Effects of ethanol dosage and ascorbic acid antioxidant therapy on embryonic chick development

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ABSTRACT

Congenital malformations are a major cause of infant morbidity and mortality, and exposure to teratogens such as ethanol/alcohol during pregnancy can have harmful effects on the developing embryo. The severity of fetal damage depends upon the amount and timing of fetal exposure to alcohol, as well as the genetic background of the fetus. Antioxidants are known to have therapeutic properties and may prevent or reduce the occurrence of malformations upon ethanol exposure during development. In this study, the effects of ethanol dosage and antioxidant therapy on embryonic development in the chick model were examined. The effect of four different ethanol concentrations, namely 0% (control), 5%, 10% and 15% ethanol was studied to examine if a dose dependence exists in the morphological effects of fetal alcohol syndrome (FAS). Additionally, ethanol-exposed embryos were treated with vitamin C to determine if it has a protective effect against ethanol-induced teratogenicity in the chick model. Embryos were examined for craniofacial malformation and abnormal limb development using staining techniques. Data was analyzed using ANOVA. Results indicate that exposure to increasing doses of ethanol has a significant impact upon the severity of phenotypic malformations produced in the developing chick embryo. Additionally, vitamin C was not found to be significantly effective in preventing ethanol-induced malformations in the developing chick embryo.

INTRODUCTION

Ethanol as a teratogen and its role in the development of fetal alcohol syndrome (FAS)

Alcohol or ethanol is one of the most common environmental teratogens. When consumed in excess during pregnancy, it is associated with a wide spectrum of embryonic and fetal malformations in humans (Wentzel and Eriksson 2006). A number of fetal alcohol spectrum disorders (FASD) can occur when alcohol is consumed during pregnancy. The spectrum of

disorders include alcohol-related birth defects (ARDB), alcohol-related neurological disorders (ARND) and fetal alcohol syndrome (FAS), (Brocardo and others 2011).

Fetal alcohol syndrome is considered to be the leading non-genetic cause of birth defects in developed countries (Memon and Pratten 2009). In the human fetus, FAS is characterized by pre- and postnatal growth retardation, central nervous system (CNS) anomalies and craniofacial malformations (Rao and Chaudhuri 2007).

The majority of ethanol embryotoxicity effects are more obvious when ethanol is consumed during the period of organogenesis, which corresponds to the period from the third to eighth week post implantation in humans (Parnell and others 2006). In the chick model, the "critical window" of sensitivity is extremely narrow, lasting only from gastrulation to cranial neural crest migration, or from 18 to 36 hours into development (Smith 1997). The chick is a suitable model for studies on FAS, since it allows for the determination of direct effects of ethanol in early pregnancy, without the influences of maternal under nutrition, concurrent drug use, acetaldehyde formation or impaired placental function (Rao and Chaudhuri 2007). Furthermore, studies have revealed that the effects of FAS in chick models are comparable to those in humans (Cartwright and Smith 1995). Thus, the chick model serves as an important research tool in studying the mechanisms of alcohol's teratogenic effects on embryonic development.

Several studies have shown that consumption of ethanol during pregnancy can produce various genotypic and phenotypic anomalies in the developing embryo. However, previous work has not presented a conclusive model for dose dependence of a single injection of alcohol. When *Xenopus* embryos were exposed to increasing alcohol concentrations from 0.3 to 1%, the frequency of tadpoles exhibiting microcephaly and growth retardation increased from 20% to

70%, respectively (Peng and others 2004). Other studies by Giles and others (2007) showed contrasting results. Chicks exposed to 50% ethanol were found to show higher mortality rates and growth retardation than those exposed to 60% ethanol. In this experiment, chick embryos were exposed to increasing ethanol concentrations (0%, 5%, 10%,15%) to test the hypothesis that increased ethanol dosage does not lead to higher occurrence of phenotypic anomalies in the developing embryo.

Effect of ethanol on craniofacial development

Neural crest cells (NCC) from the cranial region of the neural tube are crucial for the development of the facial skeleton and heart (Wentzel and Ericksson 2009). Cranial neural cells migrate from the neural tube and differentiate into a wide variety of structures. The type of body structure that the cranial neural crest cells will generate is determined before they leave the neurectoderm. Similarly, their differentiation fate (e.g. whether they develop into nerve or bone tissue) is determined before or shortly after the cells begin migration (Sant'Anna and Tosello 2006). Studies by Haworth and others (2004) revealed that fibroblast growth factors and Sonic hedgehog (*shh*) play an important role in shaping the neural crest derivatives of the head, as well as facilitation proper growth of the facial midline.

Cartwright and Smith (1995) observed increased aberrant cell death of neural crest cells after chicks were exposed to ethanol. A study by Hoffman and Kulyk (1999) showed that alcohol interferes with chick neural crest cell migration and proliferation. It was observed that instead of migrating and dividing, alcohol treated neural crest cells prematurely initiated their differentiation into facial cartilage. This research suggests that ethanol induced facial and cardiac malformations may be the result of an impaired NCC development.

Several studies have revealed that fetal alcohol exposure has a negative effect on the expression of genes required to facilitate proper embryonic development. The expression of *Msx2*, a homeobox gene, is an important factor in the development of craniofacial structure. Rifas and others (1997) discovered that mice exposed to alcohol showed a decreased expression of the *Msx2* gene. The Sonic hedgehog gene (*Shh*) plays a vital role in establishing facial midline structures. Ahlgren and others (2002) demonstrated that ethanol exposure results in a loss of *Shh* expression and transcripts in the *Shh* signaling pathway, which in turn, leads to deformities in craniofacial structure. Likewise, alcohol exposure in *Xenopus* embryos was found to reduce the expression of several key genes, of which *Xpax6* was the most vulnerable (Peng and others 2004). An alcohol concentration as low as 0.3% was found to produce more than 90% reduction of *Xpax6* gene expression. The resultant embryos were born with anomalies such as microcephaly and growth retardation (Peng and others 2004).

Limb development in the avian embryo

In the chick embryo, limb development begins when mesenchyme cells proliferate from the somatic layer of the lateral plate mesoderm and from the somites at the same level, creating a circular bulge called a limb bud (Burke and others 1995). Studies by Ohuchi and others (1997) revealed that a member of the fibroblast growth factor (FGF) family, *Fgf10*, is a key mesenchymal factor involved in the initial budding as well as the continuous outgrowth of vertebrate limbs. As development continues, the basic limb structure arises out of the limb bud system as skin, cartilage (which later develops into bone) and skeletal muscle. The development of hindlimbs and forelimbs in the chick model is specified by *Tbx4* and *Tbx5* proteins, respectively. Logan and others (1998) showed that limb buds induced by placing FGF beads

close to the hindlimb expressed *Tbx4* and became hindlimbs, whereas buds induced close to the forelimb expressed *Tbx5* and developed as forelimbs (wings). Exposure to ethanol during development is known to implicate signaling pathways involved in the regulation of embryonic limb cartilage differentiation. Ethanol affects processes such as receptor-kinase phosphorylation, adenylate cyclase and protein kinase C activation, and prostaglandin production, all of which are critical regulators of chondrocyte differentiation during embryonic limb development (Kulyk and Hoffman 1996). Hence, ethanol-treated chick embryos can be expected to show poor forelimb and hindlimb development.

Antioxidants and their role in preventing alcohol induced malformations

The precise mechanism for ethanol induced dysmorphogenesis has not yet been clarified. However, several studies have suggested that the presence of excessive amounts of reactive oxygen species (ROS) produced by the metabolism of ethanol is a major contributor towards the abnormal development of the embryo (Aberle and others 2003). Reactive oxygen species are produced as a by-product of many metabolic reactions and are regulated at physiological levels by antioxidant defense mechanisms of the body, as well as by antioxidants present in the diet (Memon and Pratten 2009). At physiological levels, ROS participate in various processes such as tissue remodeling, hormone signaling and germ cell function in adults, as well as in embryos (Hossein and others 2007). If the amounts of antioxidants are not enough to maintain their physiological limits, ROS cause malformations in the developing embryo (Jauniaux and others 2004).

Typically, levels of ROS are controlled by cellular enzymatic and non-enzymatic factors. Well characterized antioxidant enzymes are catalase, superoxide dismutase and glutathione peroxidase. Major non-enzymatic antioxidants are vitamin C, vitamin E and β-

carotene. Vitamin C or ascorbic acid is a water soluble compound synthesized by liver cells in most animals, but not in humans, so humans obtain vitamin C entirely from their diet. Fruits and vegetables are a rich source of vitamin C and it is also commercially available in tablet form. Due to its antioxidant properties, vitamin C may protect the embryo from damage caused by ROS. Peng and others (2005) showed that vitamin C inhibited ethanol- induced ROS production and protected the ethanol treated *Xenopus* embryos against microencephaly and growth retardation. Vitamin C exerts its neuroprotective effects by acting as a scavenger of ROS, reducing oxidative stress and by restoring the expression of neural marker genes such as *Pax6, Sox2, Sox3* and/or NCAM, which are all downregulated by alcohol treatment (Peng and others 2005). Furthermore, Chen and others (2004) showed that when ethanol was administered to pregnant mice in combination with antioxidant EUK-134, there was a reduced incidence of forelimb malformations and cell death in the offspring when compared with animals that received ethanol alone. Finally, treatment of ethanol-exposed pregnant dams with vitamin E was found to normalize fetal development (Wentzel and Eriksson 2006).

Despite the increasing amount of literature indicating the effectiveness of antioxidants in preventing ethanol-induced malformations, a few studies have suggested otherwise. Treatment with certain antioxidants such as NAC (Pierce and others 2006), lazaroid (Grisel and Chen 2005) and melatonin (Edwards and others 2002) has failed to prevent the ethanol-induced Purkinje cell loss in the developing rat cerebellum when these were administered concurrently with alcohol. In a different study, Tran and others (2005) also found that vitamin E did not protect against cerebellar damage nor the deficits in eye-blink classical conditioning in ethanol exposed rats. Hence, although the use of appropriate animal models has greatly improved our understanding of the mechanisms responsible for the teratogenicity of alcohol, the therapeutic value of

antioxidants in FASD remains relatively unknown. Furthermore, most studies to date have only analyzed the effects of antioxidant compounds in rodent models of FASD when these are administered concurrently with alcohol (Brocardo and others 2011). In order to ascertain the therapeutic potential of antioxidants in the prevention of ethanol-induced abnormalities, it is important to carry out studies in other animal models. In this experiment, the effect of vitamin C therapy on ethanol-exposed chick embryos was studied to test the hypothesis that antioxidants do not protect the developing chick embryo from ethanol-induced malformations.

MATERIALS AND METHODS

Treatment with ethanol and vitamin C

27- hour Single Comb White Leghorn eggs were treated with different ethanol concentrations, namely 0% (control), 5%, 10% and 15% ethanol in Howard Ringer's solution (Cartwright and Smith 1995). 0.25 ml of 5%, 10% or 15% ethanol was injected directly into the yolk of the eggs using a 1 ML syringe. A total of 6 replicates were used for each ethanol treatment. In order to examine the protective effects of vitamin C, additional eggs treated with 0% (control), 5%, 10% and 15% alcohol were injected with 0.25 mL of 200 µm vitamin C. A total of 6 replicates were used for the vitamin C treatment. All treatments were incubated at 37° C for 14 days. At the end of two weeks, the eggs were opened and embryos were examined for physical anomalies. Measurements for body mass, beak length and head diameter were recorded. Data was analyzed using two-way ANOVA test.

Organ development: chick limb development

Fixation: Following examination of the embryos, limbs were excised from each embryo and immersed in 4% phosphate buffered formalin for two days at room temperature.

Staining of cartilage and bone

Dehydration: The limb tissue was rinsed thoroughly with fresh water, transferred to 50% ethanol for two days, and then to 95% ethanol for another two days.

Staining of cartilage: Alcian Blue was used to stain cartilage (Taylor and Van Dyke 1985). The limb tissue was soaked in the staining solution for one day with gentle agitation.

Clearing (trypsin digestion): The limb tissue was placed in the clearing solution (35% saturated sodium borate and 0.25% trypsin) until about 60% clear (Dingerkus and Uhler 1977).

Staining of bone: Alizarin Red Stain was used to stain bones. Limbs were soaked in the staining solution for one day.

Limb tissues were visualized under the dissecting microscope, and cartilage and bone development of each of the different treatments were observed. Digital images were taken using an ocular microscope camera.

RESULTS

As illustrated by Figures 1-4, chick embryos in the control group (0% ethanol) were found to be in a higher developmental stage (stage 40) when compared to embryos treated with 5% (stage 38), 10% (stage 37) and 15% ethanol (stage 34). Similarly, embryos treated with vitamin C only (stage 41) developed faster than those treated with a combination of ethanol and vitamin C (Figures 5-8).

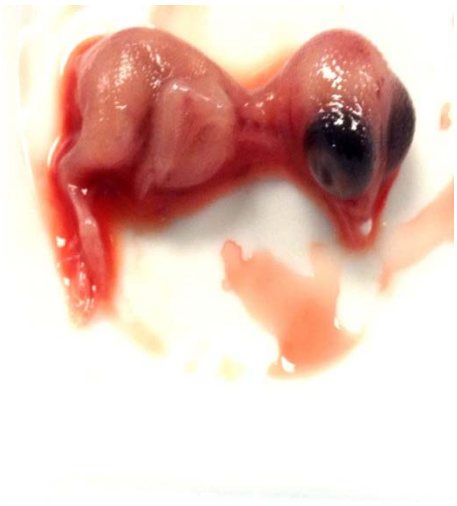

Figure 1: Chick development in 0% ethanol treatment (Average stage: 40)

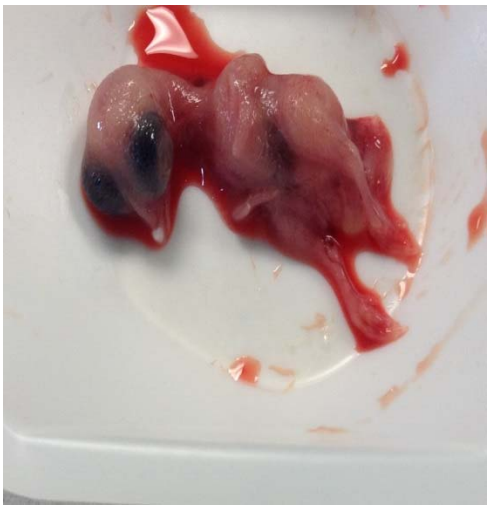

Figure 2: Chick development in 5% ethanol treatment (Average stage: 38)

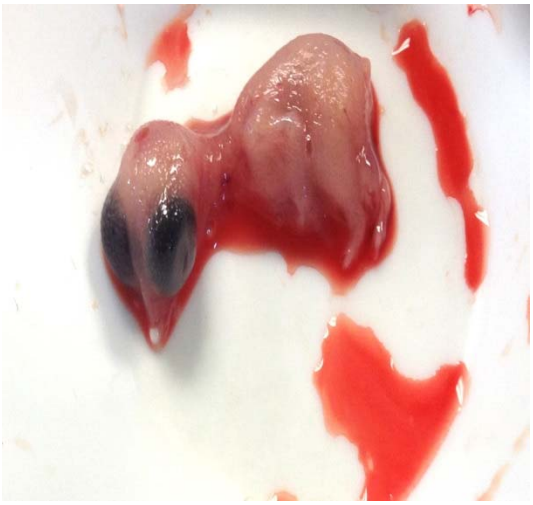

Figure 3: Chick development in 10% ethanol treatment (Average stage: 37)

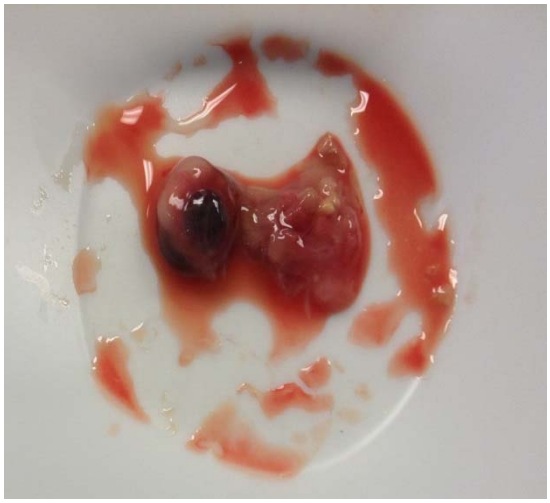

Figure 4: Chick development in 15% ethanol treatment (Average stage: 34)

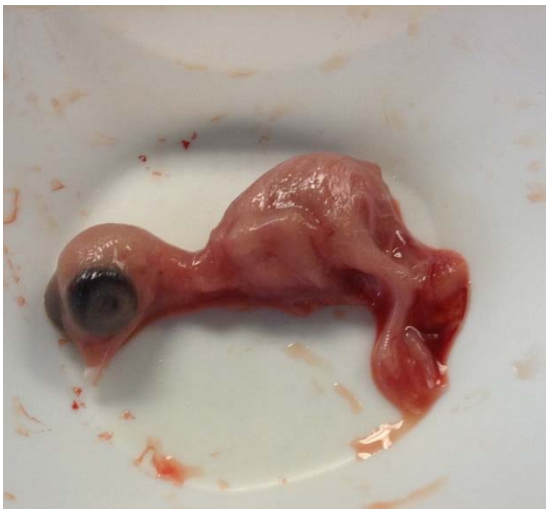

Figure 5: Chick development in 0% Etoh+ Vitamin C treatment (Average stage: 41)

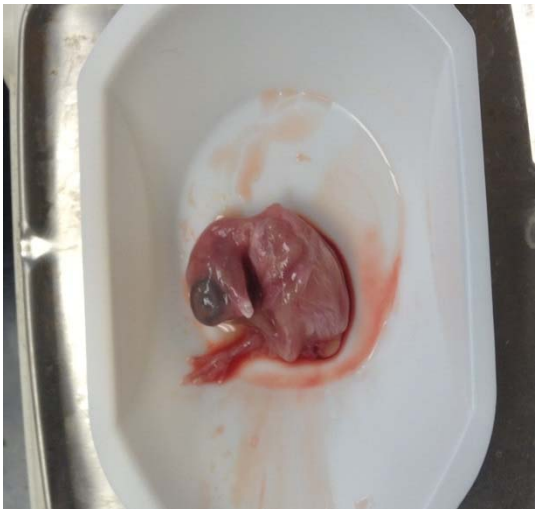

Figure 6: Chick development in 5% Etoh+ Vitamin C treatment (Average stage: 40)

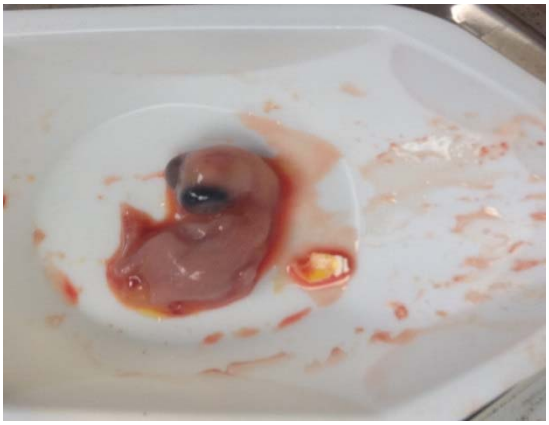

Figure 7: Chick development in 10% Etoh+ Vitamin C treatment (Average stage: 38)

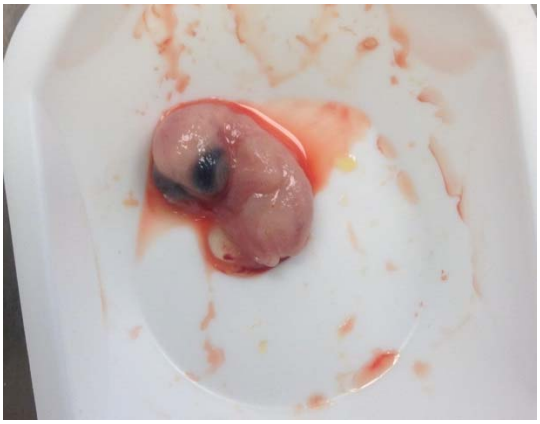

Figure 8: Chick development in 15% Etoh+ Vitamin C treatment (Average stage: 36)

Additionally, chicks in the control group (0% ethanol) showed the best development (highest body mass, beak length and head diameter) when compared to embryos injected with increasing ethanol concentrations (Figures 9-11). Similarly, embryos treated with vitamin C only were found to show higher body mass, beak length and head diameter when compared to those exposed to a combination of ethanol and vitamin C (Figures 12-14).

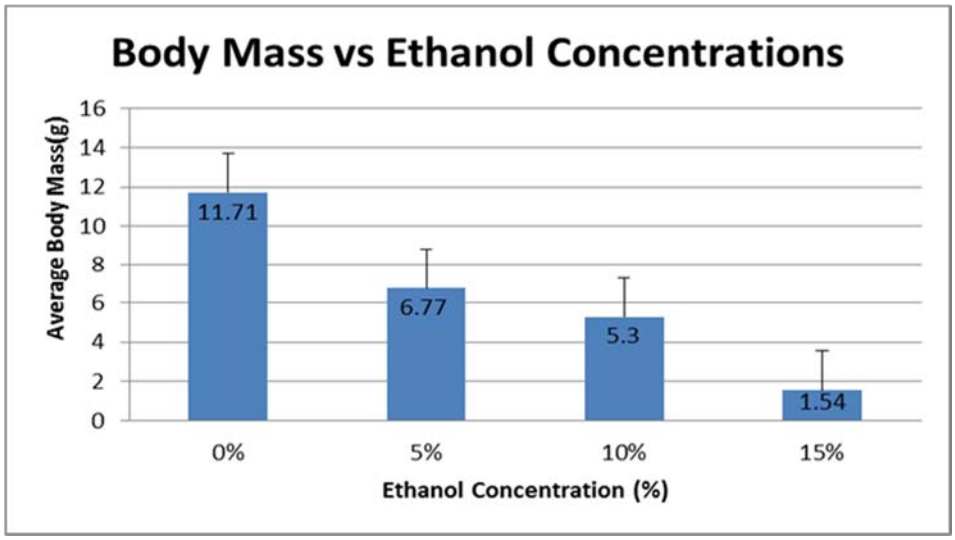

Figure 9: Average body masses of 14-day chicks at different ethanol concentrations. Bars represent the mean +/- standard deviation of measurements from six replicate samples.

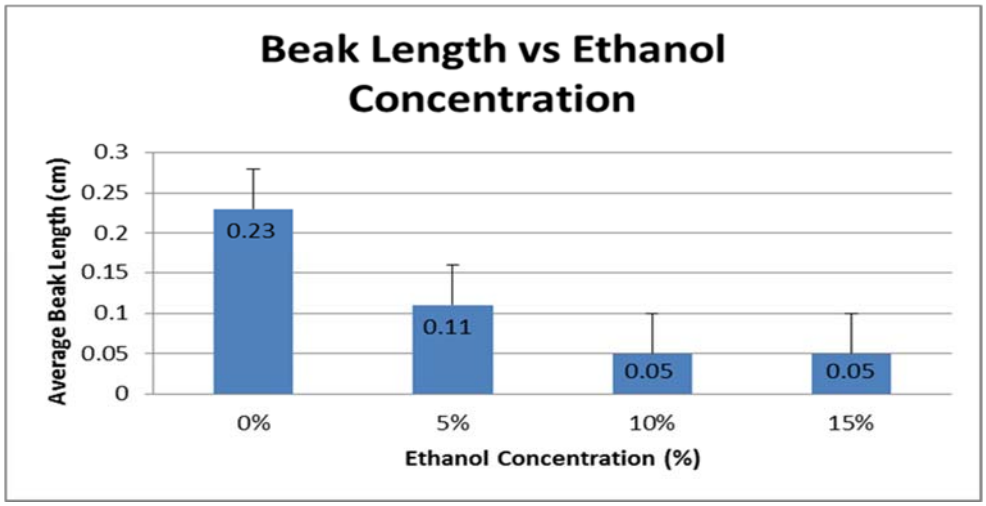

Figure 10: Average beak lengths of 14-day chicks at different ethanol concentrations
Bars represent the mean +/- standard deviation of measurements from six replicate samples.

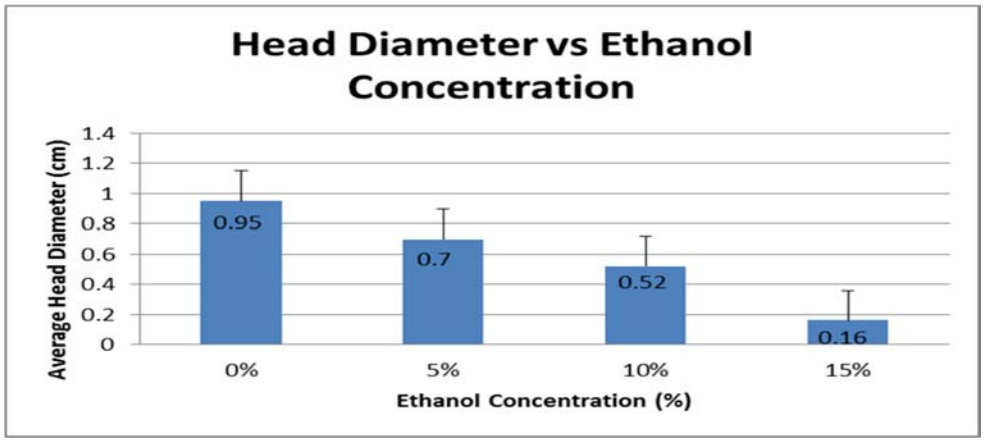

Figure 11: Average head diameters of 14-day chicks at different ethanol concentrations
Bars represent the mean +/- standard deviation of measurements from six replicate samples.

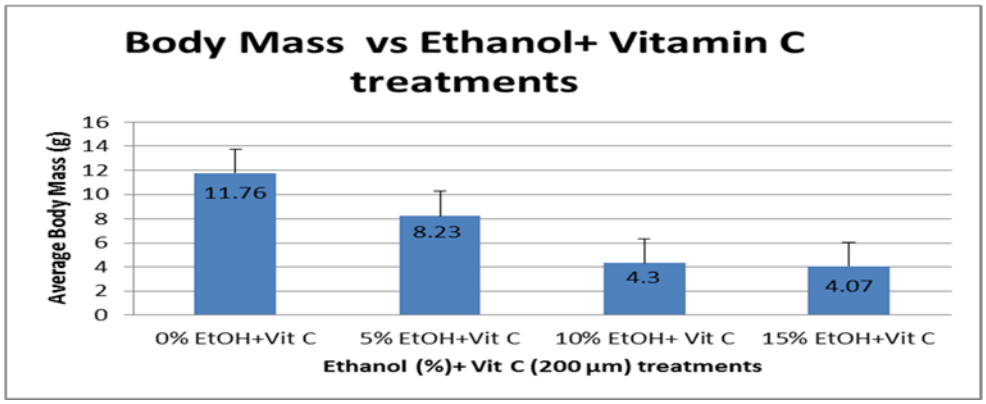

Figure 12: Average body masses of 14-day chicks at different ethanol concentrations with 200 µm vitamin C. Bars represent the mean +/- standard deviation of measurements from six replicate samples.

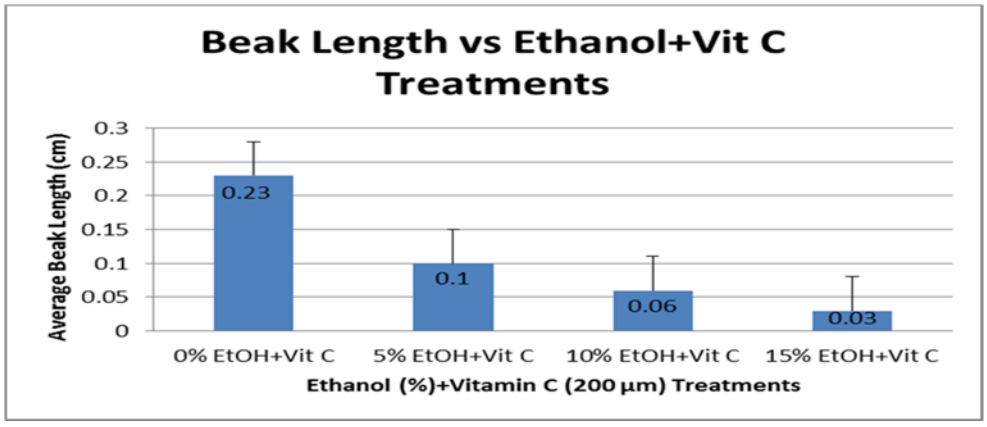

Figure 13: Average beak lengths of 14-day chicks at different ethanol concentrations with 200 µm vitamin C. Bars represent the mean +/- standard deviation of measurements from six replicate samples.

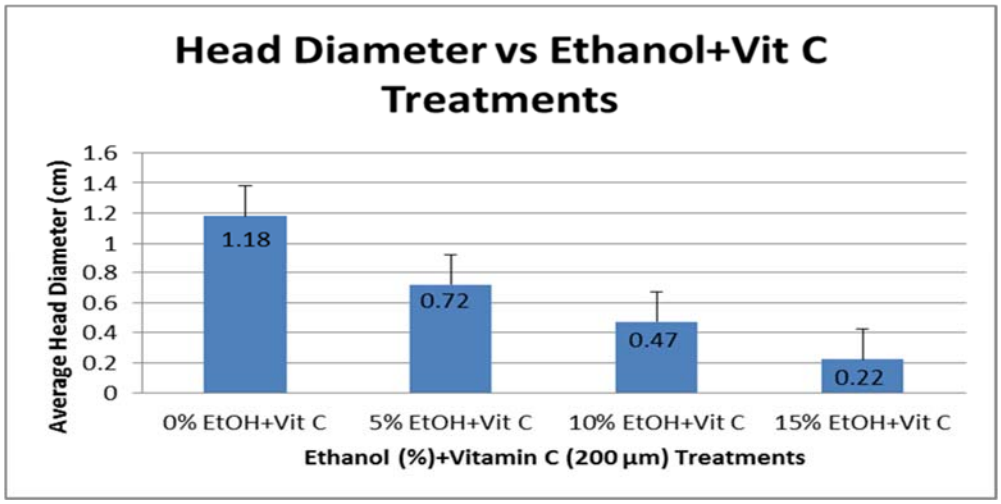

Figure 14: Average head diameters of 14-day chicks at different ethanol concentrations with 200 µm vitamin C. Bars represent the mean +/- standard deviation of measurements from six replicate samples.

Two-factor ANOVA was used to test the hypotheses for this experiment. When embryos were treated with ethanol only, body mass ($p= 0.009$), beak length ($p= 0.0003$) and head diameter ($p= 0.005$) were found to be significantly affected upon increasing the concentration of ethanol.

Upon comparing body mass ($p= 0.684$), beak length ($p= 0.894$) and head diameter ($p= 0.707$) of ethanol treated embryos with ethanol+vitamin C treated embryos, vitamin C was not found to exhibit any significant protective effect against ethanol induced teratogenicity in the chick model. A summary table of the ANOVA results obtained is shown on the next page (Table 1).

Table 1: Summary ANOVA Two-factor analysis comparing body mass, beak length and head diameter of chicks exposed to different treatments

	Source of variation	*df*	*F value*	*P value*
Body mass	Ethanol vs Vitamin C	1	0.168026	0.684059286
	Ethanol concentration(%)	3	4.377176	0.009338965
	Interaction	3	0.176833	0.911466576
Beak Length	Ethanol vs Vitamin C	1	0.017668	0.894922742
	Ethanol concentration(%)	3	7.650177	0.000371644
	Interaction	3	0.064782	0.978188674
Head Diameter	Ethanol vs Vitamin C	1	0.142748	0.707561432
	Ethanol concentration(%)	3	4.959396	0.00508545
	Interaction	3	0.134289	0.939040242

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Hindlimbs excised from embryos in the control group (0% ethanol) showed normal metacarpal formation (Figure 15 A), whereas those from embryos exposed to 15% ethanol showed incomplete and segmented development of digits (Figure 15 B) and metacarpals (Figure 15 C). Similarly, forelimbs from the control group (0% ethanol) showed normal cartilage development (Figure 16 A), whereas those from the ethanol-treated embryos (15% ethanol) showed incomplete cartilage formation (Figure 16 B).

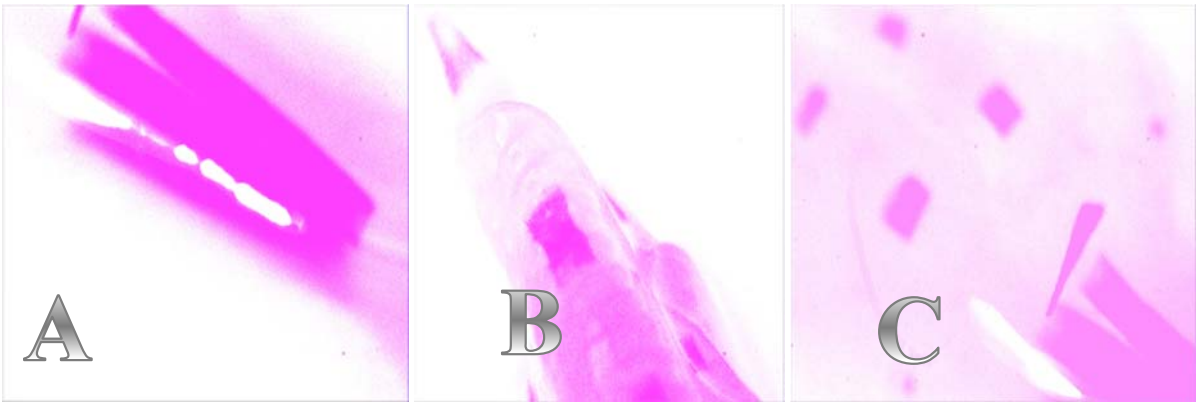

Figure 15: (A) Development of chick hindlimb metacarpals in embryo treated with 0% ethanol (control) marked by Alizarin Red stain. (B) Incomplete digit formation in hindlimb of embryo treated with 15% ethanol marked by Alizarin Red stain (C) Segmented metacarpal formation in hindlimb of embryo treated with 15% ethanol marked by Alizarin Red stain. *Mag:10X*

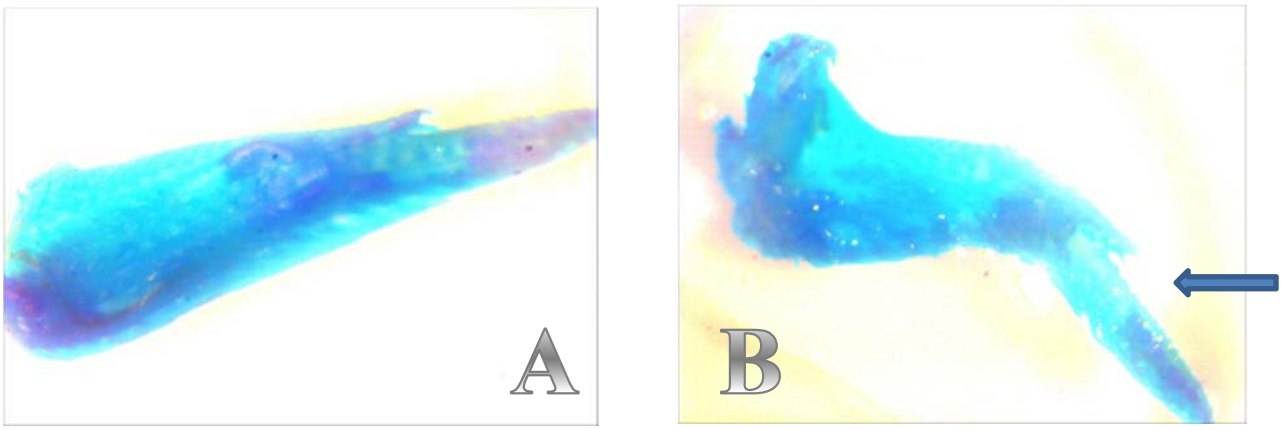

Figure 16: (A) Development of chick forelimb cartilage in embryo treated with 0% ethanol marked by Alcian Blue stain. (B) Incomplete development of chick forelimb cartilage in embryo treated with 15% ethanol marked by Alcian Blue stain. Absence of cartilage growth is indicated by the arrow. *Mag: 10X*

DISCUSSION

The results of this experiment demonstrate that there is a significant relationship between increasing ethanol concentrations and the severity of malformations produced in the developing chick embryo. Embryos in the control group (0% ethanol) showed the best development i.e. highest body mass, beak length and head diameter when compared to embryos exposed to increasing ethanol concentrations. Embryonic development was found to slow down when the concentration of ethanol was increased. These results are consistent with literature findings. Pitt and Carney (1999) found that rabbit cultures exposed to 154 mM of ethanol at gestational day 9 displayed an increase in the occurrence of facial and brain abnormalities and reduction in embryonic growth. Ranganathan and others (1987) observed increased abnormalities in *Drosophila* larvae that were reared in 14% ethanol media. Studies by Reimers and others (2004) revealed that increasing ethanol concentrations led to an increase in incidence of malformations such as craniofacial dysmorphogenesis and reduction in retinal eye diameter in zebrafish. Hence, it can be concluded that increased ethanol dosage, does indeed, lead to overall reduction in embryonic development.

Prior studies have shown that vitamin C protected ethanol treated *Xenopus* embryos against microcephaly and growth retardation (Peng and others 2005). The results of my experiment indicate that vitamin C did not exhibit any significant therapeutic effect on ethanol-exposed chick embryos. This unexpected finding may be due to the fact that the concentration of vitamin C used in my experiment (200 μm) was not enough to inhibit ethanol-induced ROS production in the developing chick embryos. This possibility may be examined by treating ethanol-treated embryos to increasing concentrations of vitamin C (>200 μm).

Limbs excised from ethanol treated embryos (15% ethanol) were found to show incomplete and segmented development of bone and cartilage. Studies on cultured neural, hepatic and other cell types have demonstrated that ethanol exposure alters the fluidity of biological membranes, thereby implicating several signaling pathways involved in the regulation of embryonic limb chondrogenesis (Kulyk and Hoffman 1996). Sensitive strains of mice exposed to alcohol have been known to exhibit limb malformations such as absence of digits, fusion of digits and less frequently, defects of more proximal limb elements (Kulyk and Hoffman 1996). Hence, it is not surprising that ethanol-exposed embryos in my study showed poor limb development when compared to those in the control group (0% ethanol).

The use of chick embryos as a model system to study the effects of prenatal alcohol exposure offers exceptional promise. Even a single episode of consuming alcohol is known to have detrimental effects on fetal development. However, it is also important to consider other factors such as timing of exposure, as well as genetic background of the fetus. Furthermore, additional research at the genetic and molecular will help examine the effectiveness of vitamin C as a therapy to reduce the occurrence of ethanol-induced embryonic malformations.

BUDGET

The experiment was performed in a Containment Level 2 laboratory at Concordia University College of Alberta. Lab space and all major equipment (egg incubator, rotary shaker, dissecting microscopes) was provided by the university. Due to the generous contribution of Concordia University's lab technician, the disposal of wastes generated in this experiment did not require any additional expenses. A list of supplies and equipment used for my experiment is provided below.

Table 2: List of supplies and equipment used for project

	Item name	Supplier	Quantity	Price
Supplies	27-hour chicken eggs	University of Alberta	6 dozen	$81
	Ethanol 5%	Concordia University College of Alberta	2.5 ML	Donated
	10%	Concordia University College of Alberta	2.5 ML	Donated
	15%	Concordia University College of Alberta	2.5 ML	Donated
	50%	Concordia University College of Alberta	100 ML	Donated
	95%	Concordia University College of Alberta	100 ML	Donated
	Vitamin C	Concordia University College of Alberta	25 g	$44.70
	4% Phosphate Buffered Formalin	Concordia University College of Alberta	100 ML	Donated
	Alcian Blue powder	Sigma-Aldrich Canada	50 mg	Donated
	Alizarin Red powder	Sigma-Aldrich Canada	2 mg	Donated
	Acetic acid	Concordia University College of Alberta	50 ML	Donated
	1% KOH solution	Concordia University College of Alberta	100 ML	Donated
	Howard Ringer's solution	Concordia University College of Alberta	50 ML	Donated
	Trypsin powder	Sigma-Aldrich Canada	50 mg	Donated
	Saturated sodium borate	Concordia University College of Alberta	35 ML	Donated
	3% Hydrogen peroxide	Concordia University College of Alberta	20 ML	Donated
	1% Potassium hydroxide	Concordia University College of Alberta	90 ML	Donated
	1 ML syringes	Sigma-Aldrich Canada	1 pkg	Donated
	Dissection kit	Concordia University College of Alberta	1	Donated
	Glass vials	Concordia University College of Alberta		Donated
	Total			**$126**
Equipment				
	Egg Incubator			Donated
	Microscope			Donated
	Rotary shaker			Donated

ETHICS STATEMENT

This project involved the use of chick embryos and will be performed following guidelines outlined by Concordia University College of Alberta's Research Ethics Committee. No harm was inflicted upon any organisms used in this study. All waste and potentially infectious material was decontaminated prior to disposal. Disposal costs of all waste material was covered by the university.

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