Original Article

Anatomy

Evaluation of chick in Ovo Culture as Screening Method for Detection of Teratogens

1. Samreen Memon 2. Umbreen Bano 3. Muhammad Yaqoob Shahani 4. Shazia Begum Shahani

1. Asstt. Prof. of Anatomy, LUM&HS, Jamshoro 2. Lecturer of Anatomy, LUM&HS, Jamshoro
3. Lecturer of Anatomy, LUM&HS, Jamshoro 4. Lecturer of Anatomy, Isra University Hyderabad

ABSTRACT

Background: Congenital malformations are the leading cause of neonatal mortality and morbidity. Out of all birth defects, congenital abnormalities of the heart are at the top. There are many etiological factors responsible for such defects. Environmental agents or teratogens are the factors which could be detected by certain screening methods and may be controlled at an early stage to minimize the chance of mortality. This study was conducted to detect the efficacy of inovo culture system as screening system for teratogenecity testing.

Study Design: Experimental study.

Place and Duration of Study: This study was conducted at the Department of Anatomy, Isra University Hyderabad for a period of six months.

Materials and Methods: White Leghorn Chicken eggs were incubated for 3 days and after 3 days eggs were injected with ethanol 10% and 20%, PBS, neutral red dye and some were left untouched. The eggs were again incubated back until day 9. On day 9 the embryos were taken out and examined for gross malformations. The hearts were then fixed and processed for H&E staining and examined under microscope.

Results: The results showed that addition of alcohol (ethanol) to chicken eggs produced many gross malformations and examination of hearts under microscope also showed malformed hearts. However other chemicals used did not show any malformations.

Conclusions: This study confirmed the teratogenecity of ethanol in chick embryos and can be used as an alternative method for screening of teratogens and toxins.

Key Words: Chick Embryos, Inovo Culture, Alcohol, Screening Method.

INTRODUCTION

Congenital anomalies, birth defects and congenital malformations are the terms currently used to describe developmental disorders present at birth[1]. These anomalies are the leading cause of infant mortality. Out of all the congenital defects 60-70% are of unknown cause, 20% are because of genetic transmission, 3-5% from chromosomal anomalies and the remaining 3-4% are due to chemicals and drugs[2]. However, many common congenital anomalies are thought to be caused by genetic and environmental factors adding together as multifactorial inheritance[1]. Environmental agents are only responsible for a small group of birth defects reported but are also the most amenable to control. For this reason much research has been focused on environmental agents as a cause of congenital defects[3]. A teratogen is any substance, agent or process that induces developmental abnormalities in the embryo/fetus as defined by the Oxford Medical Dictionary. The effect produced by a teratogen depends on the stage at which the embryo/fetus is exposed to it, the most vulnerable period is the period of organogenesis[4]. Teratology is the study of abnormal prenatal development and congenital malformations induced by exogenous chemicals or physical agents[5]. Although only some 10% of all congenital defects are caused by teratogens[6], those teratogens compromise the quality of life for millions of people worldwide and cost billions of dollars in health care every year[7]. Teratogenicity testing of different toxins, chemicals and drugs, which pregnant mothers come across in everyday life, is very essential. Screening of new chemicals with potential toxicity would allow medical experts to help pregnant women in avoiding direct contact with these potentially hazardous substances. Currently most teratogenicity assays utilize in vivo animal studies to attain the goal of detecting chemical hazards[5] The most common species used are rats, mice and rabbits. Although the rodents are the species of choice in teratology research, one should consider many physiological and biochemical differences between the animal species used in teratology research and humans, and it is not surprising that no one species can be shown to be the perfect experimental animal for developmental toxicity studies. None of the species absorb, metabolise and eliminate test substances like humans and none of them have the same placental transfer properties. Despite these liabilities, rodents have become the most commonly used species for evaluating potential human teratogens. Because of the intrinsic problems and inadequacies of teratology testing with the animals most commonly used (mouse, rat, rabbit, hamster, and monkey), scientists have tried to integrate more species into their experiments in order to find one that fulfils the criteria of an ideal animal. Dogs, cats, pigs and non-

human primates were tested with a selection of known teratogens, but none of them proved to be the appropriate animal for developmental toxicity testing. Several inter-species differences including; anatomical differences, metabolic variations, response to potential teratogens, sensitivity to environmental conditions, route of administration and vehicles used, must be considered, when designing animal developmental toxicology studies and extrapolating to humans[7]. In recent years, scientists started using *in vitro* methods to overcome the intrinsic problems and differences in animal teratology studies. These methods are now well established and invaluable for conducting these studies, and are very useful for the screening of chemicals[8]. The *in vitro* tests are less expensive, quicker, and much more reproducible. There is now an absolute need for alternatives to conventional animal-based methods due to the fact that every year hundreds of drugs are introduced to the market and pregnant women are exposed to thousands of toxic substances in everyday life[9]. Further more in recent years chick is used as an alertnative animal for screening tests and observations of developmental defects due to genetic abnormalities, because there is no need of sacrifice of mother, easy to handle and less time consuming and cheaper and give quick reproducible results and genome of chick has many similarities with human genome. This study was conducted to examine the effectiveness of chick embryo in ovo culture for detection of potential teratogens using ethanol as a test chemical.

MATERIALS AND METHODS

This study was conducted for 6 months and experiments were repeated four times for reproducible results. White leghorn fertilized chicken eggs were obtained from Henry Stewart and Co. Ltd. Eggs were randomly selected and divided in to 5 groups immediately before incubation. The first group was incubated as the non-treated group (n = 12). The rest were labelled according to different treatment modes, either PBS (vehicle control, n = 12), Neutral red dye, or EtOH solutions at different doses (10%, n = 12, and 20%, n = 12 v/v in PBS).

The day on which the eggs were incubated was counted as day zero. On day 4 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 of either neutral red dye dissolved in PBS, 10% or 20% ethanol in PBS, or PBS alone, some eggs were also injected with 50 µl of PBS using a 25- gauge needle attached to a 1 ml disposable syringe. 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. Only viable embryos were injected in each group. 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. Non treated embryos were just struck and sealed with parafilm and adhesive tape without any injection.

Sample collection and measurements: 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 any growth retardation by measuring crown rump length, and any malformations or gross abnormalities observed including limb deformities, heart defects according to the criteria shown in table 1.

Once examined grossly the hearts from all viable embryos were then taken out and fixed in 10% formaldehyde.

Paraffin tissue processing for sectioning: Fixed hearts were taken through a series of processes starting from dehydration and clearing to wax impregnation before being sectioned. All procedures were conducted in an automated tissue processor (Leica TP1020). The tissues were sectioned at 25µm by microtome and slides were prepared using glass slides.

Haematoxylin and Eosin staining: Once all the slides were prepared, they were hydrated with water and then placed into the Haematoxylin solution for 5 minutes. The haematoxylin was removed and tissues were washed with tap water. Tissues were differentiated in acid alcohol to remove excess stain before bluing the nuclei in a saturated solution of lithium carbonate. The tissues were again washed in tap water then placed in to 1% eosin for approximately 1-2 minutes. Excess stain was removed and slides were washed in tap water. Finally the tissues were dehydrated by passing through increasing concentrations of alcohol and allowed to dry before being covered with cover slips.

Statistical Analysis: All statistical analysis was performed using Prism 5 (Graphpad Software Inc. San Diego, USA). All results were compared using one way ANOVA with Dunnet's multiple comparison post hoc test, with $p < 0.05$ was considered statistically significant.

RESULTS

This investigation was performed to check the reliability of in ovo culture as toxicity screening method. Ethanol was used as a test chemical due to the fact that it is a well known toxin producing developmental defects. It was used in 2 different concentrations (10% and 20%). Embryos were scored according to the criteria shown in table 1. Embryos which were either kept untouched or only treated with PBS and neutral red dye showed no gross abnormalities. Statistical analysis showed that the

embryos treated with 10 and 20% ethanol were significantly different to other all groups in terms of crown rump length abnormalities Fig 1 (a), vittelline circulation defects figure 1 (b). Also ethanol treated embryos had flexion defects figure 1 (c) and heart malformations figure 1 (d) and limb defects fig 1 (e) as compared to controls. Histological examination of hearts showed dilated ventricles and few papillary muscles in both groups of ethanol treated embryos (fig 2 C, D), while all other groups in this study showed no histological abnormalities of the hearts (figure 2 A, B, E).

Table No.1. Morphological scoring system based on Hamburger and Hamilton staging of chick development[10].

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

DISCUSSION

This study was conducted to detect the possible role of environmental teratogens in the etiology of congenital heart defects. The chick embryo was chosen as experimental model due to the fact that chick eggs are easily available at low cost, and no licence is necessary to conduct experiments on early embryos. Moreover the stages of chick development are very well recognized[10] and there are many similarities discovered between the chicken genome and human genome. Congenital malformations are the principal cause of poor pregnancy outcome. Approximately 50% of all human concepti are lost before implantation, and of those that implant, further 15–20% are lost before term delivery. Of the fetuses which reach the end of term pregnancy, an estimated 3% are born with one or more severe congenital defects[1]. These figures show that errors that result in prenatal death or postnatal abnormalities are frighteningly common. Although rigorous research has been conducted on underlying mechanisms of many developmental defects, still many mechanisms are poorly understood. Some lifestyle factors such as a diet low in nutrients, alcohol consumption, cigarette smoking, misuse of drugs available over the counter and some maternal diseases like diabetes mellitus might play a crucial role in the etiology of congenital anomalies. Therefore identifying and adjusting these life style factors would be a step forward in detecting and preventing environmental induced defects. Alcohol, was shown to have direct impact on the development of chick hearts cultured in ovo. Chick embryos cultured in ovo showed many gross abnormalities including limb defects, head and face malformations and heart defects. It is obvious from previous data that these social drugs are most commonly consumed by women of child bearing age from low socioeconomic status, less educated, younger age and usually jobless[11]. Alcohol when consumed in excess of the recommended range produces a spectrum of defects. Ethanol or alcohol one of the major environmental toxins, when consumed in excess during pregnancy creates wide range of fetal dysmorphogenesis[12]. The fetal alcohol syndrome (FAS) is the most familiar form of ethanol teratogenesis and is characterized by growth retardation, central nervous system disorders particularly mental retardation, and a distinguishing pattern of cardiovascular, facial and limb defects. Data from

animal studies show features of fetal alcohol syndrome

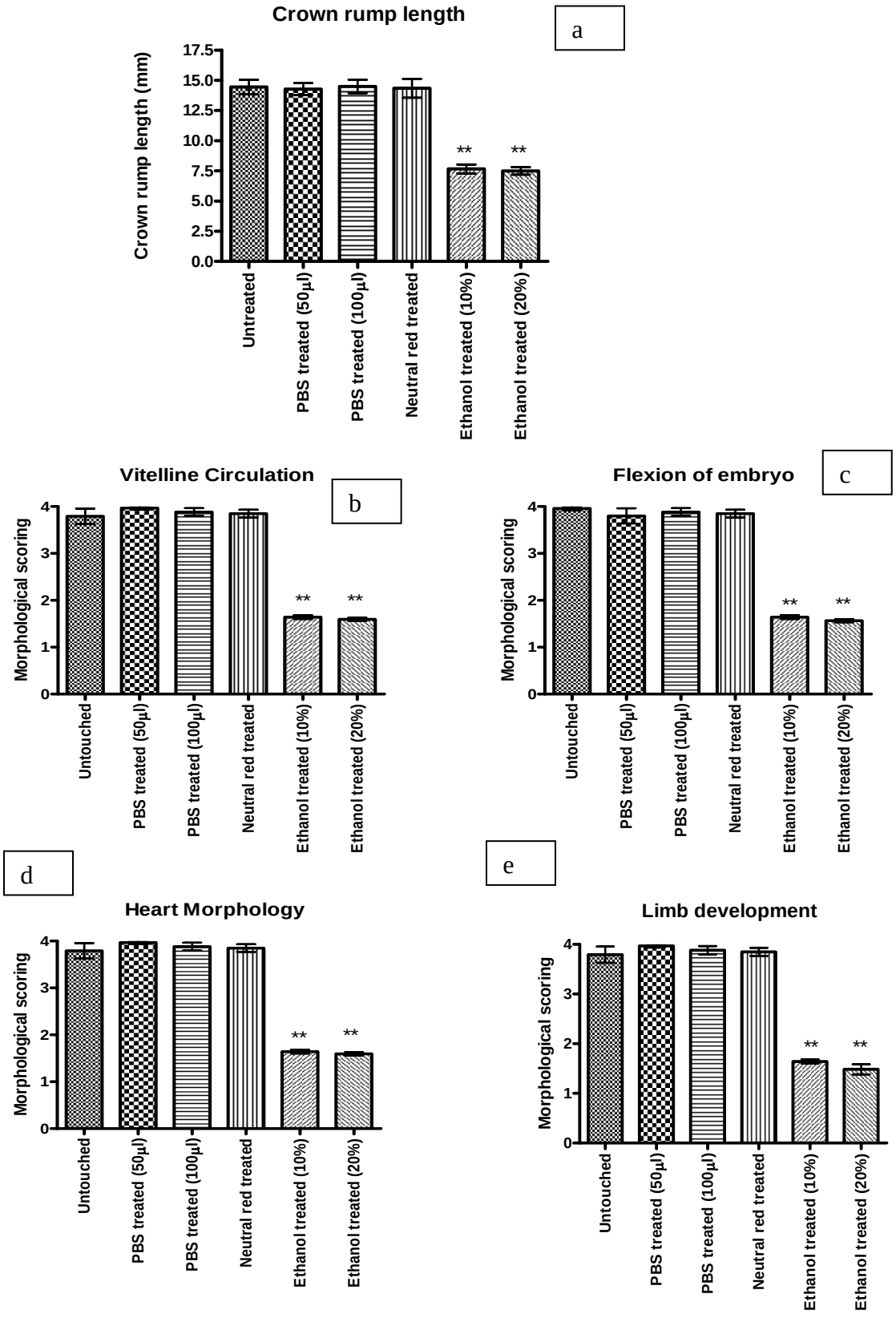

Figure1 :Showing crown rump length (a), vittelline circulation (b), flexion of embryo (c), Heart Morphology (d) and limb development (e). Of the embryos of different treatment groups

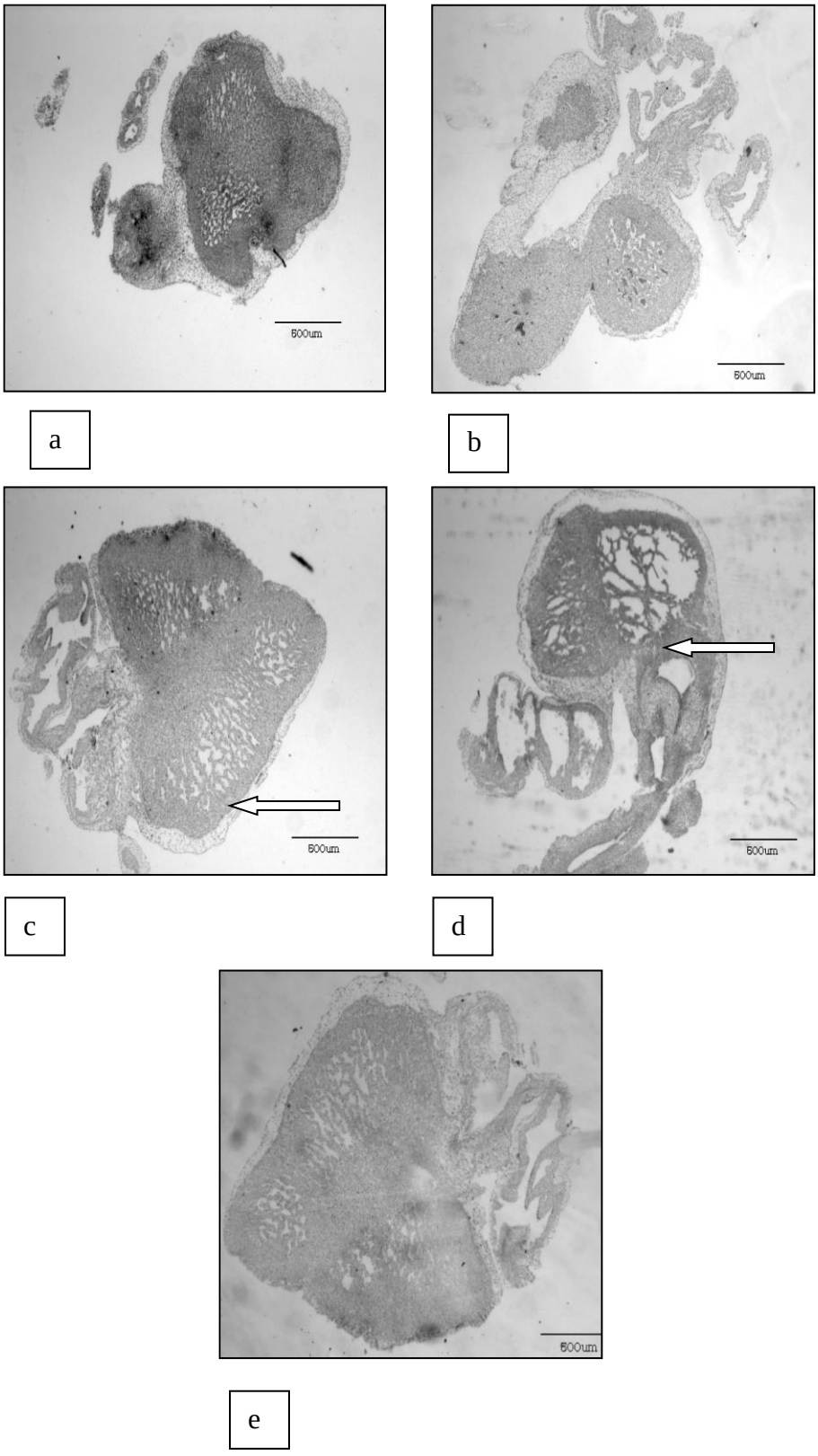

Figure2 :Showing heart sections of embryo without treatment(a), treated with PBS (b), treated with 10% ethanol (c), treated with 20 % ethanol (d) and treated with neutral red dye (e).

in embryos when treated with high doses of ethanol[13,14] However mild to moderate drinking might also produce congenital anomalies[15]. There are many possible mechanisms involved in the pathogenesis of ethanol toxicity and include increased oxidative stress, mitochondrial damage, interference with growth factor activity, effect on cell adhesion, lack of blood supply, oxidative damage and nutritional imbalance. But still the exact molecular pathways which cause the FAS are yet to be discovered. In this study the other agents used to ensure that the injection volume, PBS (vehicle control), neutral red dye (used as a guide for injections) and Para film (to seal the eggs) showed no teratogenic or toxic effects on chick embryos.

CONCLUSION

The results of this study show that in ovo culture is a good model to detect developmental defects caused by teratogens. Ethanol was used to check the reliability of this system, as ethanol is a known teratogen and proved to have toxic effects in many animal models. The chick embryos also show spectrum of malformations when treated with ethanol. Also effects of vehicle control, dye and adhesive tapes were examined and none of them showed any defects in developing embryos. This system might be chosen as an alternative method for detection of possible environmental toxins and drugs without sacrifice of mothers.

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Address for Corresponding Author:
Dr. Samreen Memon,
Assistant Professor & Head of Anatomy Department,
Liaquat University of Medical & Health Sciences
Tel:.00923443542281
Email: samreen_memon@hotmail.com