Inhibition of cyclooxygenase-2 alters cranial neural crest cell migration in the developing chick.

Bhaval Parmar, Urja Verma, Kashmira Khaire, Dhanush Danes, Suresh Balakrishnan*

Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Gujarat, India, 390 002

* Corresponding author, b.suresh-zoo@msubaroda.ac.in

ABSTRACT

A recent study from our lab revealed that inhibition of cyclooxygenase-2 exclusively reduces the level of PGE_2 amongst the prostanoids and hamper the normal development of several structures, strikingly the cranial vault, in chick embryos. In order to unearth the mechanism behind the deviant development of cranial features, the expression pattern of various factors that are known to influence the cranial neural crest cell (CNCC) migration were checked in chick embryo after inhibiting the COX-2 activity using etoricoxib. The compromised level of cell adhesion molecules and their upstream regulators, namely CDH1, CDH2, MSX1, and TGF-β, observed in the etoricoxib treated embryos indicate that COX-2, through its downstream effector PGE_2, regulates the expression of these factors perhaps to aid the migration of CNCC. The histological features and levels of FoxD3 as well as PCNA further consolidates the role of COX-2 in migration and survival of CNCC in developing embryo. The results of the current study indicate that the COX-2 plays a pivotal role in orchestrating the proliferation and migration of CNCC during embryonic development of chick.

Keywords: Cranial neural crest cells, embryogenesis, development, cell migration

INTRODUCTION

Cranial neural crest cells (CNCC) are a pool of multipotent cells and fate restricted progenitors, that can differentiate into a multitude of tissue types based on the molecular signals they receive [1]. Their precursors undergo epithelial-mesenchymal transition (EMT) and migrate from forebrain, midbrain and rhombomeres of the hindbrain to populate at pharyngeal arches and contribute in patterning of head and face structures. Once CNCC pass through EMT process they start migration, while doing that, they proliferate and increase the pool of cells. The whole

process of CNCC migration and proliferation is governed by various signaling pathways such as FGF, WNT, TGF-β and BMP [1,2]. When the migration or differentiation of CNCC is disrupted, defects of their derived tissues occur that result in craniofacial malformations, the most common birth defect in humans [3].

Based on the studies involving a wide array of model organisms, it can be construed that the molecular organizers of CNCC migration are conserved across various classes of vertebrates [2,4,5]. The canonical Wnt/β-catenin signaling pathway is reported to play a major role in the formation and progression of CNCC as it influences both delamination and migration by interreacting with BMP4 and TGF-β respectively [6,7,8,9]. Delamination is a collective effort orchestrated by downstream regulators of Wnt3a and BMP4 signaling. Interestingly, BMP also plays a pivotal role in the specification of CNCC into glial cell lineages [10]. However, the proximate regulators of TGF-β such as Snail1, Twist, MSX1, MSX2 and Sox9 maintain the pluripotency of CNCC during migration. Additionally, TGF-β decreases the levels of E-cadherin, with a concomitant surge in N-cadherin, by regulating the expression of Twist, snail, and slug which triggers the transition of cells from epithelial to mesenchymal lineage in the neural tube [11].

Among the variety of regulatory factors reported to be expressed during delamination and migration, one key molecule is COX-2, an inducible isoform of cyclooxygenase which catalyzes the formation of PGE_2 from arachidonic acid [12]. COX-2 mediated PGE_2 synthesis plays a crucial role in cellular events such as cell proliferation, migration, EMT and differentiation by modulating a myriad of signal transduction pathways such as Wnt/β-catenin, BMP and TGF-β [13,14,15]. A study by Jang and coworkers (2009) has shown that COX-2 plays a vital role in inducing EMT in the colon cancer cells by altering the expression of E-cadherin [16]. COX-2 is known to regulate the metastasis of cancerous cells by interacting with TGF-β and its downstream regulators [17].

Study from our lab has shown that inhibition of COX-2 by etoricoxib, an NSAID specific for the isoform, results in developmental defects in limbs, vascularization, tissue integrity, and in organ patterning of developing chick wherein, the most frequently occurring congenital malformations were in the craniofacial region [18]. In this study it was also observed that selective inhibition of COX-2, reduced only the level of PGE_2 while the rest of the prostanoids maintained their normal titer. Based on these findings and the reported possible interactions of COX-2 with different signaling molecules, we hypothesize that the pathways regulating CNCC

delamination and migration might be getting altered due to COX-2 inhibition, resulting in craniofacial defects in developing embryos. In order to validate this notion, in the present study, the activity of COX-2 was inhibited using etoricoxib, a selective COX-2 inhibitor and its effect on the regulators of CNCC migration was ascertained in the chicken embryo.

METHODOLOGY

Animal maintenance

Embryos used for experimentation were isolated from eggs of Rhode Island Red (RIR) breed of *Gallus gallus*. RIR eggs were obtained from the intensive poultry farm, Vadodara, Gujarat, India. Embryological studies were implemented in accordance with the purpose of control and supervision of experiments on animals (CPCSEA), protocols were approved by Institutional animal ethical committee (IAEC; No. MSU-Z/IAEC/09-2020).

Experimental design

The eggs were randomly divided into control and treatment groups. Before treatment, eggs were marked for localization of air cell by candling, thereafter eggs were wiped with an iodine solution. Dose was inserted into air cell of iodine wiped eggs in the sterile chamber (LAF). Eggs for the control group were treated with 50µl of Mili-Q water. Eggs for the treatment group were administered with same volume of 0.07mg/ml of etoricoxib prepared in Mili-Q water. Previous studies regarding dose range was done in the lab from which the 0.07mg/ml dose is selected as it is lower than LD50 value and functions as low observed effective dose (LOED) [18]. The final dose concentration injected in each egg was 3.5µg of etoricoxib in solution form. After the insertion of dose, eggs were incubated at 37±0.5°C and 67±2% relative humidity in a sterile forma environment chamber (ThermoFisher Scientific, USA) until day-1, day-2 and day-3 of embryonic development.

Technical grade etoricoxib was used for dosing of embryo. The solution of etoricoxib was prepared in Mili-Q water by sonication for two hours at room temperature. The solution of etoricoxib was administered into air cell of egg by using insulin syringe. Eggs were incubated till day-1, day-2 and day-3 then embryos of these days were collected. These embryos were analyzed for the mortality and morphological deformities. Control group had 30 eggs while 50 eggs for the treatment group to circumvent treatment induced variance. The Sun-Shepherd

formula was used to nullify the differences arise due to varied sample size such varied sample size amongst groups [19].

Histological study

Embryo isolation was carried out through filter ring method then rinsed in PBS and fixed in 10% neutral buffer formalin. The tissue was further processed and paraffin wax block of the tissue sample were prepared. Transvers section of day-2 embryo were taken using a microtome. These sections were subsequently stained with Harris Hematoxylin and Eosin (ThermoFisher Scientific, USA). The histological details of the tissue of section were visualized using Leica DM2500 microscope and pictures were captured using EC3 camera (utilizing LAS EZ software).

Western blot

The total protein isolation from hard region of embryo was done by lysis buffer with protease inhibitor under cold conditions. Bradford method was used for protein quantification. Proteins were resolved by PAGE consisting of 12% resolving and 4% stacking gels. The resolved proteins were transferred on PVDF membrane by semi dry transfer method. PVDF membrane was immune-stained for N-cadherin, E-cadherin, Vimentin and FoxD3 by using monoclonal antibodies (Sigma Aldrich, USA) diluted in assay buffer in 1:1000 ratios. GAPDH was used as an internal control for protein levels. Secondary antibody was biotinylated IgG and was used to generate colored bands on the membrane.

COX-2 activity assay

The total COX activity including both the isoform COX-1 and COX-2 was detected by using a kit-based assay (Cayman Chemical, USA) for embryos of both control and treatment group. The control, treatment, negative control and positive control were assessed as per the manufacturer's description. The specific activity was calculated by dividing the total protein values derived from Bradford assay. Significance of the data was calculated by performing multiple t-test.

RNA isolation and quantitative RT-PCR

Total RNA was isolated on day-1, day-2 and day-3 from head region of embryos using TRIzol method (Invitrogen, USA) and protocol according to manufacturer's instructions. One step cDNA synthesis kit (Applied Biosystems, USA) used to form cDNA from isolated RNA. Gene

amplification reactions were performed using LightCycler96 (Roche Diagnostics, Switzerland) for CDH1, CDH2, TGFB, WNT3A, TWIST and VIM to identify relative quantities of these in control and treated embryos (Table 1).

Table 1: Oligonucleotide primers used for RT-PCR analysis.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	NCBI ref id	Product length (bp)
WNT3A	TCGGAAACTCCCCTTTCAGC	TGCTCATCTTGCCTGGAG	NM_204675.2	106
TGFB	TCGACTTCCGCAAGGATC	CCCGGGTTGTGTTGGTT	HE646744.1	148
CDH1	GAAGACAGCCAAGGGCCTG	TCTGGTACCCTACCCTCTTG	NM_001039258.2	183
CDH2	AGCCCAGGAGTTTGTAGTG	TTTGGTCCTTTTCTGAGGCCC	XM_025147080.1	114
VIM	GACCAGCTGACCAACGA	GAGGCATTGTCAACATCC	NM_001048076.2	158
TWIST	CGAAGCGTTCACGTCGTTAC	TAGCTGCAATTGGTCCCTCG	NM_204739.2	156
MSX1	CTTACATAGGGCCGAGCCG	CAGGCACAGAACAGATCCCA	NM_205488.2	66
PCNA	TGTTCCTCTCGTTGTGGAGT	TCCCAGTGCAGTTAAGAGCC	NM_204170.2	105
CASP3	AGTCTTTGGCAGGAAAGCCA	CAAGAGTAATAACCAGGAGCG	XM_015276122.2	195
18s rRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC	NR_003278.3	144

The program was set as follows: 3 min at 95°C for initial melting, 35 cycles (each cycle of 10s at 95°C, 10s at 60°C and 10s at 72°C) and last step of 60s at 65°C for extra extension. Melting curves for each well were used to confirm the specificity of the products. 18s rRNA was used as an internal loading control. Mean Cq values of control gene expression was normalized with internal control gene expression of each group. Fold change in expression of both the genes compared to control group was calculated using $2^{-\Delta\Delta Cq}$ values as described by Livak and Schmittgen [20]. Data was analyzed by Student's t-test for significance of the mean difference (GraphPad Software Inc., USA).

RESULTS

COX-2 activity

Etoricoxib at a dose of 0.07mg/ml was administered into the air sac of a fertile egg on day-0 and to check its inhibitory effects, COX-2 activity assay was performed. Significant decrease in COX-2 activity was recorded in treated embryos, during early developmental stages namely HH 6 (day-1), HH 12 (day-2) and HH 20 (day-3), known for EMT and CNCC migration, respectively, when compared to control group (Figure 1).
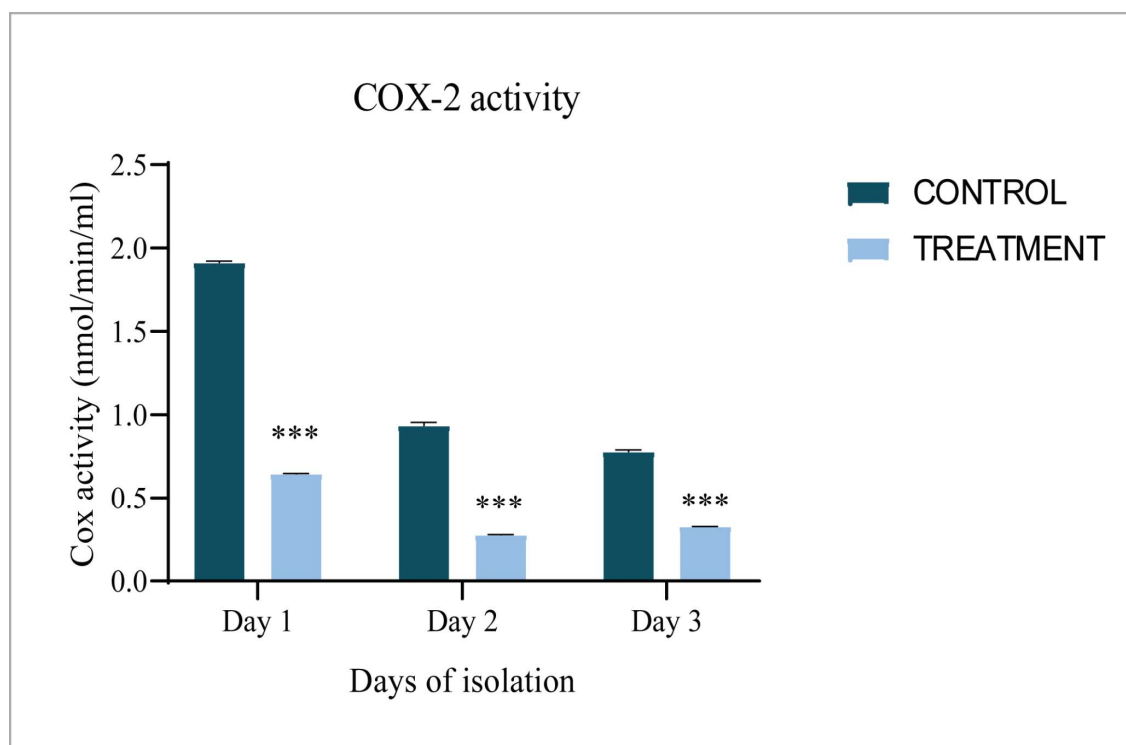
Figure 1. Activity of COX-2 during early development of chick embryo.
COX-2 activity in control and etoricoxib treated group of chick for day-1, day-2 and day-3; ***$p \le 0.001$.

Further, it was noticed that COX-2 inhibition led to marginal yet significant increase in the mortality of embryos at all the stages studied i.e. day-1, day-2 and day-3 (Table 2).

Table 2: Mortality analysis of chick embryos

Group	Day 1	Day 2	Day 3
Control	2 (1,4)***	2 (1,6)***	3 (2,7)***
Treatment	10 (8,13)	13 (8,15)	13 (9,15)

Mortality observed on day 1, 2 and 3 of embryonic development in control and Etoricoxib treated groups. The values are expressed as mode with range in parentheses; n=3 with 30 eggs per group per day; ***$p \le 0.001$.

Hence, 50 eggs were incubated for each group so as to ensure extraction of 30 live embryos for a given experiment.

Gross morphology

The sculpting of cranial features coincides with early embryogenesis and hence, morphology of the cephalic region was studied on day-1, day-2 and day-3 chick embryos. The control group embryos at day-1 showed well-formed neural tube and rhombomere (Figure 2A), whereas in treated group, head fold was disrupted while the neural tube remained distorted and incompletely formed (Figure 2B-E).
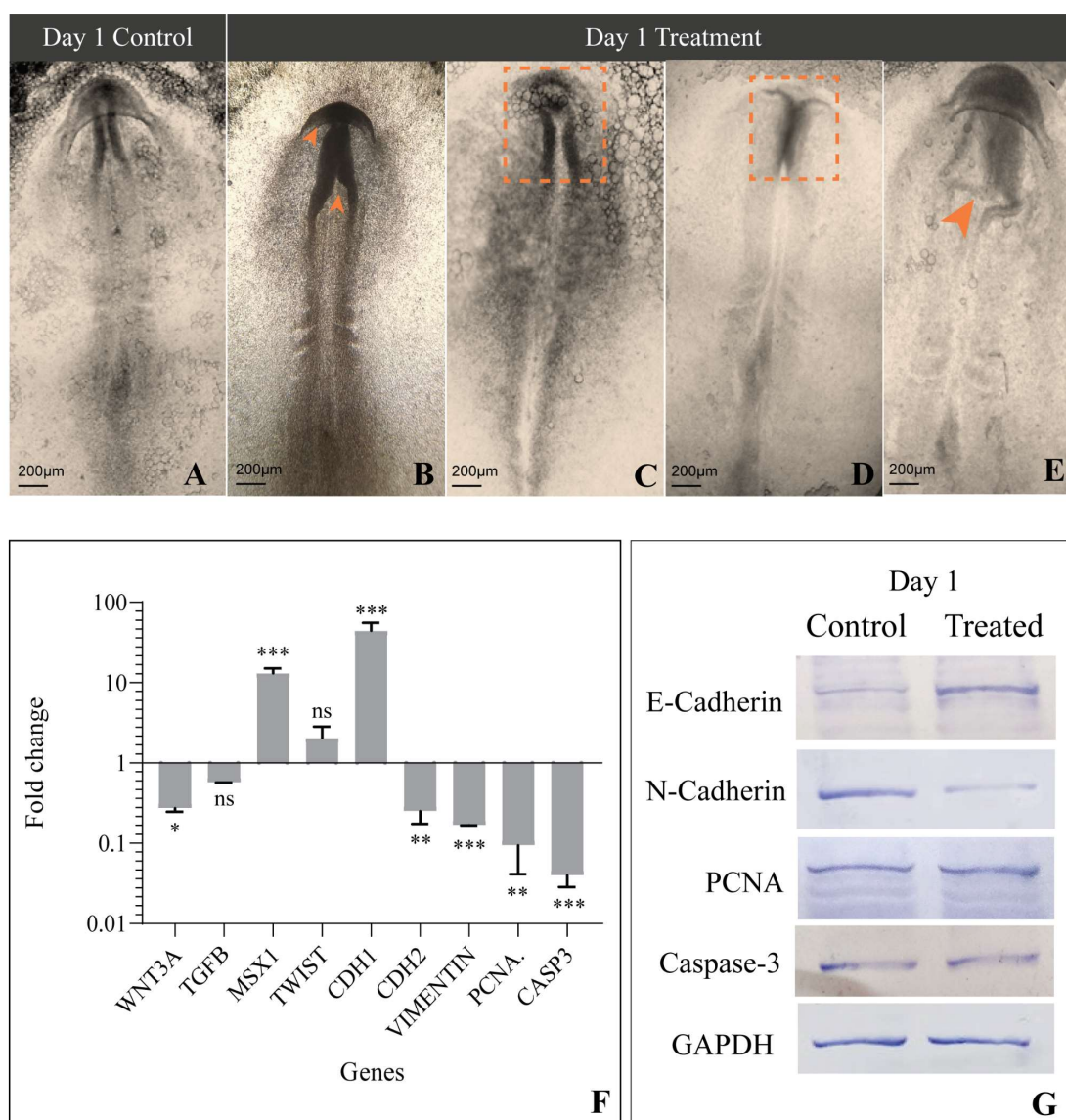
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Figure 2. Morphological and molecular aberration due to COX-2 inhibition in day-1 embryo.
(A) Control embryo with well-developed neural tube, somite and neural fold meet at level of mid brain; **(B)** Etoricoxib treated embryo have defective neural fold formation and meet at level of fore brain (orange arrowhead); **(C)** Embryo treated with etoricoxib showing delayed development and abnormal neural fold formed (orange dotted square); **(D)** The fusion of neural folds impaired in etoricoxib treated embryo (orange dotted square); **(E)** Distorted neural fold marked by orange arrow head in etoricoxib treated embryo; **(F)** mRNA expression pattern of the genes involved in regulation of neural crest cell migration for etoricoxib treated embryo. Values are expressed in fold change (Mean ± SEM). Fold change values are compared with control embryo for all the genes (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$); **(G)** western blot image showing comparative expression of various proteins: E-cadherin, N-cadherin, PCNA, Caspase-3 on day 1. GAPDH was taken as loading control, (n=30).

Further, at day-2 embryos of control group showed defined morphology, with compact neural tube, perfectly formed rhombomeres, optic vesicle and optic stalk (Figure 3A), while treated ones displayed improper closure of the neural tube, reduced number of somites along with distorted rhombomeres and optic stalks (Figure 3B-D).
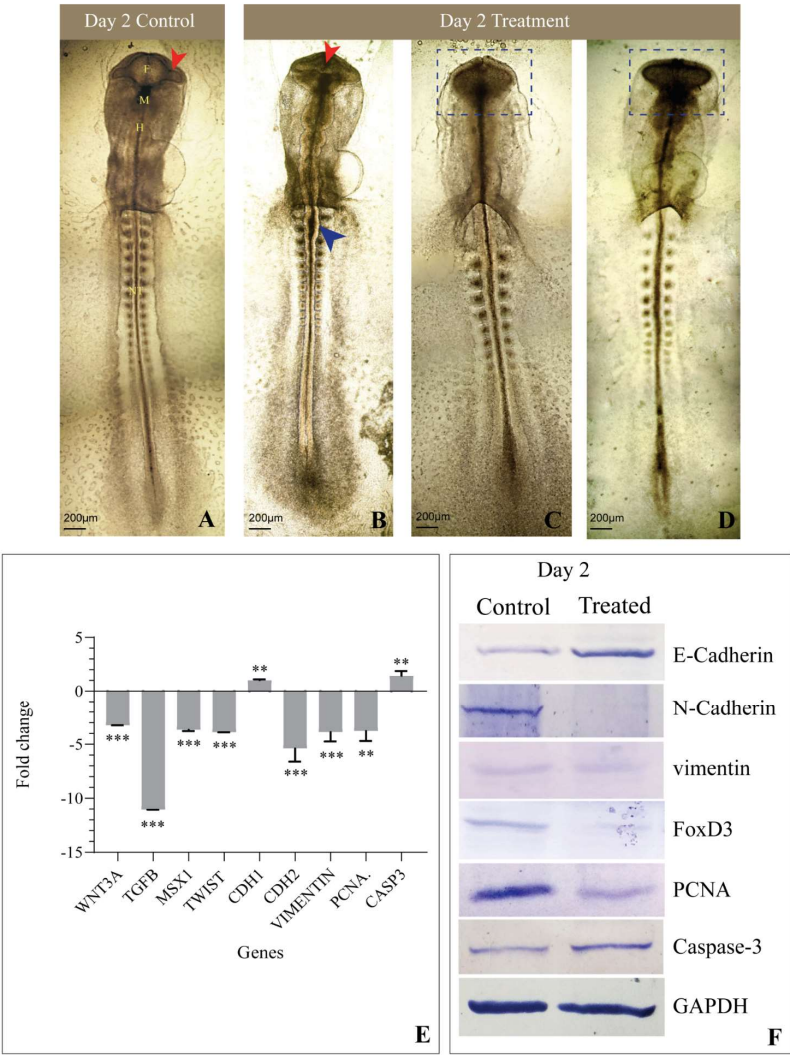

Figure 3. Structural and molecular changes under COX-2 inhibition in day-2 embryo
(A) Control embryo with well-developed primary optical vesical, optic stalk (red arrow head) and three primary brain vesicles are clearly visible (where, F-fore brain, H-hind brain, M- mid brain, NT-neural tube); **(B)** Etoricoxib treated embryo shows open neural tube (blue arrow head), incomplete fore brain and optic vesicle (red arrow head) and reduced number of somites; **(C)** The fore brain, mid brain and hind brain are not formed, absence of optic stalk and optic vesicle (blue dotted square) in etoricoxib treated embryo of day 2; **(D)** Deformed fore brain region, optic stalk and optic vesicle are not formed (blue dotted square) and less number of somites are formed in etoricoxib treated embryo. **(E)** Transcript level expression of genes involved for migration of neural crest cells in etoricoxib treated day 2 embryos. Values are expressed in fold change (Mean ± SEM). Fold change values for control embryo are compared for all the genes (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$); **(G)** western blot image showing comparative expression of E-cadherin, N-cadherin, FoxD3, Vimentin, PCNA and Caspase-3 on day-2. GAPDH was taken as loading control, (n=30).

By third day, control group embryos showed well-structured curved head, perfectly formed optic vesicles and pharyngeal arches, where neural crest cells further differentiated to form various structures such as frontonasal processes and mandible (Figure 4A). However, etoricoxib treated embryos showed malformed head with no optic vesicle and pharyngeal arches (Figure 4B-D).
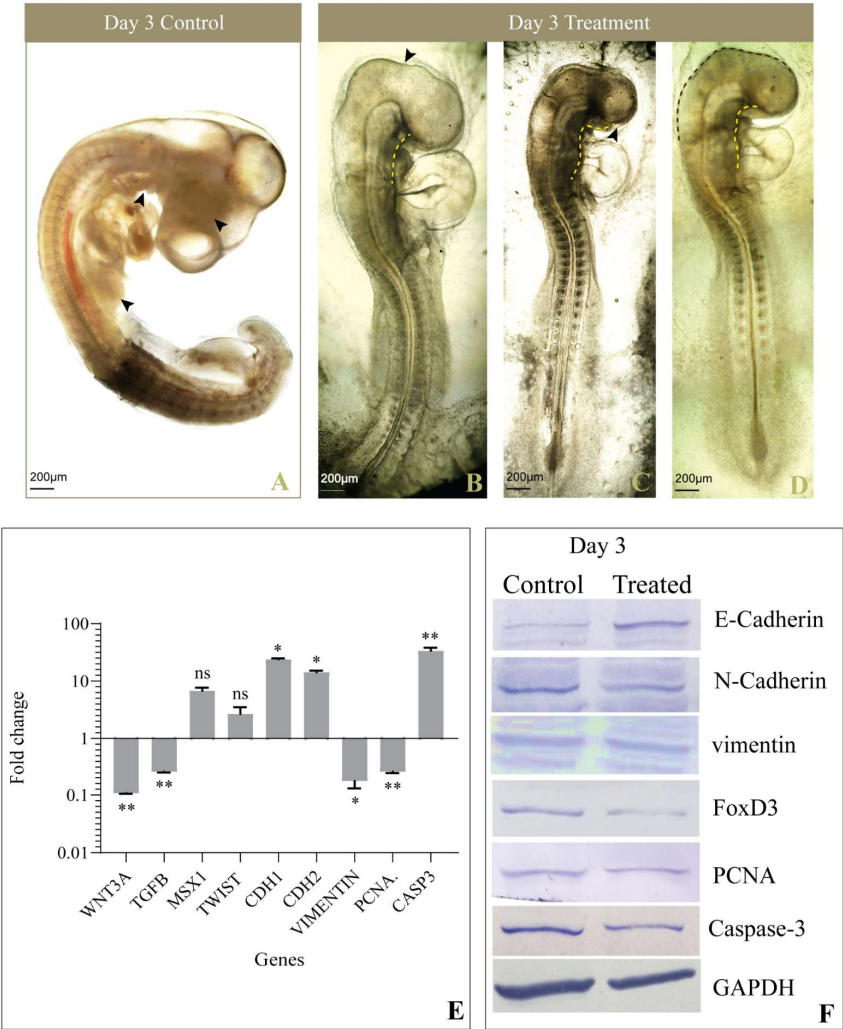

Figure 4. Morphological and molecular observations of day-3 embryo after treatment.
(A) Control embryo showing completely developed fore brain, mid brain and hind brain, visceral clefts, limb buds and primitive eye (black arrow head); **(B)** Treated embryo indicating poorly developed brain regions (black arrow head), absence of visceral cleft (yellow dotted line) and limb buds; **(C)** Etoricoxib treated embryo of day 3 showing absence of distinct brain regions, abnormal curving of body, visceral clefts not formed (yellow dotted line) and absence of primitive eye; **(D)** Under the treatment of etoricoxib embryo showing abnormal visceral cleft (yellow dotted line) and head region is defectively formed (brown dotted line); **(E)** Transcript level of genes regulating head formation and neural crest cell migration in etoricoxib treated day 3 embryos. Values are expressed in fold change (Mean ± SEM). Fold change values for control embryo is compared for all the genes (*: $p \leq 0.05$, **: $p \leq 0.01$, ns: not significant); **(F)** Western blot image showing comparative expression of E-cadherin, N-cadherin, FoxD3, Vimentin and PCNA on day 3. GAPDH was taken as loading control, (n=30).

Histological observations

The extent of hindrance in the migration of cranial neural crest cells upon COX-2 inhibition was studied by differential staining of day-2 chick embryo using hematoxylin and eosin stains. The control embryos showed well-formed CNCC, migrating towards the dorsal side of the neural tube, and a perfectly formed sclerotome (Figure 5A).
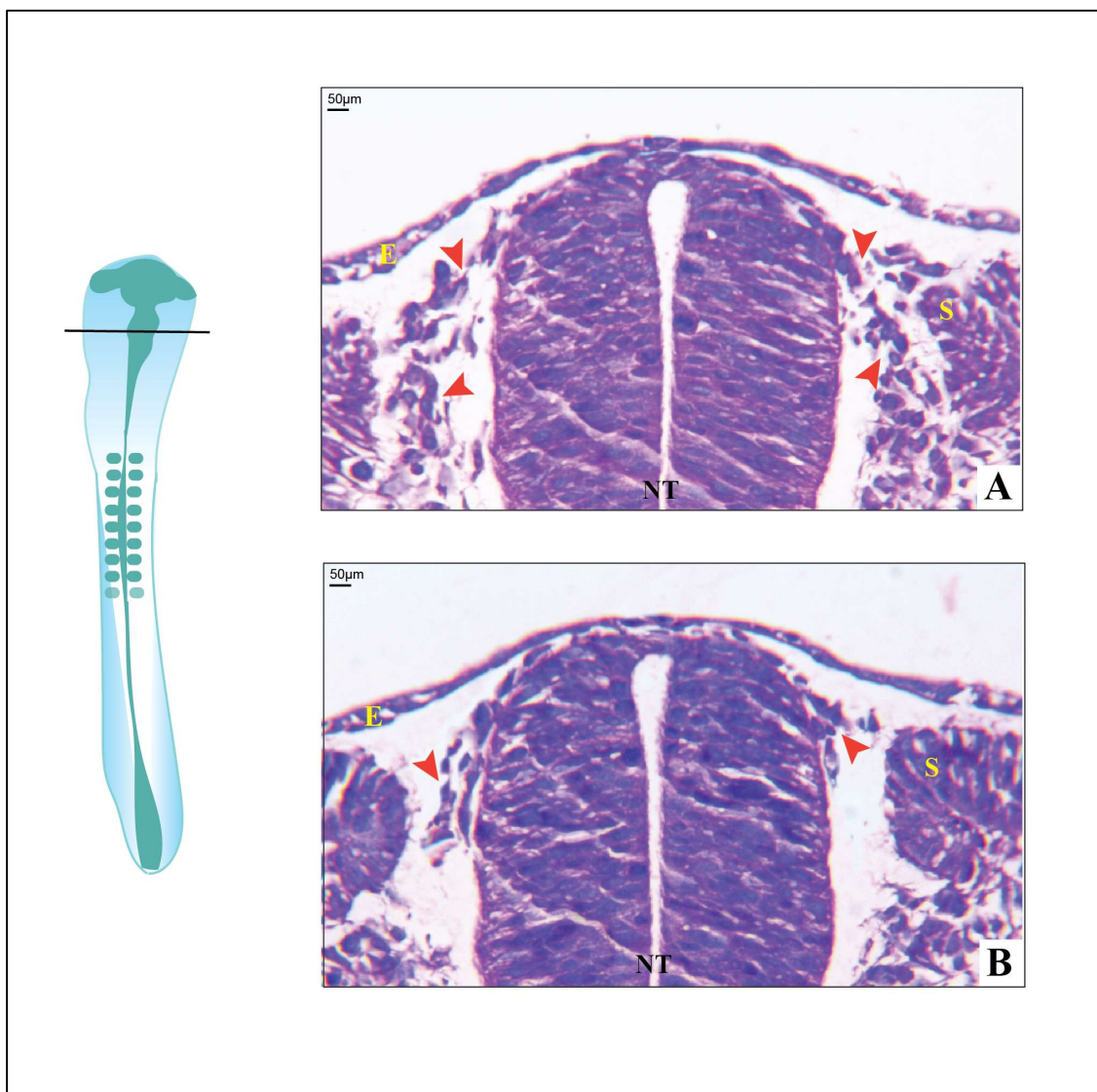

Figure 5. Histology of chick embryo in transverse section
(A) Day-2 control with well-developed neural crest cells and migrating toward ventral side of the neural tube (red arrow head); **(B)** Day-2 etoricoxib treatment number of neural crest cell are less and migration is delayed (where, E-epithelium, N-notochord, NT-neural tube, S-sclerotome).

In contrast, treated embryos showed sparsely distributed CNCC indicating delayed formation and migration. The architecture of the sclerotome too was found improper (Figure 5B).

Expression of genes involved in EMT and CNCC migration

As mentioned previously, during CNCC migration Wnt/β-catenin and TGF-β along with their downstream regulators play important role in EMT, cell survival and proliferation. Hence, the transcriptional status of WNT3A, TGFB, MSX1, TWIST, CDH1, CDH2, VIM, PCNA and CASP3 were examined across all three days in the control and treated embryos. On day-1, the treated embryos elicited a significant reduction in the expression of WNT3A while TGFB transcripts reduced marginally, when compared to control ones. Meanwhile, MSX1 showed a remarkable rise of ten folds in treated embryos. Further, TWIST transcripts increased negligibly in treated embryos along with a conspicuous increase in the CDH1 gene expression at day-1, when compared to control embryos. Further, a noticeable reduction in CDH2 and VIM gene expression was recorded in COX-2 inhibited embryo at day-1. Simultaneously, PCNA and CASP3, significantly decreased at the same time (Figure 2F). On day-2, the etoricoxib treated embryos showed a remarkable reduction in expression of migration specific genes namely, WNT3A, TGFB, MSX1, TWIST, CDH2 and VIM whereas CDH1 and CASP3 transcripts increased significantly (Figure 3E). PCNA, a known cell proliferation marker reduced noticeably at day-2, when compared to control ones. Further, on day-3, the levels of WNT3A, TGFB, VIM and PCNA transcripts decreased significantly and MSX1 expression slightly raised in the treated embryos. TWIST showed a marginal elevation at this time while significant increase in the levels of CDH1, CDH2 and CASP3 was observed (Figure 4E).

Levels of proteins involved in EMT and CNCC migration

Protein expression of key regulators of EMT, CNCC proliferation and migration like E-cadherin, N-cadherin, FoxD3, vimentin, PCNA and cleaved Caspase-3 was measured through western blot followed by the densitometric analysis of the bands. The results revealed significant upregulation of E-cadherin in the treated embryos when compared to the respective control ones, across all the three stages studied (Figure 2G, 3F, 4F). At day-1, N-cadherin reduced significantly in the treated embryos, which continued for the following stages (day-2 and day-3) as well, when compared to control. FoxD3 protein expression was not detected at day-1 in both control and treated embryos while at day-2, it reduced remarkably in the treatment group. Further at day-3 as well, the level of FoxD3 remains low in treated embryos as compared to control ones (Figure 3F, 4F). Another crucial EMT regulator namely vimentin was not recorded in both control and treated day-1 embryo while the day-2 treated ones showed no visible alteration when compared to the respective control. Although on day-3, treated

embryos expressed slight reduction in its expression when compared to day-3 control. Parallel analysis of PCNA revealed that, when compared to control, its expression remains un altered at day-1 (Figure 2G), which further plunges significantly at the following stages (day-2 and day-3) (Figure 3F, 4F) cleaved caspase 3 on the other hand reduced marginally on day-1 in treated embryos, while its level increased at day-2 significantly, when compared to the respective controls. At day-3, its level elevated negligibly when compared to respective control. GAPDH was used as an endogenous control for the western blot analysis.

DISCUSSION

Cranial neural crest cells are the most important pool of progenitors, which migrate and form various structures of the facial region such as fronto-nasal, maxillary and mandibular prominence in the developing embryos [21]. In order to understand the mechanisms underlying the CNCC migration, which plays crucial role in early development, avian model is found to be most suitable. It is closely related to mammals and migration pattern of CNCC is identical and conserved in both these classes of chordates [22]. The factors responsible for CNCC migration are widely accepted to be conserved across various classes of vertebrates. One such factor is COX-2, a member of the cyclooxygenase family involved in cellular processes such as proliferation, migration, angiogenesis and differentiation [12,13,23]. It is found to be present during the developmental period of the embryo which regulates the sculpting of various organs [18]. It is conserved amongst vertebrate species but its role in embryonic development is still not very well explored. In zebrafish COX-2 derived PGE_2, promotes embryonic vasculature maturation [24,25]. These studies substantiate that COX-2 is involved in the governance of cellular and molecular processes required during early development of embryos. Hence, in the current study involvement of COX-2 in migration of CNCC was investigated by using its specific pharmacological inhibitor etoricoxib.

COX-2 activity assay was performed to confirm the inhibition, while it was localized at the region of the neural tube in the developing embryos of day-1, day-2 and day-3 [18,26]. In the present results as well, reduction in COX-2 activity has caused alteration in levels of major regulators of the neural crest cell migration pathways in treatment group of embryos. The expression of TGF-β and Wnt3A along with their downstream signaling factors TWIST, MSX1, FoxD3, vimentin, CDH1 and CDH2 was found disturbed under the COX-2 inhibition.

Buch and his colleagues have illustrated alteration in Wnt/β-catenin signaling under the inhibition of COX-2 which hampers the regeneration of the lizard tail [27]. The involvement of Wnt3A in delamination of CNCC has been reported during early embryogenesis of chick [28,29]. In the current study, a significant reduction in the expression of WNT3A was noticed upon COX-2 inhibition. Its mRNA level decreased at day-1 and continued to stoop across day-2 and day-3 in etoricoxib treated embryos, when compared to the respective controls (Fig. 2F, 3E, 4E). Decreased WNT3A would have led to a smaller number of CNCC formed, under etoricoxib treatment. Also, histology results showed that the CNCC formed, were disoriented and lesser in number as compared to control, hence proving the impact of reduced COX-2 activity on the normal CNCC formation and function during early development.

Along with Wnt, TGF-β also showed an unstable trend at day-1, day-2 and day-3 embryos treated with etoricoxib. The gene expression of TGF-β continuously reduced across all the stages in treated embryos. Sela-Donenfeld and Kalcheim have reported that alteration in TGF-β disturbs the migration pattern, since it plays a crucial role in the switching of cadherins during CNCC delamination [30]. MSX1 and TWIST, the downstream mediators of TGF-β also showed alteration in gene expression at all the stages (day-1, day- 2 and day-3) in etoricoxib treatment group.

Among the downstream regulators of TGF-β, MSX1 controls cellular proliferation and differentiation during early embryonic development. It is highly expressed in CNCCs, which plays an important role in regulating EMT process during embryonic development [31]. In the current results significant rise in MSX gene expression at day-1 was followed by continuous reduction at day-2 and day-3 which substantiates the results obtained through histology. The CNCC might have delaminated at day-1, due to elevation in MSX1 gene expression, although they could not survive or migrate to their destined locations, under the reduced COX-2 activity. Parallel to MSX1, TWIST gene expression also showed concomitant changes at all stages observed here, which further consolidates the idea of cell-survival and cell-turnover running congruently to each other [32,33].

It is well established that epithelial cells undergo EMT process, when E-cadherin is repressed and the cell-cell adhesion is negatively affected. This further leads to cystoskeletal changes in the cells, thus allowing motility [11]. In addition, loss of N-cadherin is known to cause interruption of directed migration phenotype in Xenopus neural crest cells [34]. On the other hand, EMT is a function of well-regulated levels of E-cadherin, N-cadherin and vimentin

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[35,36,37]. Decline in CDH1 and escalation in CDH2 levels lead to the initiation of EMT in neural tube to form neural crest cells [38]. In the present study, when compared with respective controls, embryos facing compromised COX-2 activity displayed rise of E-cadherin at both gene and protein levels, along with subsequent reduction in vimentin and N-cadherin at day-1 and day-2, which visibly impeded both, CNCC formation and migration. Although, by day-3, both CDH1 and CDH2 rise back to a similar level, which could be due to the compensatory nature of the embryo. Meanwhile, gene and protein levels of vimentin remained low, even at the third day, indicating the disturbed EMT. This resulted in perturbed patterning of head region, optic vesicle and neural tube in the treated embryo.

Further the expression of FoxD3 was checked as it is a pivotal marker of CNCC, facilitating their survival [39,40]. It has been documented that FoxD3 regulates the expression of cell-adhesion molecules like E-cadherin and N-cadherin [41]. In current study its protein level expression was recorded from day-2 onwards in both control and treatment groups. This reinforces the fact that FoxD3 expression only begins post HH 8 stage in a developing chick embryo [42]. In treated embryos, FoxD3 protein expression plunged at both day-2 and day-3, when compared to the respective controls. This implies the deleterious effects of the drug on the CNCC titer and also its migration.

In order to comment upon the cell proliferation in CNCC, PCNA transcript and proteins were checked and found to be significantly reduced across all the three days, in treated embryos, compared to control ones. In accordance with this data, cleaved caspase also showed major upregulation in gene and protein expression at the three stages, in treatment group. These results direct towards the derailed cell proliferation of the delaminated CNCC due to lacking PCNA status, while analogous rise in level of caspase triggers cell death instead. Thus overall, the CNCCs are unavailable for normal patterning of frontonasal prominence, in the treated embryos due to deleterious impact of perturbed COX-2 function.

Based on our observations, it could be construed that COX-2, perhaps through its downstream effector PGE_2, regulates the temporal expression pattern of the factors responsible for CNCC formation, proliferation and migration. Any alteration in the normal titer of COX-2, by accidental prenatal exposure to its commonly used pharmacological inhibitors, would result in craniofacial dysmorphism as observed in the current study due to dysregulation of cranial patterning.

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