Original Article

Anatomy

Ethanol Induced Developmental Defects can be Prevented with Supplementation of Vitamin E

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ABSTRACT

Background: Alcohol exposure during intrauterine life produces spectrum of disorders collectively termed as fetal alcohol syndrome. Apart from craniofacial and brain defects this syndrome produces various cardiac abnormalities such as atrial and ventricular septal defects, teratology of Fallot, transposition of the great arteries, truncus arteriosus. The mechanisms behind these ethanol-induced deficits are unknown. This study was conducted to detect the preventive role of lipid soluble vitamin E in ethanol induced heart malformations in chick embryos cultured in ovo.

Study Design: Experimental study

Place and Duration of Study: This study was conducted at the in the school of Biomedical Sciences, University of Nottingham, UK for a period of 6 months.

Materials and Methods: White Leghorn chicken eggs were incubated for 3 days in 37^0C with relative humidity of 100%. Eggs were microinjected with 100µl of either PBS, vitamin E 200µM, SOD 2µM, 20% ethanol in PBS, or ethanol plus vitamin E and ethanol plus SOD dissolved in PBS. On day 9 eggs were cracked and examined in terms of their viability. The viable embryos were examined for growth retardation by measuring crown rump length, and any malformations or gross abnormalities observed including limb deformities, facial defects, heart defects and brain vesicle development

Results: Ethanol-induced alterations occurred in craniofacial development, vitelline circulation, crown rump length, facial abnormalities, brain development, which were prevented by addition of vitamin E and superoxide dismutase.

Conclusion: These results show that exposure of the chick embryos to ethanol during development result in structural changes in the heart that mimic malformations that occur in patients with fetal alcohol syndrome (FAS). These findings may be prevented with addition of vitamin E.

Key Words: Ethanol, Supplementation, Vitamin E.

INTRODUCTION

Ethanol or alcohol exposure to the developing fetus produces various birth defects in humans, which are collectively known as fetal alcohol spectrum disorders (FASD). [1] Almost all developing organs are affected by perinatal alcohol exposure but the most commonly involved areas are brain and craniofacial structures. [1,2] Alcohol also produces cardiovascular abnormalities when exposed to early developmental periods. Despite these facts many women continue to consume large quantities of alcohol (ethanol, EtOH) during pregnancy. High levels of EtOH during pregnancy can lead to congenital cardiac defects such as atrial and septal defects [3]. Furthermore, cardiac function may be affected in the absence of structural abnormalities. Previous studies have reported that exposure to EtOH induces apoptosis of cardiomyocytes both in vivo and in vitro; [4] Ethanol-induced apoptosis in different developing organs is caused by reactive oxygen species generated as a result of oxidative stress. [5,6] In our previous study, we have shown preventive effects of folic acid and vitamin C in ethanol exposed early chick embryos in vitro and in ovo cultures. [7] This study aims to detect possible preventive effects of lipid soluble vitamin E in chick embryos cultured in ovo.

MATERIALS AND METHODS

This study was conducted for a period of 6 months in the school of Biomedical Sciences, University of Nottingham, UK. White leghorn fertilized chicken eggs were obtained from Henry Stewart and Co. Ltd. Eggs were randomly selected and divided in to 6 groups immediately before incubation. The first group was incubated as the control group (PBS: n = 12). The rest were labelled according to different treatment modes, Vitamin E (trolox: n=12 w/v in PBS), superoxide dismutase (SOD: n=12 w/v in PBS), EtOH solution

(20%, n = 12 v/v in PBS), EtOH plus SOD and EtOH plus vitamin E (n=12). The eggs were incubated at 37^0C at 100% humidity.

The day on which the eggs were incubated was counted as day zero. On day 3 of incubation the eggs were taken out of the incubator 3 at a time, after being swabbed down with 70% ethanol and under sterile conditions the blunt end of eggs was struck with forceps to make a small hole. Eggs were microinjected with 100µl using a 25- gauge needle attached to a 1 ml disposable syringe of either PBS (Sigma-Aldrich, UK), vitamin E (Sigma-Aldrich, UK) 200µM, SOD (Sigma-aldrich, UK) 2µM, 20% ethanol (Chemicon, UK) in PBS, or ethanol plus vitamin E and ethanol plus SOD dissolved in PBS. All injections were under the vitelline membrane through the air sac region. After being injected the holes were sealed with parafilm and taped with insulating tape in order to avoid drying out of the embryos. All the eggs were placed blunt end up into the automated egg turner and incubated at 37.5 °C and relative humidity of 60% with 5% CO_2 in air until day 9 of incubation.

On day 9 eggs were cracked under the sterile hood and embryos were examined in terms of their viability. After removing all the membranes viable embryos were examined for growth retardation by measuring crown rump length, and any malformations or gross abnormalities observed including limb deformities, facial defects, heart defects and brain vesicle development etc according to the criteria shown in table 1. Table 1 was formulated on the basis of scoring system which was developed with reference to Hamburger and Hamilton [8] developmental series.

Statistical Analysis: Statistical analysis was performed using Prism 5^{TM} software. The data was analysed using one-way ANOVA with the post hoc Dunnets test. In all cases p value <0.05 was considered significant.

RESULTS

This investigation was performed to detect the preventive role of vitamin E in ethanol induced malformations in chick embryos. Embryos were scored according to the criteria shown in table 1. Figure 1 a shows the photograph of embryos treated with PBS, figure 1 b shows photograph of embryos treated with ethanol and figure 1 c and 1 d show photos of embryos treated with ethanol plus vitamin E and ethanol plus SOD. The embryos treated with ethanol show gross malformations including brain, facial and limb defects as clearly shown in photos and are short as compared to the rest of groups. Statistical analysis showed that the embryos with ethanol (20%) treatment were smaller in length figure 2, table 2, had less vitelline circulation figure 3, table 3 and less flexion fig 4, table 4. Furthermore the embryos treated with 20% ethanol were significantly different to other all groups in terms of heart development figure 5, table 5, brain vesicle defects figure 6, table 6, limb and craniofacial developmental abnormalities Fig 7, 7, table 8, figure 8 respectively.

Table No.1: Morphological scoring system based on Hamburger and Hamilton staging of chick development [8].

Embryonic feature	0	1	2	3	4
Vitelline circulation			5-6 large vitelline vessels	Extensive network of vessels	Entire vitelline membrane well supplied
Flexion	None	Cranial flexure visible	Trunk rotation in addition to cranial flexion visible	Trunk rotation and tail bud rotation visible	Cranial flexure, trunk rotation and tail bud bending visible
Heart	No beating	Paired heart primordia	Beating heart bent to right	Atrioventricular canal and ventricular loop	Four-chambered appearance
Brain	Primitive streak	Neural folds visible	Brain vesicle, Anterior neuropore closed	Telencephalon enlarged, Forebrain forms right angle with hindbrain	Enlarged and transparent, Forebrain parallel to hindbrain
Gross Facial Deformities	None	Primary optic vesicles	Optic vesicles constricted at base	Optic cup completely formed on one side	Optic cup completely formed on both sides + beak is fully formed
Limbs	None	None	None	Digits and toes visible	Digits and toes visible on both sides

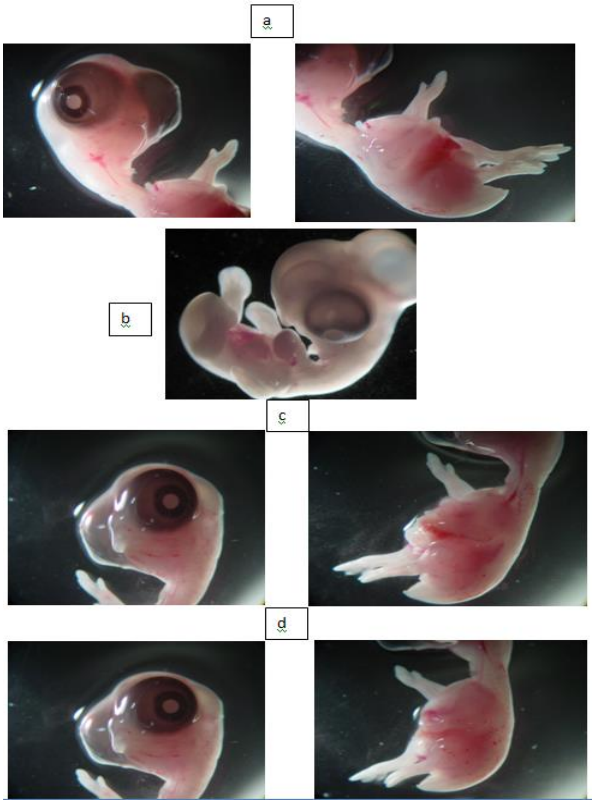

Figure No.1: Shows photographs of chick embryos exposed to PBS (control) a, ethanol (b), Ethanol plus vitamin E (c) and ethanol plus SOD (d). Note the ethanol exposed embryos are smaller in size and have gross craniofacial and limb abnormalities, while addition of vitamin E and SOD prevented these defects in chick embryos.

Table No.2: Shows statistical analysis of crown rump length of different treatment groups

Treatment Groups	Mean Diff.	Significant? P < 0.05?	95% CI of diff
PBS treated vs Vitamin E treated	0.1655	No	-1.917 to 2.248
PBS treated vs SOD treated	-0.04545	No	-2.128 to 2.037
PBS treated vs Ethanol treated	6.948	Yes	4.865 to 9.031
PBS treated vs vitamin E + ethanol	0.1005	No	-1.982 to 2.183
PBS treated vs SOD + ethanol	-0.3855	No	-2.468 to 1.697

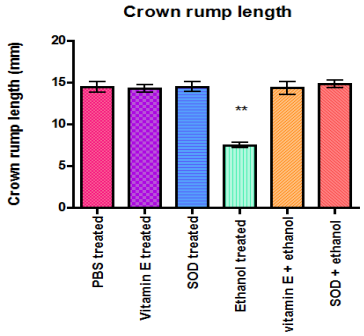

Figure No.2: Shows crown rump length of different groups of day 9 old chick embryos

Table No.3: Shows vitelline circulation of different treatment groups of chick embryos

Treatment Groups	Mean Diff.	Significant? P < 0.05?	Summary	95% CI of diff
PBS treated vs vitamin E treated	0.1583	No	ns	-0.2419 to 0.5585
PBS treated vs SOD treated	0.07500	No	ns	-0.3252 to 0.4752
PBS treated vs Ethanol treated	2.392	Yes	***	1.992 to 2.792
PBS treated vs Vitamin E+ ethanol	0.1083	No	ns	-0.2919 to 0.5085
PBS treated vs SOD+ ethanol	0.1917	No	ns	-0.2085 to 0.5919

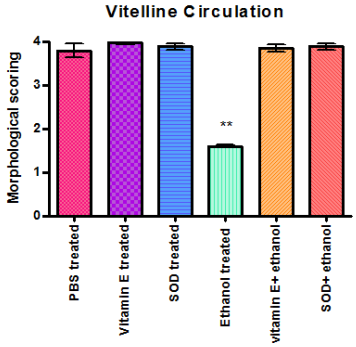

Figure No.3: Shows vitelline circulation of different treatment groups of chick embryos

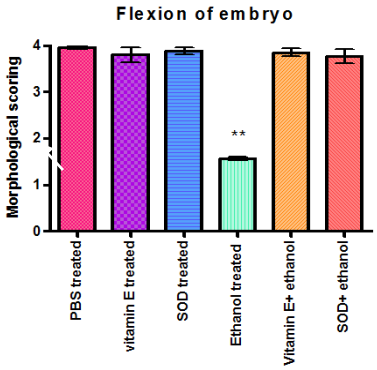

Figure No.4: Shows flexion of different treatment groups of chick embryos

Table No.4: Shows flexion of different treatment groups of chick embryos

Treatment Groups	Mean Diff.	Significant? P < 0.05?	Summary	95% CI of diff
PBS treated vs Vitamin E treated	-0.1750	No	ns	-0.5159 to 0.1659
PBS treated vs SOD treated	0.09167	No	ns	-0.4326 to 0.2493
PBS treated vs Ethanol treated	2.197	Yes	***	1.856 to 2.538
PBS treated vs vitamin E+ ethanol	-0.05833	No	ns	-0.3993 to 0.2826
PBS treated vs SOD+ ethanol	0.09167	No	ns	-0.4326 to 0.2493

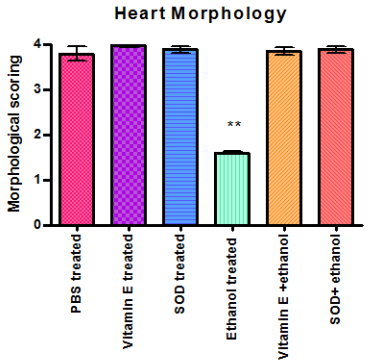

Figure No.5: Shows Heart Morphology of different treatment groups of chick embryos

Table No.5: Shows Heart Morphology of different treatment groups of chick embryos

Treatment Groups	Mean Diff.	Significant? P < 0.05?	Summary	95% CI of diff
PBS treated vs Vitamin E treated	-0.1750	No	ns	-0.5159 to 0.1659
PBS treated vs SOD treated	-0.09167	No	ns	-0.4326 to 0.2493
PBS treated vs Ethanol treated	2.197	Yes	***	1.856 to 2.538
PBS treated vs Vitamin E +ethanol	-0.05833	No	ns	-0.3993 to 0.2826
PBS treated vs SOD+ ethanol	-0.09167	No	ns	-0.4326 to 0.2493

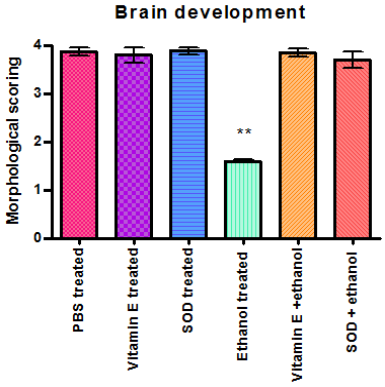

Figure No.6: Shows brain development of different treatment groups of chick embryos

Table No.6: Shows brain development of different treatment groups of chick embryos

Treatment Groups	Mean Diff.	Significant? P < 0.05?	Summary	95% CI of diff
PBS treated vs Vitamin E treated	0.07500	No	ns	-0.3513 to 0.5013
PBS treated vs SOD treated	-0.008333	No	ns	-0.4346 to 0.4180
PBS treated vs Ethanol treated	2.280	Yes	***	1.854 to 2.707
PBS treated vs Vitamin E +ethanol	0.02500	No	ns	-0.4013 to 0.4513
PBS treated vs SOD + ethanol	0.1750	No	ns	-0.2513 to 0.6013

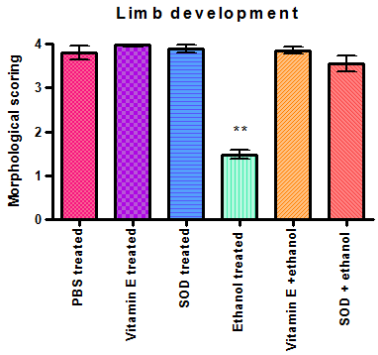

Figure No.7: Shows limb development of different treatment groups of chick embryos

Table No. 7: Shows limb development of different treatment groups of chick embryos

Treatment Groups	Mean Diff.	Significant? P < 0.05?	Summary	95% CI of diff
PBS treated vs Vitamin E treated	-0.1750	No	ns	-0.6286 to 0.2786
PBS treated vs SOD treated	-0.09167	No	ns	-0.5452 to 0.3619
PBS treated vs Ethanol treated	2.308	Yes	***	1.855 to 2.762
PBS treated vs Vitamin E +ethanol	-0.05833	No	ns	-0.5119 to 0.3952
PBS treated vs SOD + ethanol	0.2417	No	ns	-0.2119 to 0.6952

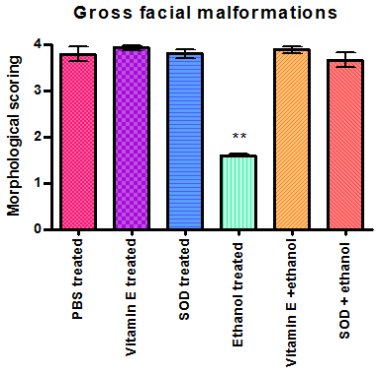

Figure No.8: Shows gross malformation of different treatment groups of chick embryos

Table No.8: Shows gross malformation of different treatment groups of chick embryos

Treatment Group	Mean Diff.	Significant? P < 0.05?	Summary	95% CI of diff
PBS treated vs Vitamin E treated	-0.1333	No	ns	-0.5532 to 0.2865
PBS treated vs SOD treated	-0.008333	No	ns	-0.4282 to 0.4115
PBS treated vs Ethanol treated	2.197	Yes	***	1.777 to 2.617
PBS treated vs Vitamin E +ethanol	-0.09167	No	ns	-0.5115 to 0.3282
PBS treated vs SOD + ethanol	0.1250	No	ns	-0.2949 to 0.5449

DISCUSSION

This study was conducted in sequence to our previous work to detect the preventive role of antioxidants in ethanol induced embryonic malformations. In previous study two water soluble vitamins (vitamin C and folic acid) with antioxidant properties were used [7]. It was observed that administration of antioxidants reversed the effects of ethanol in chick embryos cultured in vitro as well as in ovo. Ethanol or alcohol when consumed in excess during pregnancy produces drastic malformations in embryos. Studies on different animal species and humans proved the role of ethanol in congenital malformations of major organ, systems of the body. The major target areas of the embryo affected by ethanol are brain vesicle, craniofacial skeleton and cardiovascular system.[7] In this study chick embryos of day 3 were exposed to 20% ethanol, which produced severe craniofacial deformities, brain vesicle abnormalities and limb defects when examined grossly. These results are in line with our previous work .[7] Vitamin E is a biological antioxidant known to prevent lipid peroxidation of cellular membranes by quenching free radicals thus it functionally protects the cells and tissues from free radical derived damage. It is also required by many animal species such as rats, sheep and chickens to maintain normal growth rate [9]. Apart from its antioxidant functions, vitamin E, in particular α-tocopherol, also plays a significant role in cell signalling, it inhibits smooth muscle cell proliferation, increases phosphoprotein phosphatase 2A activity, and decreases protein kinase C activity. Its effects on protein kinase C inhibition have been reported in human platelets and monocytes. Vitamin E also plays a critical role in the male reproductive system [10]. It prevents loss of spermatogenesis and thus helps in prevention of male infertility. The deficiency of vitamin E in humans causes abnormalities in neuromuscular systems and is characterized by ataxia, peripheral neuropathies and myopathies [11]. These are likely to occur due to free radical damage to these tissues. Patients with familial vitamin E deficiency present with many neurological symptoms such as cerebellar ataxia, absence of deep tendon reflexes, vibratory and proprioceptive sensory loss, dysarthria, and +ve Babinski's sign. These symptoms can be ameliorated with up to 2000 mg of vitamin E per day [12]. The ethanol induced malformations are due to oxidative stress and addition of vitamin E protected the deleterious defects of ethanol induced malformations [13,14]

CONCLUSION

Ethanol is a known teratogen and has shown to be toxic in many animal studies. In this study the ethanol showed gross anomalies in chick embryos. Addition of vitamin E a lipid soluble vitamin with antioxidant properties reversed the tertogenic effects of ethanol induced malformations.

REFERENCES

1. Guerri C, Bazinet A, Riley EP. Foetal Alcohol Spectrum Disorders and alterations in brain and behaviour. Alcohol Alcohol 2009;44:108-114.
2. Bailey BA, Sokol RJ. Pregnancy and alcohol use: evidence and recommendations for prenatal care. Clin Obstet Gynecol 2008;51:436-444.
3. Serrano M, Han M, Brinez P, Linask KK. Fetal alcohol syndrome: cardiac birth defects in mice and prevention with folate. J Obstet Gynecol 2010;203: 77-75.
4. Patten RD, et al. 17β-Estradiol Reduces Cardiomyocyte Apoptosis In Vivo and In Vitro via Activation of Phospho-Inositide-3 Kinase/Akt Signaling. Circ Res 2004;95:692-699.
5. Chu J, Tong M, de la Monte SM. Chronic ethanol exposure causes mitochondrial dysfunction and oxidative stress in immature central nervous system neurons. Acta Neuropathol 2007;113: 659-673.
6. Ikonomidou C, Kaindl AM. Neuronal death and oxidative stress in the developing brain. Antioxid Redox Signal 2001;14:1535-1550.
7. Memon S, Pratten MK. Developmental toxicity of ethanol in chick heart in ovo and in micromass culture can be prevented by addition of vitamin C and folic acid Reproductive Toxicology 2009;28: 262-269.
8. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. J Morphol 1951;88:49-92.
9. Brigelius-Flohe R, et al. The European perspective on vitamin E: current knowledge and future research. Am J Clin Nutr 2002;76:703-716.
10. Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radical Biology and Medicine 2007;43:4-15.
11. Bieri JG, Corash L, Hubbard VS. Medical uses of vitamin E. New Engl J Med 1983;308:1063-1071.
12. Ouahchi K, et al. Ataxia with isolated vitamin E deficiency is caused by mutations in the −tocopherol transfer protein. Nature Genetics 1995;9:141 - 145.
13. Botto LD, Mulinare J, Erickson JD. Do multivitamin or folic acid supplements reduce the risk for congenital heart defects? Am J Med Genetics 2003;121: 95-101.
14. Willcox BJ, Curb JD, Rodriguez BL. Antioxidants in cardiovascular health and disease: key lessons from epidemiologic studies. Am J Cardiol 2008; 101:75-86.