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The impact of ethanol exposure during fetal development on the retina of chicken embryos

El impacto de la exposición al etanol durante el desarrollo fetal en la retina de embriones de pollo

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ABSTRACT

It is common knowledge that excessive consumption of alcohol by pregnant women may lead to neonatal mortality, a delay in fetal development and serious malformations. In this study, we used the chicken-embryo model to demonstrate the ethanol teratogenic effect on the development of the retina. Forty-nine fertilized eggs were employed in our experiment. As a result, the chicken embryos were injected with varying amounts of ethanol in the air space (10%, 30%, and 50%). The eggs were kept in a humid incubator with a temperature of 37 °C and a humidity level of 60–65%. On the 20th day of incubation, the eggs were cracked and the chicks were examined with their length and body weight taken into account. These operations were followed by the resection of the ocular globes for a histological examination. The eyes were fixed with formalin at 10%. The right eye was sectioned at 5µm with a Leica microtome. Hematoxylin-Eosin staining was, then, applied on the slides, which were also observed under a microscope. The optic-microscopic observation of the ocular samples of each group has revealed that the *in ovo* ethanol exposure has caused retina changes, including the internal plexiform and the ganglion-cell layers. In treated groups the thickness of these two layers decreased in comparison with the control group. In conclusion, the current findings indicated that treating chicken embryos with ethanol at an early stage resulted in significant abnormalities and a lower embryo survival rate. Ethanol has harmful effects on the embryonic development including growth retardation and ocular abnormalities; this effect is dose-dependent.

Key words: Alcohol; chicken embryo; malformation; retina; teratogenic effect

RESUMEN

Es bien sabido que el consumo excesivo de alcohol por parte de las mujeres embarazadas puede provocar mortalidad neonatal, retraso en el desarrollo fetal y malformaciones graves. En este estudio, utilizamos el modelo de embrión de pollo para demostrar el efecto teratogénico del etanol sobre el desarrollo de la retina. En esta investigación experimental se utilizaron 49 huevos fecundados. Para ello, se inyectaron diferentes dosis de etanol en los embriones de pollo al (10%, 30% y 50%) en el espacio aéreo. Los huevos se incubaron en una incubadora húmeda a una temperatura de 37 °C y a un 60-65% de humedad. Al vigésimo día de incubación, se cascaron los huevos y se examinaron los pollitos teniendo en cuenta su longitud y su peso corporal. A estas operaciones siguió la resección de los globos oculares para un examen histológico. Los ojos se fijaron con formol al 10%. El ojo derecho se seccionó a 5µm con un micrótomo Leica. A continuación, se aplicó la tinción de hematoxilina-eosina en los porta objetos, que también se observaron al microscopio. La observación óptico-microscópica de las muestras oculares de cada grupo ha revelado que la exposición *in ovo* al etanol ha provocado cambios en la retina, incluidas las capas plexiforme interna y ganglionar. El grosor de estas dos capas disminuye en comparación con el grupo de control. En conclusión, los hallazgos actuales indican que el tratamiento de embriones de pollo con etanol en una fase temprana provoca anomalías significativas y una menor tasa de supervivencia embrionaria. El etanol tiene efectos nocivos en el desarrollo embrionario, como retraso del crecimiento y anomalías oculares; este efecto depende de la dosis.

Palabras clave: Alcohol; embrión de pollo; malformación; retina; efecto teratogénico

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INTRODUCTION

Among the various teratogenic agents, alcohol is considered the most prevalent worldwide. Maternal alcohol consumption during pregnancy has been widely documented as a major risk factor for developmental abnormalities, notably leading to fetal alcohol syndrome (FAS) and fetal alcohol spectrum disorders (FASD). Given the significance of this issue, any statement regarding the prevalence or impact of teratogens should be supported by quantitative data, and ideally complemented by comparative analyses highlighting their principal causes [1].

Given the growing number of women who drink alcohol globally [2], this is a very significant situation. Despite the fact that the rate of alcohol use has been largely stable over the past 30 years, pregnant women's alcohol consumption increased from 12.4% in 1991 to 16.3% in 1995. FAS cases also doubled in number between 1979 and 1993, with an incidence rate of 6.7% per 10.000 live births in 1993 [3].

An elevated infant mortality, head and facial malformations, a microcephaly, and organ malfunction are just a few of the potentially harmful clinical effects of alcohol use during pregnancy [4 , 5].

Retinal degeneration and optic nerve hypoplasia are the most often seen defects in children with FAS. Alcohol disrupts the development of the retina, resulting in a delayed and faulty cell migration as well as a loss of neurons in the interior layers of the retina, especially the ganglion cell layer. The number of myelin-axons in the layer of optic nerve fibers has been significantly decreased [6].

Researchers have utilized a variety of animal models to investigate the causes and consequences of various alcohol-related disorders. Such models have aided researchers in their exploration of the ways that both short- and long-term alcohol intakes can obstruct the normal development of embryos [7].

An incredibly helpful model in developmental biology, embryology, and teratology is the chicken embryo (*Gallus gallus domesticus*). There are many sources that provide an accurate and comprehensive description on the growth of chicks [8].The use of chicken models has a number of benefits for developmental study. Fertilized eggs are readily available, affordable, and don't need an incubator to grow. To directly view or manipulate the embryo, a hole can be simply made in the eggshell. The eggshell can potentially be simply destroyed to continue developing [9].

Because there is no in ovosystem in the maternal metabolism, teratogenic effects cannot be directly isolated. Briefly stated, the chicken-embryo model provides a realistic and affordable model in which modern experimental tools may be used to understand how a teratogen affects particular pathways underpinning organogenesis and morphogenesis [8].

The chicken eye has become a preferred animal model in experimental ophthalmology because of its quick growth, superior visual abilities, and ease of general manipulation. It is frequently employed in the research of parasympathetic functions in the development and ageing [10].

The purpose of this study is to examine the teratogenic effects of *in ovo* ethanol exposure on the retinal development in a system of chicken embryos, taking into account the injected ethanol dose and the stage of embryonic development.

MATERIAL AND METHODS

Ethical approval

Throughout the trial, the institutional animal ethics committee and national animal welfare requirements were adhered to accordingly. Guidelines of the Local Ethical Committee. The study is registered under the reference 03/2024.

Experimental design

The experimental study was done on a set of 49 embryonated eggs of the broiler chickens (Hubbard F15) strain collected at the private hatchery of Agricultural Cooperative Errakhaà Walzddihar, located in Theniyetsaida (Ain Yagout – Batna, Algeria). The eggs were divided into five groups: untreated group, group treated by 0.9% NaCl and three groups exposed to ethanol at different concentrations (10%, 30% and 50%) (TABLE I). The fertilized chicken eggs of each group were injected once with100µl of ethanol.

TABLE I
Treatments and number of eggs in each group of study

Group	Treatment	Number of eggs
Untreated group	No-treated	7
NaCl-treated	NaCl (0.9%)	9
Treated; ethanol	EtOH (10%)	10
	EtOH (30%)	11
	EtOH (50%)	12

NaCl: Sodium Chloride, EtOH: Ethanol.

Injection of substances

This particular process is essential and demands the highest level of sterility. In order to inject the chemical into the air sacs using a graduated syringe in microlitre, a Bunsen burner (OMM, Italy), equipped with a tap and a flame stabilizer, was used [11 , 12 , 13].

Incubation

The eggs were automatically turned every four hours (h) during the 480 h (20-day) incubation period in a humid incubator (Real 49 automatic, Italy) set at 37 °C and 60–65% humidity [6 , 11].

Overall length and body weight measurement of the chicks

On the 20th day of incubation, the eggs were cracked and the embryos were examined.Their lengths were measured using a caliper (RS PRO, China), and their body weights were determined

with a balance (KERN, Germany). To identify the overall length and body weight of the chicks, the extra-embryonic membranes were removed and the samples were placed on absorbent paper during 2 to 3 min. Each measure was repeated four times.

Histological examination

The samples (eyes) were fixed in buffered formalin at 10% passed through a series of ethanol showers from 80 to 100%, then cleared with Xylene during one h in each ethanol shower and then incorporated in paraffin Wax (Histosec Pastilles) Sigma-Germany bath at 58 °C [14 , 15]. 5 µm sections of the right eye were taken with a microtome (Leica Jung-histocut 820, Germany), Following hematoxylin and eosin staining (H&E), the slides were examined using a Carl Zeiss Axioskop 20 optical microscope (Germany) that was attached to a DOM 300 digital video camera [16 , 17 , 18 , 19]. The image was viewed using Oasis software (Oasis Scientific Inc.) and analyzed using Image J, a powerful image analysis program developed by the National Institute of Health [20].

Statistical analysis of the experimental data

The statistical analysis of the experimental data was carried out using Microsoft Excel 2010 Software and Graph Pad Prisme 5.01.

The data concerning the chicks' viability were analyzed using the chi-square test.

The data concerning the chicks' body weight and length, and the retina layers thickness were analyzed using the Mann-Whitney U test. The data were presented in average ± standard deviation (SD). A difference of $P<0.05$ between the ethanol-exposed and control groups was deemed statistically significant.

RESULTS AND DISCUSSION

The effects of ethanol on chicks' viability

The results have revealed a slight difference in the survival rate of chicks treated with 0.9% NaCl (77.8%) and 10% ethanol (70%) compared to the untreated group (85.7%). When the ethanol dose was increased to 30% and then to 50%, the survival rate further reduced to (54.5%) and (50%), respectively (TABLE II). This indicates that the mortality of 20-day-old chicks varies between groups and that their viability declines in a dose-dependent manner. These data clearly show that ethanol exposure significantly increased early embryo mortality. This finding is consistent with previous studies by Boussouar *et al*. [21]; Kamran *et al*. [22]; Shabtai and Fainsod [23]; and Laghari *et al*. [24], which also reported that early-stage ethanol treatment reduces the survival rate of chicken embryos.

TABLE II
Average of survival rates of chicks treated with ethanol

Groups	Treatment	Number of eggs	Number of living chicks	Living chicks (%)
Untreated group	No-treated	7	6	85.7
NaCl-treated	NaCl (0.9%)	9	7	77.8
Treated; ethanol	Ethanol (10%)	10	7	70
	Ethanol (30%)	11	6	54.5
	Ethanol (50%)	12	6	50

NaCl: Sodium Chloride

The effects of ethanol on body weight of the chicks

Concerning the measurements, the results revealed a significant decrease in the body weight of chicks exposed to high doses of ethanol (30 and 50%), with mean values of (27.77 ± 2.15 and 24.82 ± 2.33g), respectively. These values were significantly lower ($P<0.05$) than those recorded in the group treated with 0.9% NaCl, which showed averages of (30.94 ± 2.34 and 30.31 ± 2.69g) (TABLE III). This dose-dependent reduction in body weight indicates that ethanol exposure leads to a marked delay in prenatal growth. These findings are in agreement with Naqi *et al*. [25], who reported a similar decline in chick body weight following ethanol treatment. Furthermore, they confirm previous observations by Tufan and Satiroglu-Tufan [11], as well as studies by Rao and Chaudhuri [26]; Smith *et al*. [27]; and Laghari *et al*. [24], which demonstrated that ethanol exposure even during the pre-hatching stage can negatively affect the health and development of avian embryos. Commonly observed effects include teratogenic effects, reduced hatchability, lower hatch weight, and impaired post-hatch growth. In addition, Munir *et al*. [28] reported that even low doses of ethanol do not improve poultry productivity and that moderate to high doses may negatively affect welfare and performance traits.

TABLE III
The body-weight and length averages of the chicks

Groups	Body weight of the chicks (Average ± SD)	Length of the chicks (Average ± SD)
Untreated group	30.94 ± 2.34	14.97 ± 0.68
NaCl (0.9%)	30.31 ± 2.69	14.50 ± 1.63
EtOH (10%)	28.27 ± 4.61	13.97 ± 1.43
EtOH (30%)	27.77 ± 2.15*	13.48 ± 0.43*
EtOH (50%)	24.82 ± 2.33*	13.37 ± 0.80*

NaCl: Sodium Chloride, EtOH: Ethanol, SD: Standard deviation,*: values in the same column are significantly different ($P<0.05$).

The effects of ethanol on body length of the chicks

Also, a significant decrease in the overall length of the chicks was observed in the ethanol-exposed groups, particularly those treated with (30 and 50%) ethanol, which showed average lengths of (13.48 ± 0.43 and 13.37 ± 0.80 cm), respectively. These values were significantly lower ($P<0.05$) compared to the untreated group. Ethanol thus appears to reduce chick embryo body length in a dose-dependent manner. These findings are in agreement with those reported by Afzal *et al*. [29].

The effects of ethanol on the retina microscope structure

The microscopic examination of retinal samples from each group using an optical microscope revealed notable histological changes depending on ethanol concentration. In the control groups (untreated and 0.9% NaCl-treated), the two retinal layers (the internal plexiform layer and the ganglion-cell layer) showed normal organization. However, in the group exposed to 10% ethanol, there was a significant reduction ($P<0.05$) in the thickness of both the internal plexiform layer (54.67 ± 0.69 µm vs. 60.31 ± 0.49 µm in controls) and the ganglion-cell layer (22.11 ± 0.56 µm vs. 28.70 ± 0.32 µm in controls) (FIG. 1).

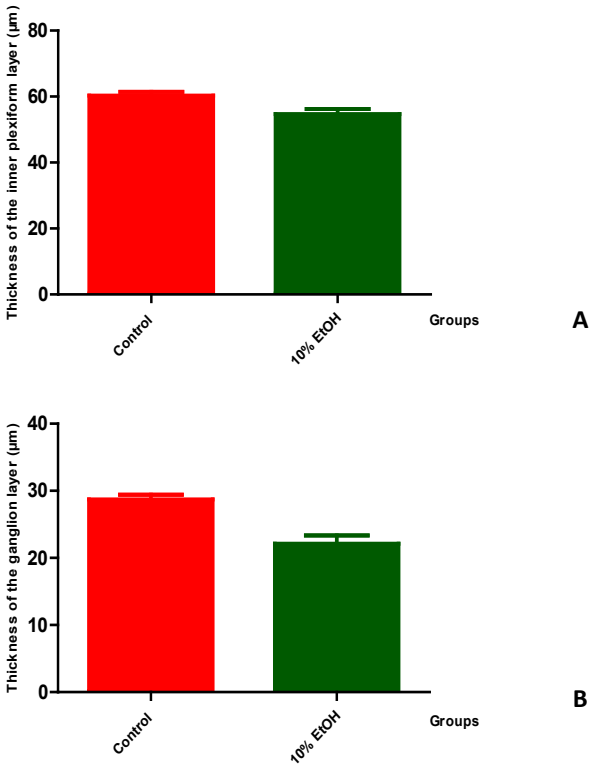


FIGURE 1. Effect of exposure to ethanol (10%) on the chick's retina (Hubbard F15), Algeria. A.Effect of EtOH (10%) on the internal plexiform layer; B. Effect of EtOH (10%) on the ganglion layer.

These alterations became more pronounced in the group exposed to 30% ethanol, which exhibited a marked decrease ($P<0.01$) in the thickness of the internal plexiform layer (42.89 ± 1.19 µm) and ganglion-cell layer (18.99 ± 0.54 µm) compared to controls (FIG. 2).

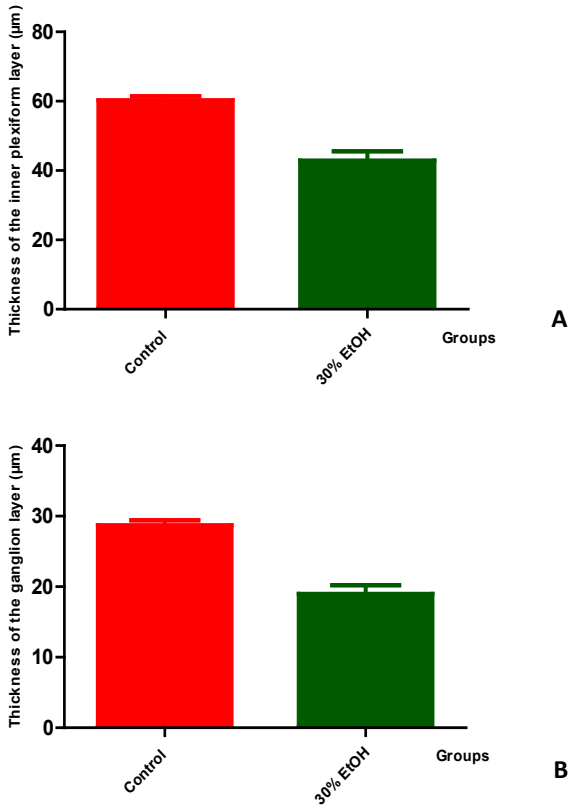
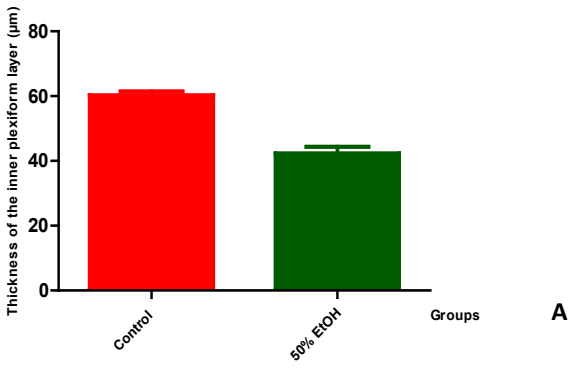


FIGURE 2. Effect of exposure to ethanol (30%) on the chick's retina (Hubbard F15), Algeria. A. Effect of EtOH (30%) on the internalplexiform layer; B. Effect of EtOH (30%) on the ganglion layer.

The most severe changes were observed in the 50% ethanol group, where the internal plexiform layer and ganglion-cell layer showed significant thinning (42.33 ± 0.92 µm and 19.92 ± 0.27 µm, respectively; $P<0.001$) relative to controls (FIG. 3).



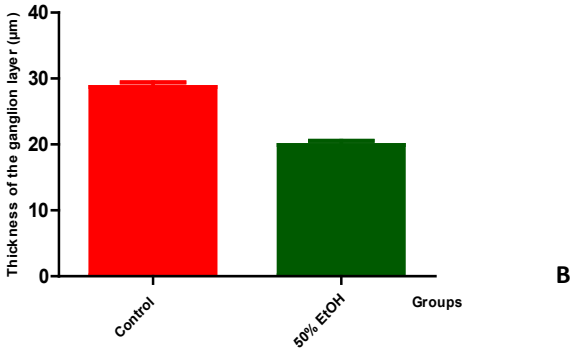


FIGURE 3. Effect of exposure to ethanol (50%) on the chick's retina (Hubbard F15), Algeria. A. Effectof EtOH (50%) on the internal plexiform layer; B. Effect of EtOH (50%) on the ganglion layer.

Overall, these findings demonstrate that *in ovo* exposure to ethanol leads to retinal degeneration in a dose-dependent manner, particularly affecting the internal plexiform and ganglion-cell layers, which supports the observations reported by Tufan *et al*. [12]; Guerri and Pascual [30] and Holahan *et al*. [31].

CONCLUSION

This study aimed at clarifying the teratogenic effect of prenatal ethanol exposure at early stages of the development on the retina in a chicken-embryo model. Ethanol has a direct effect on embryonic viability, causing a dose-dependent decrease in embryo survival. Ethanol caused a significant delay in prenatal growth, as shown by dose-dependent measurements of mean body weight and chick length. The chicks exposed to ethanol showed a change in microscopic structure of the retina. This change has been observed at the level of the internal retina layers; in particular, the internal plexiform and the ganglion-cell layers whose thickness has decreased in comparison to the control group. This decrease is closely correlated to the delivered dose. In conclusion, ethanol negatively impacts embryonic development, major abnormalities in the structure of the retina and growth retardation.

Conflict of interests

The authors have no conflict of interest to declare in regard to this publication.

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