the actual weight gain of the pups of the alcoholized group on the 36th postnatal day is in disagreement with the paper published by Boggan et al.[27], who observed an increased weight gain of 7.14% on the 15th postnatal day of male pups in the alcoholized group in their study.

The choice of the period of alcoholization during pregnancy and the study of the effects in offspring was based in the study of Wainwright et al.[26], who showed reduction in the number of live offspring due to chronic use of ethanol, as well as decreased number of effective pregnancies and teratogeny, and decreased weight in the adult animal.

In the present study model of chronic exposure to ethanol it was observed a reduction of live pups and the presence of teratogeny. The model of chronic and daily exposure of ethanol during the pregnancy of mice is more adequate to study FAS, comparing with the acute exposure[27]. However, teratogeny in central nervous system, locomotor system and urinary tract can also occur with a single high dose of ethanol.

The majority of the malformations found in some pups of the ethanol group are described in the literature, specially those referred as abnormalities of the central nervous system, extremities and urinary tract. In the present study, microcephaly, microphtalmy and reduction in size of paws were also found, as described in the literature in other experimental models of maternal-fetal intoxication with ethanol. The presence of a stillborn with gastroschisis has not yet been described in mice.

It was found in the present study that dopaminergic cells in the offspring substantia nigra of the diet group

showed a significant decrease of those cellular profiles in comparison with the control group, but the remaining dopaminergic neurons showed increased cell volume in the adult offspring, when compared to the control group, indicating the possibility of local trophic responses, necessary to keep these neurons alive and functioning.

This study model has used chronic administration of ethanol to pregnant mice. However, it has been demonstrated that a single exposure of infant rats or mice to ethanol during synaptogenesis (corresponding to mid-late pregnancy for humans) can be responsible for apoptosis of neurons on a massive scale as well as in a wide distribution[28].

Based on the fact that the results here obtained were due to the utilization of immunohistochemical methods and stereological quantification, it is possible to speculate that there is a reasonable difficulty in postnatal recovery of neurons developed in a regimen of gestational ethanol, specially in their morphological analysis.

In humans, various anatomical alterations have been demonstrated including gross reductions in brain size, emphasizing that prenatal alcohol exposure is responsible for the impairment of cognitive and behavioral function[29]. The present findings demonstrate the usefulness of an animal model for studying the neurotoxic effects of ethanol on the developing central nervous system.

CONCLUSIONS

1. Ethanol administered during pregnancy induced teratogeny in the offspring of female mice and increase natimortality; however, the weight gain of the surviving pups was not altered significantly.
2. Ethanol during pregnancy induced a lesser dopaminergic neuron number in substantia nigra, when counted in the adult offspring but the remaining dopaminergic neurons showed increased cell volume in the adult offspring, when compared to the control group.
3. The dopaminergic terminals marked in the offspring striatum showed decreased immunoreactivity in animals treated with MPTP. However, MPTP did not provoke additional decrease in the number of dopaminergic neurons in substantia nigra of the adult offspring that received ethanol during pregnancy.

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