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Original Article

Myelin degeneration in peripheral nerve in chick embryos following continuous ethanol exposure during early gestational period: a preliminary report

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ABSTRACT

Fetal alcohol syndrome (FAS), a condition occurring in some children of mothers who have consumed alcohol during pregnancy, is characterized among other features by motor and sensory derangements and neurobehavioral deficits. However, no specific changes in the brain and spinal cord that could conclusively explain these neuronal defects have been reported.

The aim of this study was to investigate the effects of continuous ethanol exposure during early gestation on the peripheral nerve, towards the understanding of neuronal disorders in FAS. A chick model of FAS was used and embryos were exposed to 5%, 10% and 15% ethanol on embryonic days 1-8. The effects on general growth and development, and on the peripheral nerve of ethanol-exposed embryos were examined following the full period of gestation.

There was significant reduction in crown rump length, head circumference and body weight in ethanol exposed chicks when compared with appropriate control groups. The growth retardation among the different alcohol exposed groups was dose dependent and prenatal mortality of embryos was seen in embryos exposed to 10% and 15% ethanol. Features of myelin degeneration were observed in the peripheral nerve in majority of chick embryos exposed to 10% and 15% ethanol as compared to embryos exposed to 5% ethanol as well as in the control groups. Thus, this study demonstrates the dose dependent effects of continuous prenatal ethanol exposure in a chick model of FAS. More significantly, it demonstrates the detrimental effects of prenatal alcohol exposure on the developing peripheral nerve. ***Neuroanatomy; 2006; 5: 50–55.***

Key words [fetal alcohol syndrome] [FAS] [myelin degeneration] [peripheral nerve]

Introduction

Fetal alcohol spectrum disorders (FASD) are caused by maternal alcohol consumption during pregnancy. Its most commonly recognizable clinical presentation, Fetal Alcohol Syndrome (FAS), is a serious health concern in the United States. Surveys have revealed that about 16% of children have been prenatally exposed to alcohol, making it the most common neurobehavioral teratogen [1]. While earlier studies had associated FASD with high alcohol consumption during pregnancy, recent studies have shown that even the smallest amounts of alcohol can adversely affect the developing fetus [2, 3]. The prevalence of FAS in the United States has been estimated at 1-3 per 1000 live births [4-6] and the rate of FASD as 9.1 per 1000 live births, though the incidence could possibly be higher as the diagnosis may often be overlooked [7].

FASD is a broad term that describes the range of effects that can occur in an individual whose mother consumed alcohol during pregnancy. It is the most common cause of preventable mental retardation in children, and its effects may include permanent physical, mental, behavioral and learning disabilities. The Centers for Disease Control and Prevention diagnostic criteria for FAS include three specific facial findings namely, smooth philtrum, thin vermilion border of the upper lip and short palpebral fissures, or the presence of one or more facial features along with deficit in body weight or central nervous system abnormalities [8].

A significant clinical feature in FAS is motor and sensory derangements, and neurobehavioral deficits that have been directly linked to the quantity of alcohol consumed by the mother during pregnancy [9]. Reported abnormalities include microcephaly, partial or complete agenesis of the corpus callosum and cerebellar hypoplasia, with or without neurological 'hard or soft signs' such as impaired fine motor skills, neurosensory hearing loss, poor tandem gait and deficient eye-hand coordination [10-12].

The brain and spinal cord have been examined in children exposed to alcohol in the gestational period, and in experimental models of FAS [13-22]. As no significant changes have been observed in these regions that could convincingly explain neuronal deficits in FAS, it has been hypothesized that alterations in the peripheral nervous system could be responsible for some of the neuronal disorders.

In this context, the tibial and saphenous nerves have been examined in a mouse model of FAS, but no significant changes were observed [23]. This study examined the effects on peripheral nerves following a single instance of ethanol exposure during gestation, thus mimicking a binge pattern of drinking in humans. However, survey of the drinking patterns of pregnant women show that more women consume small to moderate amounts of alcohol before and during pregnancy, rather than indulge in bouts of heavy binge drinking [24-26]. Further, except for one study population where binge drinking in pregnancy resulted in significant clinical deficits in offspring [27],

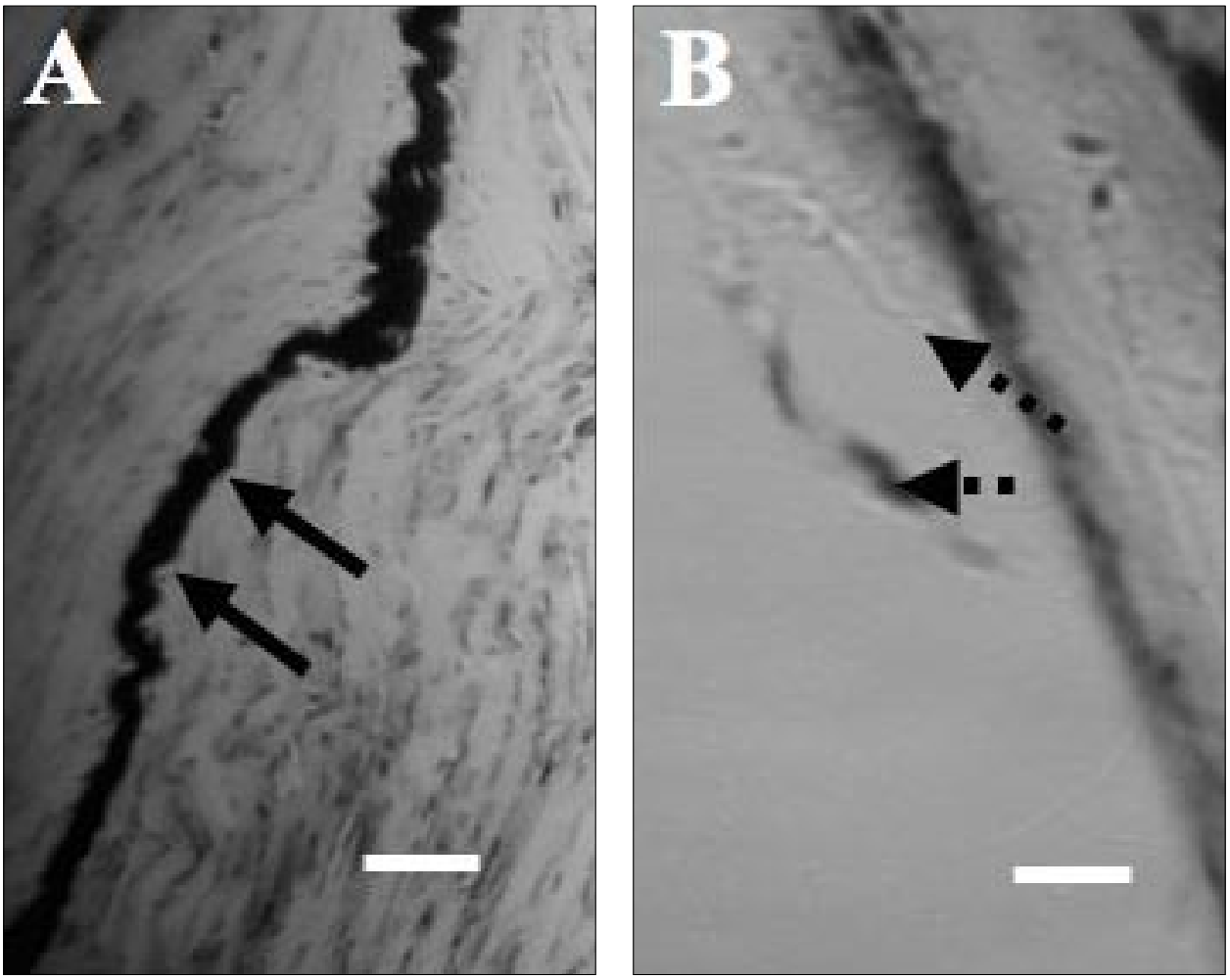

Figure 1. Myelin degeneration in peripheral nerve. Myelin degeneration of the peripheral nerve (arrows) supplying the quadriceps muscle in chick embryos exposed to 10% ethanol during the gestational period (Panel ***A***). Panel ***B*** depicts normal myelin (dashed arrows) in peripheral nerve of control chick embryos that were not exposed to alcohol. The Swank-Davenport modification of Marchi's method was used for detection of degenerated myelin. Scale bar =10 μm.

most studies have reported little effects of binge drinking during pregnancy on birth height and weight, head circumference, gestational age and Apgar score [28-30] or on the incidence of malformations [31, 32].

Therefore, the investigation of the effects of continuous ethanol exposure throughout the pregnancy on the developing peripheral nerve would be more relevant in the context of FAS. Mithen et al., have examined the effects of continuous ethanol on a peripheral nerve cell culture model of FAS, and reported features of disrupted Schwann cell proliferation and defective myelination [33]. In the human context, a study by Avaria et al., of newborn children exposed continuously to alcohol during their gestational period showed a significant impairment in nerve velocity and amplitude changes in peripheral nerves [34]. This effect persisted beyond one year, suggesting that prenatal alcohol exposure can cause persistent impairment of peripheral nerve functions. The authors have hypothesized it could possible be due to axonal disruption or myelin degeneration. However, the design of the study did not allow the investigation of these factors, though the findings of impaired peripheral nerve function in FAS is significant as it directly links gestational alcohol exposure with peripheral neuropathy, and provides a possible explanation for some of the neurological deficits observed in FAS.

Thus, the aim of this study was to examine the specific effect of continuous ethanol exposure during early pregnancy on the developing peripheral nerve in a chick model of FAS. The chick is a convenient model for studies on FAS, since it allows for the determination of the direct effects of ethanol in early pregnancy (when administered before day 10), without the influences of maternal under nutrition, concurrent nicotine or drug use, acetaldehyde formation or impaired placental function [35, 36]. Further, studies on early neural growth and development in chick embryos are feasible due to a relatively low mortality rate, and since ethanol-exposed chicks demonstrate growth suppression early in development [37], and experience learning deficits after hatching even without evident growth suppression [38].

Material and Methods

Freshly fertilized White Leg Horn chick eggs, type Single Comb (SC) were obtained form the local hatchery and divided into three groups I, II and III. The weight of the eggs ranged from 55 to 59 g with a mean weight of 56 grams. There was no variability in the weight of the eggs, and 45 eggs were randomly assigned to group I, while groups II and III consisted of 180 eggs each. The eggs in groups I were further randomly divided into three subgroups of 15 eggs each, designated as A1, B1 and C1. The eggs in groups II and III were also randomly divided into three subgroups of 60 eggs each and designated as A2, B2 and C2, and A3, B3 and C3 respectively.

The fertilized eggs were obtained within 6 h of the being laid, and cleaned with 70% ethanol. A small hole was made at the blunt end of each egg, under a laminar flow hood, and 250 μl of a 5%, 10% and 15% solution of ethanol in chick Ringer's solution [sodium chloride (123 mM), potassium chloride (5 mM), and calcium chloride (1.6 mM), pH 7.2] was injected directly into the air sac of the eggs in subgroups A1, A2 and A3 respectively. The injections were carried out on embryonic days 1-8 (E1-E8), the day the eggs were laid being considered as embryonic day 0 (E0). All injections were carried out in a horizontal position, so that ethanol exposure in the chick embryos was diffusion mediated. The ethanol used for injection was the one used in a previous study in this laboratory [2], and conformed to the requirements of absolute (anhydrous) alcohol, Indian Standard 321 of 1964, and was obtained from a commercial supplier (Ishika International, Mumbai, India). The eggs in subgroups B1, B2 and B3 were injected with 250 μl of chick Ringer's solution on E1-E8. The openings in the eggshells were sealed with wax. Eggs in subgroups C1, C2 and C3 were left untouched and constituted the absolute control group.

Eggs were incubated in an egg incubator (Model EI 1999, Capital Engineering Corporation, New Delhi, India) in a horizontal position at 37ºC and relative humidity of 70% for 3 weeks, and the eggs were manually rotated twice a day. The guidelines set up by the All India Institute of Medical Sciences Animal Care and Use Committee was followed concerning the treatment and disposal of embryos.

Examination of chicks

Chicks from all groups (i.e. I, II and III) were recovered after full gestational period of 21 days. The eggshells were manually cracked, and the chicks were allowed to emerge from their shells. The chicks were examined for gross deformities, and body weight, crown rump length and head circumference was measured, according to the procedure described by Pennington and Kalmus [39].

The chicks were killed by decapitation and the quadriceps muscle was carefully dissected out from both limbs and about 2 mm thick horizontal slices were prepared for staining by the Swank–Davenport modification of Marchi's method [40]. The slices were rinsed in 1% potassium chlorate for 7-8 minutes, and transferred to the impregnating solution (20 ml 1% osmium tetroxide, 60 ml 1% potassium chlorate, 12 ml formaldehyde and 1 ml of glacial acetic acid). The slices were maintained in the impregnating solution at room temperature in the dark for 10 days, and then washed in running tap water for one day. Frozen sections 30 μm thick were cut on a cryotome and dehydrated, cleared and mounted in Canada balsam. A total of 30 serial sections were cut from each quadriceps muscle and every fifth section was examined. Thus, a total of six sections of the peripheral nerve innervating the quadriceps muscle were examined. In sections where no unusual features were observed, all the 30 sections were examined. Sections were photographed using Olympus microscope (Model AX 70).

Statistical analysis

One way analysis of variance (ANOVA) was used to test for differences in crown rump length, head circumference, and body weight among ethanol-treated, chick Ringer's solution-treated and untreated groups. A p value of <0.05 was considered statistically significant.

Results

Gross anomalies and body parameters

After hatching, all the chicks were examined for gross anomalies and mean body weight, crown rump length, and head circumference was recorded. As no significant differences were observed in body parameters among chicks obtained from each of the subgroups, a single representative mean of the measurements is presented in Table 1.

Group I (5% ethanol exposure). No premature mortality or gross anomalies were observed in any chicks recovered from all the three subgroups. However, infertility was noted in all subgroups, and 12, 12 and 13, chicks were obtained from subgroups A1, B1 and C1 respectively, as the rest of the eggs were infertile. Chicks exposed to 5% ethanol demonstrated a significant ($p<0.05$) reduction in body parameters when compared with chicks exposed to chick Ringer's solution and in those where no manipulations were carried out.

Group II (10% ethanol exposure). In this group 52, 57 and 55 chicks were recovered from subgroups A2, B2 and C2 respectively. In chicks exposed to 10% ethanol, eight embryos (15.4%) were dead in various stages of development and necrotic features were observed. No gross anomalies were, however, observed in any of the viable chicks in this subgroup. In the subgroups of chicks exposed to Ringer's solution and in those in whom no manipulations were carried out, no mortality or gross deformities were observed.

In comparison with the chicks where no manipulations were carried out and in chicks exposed to Ringer's solution, there was a significant reduction ($p<0.05$) in all body parameters in chicks exposed to 10% ethanol in early gestation. Moreover, there was a significant reduction ($p<0.05$) in the body parameters of chicks exposed to 10% ethanol when compared with those exposed to 5% ethanol.

Group III (15% ethanol exposure). In this group, 58, 55 and 58 embryos were recovered from subgroups A3, B3 and C3 respectively. Significant mortality was observed

Table 1. Body parameters (mean ± standard deviation), infertility, and mortality of chick embryos exposed to ethanol. I, II and III represent the 5%, 10%, and 15% ethanol exposure study groups respectively. A1, A2 and A3 represent chicks exposed to 5%, 10%, and 15% ethanol respectively. B1, B2 and B3, C1, C2, C3 groups represent chicks exposed to Ringer's solution and chick in which no manipulations (other than rotation of eggs) were carried out.

	I			II			III		
Subgroups	A1 (n=15)	B1 (n=15)	C1 (n=15)	A2 (n=60)	B2 (n=60)	C2 (n=60)	A3 (n=60)	B3 (n=60)	C3 (n=60)
Head circumference (cm)	3.9 ± 0.2 [a]	4.7± 0.1	4.7± 0.2	2.9 ± 0.2 [a, b]	4.5± 0.3	4.7± 0.1	2.1± 0.2 [a, c]	4.9± 0.2	4.5± 0.1
Body weight (g)	16.2 ± 1.7 [a]	22.1± 1.2	22.9± 1.5	10.8± 0.9 [a]	21.9± 1.5	22.4± 0.9	8.7± 0.7 [a]	21.7± 1.6	22.4± 0.9
Crown rump length	6.1± 0.3 [a]	8.5± 0.1	8.4± 0.1	3.9± 0.2 [a]	7.9± 0.4	8.2± 0.2	2.9 ± 0.3 [a]	8.1± 0.2	7.9± 0.2
Percentage of unfertile eggs	20	20	13.3	13.3	5	8.3	3.3	8.3	3.3
Percent mortality	0	0	0	15.4	0	0	59.6	0	0

[a] *Significantly different from values for chicks in the same subgroups in which no manipulations were carried out and in those exposed to equivolume chick Ringer's solution (P<0.05)*
[b] *Significantly different when values were compared with chicks exposed to 5% ethanol (P<0.05)*
[c] *Significantly different when values were compared with chicks exposed to 5% and 10% ethanol (P<0.05)*

in chicks exposed to 15% ethanol. Forty-nine embryos (84.5%) were dead at various stages of development and necrotic features were seen. Nine embryos (15.5%) were viable and did not show any gross abnormalities on naked eye examination. These were further examined, and their body parameters recorded. In comparison with chicks in other subgroups, a significant ($p<0.05$) reduction in body parameters was observed in viable ethanol exposed chicks as compared with controls. There was also a significant reduction in body parameters in these chicks when compared to chicks exposed to 5% and 10% ethanol.

Microscopic examination of peripheral nerve

Sections of the quadriceps muscle were examined for features of myelin degeneration in peripheral nerves. In chick embryos exposed to 5% ethanol, the myelin of peripheral nerves appeared colorless or light brown in color, which is characteristic of normal myelin stained by the Swank–Davenport modification of Marchi's method [40]. In chicks exposed to 10% ethanol (Group II), only those that were viable and appeared normal on gross examination were examined. An intense black coloration of the myelin of peripheral nerves, characteristic of degenerated myelin, was observed in 31 out of the 52 (59.6%) chick embryos examined (Figure 1). In the nine viable embryos exposed to 15% alcohol (Group III), eight embryos showed the characteristic intense black discoloration of myelin. There were no features of myelin degeneration in embryos recovered from all the subgroups exposed to chick Ringer's solution, or in those in which no manipulations had been carried out. The myelin in the peripheral nerve in these chick embryos appeared colorless or light brown in color. As previously mentioned, when examination of six sections of the quadriceps muscle from any of the experimental or control animals did not reveal any features of myelin degeneration, all the sections were examined to rule out observation errors.

Discussion

The results of the present study demonstrate the effects of continuous ethanol exposure during early gestation on developing chick embryos. Chick embryos exposed to 5%, 10% and 15% ethanol demonstrated significant growth retardation, as compared to chick embryos in the control group and those exposed to chick Ringer's solution. The reduction in body parameters was directly proportional to increasing ethanol concentrations, with marked prenatal mortality in embryos exposed to 10% and 15% ethanol. Further, majority of chick embryos exposed to 10% and 15% ethanol showed degeneration of myelin of peripheral nerves.

Methodological considerations

In this study, the Swank–Davenport modification of Marchi's method was used for detection of myelin degeneration in the peripheral nerve of chick embryos [40]. The method is based on the property of all ethylenic (double) bonds, such as in fatty acids of lipids, to reduce osmium tetroxide to lower oxides and black metallic osmium. However, this property of ethylenic bonds is lost on previous or simultaneous oxidation. Normal myelin, in contrast to degenerated myelin, does not contain any hydrophobic neutral lipids such as triglycerides and cholesterol esters. Therefore when degenerated (hydrophobic) and normal (hydrophilic) myelin are exposed to aqueous potassium chlorate, only ethylene bonds in normal myelin are affected. Consequently, the unaffected bonds of degenerated myelin are still free to reduce osmium tetroxide, and thus degenerated myelin and neutral lipids appear black, while normal myelin is usually unstained or appears light brown in color, when stained by this method.

The reliability of staining for degenerated myelin by this method has been discussed by Strich and Fraser [41, 42]. More recently it has been used to study myelin degeneration in the spinal cord in guinea pigs [43], cats [44] and rabbits [45] and in a rat model of FAS [46].

Implications of results

In this study, ethanol was injected into the air sac of fertilized chick eggs on days E1-E8. This demonstrated the direct effects of ethanol, since till E10 chick embryos lack the capacity to produce acetaldehyde. The daily amounts of ethanol injected into fertilized eggs in this study were similar to an earlier study carried out in this laboratory [2]. However in the earlier study, single doses of 5%, 10% and 15% ethanol were injected on day E0, while in this study daily injections were carried out from days E1-E8, thus replicating a model of early gestational ethanol exposure. Continuous ethanol exposure resulted in a significant increase in prenatal mortality in chick embryos exposed to 10% ethanol, in contrast to the previous study [2], where a single injection of 10% ethanol did not cause any prenatal mortality. Moreover, in embryos exposed to 15% ethanol also, there was significantly increased mortality as compared to the previous study. However, as the present findings of dose dependent prenatal growth retardation and mortality are similar to those previously reported in other experimental models of FAS [47-53], as well as in humans [54, 55], they will not be discussed further.

Based on this study, it is not possible to speculate on the mechanisms of ethanol induced growth retardation in embryos following prenatal exposure. Repeated observations of growth retardation in ethanol exposed animal models, have led investigators to suggest that defects in intestinal metabolism [47, 56, 57], skeletal muscle growth [58, 59], or disturbances in hormonal function [60] could be contributory, though definite conclusions are still unavailable.

The significant finding in this study is myelin degeneration in the peripheral nerve following ethanol exposure in early gestation. This is in contrast to an earlier report that concluded that prenatal ethanol exposure does not affect the peripheral nervous system [23]. However, while that study examined the effects of a single dose of ethanol, the present study examined the effects of continuous ethanol exposure during early gestation, which is more representative of the current pattern of female alcohol consumption.

Myelin is a highly specialized multilamellar membrane that results from the elaboration of plasma membranes of oligodendrocytes in the central nervous system, or Schwann cells in the peripheral nervous system. Myelination, one of the important developmental events in the nervous system, is essential for rapid propagation of action potentials and for normal neurological function [61]. This process is associated with increase in axonal caliber [62], and is a major determinant of neuronal conduction velocity [63].

Previous studies have demonstrated derangements in myelination in the central nervous system following prenatal ethanol exposure. Offspring of ethanol-exposed dams have been reported to have lower brain myelin concentrations [16], while delayed myelination has been noted in pups of pregnant rats exposed to ethanol [15, 64]. Low myelin basic protein (MBP) levels in oligodendrocytes have also been noted in mice exposed to ethanol in the prenatal period [65], which has later been confirmed in a cell culture model of FAS [66]. In studies on the peripheral nerve in FAS, prenatal ethanol has been demonstrated to disrupt Schwann cell function and myelination in peripheral nerve culture models of FAS [33]. More significantly, decreased nerve conduction velocity in peripheral nerves has been observed in children exposed to prenatal alcohol [34]. Thus the findings of myelin degeneration in this study provide an anatomical basis for the impaired nerve function observed in these children. Moreover, myelin disruption could also be a possible explanation for other neuronal deficits observed in FAS. The mechanisms of myelin degeneration have not been examined in this study. However, new assays in neuroscience and the advancement of microtechnology have suggested possible mechanisms for ethanol induced myelin toxicity. These include the formation of damaging free radicals and derangement of antioxidant defense systems [67], or lack of adaptive mechanism for alterations in the microenvironment induced by ethanol exposure [68], or changes in the cranial neural crest area from which Schwann cells are derived [69].

In conclusion, this study demonstrates that continuous ethanol exposure during early gestation can cause significant growth retardation, increased mortality and myelin degeneration in a dose dependent manner.

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