Effect of a single dose of ethanol on developing peripheral nerve of chick embryos

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SUMMARY

Fetal Alcohol syndrome (FAS) is a condition occurring in some children of mothers who have consumed alcohol during pregnancy. It is characterized by physical and mental growth retardation, craniofacial anomalies and derangements of numerous body systems. A particular distressing feature of FAS is affection of the nervous system, which manifest as 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 a single high dose of ethanol exposure during early gestation on the peripheral nerve, with a view to understanding the neuronal disorders appearing in FAS. A chick model of FAS was used and embryos were exposed to 5%, 10% and 15% ethanol immediately after hatching. 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 a significant reduction in crown-rump length, head circumference and body weight in ethanol-exposed chicks as compared with appropriate control groups. The degree of growth retardation among the different alcohol exposed groups was dose dependent and significant prenatal embryo mortality was seen in those exposed to 15% ethanol. In embryos that survived the exposure to 15% ethanol, features of myelin degeneration were observed in the peripheral nerve in the majority of cases. No features of myelin degeneration were observed in embryos exposed to 5% and 10% ethanol or in the control groups. This study demonstrates the direct toxic effects of a single high dose of ethanol on developing embryos in general and the peripheral nerves in particular. Accordingly the pathological changes observed in peripheral nerves could account for some of the neurobehavioral deficits observed in FAS.

INTRODUCTION

Fetal alcohol spectrum disorders (FASD), and their clinical presentation- Fetal Alcohol Syndrome (FAS), is a condition occuring in some children due to maternal alcohol consumption during pregnancy. Some surveys have revealed that about 16% of children are

prenatally exposed to alcohol, making it the commmonest neurobehavioral teratogen (Smith, 1980). 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 (Chaudhuri, 2004; Jacobson and Jacobson, 1994).

Current diagnostic criteria for FAS include three specific facial findings namely, a smooth philtrum, a thin vermilion border of the upper lip and short palpebral fissures, and the presence of one or more facial features along with a deficit in body weight or central nervous system abnormalities (see Wattendorf and Muenke, 2005 for details). A significant clinical feature in FAS is motor and sensory derangements, which include impaired fine motor skills, neurosensory hearing loss, poor tandem gait and deficient eye-hand coordination (Chudley et al., 2005; Livy and Elberger, 2001; Sood et al., 2001). However, no changes have been observed in the brain and spinal cord in children with FAS or in experimental models of FAS (Sluyter et al., 2005; Farber and Olney, 2003; Norton and Kotkoskie, 1991; Ledig et al., 1988; Miller, 1987; Rudeen and Guerri, 1985; Lancaster et al., 1984; Stolenburg-Didinger and Spohr, 1983; Lancaster et al., 1982; Barnes and Walker, 1981) that might possibly explain the neuronal deficits in FAS. Thus, it has been hypothesized that alterations in the peripheral nervous system could be responsible for some of the observed neuronal disorders.

In this context, the tibial and saphenous nerves were examined in a mouse model of FAS following a single instance of ethanol exposure during midgestation (Parson and Sojitra, 1995). No significant changes were observed in these nerves although, interestingly the optic nerve showed a significant disruption of myelination, leading the authors to suggest that prenatal ethanol exposure is not detrimental to peripheral nerves. In contrast, a recent study by Avaria et al. (2004) of newborn children exposed continuously to alcohol during their gestational period revealed a significant impairment in nerve conduction velocity and amplitude changes in peripheral nerves. This effect persisted beyond one year of life, suggesting that prenatal alcohol exposure can cause persistent impairment of peripheral nerve functions. In that study the authors hypothesized that it could be due to axonal disruption or myelin degeneration,

although the design of the study did not allow these factors to be investigated. However, impairment of nerve conduction velocity is strongly suggestive of impaired myelination and consequent sensory and motor deficits (Colello and Pott, 1997). This is significant because it directly links gestational alcohol exposure with peripheral neuropathy and provides a possible explanation for some of the neurological deficits observed in FAS.

Hence, the aim of this study was to examine the specific effect of a single high dose of ethanol during early pregnancy on developing myelin in the peripheral nerve in a chick model of FAS. The choice of a single dose of ethanol during early gestation was based on surveys of the drinking pattern of pregnant women, many of whom consume alcohol during early pregnancy without realizing that they are pregnant (Floyd et al., 1999). Further, as some studies have also shown that a single dose of ethanol maybe be detrimental to developing skeletal muscle (Chaudhuri, 2004), it is possible that a similar effect could also be induced in the peripheral nervous system. This considers that the process of myelination is more vulnerable during the initial phase of gestation, when reciprocal interactions between differentiating glial cells and neurons define the course of myelination even before these cell types are definitely recognizable (Barone et al., 2000).

The chick is a convenient model for studies on FAS since it permits for the determination of the direct effects of ethanol when administered before day 10, without the influences of maternal undernutrition, concurrent nicotine or drug use, acetaldehyde formation, or impaired placental function (Bupp-Becker and Shibley, 1998; Wilson et al., 1984). Further, studies on the effects of early ethanol exposure on chick embryos are feasible owing to a relatively low mortality rate, and because ethanol-exposed chicks demonstrate growth suppression early on in development (Pennington 1990), and show learning deficits after hatching even without evident growth suppression (Means et al., 1989).

Material and Methods

Freshly fertilized White LegHorn chick eggs, type Single Comb (SC), were obtained from the local hatchery and divided into three groups I, II and III. The weight of the eggs ranged from 0.052 to 0.057 kg with a mean

weight of 0.055 kg. There was no variability in the weight of the eggs, and 30 and 60 eggs were randomly assigned to groups I and II, while group III consisted of 120 eggs. The eggs in group I were further randomly divided into three subgroups of 10 eggs each, designated as A1, B1 and C1. The eggs in group II were also randomly divided into three subgroups of 20 eggs each and designated as A2, B2 and C2, while the eggs in group III were divided into subgroups of 40 eggs each and designated A3, B3 and C3 respectively.

The method of ethanol exposure was similar to that used in a previously published study (Chaudhuri et al., 2004).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 (123mM), potassium chloride (5mM), and calcium chloride (1.6mM), pH 7.2] was injected directly into the air sac of the eggs in subgroups A1, A2 and A3 respectively. All injections were carried out in the horizontal position, so that ethanol exposure in the chick embryos would diffusion-mediated. The ethanol used for injection was same as that used in a previous study in this laboratory (Chaudhuri, 2004), 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. The openings made in the egg shells were then sealed with wax. The eggs in subgroups C1, C2 and C3 were left untouched and formed the absolute control group.

The eggs were incubated in an (egg) incubator (Model EI 1999, Capital Engineering Corporation, New Delhi, India) in the horizontal position at 37ºC and a relative humidity of 70% for 3 weeks, and 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 the full gestational period of 21 days. The egg shells were cracked manually, 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 (1987).

The chicks were killed by a prolonged overdose of carbon dioxide, 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 (Swank and Davenport, 1953). 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 an 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

3.1. 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 were recorded. Since no significant differences were observed in body parameters among chicks obtained from each of the subgroups, a single representative mean of the measurements is given in Table 1.

3.1.1. Group I (5 % ethanol exposure)

No premature mortality or gross anomalies were observed in any of the chicks recovered

from all the three subgroups. However, infertility was noted in all subgroups, and 8, 8, and 9 chicks were obtained from subgroups A1, B1 and C1 respectively, because the rest of the eggs were infertile. Chicks exposed to 5% ethanol demonstrated a significant (P<.05) reduction in body parameters as compared with chicks exposed to chick Ringer's solution and in which no manipulations were carried out.

3.1.2. Group II (10% ethanol exposure)

In this group 18, 17, and 17 chicks were recovered from subgroups A2, B2 and C2 respectively. No gross anomalies or premature mortality were observed in any of the viable chicks in this subgroup. In the subgroup of chicks exposed to Ringer's solution and in those in which no manipulations were carried out, no gross deformities or mortality were observed. In comparison with chicks in which 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 the chicks exposed to 10% ethanol during early gestation.

3.1.3. Group III (15% ethanol exposure)

In this group, 37, 36 and 37 embryos were recovered from subgroups A3, B3 and C3 respectively. A significant mortality was observed in chicks exposed to 15% ethanol. Sixteen (43.2%) % were dead at various stages of development and necrotic features were seen. Twenty-one embryos (56.8%) were viable and did not show any gross abnormalities upon visual examination. These were further examined, and their body parameters were recorded. In comparison with the chicks in the 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 those exposed to 5% and 10% ethanol.

3.2. Microscopic examination of the peripheral nerves

Sections of the quadriceps muscle were examined for features of myelin degeneration in peripheral nerves. In chick embryos exposed to 5% and 10% ethanol, the myelin of the peripheral nerves appeared colorless or light-brown in color, which is characteristic of normal myelin as stained by the Swank-Davenport modification of Marchi's method. In chicks exposed to 15% ethanol an intense black coloration of the myelin of the peripheral nerves, characteristic of degenerated myelin, was observed in 13 out of the 21 (61.9 %) viable chick embryos examined (Figure 1). There were no features of myelin degeneration in embryos recovered from any of the subgroups exposed to chick Ringer's solution, or in those in which no manipulations had been carried out. The myelin in the peripheral nerves 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 failed to 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 a single high dose of ethanol 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 increase in ethanol concentrations, with a marked prenatal mortality in embryos exposed to 15% ethanol. Further, the majority of viable chick embryos exposed to 15% ethanol showed myelin degeneration in the peripheral nerves.

Methodological considerations

In this study, the Swank-Davenport modification of Marchi's method was used for the detection of myelin degeneration in the peripheral nerves of chick embryos (Swank and Davenport, 1953). The method is based on the property of all ethylene (double) bonds, such as in the fatty acids of lipids, to reduce osmium tetroxide to lower oxides and black metallic osmium. However, this property of ethylene bonds of reducing osmium tetroxide is lost upon previous or simultaneous oxidation. Normal myelin, in contrast to degenerated myelin, does not contain any hydrophobic neutral lipids. Therefore, when
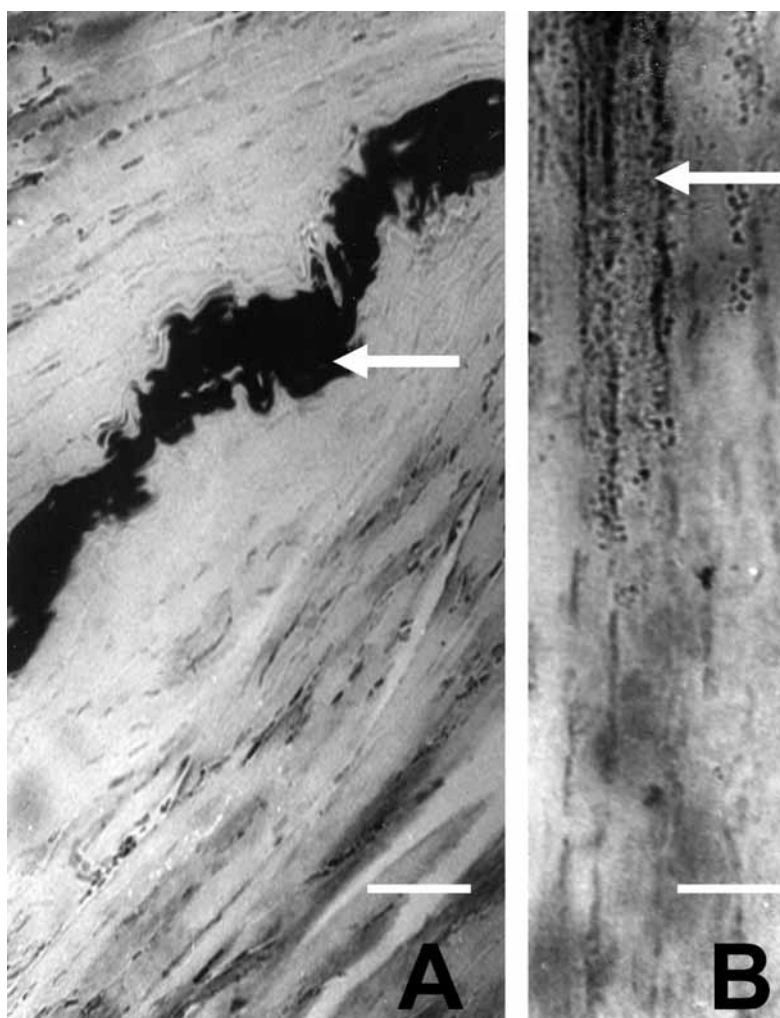
Figure 1. Myelin degeneration of a peripheral nerve (arrows) supplying the quadriceps muscle in chick embryos exposed to 15% ethanol during the gestational period (Panel **A**). Panel **B** depicts normal myelin in the peripheral nerves of control chick embryos that were not exposed to alcohol. The Swank-Davenport modification of Marchi's method was used for the detection of degenerated myelin. Scale bar =10 μm

degenerated (hydrophobic) and normal (hydrophilic) myelin are exposed to aqueous potassium chlorate, only the ethylene bonds in normal myelin are affected. Consequently, the unaffected bonds of degenerated myelin are still free to reduce osmium tetroxide, and hence degenerated myelin appears 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 using this method has been discussed by Fraser (1972) and Strich (1968). More recently, the Swank-Davenport modification of Marchi's method has been used to study myelin degeneration in the spinal cord in rabbits (Corneliuson et al., 1987), cats (Berthold and Carlstedt, 1977) and guinea pigs (Hildebrand and Aldkogius, 1976), and in a rat model of FAS (David and Subramaniam, 2005).

Implications of the results

In this study, ethanol was injected into the air sac of fertilized chick eggs within 6 hours of hatching, thus demonstrating the direct

Table 1. Body parameters (mean ± standard deviation), infertility, and mortality of chick embryos exposed to ethanol.

Groups	I			II			III		
Subgroups	A1 (n=10)	B1(n=10)	C1(n=10)	A2(n=20)	B2(n=20)	C2(n=20)	A3(n=40)	B3(n=40)	C3(n=40)
Head circumference (cm)	4.1 ± 0.2*	4.5 ± 0.1	4.7 ± 0.2	3.8 ± 0.1*	4.7 ± 0.2	4.6 ± 0.2	3.1 ± 0.1**	4.8 ± 0.2	4.6 ± 0.2
Body weight (g)	16.1 ± 1.6*	21.8 ± 1.3	21.9 ± 1.4	15.9 ± 0.9*	21.8 ± 1.4	21.9 ± 0.8	11.8 ± 0.7 **	21.9± 1.5	22.4± 1.1
Crown rump length	6.1 ± 0. 2*	8.6 ± 0.2	8.5 ± 0.1	5.8 ± 0.2*	8.1 ± 0.2	8.2 ± 0.1	3.2 ± 0.3**	8.1 ± 0.3	8.1 ± 0.1
Percentage of unfertile eggs	10	10	5	10	15	10	7.5	20	7.5
Percent mortality	0	0	0	15.4	0	0	43.2	0	0

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, and C3 groups represent chicks exposed to Ringer's solution and chicks in which no manipulations (other than rotation of eggs) were carried out.

* Significantly different from values for chicks in which no manipulations were carried out and in those exposed to equivolume chick Ringer's solution ($p<0.05$)
** Significantly different when values were compared with those of the chicks in all the other groups ($p<0.05$)

effects of ethanol, since till embryonic day 10 chick embryos lack the capacity to produce acetaldehyde. The daily amounts of ethanol injected into fertilized eggs in this study were similar to those used in an earlier study carried out in this laboratory (Chaudhuri, 2004). Since the present findings of dose-dependent prenatal growth retardation and mortality are similar to those previously reported in other experimental models of FAS (Bhalla et al., 2004; Wang and Kernell, 2001; Becker et al., 1996; Zajac and Abel, 1992; Ernhart, 1991; Clarren et al., 1988; Lee and Leichter, 1980), as well as in humans (Cornelius et al., 2002; Day et al., 2002), they will not be discussed further.

The significant finding in this study is the myelin degeneration in peripheral nerves following a single instance of ethanol exposure during early gestation. This is in contrast to the findings of an earlier study, which concluded that prenatal ethanol exposure does not affect the myelin of peripheral nerves (Parson and Sojitra, 1995). This finding seems fortuitous, considering that prenatal ethanol is detrimental to myelination in central nervous systems. This is primarily because despite variations in the process of myelination in the central and peripheral nervous systems, inherent similarities also exist between the two processes. Previous studies on the effects of ethanol on central nervous myelination have demonstrated lower total brain myelin concentrations (Lancaster et al., 1984), significant delays in myelination (McNeill et al., 1991; Lancaster et al., 1982), and low myelin basic protein (MBP) levels in oligodendrocytes in mice (Ozer et al., 2000) and cell culture models of FAS (Bichenkov and Ellingson, 2001). It has also been demonstrated that ethanol causes a disruption of Schwann cell function and myelination in peripheral nerve culture models of FAS (Mithen et al., 1990). More significantly, a decreased nerve conduction velocity in peripheral nerves has been observed in children exposed to prenatal alcohol (Avaria et al., 2004). The design of this study did not allow us to investigate whether axonal or Schwann cell function was disrupted, since the formation of peripheral myelin is a complex and dynamic process involving the continued interactions between neurons and Schwann cells controlling the various stages of myelination. Thus, the cumulative evidence suggests that prenatal ethanol could be detrimental to peripheral myelin formation. Since the find-

ing of this study of a derangement of peripheral myelination is in contrast to that reported in a previous study (Parson and Sojitra, 1995), certain possibilities exist to account for the observed differences, the main one being the time point of ethanol exposure during gestation. In the study by Parson and Sojitra (1995), pregnant female mice were exposed to ethanol in midgestation, whereas in this study chick eggs were exposed to ethanol immediately after hatching. This is significant since a temporal sequence of events has been reported to exist in the myelination of peripheral nerves (D'antonio et al., 2006; Rees and Inder, 2005) and consequent nerve conduction velocities (Waxman, 1980). For example, a significant difference in levels of myelin proteins between embryonic days 16 and 18 has been described (Leblanc et al., 1989). Thus, based on the findings of this study it may be concluded that critical periods of vulnerability exist in the process of myelination of peripheral nerves. We contend that the myelination degeneration observed in this study would be due to exposure of chick embryos to a more vulnerable period in development, in comparison with the results of the earlier study by Parson and Sojitra (1995).

In conclusion, this study demonstrates that a single high dose of ethanol during early gestation can cause significant growth retardation, increased mortality and myelin degeneration. This offers a possible explanation for the motor and sensory disturbances observed in FAS, more specifically for the impaired nerve conduction in the peripheral nerves of children with FAS.

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