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# Effect of alloxan-induced diabetes *mellitus* and ethanol on pregnancy outcome in mice

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## Efeito do diabetes mellitus induzido por aloxana e etanol na gestação de camundongos

Luiz Cesar Peres<sup>1</sup>; Camila Nunes de Moraes Ribeiro<sup>2</sup>; Cristiane Minot Gutierrez<sup>3</sup>; Milton Cesar Foss<sup>4</sup>

key words	abstract
<p><b>Diabetes mellitus</b></p> <p><b>Ethanol</b></p> <p><b>Neural tube defects</b></p> <p><b>Fetus mice</b></p>	<p><b>Introduction and objectives:</b> To investigate the effects of ethanol, diabetes <i>mellitus</i> and the combination of both on mouse fetuses. <b>Methods:</b> We used 24 female Swiss mice, dividing them into four groups of 6 each: control (C), ethanol (E), diabetes (D) (blood glucose &gt; 200 mg/dL) and diabetes + ethanol (DE). Diabetes was induced by alloxan (40 mg/kg) on day 7 of pregnancy. Groups E and DE received 4 g/kg of 25% v/v ethanol intraperitoneally, whereas groups C and D received saline. On day 18, all fetuses were harvested. <b>Results:</b> In group DE the following anomalies were found: exencephaly, <i>situs inversus totalis</i>, <i>situs inversus partialis</i>, eyelid skin tag and one animal from group E had pulmonary artery hypoplasia. Ethanol administration partially reverted diabetes-fetal resorption caused by diabetes, yet it induced late fetal death. Both diabetes and ethanol reduced placental diameter and increased its weight. Ethanol had more effect on fetal length in males than in females, however, such bias was not found for diabetes. Ethanol prevented diabetes-induced tail shortening in both genders. <b>Conclusions:</b> These results show that, although ethanol might improve energy metabolism in early gestation, it causes cell damage that leads to cardiovascular, limb and neural tube defects, late fetal death and reduced placental size.</p>

resumo	unitermos
<p><b>Introdução e objetivos:</b> Investigar o efeito do etanol, do diabetes mellitus (DM) e da associação de ambos sobre os fetos de camundongo. <b>Métodos:</b> Foram utilizadas 24 fêmeas de camundongos Swiss divididas em quatro grupos de seis animais cada: controle (C); etanol (E); diabetes (D) (glicemia &gt; 200 mg/dl), e diabetes + etanol (DE). O diabetes foi induzido pela aloxana (40 mg/kg) no dia 7 da gestação. Os animais dos grupos E e DE receberam 4 g/kg de solução a 25% v/v de etanol intraperitoneal (IP), enquanto os animais dos grupos C e D receberam salina. No dia 18, todos os fetos foram coletados. <b>Resultados:</b> Foram encontradas as seguintes anomalias no grupo DE: exencefalia, <i>situs inversus totalis</i>, <i>situs inversus partialis</i> e apêndice cutâneo palpebral. Um animal do grupo E apresentou hipoplasia da artéria pulmonar. A administração de etanol reverteu parcialmente a reabsorção fetal induzida pelo diabetes, porém aumentou a morte fetal tardia. Ambos, diabetes e etanol, reduziram o diâmetro placentário e aumentaram o seu peso. O etanol teve mais efeito no comprimento de fetos machos, contudo isso não ocorreu com o diabetes. O etanol preveniu a redução da cauda induzida pelo diabetes em ambos os sexos. <b>Conclusão:</b> Esses resultados indicam que, embora o etanol possa melhorar o metabolismo energético no início da gestação, ele causa lesão celular que leva a defeitos cardiovasculares, dos membros e do tubo neural, além de morte fetal tardia e redução do tamanho da placenta.</p>	<p><b>Diabetes</b></p> <p><b>Defeitos do tubo neural</b></p> <p><b>Feto</b></p> <p><b>Camundongo</b></p>

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## Introduction

Major congenital anomalies are found in 2% to 3% of liveborn infants. The prevalence of such anomalies has been increasing in parallel with the decline in infant mortality rates, which is related to the number of diseases that are currently preventable. Among the congenital anomalies classified as isolated defects, those affecting the central nervous system are second only to cardiovascular anomalies<sup>(30)</sup>.

Periconceptional folic acid supplementation has been shown to reduce significantly the first occurrence and recurrence of neural tube defects (NTDs) in humans, pointing to its role in the pathogenesis of this condition. However, in experimental animals, a variety of environmental agents have been implicated, including ethanol<sup>(10, 28)</sup>, inhibition of cholesterol synthesis<sup>(6, 10)</sup>, retinoic acid<sup>(45)</sup>, vitamin A<sup>(23)</sup>, hyperglycemia<sup>(12, 43)</sup>, hypoxia<sup>(2, 13)</sup>, ionizing radiation/hyperthermia<sup>(30)</sup> and nicotine<sup>(18, 24)</sup>.

It is well recognized that babies born to diabetic mothers are 3 to 4 times more prone to present congenital anomalies than is the general population<sup>(21, 34)</sup>. Such anomalies have been correlated with inefficient blood glucose control<sup>(34, 43)</sup> which may induce diabetic embryopathy, which encompasses NTDs and other system defects<sup>(12, 16)</sup>.

Ethanol, which is widely consumed worldwide, has been implicated in the etiology of NTDs and of heart and craniofacial defects<sup>(27, 40)</sup>. According to 1997 data provided by the United States Centers for Disease Control and Prevention<sup>(3)</sup>, approximately 50% of all women of childbearing age drink alcoholic beverages. Additionally, 60% of pregnant women may not realize they are pregnant until the fourth week of gestation<sup>(13, 28)</sup>.

Animal models of ethanol-induced congenital anomalies have provided invaluable data regarding critical developmental periods and pathogenesis<sup>(37)</sup>. Administering a dose of ethanol corresponding to the limit of human tolerance to C57B/6J mice during gastrulation has been shown to result in craniofacial defects, including midfacial hypoplasia and prosencephalic anomalies<sup>(38)</sup>.

Recently, Padmanabhan and Shafiullah<sup>(28)</sup> demonstrated an increase in resorptions, intrauterine growth restriction and congenital anomalies in the litters of TO mice treated with streptozotocin-induced diabetes *mellitus* receiving a single dose of ethanol on gestational day 7 or 8.

The combination of experimental maternal diabetes *mellitus* and ethanol has received little attention. Therefore,

it is important to determine how these two common environmental teratogenic factors interact. This approach is relevant since diabetes *mellitus* is quite common<sup>(15, 28)</sup> and ethanol is widely consumed by young women of reproductive age<sup>(5, 28)</sup>.

## Material and methods

This study was approved by the University of São Paulo Committee on Ethics in Animal Research (Process nº 010/2004).

A total of 24 young nulliparous female Swiss mice weighing 40 to 50 g were used. The animals were housed in appropriate individual plastic cages in a 22°C, 12-h light/12-h dark environment and given ad libitum access to tap water and commercial mouse chow throughout the experiment.

Alloxan (40 mg/kg) was injected into the dorsal vein of the tail of 12 randomly-selected mice. The remaining 12 mice received similar injections of saline. The diabetic mice were treated with subcutaneous injection of 0.25 IU of insulin (10 ml of Humulin® and 100 U/ml of neutral protamine hagedorn (NPH) human insulin; Eli Lilly, Indianapolis, IN, USA) until mating. Beginning at four days after the induction of diabetes, five females were caged together with 1 male for two hours daily, from 9:00 to 11:00 am. Day 0 of pregnancy was determined by the presence of a vaginal plug. Pregnant mice were divided into four groups of 6 mice each: control (C); ethanol (E); diabetes (D) and diabetes plus ethanol (DE). Diabetic mice were considered those which presented blood glucose of over 200 mg/dl.

On day 7 of pregnancy, the mice in groups E and DE received an intraperitoneal injection of 25% ethanol (4 g/kg) in saline (v/v) and those in groups C and D received the same volume of saline.

Blood glucose from the tail was measured after a fasting time of 12 hours one day before treatment with alloxan or saline, three days after treatment and on the day of sacrifice using a MediSense Optium blood glucose meter (Abbott Laboratories, Abbott Park, IL, USA) and MediSense Optium blood glucose test strips (MediSense UK Ltd, Cambridge, England, UK).

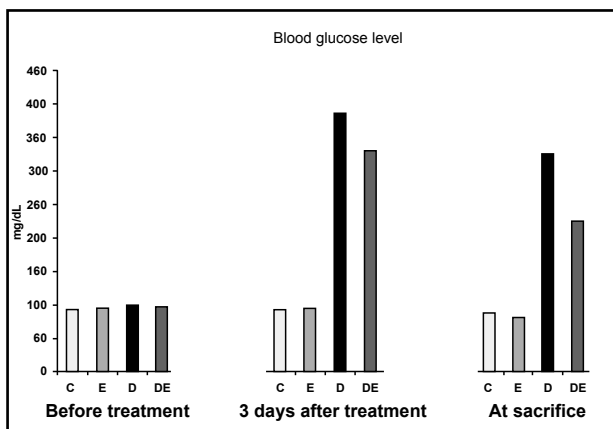
On day 18 of pregnancy, the animals were killed in a CO<sub>2</sub> chamber, and all fetuses were harvested by caesarean section. The fetuses were counted and the following data

were recorded: gender; body weight, crown-rump length and tail length. Placentas were also weighed and its diameter measured. Fetal weight and measurements were considered only for live fetuses. The number of resorptions (representing early fetal death), late fetal deaths (considered those fetuses that have completed their development and were in a process of degeneration), normal live fetuses and fetuses with NTD, as well as the total number of all anomalies, were recorded. Implants were defined as the sum of all resorptions, late fetal deaths and live fetuses with or without any anomaly. After fixation in buffered formalin, all fetuses were submitted to autopsy according to Sterz and Lehmann *in situ* sectioning method<sup>(35)</sup> using a stereomicroscope coupled to a digital camera.

All data were statistically analyzed using the GraphPad Prism 4.0 program for analysis of variance (ANOVA), the Kruskal-Wallis test, the Mann Whitney test and the Cytel StatXact® 7 program for the Fisher-Freeman-Halton test. Values of  $p < 0.05$  were considered statistically significant.

## Results

There was no difference in blood glucose level among the groups before treatment. After alloxan administration, however, blood sugar levels characteristic of diabetes were observed in groups D and DE (**Figure 1**).



**Figure 1** – Blood glucose level in mg/dl in the different groups and in different moments

There was no difference among the groups in terms of the number of implants. However, although the number of normal live fetuses did not differ between groups C (110) and E (94), there was a difference between these two groups and groups D and DE (**Table 1**).

**Number of implants, normal live fetuses, resorptions, live fetuses with congenital anomalies and late fetal death of groups C, E, D and DE (Fisher-Freeman-Halton Test)**

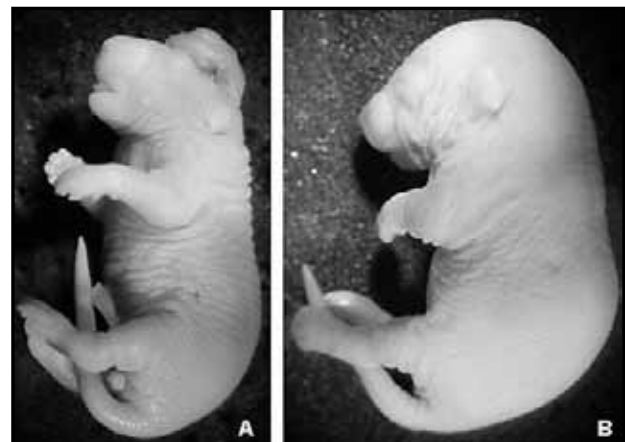
**Tabela 1**

Findings	Groups (n = 6)				p value
	Control	Ethanol	Diabetes	Diabetes + ethanol	
Implants	119	106	89	96	$p = 0.049$
Resorptions	8	9	19*	11	$p = 0.002$
Late fetal death	1	3	4	8*	$p = 0.002$
Normal live fetuses	110	94	66*	65*	$p = 0.003$
Live fetuses with congenital anomalies	0	1	0	12*	$p = 0.001$

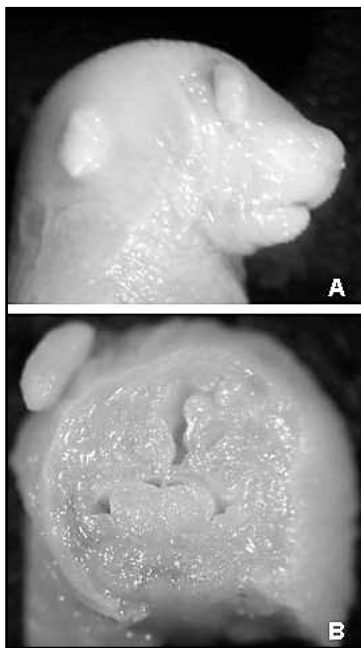
\*Statistically different from control.

Resorption, the result of early fetal death, was significantly higher in group D animals, whereas late fetal death was higher in group DE ones (Table 1).

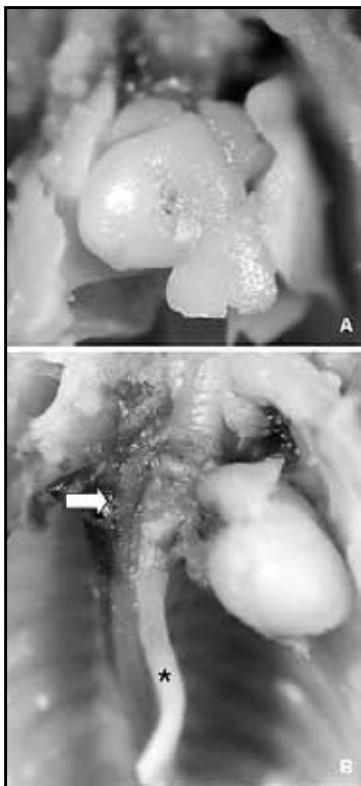
There were less normal live fetuses in groups D and DE and congenital anomalies were confined to groups DE and E, which received ethanol (Table 1). The congenital anomalies found were exencephaly (nine fetuses from group DE) (**Figure 2A**, **Figure 2B** is a control fetus), cutaneous tag over the eyelid (1 fetus from group DE) (**Figures 3A** and **3B**), *situs inversus totalis* (**Figures 4A** and **4B**) and *situs inversus partialis* (**Figures 5A** and **5B**) (1 fetus each from group DE) and pulmonary artery hypoplasia (one fetus from group E) (**Figures 6A** and **6B**).



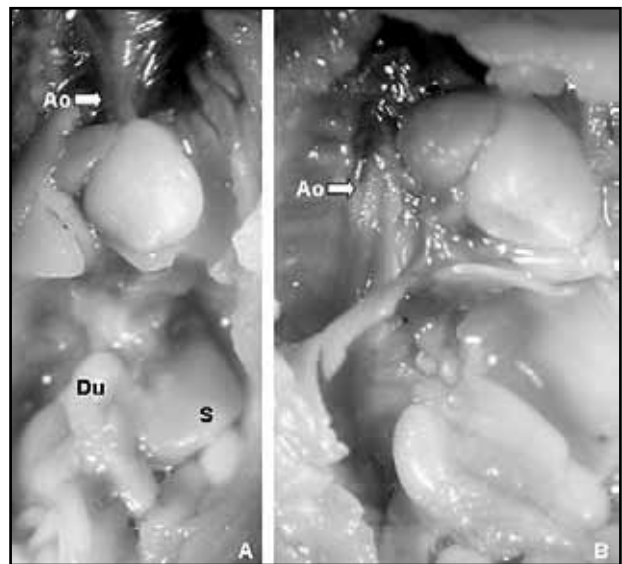
**Figure 2** – Photograph of mouse fetuses. Neural tube defect is clearly seen in a group DE fetus (A). A normal control group fetus is shown in B



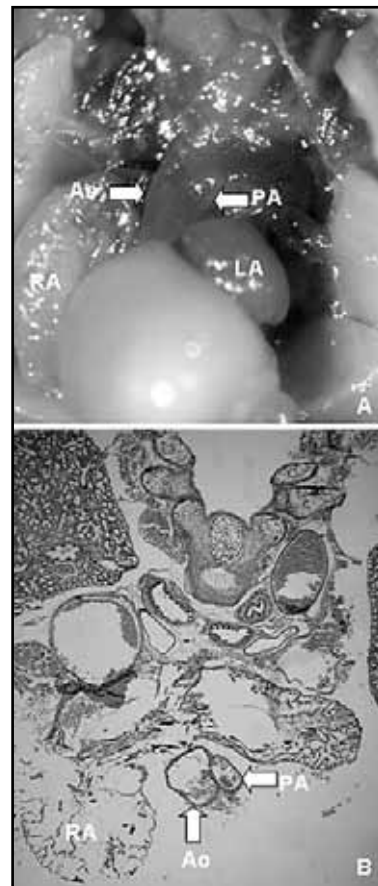
**Figure 3** – A cutaneous appendage on the right eyelid is seen in one fetus of group DE (A and B)



**Figure 4** – Macroscopic pictures of the other anomalies found in a group DE fetus with situs inversus totalis. Note in A the apex of the heart turned to the right. After trimming off the lungs and displacement of the heart to the left, one can clearly see the aorta running along the right side of the vertebral column (arrow) in B. The tube running above the column is the esophagus (asterisk)



**Figure 5** – A and B is from the autopsy of a group DE fetus with situs inversus partialis. Note the right aortic arch (Ao) and descending aorta (Ao), dextrocardia and the normally positioned duodenum (Du) and stomach (S)



**Figure 6** – Photograph of the autopsy of a group E fetus. Note the severe hypoplasia of the pulmonary artery (PA) compared to the aorta in A. Histologic section at the level of the base of the heart depicts the hypoplastic pulmonary artery (PA) close to the normal sized aorta (Ao) in B (hematoxylin and eosin, 50x original magnification) RA: right atrium, LA: left atrium.

A gender-based difference was found in terms of fetal length. Male crown-rump length was reduced in animals from groups E, D and DE, whereas female fetal crown-rump length

was decreased only in groups D and DE. Male fetuses of group DE presented less crown-rump reduction than female ones and the most severely affected group of all was group D (**Table 2**).

**Median fetal crown-rump length of Swiss mouse fetuses recovered on gestational day 18 from dams which received saline (control), ethanol 4 mg/kg (ethanol), alloxan-induced diabetes + saline (diabetes) and alloxan-induced diabetes + ethanol 4 mg/kg on gestational days 7, 8 and 9 (One way ANOVA, Kruskal-Wallis Test)**

**Tabela 2**

	Control		Ethanol		Diabetes		Diabetes + ethanol	
		<i>n</i>		<i>n</i>		<i>n</i>		<i>n</i>
Crown-rump length (male + female) (cm)	2.5	110	2.4	94	2	74	2.2	74
Male crown-rump length (cm)	2.5	56	2.4	49	2	38	2.2	29
Female crown-rump length (cm)	2.5	54	2.4	45	2	36	2.1	45

Dunns multiple comparison test for fetal crown-rump length			
	Male + female	Male	Female
Control vs. ethanol	$p < 0.05$	$p < 0.001$	$p > 0.05$
Control vs. diabetes	$p < 0.001$	$p < 0.001$	$p < 0.001$
Control vs. diabetes ethanol	$p > 0.05$	$p < 0.001$	$p < 0.001$
Ethanol vs. diabetes	$p < 0.001$	$p < 0.001$	$p < 0.001$
Ethanol vs. diabetes ethanol	$p > 0.05$	$p > 0.05$	$p < 0.01$
Diabetes vs. diabetes ethanol	$p < 0.001$	$p > 0.05$	$p > 0.05$

Fetal body weight was equally decreased in groups E, D and DE for both sexes and this finding correlated with IUGR (**Table 3**).

Tail length reduction was observed in both male and female fetuses from groups D and DE, although less intensely in the latter (**Table 4**).

Placental diameter was reduced in groups E, D and DE whereas placental weight was increased in groups E and DE (**Table 5**).

## Discussion

The reproductive outcome is affected by different conditions that can be endogenous or exogenous. Maternal diabetes is a well known metabolic endogenous factor associated with poor pregnancy outcome, inducing

congenital anomalies. Ethanol, on the other hand, is a common exogenous offender, recognized by its many deleterious actions on the developing fetus. When associated, diabetes and ethanol are expected to induce more harmful effects due to their synergistic action.

In the present study, Swiss mice became diabetic after alloxan administration before mating but were supplemented with insulin until they became pregnant. This approach was effective since we avoided any influence of alloxan on the fetuses at the same time fertility was kept unaltered with insulin treatment, which is reflected by the absent difference regarding implant number among all groups.

Although both ethanol and diabetes are detrimental to the fetuses, diabetes seems to be worse than ethanol on early fetal development due to the increased number of early fetal death observed in group D, whereas ethanol is worse on late fetal development, which is reflected by the

**Median fetal body weight and percentage of intrauterine growth restriction (IUGR) of Swiss mouse fetuses recovered on gestational day 18 from dams which received saline (control), ethanol 4 mg/kg (ethanol), alloxan-induced diabetes + saline (diabetes) and alloxan-induced diabetes + ethanol 4 mg/kg on gestational days 7, 8 and 9 (One way ANOVA, Kruskal-Wallis Test)**

**Tabela 3**

	Control		Ethanol		Diabetes		Diabetes + ethanol	
		<i>n</i>		<i>n</i>		<i>n</i>		<i>n</i>
Body weight (male + female) (g)	1.55	110	1.31	94	0.99	74	1.2	60
IUGR (male + female) -2 SD (%)	1.82	110	13.8	94	68.9	74	43.3	60
Male weight (g)	1.59	56	1.27	49	1.08	38	1.22	29
IUGR (male) -2 SD (%)	1.75	56	40.8	49	74.5	38	68.9	29
Female crown-rump length (cm)	1.47	54	1.37	45	0.96	36	1.15	46
IUGR (female) -2 SD (%)	1.85	54	8.8	45	61.1	36	37.7	46

SD: standard deviation.

## Dunns multiple comparison test for fetal crown-rump length

	Male + female	Male	Female
Control vs. ethanol	$p < 0.001$	$p < 0.001$	$p < 0.01$
Control vs diabetes	$p < 0.001$	$p < 0.001$	$p < 0.001$
Control vs. diabetes ethanol	$p < 0.001$	$p < 0.001$	$p < 0.001$
Ethanol vs. diabetes	$p < 0.001$	$p < 0.001$	$p < 0.001$
Ethanol vs. diabetes ethanol	$p < 0.001$	$p < 0.001$	$p < 0.01$
Diabetes vs. diabetes ethanol	$p > 0.05$	$p > 0.05$	$p > 0.05$

increased late fetal death in group DE. It may be speculated that ethanol provides enhanced caloric availability in early gestation and thus prevents early fetal death. This may also be the explanation why IUGR, fetal body weight, crown-rump length and tail length were less severely affected in group DE than in group D.

The mechanism involved in IUGR seen with both diabetes and ethanol is the production of reactive oxygen species and free radicals<sup>(28)</sup>, which damage tissues, inducing apoptosis. It is postulated that the oxidative stress, possibly more intense with ethanol administration, may induce late fetal death and congenital anomalies. In fact, this was observed in the present study, since congenital anomalies were only observed in groups which received ethanol, either alone, group E or associated with diabetes, group DE, and late fetal death was higher in the latter. Additionally, cases of NTD were found only in

the diabetic animals receiving ethanol, indicating that the combination of both factors is essential. This finding can be interpreted in the light of epidemiological studies which have found an increased risk for major systemic anomalies in human maternal diabetes without an overall incidence of anomalies, suggesting that diabetes is not a primary teratogen but acts by promoting an initial insult and potentializing the detrimental effects of other teratogens<sup>(22)</sup>. The reverse has also been proposed, i.e., that ethanol potentiates the effects of diabetes or that anomalies are attributable to the interaction between ethanol and diabetes-induced metabolic disorders<sup>(19)</sup>. Apoptosis and decreased migration and differentiation of neural crest cells<sup>(4)</sup> may be implicated in NTD. Therefore, the combination of the two teratogens would increase not only the risk of congenital anomalies but also that of intrauterine growth restriction (IUGR)<sup>(28)</sup>.

**Median fetal tail length of Swiss mouse fetuses recovered on gestational day 18 from dams which received saline (control), ethanol 4 mg/kg (ethanol), alloxan-induced diabetes + saline (diabetes) and alloxan-induced diabetes + ethanol 4 mg/kg on gestational days 7, 8 and 9 (One way ANOVA, Kruskal-Wallis Test)**

**Tabela 4**

	Control		Ethanol		Diabetes		Diabetes + ethanol	
		<i>n</i>		<i>n</i>		<i>n</i>		<i>n</i>
Tail length (male + female) (cm)	1.3	110	1.3	94	1.2	74	1.3	74
Male tail length (cm)	1.3	56	1.3	49	1.15	38	1.3	29
Female tail length (cm)	1.3	54	1.3	45	1.2	36	1.2	45

## Dunns multiple comparison test for fetal tail length

	Male + female	Male	Female
Control vs. ethanol	$p > 0.05$	$p > 0.05$	$p > 0.05$
Control vs. diabetes	$p < 0.001$	$p < 0.001$	$p < 0.001$
Control vs. diabetes ethanol	$p > 0.05$	$p > 0.05$	$p > 0.05$
Ethanol vs. diabetes	$p < 0.001$	$p < 0.001$	$p < 0.001$
Ethanol vs. diabetes ethanol	$p > 0.05$	$p > 0.05$	$p > 0.05$
Diabetes vs. diabetes ethanol	$p < 0.001$	$p < 0.001$	$p < 0.05$

**Median placental weight and median placental diameter of Swiss mouse fetuses recovered on gestational day 18 from dams which received saline (control), ethanol 4.0 mg/kg (ethanol), alloxan-induced diabetes + saline (diabetes) and alloxan-induced diabetes + ethanol 4 mg/kg on gestational days 7, 8 and 9 (One way ANOVA, Kruskal-Wallis Test)**

**Tabela 5**

	Control		Ethanol		Diabetes		Diabetes + ethanol	
		<i>n</i>		<i>n</i>		<i>n</i>		<i>n</i>
Placental weight (g)	0.107	110	0.12	94	0.11	74	0.111	74
Placental diameter (cm)	0.8	110	0.7	94	0.5	74	0.6	74

## Dunns multiple comparison test for placental weight and diameter

	Placental weight	Placental diameter
Control vs. ethanol	$p < 0.001$	$p < 0.001$
Control vs. diabetes	$p > 0.05$	$p < 0.001$
Control vs. diabetes ethanol	$p < 0.01$	$p < 0.001$
Ethanol vs. diabetes	$p > 0.05$	$p < 0.001$
Ethanol vs. diabetes ethanol	$p > 0.05$	$p > 0.05$
Diabetes vs. diabetes ethanol	$p > 0.05$	$p > 0.05$

Swiss mouse seems to be less affected by diabetes and/or ethanol than do other strains, such as TO<sup>(28)</sup> and C57BL/6J<sup>(39)</sup>. The latter is particularly prone to the teratogenic effects of ethanol, resulting in the facial dysmorphisms seen in the fetal alcohol syndrome (FAS)<sup>(39)</sup>, which were not found in the swiss mouse fetuses. However, it is not possible to assure that in the diabetic group not receiving ethanol there were no NTDs or other major defects.

One of the fetuses of group E presented pulmonary artery hypoplasia. This finding may be interpreted in the light of the studies that present congenital heart disease as part of FAS, what has already been demonstrated in animal models as well<sup>(5)</sup>.

*Situs inversus, totalis* or *partialis*, a congenital defect characterized by the mirror image of viscera, is found in humans<sup>(32, 41)</sup> and animals<sup>(8, 42)</sup>. This defect can be accompanied by other anomalies<sup>(20, 32)</sup> and is 84 times more common in infants born to diabetic women<sup>(14)</sup>. *Situs inversus* is rare in mouse strains, including C57BL/6J<sup>(25)</sup>. Recently, a transgenic mouse strain (inv/inv mice) was reported, in which almost 100% of the homozygous individuals were characterized by *situs inversus totalis* as a consequence of insertional mutagenesis of the inversin gene<sup>(44)</sup>. Therefore, the cases occurring in our study were probably related to diabetes although it is not possible to exclude any participation of the ethanol since it was found in group DE. In the same group, we found a case of a cutaneous appendage on the eyelid. No such defect has been reported previously, although there is evidence that ethanol interferes with ocular development, inducing anophthalmia, microphthalmia, hypo- and hypertelorism and cyclopia<sup>(33, 38)</sup>.

IUGR was observed in all experimental groups, with increasing frequency in groups E, DE and D. The results of clinical studies have demonstrated that the development of embryos of diabetic mothers is impaired, and that growth restriction is a risk factor for congenital anomalies<sup>(29)</sup>. Lin *et al.*<sup>(19)</sup> showed that rat infants born to diabetic dams receiving or not receiving ethanol weighed less than did those born to dams receiving ethanol or receiving no treatment. Although ours was a mouse model, this is in agreement with our findings. Those authors stated that fetal growth is accelerated after gestational day 11 in the rat, and that using a single dose prior to that moment, as was done in the present study, would therefore have a less intense effect at the end of gestation. In addition, Padmanabhan and Shafiullah<sup>(28)</sup> showed that the IUGR induced by ethanol injection on

gestational day 8 in a mouse model of diabetes was less intense than that caused by ethanol injection on day 7, indicating that the effect is gestational stage-dependent.

In group E, fetal weight of male and female fetuses was lower than that seen in group C. This finding is in accordance with those previously described<sup>(11, 36)</sup>. Diabetes also induced IUGR that was more pronounced than that induced by ethanol.

We found that diabetes impaired tail growth, although the same was not found for ethanol. Once again, ethanol was found to block further reductions in tail length in male and female infant mice born to diabetic dams, possibly because of the higher calorie provided by ethanol.

Although the combination of ethanol and diabetes induced less IUGR and tail growth, it was the cause of the NTDs and other major anomalies seen. Therefore, the net result is decidedly negative.

Contrary to diabetes, which affects both genders in a similar way, ethanol affects male fetuses crown-rump length more intensely than female ones.

The influence of ethanol alone or in association with diabetes on the placenta was more marked, resulting in a heavier placenta. The correlation between IUGR and loss of placental size might constitute a cause and effect relationship or might be the net result of the deleterious effects that both diabetes and ethanol have on the fetuses and placentas simultaneously. In humans, the placenta is usually larger and heavier in diabetic women, possibly related to the chronically reduced blood flow<sup>(17)</sup> caused by diabetes-induced systemic hypertension, which in turn might be linked to the diabetic embryopathy<sup>(31)</sup>. Although we have not tested this hypothesis, it is possible that an abnormal uterine environment is the cause of the placental size reduction seen in our animals.

The perinatal deaths observed in the present study indicate placental insufficiency, since the apparent cause was asphyxia, as it typically is in humans<sup>(9)</sup>. In a rat model of preeclampsia, diabetes-induced oxidative stress was the cause of the placental insufficiency<sup>(26)</sup>. Akay and Koçkaya<sup>(1)</sup> showed that fibrin deposition, inflammation, fibroblast proliferation and synthesis of the extracellular matrix in the placentas of rats exposed to ethanol, that could explain the weight increase in groups E and DE placentas, together with the diabetes-related impairment of the influx of nutrients from the maternal circulation<sup>(7)</sup>, contribute to IUGR and late fetal death. These findings

explain at least in part the larger number of perinatal deaths observed in the diabetic groups, all of which presented reduction of placental size.

In summary, the present study shows that, although a single high dose of ethanol administered to Swiss mice with alloxan-induced diabetes *mellitus* on gestational day 7 has some favorable effects in terms of less IUGR and tail

reduction, it induces congenital anomalies, cardiovascular defects and NTDs in particular, reduction of the size and increase in the weight of the placenta and late fetal death. The synergism of the two teratogenic factors is a relevant issue and, despite interspecies differences, can serve as a warning of the risk that this combination poses to human population.

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