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Roles of cyclooxygenase-2 in cardiac neural crest cells differentiation and patterning of the heart tube

INTRODUCTION

Congenital heart defects (CHD) are heart abnormalities that occur before birth while the embryo is still developing in the uterus. CHDs occur in more than 1% of live births and are the leading cause of infant mortality and a risk factor for cardiovascular disease later in life (Pierpont et al., 2007). These defects can be mild, initially not affecting the heart's function, but leading to cardiac complications later in life, or life-threatening, contributing to the majority of mortalities. The spectrum of CHD is extremely broad and can result in outflow tract (OFT), chamber separation, and disrupted valve formation. Out of all these defects, OFT covers about 20–30% of all CHD cases (Neeb et al., 2013). In OFT defects, the most severe is Persistent Truncus Arteriosus (PTA), defined as a complete absence of aortopulmonary septation, leading to OFT narrowing.

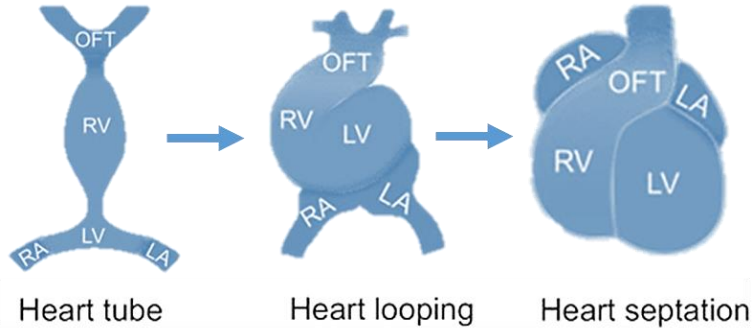

Figure 4.1: Early development of vertebrate heart showing heart tube looping and septation for chamber formation (OFT-outflow track, RV-right ventricle, LV-left ventricle, RA-right auricle, LA-left auricle)

The cause of OFT septation defects is cardiac neural crest cell ablation, which alters ventricular functions and decreases ventricle ejection fraction (Creazzo et al., 1998). The vertebrate heart develops from the lateral plate mesoderm that converges at the embryonic

midline and fuses to form the heart tube. Looping of the heart tube leads to remodelling and forms heart chambers; two atria and two ventricles (Kathiriya & Srivastava, 2000). The looped heart tube connects to the developing lung through the truncus arteriosus, while initially it is a single vessel destined to form the OFT (Fig 4.1). Cardiac neural crest cells are essential in OFT and Secondary heart field formation. The OFT septate into different vessels, which are important for blood exchange in the pulmonary circulation (Knierbein et al., 1992).

Neural crest cells (NCCs) initially reside in the neural plate border territories and develop in the lateral part of the ectodermal neural plate while in contact with the neural epidermis. During neurulation, neural plate borders fuse, a neural tube is formed, and nascent NCC migrates from the dorsal region of the neural tube (Morales et al., 2005). Neural crest cells arise from the border of neural plate and epidermal ectoderm in rostrocaudal manner and migrate to prosencephalon (Forebrain), mesencephalon (Middle brain), and rhombencephalon (Hindbrain). The NCC population migrate to various regions of developing embryo and is composed of three types: Cranial neural crest cells, Cardiac neural crest cells and trunk neural crest cells. It has been shown that NCCs originating from distal cervical rhombomeres provide the majority of cardiac NCC, contributing to cardiac structure development such as OFT (Kirby et al., 1983; Kirby and Hutson, 2010).

Cardiac neural crest and patterning of the heart

A sub-region of cranial neural crest ablated by Dr Kirby has been called the ‘‘cardiac neural crest’’, not because the cells of this region migrate solely to the heart but for the importance of crest-derived ectomesenchyme in cardiovascular development (Waldo et al., 1998). Cardiac neural crest cells originate from the dorsal neural tube between the mid-otic placode to the posterior border of somite three (Fig 4.2). Several days after the neural crest cells have emigrated from the dorsal neural tube to the pharynx, a subpopulation of cells migrate into the cardiac outflow cushions (Fig 4.2). Here then condense to form the aorticopulmonary septation complex in OFT (Waldo et al., 1998; Waldo et al., 1999), dividing the common arterial outflow into the aorta and pulmonary trunk (Fig 4.2).

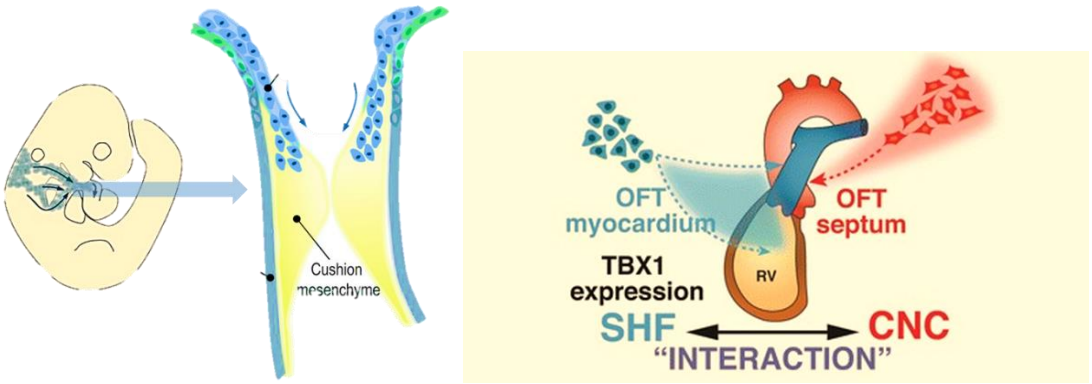

Figure 4.2: Cardiac neural crest cells migrate in the developing heart tube, forming OFT and SHF (Kodo et al., 2021)

The first classical chick embryological experiments showed that surgical removal of cardiac neural crest cells leads to phenocopies of human PTA (Besson et al., 1986, Kirby & Waldo, 1995). Along with OFT formation, cardiac neural crest cells also help in the looping of the heart tube in chick embryo (Yelbuz et al., 2002). Initially, the straight heart tube bends and forms a C-shaped tube which is very important for the alignment of cardiac septa. It has been postulated that the differential rate of proliferation of pre-cardiomyocytes and regional differences in actin bundles alter cell adhesion across the tube and leads to a change in the shape of the heart tube (Fig 4.3), which is known as looping (Cluzeaut & Maurer-Schultze, 1986).

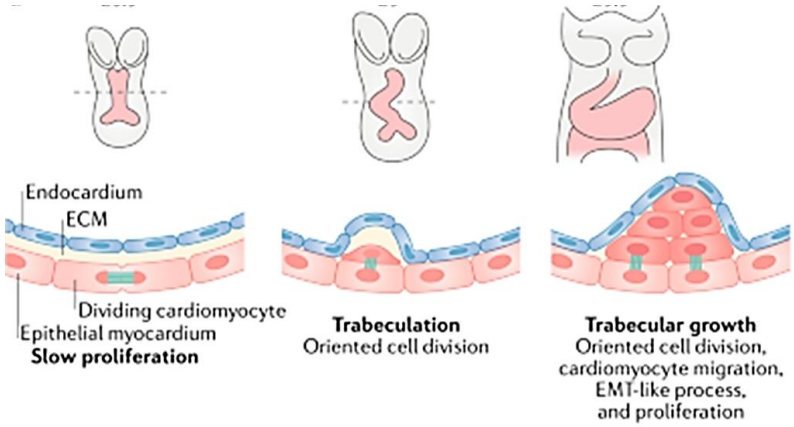

Figure 4.3: Cellular events during heart development (MacGrogan et al., 2018)

A combination of embryology and cellular approaches has led to a clearer picture of signalling pathways essential for heart patterning. Various signalling pathways are critical for a developing heart in vertebrates. These pathways involve the interactions between signalling molecules and their receptors, changing the expression pattern of transcripts, proteins and cellular behaviour.

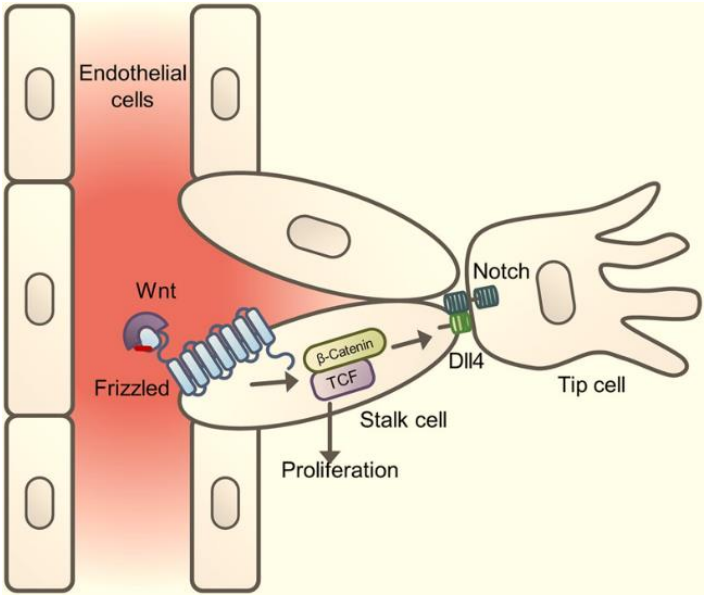

Figure 4.4: Cross-talk between Wnt signalling and endothelial cells during heart formation (MacGrogan et al., 2018)

Wnt signalling plays a crucial role in the formation of the heart during gastrulation (Marvin et al., 2001). *WNT3a, WNT5* and *WNT11* signalling are involved in several aspects of heart development, including the formation and proliferation of cardiac progenitor cells, specification of cardiac cell fate, and patterning of the heart tube (Fig 4.4) (Gessert & Kuhl, 2010). *WNT11* has been shown to promote the differentiation of cardiac progenitor cells into cardiomyocytes by upregulating the expression of *GATA4* (Afouda et al., 2008). *WNT11* signalling is involved in multiple aspects of heart development, including the formation and proliferation of cardiac progenitor cells, the cardiac cell fate specification, and the heart tube patterning (Pandur et al., 2002). In particular, *WNT11* has been shown to promote the differentiation of cardiac progenitor cells into cardiomyocytes comprising the heart muscles. In mice, Wnt and BMP control the differentiation of cardiac progenitor cells into cardiomyocytes (Klaus et al., 2007).

A member of TGF-β family of signalling molecules, BMPs are also crucial in the early stages of heart development. BMPs are secreted proteins that regulate the differentiation of the cardiac mesoderm into the different regions of the heart. Studies in vertebrates have shown that three members of BMP family (*BMP2, BMP4* and *BMP10*) express within the cardiogenic region of the primitive streak in chick embryos (Schultheiss et al., 1997). *BMP4* signalling is involved in the specification and differentiation of cardiac progenitor cells, which give rise to the different cell types in the heart. *BMP4* promotes the differentiation of cardiac progenitor cells into myocardial and heart muscle cells by

regulating *GATA4* expression in mice (Nemer & Nemer, 2003). *BMP4* also plays a role in the formation and patterning of the heart tube. Studies in the chick embryo showed that tissue-specific inactivation of BMP receptors causes the failure of OFT due to defects in cardiac neural crest survival and migration (Boot et al., 2003).

SHH promotes the differentiation of cardiac progenitor cells into the endocardial and myocardial cell lineages. Shh regulates the expression of *Gli, NKX2.5* and *TBX20* transcription factors involved in the differentiation of the cardiac mesoderm and the formation of the heart chambers. Shh helps the cardiac neural crest cells to survive and populate them in OFT. Studies have shown that SHH signalling controls the proliferation and differentiation of cardiac cells during embryonic development (Tsukui et al., 1999).

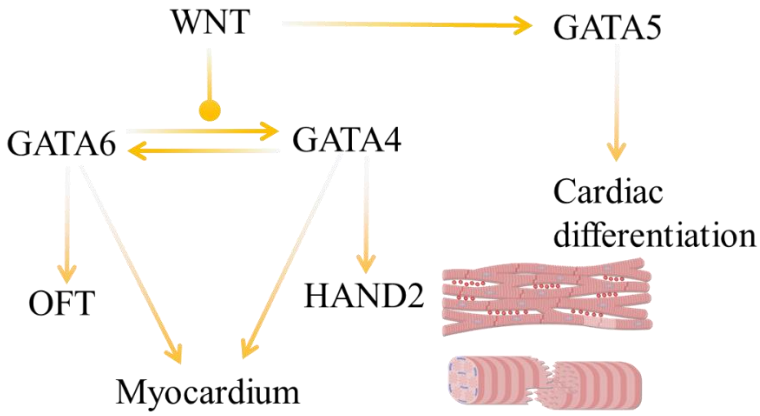

Figure 4.5: Molecular interaction of cardiac cell differentiation (Koshiba et al., 2016)

Along with major signalling molecules, transcription factors such as *GATA4*, *GATA5*, and *GATA6* play a crucial role in heart development. *GATA5* is expressed in a developing heart during the formation of the atria and ventricles. It is also expressed in the endocardium, the innermost layer of the heart (Peterkin et al., 2005). BMP signalling promotes the expression of GATA5 in the endocardium and is required to properly develop the heart valves (Reiter et al., 1999). Further, Wnt signalling promotes the expression of *GATA5* in the developing heart and is involved in the formation of the atrioventricular canal (Martin et al., 2010). Studies revealed that mutation in GATA5 prevents the formation of cardiac and endothelial tissue in mouse (Jiang et al., 2013). *GATA6* is essential for the differentiation and proliferation of cardiac progenitor cells (Fig 4.5). The expression of *GATA6* is induced by the Wnt signalling pathway and the transcription factor *Tbx5* (Laforest & Nemer, 2011).

These signalling molecules are critical for properly developing the heart in vertebrates. Interactions between these signalling pathways result in necessary gene activation and inhibition, which leads to cellular behaviour necessary for the formation of the different regions of the heart and the heart valves. A plethora of potent signalling pathways have been implicated in cardiac neural crest cell migration and heart development; one more such signalling molecule, cyclooxygenase-2 (COX-2), was studied here in the patterning of the heart.

The literature talks about a plethora of molecules involved in orchestrating the development of the heart by facilitating neural crest cell migration and heart development. In this work, we tried to unravel the potential role of one such signalling molecule named Cyclooxygenase-2 (COX-2) during heart tube patterning.

COX-2 is an enzyme that plays a role in the inflammation response and is expressed in various tissues, such as the kidney and bladder, in adults, but their presence during development is unexplored. Studies have shown that COX-2 is expressed in developing embryos and upregulated in zebrafish's developing heart regions (Cappon et al., 2003). COX-2 regulates various aspects of cellular events, such as proliferation, migration and differentiation in adults during inflammation (Nakanishi et al., 2001; Singh et al., 2005; Zhang et al., 2002). Specifically, COX-2 has been shown to play a role in the differentiation of cardiac progenitor cells into cardiomyocytes and in regulating cardiac contractility in adult mice (Zhang et al., 2016). Despite its potential role in heart disease, the exact mechanisms by which COX-2 affects heart development and function still remains an active area of research. That is why a study was conducted to understand the role of COX-2 in cardiac neural crest cells and early patterning of the heart using the chick as a model system. Further understanding of the regulation and function of COX-2 in the early patterning of the heart may lead to unearthing the reasons for CHD and help to find a therapeutic approach.

MATERIALS & METHODS

The study was aimed to determine the role of COX-2 in the patterning of vertebrate hearts. Development of the heart starts from the HH10 stage, at which time the heart field defined in chick embryos. Subsequent stages from HH12 to HH20 stages, are crucial for various

processes of heart development such as looping, OFT formation, wedging and trabeculation. At the same stages, the cardiac neural crest cells migrate and reside in pharyngeal arches, forming OFT (Hutson & Kirby, 2007).

Insitu hybridization was performed to detect the presence of COX-2 mRNA in the heart tube region. The nucleotide sequence for chick COX-2 was obtained from the NCBI database. A nucleotide blast was used to generate a COX-2 template. The template for probe generation was obtained by RT-PCR and cloned into a pGEM-T vector using a cloning kit (Promega, USA). Competent DH-alpha cells were transformed with construct and screened for the presence of the plasmid with insert. The orientation of the insert was determined by using restriction enzyme and DNA sequencing. Plasmid was linearized, and the RNA probe was generated by invitro transcription using a DIG-labeled kit (Roche, USA). The prepared DIG-labeled probes were quantified and processed for hybridization. The embryos were fixed in 4% PFA, digested with Proteinase K for 5 min, washed and incubated in a hybridization solution with appropriate probes. To detect bound probe, embryos were incubated in BCIP and NBT solution.

The freshly laid eggs were cleaned and divided into control and treatment (etoricoxib) groups. The treatment group of eggs was dosed after an incubation of 24hrs to avoid the effect on the neural crest cell migration. Dosing was done as explained in the material and methods section of the thesis. Heart tissue from the embryos was collected on HH12 and HH20 developmental stages and further analyzed for COX-2 activity and localization of COX-2. The morphology of embryos was checked in both groups, and heart tubes were dissected out to understand the effect of etoricoxib.

Further histological analysis was carried out by cryo-sectioning the developing heart tube and staining (H & E). Moreover, to understand the effects of COX-2 inhibition at the molecular level, transcripts and protein levels of heart patterning molecules were analyzed using q-RT PCR and western blot. All the data were statistically analyzed and represented in the form of graphs.

RESULTS

Presence of COX-2 in developing heart

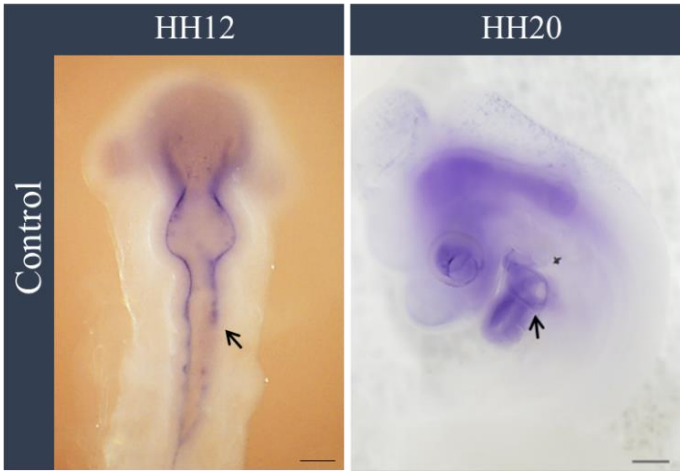

Figure 4.6: *In situ* hybridization on HH12 and HH20 stage of development of control chick embryos. Blue-stained regions indicate the presence of COX-2 mRNA

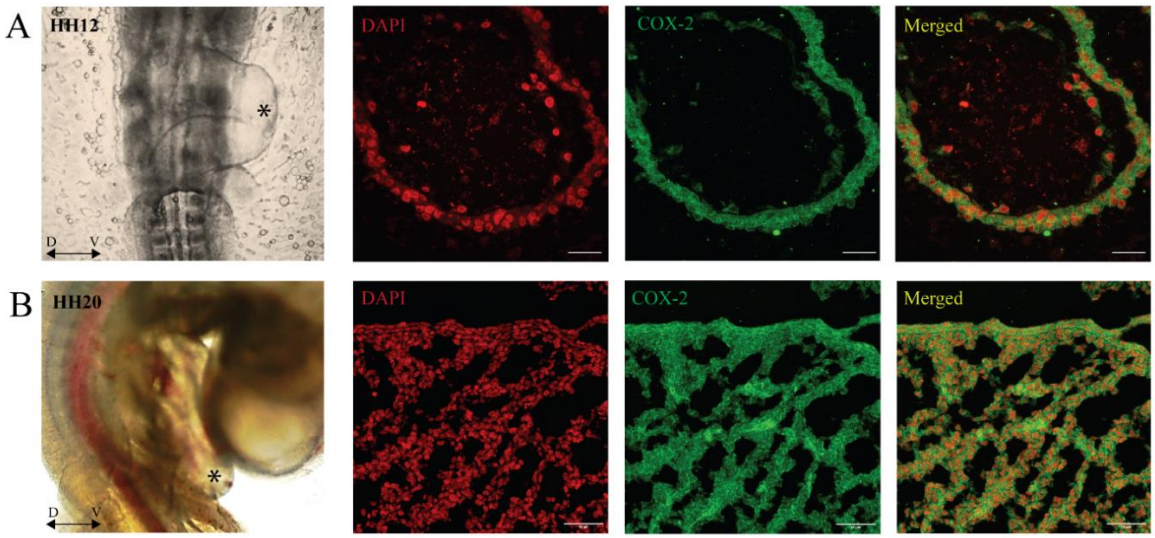

Figure 4.7: Immunolocalization of COX-2 in HH12 and HH20 heart sections of control embryos (A) In HH12 (day 2), control embryo displayed COX-2 localization in the cytoplasm of a single layer of cardiomyocytes forming the developing heart tube on the ventral side. The black asterisk indicates the section plane within the heart region (40X). The black asterisk indicates the section plane of the heart region. (B) HH20 (day 3) the control embryo exhibited COX-2 protein expression in the cytoplasm of cells located on the outer wall of the heart, while COX-2 was also present in the trabecula (40X).The black asterisk shows the plane of section of the heart region, D ↔ V: represent Dorsal-Ventral orientation

In situ hybridization shows the presence of mRNA of COX-2 in developing heart tubes on the HH12 and HH20 stages of chick embryos (Fig 4.6). Moreover, immunolocalization of

COX-2 indicates the COX-2 protein is expressed in the cells of developing heart on HH12 stage of development and is also expressed in myocardial cells of the heart on the HH20 stage of developing chick embryo (Fig 4.7).

Inhibition of COX-2

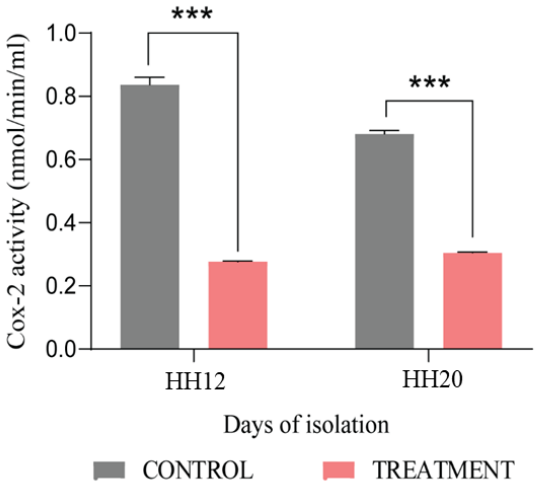

Figure 4.8: COX-2 activity analysis on HH12 and HH20 stage of development in embryos of the control and treatment group. ***p≤0.001

Etoricoxib was used to inhibit the activity of COX-2 in developing embryos. COX-2 activity assay was carried out to confirm the inhibition in both the group of embryos on HH12 and HH20 stages of development. The COX-2 activity assay indicates that embryos treated with etoricoxib exhibit significantly decreased COX-2 activity on the development stages HH12 and HH20 (Fig 4.8, Table 4.1).

Morphology of developing heart

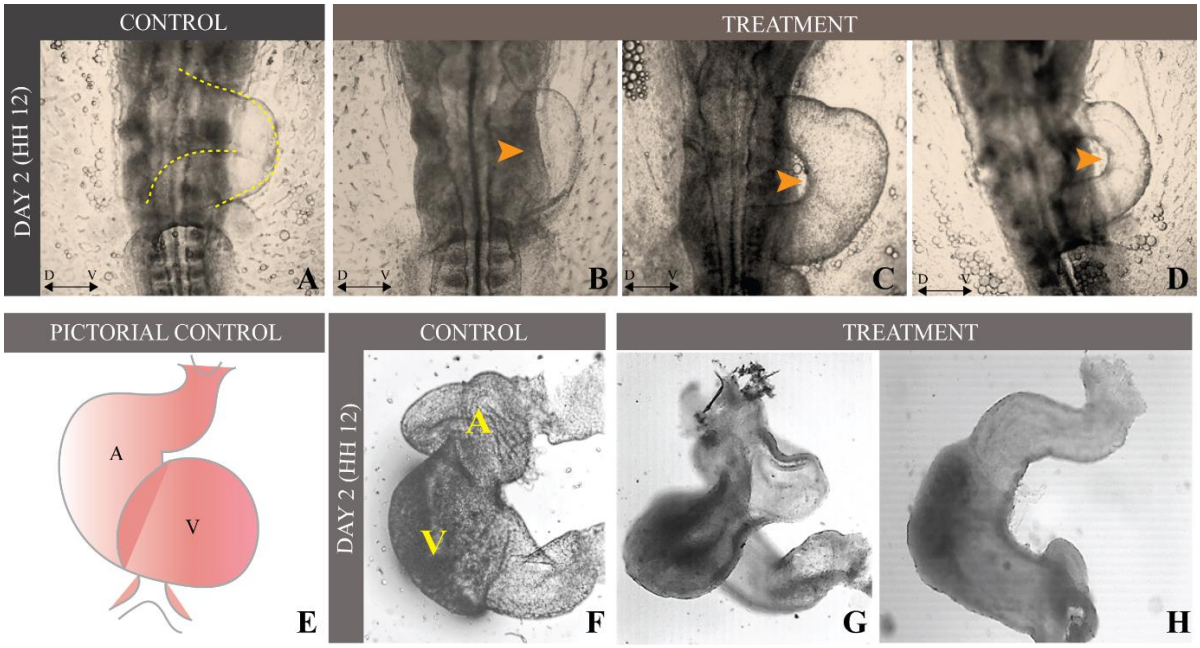

Figure 4.9: Morphology of the developing heart on HH12 stage of control and etoricoxib treated embryos. The yellow dotted line and orange arrowheads display the looping pattern of the heart in the developing chick embryos and the yellow alphabets A and V depict atrium and ventricle respectively

The morphology of the HH12 stage exhibited a typical S-shaped heart tube in the control group embryo (Fig 4.9A). The embryos from the treatment group displayed defects in the heart tube looping. Furthermore, the treated embryos had a considerably higher rate of heart tube loop abnormalities. In embryos treated with etoricoxib, abnormalities, such as an underdeveloped heart, a distorted heart tube, and a faulty heart tube curvature, were frequently reported (Fig 4.9B-D). A visual examination of the heart tissues extracted from the control group and the treatment group of HH12 staged embryos indicated the presence of a heart-loop abnormality (Fig 4.9F-G).

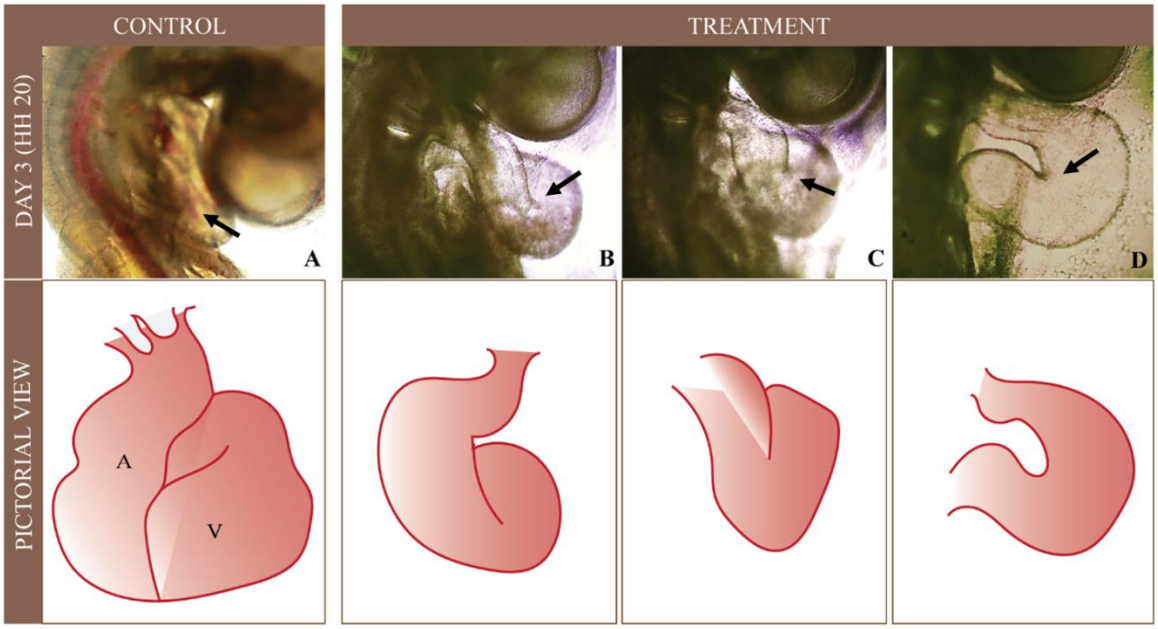

Figure 4.10: Morphology of developing heart on HH20 stage of development in control and a treatment group of embryos. (A) Control embryo has thickening at ventricle walls (black arrowhead), (B-D) Treatment group of the embryo has delayed development of the heart tube and uneven thickening of ventricular walls (black arrowhead); the lower panel shows a pictorial representation of day three heart for normally developing embryo and under the exposure of etoricoxib. A: Auricle, V: Ventricle

The control group of embryos on HH20 stage showed normal development and attained the distinctive conical heart form, with auricles and ventricles clearly distinguished (Fig 4.10A). In contrast, the treated embryos displayed numerous abnormalities, including an irregular heart tube bend and a straight heart tube. Additionally, compared to control embryos, the heart chambers, atria, and ventricles were not fully developed in treated embryos (Fig 4.10B-D)

Transcript level analysis

In order to determine heart-loop deviation, a series of studies involving gene expression was carried out on HH12 and HH20 stages of the developing embryos. The mRNA levels of genes that play a significant role in heart tube patterning and looping, including *WNT11*, *GATA5*, *GATA6*, and *BMP4*, were drastically decreased in embryos treated with etoricoxib (Fig 4.11A) in the HH12 stage. However, the ablation of COX2 activity showed an abnormal expression pattern in the transcript levels of genes involved in the maturation and looping of cardiomyocytes. *SHH*, *PCNA*, *GATA4*, *TBX20*, and *CASP3* mRNA levels increased, whereas *HAND2* mRNA levels decreased in treated embryos compared to untreated embryos.

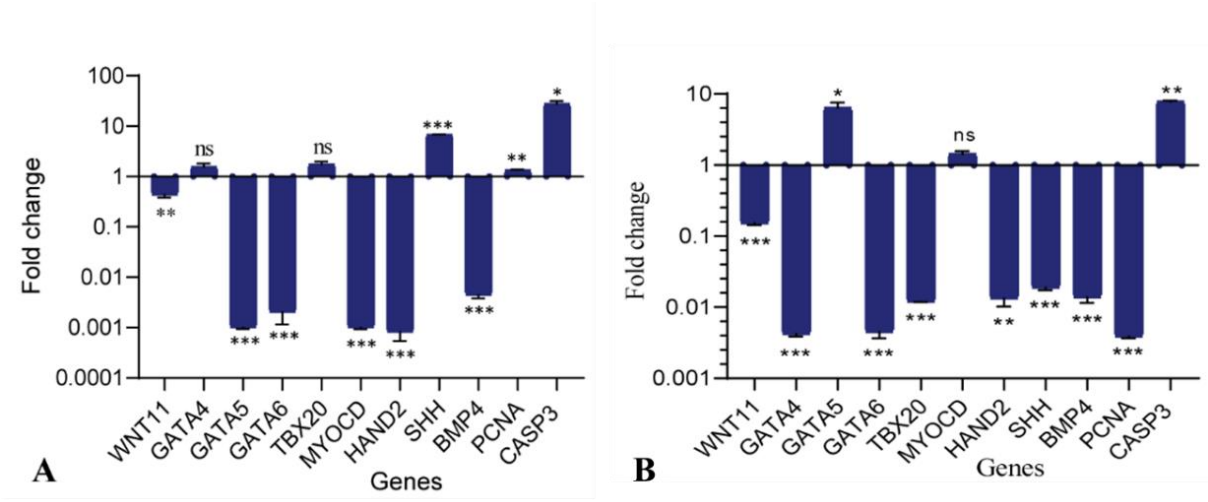

Figure 4.11: Transcript levels of major regulators in heart development under etoricoxib mediated inhibition of COX-2. (A) Fold change value of genes on HH12 stage of development; (B) Fold change value of genes on HH20 stage of development. The fold change value for the control embryo is 1.0 for all the genes, n=3 with 30 eggs per group per stage; ns= non significant *p≤0.05; **p≤0.01; ***p≤0.001

The relative mRNA expression levels of the cardiomyocyte-specific gene, *MYOCD*, decreased significantly with etoricoxib treatment (Fig 4.11A, Table 4.2). Further, the relative mRNA expression levels of *WNT11*, *GATA4*, *GATA6*, *TBX20*, and *BMP4* were significantly downregulated in embryos treated in the HH20 stage. Similarly, the *PCNA*, *SHH*, and *HAND2* transcript levels in treated embryos were considerably reduced (Fig 4.11B, Table 4.3). In contrast, q-RT PCR examination of *GATA5*, *MYOCD*, and *CASP3* transcripts revealed significantly increased expression levels.

Protein level analysis

To further validate the data of mRNA expression, a Western blot was performed for cardiac patterning and looping responsible proteins. Western blotting assessed the protein expression of regulatory molecules of cardiomyocyte proliferation and migration, including Shh, MyoCD, Cl.caspase-3, and PCNA, followed by densitometric analysis of band intensities.

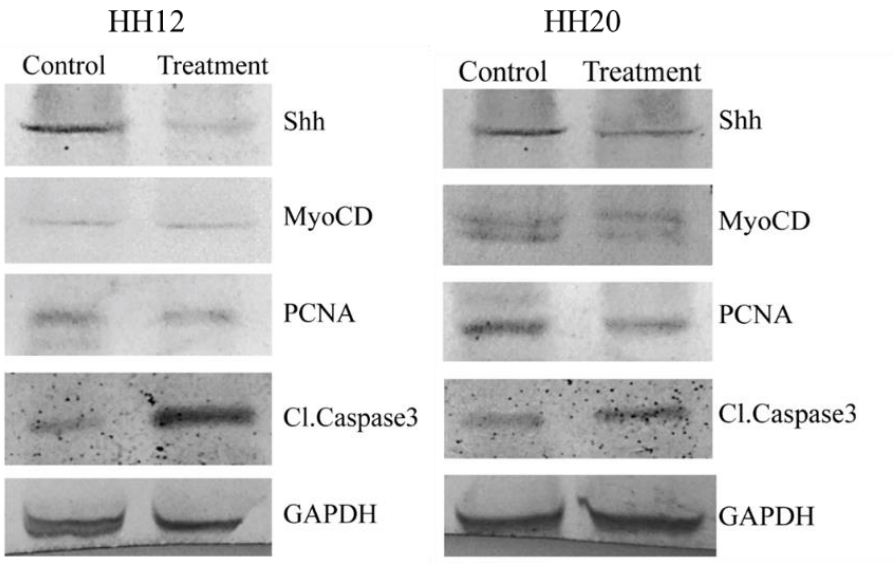

Figure 4.12: Western blot of regulatory proteins of myocardial patterning on HH12 and HH20 stages of developing control and treated embryos

On the HH12 stage, concurrent protein immunoblot analysis showed that treated embryos had lower levels of SHH, MyoCD, PCNA, and Cl.Caspase3 than control embryos (Fig 4.12, Table 4.4). Densitometric examination of Shh, MyoCD, and PCNA blots showed a significant decrease in their levels in response to etoricoxib treatment on the HH20 stage. Cl.Caspase3, an apoptotic marker, is expressed at exceptionally elevated levels in the embryos of the treatment group (Fig 4.12, Table 4.4).

Histological analysis

The histological extent of abnormalities during heart patterning on HH20 embryos was evaluated to understand the tissue-level defects caused by the inhibition of COX-2 in developing embryos. The ventricular wall of control embryos was functionally competent and exhibited usual compaction and trabeculation (Fig 4.13A & C). In contrast, the treatment group displayed evident ventricular non-compaction and defective trabeculation (Fig 4.13B & D).

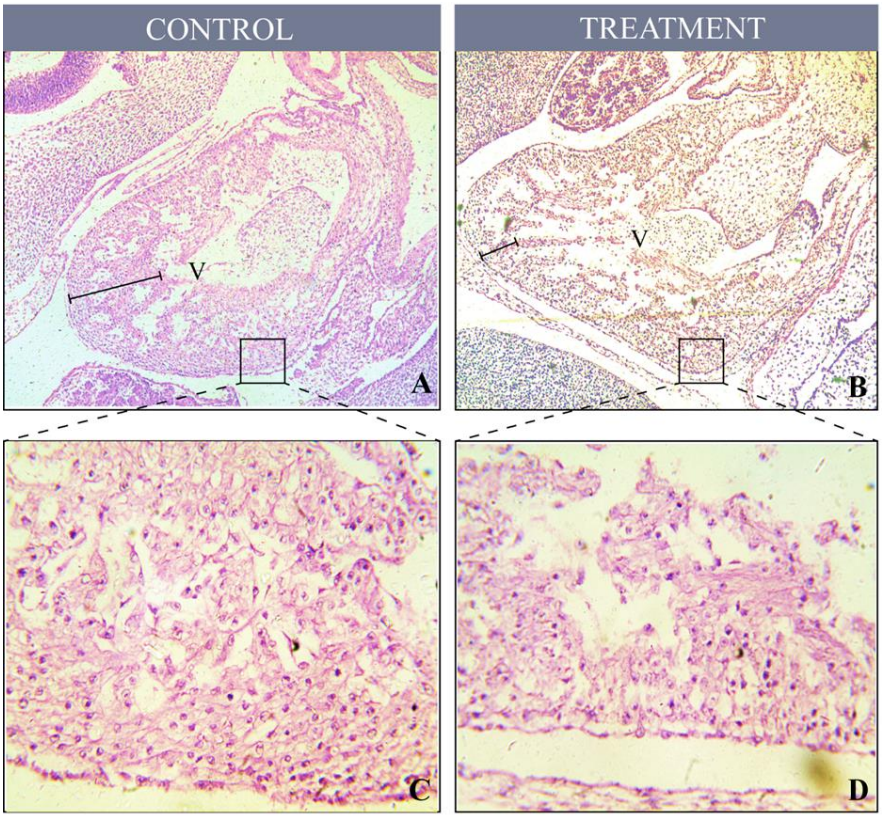

Figure 4.13: Histology of heart tube on HH20 stage of heart development (A) Control heart, observed at a lower resolution (10X), has a triangular shape and dense ventricular walls, as indicated by the black line segment (B) Etoricoxib treated heart exhibits thin ventricular walls, which are visible at 10X resolution, as shown by the black line segment. (C) Control heart, magnified at 40X, reveals tightly packed cardiomyocytes with normal trabeculation in the control group (D) Treatment group, the inset image of the heart illustrates a lack of cardiomyocyte packing in the ventricular wall. This loose arrangement of cells in the treated embryonic heart confirms the trabeculation defect at the same stage compared to the control group

The localization of Cl.caspase3 was carried out on HH12 control and etoricoxib-treated embryo sections. In control sections, the Cl.caspase3 levels were deficient, whereas the treated group section had high expression of Cl.caspase3 in the outer wall of the developing heart tube (Fig 4.14), which was correlated with western blot results. Due to the high level of Cl.caspase3, the shape of the developing heart tube was distorted compared to the control.

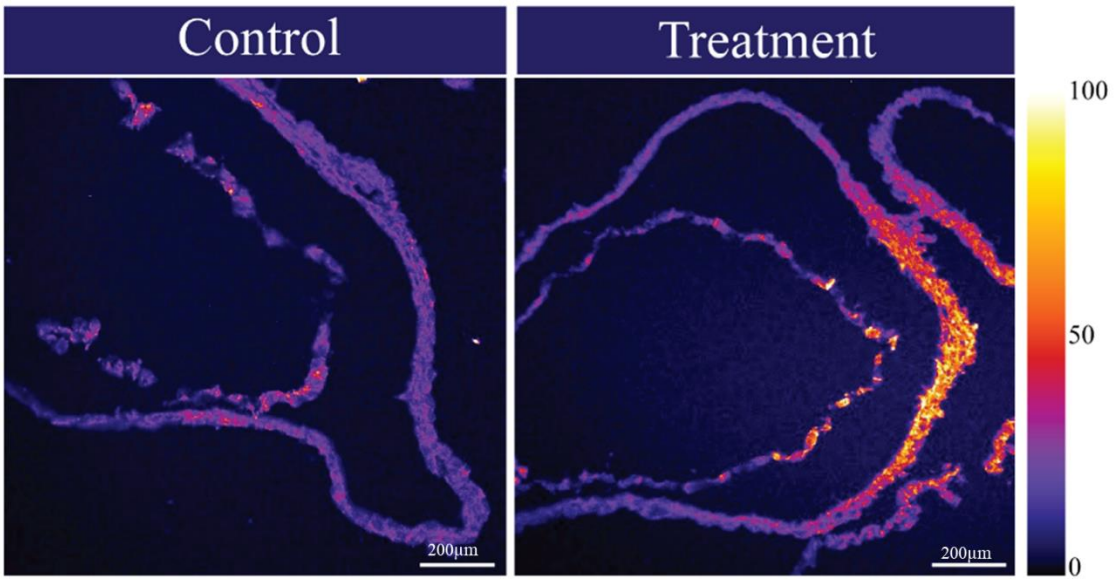

Figure 4.14: Cl.caspase3 localization on HH20 stage of development of control and treated embryos

DISCUSSION

Numerous studies in mammals and other animal models have demonstrated that COX-2-derived PGE_2 is an essential mediator of various physiological and pathological processes (Bondesen et al., 2006; Pakrasi and Jain, 2008; Nørregaard et al., 2015). Although earlier research has suggested a potential relationship between COX-2 derived PGE_2 and an embryonic abnormality, its significance in the development of an embryonic heart or organogenesis remains uncertain (Randall et al., 1991). The overall objective of the study was to understand the importance of COX-2 in the patterning of the heart using vertebrate animal model- chick embryos. Single knockout of COX-2 in mice fetuses showed severe defects related to the axial skeleton and died after birth due to respiratory distress (Shim et al., 2010).

In a normally developing embryo, COX-2 is expressed in developing organs; on HH12 stage of development, COX-2 is present in the heart tube region and on HH20, it is expressed in the trabecula (Parmar et al., 2022). The present study showed that COX-2 inhibition had hampered heart development by altering cardiomyocyte formation and differentiation in the heart tube. Additionally, condensation of cardiomyocytes and defective trabeculation leads to erroneous heart chamber formation under the inhibition of COX-2. The canonical and non-canonical Wnt signalling regulates the patterning of the

developing heart by influencing the fate of cardiomyocytes (Pandur et al., 2002; Piven and Winata, 2017). *Wnt11* is expressed very early during development, beginning from differentiation of paraxial mesoderm for heart patterning. In a study conducted using a quail embryo, the dominant negative *Wnt11* construct decreased the expression of cardiac markers; hence, the cells could not differentiate into cardiomyocytes (Eisenberg & Eisenberg, 1999). At the later stage of heart development, *Wnt11* expression in the outflow tract region, along with *Wnt5a*, played a role in the morphogenesis of the heart (Cohen et al., 2012). On HH12 and HH20 stages of development, we observed a significant decrease in Wnt11 expression in the heart region of etoricoxib-treated embryos. Decreased levels of Wnt11 affects the downstream molecules, such as Twist and GATA family members in the treatment group of embryos.

Furthermore, specific genes and proteins were analyzed to understand the molecular aspect of physiological defects observed under COX-2 inhibition. One such transcription factor that controls the number of myocytes during cardiac development is *GATA5*. The formation of cardiomyocytes is hindered in the treatment group of embryos when *GATA5* levels drop at the HH12 stage but gradually rise at the HH20 stage, possibly due to a feedback loop (Peterkin et al., 2005). Abnormal *GATA5* expression has been linked to mesenchymal malformation, epicardia, trabeculation deficiencies, and valve dysfunction in chick embryos (Chen & Fishman, 2000; Laforest & Nemer, 2011). A similar trend of GATA5 expression was observed in the study, along with the defective heart tube formation on the HH12 stage of dvelopment. However, the levels of GATA5 rise back by HH20 stage under inhibition of COX-2, but defective trabeculation was still observed in the histology of the heart tube.

Another transcription factor that aids in cardiomyocyte migration is GATA4. As per reports, GATA4 is expressed during the earliest phases of heart development, from the migration of precardiac cells to the construction of the entire heart tube (Lopez-Sanchez et al., 2009). In homozygous GATA4 null mice, the erroneous migration of pro-myocardial primordia resulted in the formation of two separate heart tubes (Molkentin et al., 1997). In the present study GATA4 expression was altered in embryos treated with etoricoxib compared to control embryos. COX-2 inhibition initially enhanced GATA4 expression on the HH12 stage; however, at the HH20 stage, GATA4 levels decreased. According to a report, the GATA4 and GATA5 genes in zebrafish interact to regulate myocyte population and carry out processes, including liver and heart organogenesis (Singh et al., 2010). There

is abundant evidence that the other GATA gene compensates for the expression of each GATA gene if one gene is downregulated (Laforest & Nemer, 2011; Charron & Nemer, 1999). The antagonistic expression pattern observed for *GATA4* and *GATA5* for day two and day three treated embryos agrees with the above notion.

Another member of the GATA family, the GATA6 is also essential for cardiac neural crest cell migration and patterning of cardiac outflow track in developing vertebrate embryos. Conditional deletion of GATA6 in mice leads to defective cardiac outflow track formation (Lepore et al., 2006). In our study, inhibition of COX-2 by etoricoxib leads to a decrease in the expression of *GATA6* transcription factor on HH12 and HH20 stages of developing embryos. The decreased level of *GATA6* affects the outflow track formation, which was clearly observed in the histology of HH20 staged embryos. In addition, it has been claimed that an increase in *GATA6* levels may compensate for the loss of *GATA4* in chick embryos during heart formation (Narita et al., 1997).

Similar outcomes were observed in our experiments on the HH20 stage treatment group of embryos.

Moreover, the T-box transcription factor (TBX) is also a crucial protein for heart morphogenesis. Members of the TBX family interact with GATA factors and collectively contribute to the development of the vertebrate heart (Olson, 2006). A report states that when TBX20 mutates, heart development is stopped at the tube stage and does not continue in mice embryos (Plageman & Yutzey, 2004). Furthermore, it has been found that TBX20 regulates myocyte population by co-expressing with GATA5 (Laforest & Nemer, 2011). Morphological observations of HH12 stage embryos of the treatment group showed a looping defect in the heart tube. The transcript level of *TBX20* showed high expression, while *GATA5* reduced at the HH12 stage in the treated embryo group. It is widely known that TBX20 regulates the transcription of the ANF gene (atrial natriuretic factor).

Nonetheless, the ANF promoter region is susceptible to other stimuli, including TBX5 (Horb & Thomsen, 1999). During cardiac morphogenesis, this complicated interaction between T-box transcriptional factors and downstream genes enables the formation of inflow and outflow tracks, atrioventricular cushions, and valves (Brown et al., 2005). The lowered expression of *TBX20* hinders the sculpting of the heart in developing embryos despite having a high level of GATA5 in HH20 stage treated embryos.

In addition, the *MYOCD* gene, which directs myocardin protein production, is expressed in the heart field of early embryos (Huang et al., 2009). Myocardin is detected in developing chicks from the HH9 stage when cardiomyocyte-fated progenitors merge until the valves and nodes of a fully formed heart are formed (Brand, 2003). Myocardin is vital for maintaining the contractile function of cardiomyocytes. It activates genes that regulate calcium handling and energy metabolism, which is essential for proper cardiac function. Many structural and functional roles of MYOCD protein in heart development, including cell fusion and cardiomyocyte contractility, have been reported (Raphel et al., 2012). In our study, inhibition of COX-2 caused the downregulation of MYOCD in HH12 stage embryos relative to controls. It has been reported that in a mouse model of hindbrain ischemia, MYOCD overexpression inhibits cell death and stimulates tissue healing (Madonna et al., 2013). Hence, it is believed that decreased MYOCD expression may contribute to apoptosis and altered morphogenesis in the developing heart tube when COX-2 activity is inhibited in HH12 stage embryos.

Further, the transcription factor HAND2 has been identified in the early heart field at HH16, and its downregulation results in defective ventricle development (Srivastava et al., 1997). In later stages of development, HAND2 moves to the outflow tract with cardiac neural crest cells (Tsuchihashi et al., 2011). In this study, etoricoxib treatment decreased HAND2 expression in embryonic heart tissue, which, along with a lowered level of MYOCD, would account for the looping anomalies found in the heart of chick embryos on the HH12 and HH20 stages.

SHH is an additional factor that regulates the right and left looping of the heart in embryos. It orients the heart tube in the correct direction, further interacts with HAND, and promotes the maturation of the heart chambers (Tsuda et al., 1998). At the HH12 stage, expression of SHH increases under COX-2 inhibition, while in the HH20 stage, SHH levels decreased compared to respective control embryos. SHH signalling pathway is critical for regulating cardiomyocyte proliferation and migration rate; deviant expression of SHH leads to congenital heart defects in developing embryos (Dyer & Kirby, 2009). The altered expression of SHH leads to defective looping of the heart tube, which is visible in the HH12 stage of etoricoxib-treated embryos in the study.

In the cellular event of heart development, TGF-β family member BMP4 regulates cardiomyocyte differentiation and proliferation by interacting with WNT signalling (Ye et

al., 2019). A study in zebrafish showed that BMP4 provides a left-right configuration for developing hearts (Chen et al., 1997). The expression of BMP4 was found to decrease in the treated group of embryos of HH12 and HH20 stages. Reduced BMP4 levels suggest less interaction with Wnt and their downstream molecules, resulting in aberrant heart tube patterning.

The cardiomyocytes proliferate constantly to form heart tube loop and subsequently lead to trabeculation of ventricles during embryogenesis (Linask et al., 2005). Initially, when a heart tube is formed, the apoptosis rate in cardiomyocytes is low; however, as the heart tube differentiates into chambers, the apoptosis rate increases (Zhao & Rivkees, 2000). The ratio of proliferation and apoptosis maintains the cardiomyocyte population and progresses heart formation. As substantiated by the histology of HH20 stage heart sections, the level of PCNA in embryos treated with etoricoxib reduced significantly, whereas the level of Cl.Caspase3 increased, resulting in the death of more heart cells.

Overall, COX-2 suppression affected a pivotal signalling molecule, Wnt11, as well as its downstream targets, GATAs and TBXs, which are interrelated for the patterning of the heart tube. These change in expression pattern of regulatory molecules may have led to the malformation of the embryonic heart of the chick. HH12 and HH20 chick embryos exhibited altered heart tube looping and poor trabeculation in the ventricular area due to COX-2 suppression. When COX-2 levels declined, the regulatory molecules were altered. The morphology of the developing heart reflected changes at the gene and protein levels. Our observations with chick embryos indicated that COX-2 is essential for the development of the heart in vertebrates.

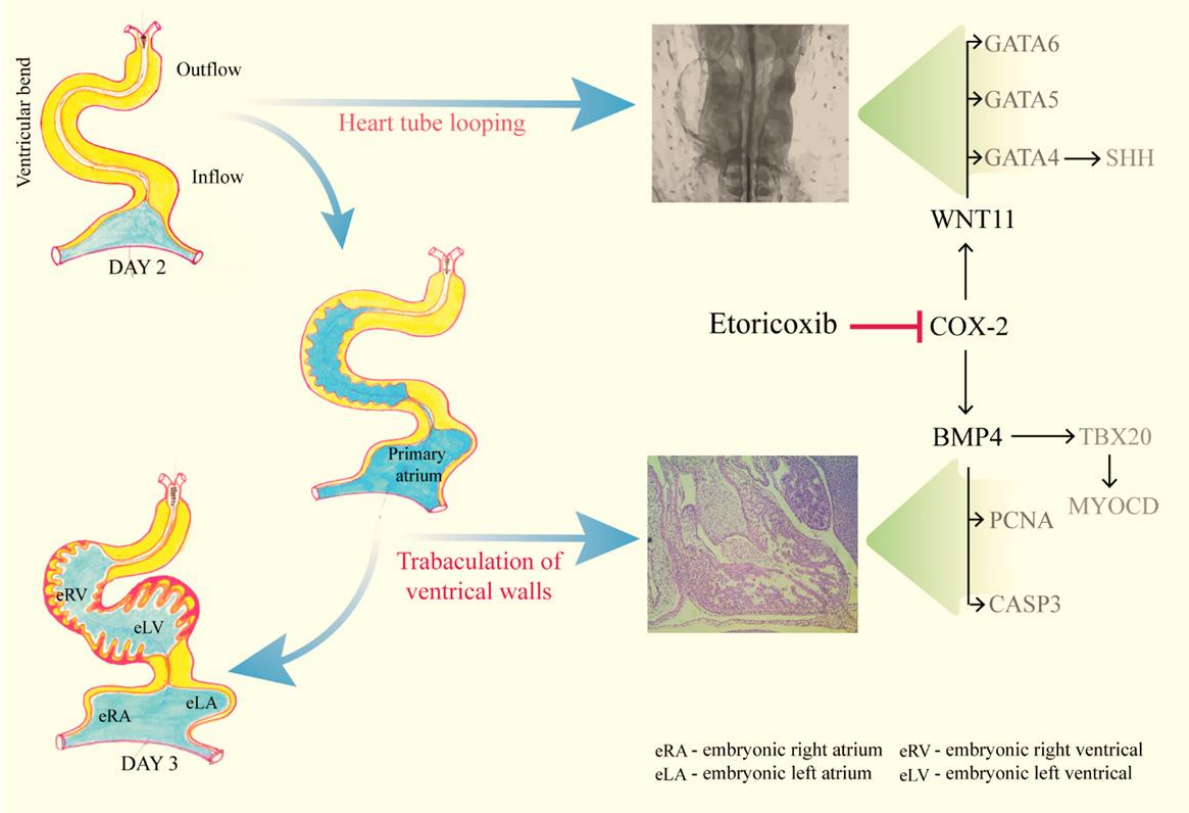

Figure 4.14: Summary image. The illustration describes the effect of etoricoxib mediated inhibition of COX-2 that ablates the normal heart development by influencing major effoector molecules like the GATA family molecules, PCNA, CASP3, MYOCD, TBX20, WNT11, BMP4 and SHH. The major defects were defunct heart tube looping and improper trabeculation of ventricular walls

'*The heart's journey from a simple tube to a complex four-chambered organ is a testament to the intricacy of developmental biology*.'

Tables:

Table 4.1: COX-2 activity of control and treated embryos during HH12, and HH20 stages of development.

Days of isolation	Activity (nmol/min/ml)	
	Control	Treatment
HH12	0.953±0.0004	0.682±0.0121***
HH20	0.958±0.0029	0.149±0.0094***

Enzyme activity are expressed as Mean±SEM, n=3 with 30 eggs per group per day; ***p≤0.001

Table 4.2: Transcript levels on HH12 stage of development in control and etoricoxib treated embryos

Gene	Fold change
WNT11	0.4365±0.0523**
GATA4	1.5510±0.2639
GATA5	0.0010±6.40924E-05***
GATA6	0.0020±0.0009***
TBX20	1.7421±0.2441
MYOCD	0.0010±7.81147E-05***
HAND2	0.0008±0.0003***
SHH	6.8062±0.0782***
BMP4	0.0044±0.0006***
PCNA	1.3416±0.0276**
CASP3	27.6380±3.7487*

Fold changes are expressed as Mean±SEM. Fold change values for the control embryo is 1.0 for all the genes, n=3 with 30 eggs per group per day; *p≤0.05; **p≤0.01; ***p≤0.001

Table 4.3: Transcript levels on HH20 stage of development in control and etoricoxib treated embryos

Gene	Fold change
WNT11	0.1520±0.0095***
GATA4	0.0042±0.0003***
GATA5	6.3628±1.2148*
GATA6	0.0045±0.0008***
TBX20	0.0119±0.0001***
MYOCD	1.4298±0.1344
HAND2	0.0133±0.0030***
SHH	0.0189±0.0015***
BMP4	0.0138±0.0024***
PCNA	0.0039±0.0002***
CASP3	7.7777±0.3395**

Fold changes are expressed as Mean±SEM. Fold change values for the control embryo is 1.0 for all the genes, n=3 with 30 eggs per group per day; *p≤0.05; **p≤0.01; ***p≤0.001

Table 4.4: Spot densitometry analysis of the western blot bands on HH12 and HH20 stage of development in control and treated embryos

Protein	Band intensity in arbitrary units			
	HH12		HH20	
	Control	Treatment	Control	Treatment
Shh	79.5858±1.1635	35.6279±0.4523***	72.6819±2.3640	61.5498±0.9568**
MyoCD	35.9690±0.6264	33.4221±0.5584*	65.9210±0.5056	59.0426±0.7206***
PCNA	57.8709±2.0021	43.9562±1.0054**	73.1841±1.8388	51.2015±1.7802***
Cl.Caspase3	71.9451±0.4546	106.7004±3.4319***	64.7411±0.7768	78.9790±0.4428***

The values are expressed as Mean±SEM; n=3 with 30 eggs per group per experiment; *p≤0.05, **p≤0.01, ***p≤0.001