ABSTRACT

Objective: To compare the incidence of congenital malformations among the offspring of female mice exposed to alcohol or alcohol plus nicotinamide. Methods: Three groups of pregnant C57BL/6J mice were studied; G1 received alcohol (5 g/kg) in saline solution (20% - vol/vol); G2 received nicotinamide, 50 mg/ml associated to alcohol; and G3, only saline solution; all by intraperitoneal injection on the seventh day of pregnancy. The animals were killed in a CO₂ chamber on day 18 of pregnancy. The intrauterine content was assessed and the number of complete and reabsorbed fetuses was counted. The complete fetuses had their weight and crown-rump length measured and malformations were identified. Results: G1 showed the highest number of malformations: micrognathia, low set ears, hypertrophic nose, scoliosis, and atrophy of the lower and upper limbs. Weight was significantly different among the groups (p = 0.0139), and in G1 it was below average as compared to G3 (p = 0.3133). As for length, the lowest values were found in G2 and G3 showed the highest ones. There was a significant difference among the groups (p = 0.0145). Conclusions: Ethanol, when administered to pregnant mice was teratogenic. However, length of G1 fetuses was, in average, higher than that of other groups. Nicotinamide decreased the number of malformations and may be a possible protector against alcohol effects.

Keywords: Congenital abnormalities; Alcoholism; NAD

INTRODUCTION

Ethanol may interfere in the morphogenesis of several organs, incurring in permanent and irreversible physical, cognitive and behavioral changes, which characterize the Fetal Alcohol Syndrome (FAS) (1). Ethanol effects during pregnancy vary according to several factors, such as the top blood alcohol levels attained, pattern...
of consumption, species and individual susceptibility, among others\(^5\).

There is no cure for the FAS, but it can be fully prevented. Healthcare professionals discourage the use of alcoholic beverages by women intending to get pregnant and by those sexually active not using contraceptive methods. The reason for this is that some studies say that there is no safe alcohol dose\(^3\). The incidence of the FAS in concepts of mothers who use alcohol vary from 30 to 50\%, in different populations\(^4\), with estimated 1-3:1000 live births in the United States of America and 1:600 in Sweden\(^6\).

The lack of symptom specificity and the absence of definite diagnostic criteria hamper investigation and classification of this syndrome\(^7\). According to the Research Society on Alcohol, three criteria must be considered during evaluation: a specific pattern of facial anomalies, which include small palpebral fissures, ptosis, flat hemiface, anteverted nostrils, smooth and thin philtrum, micrognathia; pre- or postnatal growth deficit; motor or behavioral evidence of central nervous system dysfunction\(^8\)-\(^9\).

Because of the great importance of this syndrome, investigators have been concentrating efforts in an attempt to elucidate the mechanisms of action of alcohol in the developing fetus. Much of what is known about FAS and on the effects of prenatal exposure to alcohol derives from animal studies. These models have contributed to understand the mechanisms leading to morphological and behavioral changes found in fetuses exposed to alcohol, and have also suggested some preventive actions for FAS. The most promising form of prevention is nicotinamide, used in the treatment of auto-immune disorders, recently described as a potential therapy to avoid the development of FAS\(^10\)-\(^11\).

Nicotinamide is a soluble B vitamin (vitamin B3); it prevents the nicotinamide adenine dinucleotide (NAD+) wasting and protects against the low protection of adenosine triphosphate (ATP), in addition of having neuroprotective action against injuries caused by neurochemical toxins in rodent brains. This medication was studied at Cornell University, in the United States of America; it was demonstrated that this drug could be used to reduce damage caused in children whose mothers drank alcohol during pregnancy\(^10\)-\(^11\).

**OBJECTIVE**

Because FAS is a major public health problem and knowledge about its prevention is still limited, the objective of the present study was to compare the incidents of congenital malformations between the offspring of pregnant mice exposed to alcohol, or to alcohol plus nicotinamide.

**METHODS**

**Sample**

For this study C57BL/6J mice from the animal center of Universidade Estadual de Campinas kept in the animal laboratory of Faculdade de Medicina de Jundiaí were used. Animals of both sexes, three to five month-old were kept in cages at controlled temperature (25 ± 2 °C) and exposed to light for 12 hours (7 AM to 7 PM). Water and commercial chow were given *ad libitum*. The study was approved by the Ethics Committee of Faculdade de Medicina de Jundiaí. They were sorted into three groups: a Control Group (30 animals); one group receiving alcohol (35 animals); one group receiving alcohol + nicotinamide (13 animals).

**Mating**

The female C57BL/6J mice were placed with male mice of the same strain and the presence of vaginal tampon was observed every morning. The day the tampon was identified was considered as the first day of pregnancy.

**Alcohol and nicotinamide administration**

The pregnant female mice were sorted into the three study groups, as follows:
- **Group 1 (G1):** intraperitoneal alcohol injection, on the seventh day of pregnancy;
- **Group 2 (G2):** intraperitoneal alcohol and nicotinamide injection, on the seventh day of pregnancy;
- **Group 3 (G3):** Control; intraperitoneal saline injection, on the seventh day of pregnancy.

G1 was injected with a single dose of ethanol (5 g/kg) in saline solution (20% - vol/vol); the addition of nicotinamide 50 mg/ml to the alcohol dose was injected in G2. G3 was injected with saline solution alone (same volume as the other groups), simulating the injection stress only.

**Collection and analysis of the fetuses**

The pregnant mice were killed in CO\(_2\) chamber on the 18th day of pregnancy (three days prior to term). The uterus was dissected and transferred to a glass dish containing saline solution. The uterine content was assessed and the number of complete
and reabsorbed fetuses was counted. The complete fetuses were weighed and their crown-rump length measured. Then the congenital malformations were identified, with emphasis in cranio-facial, limb and spinal anomalies.

Analysis and photographic documentation of the samples

Fetuses with malformations were photographed with a Canon Power Shot A80 camera (Figures 1 to 10). The data were displayed in tables (Tables 1, 2 and 3). Means, standard deviations and minimum and maximum values are presented.

Figure 1. Fetus with micrognathia (arrow) (G1)

Figure 2. Fetus presenting mouth laceration (arrow) (G2)

Figure 3. Normal fetus (G3)

Figure 4. Fetus with low-set ears (arrow) (G1)

Figure 5. Normal fetus, ear setting at eye-level (arrow) (G3)
Weights and lengths were compared among the groups using ANOVA, and since the results were significant, the Tukey test for multiple comparisons was applied. The SAS version 9.02 package was used and the level of significance adopted was 5% (p < 0.05).

RESULTS

The teratogenic effects of ethanol observed were micrognathia, low set ears, hypertrophic nose, atrophy of the upper and lower limbs and skeletal deformities (scoliosis). These deformities were less frequent in G2.

The highest proportion of mouth laceration and paranasal hyperpigmentation were observed in G2; in G3 nose hyperpigmentation was frequent and not
found in the other groups. The highest proportion of reabsorbed fetuses occurred in G2.

Weight was significantly different among the groups \((p = 0.0139)\). The Tukey test for multiple comparisons was then performed and revealed that G2 was statistically different from G3 \((p = 0.102)\). G1 fetuses weighed less than the G3 mean weight \((p = 0.3133)\).

As for length, the highest was in G1 and the lowest in G2. The two groups were significantly different \((p = 0.0145)\). The Tukey test for multiple comparisons was then performed and it showed that G2 was statistically different than G1 \((p = 0.0363)\) and that G1 was different from G3, although the \(p\) value was close to 0.05, but below this value, therefore, significant.

Thus teratogenesis was lower, in general, in G2 than in the group exposed to alcohol.

**DISCUSSION**

There are many studies in the literature showing the effects of ethanol on the offspring of alcohol-treated female mice, clearly demonstrating the association between alcoholism during pregnancy and teratogenic defects found in the fetus. The C57B/6J female mice were chosen for the present study due to their small size, low cost and easy handling. The comparison between the findings in human and murine offsprings exposed to ethanol in uterus is quite consistent and justifies the choice of such species for FAS animal studies\(^{12}\).

Skeletal teratogenesis by ethanol was found only in G1, in accordance with the literature\(^{13}\), showing that alcohol consumption during pregnancy adversely affects fetal skeleton development. In this experimental group, a higher proportion of micrognathia was also found, as in other studies\(^{14}\). It may be assumed that nicotinamide had a protective effect, because of the lower rate of this malformation.

The proportion of other teratogenic effects of alcohol, such as low set ears, hypertrophic nose, and atrophy of the upper and lower limbs was lower in the group receiving nicotinamide, suggesting the likely protective role of this vitamin. However, in the nicotinamide treated group, the highest proportions of mouth lacerating and paranasal hyperpigmentation were not observed in other groups. The scarcity of studies analyzing these parameters makes the comparison between the findings of the present study and the literature difficult; lacerations may be explained by an artifact originated at the time of collecting and handling of the fetuses.

**Table 1. Description of absolute and relative frequencies of malformation in fetuses**

<table>
<thead>
<tr>
<th>Malformation</th>
<th>Saline (Control group)</th>
<th>Alcohol</th>
<th>Alcohol + nicotinamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 30)</td>
<td>(n = 35)</td>
<td>(n = 13)</td>
</tr>
<tr>
<td></td>
<td>(n)</td>
<td>%</td>
<td>(n)</td>
</tr>
<tr>
<td>Micrognathia</td>
<td>2</td>
<td>6.7</td>
<td>6</td>
</tr>
<tr>
<td>Low-set ears</td>
<td>-</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>Hypertrophic nose</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Upper limb atrophy</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Lower limb atrophy</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Skeletal deformities (scoliosis)</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Mouth laceration</td>
<td>1</td>
<td>3.3</td>
<td>1</td>
</tr>
<tr>
<td>Reabsorbed fetus</td>
<td>5</td>
<td>16.7</td>
<td>-</td>
</tr>
<tr>
<td>Pigmented nose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pigmented paranasal region</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total number of fetuses</td>
<td>30</td>
<td>35</td>
<td>13</td>
</tr>
<tr>
<td>Total number of mice</td>
<td>4</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Mean number of fetuses/mouse</td>
<td>7.5</td>
<td>7.0</td>
<td>4.3</td>
</tr>
</tbody>
</table>

**Table 2. Comparative study on fetal weight**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>Standard-deviation</th>
<th>Minimum</th>
<th>Median</th>
<th>Maximum</th>
<th>(p)-value (ANOVA)</th>
<th>Tukey test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinamide</td>
<td>0.7667</td>
<td>0.1111</td>
<td>0.5921</td>
<td>0.7553</td>
<td>0.9753</td>
<td>0.0139</td>
<td>Nicotinamide versus Saline solution 0.0102</td>
</tr>
<tr>
<td>Saline solution</td>
<td>0.8517</td>
<td>0.0636</td>
<td>0.6988</td>
<td>0.8507</td>
<td>0.9629</td>
<td>0.1345</td>
<td>Nicotinamide versus Alcohol 0.1345</td>
</tr>
<tr>
<td>Alcohol</td>
<td>0.8206</td>
<td>0.0911</td>
<td>0.6574</td>
<td>0.2815</td>
<td>0.9848</td>
<td>0.3133</td>
<td>Alcohol versus Saline solution 0.3133</td>
</tr>
</tbody>
</table>

**Table 3. Comparative study on fetal length**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>Standard-deviation</th>
<th>Minimum</th>
<th>Median</th>
<th>Maximum</th>
<th>(p)-value (ANOVA)</th>
<th>Tukey test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinamide</td>
<td>1.877</td>
<td>0.130</td>
<td>1.700</td>
<td>1.900</td>
<td>2.200</td>
<td>0.0145</td>
<td>Nicotinamide versus Saline solution 0.7821</td>
</tr>
<tr>
<td>Saline solution</td>
<td>1.903</td>
<td>0.125</td>
<td>1.500</td>
<td>1.900</td>
<td>2.100</td>
<td>0.0363</td>
<td>Nicotinamide versus Alcohol 0.0363</td>
</tr>
<tr>
<td>Alcohol</td>
<td>1.974</td>
<td>0.109</td>
<td>1.800</td>
<td>2.000</td>
<td>2.200</td>
<td>0.0490</td>
<td>Alcohol versus Saline solution 0.0490</td>
</tr>
</tbody>
</table>
As for the higher number of pups in G1, it does not agree with the literature findings\(^{(12)}\). In addition, the weight of G1 fetuses was lower than the G3 mean weight, as also observed in a Brazilian study\(^{(12)}\), although not found by other studies\(^{(14)}\). G2 fetuses, unexpectedly, weighted less than those of the other groups, which might be due to the small number of animals in this group.

Regarding the number of reabsorbed fetuses, the present study does not agree with the literature findings\(^{(14)}\), which show reduced fetal viability and increased number of reabsorbed fetuses, while G3 in this study had a rate higher than G1. However, the highest proportion was found in G2.

Recording the length of the fetuses, G1 was significantly different from G3 and not similar to other studies\(^{(14)}\). The shortest lengths were found in G2.

Lastly, it is important to stress that the objective of the present study was not to propose an alternative for alcohol intake during pregnancy, but to propose a possible strategy to reduce the effects of this drug on the offspring when information and medical follow-up are not sufficient to avoid the use of alcohol by the pregnant woman, as well as in chronic alcoholics.

**CONCLUSIONS**

Nicotinamide reduced the number of malformations and it may be a protective factor for attenuating the effects of alcohol. Based on the results of this study, it may be said that the best proposal for preventing FAS is still to avoid the use of alcoholic beverages during pregnancy. Larger studies on the effects of nicotinamide are necessary for enhancing knowledge on the action of this vitamin.

**ACKNOWLEDGEMENTS**

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**REFERENCES**