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Effect of Diclofenac Sodium in Developing Chick Embryo, *Gallus Gallus domesticus* (Linn, 1758)

Samir Terdalkar

Author's Affiliation:
Assistant Professor, Department of Zoology, Fergusson College (Autonomous), Pune, Maharashtra 411004, India

***Corresponding author:**
Samir Terdalkar,
Assistant Professor, Department of Zoology, Fergusson College (Autonomous), Pune, Maharashtra 411004, India
E-mail: sterdalkar@fergusson.edu

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ABSTRACT:
Diclofenac sodium, a non steroidal anti-inflammatory drug (NSAID) is an acetic acid derivative with generic name Diclofenac. NSAIDs represent some of the oldest medicines with recorded history of use of people. However, it is also known to inhibit implantation and embryonic development in rats and mammals. It is widely prescribed in India to women of child bearing age for the treatment of various conditions including arthritis, masculo-skeletal pain etc. Research has shown that it also crosses human placenta readily and potentially teratogenic. The present study focuses on assessing the embryonic development in chick under varied dosages of diclofenac sodium at different developmental stages. The results showed teratogenic effects such as omphlaocoele, reduced body size, delay in hatchability and angiogenesis with irregular heartbeats.

Keywords: NSAID, Diclofenac Sodium, Toxicity, Embryology, Non Steroidal Anti-Inflammatory Drug

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INTRODUCTION

Diclofenac, a non steroidal, anti-inflammatory drug (NSAID) is an acetic acid derivative, with generic name Diclofenac. NSAID's represent some of the oldest medicines with the recorded history of use by people. Nonsteroidal anti-inflammatory drugs (NSAID) have been widely used for their anti-inflammatory and analgesic properties. Diclofenac originated from Ciba-Geigy (now Novartis) in 1973 and was introduced in the United Kingdom in 1979.Use of Diclofenac in animals has been reported to have led to a sharp decline in the vulture population in the Indian subcontinent, 95% decline in 2003, 99.9% decline in 2008(as reported by a documentary film from Gov. of INDIA). In Pakistan 35%-95% vulture decline was seen (Lindsay et al., 2004). The mechanism is probably renal failure, a known side effect of Diclofenac. Vultures eat the carcasses of livestock which have been administered veterinary Diclofenac, and are poisoned by the accumulated chemical.

Diclofenac is used widely in the women of child bearing age for the treatment of gynecological problems. Toxicity of Diclofenac has been confirmed on rat embryos (Chan *et al.,* 2001). The chick embryo has been found to be a suitable model system for assessing teratogenesis as it is sensitive to teratogenic agents (Maci, 1980, Carp *et al.,* 1988, Bogdanenko *et al*., 1999). The chick embryo is widely used in studying drugs for teratogenic activity. Developmental abnormalities in chick embryo

have also been reported by Murphy *et al.,* 1956. The effects of many drugs, such as insulin, azaserine, sulfanilamide, thallium, lead, boric acid have been reviewed by various researchers. They also become easy for observations at desired stages and results can also be well documented.

The main objectives of the present studies were to assess the effect of the diclofenac sodium in developing chick embryo, record morphological alterations during the development of chick embryo by comparing the treated and control embryos.

MATERIALS AND METHODS

A total of 170 fertilized eggs divided into two groups as control and treated group were used in this study. The eggs were cleaned with alcohol (70%) and incubated at 37.5^{0}C after marking them as 'control' and 'treated'. Eggs were turned twice a day, as turning keeps the embryo from floating and coming in contact with shell to which it may stick. (Shrack and Darre). Diclofenac Sodium (DS) (Voveran, SR 100 IP) tablets manufactured by Novartis pharmaceutical company were procured commercially from medical stores. The tablet chosen was containing only diclofenac sodium salt and a colouring agent- iron oxide and titanium dioxide. The tablet was found to be completely water soluble. A single tablet of 100mg was dissolved in 100ml of distilled water so as to have 1mg/ml of stock solution. The solution was kept on magnetic stirrer for obtaining a saturated solution. Different concentrations of diclofenac sodium were prepared from the stock solution. For the control group of eggs, 0.9% saline solution (avian saline) was prepared and injected into the eggs. Prior to administering the drug, a minute opening was made into the air space of egg by using a 28 gauze size needle and drug was injected only once by using hypodermic needle (1ml). 150 µl of desired concentration was injected. The details of dosage and duration of incubation are given in Table 1.

Table 1: Experimental Groups (Dosage and Time of Exposure)

Set No.	Dosage (mg/ml)	Duration of Incubation (Hrs.)
1	0.5	96
2	0.4	96
	0.3	96
	0.2	96
3	0.2	48-96
4	0.3	48-96
5	0.3	48 Hrs-Hatching
6	0.03	48 Hrs-Hatching
7	0.03	48 Hrs-Hatching
8	0.03	48 Hrs-Hatching
9	0.03	48 Hrs-Hatching

The embryos of the desired hours of incubation were harvested and the embryos were taken on the slides by the filter paper ring technique for which Whatman paper No. 1 was used. After taking the embryos on the slides, they were kept in acetic alcohol for one hour for the fixation. After the fixation process, embryos were kept in alcohol grades of 70%, 50%, and 30% respectively for ten minutes in each grade, so as to hydrate it. Then they were stained with alum

carmine for 30 to 40 seconds. After staining again they were kept in the alcohol grades of 30%, 50%, 70%, 95%, and absolute alcohol respectively for ten minutes, so as to remove excess stain and to dehydrate the embryo. Embryos were again kept in absolute alcohol for ten minutes to ensure their dehydration. The slides were then kept in xylene for ten minutes and were mounted in DPX. Embryos of more than 5 days were harvested, and kept in acetic alcohol for 60 minutes for fixation. After this they were stored in appropriate vials/bottles containing 10% of formalin in it.

The morphological assessment was done on the basis of following parameters such as formation and branching of blood vessels**,** average count of heart beats**,** anterior to Posterior length of the embryo**,** measurements of head region, beak and limbs, diameter of eye and neck region**,** abdomen region and presence of feather germs

RESULTS AND DISCUSSION

The pilot experiments carried out showed that, administration of the drug after 24 Hrs of incubation ensured growth of the embryo, as earlier results without 24hrs incubation showed mortality. The dosage of 0.3mg/ml of Diclofenac was administered, but the dosage was high and the embryos could not survive after 11 days. Hence, the dosage was diluted 10 times ie., 0.03mg/ml, which showed results and the embryos could hatch out. The details of morphometric measurements are provided in Table-2.

Table 2: Morphometric Measurements

Incubation	AP Length (cm)	Eye Diameter(cm)	Head Width(cm)	Hind Limbs(cm)	Wings(cm)
06 Days	C=1.3 T=1.1	C=0.4 T=0.2	C=0.5 T=0.3	_____	_____
08 Days	C=4.3 T=2.0	C=0.7 T=0.4	C=1.0 T=0.4	C=1.2 T=0.5	C=1.0 T=0.7
10 Days	C=5.4 T=3.8	C=1.0 T=0.6	C=1.6 T=1.0	C=2.3 T=1.3	C=1.8 T=1.3
14 Days	C=6.3 T=5.8	C=1.3 T=1.1	C=2.6 T=2.4	C=3.0 T=2.8	C=2.6 T=2.4
Hatch Out	C=10.1 T=9.7	C=0.6 T=0.4	C=2.4 T=2.1	C=4.5 T=3.9	C=4.4 T=3.9

AP: Anterior – Posterior Length
C: Control Embryos
T: Treated Embryos

After 48hrs of incubation, the cranial and cervical flexures were not seen making broad curves in treated embryos. Blood vessel formation was seen but the development was poor in treated embryos as compared to control ones. Branching of blood vessels was also poor in treated embryos. Heart beat count was lesser (55bpm) in treated as compared to 78bpm in control ones. After 72 hrs. of incubation.

The aim of the present study was to investigate the effects of Diclofenac sodium salt on the embryogenesis of chick. The chick embryo happens to be one of the most preferred animal model for such kind of studies (Drake *et al*., 2006 and Stern, 2004). It has been confirmed that, diclofenac can cross human placenta readily due to its low molecular weight (Siu *et al.,* 2000), following which the drug was administered. Diclofenac is almost completely absorbed, highly protein –bound, penetrates well into synovial fluid, and is extensively metabolized. This may be the reason why only single exposure of the drug was potent to show effects for longer time.

The animal studies have shown that administration of the drug in early gestation,

inhibits implantation and embryonic development (Syed Zaki, 2011). Probably this may be the reason why the embryos exposed to the drug within first 24 Hrs of incubation could not develop further. However incubating the embryos for 24 Hrs and then administrating the drug ensured the survival and growth of the embryos.

Comparative studies suggest that DS has a favourable side effect profile, excellent patient tolerability when compared with Aspirin and other NSAIDs (Skoutakis *et al*., 1988). The results showed that treated embryos had variable heart beats, omphalocele and reduced body size and delay in development as well as hatchability.

The above mentioned defects in developing embryos can be categorized as developmental birth defects which result from interference with the extrinsic breakdown of an originally normal prenatal development of arteries, veins, capillaries. Vaso constriction of embryonic vessels, hypoperfusion and obstruction may cause reduced supply of nutrients (here in the form of yolk) to embryonic tissues which affect development and growth of embryonic structures or result in the tissue loss. (Human Reproduction Update, 2010, Teratogenic Mechanism of Medical Drugs)

Abnormalities seen in heart beat counts may be due to Diclofenac effect on the cardiac muscles by inhibiting the Na^+ current. Diclofenac can inhibit the Na^+ reversibly and the L-type Ca^+ currents irreversibly. More than 10 µM diclofenac can also block Na^+ channels which can cause action potentials. This drug may depress cardiac excitability and contractibility simultaneously (Oleg *et al.,* 2009).

Apart from the above, present study observed that, the abdominal contents in the embryos injected with Diclofenac have actually overgrown / come out of the developing body wall. The thoracic and abdominal regions showed defect in their closure pattern as compared to normal embryos. This is usually referred as Omphalocele. This is due to ventral body wall defects (Mubarak and Asmari, 2011). It is also known as exomphalous (outside the abdominal cavity). Herniation of abdominal contents has also been seen due to effect of calcium salts as studied by Grabowski, 1966. This is a rare congenital abdominal wall defect, where the abdominal organs like small intestine, large intestine, liver (usually), spleen and gonads (occasionally) lie outside abdominal cavity. These are covered with a thin membrane or sac. In case of humans it affects approximately 1 in 5,000 live babies, as in most cases babies get normally aborted because of severe abnormality or are sometimes misadvised termination of pregnancy by gynecologist. While it is normal for organs to develop outside the abdomen of the fetus until the tenth week in-utero, an Omphalocele might develop if they do not return to abdomen after initial period.

The reduction in body size of treated embryos as compared to normal embryos, may be due to Diclofenac inhibitory effect on metabolism. The retardation in the growth of Rats due to diclofenac has also been reported by Carp *et al*., 1988, wherein the diclofenac treated host mothers, 34% of the embryos were growth retarded. However general development of the treated embryos was seen. The delay in growth development has also been seen in diclofenac treated fetuses of laboratory mice (Bogdanenko *et al.,* 1999). The findings from present study show that diclofenac may directly interfere with the normal development of chick embryo and cause growth retardation. The NSAID's are characterized by different rates of metabolism, which is most important in functioning of drugs, the duration of action of NSAID's is related in part, to the high degree of protein binding of drug (Skoutakis *et al*., 1988). As this drug binds to protein, this has advantage that a single exposure to the drug will result in a long term effect rather will affect all the developmental stages.

Almost all NSAID's irritate gastric mucosa and enhance ulceration by blocking the protective action of prostaglandins (ducto inhibition of

Cox) on gastric mucosa causing ulcer formation not only in pyloric sphincter, but also in lesser curvature of stomach where they are called as gastric ulcers (Derle *et al*., 2006).

The treated embryos hatched out showing delay of one day. Such delay in hatching out was also seen in diclofenac treated medaka fishes. The diclofenac shows toxic effects on medaka embryos during embryonic development and around the day of hatching (Naseef *et al.,* 2010).

In the present studies, it has been found that, Diclofenac shows direct teratogenic effect on chick embryos. The teratogenecity of Diclofenac has also been seen on rat embryos (Chan *et al.,* 2001)

CONCLUSION

The above mentioned defects in developing embryos can be categorized as structural birth defects which result from interference with the extrinsic breakdown of an originally normal prenatal development of arteries, veins, capillaries. In the embryo, disruption of newly formed blood vessels/external compression, embolic events, premature regression of embolic vessels, abnormal regulation in vessel formation leads to vascular disruption. Vaso constriction of embryonic vessels, hypoperfusion and obstruction may cause reduced supply of nutrients, here in the form of yolk, to embryonic tissues which affect development and growth of embryonic structures or result in the tissue loss. Overall, it can be concluded that, Dichlofenac induces teratogenicity in chick embryo giving rise to structural deformities.

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