Efeitos da exposição pré-natal ao etanol sobre o desenvolvimento e a atenção de ratos Wistar

Ivani Brys

Dissertação de Mestrado

Porto Alegre
2011
Efeitos da exposição pré-natal ao etanol sobre o desenvolvimento e a atenção de ratos Wistar

Ivani Brys

Dissertação apresentada como requisito parcial para obtenção do Grau de Mestre em Psicologia sob orientação da Profª Dra Lisiane Bizarro

Universidade Federal do Rio Grande do Sul
Instituto de Psicologia
Programa de Pós-Graduação em Psicologia
Fevereiro, 2011
AGRADECIMENTOS

Gostaria de agradecer com muito carinho à Lisiane Bizarro pela excelente orientação, pela relação de confiança que construímos e por todos os momentos de convivência. Sou muito grata por tua disponibilidade, tua atenção e por ter sempre me feito sentir tão bem em nossas supervisões, ao proporcionar momentos de aprendizado, descontração e alegria. Um agradecimento especial pelo suporte incondicional e pela certeza de poder contar contigo do começo ao fim desta trajetória. Me faltam palavras para expressar meu reconhecimento por tudo isso e para descrever a tua importância em minha formação profissional e pessoal. Muito obrigada.

À equipe da Unidade de Experimentação Animal do HCPA: Marta Cioato, Fabíola Meyer, Carolina Mello e especialmente Eduardo Silveira pela acolhida e pelo excelente cuidado oferecido aos animais.

Ao CNPq pelo apoio financeiro e à Wellcome Trust por proporcionar estrutura e materiais de excelente qualidade para a realização deste trabalho.

À professora Carla Dalmaz por sempre me receber de portas abertas em seu laboratório e por compartilhar seu conhecimento e experiência.

À Rosa por suas contribuições sempre bem humoradas.

Aos colegas Taís, Renata e Roberto pelo interesse e pelos momentos de companhia no laboratório. Ao João e ao Guto pela cooperação e pelas valiosas contribuições técnicas.

À minha mãe pelo apoio, pelo amor e por sempre valorizar minhas escolhas.

À Kati e à Franci por estarem presentes em tudo o que faço.

E, por fim, um agradecimento especial ao Stéfano por compartilhar comigo este projeto e torná-lo completo. Tua contribuição foi essencial para a realização deste trabalho, que é resultado de dedicação e esforço conjuntos. Posso dizer que tive muita sorte por nossas trajetórias terem se cruzado e por termos compartilhado tantas ideias e tantos momentos de alegria e inquietação. Sou muito grata pelas vezes em que me tornou mais forte e me ajudou a enxergar que os sonhos são bem mais possíveis do que distantes. Muito obrigada pela presença, pela parceria e por acreditar em nosso sucesso na realização deste e de outros projetos que nos desafiam pela frente.
SUMÁRIO

LISTA DE TABELAS..........................................................................................................................5
LISTA DE FIGURAS...........................................................................................................................6
RESUMO..............................................................................................................................................7
ABSTRACT..........................................................................................................................................8
INTRODUÇÃO.....................................................................................................................................9
CAPÍTULO I
Role of dose in developmental abnormalities and increased locomotor activity in rats prenatally exposed to ethanol
Abstract................................................................................................................................................11
Introduction.........................................................................................................................................12
Method
   Subjects...................................................................................................................................15
   Procedures..............................................................................................................................15
   Data analysis..........................................................................................................................17
Results
   Gestational data......................................................................................................................17
   Litter weight...........................................................................................................................18
   Negative geotaxis..................................................................................................................19
   Grip Strength..........................................................................................................................20
   Locomotor Activity...............................................................................................................21
Discussion...........................................................................................................................................22
CAPÍTULO II
Specific attentional deficit of Wistar rats prenatally exposed to ethanol in the Five choice serial reaction time task
Abstract................................................................................................................................................25
Introduction.........................................................................................................................................26
Method
   Subjects..................................................................................................................................28
   Test apparatus.........................................................................................................................29
   Behavioral procedures.........................................................................................................30
Baseline acquisition and performance..............................................................................31
Challenges of attention......................................................................................................31
Data analysis....................................................................................................................32

Results

Body weight.......................................................................................................................33
Baseline acquisition and performance............................................................................33
Challenges of attention......................................................................................................35

Discussion..........................................................................................................................38
CONSIDERAÇÕES FINAIS................................................................................................42
REFERÊNCIAS..................................................................................................................44
ANEXO A...........................................................................................................................51
LISTA DE TABELAS

Tabela 1 - Data regarding the gestational period.................................................................18

Tabela 2 - Mean weight of female and male pups in postnatal......................................19
days 1, 7, 14, 21 and 40

Tabela 3: Mean performance in the 5-CSRTT over 8 consecutive...................................34
baseline days

Tabela 4: Mean performance in test sessions with manipulations.................................36
of ITI and stimulus duration
LISTA DE FIGURAS

Figura 1: Mean performance in the negative geotaxis .......................................................20

Figura 2: Mean performance in the grip strength.................................................................21

Figura 3: Distance walked by male rats in a 15-min locomotor ........................................23
    activity task, divided in three 5-min blocks

Figura 4: Accuracy and percentage of omissions in the .................................................34
    5-CSRTT over 8 consecutive baseline days

Figura 5: Mean percentage of omissions in sessions whit .............................................37
    ITI manipulations

Figura 6: Mean percentage of omissions in test..............................................................38
    sessions with 0.25s and 0.5s SD
RESUMO

Este trabalho teve como objetivo investigar os efeitos da exposição pré-natal a duas doses de etanol sobre o desenvolvimento e a atenção de ratos. Método: 49 ratas Wistar prenhes distribuídas em quatro grupos de acordo com o tratamento gestacional: A35 – dieta líquida com 35% de calorias derivadas do etanol (CDE); A10 – dieta líquida com 10% CDE; Controle – dieta líquida sem etanol; Chow – livre acesso à água e ração. No estudo I, foram avaliados o peso, os reflexos (geotaxia negativa e força de agarrar) e a atividade locomotora dos filhotes. No grupo A35, apenas 43% dos filhotes sobreviveram. Os animais desse grupo apresentaram menor peso que os demais, prejuízos na tarefa de força de agarrar e maior atividade locomotora. No estudo II, 12 filhotes machos adultos de cada grupo foram testados na Tarefa serial de cinco escolhas. Os animais do grupo A35 apresentaram maior número de omissões, principalmente no final das sessões. Conclusão: Efeitos do etanol são dose-dependentes e podem estar associados com um prejuízo atencional específico.

Palavras-chave: Exposição pré-natal ao etanol, álcool, síndrome alcoólica fetal, desenvolvimento, atenção
**ABSTRACT**

**Effects of prenatal ethanol exposure on developmental milestones and attention of Wistar rats**

The objective of this study was to investigate the effects of prenatal exposure to two doses of ethanol on developmental milestones and attention of rats. **Method:** 49 pregnant Wistar rats were assigned to one of four gestational treatments: A35 - liquid diet with 35% ethanol-derived calories (EDC); A10 - liquid diet with 10% EDC; Control – ethanol-free liquid diet; Chow – laboratory chow and water. In study I, litters were tested for body weight, negative geotaxis, grip strength and locomotor activity. Only 43% of A35 pups survived. A35 weighed less than other groups, performed worse in grip strength and presented more locomotor activity. In study II, 12 male two-month old rats from each group were tested in the Five choice serial reaction time task. A35 performed more omission errors than other groups, mainly during the final part of the sessions. **Conclusion:** Ethanol effects are dose-dependent and might be associated with a specific attentional deficit.

**Key words:** Prenatal exposure to ethanol, alcohol, fetal alcohol syndrome, development, attention
INTRODUÇÃO

O consumo de álcool durante a gestação tem sido associado com diversos prejuízos para o desenvolvimento do feto. O etanol é um agente teratogênico e o desenvolvimento neural é especialmente sensível a seus efeitos durante a gestação (Roebuck, Mattson, & Riley, 1998). Uma das consequências mais conhecidas é a Síndrome Alcoólica Fetal (SAF), caracterizada por retardo no crescimento, distinção da aparência crânio-facial, prejuízos em funções cognitivas e impulsividade (Alderazi & Brett, 2007).

A gravidade dos danos causados ao feto está associada com o consumo crônico de altas doses de álcool (Riley & McGee, 2005), porém não há consenso na literatura sobre a existência de uma dose segura de consumo dessa droga durante a gestação. A exposição a níveis baixos e moderados de etanol também tem sido associada com prejuízos cognitivos e comportamentais (Sutherland, McDonald & Savage, 1997). Atualmente, a literatura reconhece a existência de um espectro de prejuízos que pode resultar da exposição pré-natal ao álcool que não engloba necessariamente todos os critérios necessários para o diagnóstico de SAF (Astley et al., 2009; Sokol, Delaney-Black, & Nordstrom, 2003). Esses prejuízos incluem problemas de aprendizado (Streissguth, Bookstein, Sampson, & Barr, 1995) e de memória (Streissguth et al., 1994), maior propensão para o desenvolvimento de transtornos de conduta (Disney, Iacono, McGue, Tully, & Legrand, 2008) e de adição a drogas na idade adulta (Baer, Sampson, Barr, Connor, & Streissguth, 2003; Yates, Cadoret, Troughton, Stewart, & Giunta, 1998).

Em tarefas que avaliam atenção, crianças expostas ao álcool no período pré-natal apresentam desempenho prejudicado de forma similar a crianças diagnosticadas com Transtorno de Déficit de Atenção e Hiperatividade (TDAH) (Coles et al., 1997; Nanson & Hiscock, 1990). Esses prejuízos incluem declínio do desempenho ao longo da duração do teste, maior número de erros, maior variabilidade na latência de resposta, maior tempo de reação e maior ocorrência de omissões (Coles et al., 1997; Kooistra, Crawford, Gibbard, Ramage, & Kaplan, 2009).

Esses dados sugerem que a ingestão de álcool durante a gestação pode ser considerado um fator de risco para o desenvolvimento de TDAH na infância (Brown et al., 1991; Streissguth et al., 1994; Mick, Biederman, Faraone, Sayer, & Kleinman, 2002). No
entanto, essa relação tem sido pouco estudada em modelos animais. Esse tipo de estudo é importante, pois permite maior controle de variáveis, tais como dose, frequência de consumo, uso concomitante de outras drogas e nutrição.

Em um dos poucos estudos que investigaram a relação entre exposição pré-natal ao etanol e prejuízos atencionais em roedores, Hausknecht et al. (2005) avaliou o desempenho de ratos adultos na tarefa *Choice Reaction Time (CRT)*. Os resultados mostraram que os animais tratados com etanol exibiram maior variação no tempo de reação e maior índice de respostas prematuras, indicando possíveis lapsos de atenção e impulsividade.

A fim de contribuir para o entendimento do tema, serão apresentados a seguir dois artigos empíricos a respeito dos efeitos do etanol sobre o desenvolvimento e a atenção de ratos Wistar. No Capítulo I, será apresentado o artigo sobre o papel da dose de etanol nos prejuízos de desenvolvimento e atividade locomotora dos animais. No Capítulo II, será apresentado o artigo sobre os efeitos da exposição pré-natal ao etanol sobre a atenção, avaliada através da Tarefa Serial de Cinco Escolhas.
Role of dose in developmental abnormalities and increased locomotor activity in rats prenatally exposed to ethanol

Abstract

Objective: There is a scarcity of experimental studies investigating the teratogenic effects of alcohol in small quantities. To address that, we investigated the effects of a prenatal exposure to a standard and a lower dose of ethanol in Wistar rats. Method: Pregnant rats were allocated to 4 weight-matched groups that were assigned either to liquid diets (containing 35%, 10% or 0% ethanol-derived calories [EDC]) or free access to laboratory chow and water. Developmental milestones (weight, negative geotaxis, grip strength) and locomotor activity were evaluated in the offspring of these females. Results: Teratogenic effects of prenatal ethanol were observed only in the group treated with the standard dose. Abnormalities included a lower weight gain, less muscular strength and increased locomotor activity. Conclusion: By limiting the amount of liquid diet consumed by each weight-matched triplet, we were able to estimate the effect of standard (35% EDC) or lower (10% EDC) doses of ethanol during pregnancy on their offspring on developmental milestones. These results stress the role of the dose of prenatal ethanol in the impairments caused by this teratogen, suggesting that, in very small doses, it might not cause detectable negative developmental consequences to rats.

Keywords: Prenatal Exposure to Ethanol, Dose, Alcohol, Fetal Alcohol Syndrome, Blood Alcohol Concentration, Negative Geotaxis, Grip Strength, Locomotor Activity
Introduction

Ethanol is a well-known teratogenic agent that produces a set of physical and cognitive impairments known as Fetal Alcohol Syndrome (FAS), a severe condition that results in mental retardation, facial dismorphisms and impaired motor coordination (Jones & Smith, 1973), as well as lower birth weight and Apgar scores (Bagheri, Burd, Martsolf, & Klug, 1998). Currently, the literature recognizes a spectrum of impairments that might result from prenatal ethanol exposure that do not necessarily achieve the criteria for a diagnosis of FAS (Sokol, Delaney-Black, & Nordstrom, 2003). This spectrum includes increased propensity for learning problems (Streissguth et al., 1995), deficits in attention and memory (Streissguth et al., 1994), increased incidence of conduct disorders (Disney et al., 2008) attention-deficit hyperactivity disorder (Mick et al., 2002) during school years and development of addiction to alcohol (Baer et al., 2003), nicotine and other drugs in adulthood (Yates et al., 1998).

However, so far, it is uncertain if some amount of ethanol can be ingested without causing teratogenic effects. This issue has resurfaced recently, when the United Kingdom decided to revise its guidelines for alcohol consumption during pregnancy. It now advises total abstinence, thus sparking a debate over the available evidence (O'Brien, 2007; Nathanson, Jayesinghe, & Roycroft, 2007). Due to the inexistence of a reliable biological marker for moderate alcohol consumption, the majority of studies with humans are based on interviews, in which mothers self-report, often years later, if they did or did not consume ethanol during pregnancy (Sokol Delaney-Black, & Nordstrom, 2003). Perhaps due to the difficulty in adequately controlling for factors such as dose, genetics and bias (Huizink, 2009), the literature is controversial as to the effects of small (one to two drinks a week) doses of ethanol on the offspring. There are studies that support (Sood et al., 2001; Huizink, 2009; Abate, Pueta, Spear, & Molina, 2008) or oppose (Polygenis et al., 1998; Kelly et al., 2008; Henderson, Gray, & Brocklehurst, 2007) the notion that consuming even small amounts of ethanol can be detrimental to the offspring.

In contrast, animal models allow greater experimental control over dose and period of exposure. Indeed, there is a vast range of work in both rodents and primates that corroborate the cognitive deficits found in human (see Cudd, 2005). Animals also provide a way to study more clearly the biochemical and neuroanatomical effects of prenatal ethanol exposure. Impairments such as hypothalamic oxidative stress (Dembele, Yao, Chen, & Nyomba, 2006),
a lower overall brain weight (Ikonomidou et al., 2000), lower number of serotonergic neurons in the brainstem (Sari & Zhou, 2004) and a reduced number of neurons in the prefrontal cortex (Burke, Palmour, Ervin, & Ptito, 2009) are examples of the possible physical substrates of the cognitive and behavioral abnormalities found in humans and other animals.

One of the sources of variability in the outcomes produced by different levels of prenatal exposure to ethanol is the wide range of methods of administration used in studies with animals. In comparison to injections or oral/intragastric gavage, the use of liquid diets (nutritional preparations containing differing amounts of ethanol) are less stressful and allow for more stable levels of blood alcohol concentration (BAC). The most widely used procedure involving liquid diets entails the addition of 67ml of ethanol for each litre of diet, leading to 35% of ethanol-derived calories (EDC) in the diet, which produces a BAC of a little over 100mg/dl for much of the dark cycle (Driscoll, Streissguth, & Riley, 1990). Compared with the peak BACs produced by intraperitonial injections or gavage (which often reach over 300 mg/dl), and the fact that a chronic administration of ethanol produces lower BACs in contrast to an acute one (Bonthius & West, 1990; Bielawski & Abel, 2002), this could be considered a moderate level of exposure, even though the total amount of alcohol consumed in a day might be similar or higher than in other methods.

Although the BAC produced is not too high, the standard 35% EDC dose is still often associated with a high number of stillborn pups and other gross impairments (Bond & Di Giusto, 1977). The prenatal exposure to this dose results in less weight in male pups (Riley, Shapiro, & Elizabeth, 1979), impaired reflexes and poor muscular strength in the negative geotaxis and grip strength tasks (Hannigan, 1995). Additionally, these rats tend to walk for longer distances in an open field test when young (Bond & Di Giusto, 1976; Bond & Di Giusto, 1977).

The consequences of exposure to even lower doses of ethanol are still relatively little known. Most studies in this area were produced by two researchers, Daniel Savage and Edward Riley. The group led by Savage has focused on the hippocampal abnormalities produced by liquid diets with doses most commonly ranging from about 15% to 26% EDC (Reyes et al., 1989; Farr, Montano, Paxton, & Savage, 1988; Tan, Berman, Abel, & Zajac, 1990; Savage, Montano, Otero, & Paxton, 1991; Martin, J. C. Martin, D. C., Sigman, & Radow, 1978; Savage, Cruz, Duran, & Paxton, 1998). This range produces a BAC of roughly 30 to 80mg/dl, respectively. In their only study investigating doses lower than that range,
(Savage, Becher, de la Torre, & Sutherland, 2002) compared the effects of different doses using groups exposed to liquid diets with about 11%, 16% or 26% EDC in contrast to control groups for each dose, and found a dose-dependent effect of prenatal ethanol on synaptic plasticity. Those fed with about 11% EDC (with a BAC of ~7mg/dl) showed no effects, whereas plasticity was reduced in the groups fed with 16% and 26% EDC. None of these groups showed any difference in weight or neonatal mortality.

The group led by Riley introduced the important step of limiting the amount of liquid diet consumed by each dam in groups fed with control diets (0% EDC) or lower doses of ethanol to the amount consumed by their weight-matched dam in the higher dose group. By controlling this, the number of calories consumed is necessarily the same in all groups, and the proportion of ethanol contained in the diet is fixed, while also reducing the need for additional control groups and animals. In studies using this method and liquid diets ranging from 8% to 35% EDC, his group demonstrated that alcohol dose-dependently increased the response rate in two separate tasks that measured spontaneous nose-poking and head-dipping behaviors (Riley, Shapiro, & Lochry 1979a). Although no direct comparisons between groups were made, the article implies that the 12% EDC group (mean BAC ~30mg/dl) differed significantly from controls. In another study (Lochry & Riley, 1980), with the same levels of exposure, all groups exposed to ethanol weighed less than controls, although, again, the statistical significance is derived from an indirect comparison. Riley, Lochry and Shapiro (1979b) provided evidence that even rats exposed to a liquid diet with 8% EDC needed more trials to learn a passive avoidance task in relation to controls. This exposure produced a BAC of only 4mg/dl, the lowest ever reported in the literature. Both this study and a subsequent one (Lochry and Riley, 1980), with a similar design, found that groups with intermediate degrees of exposure (17.5%, 19% and 32% EDC) had learning and retention deficits on the same task.

Clausing et al. (1995, 1996) found that 35% but not 18% EDC, administered in the same way as in Riley’s studies, showed lower body weight and impaired taste aversion and timing behavior. Vaglenova and Petkov (1998) administered 1g/kg of ethanol a day via peroral intubation, producing a BAC of 36mg/dl, which resulted in a higher mortality in comparison to controls. With a similar method and even lower doses (0.15 and 0.30g/kg), Abel (1996) found no differences in birth weight.

Therefore, we identify a need for better information regarding the teratogenic effects of small doses of ethanol. Our goal was to investigate further the role of dose of ethanol exposure
during pregnancy on possible developmental impairments in the offspring of rats in a controlled, experimental study. Using liquid diets with a methodology adapted from Riley, Shapiro, and Lochry (1979a), we compared the effects of a standard (35% EDC) and a lower dose (10% EDC) of prenatal ethanol on developmental milestones - weight, negative geotaxis, grip strength - and locomotor activity in both male and female pups.

**Method**

**Subjects**

Wistar rats (80 females and 40 males, Fundação Estadual de Produção e Pesquisa em Saúde, Rio Grande do Sul, Brazil) were two to three months old at the beginning of the study and were housed at the Unidade de Experimentação Animal at Hospital de Clínicas de Porto Alegre, in a 12 h light/dark cycle (lights on from 0700 h to 1900 h), and a temperature of 22-24°C. After two weeks of acclimation to the laboratory, they were singly housed in plastic cages (24 cm x 38 cm x 15 cm). Dams were assigned to one of the four weight-matched groups: (1) A35-liquid diet with 35% ethanol-derived calories (EDC), or 6.7% v/v, (2) A10-liquid diet with 10% EDC, or 1.8% v/v (3) Control - liquid diet without ethanol and (4) Chow - free access to laboratory chow and water. Group A35 had access to 150 ml of liquid diet each day, and the amount consumed by each dam at a given gestational day was the limit provided to its weight-matched counterparts in the A10 and control groups on the same gestational day. To make the A10 diets isocaloric compared with the other groups, maltodextrin was added according to the guidelines of the diet provider (BioServ, Frenchtown, United States. National and institutional guidelines for animal welfare were followed and all procedures were approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre.

**Procedures**

A week before initiating the breeding procedures, the females were habituated to the liquid diet. To minimize effects due to the possibly aversive taste of ethanol, its concentration was increased progressively for animals in the A35 group, up to the final dose containing 35% EDC. The animals in groups A10 and Control received the liquid diet in the same constitution as that during pregnancy (10% and 0% EDC, respectively). During breeding, all animals had free access to water and chow.
The animals were mated by housing each female with a male throughout the dark phase of the day-night cycle whenever a vaginal smear in the morning of that day indicated the animal was in proestrus or diestrus. The presence of sperm in the vaginal smear in the morning following mating was considered a confirmation of pregnancy, and the treatment with diets reinitiated. Rats with no confirmation of pregnancy were reintroduced to the mating procedures. The breeding period lasted two weeks.

During pregnancy, the animals were weighted three times a week. The liquid diet was replenished daily in graduated feeding tubes between 1400 h and 1600 h and the amounts of liquid food consumed were recorded. Immediately after giving birth, the groups fed with liquid diet were switched to water and chow *ad libitum*. The litters were culled, whenever possible, to 8 pups (4 males, 4 females). All behavioral tests were conducted during the second half of the light phase of the cycle. With the exception of the locomotor activity test, all tests were conducted in the room where the litters were raised. The pups were briefly separated from their mothers for all tests, and returned to their home cages immediately after finishing them.

*Weights*

The pups were weighed at postnatal days (PND) 1, 7, 14, 21 and 40.

*Negative Geotaxis*

Subjects were tested for geotactic reflexes on PNDs 7–10. During this task, each pup was placed face-downward on a plain wooden board with a 30° slant. Latency to rotate 180° was measured, with a maximum of 30 seconds allowed per trial, with two trials per day. If the pup completed only one trial within the allotted time, data from it was its score for the day. If, however, a pup was able to complete the rotation within the allotted time in both trials, the mean latency of these two trials was used for analysis.

*Grip Strength*

The rat was positioned vertically a few centimeters above the floor, until its paws grasped a steel rod (diameter 0.2 cm), fixed at a height of approximately 20cm in parallel to the floor. When it grasped the rod, the rat was released, and the time the rat held on to the rod before falling was recorded, with a maximum of 20 seconds per trial, in two trials per day. The
mean latency of both trials was the measure analyzed at each day. This test was performed on PND 14 and PND 17.

**Locomotor Activity**

Subjects were placed individually for 15 min in activity chambers (34.5 cm height x 45 cm depth x 45 cm width) (Insight Ltda, Ribeirão Preto), equipped with photocells and 16 infrared light beams. Data were recorded on a Windows PC using proprietary software (Monitor de Atividades) provided by the manufacturer of the boxes. Horizontal locomotor activity was estimated from the number of beam breaks, and converted by the software to distance walked in periods of 5 minutes. This test was performed on PND 19 with two male pups from each litter, randomly selected.

**Data Analysis**

Weight and latency data were subjected to analysis of variance (with post-hoc Tukey HSD tests where applicable); comparisons such as the number of rats to complete the negative geotaxis and grip strength test on a particular day were made by Pearson’s chi-square test. Latencies were log-transformed before analysis in order to correct for the skew imposed by the time limit. For weight, the unit of analysis was the litter (that is, the mean weight of its members, separated by gender). Whenever possible, male and female rats were analyzed separately, in order to account for possible differences in the ability to perform a task and to identify possible sex-specific effects of the treatment. The significance criterion was p<0.05. All data was analyzed using the software SPSS for Windows, version 16.

**Results**

**Gestational data**

The amount of liquid diet consumed daily during pregnancy was approximately 70 ml per dam. Gestational data is summarized in Table 1. Weight increased progressively [F(3,132)=544.7, p<0.001], but dams fed with liquid diets gained less weight than chow-fed dams [F(3,44)=4.9, p=0.005]. This difference between groups became more evident in the last weeks of pregnancy [F(9,44)=4.7, p<0.001], given that the chow group gained more weight than the control group treated with liquid diet from days 7 to 21 (p<0.05). Only groups treated with ethanol gave birth to stillborn pups. The A10 group had a single observed stillbirth. In the
A35 group, there were 18 of these cases and 19 other pups died during PND 1-7, a survival rate of 43% (Table 1).

Table 1

*Data regarding the gestational period*

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>Control</th>
<th>A10</th>
<th>A35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of dams</td>
<td>12</td>
<td>11</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Initial weight of dams (grams) – Mean</td>
<td>218.5±6.5</td>
<td>213.1±4.1</td>
<td>214.6±4.1</td>
<td>218.2±5.3</td>
</tr>
<tr>
<td>Weight gain of dams (grams) – Mean</td>
<td>114.4±7.7*</td>
<td>81.4±5.3</td>
<td>87.0±4.4</td>
<td>84.1±9.1</td>
</tr>
<tr>
<td>Number of stillborn pups</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>Number of pups that died during PNDs 1-7 (not counting stillborns)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>Number of live pups</td>
<td>86</td>
<td>76</td>
<td>117</td>
<td>28</td>
</tr>
<tr>
<td>Percentage of live male pups</td>
<td>51.1%</td>
<td>44.7%</td>
<td>49.6%</td>
<td>67.8%</td>
</tr>
</tbody>
</table>

*Dams in the liquid-diet groups had significantly less weight gain than their counterparts in the Chow group (p=0.005)*

**Litter weight**

An analysis of variance showed no statistically significant difference between the birth weights of the different groups of male [F(3,39)=2.0, p=0.1] or female [F(3,38)=2.5, p=0.68] pups. However, during the subsequent postnatal days, weight differences emerged.

For females, a repeated measures analysis of variance showed an effect of PND [F(4,136)=5232.4, p<0.001] and group [F(3,34)=2.8, p=0.054], and an interaction between these factors [F(12,136)=2.7, p=0.042]. For males, a repeated measures analysis of variance found an effect of PND [F(4,148)=6225, p<0.001], group [F(3,37)=5, p=0.005] and an interaction between these factors [F(12,148)=3.5, p=0.013]. In most days, post hoc tests evidenced that the A35 groups had less weight in both genders (Table 2).
Table 2

*Mean (SEM) weight (grams) of (A) female and (B) male pups in postnatal days 1, 7, 14, 21 and 40*

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>Control</th>
<th>A10</th>
<th>A35</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FEMALES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 1</td>
<td>6.4 (0.3)</td>
<td>5.9 (0.1)</td>
<td>5.7 (0.1)</td>
<td>5.6 (0.3)</td>
</tr>
<tr>
<td>PND 7</td>
<td>15.9 (0.4)</td>
<td>15.8 (0.5)</td>
<td>15.40 (0.4)</td>
<td>11.5 (1.5) *</td>
</tr>
<tr>
<td>PND 14</td>
<td>29.1 (0.9)</td>
<td>29.9 (1.2)</td>
<td>28.0 (0.7)</td>
<td>22.9 (1.8) *</td>
</tr>
<tr>
<td>PND 21</td>
<td>45.3 (1.5)</td>
<td>46.3 (1.2) a</td>
<td>43.6 (1.2)</td>
<td>38.6 (3.3) a</td>
</tr>
<tr>
<td>PND 40</td>
<td>137.4 (2.7) a</td>
<td>132.6 (2.1)</td>
<td>131.6 (2.4)</td>
<td>120.9 (2.6) a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>Control</th>
<th>A10</th>
<th>A35</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MALES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 1</td>
<td>6.7 (0.2)</td>
<td>5.6 (0.6)</td>
<td>6.0 (0.1)</td>
<td>5.7 (0.3)</td>
</tr>
<tr>
<td>PND 7</td>
<td>16.5 (0.3)</td>
<td>16.6 (0.6)</td>
<td>15.57 (0.3)</td>
<td>12.2 (1.4) *</td>
</tr>
<tr>
<td>PND 14</td>
<td>29.8 (0.8) b</td>
<td>31.1 (1.2) a</td>
<td>28.8 (0.8)</td>
<td>24.5 (2.0) a,b</td>
</tr>
<tr>
<td>PND 21</td>
<td>46.6 (1.3)</td>
<td>48.2 (1.5) a</td>
<td>44.9 (1.4)</td>
<td>39.5 (2.9) a</td>
</tr>
<tr>
<td>PND 40</td>
<td>155.9 (3.1)</td>
<td>157.9 (2.4)</td>
<td>153.6 (2.9)</td>
<td>137.7 (3.9) *</td>
</tr>
</tbody>
</table>

*Represents a statistically significant difference (p<0.05) compared with all other groups in that day. a, b Represents a statistically significant difference (p<0.05) between the respective groups in that day.

**Negative geotaxis**

A Pearson’s chi-square analysis showed no significant difference between groups in failures to complete at least one trial on days 7, 8, 9 and 10 (p>0.5). Using the mean time to complete both trials within a day as the dependent measure, a two-factor repeated measures ANOVA found an effect of Time (males, [F(3,114)=18.8, p<0.001]; females,[F(3,117)=10.9, p<0.001]), with the time to complete trials decreasing as days passed (Figure 1). However, no effect of Group was found for males [F(1,38)=0.3, p=0.8] or females [(1,39)=0.38, p=0.76]. No significant interaction was found for either gender.
Figure 1. Mean performance in the negative geotaxis. Mean (SEM) of the time, in seconds, spent by (A) female and (B) male pups to complete a 180° turn on a slope (less is better). No significant difference between groups was found.

Grip Strength

For both males and females, a Pearson’s chi-square analysis showed that, on both days of test, Group was not a significant factor in determining whether or not the rat was able to complete at least one of the trials (p>0.05). It was, however, a significant factor in the time to complete those trials. Using two separated one-way ANOVAs, we observed that, on the first (PND 14) but not the second (PND17) day of this task, both male [F(3,37)=3.8, p=0.018] and female [F(3,38)=4.6, p=0.007] rats from the A35 group showed impaired grip strength when compared to control and chow groups (Figure 2). No other significant difference was found between groups in either day of the task.
**Figure 2.** Mean performance in the grip strength. Female (A) and male (B) rats in the A35 group were able to support their weight by grasping in a metal rod for significantly less time at PND 14 (more is better). *Represents a statistically significant difference (p<0.05) compared with all other groups. a, b Represents a statistically significant difference between two groups.

**Locomotor Activity**

In PND 19, an One-way Anova showed that the A35 group displayed an increased locomotor activity in a 15-min session [F(3,89)=3.7, p=0.013]. The post hoc tests indicate that the A35 Group walked for a longer distance compared with groups A10 (p=0.011) and control (p=0.049). Breaking down the session in three 5-minute blocks, and analyzing each block separately with a one-way ANOVA, the Group factor was significant in the first [F(3,93)=3.7, p=0.14] and second [F(3,93)=2.9, p=0.38], but not in the last block [F(3,93)=1.1, p=0.327].
Post hoc tests indicated that the A35 animals walked a significantly greater distance compared to A10 in the first block (p=0.12) and control in the second (p=0.30) (Figure 3).

**Figure 3.** Distance (cm) walked by male rats in a 15-min locomotor activity task, divided in three 5-min blocks. (a, b) Indicates a significantly different performance between groups (p<0.05) in each block.

**Discussion**

As previously reported in the literature, liquid diets prepared with ethanol at standard doses (35% EDC) caused severe consequences for litter development. The high mortality and the impaired weight gain observed in this study with that dose are consistent with earlier studies (Vorhees, 1989; Lopez-Tejero, Ferrer, Llobera, & Herrera, 1986). In contrast, the group exposed to a lower quantity of ethanol during gestation (10% EDC) did not differ from control groups in any of the tests performed, an original finding for this range of exposure in all these tests, and one that serves to better detail the consequences of such small quantities of ethanol during pregnancy.

In the negative geotaxis task, there was no effect of prenatal ethanol exposure on the ability or latency to complete the task. Some studies have reported an impact of ethanol on negative geotaxis (Hannigan, 1995; Thomas, Abou, & Dominguez, 2009), while others have not (Vaglenova et al., 2007). These studies used a variety of doses, and there’s no consistent
dose-response curve one could extrapolate from them. Therefore, this discrepancy is likely due to methodological differences between the experiments. Thomas, Abou, and Dominguez (2009), for instance, used the task in PNDs 7 to 16, and found a significant difference only on day 7. In their experiment, however, the maximum allotted time per trial was 3 minutes and the slant of the board was 45°, in contrast to the 30 seconds and 30° slant used in our study. (Vaglenova et al., 2007) used the negative geotaxis task in PNDs 3 to 8, with a duration of 45 seconds per trial and a slant of 30°, and also found no difference between alcohol-treated and control groups.

In the grip strength task, only the offspring (both male and female) of dams treated with higher doses of ethanol held on for significantly less time on the first day of task (PND 14), and this effect disappeared on PND 17. This suggests an impairment in the development of muscular strength and the suspension reflex, a result that has been observed before (Hannigan, 1995), and which corroborates the findings of Thomas, Abou, and Dominguez (2009), who also found that the ability to complete the task was impaired around PND 14, but not afterwards. Prenatal exposure to ethanol has been found to reduce somatosensory (Margret et al., 2006) and motor (Xie, Yang, Chappell, Li, & Waters, 2010) cortical representation of rats’ forelimbs (i.e. the cortical area identified by direct electrical stimulation), which may underlie the verified deficits in this kind of reflex development. It seems that the smaller dose used in our study might not interfere with these aspects of development, as it produced no impairment on the grip strength task.

As for the locomotor activity task, the increased ambulation we found in the group receiving 35% EDC diets is also found in other studies with this level of exposure (Bond and Di Giusto, 1976), although it is likely that this effect disappears with age (Bond and Di Giusto, 1977). We were the first to report that these differences are mostly dependent on the performance in the initial part of the task, as their hiperactivity was no longer discernible after a period of 10 minutes, suggesting that their increased locomotion might be triggered by novelty. Here, again, the rats exposed to 10% EDC had a performance indistinguishable from controls.

Our work provides evidence that the known deleterious effects of a larger dose of ethanol in weight, reflex development, muscular strength and locomotor activity cannot be observed when chronically administering a very small dose (10% EDC on liquid diets).
Further studies with low to moderate doses should investigate other behavioral and neuroanatomical effects of exposure in the aforementioned range.

The experiments described so far suggest that, in rats, the threshold for the teratogenic effects of ethanol can be as low as a BAC of 4mg/dl (Riley, Lochry, & Shapiro 1979b). However, as our experiments demonstrated, in accordance with a previous observation (Savage, Becher, de la Torre, & Sutherland, 2002), these effects might not be reliably observed in all tests, which reinforce the idea that different doses may affect the brain in particular ways. The discussion about a threshold for the teratogenic effects of ethanol should take this into consideration, moving away from the concept of determining a single “safe level” of exposure (which, in rats, do not appear to exist in any practical sense), recognizing instead several different thresholds for the variety of effects it might cause.
Abstract

Introduction: Prenatal exposure to alcoholic beverages has been associated with attentional deficits found in fetal alcohol syndrome (FAS) and attentional-deficit/hyperactivity disorder (ADHD). However, it is difficult to disentangle the pharmacological effects from nutritional, genetic and environmental ones. Objective: To investigate inattentive and impulsive responses in rats prenatally exposed to ethanol using the 5-choice serial time task (5-CSRTT). Method: Subjects were 48 adult male Wistar rats, born from dams exposed to one of four treatments during pregnancy: A35 - liquid diet with 35% ethanol-derived calories (EDC) (6.7% v/v); A10 - liquid diet with 10% EDC (1.8% v/v), isocalorically pair-fed with the A35 group; CONTROL – pair-fed with liquid diet without ethanol; CHOW - free access to laboratory chow and water. Rats were trained in 5-CSRTT with food rewards to detect and respond to brief (1s) visual stimuli presented in 5s inter-trial intervals in one of five holes until a stable baseline was achieved. Afterwards, four test sessions were performed, manipulating stimulus duration (SD: 0.5s, 0.25s) and inter-trial-intervals (ITI: 7s, 2s). Results: All groups acquired the task, and had similar accuracy, latency to respond, latency to collect reward, premature, incorrect and perseverative responses in baseline and test sessions. But A35 had a persistent higher percentage of omission errors than other groups in baseline and test sessions SD 0.5, ITI 7s and ITI 2s. A10 did not present any detectable impairment. Conclusion: These results suggest that a prenatal exposure to ethanol may cause a dose-dependent specific attentional deficit.

Key words: Prenatal, ethanol, alcohol, attention, 5-CSRTT, omissions, ADHD, FASD
Introduction

Ethanol ingestion during pregnancy is associated with several abnormalities and developmental impairments in the offspring. The most widely known consequence of prenatal alcohol exposure in humans is the Fetal Alcohol Syndrome (FAS). It is characterized by growth retardation, distinct facial deformities and central nervous system dysfunctions (Jones & Smith, 1973). However, this syndrome is not the only possible outcome of a prenatal exposure to ethanol (Alderazi & Brett, 2007). Currently, the term Fetal Alcohol Spectrum Disorder (FASD) is used to describe a broad range of impairments that might affect individuals exposed to ethanol in uterus (Riley & McGee, 2005).

It is uncertain if there is some secure amount of ethanol that might be ingested during pregnancy without causing impairments in the offspring. In general, studies with humans are based on interviews with mothers after delivery (Sokol, Delaney-Black, & Nordstrom, 2003), making it difficult to control factors such as dose, genetics and concomitant use of other drugs during pregnancy in these studies (Huizink, 2009).

Cognitive deficits, including problems with learning, memory (Streissguth et al., 1994; Streissguth, Barr & Sampson, 1990) and attention (Coles, Platzman, Lynch, & Freides, 2002) (O'Malley & Nanson, 2002) have frequently been reported to be associated with FASD. Children prenatally exposed to ethanol showed a higher reaction time during the Simple Reaction Time (SRT) and Choice Reaction Time (CRT) tasks, suggesting reduced speed of information processing and delays in responding (Simmons, Wass, Thomas, & Riley, 2002).

Notably, some of these characteristics are also usually present in children diagnosed with Attention Deficit and Hyperactivity Disorder (ADHD) (Coles et al., 1997; Nanson & Hiscock, 1990). Adolescents with ADHD showed fewer correct responses and higher error rates, especially more omission errors in the Continuous Performance Task (CPT) in comparison with control subjects (Coles, Platzman, Lynch, & Freides, 2002). Indeed, the ingestion of ethanol during pregnancy is a risk factor for the diagnosis of ADHD during childhood (Brown et al., 1991; Streissguth et al., 1994; Mick, Faraone, Sayer, & Kleinman, 2002). This suggests that the teratogenic action of ethanol in the developing brain might cause long-lasting damage to areas involved with attention and impulse control.

The attempts to verify this idea in animal models have not been entirely successful. Researchers have found that the offspring of rats exposed to ethanol during pregnancy had
lower overall brain weights (Ikonomidou et al., 2000), lower number of serotonergic neurons in the brainstem (Sari & Zhou, 2004), a reduced number of neurons in the prefrontal cortex (Burke, Palmour, Ervin, & Ptito, 2009) and several abnormalities in hippocampal structure (Sutherland, McDonald, & Savage, 1997). In behavioral tests, these rats also showed a poorer performance in spatial memory tasks (Savage et al., 2010; Savage, Becher, de la Torre, & Sutherland, 2002) However, crucially, very few specific tests of attention were performed in rats prenatally exposed to ethanol.

The first two studies that evaluated the effects of a prenatal exposure to ethanol on attention in rats investigated the heartbeat orienting response produced by a novel auditory (Caul, Fernandez, & Michaelis, 1983) or olfactory (Hayne, Hess, & Campbell, 1992) stimulus. Both studies did not find evidence for any attentional abnormalities. After this, Hausknecht et al. (2005) examined the attention of rats prenatally exposed to ethanol using the Choice Reaction Time Task, an adaptation of the human Continuous Performance Task for animals. The performance of rats treated with ethanol was characterized by a more variable reaction time distribution, and more false alarms, suggesting lapses of attention that are similar to children with FASD and ADHD.

Alongside the need for more studies directly measuring attention in animal models of FASD, another point of contention in the literature is concerned with the route of administration and dosage of ethanol necessary to cause structural and/or behavioral consequences. The discrepancy in the results from these previous studies might be due to the different levels and methods of ethanol exposure. In the only study that used liquid diets, dams received a diet prepared containing 35% ethanol derived calories from gestational day 6 to 20 (Hayne et al.). The other two studies used intragastric intubations, either twice a day in gestational days 10 to 14 (doses of 8, 4 or 0g/kg) (Caul, Fernandez, & Michaelis., 1983) or in days 8 to 20 (doses 0 or 3g/kg twice a day in weekdays, single 0 or 4g/kg during weekends)(Hausknecht et al., 2005).

The use of liquid diets prepared with different amounts of ethanol might be advantageous for several reasons. It allows a more distributed pattern of alcohol intake in comparison with intubation and other methods, causing relatively stable levels of blood alcohol concentration (Uzbay & Bizarro, 2010; Driscoll, Streissguth & Riley, 1990). Also, this procedure includes two control groups, one with free access to water and standard lab chow and the other pair-fed to an alcohol-exposed group. This pair-fed group achieves similar
caloric and nutritional intake because alcohol-derived calories are replaced by maltodextrine. Therefore, even if the alcohol-exposed group ingests less calories in comparison with free-fed dams, as expected, its nutritional condition will be replicated in the liquid diet control group, and the possible effects of this reduced caloric intake can be compared with the free-fed group. Moreover, the use of liquid diets is less stressful than injections or intragastric intubation, which is crucial as prenatal stress itself can be a teratogen (Welberg & Seckl, 2001).

Furthermore, a more robust test of attention should be used. Currently, the most widely used task for evaluating attention in rodents is the Five Choice Reaction Time Task (5-CSRTT), considered one of the most reliable tests of attention and impulsivity in animal models (Bari, Dalley, & Robbins, 2008). To perform this task, it is required that the animal continually scans a horizontal array of five holes, in order to nose-poke it soon after the presentation of a light. Therefore, this task evaluates the ability of the rodent to sustain attention, divided across the five holes over a large number of trials (typically, the task is performed in 5-6 30-minute sessions a week, over a few months). Other than the number of correct responses, other parameters, such as the number of premature responses - those made before the presentation of stimulus – and the response latencies are also recorded, providing auxiliary measures of impulsivity and motivation (Robbins, 2002).

The range of parameters assessed in the 5-CSRTT can provide a clearer idea of the cognitive impairments produced by prenatal alcohol exposure. This is clinically relevant to avoid preventable cognitive deficits, particularly to attention. Also, the use of liquid diets as a route for delivering alcohol to pregnant rats can help to disentangle alcohol effects from those produced by nutritional deficits or stress. In the present experiment, we investigated the consequences of prenatal exposure to two different doses of ethanol using liquid diets on acquisition and performance of rats under manipulations that challenge attention in the 5-CSRTT.

Method

Subjects

Subjects were 48 adult male Wistar rats born from dams exposed to one of four maternal treatments during pregnancy: (1) A35-liquid diet with 35% ethanol-derived calories, (2) A10-liquid diet with 10% ethanol-derived calories (3) Control-liquid diet without ethanol
and (4) Chow- free access to laboratory chow and water. Diets offered to groups A10 and Control were supplemented with maltodextrine, in order to match isocalorically the diet fed to the group A35. The amount of available liquid diet was limited via pair feeding to a female with similar weight (at the beginning of the gestation) in the A35 group, in order to eliminate differences in calories ingested, and appropriately control ethanol intake (Clausing, Ferguson, Holson, Allen, & Paule, 1995). We used one animal chosen at random from each litter in Chow, Control and A10 groups in order to have as much of a genotypic diversity as possible. In the A35 group, two or three animals from each litter were used due to the high mortality caused by this dosage, as reported previously (see Capítulo I).

Initially, the animals were housed in groups of 5 in plastic cages (24cm x 38cm x 15cm) acconditioned in ventilated racks (Alesco, Brazil) at the Laboratório de Psicologia Experimental, Neurociências e Comportamento at the Universidade Federal do Rio Grande do Sul, with lights on from 7h to 19h, and temperature ranging from 22°C to 24°C. After two weeks of acclimation to our laboratory, animals (2 months old, weighting 319g on average) were pair housed. Rats had free access to water and were placed on a food-restricted diet to maintain them at 85% of the mean weight of six free-feeding rats. These weight control rats were males derived from different litters of Chow groups that were not included in the experiment. They were maintained in the same housing conditions of the experimental subjects. National and institutional guidelines for animal welfare were followed and all procedures were approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre.

**Test Apparatus**

**Five-choice serial reaction time task**

Eight 5-CSRTT chambers (Med Associates, St Albans, Vermont, USA) were used. The test apparatus consisted of a 25 cm × 25 cm aluminium chamber illuminated by a house light, enclosed in a sound-isolated and ventilated box. The curved left wall of each chamber contained five 2.5 cm$^3$ cubic apertures located 2.5cm above a stainless steel grid floor. Each aperture contained a small lamp located at its rear and an infrared photocell beam to record nose pokes. On the right wall, a food dispenser of 45mg sucrose pellets (BioServ, USA) was connected to a receptacle located at the center of the wall, which also had an infrared photocell beam crossing its entrance to record head entries. The chambers were automatically controlled
by a program, and data was collected via a computer using Med-PC interface and software (Med Associates Inc., St Albans, Vermont, USA).

**Behavioral procedures**

**5-CSRTT Training**

The training procedure was similar to that described by Bari, Dalley, and Robbins (2008). Rats were trained with food rewards to detect and respond to flashes of light of one second of duration, presented randomly in one of the five holes of the apparatus after an inter-trial-interval (ITI) of 5 seconds. On the first three days of habituation to the testing apparatus, rats were placed in the chambers for a short session of 15 min with all lights lit, and 2 pellets were placed in each hole and 10 pellets in the food tray. Once the rats were able to eat all the pellets provided within the allotted time, the 5-CSRTT training started. Each session of training began with the illumination of the chamber by the house light and the delivery of a free food pellet. Each trial was initiated by a head entry of the animal in the receptacle linked to the food dispenser. After a fixed ITI of 5s, one of the opposite apertures was illuminated and the rat was required to respond with a nose-poke in the appropriate aperture. Responses on this aperture while it was lit or within the 5s after the light was turned off (limited hold-LH) were registered as correct responses, and rewarded with a pellet. Incorrect responses (responses in any of the non-illuminated apertures), omissions (failure to respond within the 5s limited hold) and premature responses (responses within the ITI period) were registered and punished with a 5s time-out, during which the house light was extinguished and no other stimulus was presented. After one correct response, entries in the same hole during the limited hold were recorded as perseverative responses, which were not punished. The latencies to correct and incorrect responses, and to collect the reward – the time between a correct response and a nose-poke in the feeder receptacle - were registered. Accuracy was calculated as the number of correct responses x 100/(correct + incorrect responses).

Each session ended after 30 minutes or 100 trials, whichever occurred first. The sessions were performed 5 times a week, during 16 weeks of training, when final baseline was
stable. The initial parameters used were stimulus duration (SD) 30s, ITI 2s, LH 30s. The stimulus and LH duration were decreased throughout 6 training stages, depending on the rats’ individual performances. In the first two stages (stage 2: SD 20s, ITI 2s, LH 20s) of training the criterium required to move to the next stage was a minimum of 30 correct trials. To move from the third (SD 10s, ITI 5s, LH 10s) to fourth (SD 5s, ITI 5s, LH 5s) stage, a minimum of 50 correct trials were required. The animals progressed to fifth stage (SD 2.5s, ITI 5s, LH 5s) when they completed at least 50 correct trials and had 80% accuracy. To move from the fifth to sixth (SD 1.25s, ITI 5s, LH 5s) stage, at least 50 correct responses, 80% accuracy and less than 20% omissions were required. When 80% of the animals showed reliably stable performance (without statistically significant difference among groups in the accuracy) on 7 consecutive sessions with the target parameters (SD 1s, ITI 5s, LH 5s), all animals were moved from the sixth to the last stage. Rats were then trained until a stable baseline performance (when an ANOVA for repeated measures did not show an effect of Session for accuracy and animals had less than 25% of omissions over 8 consecutive sessions. Two animals (one from Control, and one from the A35 groups) were excluded because they were unable to achieve these criteria after several weeks of training.

**Baseline acquisition and performance**

In this experiment, the last 8 consecutive sessions using the final parameters of training (baseline) were analyzed to compare final performance of the different groups. The acquisition of the task was recorded as the number of sessions necessary to reach the baseline.

**Challenges of attention**

The objective of this experiment was to investigate the effects of several manipulations of ITI and SD in different test sessions in the performance of the groups. After all animals showed a stable baseline performance in the parameters of training, four test sessions were performed, with manipulations of ITI (short 2s, long 7s) and SD (0.5s, 0.25s) in a randomized order. These sessions were performed on Tuesdays and Fridays. On the other days – Monday, Wednesday and Thursday – baseline sessions were performed.

In test sessions with shorter SDs, the sessions were terminated after 30min or 100 trials. For both manipulations of ITI duration, the length of the session was increased to 45min. With a 7s ITI, the session was terminated after 45min or 100 trials, whichever occurred
first. When the ITI was 2s, the session ended after 45min or 200 trials. These modifications were necessary because, in the first case, the animals would need more time to complete 100 trials and, when the ITI was shorter, the intention was to provoke a large number of responses (Bari Dalley, & Robbins, 2008).

All measures of the test sessions were recorded for the entire session. Data could also be separated into three successive time periods of 10 minutes for sessions that lasted 30 minutes, and five successive time periods of 9 minutes for sessions that lasted 45 minutes, which allowed the analysis of attentional performance in separate blocks when necessary.

**Data Analysis**

The following variables were analyzed in this study:

*Accuracy* – 100 x correct responses/(correct + incorrect responses)

*Percentage of omissions* – 100 x number of omission errors/total number of stimulus presentation

*Percentage of premature responses* – 100 x number of premature responses/sum of emitted responses (correct, incorrect, premature and perseverative responses)

*Perseverative responses* – number of nose-poke responses performed in unlit orifice (ITI excluded)

*Correct latency* – mean latency to attend to the correct orifice during a trial

*Reward latency* - mean latency to collect the rewards in the feeder orifice

Baseline data were obtained from mean performance over 8 last consecutive baseline sessions. Baseline and test sessions were analyzed using one-way ANOVAs, with Group (A35, A10, Control, Chow) as a factor. Data from 8 last consecutive baseline daily sessions were also analyzed using repeated measures ANOVA with Group (between) and Day (within) factors. Separately, test sessions with blocks of 10 and 9 minutes were analyzed separately by a two-way ANOVA for repeated measures with Group (between) and Block (within) as factors. Body weights were compared using a repeated measures ANOVA with Group
(between) and day (within) factors. All data involving percentages were arcsine transformed, and data involving latencies were log-transformed. Post Hoc analyses were carried out using a Tukey post hoc test. The significance criterion was p<0.05. All analyses were performed using the software Statistical Package for the Social Sciences (SPSS), version 17.

Results

Body weight

A repeated measures ANOVA showed that since a day before the beginning of restriction food and throughout the experiment, the body weight from the A35 group was significant different from Chow [F(3.44)=3.2, p=0.03], but not from other groups (p>0.05). As expected, the body weight of all groups increased with time [F(2.2, 100.5)=638.8, p<0.001] but there was no interaction between factors Time and Group [F(18, 100.5)=0.9, p=0.5].

Baseline acquisition and performance

All groups were able to learn the task just as quickly. Stable baseline performance with the final parameters of training was reached by the four different groups within 68 sessions after the start of training, considering the criteria used in each stage of training.

There was no effect of Time [F(7,294)=0.9, p=0.4], Group [F(3,42)=0.5, p=0.6] nor an interaction between these factors [F(21,294)=0.6, p=0.8] regarding accuracy over the 8 consecutive baseline sessions, Figure 4A. However, the groups differed in percentage of omissions [F(3,42)=4.1, p=0.01]. Animals from the A35 Group showed more omissions errors than Control (p=0.02) and Chow (p=0.01), Figure 4B. The repeated measures ANOVA showed also a main effect of days of baseline sessions [F(7, 294)=6.9, p<0.01] but there was no interaction between group and day factors. The animals did not differ in any other variable analyzed (Table 3).
Figure 4. Accuracy (A) and percentage of omissions (B) in the 5-CSRTT throughout 8 consecutive baseline days. Mean (SEM) is depicted. Inserted graphs show mean accuracy and mean of percentage of omissions over 8 days, bars represent Chow (white), Control (dots), A10 (stripes) A35 (black). The A35 group showed more omission errors than all other groups (p<0.05) (One-way ANOVA/Tukey).

Table 3

Mean performance in the 5-CSRTT over 8 consecutive baseline days

<table>
<thead>
<tr>
<th>5-CSRTT parameters - baseline</th>
<th>Chow</th>
<th>Control</th>
<th>A10</th>
<th>A35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of trials</td>
<td>99.6(0.3)</td>
<td>99.6(0.2)</td>
<td>99.1(0.7)</td>
<td>99.3(0.6)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>72.2(1.7)</td>
<td>69.5(2.5)</td>
<td>68.3(2.7)</td>
<td>70.6(1.6)</td>
</tr>
<tr>
<td>% Omission</td>
<td>4.2(0.5)</td>
<td>4.2(1.0)</td>
<td>4.8(0.8)</td>
<td>7.9(0.9)*</td>
</tr>
<tr>
<td>% Premature responses</td>
<td>6.6(1.0)</td>
<td>7.5(1.2)</td>
<td>6.7(2.0)</td>
<td>6.2(1.4)</td>
</tr>
<tr>
<td>Perseverative responses</td>
<td>5.3(0.7)</td>
<td>5.9(1.7)</td>
<td>5.7(1.7)</td>
<td>4.6(0.6)</td>
</tr>
<tr>
<td>Correct latency</td>
<td>0.899(0.04)</td>
<td>0.896(0.05)</td>
<td>0.964(0.03)</td>
<td>0.969(0.04)</td>
</tr>
<tr>
<td>Reward latency</td>
<td>1.4(0.06)</td>
<td>1.4(0.07)</td>
<td>1.3(0.03)</td>
<td>1.5(0.05)</td>
</tr>
</tbody>
</table>
Challenges of attention

Manipulations of ITI

In the session with a longer (7s) ITI, a one-way ANOVA indicated an effect of Group in the percentage of omissions [F(3,42)=3.7, p=0.018]. The animals from the A35 Group showed more omission errors than Control (p=0.01) and A10 (p=0.04) groups (Table 4, Figure 5). In the test session in which the ITI was shortened to 2s, the animals from A35 Group also showed more omissions errors than other groups, but no significant difference was found [F(3,42)=1.9, p=0.1] (Figure 5). The groups did not differ in any other variable analyzed (Table 4).

For both manipulations of ITI (2s and 7s), half of the animals finished the session before completing the fifth 9-min block. The median duration of these sessions was 40 min for the shorter ITI and 37 min for the longer. Due to this, the analysis of the successive blocks included only the first four 9min blocks in sessions in sessions with shorter or longer ITI.

A repeated measures ANOVA of the performance in the successive blocks of 9 minutes from sessions with longer ITIs (7s) indicated that percentage of omissions tended to be larger as the blocks progressed [Time, F(2.1,83.7)=3.5, p=0.03], but there was no effect of Group [F(3,39)=2.2, p=0.9]. This effect was associated with an interaction between Time and Group [F(6.4,83.7)=2.2, p=0.04]. A one-way ANOVA indicated that the A35 group performed more omission errors [F(3,42)=4.1, p=0.1] than Control (p=0.01) and Chow (p=0.04) groups at the fourth block. These analysis included only animals that completed more than one trial in each time period (A35: n=9; A10: n=12; Control: n= 11, Chow: n=11) (Figure 5A).

In the successive blocks of 9 minutes from sessions with shorter ITIs (2s), there were main effects of Time [F(2.1,84.9)=18.7, p<0.01] and Group [F(3,40)=3.4, p=0.02]. Similarly to what occurred in the session with a longer ITI, there was an increase in the percentage of omissions for the A35 group at the fourth block [Group x Time, F(6.3,84.9)=2.3, p=0.04]. A one-way ANOVA showed that this group had more omission errors [F(3,42)=4.3,p=0.01] than Control (p=0.02) and Chow (p=0.01) groups in that block (Figure 5B). The animals did not differ in any other variable analyzed in the successive blocks (p>0.05).
Table 4

Mean performance in test sessions with manipulations of ITI (7s and 2s) and stimulus duration (0.5s and 0.25s)

<table>
<thead>
<tr>
<th>7s ITI</th>
<th>Chow</th>
<th>Control</th>
<th>A10</th>
<th>A35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of trials</td>
<td>92.5(4.4)</td>
<td>97.0(2.0)</td>
<td>97.3(2.6)</td>
<td>93.2(6.1)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>58.4(2.1)</td>
<td>58.8(2.9)</td>
<td>63.3(2.6)</td>
<td>61.7(2.0)</td>
</tr>
<tr>
<td>% Omission</td>
<td>4.6(1.2)</td>
<td>3.1(0.6)b</td>
<td>3.9(1.1)b</td>
<td>8.4(1.5)a</td>
</tr>
<tr>
<td>% Premature Perseverative Correct latency Reward latency</td>
<td>39.0(2.3)</td>
<td>39.4(2.9)</td>
<td>34.7(3.8)</td>
<td>35.2(5.6)</td>
</tr>
<tr>
<td>0.5s SD</td>
<td>Chow</td>
<td>Control</td>
<td>A10</td>
<td>A35</td>
</tr>
<tr>
<td>Number of trials</td>
<td>98.7(1.2)</td>
<td>100(0.0)</td>
<td>99.1(0.6)</td>
<td>98.2(1.8)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>60.6(2.2)</td>
<td>59.3(2.8)</td>
<td>59.1(2.4)</td>
<td>58.7(4.0)</td>
</tr>
<tr>
<td>% Omission</td>
<td>3.6(0.9)</td>
<td>3.3(0.7)</td>
<td>5.9(1.6)</td>
<td>13.9(2.3)*</td>
</tr>
<tr>
<td>% Premature Perseverative Correct latency Reward latency</td>
<td>10.5(2.2)</td>
<td>9.2(1.8)</td>
<td>8.5(2.0)</td>
<td>6.5(1.2)</td>
</tr>
<tr>
<td>0.25s SD</td>
<td>Chow</td>
<td>Control</td>
<td>A10</td>
<td>A35</td>
</tr>
<tr>
<td>Number of trials</td>
<td>96.2(2.7)</td>
<td>100(0.0)</td>
<td>100(0.0)</td>
<td>100(0.0)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>51.1(2.2)</td>
<td>48.6(2.5)</td>
<td>49.3(2.3)</td>
<td>48.9(1.6)</td>
</tr>
<tr>
<td>% Omission</td>
<td>7.3(1.9)</td>
<td>4.5(0.8)</td>
<td>6.7(1.2)</td>
<td>9.4(1.4)</td>
</tr>
<tr>
<td>% Premature Perseverative Correct latency Reward latency</td>
<td>10.8(1.8)</td>
<td>10.9(2.5)</td>
<td>9.0(1.0)</td>
<td>9.9(2.8)</td>
</tr>
</tbody>
</table>

Mean (SEM) is depicted. * Represents a statistically significant difference (p<0.05) compared with all other groups; a Represents a statistically significant difference (p<0.05) compared with b marked groups (One-Way ANOVA/Tukey).
Figure 5. Mean percentage of omissions in sessions with ITI manipulations. Mean (SEM) is depicted. A: Mean percentage of omissions in four successive 9min blocks from a test session with a 7s ITI. B: Mean percentage of omissions in four successive 9min blocks from a test session with a 2s ITI. The inserted graphs show mean percentage of omissions from all sessions, bars represent Chow (white), Control (dots), A10 (stripes) A35 (black). Group A35 performed more omission errors than Control and A10 groups in the test session with 7s ITI (p<0.05, One-way ANOVA/Tukey).

Shorter SD

The percentage of omissions from A35 was greater than all other groups in the session in which the SD was reduced to 0.5s [F(3,42)=8.9, p<0.001, Tukey p<0.05). When the SD was reduced to 0.25s, a one-way ANOVA did not evidence a significant difference between groups in omissions [F(3,42)=1.7, p=0.1] (Table 4, Figure 6).

For both shorter SDs, half of the animals finished the session before completing the third 10 minute block. The median duration of these sessions were 21 min for a SD of 0.5s, and 22 minutes for a SD of 0.25s. Due to this, the analysis of the successive blocks included only the first two 10 minute blocks.
A repeated measures analysis of variance using the successive blocks of 10 minutes from these sessions showed an effect of Group on the percentage of omissions \([F(3,42)=11.3, p<0.001]\) in session with a 0.5s SD. The number of omission errors in the A35 was greater than in all other groups \((p<0.001)\). There was no effect of Time \([F(1,42)=0.2, p=0.6]\) nor interaction between Time and Group factors \([F(3,42)=0.2, p=0.8]\). When the stimulus was decreased to 0.25s, there was no significant effect of Time \([F(1,42)=1.5, p=0.2]\), Group \([F(3,42)=1.2, p=0.3]\) nor interaction between factors \([F(3,42)=0.8, p=0.5]\) on the performance of animals throughout blocks.

![Graph showing percentage of omissions](image)

*Figure 6.* Mean percentage of omissions in test sessions with 0.25s and 0.5s SD. *Represents a statistically significant difference \((p<0.05)\) from all other groups (One-way ANOVA/Tukey).

**Discussion**

The present results demonstrated that the A35 group performed more omission errors than all other groups in baseline parameters, and with most manipulations of ITI and SD duration used in test sessions. The A10 group was not distinguishable from control groups in percentage of omissions. Additionally, in any other variable analyzed, both ethanol-exposed
groups did not differ from Control and Chow groups in any test condition, evidencing that the attentional deficit caused by a prenatal exposure to ethanol seems to be very specifically related to omission errors, and only perceptible after a certain level of exposure.

In a way, this specificity was interesting. Given the several abnormalities already documented in previous studies with prenatal alcohol exposure, a more global effect of this regimen on a 5-CSRTT was expected. Accuracy, which indicates the capacity to select and attend to a brief visual stimulus, was unimpaired, suggesting that structures and pathways associated with selective attention might be intact. The fact that prenatal alcohol produced no alterations in premature responding, which is commonly used as a measure of impulsivity, was also an important result. Furthermore, the latencies to respond and collect rewards were also unimpaired, a fact that allows us to interpret the increase in omissions not as an altered state of arousal and/or satiation, but as evidence of a specific attentional problem related to sustained attention and vigilance.

There is some evidence to suggest that the attentional deficits presented by the A35 group were not significant at the beginning of a session, but only at the end, particularly when its duration surpassed the usual 20-30 minutes. However, as a confounding factor, this result was observed only when the session duration was increased and the ITI was manipulated. Unfortunately, we did not have records of the performance of animals by time blocks in the baseline sessions. In test sessions in which the SD was manipulated, no effect of time nor an interaction between Time and Group was observed in 10 min time blocks.

The possibility that our results could be the consequence of gross physical impairments, relatively prevalent in individuals with FASD, also needs to be addressed. Indeed, using rats from the same litters as the ones in this study, we found that the A35 group had a slower weight gain until PND 40, less muscular strength at PND 14 (but not PND 17) and a higher degree of ambulation at PND 19 compared to controls (see Capítulo I). However, throughout this study, the body weight of A35 rats was only different from the Chow group.

Another hypothesis is that ocular malformations and visual deficits that have been identified in subjects with FASD (Gonzalez, 1981; Strömland, 1987; Katz & Fox, 1991) could be associated with the difficulty of animals prenatally exposed to ethanol to perform a task of visual attention. However, in our study, the accuracy of the ethanol-exposed animals was similar to control groups in every condition, even when the stimulus duration was shortened by a considerable degree, strongly suggesting that their vision was unimpaired. Additionally,
the fact that the analysis of time-limited blocks suggests that the number of omission errors of these groups did not differ from Control and Chow at the beginning of sessions is another evidence that a visual impairment might not be a good explanation for our results. Moreover, similar latencies in the 5-CSRTT also evidence that all groups moved with comparable speed, suggesting equal locomotor capabilities.

Therefore, having ruled out these possible explanations, it seems reasonable to conclude that there was a decrement of vigilance by the end of the session, a common effect in the 5-CSRTT as discussed by Robbins (2002). As this decrement was particularly strong in the A35 group in most test conditions, it seems that the effects of prenatal ethanol on attention, on a dose equivalent to what the A35 group received, are manifested only on vigilance and sustained attention.

In humans, the impairments caused by a prenatal exposure to ethanol are also sometimes specifically related to sustained attention and an inability to attend to tasks that last for long periods of time. In a study that compared the performance of children with ADHD and FASD in the CPT, subjects from both groups showed declines in task performance as a function of time, with increased latency to respond, more variability in latency and more omission errors at the final part of the task (Kooistra et al., 2009). Coles et al. (1997) also found an effect of prenatal ethanol on higher omissions and errors in a CPT. Nevertheless, other studies found no specific problem in vigilance/sustained attention in individuals with FASD (Olson, Feldman, Streissguth, Sampson, & Bookstein, 1998). Some authors suggest that this deficit might arise only when active cognitive processing is required (J. L. Jacobson & S. W. Jacobson, 2002). In our study, the animals did not differ during the acquisition of the task, but differences emerged when the final parameters of training were achieved, and when ITI and SD duration were manipulated. Similar to what happens with humans, the impaired performance of the A35 Group seems to be associated with an increase in attentional demand.

In general, studies with ethanol exposure in humans are somewhat unreliable, because the consumption of ethanol during pregnancy might be associated with the use of other drugs and socioeconomical status. Other factors, such as the nutritional status of the mother and the unreliability of self-reported levels of consumption (the main source of data of studies with humans) might also be important. Studies with animals are able to reduce the number of interventional variables. In our study, the use of liquid diets with a pair-fed Control group means that secondary factors, such as malnutrition, cannot explain the results. This allows us to
conclude that the attentional impairments observed in the A35 group are most likely due to ethanol exposure itself.

Our study reinforces the findings of the previous study from Hausknecht et al. (2005), which demonstrated that prenatal exposure to ethanol might results in attentional impairments similar to those observed in children with FASD and ADHD. By using a popular task to measure attention in rats, the 5-CSRTT, we evaluated independent measures of behavioral control, such as impulsivity, accuracy, omissions and response latencies (Bari, Dalley, & Robbins, 2008). As mentioned before, our results suggest that the impairments caused by a prenatal exposure to ethanol are specifically related with an increase in the number of omissions. Furthermore, we compared the effects of two different levels of prenatal ethanol in order to investigate if attentional effects could be associated with dosage of exposure. Only the group exposed to a higher dosage of ethanol presented attentional impairment. This suggests that, in rats, the threshold for attentional impairments is likely between the range of blood alcohol concentrations caused by 10 to 35% EDC and more studies are needed to investigate this. In rodents, in particular, the use of a task more specifically suited for testing vigilance, such as the adaptation of the 5-CSRTT proposed by Young, Light, Marston, Sharp, and Geyer (2009), as well as a closer investigation of brain structures and pathways underlying the attentional deficits might be useful to clarify the effects of prenatal ethanol exposure in this cognitive function.
CONSIDERAÇÕES FINAIS

Nossos estudos tiveram como objetivo comparar os efeitos da exposição pré-natal a duas doses de etanol sobre o desenvolvimento, a atividade locomotora e a atenção de ratos Wistar. Em ambos os estudos, a exposição à dose mais alta de etanol (35% EDC) resultou em prejuízos no desempenho dos animais em todas as tarefas (com exceção da geotaxia negativa). Por outro lado, o grupo exposto à dose mais baixa de etanol (10% EDC) não apresentou nenhum prejuízo detectável.

Para a exposição ao etanol, foram utilizados dois grupos controles em nosso estudo: Controle dieta líquida – animais receberam dieta líquida isocalórica e na mesma quantidade que seus pares nos grupos álcool; Chow – animais tiveram livre acesso à ração padrão de laboratório e água durante a gestação. Dessa forma, foi possível controlar os efeitos da redução de calorias ingeridas pelas ratas dos grupos álcool. Além disso, a utilização de um modelo animal nos permitiu controlar outras variáveis que geralmente não são controladas em estudos com seres humanos, tais como: dose ingerida, uso concomitante de outras drogas, condições ambientais e exposição a estressores. Com isso, foi possível observar os prejuízos ocasionados pela exposição ao etanol de forma separada daqueles decorrentes das variáveis citadas.

Uma limitação importante dos nossos estudos diz respeito ao fato de não termos uma medida de concentração sanguínea de etanol das duas doses. Essa medida deveria ter sido realizada em diferentes momentos do dia em um grupo separado de ratas (prenhas ou não), uma vez que a dieta ficava à disposição dos animais continuamente. De acordo com Driscoll, Streissguth e Riley (1990), o padrão de consumo da dieta resulta em níveis de concentração sanguínea de etanol relativamente mais estáveis que métodos como gavagem ou injeções. Esses últimos geralmente resultam em picos altos, mas agudos de concentração da droga. Essa medida seria importante para descrevermos o quanto da droga foi absorvida pelos animais e para facilitar a comparação de nossos achados com estudos que utilizaram diferentes métodos de administração de etanol. No entanto, por limitações técnicas, não dispomos desses dados.

Por fim, é importante mencionar que a extrapolação dos nossos resultados para seres humanos deve ser feita com cautela. Embora o grupo exposto à dose mais baixa de etanol não tenha apresentado nenhum prejuízo detectável em nossos estudos, não é possível concluir sobre a existência de uma dose segura de consumo de etanol durante a gestação. Diferenças de
metabolismo da droga e de desenvolvimento fetal podem comprometer essa extrapolação. Além disso, é possível que o etanol nessa dose tenha afetado parâmetros não avaliados nesse estudo, tais como, memória e responsividade ao estresse, que poderiam ser investigados em estudos futuros.
REFERÊNCIAS


Savage, D. D., Rosenberg, M. J., Wolff, C. R., Akers, K. G., El-Emawy, A., Staples, M. C.,


Tan, S., Berman, R. F., Abel, E., & Zajac, C. Prenatal alcohol exposure alters hippocampal slice


ANEXO A

Carta de aprovação do projeto no Comitê de Ética do Hospital de Clínicas de Porto Alegre

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
Grupo de Pesquisa e Pós-Graduação
COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB00000921) analisaram o projeto:


Pesquisadores:
LISIANE BIZARRO ARAUJO
STEPHANO PUPE JOKANN
IVAMI BRYS
GIOVANA BROLESE

Título: EXPOSIÇÃO PRÉ-NATAL AO ETANOL EM RATOS E SEUS EFEITOS NA ATENÇÃO, MEMÓRIA, ADIÇÃO E DESENVOLVIMENTO

Este projeto foi Aprovado em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Internacionais e Nacionais. Os membros do CEP/HCPA não participaram do processo de avaliação dos projetos onde constam como pesquisadores. Toda e qualquer alteração do Projeto deverá ser comunicada imediatamente ao CEP/HCPA.

Porto Alegre, 24 de agosto de 2009.

Prof. Nadine Clausell
Coordenadora do FPPG e CEP-HCPA