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Toxicity Study of Chlorzoxazone and Isosorbide Dinitrate using Chick Embryo

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Number of potential drugs are underutilized due to a lack of availability of teratological data. Isosorbide Dinitrate is a saviour drug in angina prophylaxis while chlorzoxazone is a skeletal muscle relaxant and there is no adequate teratogenic study performed till date. This study aims to assess the teratological effect of these drugs on vital organs using the chick embryo model. White Leghorn's (Gallus gallus domesticus) fertilised chicken eggs were acquired from shivneri agro and hatcheries Nashik and divided into five groups (n=10) as Control, non-teratogenic, teratogenic, chlorzoxazone, and Isosorbide Dinitrate. The drug was injected via yolk inoculation and after inoculation; the eggs were re-incubated at 37.5-37.8°C and 50-60% RH for 21 days. Then the embryos were harvested and evaluated for morphological and histopathological changes. The gross macroscopic examination of Isosorbide Dinitrate and chlorzoxazone treated chicks were normal. The development of the embryo was found shunted in Isosorbide Dinitrate treated group. Microscopic abrasions found in Isosorbide Dinitrate treated group are myocardial congestion, hemorrhage, hydropic degeneration, dislocation of the nucleus, splitting of cells, and infiltration of cells at all three doses. No teratogenic response was observed in chlorzoxazone treated group hence found to be safe. Teratogenic effect of Chlorzoxazone and isosorbide dinitrate in chick embryo provided notable details. Chlorzoxazone was found to be safe in chick embryos in the developmental phase, While Isosorbide dinitrate at highest dose was found toxic and so, it is inadvisable for its utilization in pregnancy.

Keywords: Chick embryo; chlorzoxazone; Hepatotoxicity; Isosorbide dinitrate; Teratogenicity.

Drug safety monitoring during pregnancy is important to prevent fetal death and also to avoid abnormality in fetus which remains lifelong. The general health issues observed during pregnancy are anemia, nausea, depression, preeclampsia, miscarriage, gestational diabetes and many more.[1]

Chlorzoxazone is promising muscle relaxant but not utilize due to lack of its teratological information. Chlorzoxazone prevents the production of histamine and slow-reacting substance of anaphylaxis (SRS-A), mediators of type I allergic reactions, by inhibiting the degranulation of mast cells. The release of inflammatory leukotrienes may also be decreased by chlorzoxazone. It is also known to prevent calcium and potassium influx, which block neurons and relax muscles. chlorzoxazone largely suppresses multisynaptic reflex arcs responsible for skeletal muscle spasm production and maintenance in the spinal cord and subcortical regions of the brain (fig.1).[2]

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Isosorbide Dinitrate (ID) is used in cardiovascular diseases as a vasodilator and it corresponds to nitrate class.[3]Nitric oxide (NO), a strong vasodilator gas that is generated when the isosorbide dinitrate is digested (fig.2). Vascular endothelial cells contains soluble guanylylcyclase which is activated by NO, which raises the intracellular concentrations of cyclic GMP (cGMP). Protein kinases G and I, which are cGMP-dependent, are activated by cGMP, and this triggers the intracellular cascades which includes the IP3-mediated pathway's inhibition, phosphorylation of the large calcium-activated potassium channel, cell hyperpolarization, and decreased calcium influx, as well as increased calcium efflux via the Ca2+-ATPase pump, all contribute to lower intracellular calcium concentrations.[4] Myosin light chains are dephosphorylated and smooth muscle cells relax as a result of lower intracellular calcium levels.[5] It can be used in preeclampsia. As per current literature survey there is no any teratological data available for both the drugs till date.

Chick embryo model is cheap, worthy technique. Hence, efforts are made to explore teratogenicity of caliber drugs like above mentioned.[6]

MATERIAL AND METHODS

Test chemicals

Chlorzoxazone was obtained from Bimal Pharma PVT.LTD, Mumbai-400 097 (CLX/20/0307) with the purity of 99%. Isosorbide dinitrate, paracetamol and thalidomide was obtained from local store.

Test animals

Gallus gallus domesticus strain was used for this experiment. Eggs weighing greater than equal to 50g were selected and the eggs were obtained from the Shivneri Agro and hatcheries, Nashik. The eggs were placed in plastic tray in incubator at 37.5-37.8°C and 50-60% RH (Relative humidity). This experiment was approved by IAEC (Institutional animal ethics committee) Approval no. (IAEC/2020-2021/05)

Method

After stabilization of eggs, they were candled and weighed. The rotten eggs were removed (fig. 3). The doses for the eggs were calculated based on LD_{50} (Lethal dose) of each drug. As drugs were slightly soluble in water, suspension was prepared with 1% CMC (Carboxymethyl cellulose) as a suspending agent.[7] On third day of incubation, drug was injected through amniotic inoculation route. The drug was administered by windowing to egg shell using scissor tip or concentrated HCL. After drug injection the window was sealed using paraffin wax after those eggs were re-incubated. On every 5th, 10th, 15th, 20th day of incubation candling was done to remove rotten eggs and also to observe development of chick embryo. When 21 days of incubation completed, eggs were removed and manually hatched and chicks were observed whether they alive or dead. Various evaluation parameters were performed and noted. The all data were extrapolated on control, teratogenic and safe group and toxicity of drug was observed based on morphology and histopathology of chick. The organs were dissected weighed and sent to pathology laboratory to prepare slides and blocks.

Experimental design

The eggs were incubated for 21 days to observe developmental changes. Eggs were divided into nine groups each containing 10 eggs. Following are the groups:

Group I: 0.9% NaCl, Group II: 150 mg/ Kg egg-weight Paracetamol, Group III: 10 mg/ Kg egg-weight thalidomide, Group IV: 88ìg/ Kg egg-weight chlorzoxazone, Group V: 176ig/ Kg egg-weight chlorzoxazone, Group VI: 352ig/ Kg egg-weight chlorzoxazone , Group VII: 9mg/ Kg egg-weight Isosorbide Dinitrate , Group VIII: 17.9mg/ Kg egg-weight Isosorbide Dinitrate and Group IX: 35.86mg/ Kg egg-weight Isosorbide Dinitrate.

Drug administration in chick embryos

Drug was administered to embryo through amniotic inoculation route. Based on the lethal dose of drugs, dose for chick embryo was calculated. The eggs were randomly assigned into nine groups (n=10). Hatch able eggs were placed at 37.5 °C temperature and 50-60% relative humidity in incubator (META-LAB) (fig.4).[8]

Assessment of pathological changes

Eggs were artificially hatched and embryos were evaluated for its gross and histopathological injuries on 21st day of development. The embryos were also observed for any gross lesions, then after embryos were dissected and organs were isolated.

The organs were then preserved in 10% formalin to prepare paraffin-embedded tissues and then it was stained with hematoxylin and eosin.

Measurements

The body length was calculated by scale and head diameter (crown rump length) was calculated by using vernier caliper (Advance Company and range 0-150mm). Body length was measured from angle of the head to the apex of the tail.

Statistical analysis

All observations were presented as mean ± standard error mean. The data was analyzed by one way ANOVA followed by Tukey's test. P-value < 0.05 was considered statistically significant. For the statistical analysis Graph pad prism (version 5.00), United States was utilized.

RESULTS

Macroscopic Findings

The all groups of embryo were evaluated for any lesions and abnormalities on external body surface.

The embryos of all three experimental chlorzoxazone groups (a, b & c) were found to be normal as compared to control and paracetamol groups and no any abnormalities were observed externally. The embryos of ISD groups (ISDA, ISDB & ISDC) showed growth retardation and at highest dose 100% mortality was observed.

Effect of drugs on various parameters of chick embryo morphology:

Body weight:

The statistical data of all groups regarding the weight of embryos were found that Ca and Cb group has shown non-significant results with control group while Cc was statistically significant with control a P value of (**P<0.01). Chlorzoxazone groups found to be statistically non-significant with Paracetamol while significant with thalidomide (***P<0.001). Ia, Ib, and Ic groups had significant results with control group and Paracetamol group a P value of (***P<0.001) while non-significant with thalidomide. (Figure 6)

CRL

The statistical data of all groups regarding the CRL were found that Ca, Cb and Cc groups had shown non-significant results with control and Paracetamol group while significant with

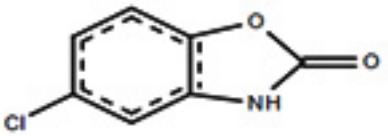

Fig. 1. Chlorzoxazone

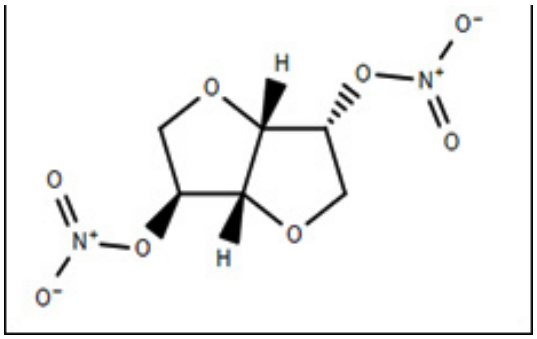

Fig. 2. Isosorbide Dinitrate

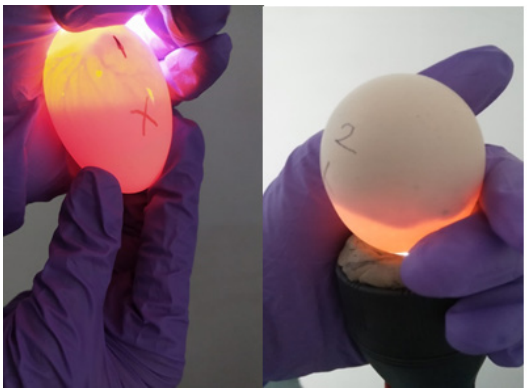

Fig. 3. (a). Hatach-able egg (b). Rotten eggs

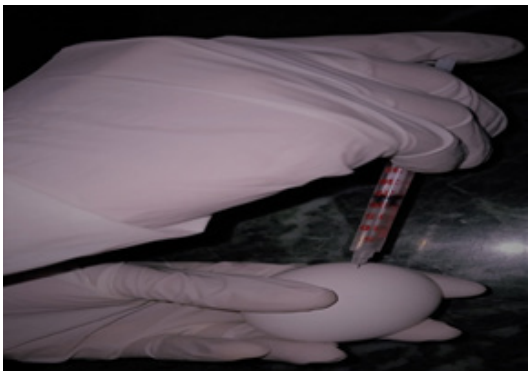

Fig. 4. Drug administration to chick embryos

thalidomide (***P<0.001). Ia, Ib, and Ic groups had significant results with control and Paracetamol group a P value of (***P<0.001) while non-significant with thalidomide. (Fig.7)

Body Size

The statistical data of all groups regarding the body size were found that Ca, Cb and Cc groups had shown non-significant results with control and Paracetamol group while significant with thalidomide (***P<0.001). Ia, Ib, and Ic groups had significant results with control and Paracetamol group a P value of (***P<0.001) while non-significant with thalidomide. (Fig. 8)

Length of beak

The statistical data of all groups regarding the length of beak were found that Ca, Cb and Cc groups had shown non-significant results with control and Paracetamol group while significant with thalidomide a P Value of (***P<0.001). Ia, Ib, and Ic groups had significant results with control

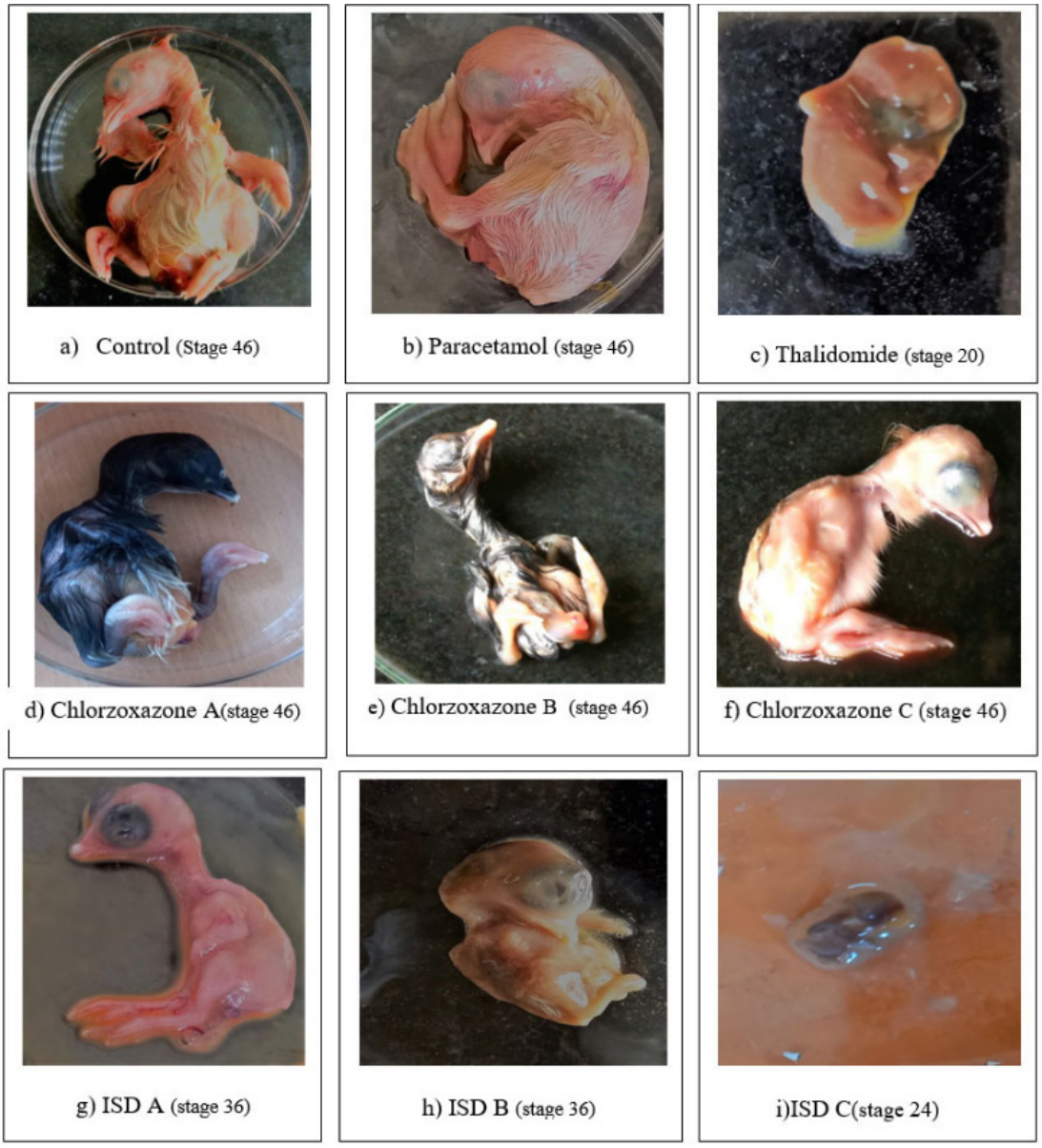

Fig. 5. Macroscopic findings

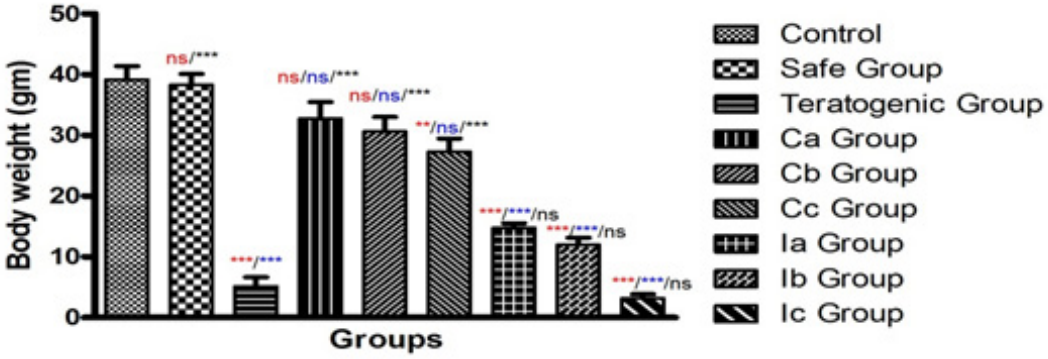

Fig. 6. Effect of treatment on body weight of chick embryo

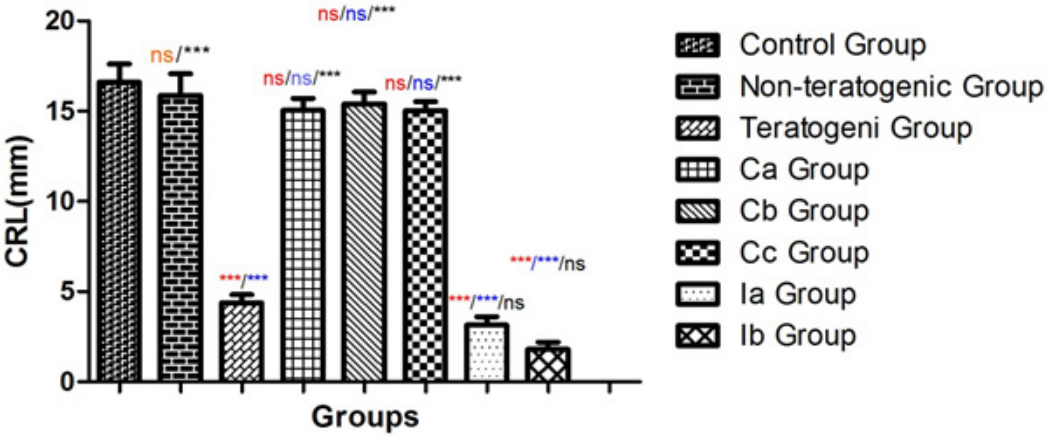

Fig. 7. Effect of treatment on CRL of chick embryo

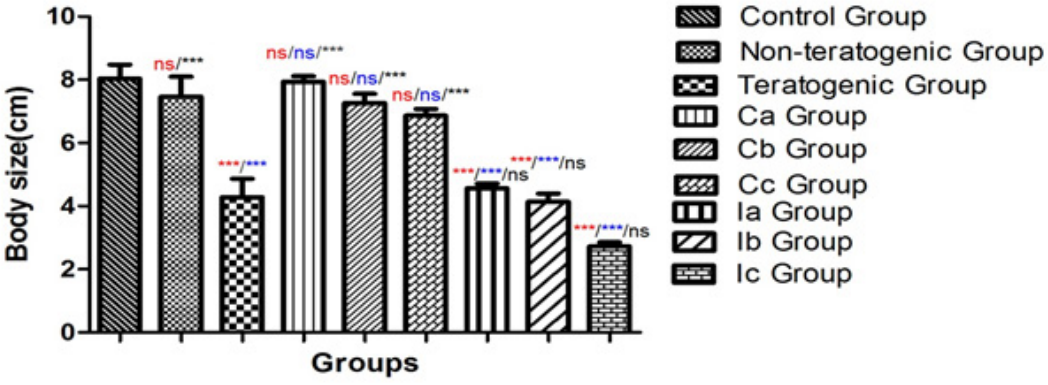

Fig. 8. Effect of treatment on body size of chick embryo

and Paracetamol group a P value of (***P<0.001) while non-significant with thalidomide. (Fig. 9)

Torso/Head ratio

The statistical data of all groups regarding Torso/Head ratio were found that Ca, Cb and Cc groups had shown non-significant results with control and Paracetamol group while significant with thalidomide (***P<0.001). Ia, Ib, and Ic groups had significant results with control and Paracetamol group, P value of (***P<0.001) while non-significant with thalidomide. (Fig. 10)

Viability study

Viability study was performed to find the compatibity of eggs with the experimental condition and survival rate. 90 % of the eggs from control, non teratogenic and chlorzoxazone A and B group was observed. The ISD treated group showed higher mortality rate i.e. 0 % survival. (Table no.1)

Microscopic findings

Histopathology of Brain

Normal microanatomy of rat was observed in Control, non teratogenic and chlorzoxazone B group rat brain while in chlorzoxazone A, marked changes in anatomy of brain were observed i.e increase in intercellular area along with granular layer. Histology of brain of isosorbide groups was not performed as the embryo was not fully developed.

Histopathology of Heart

Control group and nonteratogenic group showed normal morphology of cardiomyocyte. Teratogenic group showed dislocation of nucleus, and myocardial degeneration. Chlorzoxazone group showed normal histological changes as compared to control and nonteratogenic group while isosorbide group showed splitting of cardiac

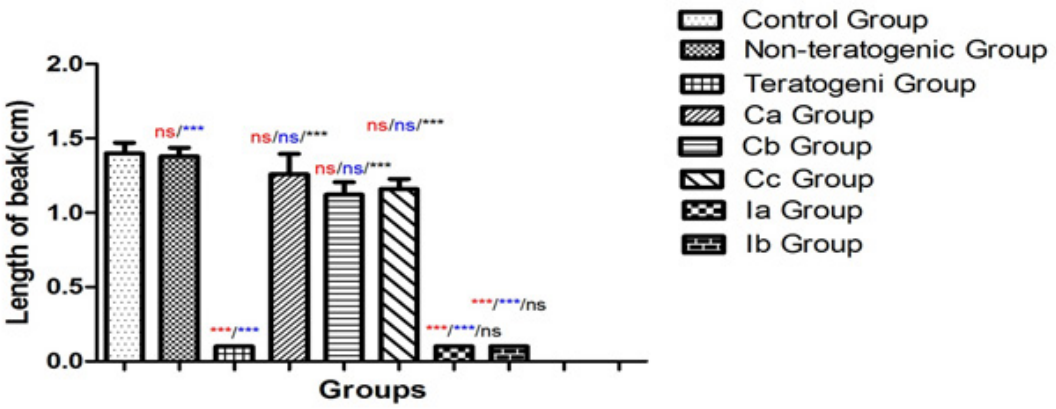

Fig. 9. Effect of treatment on length of beak of chick embryo

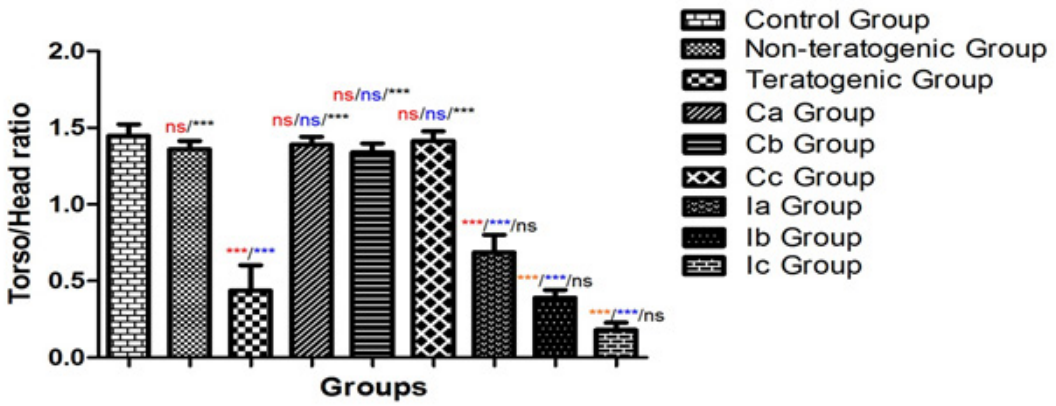

Fig. 10. Effect of treatment on torso/head of chick embryo

cells, dislocation of nucleus and infiltration of neutrophils.

Histopathology of Liver

Control and non-teratogenic group showed normal sinusoids and cellular anatomy of liver. Teratogenic group showed infiltration of cells (a) and hemorrhage (b). chlorzoxazone A group liver was observed with inflammatory cell infiltrate. Moderate dose of chlorzoxazone B and C reflected with chronic observation of inflammatory cell infiltrate (a) and hepatic necrosis (c). Isosorbide dinitrite treated rats showed hydropic degeneration of hepatocytes and hemorrhage (b) in liver.

Histopathology of Intestine

Large intestine was studied microscopically which showed normal anatomy in all group except isosorbide and Chlorzoxazone B group.

Morphological parameters

Morphological parameters like Body weight, size, Crown-rump length, length of beak and torso/head ratio were measured. The teratogenic group showed significant ($p<0.001$) pathological changes in all the parameters as compared to control group. Chlorzoxazone and non-teratogenic group showed significant difference ($p<0.001$) in morphology while no significant change was observed with isosorbide treated group as compared to teratogenic group.

DISCUSSION

Toxicological profile of number of potential drugs is not available and so that they are underutilized, hence an attempt was made to reveal the toxicological data of Chlorzoxazone and Isosorbide dinitrite. Apart from being cheap and easy availability, chicken embryo can reveal detrimental effects and thus, not to be followed up in rats, this can help to minimize the number of mice necessary in preliminary drug screening and also can provide some insight into probable mechanisms of action of the medication as well as safety.[9] Chlorzoxazone is a muscle relaxant drug that can be used relieve abdominal muscle cramps which is the common symptom in pregnancy. Data available from animal experiments as well as human studies indicate that chlorzoxazone acts primarily at the level of the spinal cord and

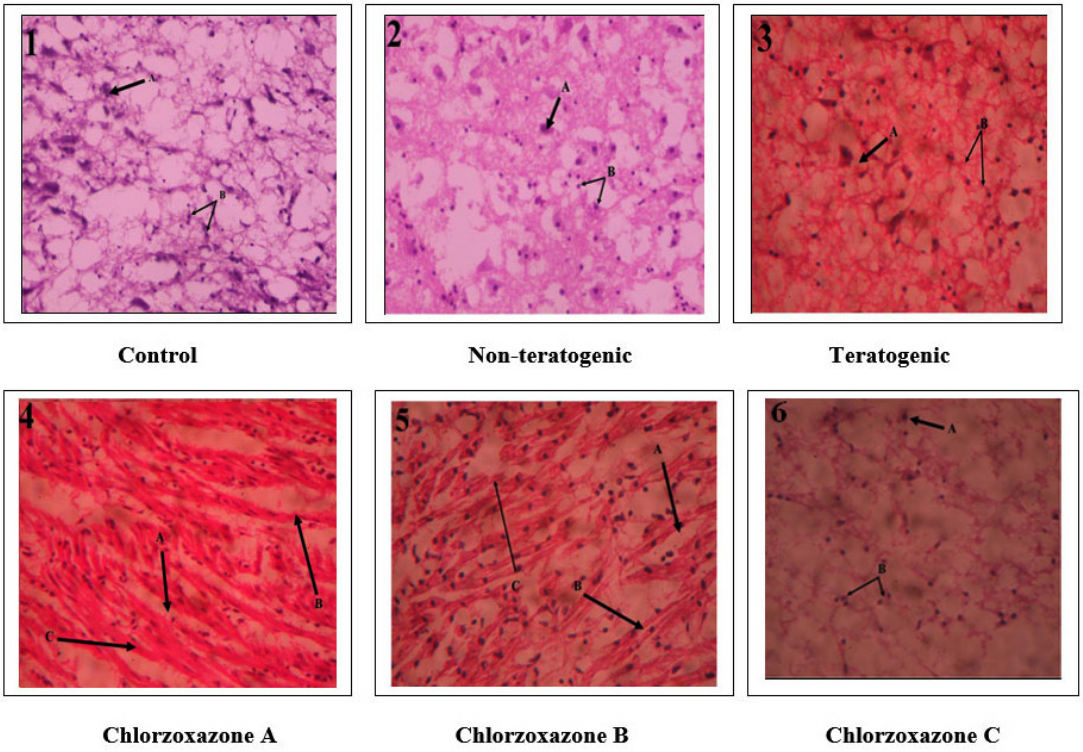

Fig. 11. Histopathology of Brain (45x)

subcortical areas of the brain where it inhibits multisynaptic reflex involved in producing and maintaining skeletal muscle spasm of varied etiology. The clinical outcomes reveal a reduction of the skeletal muscle spasm with relief of pain and increased mobility of the involved muscles.[10]

Isosorbide dinitrate is a part of the class of drugs known as nitrates. It is used in the prophylaxis of angina as a vasodilator. It functions by widening the blood arteries, boosting the flow of blood and oxygen to the heart, and lightening the workload on the organ. It can be used in preeclampsia.[11] Un-avaibility of teratological data of Isosorbide Dinitrate & chlorzoxazone promoted us to include these drugs in the present research.

Morphological parameters of the chick embryo reveals whether test regimens affects the development of embryo. Various morphological parameters were studied like body weight, body size, beak length, crown-rump length, and torso to the head ratio. Significant changes in test group from the control and Paracetamol group indicated that the drug is affecting the developmental stages of the embryo. Chick embryos of the chlorzoxazone group were found to be normal with control and Paracetamol group. Isosorbide dinitrate has found have effects on the chick embryo as morphological development was seen in lower dose group (Isosorbide Dinitrate A) but it didn't signifies that

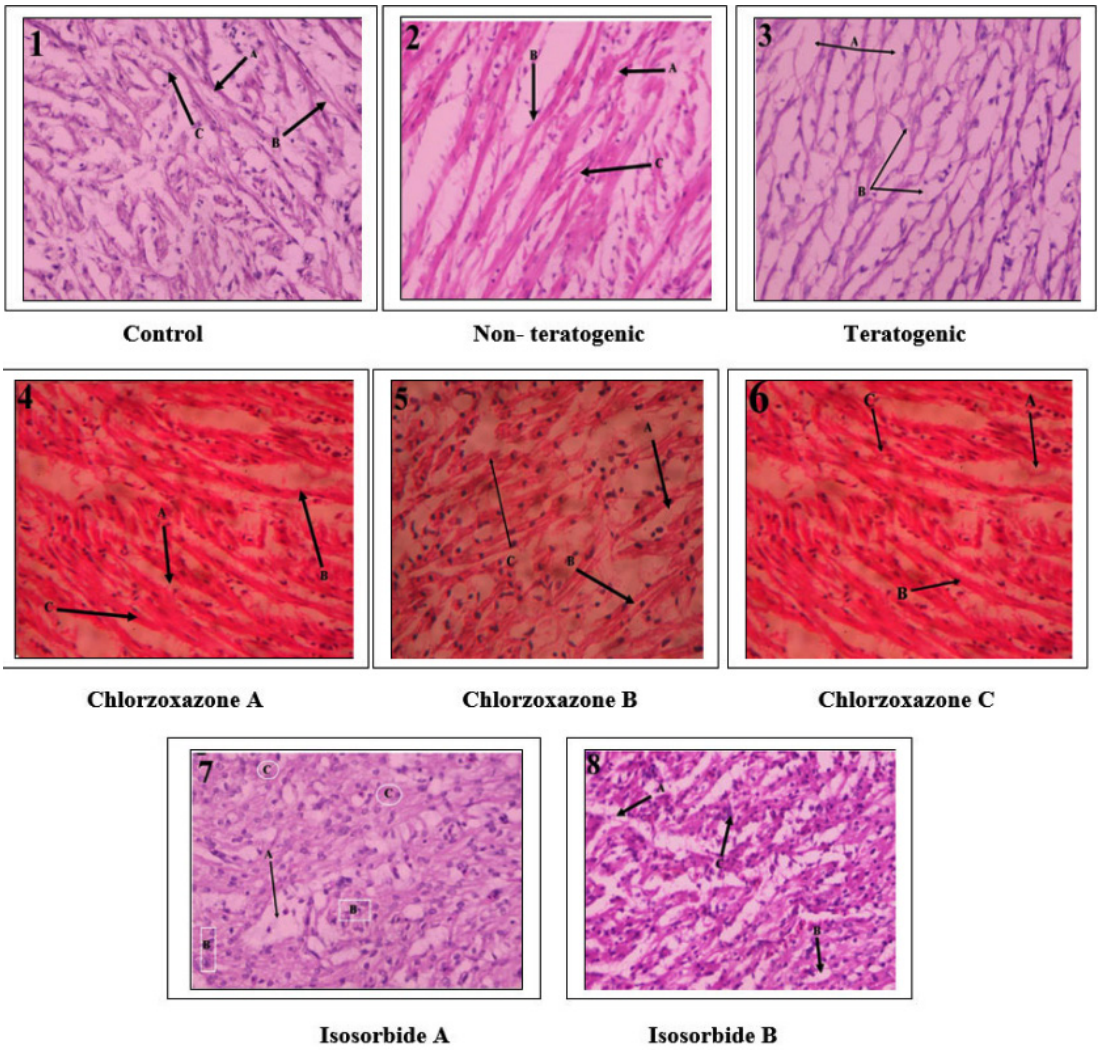

Fig. 12. Histopathology of Heart (45X)

ISD is safe as remaining groups of ISD showed higher death range compared to control group.

The gross anatomical study of the chlorzoxazone group did not showed significant results as compared with the control and Paracetamol group while significant results were observed with the thalidomide group. Isosorbide Dinitrate group had significant results with control and non-teratogenic group i.e. Paracetamol group and according to morphological parameters that may suggest that ISD may affect the metabolic system of the chick embryo so that embryo was unable to use its yolk and indirectly affects the development of the embryo. ISD C group was found to have 100% mortality as the dose increases toxicity levels also increased.

The percent survival rate of the chlorzoxazone group was high as compare to the ISD group. The higher dose group of ISD Isosorbide dinitrate C found to cause 100% mortality.

The avian brain consists of three basic parts cerebellum, cerebrum, and medulla oblongata. The cerebral cortex is characterized by a greater number of pyramidal cells. The abundance of pyramidal cells in the cortex suggests the wellbeing of cognitive process[12]. Chlorzoxazone found to have no effect on brain of chick embryo and showed normal structure of pyramidal cells and purkinje cells.

The histopathological alterations indicated that the chick embryo is susceptible to the application of Isosorbide Dinitrate during embryonic development. Neutrophil cell infiltration affects myocardial cells and produced congestion of cells. The ISD affects heart by causing myocardial

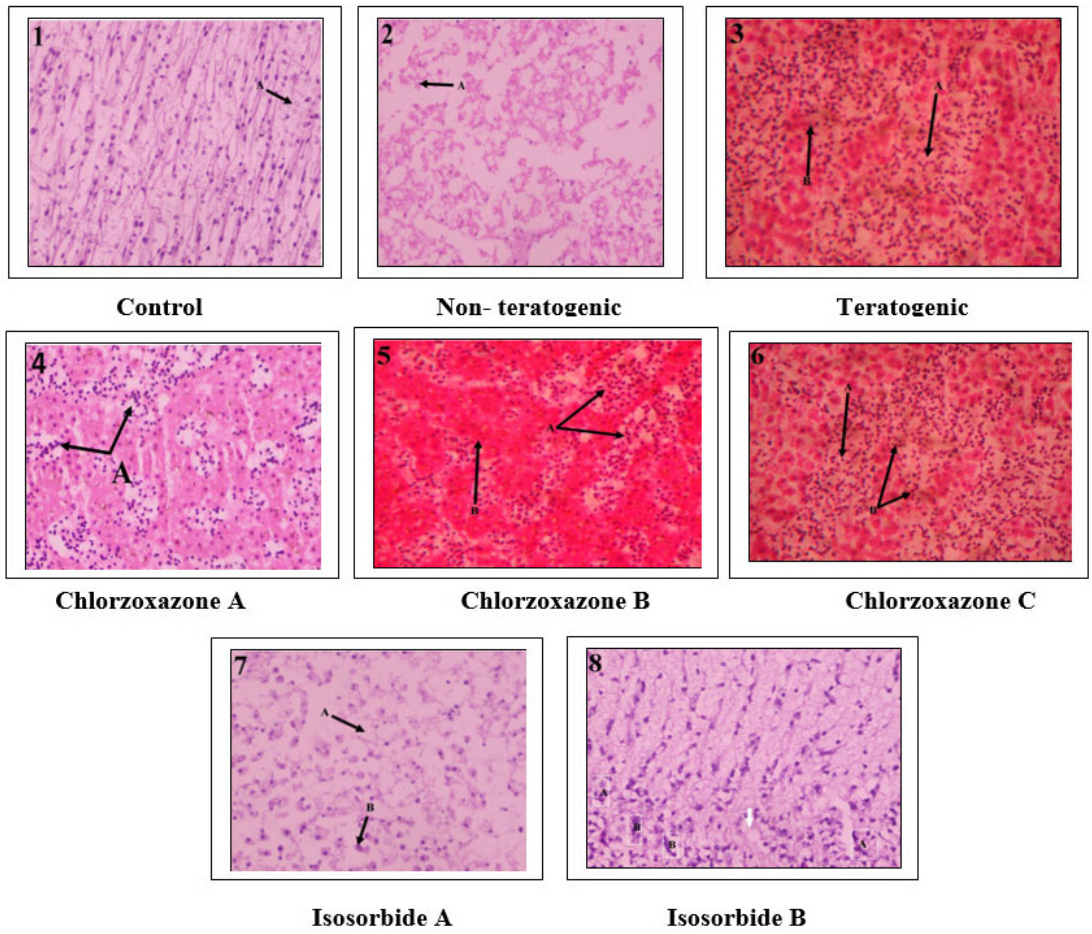

Fig. 13. Histopathology of Liver (45X)

congestion, infiltration of cells, dislocation of the nucleus, and slitting of cells[13] which need to be given attention as ISD is used in Angina prophylaxis.

Lobules are the functional unit of liver within these polygonal hepatocytes is arranged in row with large central nucleus and sinusoids. ISD treatment showed histopathological lesions in the liver as infiltration of cells, Hydropic degeneration of hepatocytes and hemorrhage[14]. Chlorzoxazone was found to be hepatotoxic in the chick embryo and this finding was also observed in one of the clinical studies[15]. Contrarily histological data disclosed no any malformations and found highly significantly safe as compared to the thalidomide group.

Intestinal lining is made up of muscularis mucosa, sub mucosa, muscularis externa and serosa. Intestinal villi lined by simple columnar epithelium and the superficial differences in villi used to identify the specific section of intestine[16]. Chlorzoxazone did not cause any structural changes in intestine.

Jelinek and Marhan (1994)[17] reported on the predictive value of the CHEST assay in comparison to the conventional rat and rabbit procedures. They compared the results of 50 different chemicals and found 80% congruency. They suggest that the CHEST assay can be used as a predictive tool not for replacing the official routine rodent and rabbit procedure.

Table 1. Viability study

Viability	Control Group	Non-teratogenic Group	Teratogenic Group	Drug A Group (µg/ Kg egg-weight)			Drug B Group (mg/ Kg egg-weight)		
				88	176	352	9	17.9	35.86
No. of eggs	10	10	10	10	10	10	10	10	10
Survival %	90	90	0	90	90	80	10	0	0

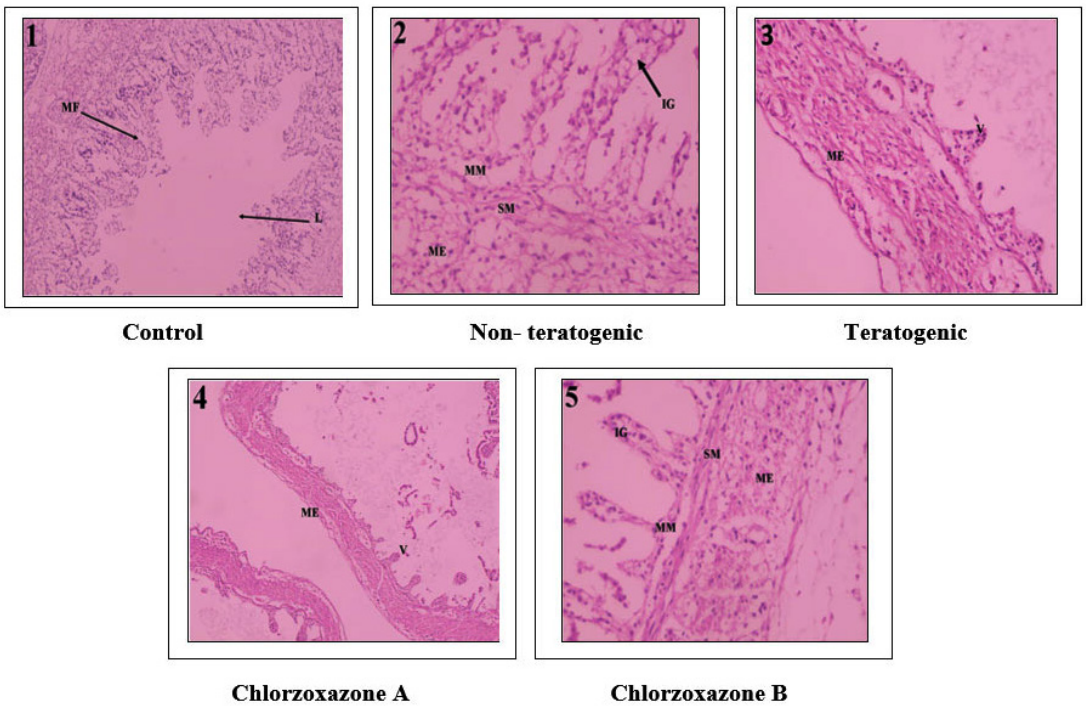

Fig. 14. Histopathology of intestine (45X)

Table 2. Effect of drug on various morphological parameters of chick embryo

Parameters	Control Group	Non-teratogenic group	Teratogenic group	Ca group	Cb group	Cc group	Ia group	Ib group
Body weight	39.20 ±2.209	38.3 ±1.80 ***	5.14 ± 1.51$^{###}$	32.8 ± 2.74***	30.6 ± 2.37***	27.29 ± 2.19***	14.75 ± 0.79 ^{ns}	12± 1.13 ^{ns}
Body size	8.040 ±0.437	7.46 ±0.63***	4.28 ±0.58$^{###}$	7.94 ± 0.17***	7.26 ± 0.29***	6.68 ± 0.21***	4.56 ± 0.15^{ns}	4.14 ±0.25 ^{ns}
CRL	16.6 ± 1.01	15.87 ±1.21***	4.37 ±0.46$^{###}$	15.04 ± 0.66***	15.41 ± 0.65***	15.03 ± 0.50***	3.15 ± 0.45^{ns}	1.816 ±0.37 ^{ns}
Length of beak	1.400 ±0.07	1.38 ±0.06***	0.10 ±0.0$^{###}$	1.26 ± 0.136***	1.12 ± 0.07***	1.16 ± 0.07***	0.10 ±0.0^{ns}	0.10 ±0.0^{ns}
Torso/Head ratio	1.448 ±0.07	1.36 ±0.05^{ns}	0.437±0.16$^{###}$	1.39 ± 0.05***	0.07***	1.41 ± 0.06***	0.68 ± 0.11^{ns}	0.391 ±0.04^{ns}

All data are represented as mean ± SEM.* denotes Group Ca, Cb, Cc, Ia, Ib and non teratogenic group is compared with teratogenic group, while # denotes teratogenic group is compared with control group. ***/### signifies $P < 0.001$ and ns as non significant.

During the conduction of research some findings were observed like for hard shell an egg candling was difficult, fungal infection of one egg get spread to other eggs due to this one whole batch was discarded and Egg fluid oozed out due to increasing in internal pressure.

This study can be extended as biochemical study for chick embryo, as it was not performed and also to study mechanism of action of toxicity.

CONCLUSION

Chick embryo model should be utilized routinely in drug discovery and developmental studies before going for any animal studies as it showed robust result in present study. Chlorzoxazone, in chick embryo is found non-teratogenic and can be utilized in pregnancy after appropriate clinical investigation. Isosorbide Dinitrate, induced weight reduction and mortality with increase in dose, which represents teratogenicity with specific etiology.

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Conflict of Interest

Authors declared no conflict of interest.

Ethical Statement

Before the commencement of study, permission from MET Institute of pharmacy Institutional Animal Ethics Committee was taken.

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