Material & Methods

The laid objectives were achieved using several methods in various sets of experiments. Some of the procedures were commonly performed as and when the eggs were procured from the poultry unit. These were – sterilization of the eggs externally, candling, random grouping of the eggs, their treatment with particular concentration of test chemical or vehicle, and incubation. These basic procedures were then followed by more specific methodologies adopted for particular experiments namely morphometry, histopathology, immunohistochemistry, activity assays, gene expression analysis, protein expression analysis, and metabolite quantification. However, each of these experiments needs a pre-derivation of concentration of test chemical to be used for the experimental group every time. Such a dose was decided based on the dose-range analysis of the test chemical. The procedural details, as well as sample preparation steps for each of these experiments are described in the following section.

EGG PROCUREMENT AND ETHICAL STATEMENT

Fertile eggs of *Gallus domesticus* of Rhode Island Red (RIR) breed were used for this research work. All the eggs used for the experimentation were procured from the government intensive poultry's Vadodara unit. All the experimental protocols were approved by a CPCSEA, New Delhi approved Institutional Animal Ethics Committee (IAEC) under the protocol numbers MSU-Z/IAEC/13-2017 and MSU-Z/IAEC/05-2019. The collected eggs were immediately used for the study.

IN OVO TREATMENT

The eggs brought as soon as possible from the poultry unit post-laid, were candled to mark the air cell. They were treated according to the method described by Blankenship and co-workers inside a sterile laminar air flow chamber (Blankenship et al., 2003). In brief, the air cell was punctured with the help of sharp and thin sterile piercing needle. The required substance was injected using 0.5 mL sterile insulin syringe with 31-gauge needle (Becton, Dickinson and Company, Haryana). The hole was immediately sealed by molten paraffin

wax with the help of a sterile scalpel. The treatment substance was either water (vehicle for test chemical) or the test chemical, which was etoricoxib. The concentration of test chemical for all the experiments was decided based on the result derived from the dose range study. For the dose-range study, eggs were divided into three groups, namely untreated control, vehicle control, and experimental groups. Untreated group eggs were not treated with any material, and therefore were not punctured as well. The vehicle control eggs were treated with water. The experimental group eggs were treated with various concentrations (described in dose range methodology) etoricoxib. The dose range study results proved no significant mortality in the vehicle control group as compared to untreated control group. Therefore, all the further experiments were performed using only two groups, wherein the control group was treated with water and experimental was treated with etoricoxib. The selection of eggs for either control or experimental groups was done stochastically.

TEST CHEMICAL: ETORICOXIB

Pharmaceutical grade etoricoxib was received as a kind gift from Sun Pharma Advanced Research Company, Vadodara. The physical and chemical properties of etoricoxib is as follows.

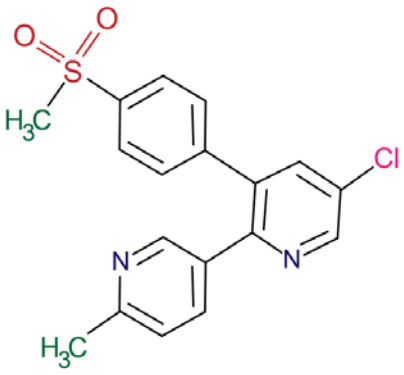
Chemical name	5-Chloro-6'-methyl-3-[4-(methylsulfonyl)phenyl]-2,3'-bipyridine
Manufacturer	Sun Pharmaceuticals Industries Ltd.
Physical appearance	Amorphous solid
Storage condition	In a dark container at 4 ºC
Chemical Structure	
Molecular formula	$C_{18}H_{15}ClN_2O_2S$
Solubility	Slightly soluble in aqueous solvents, Slightly soluble in organic solvents

Table 3: *Physicochemical properties of Etoricoxib*

Etoricoxib is soluble in water at very less concentrations until 80 µg/mL. A dose-range experiment was preceded by an experiment to decide the vehicle for etoricoxib as it is easily soluble in neither water nor oil. Total five solvents in various combinations were used for this purpose. Addition of some solvents like PEG-400 increases its solubility at room temperature to 2 mg/mL (Nayak and Panigrahi, 2012). Therefore, in the current research, several vehicles were used for increasing solubility of etoricoxib initially. Carboxymethyl Cellulose (CMC), Polyethylene Glycol (PEG-400), Dimethyl Sulfoxide (DMSO), and acetone were used to improve the solubility. Combined effect of etoricoxib and one of these solvent mixtures were checked on the embryos for mortality rates before deciding the solvent for further use. Each of these solvents (0.01 %) were added in water as well as PBS to check any differing effect due to PBS. The results proved water as a best solvent for the current study, owing to the negligible mortality in the vehicle control group embryos as discussed in chapter 1 in detail. Later, the dose range study was carried out to identify the dose to be used for all further experiments in this work.

Preparation of etoricoxib in water needs a special methodology due to its poor solubility index. The steps followed are appended below.

- Deionized water (450 mL) was placed on magnetic stirrer on medium speed at room temperature,
- The desired grams of etoricoxib was weighed in one-mL vial, and an mL deionized water was added to it,
- The mixture was vortexed for five minutes,
- The mixture was added in the water placed on magnetic stirrer drop by drop (using a micropipette),
- The mixture was allowed to stir for at least two hours. The remaining volume was made up by using deionized water. This solution was stored at 4 °C until use in a tightly sealed bottle, for maximally one months' time.

The solution derived in this manner was used for injection with the help of the air cell method as described in the previous section. The control and experimental group eggs were then shifted to the incubator for allowing the growth of the embryos.

EGG INCUBATION

The eggs were immediately used for the experimentation. They were stored at 12 °C post-collection for a while, when preparations were done for experimentation. Prior to starting any experiment, eggs were wiped with 10 % w/v povidone-iodine solution for which, commercially available formulation Betadine (Win-Medicare, New Delhi) was used. They were placed in incubator (Scientific equipments works, New Delhi) after treating with appropriate substances. The incubator was set to maintain a temperature of 37.5 ± 0.5 °C and 75 – 80 % relative humidity for the required number of days according to the stage of isolation. The eggs were turned manually at an interval of two hours to an angle of 45 degrees till the isolation time. Turning was necessary to avoid sticking of embryos to the inner shell membrane, which can possibly lead to stunted development and subsequent death of the organism. The embryos were isolated using particular method as per the age of the embryo as described below.

ISOLATION OF EMBRYOS

The embryos were isolated at different days according to the requirement of the experiment based on the part of specific aim under study. The embryos were isolated using blunt forceps after cracking the egg-shell. However, a special technique was followed for the embryos incubated for less than or equal to 3 days. The methodology followed to isolate such early embryos was a modified version of that published by Chapman and co-workers (Chapman et al., 2001). The methodology is called the filter-ring method. The methodology is described as follows.

- Egg was placed in the petri dish (90 mm, Tarson, India) on its rolling surface for five to fifteen minutes. The upper surface of the placed egg was marked with pencil.
- It was held at its long axis using both the hands, and was broken into a petri plate keeping the marked surface up by tapping the lower surface in the plate. The content was released slowly by pulling the blunt and sharp ends of the egg apart from each other.
- The embryo was seen placed upward, which was shifted by chalazae during the first step of incubation.
- The thick albumen from around the embryo was removed using the Whatman filter paper (grade 3, Z240478, Sigma-aldrich, USA) by pulling the albumen layer away

from the blastoderm. This step exposed the vitelline membrane for optimum adherence to the filter ring.

- A filter ring specific for the embryo size was placed on the vitelline membrane such as the aperture was superimposed to the embryo-proper. (ring sizes: aperture diameter 1 cm in a square with 2 cm long sides – for stages till HH 13; aperture diameter 2 cm in a square with 3 cm long sides – for stages till HH 20). The ring was kept there for about half a minute for optimum adherence to the vitelline membrane.
- The membrane around the attached ring was then cut at the edges of the filter ring.
- The embryo attached to vitelline membrane was transferred to the ring at this time point, which was slowly harvested by sliding it away from the yolk in an oblique manner.

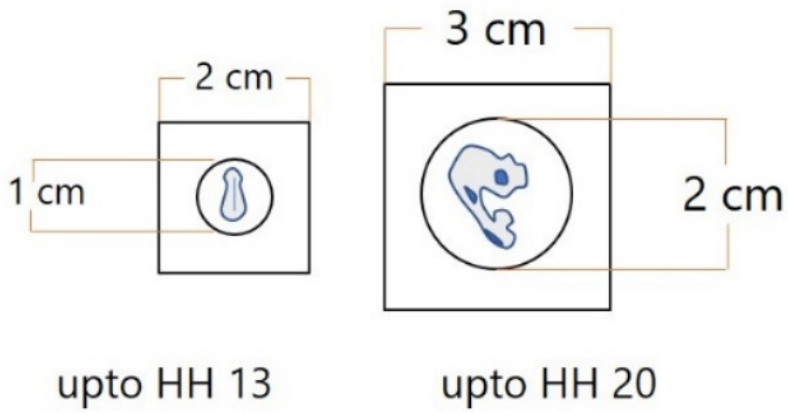


Figure 4: *Dimensional details of Whatman filter paper rings for isolation of chick embryos*

The isolated embryos were used for further analysis after a proper storage step. However, first of all the experiments, dose range study was performed to identify etoricoxib concentration to be used for all further experiments in the experimental group.

DOSE RANGE STUDY

Eggs were divided into 13 groups for the dose range study. One group for control mortality analysis was having 30 eggs per group. All the other 12 groups were having 50 eggs each. Per group the eggs were treated with 50 µL of one of these dose concentrations: 0, 10, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/mL etoricoxib prepared in Milli-Q water. Vehicle control group was added with 0 µg/mL, meaning the treatment of only water without

the drug. Other control group eggs were kept **untreated** in this experiment. All the eggs – belonging to the untreated control, vehicle control, and experimental groups were opened and embryos were isolated at 10-day stage (HH 36) and were examined whether alive or dead based on the heartbeats. Morphological defects were noted and photographed for both control and treated groups so as to understand dose-based malformations in them.

Due to the observation of less mortality in control and high mortality in treated groups, few numbers of eggs were treated with vehicle and a greater number of eggs was treated with each concentration of etoricoxib doses, so as to avoid gratuitous sacrifice of embryos. Total 30 eggs were taken as control group and 50 for each treatment group to circumvent plausible enormous variance. Control correction for such varied sample sizes amongst groups can be done by the Sun-Shepard's formula (Parsad, 2010).

$$Corrected\ \% = \frac{Mortality\ \%\ in\ treated\ plot - Change\ \%\ in\ control\ plot\ population}{100 - Change\ \%\ in\ control\ plot\ population}\ x\ 100$$

Corrected mortality was subtracted from 100, which yielded % survival in various treatment groups, these were used for non-linear fit analysis and LD_{50} was computed to be 144.4 µg/mL etoricoxib. All other experiments were carried out with 72.2 µg/mL, which was a Least Observed Effect Concentration (LOEC). At this dose, % survival was 70 ± 4 (mean ± SD, n = 30). It was observed that the eggs receiving only water and those being untreated had no significant difference between the mortality rate. Therefore, after the dose range study all the other experiments were carried out with 50 µL water treated eggs as control eggs. The morphological defects were observed and classified first.

MORPHOMETRY

Effects of COX-2 inhibition was first identified by the visual analysis of control and experimental embryos. Gross structural deformities were noted and counted for their occurrence rate. The observed malformations were divided into four classes for identifying the most frequent one. All the head, eye, and beak related deformities were classified under craniofacial deformities. Varied limb lengths and web digestion were classified under limb deformities. Embryos possessing omphalocele or gastroschisis were counted under visceral deformities class. Embryos possessing haemorrhages were classified separately. Sample sizes

were 30 eggs for control and 50 for treated in each of the three replicates of this experiment. Some of the embryos were checked for their histological details to identify histological differences as well.

After initial studies of morphologically observed defects, limb related defects were further studied in detail. The lengths of limb buds and limbs were measured using several techniques in control and experimental groups. The limb buds at day-3 (HH 20) and day-4 (HH 24) were observed and photographed using the inverted biological microscope (LM-52-3501, Lawrence & Mayo, India) and ISCapture software. Their lengths were determined using the same software. The later stages such as days 5 to 10 (HH 27 to 36) possessed large enough limbs to be measured using Vernier callipers. The data was analysed using individual sample t-test for each experimental limb length in comparison to the corresponding control limb length.

Observation of morphological defects and developmental delay in the experimental group embryos led to a necessity of verification for etoricoxib in the treated embryos. This was done by using LC-MS technique.

LC-MS OF ETORICOXIB

Etoricoxib presence in treated samples and its endogenous absence in control samples were identified by mass spectrometric approach. Thirty embryos of day-2 were collected for methanol extraction of control and treated groups. Samples were homogenised in pre-chilled mortar and pestle in 90 µL cold methanol per each mg of tissue weight. Homogenate was vortexed for a minute, followed by sonication for 5 minutes. The liquefied homogenate was then centrifuged for 7 minutes at 12000 *g* at 4 ºC. The supernatant was collected and filtered via 0.4 µm filters. These samples were kept at -80 ºC until used. For mass spectrometry, the mobile phase was acetonitrile-water (90:10). The flow rate was 0.3 mL/min., with an injection volume of 15 µL. The standard solution was prepared with 70 µg/mL etoricoxib and was vortexed, sonicated, and centrifuged in a similar way to that of the samples to understand any procedural errors. The separation was carried out on an Eksigent Ekspert UltraLC 100 machine coupled with an ABSciex 3200 Q Trap machine. Data were analysed on the Analyst Pro software (version 1.6.2).

After verification of etoricoxib being present in treated embryos, the activity of both COX isoforms and total COX activity was evaluated to check if there was a significant difference between control and treated groups.

COX ACTIVITY ASSAY

This assay was used to determine the extent of total and isoform-wise COX activities due to addition of etoricoxib. A kit-based assay was used to detect COX-1, COX-2, and total COX activity in control as well as experimental embryonic samples of day 1 to 10. The negative control, standards, controls, and treatment samples were assayed as per the manufacturer description. Specific activities were calculated by dividing total protein values derived from Bradford assay (Bradford, 1976). Multiple t-test was performed to pick the significant values from the data obtained after three trials of the same.

It was obvious that COX-2 had several roles to play in the embryonic life of chick. These roles were related to the organs where morphological defects were found in the earlier experiment. Further analysis of its roles at molecular level was done using gene expression and protein analyses.

STORAGE OF ISOLATED EMBRYOS

The embryos were stored in appropriate solution as per the plan of further usage. In case of morphological analysis, they were cleaned free of the blood droplets around them, and were immediately photographed. Morphometric analysis such as calculation of number of deformities and/or measurements of organ lengths were also carried out in the non-fixed embryos. The embryos were collected in TRIzol without the washing step at 25 mg tissue per ml of TRIzol ratio. Step of washing is avoided to circumvent the plausible loss of RNA due to RNase contents of washing buffers, as well as to avoid temperature fluctuations. In case of protein isolation, the embryos were chopped and stored in lysis buffer (1 mL for 25 mg tissue weight) containing protease inhibitor. The samples were collected in 10 % neutral buffered formalin [100 mL Formalin (37 – 40 % stock solution), 900 mL Water, 4 g/L NaH_2PO_4 (monobasic) and 6.5 g/L Na_2HPO_4 (dibasic/anhydrous)] for sectioning. The samples stored with one of these methods were used for one of the following processes.

LC-MS/MS OF PROSTANOIDS

The four main prostanoids, namely PGD_2, PGE_2, $PGF_{2\alpha}$, and TXA_2 were checked for their quantities in control and experimental groups. This was done using LC-MS/MS methodology standardized and performed at University of Alberta. The novel parameters of both LC and MS systems are discussed in a later chapter in detail. The result showed that only PGE_2 decreased in treated group, which fed the hypothesis of it being the COX-2 effector in the embryogenesis.

PGE_2 ACTIVITY ASSAY

Roles of COX-2 as identified by morphological data and deformities calculations, had to be taken ahead for understanding of the effector prostanoid molecule. Here, the hypothesis was that the embryonic effector of COX-2 could be PGE_2. This hypothesis was checked by deriving the concentration of PGE_2 in both the control and experimental embryos. Isolation, purification, and quantification of PGE_2 were carried out following a sandwich ELISA kit as per its protocol book suggestions (R&D Lab systems, USA) from day-1 (HH 6) to day-10 (HH 36) embryos. Embryos were weighed, and about a gram of embryos per stage (of days 1 to 10) were homogenised in pre-chilled mortar-pestles and were centrifuged at 5000 *g* for 15 minutes. A clear homogenate was carefully pipetted out for the assay avoiding whole cells and tissue chunks. An mL of homogenate was used for the assay. Concentration of PGE_2 in the samples were derived using the four-parameter algorithm graph (GraphPad Software).

GENE EXPRESSION ANALYSIS

Study of the expression pattern for any gene, was done by following the standard steps like RNA isolation, RNA quality check, RNA quantification, complimentary DNA (cDNA) synthesis, primer designing, reverse transcriptase polymerase chain reaction (RT-PCR), densitometric analysis, quantitative RT-PCR (qRT-PCR), its analysis, and statistical evaluation of derived data. The essential steps of preparation of reagents and procedural details are described hereunder in a sequential manner.

I. RNA isolation

A clean pair of purple nitrile gloves was used while carrying out the whole procedure of RNA isolation. The working table was completely sterilized using 70 % isopropanol. The vessels used for preparation of material were autoclaved. Solutions were prepared in RNase-free water (DEPC water). The floor was wiped with RNaseZap (Sigma-aldrich, USA) once in each month which keeps the working substratum free of RNase enzyme.

i. Steps for RNA isolation

Cell lysis: 1 mL TRIzol reagent was used to homogenise 25 mg tissue with the help of a pre-chilled pair of mortar-pestle manually. The chunk-less solution was transferred to a new centrifuge tube. Whole procedure was carried at 4 ºC to prevent the temperature-labile RNA from degradation. The tube was spinned for 20 minutes at 4 ºC and 5000 *g*.

Phase separation: Supernatant was collected in new centrifuge tube containing 200 µL chloroform. It was allowed to stand for 15 – 20 minutes with occasional gentle shaking for adequate disunion of phases. Mixture was centrifuged at 12000 *g* for 15 minutes. The upper clear layer of solution contains the RNA which was collected in a different vial [care was taken not to aspirate the intermediate DNA containing layer as well as proteins and lipid rich bottom organic layer]. This step was repeated if supernatant looked turbid or intermediate layer accidently got mixed with the upper layer.

RNA precipitation: Collected upper layer of solution was incubated with 500 µL of isopropanol for 1 hour with occasional gentle shaking. The mixture was spinned at 12000 *g* for 20 minutes to derive RNA in the form of a pellet. Supernatant was discarded and pellet was washed several times, depending upon the visible debris. In most cases, two washes were enough to wash off the debris.

Wash: RNA pellet was added with 75 % ethanol (prepared in DEPC water) and was detached from tube wall with the help of brief vortex, for thorough wash. Pellet was settled again by spinning at 7500 *g* for 10 minutes. The washing step was repeated twice and pellet was allowed to air dry for up to 10 minutes. Excess drying was avoided due to the decreased solubility of over-dried pellets. The pellet was dissolved in DEPC water (approximately 15 to 30 µL, depending upon the pellet size) and stored in -20 ºC until used for the preparation of cDNA. Freeze-thaw cycles were avoided for RNA samples and were used to prepare cDNA right after quality-check, for most of the times.

ii. Quality-check of RNA

2 µL of isolated RNA sample along with RNA loading dye was allowed to run in 1 % agarose gel matrix added with 5 µL of 25 mM Ethidium Bromide (EtBr) under the influence of 50 volts power supply. Bromophenol containing loading dye also allows tracking of RNA sample. The samples were allowed to reach 80 % of total gel length and were observed under UV light in the gel-documentation unit (GeNei, India). The gel matrix was prepared according to the following procedure.

Preparation of gel: Gel was prepared from Agarose obtained in powder form and TBE buffer in w/v percentage solution. According to percentage (1 % for RNA qualification and 2 % for PCR products), agarose was weighed in Erlenmeyer flask. TBE buffer was added to the required calculated quantity and flask was stirred. The buffer used for preparation of RNA was the same as the one used as running buffer during electrophoresis. This mixture was added with 5 µL of 25 mM EtBr. The flask was then warmed over Bunsen flame until agarose was completely dissolved. At intervals of 30-40 seconds flask was swirled for proper mixture of contents. Care had to be taken while warming the flask as it contains EtBr which is a potent carcinogen if inhaled.

iii. Quantification of RNA

Quantity of RNA was determined by QuantiFluor ssdna assay (Promega, USA) with the help of Qubit 3.0 instrument (Life technologies, USA). RNA samples were diluted twice for this fluorometric quantification. The dilution factor was multiplied with the qubit reading in the end. The reagents were prepared as per the manufacturer's guidelines. Briefly, working solution of TE buffer was prepared by diluting the stock solution provided as 20x concentration. The dye was mixed with working TE buffer solution in a ratio of 1:400. This solution was referred to as a dye mixture. The samples were diluted in nuclease-free distilled water (MilliporeSigma, Germany). A microlitre of the diluted sample was added to a specific scratch-free qubit vial pre-added with 99 µL of working TE buffer. The dye-mix 100 µL was added to this and the vial was vortexed briefly. The content was allowed to stand for 2 minutes and the reading was taken using the ssdna (single-stranded DNA) option of qubit instrument. Reading was taken at least thrice to assure the constant value of the same sample. The qubit instrument was calibrated once in each month to omit the instrumental errors. The calibration was done using the nuclease-free deionized water as blank and the RNA standard provided with the other reagents. From each sample, 10 µL of 1 µg/µL RNA was harvested

and used for cDNA preparation. The concentrated samples were diluted to adjust the concentration.

II. cDNA synthesis

Synthesis of cDNA was carried out using a commercial kit (Applied biosystems, USA). Freeze-thaw cycles were avoided for cDNA sample once prepared. Following material was mixed in the mentioned ratio for preparation of cDNA synthesis vials.

Material	**Volume per vial (µL)**
10x RT Buffer	02.0
25x dNTP mix	00.8
10x RT Random Primer	02.0
RT enzyme	01.0
Nuclease-free H_2O	04.2
RNA sample	10.0
Total system volume	20.0

The vials containing this mixture was labelled individually and the following protocol was set in thermal cycler (BioRad T100^{TM}) for the synthesis reaction.

Settings	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Temperature	25 °C	37 °C	85 °C	12 °C
Time (minutes)	10	120	5	∞

The vials were kept in crushed ice, during the mixture preparation, to maintain at least 4 °C before the synthesis starts. They were briefly centrifuged in a short-spin (RM-02 plus, Remi, India) to get rid of the air bubbles before placing them in the thermal cycler. After the reaction is over, vials were immediately stored at -20 °C.

III. Reverse transcriptase PCR (RT-PCR)

Thermal cycler (BioRad, India) was used for the RT-PCR reaction. The prepared cDNA samples served as substrates. A PCR ready-mix (Sigma-aldrich, USA) was used, which contained all the components such as DNA polymerase enzyme, buffer, dNTPs, and $MgCl_2$, and bromophenol blue. Other than PCR mix and substrate (cDNA), primers were used to prime the extension step carried out by the thermostable DNA polymerase. The ncbi (national centre for biotechnology information) database for chicken genome was used to select the

primer sequences from suggested primers in 'pick primers' section. The primers which were overlapping the exon-exon junctions were preferred so as to avoid the DNA contamination in the prepared cDNA which could have come since the RNA isolation steps. Nearly 50 % GC content, melting temperature between 58 and 62 °C, and less self-complementarity – was preferred while selecting the sequences. The primers were received in a lipophilic form. They were reconstituted using nuclease-free water so as to prepare 100 µM concentrated stock. This was diluted twenty times using sterile distilled water which was called as a working dilution of a primer. Such working dilutions were stored at 8 – 12 °C for less than 4 months, after which they were re-prepared. Using these reagents, the RT-PCR vials were prepared using following volumes of reagents.

Material	**Volume per vial (µL)**
Substrate (cDNA sample)	01.0
PCR master-mix (2x)	05.0
Forward Primer (5 µM)	01.0
Reverse Primer (5 µM)	01.0
Nuclease-free H_2O	02.0
Total system volume	10.0

The protocol ran in the thermal cycler BioRad T100 was set as follows.

Parameters	**Step 1**	**Step 2**	**Step 3**	**Step 4**	**Step 5**
Temperature (ºC)	95	95	60	72	4
Time (seconds)	180	10	30	30	∞
cycles		35 cycles			

PCR product was loaded on the horizontal gel to allow the separation of differently sized components of each sample.

IV. Horizontal gel electrophoresis

The RT-PCR resulting solutions were evaluated using the electrophoretic technique. The agarose (Molecular grade, Takara, USA) was used to resolve the product DNA fragments. 2 % agarose solution was prepared in 1x TBE buffer and 4 µL EtBr was added to 100 mL agarose solution. EtBr has capability of intercalating with nucleic acids, thus was used for the later visualization of DNA under the ultraviolet light. The assembly of gel electrophoresis was set. The PCR product solution was added to the wells and were allowed to migrate under

the force of 50 volts electric current. The gel was observed in gel documentation unit (GeNei, India) and was picturized. The images were then added to doc-ItLs software for band intensity analysis. The band intensities of each gene in each sample was divided by that of corresponding housekeeping gene. This calculation yielded the relative band intensities in arbitrary units. Statistical analysis for three repetitions of each gene's band intensity (technical replicates) was done using the multiple t-tests (GraphPad Prism Inc., USA).

V. Real-time quantitative Reverse Transcriptase PCR (qRT-PCR)

Absolute and relative quantities of the transcripts of the genes in embryos of various stages were found using qRT-PCR. SYBR Green was used as a fluorescent dye which intercalates to the minor groove of DNA double helix. During an elongation phase in PCR, it binds to the DNA double helices being formed. The emission wavelength of the dye-DNA complex is reported to be 520 nm which is a green light (Navarro et al., 2015). The new DNA strands formed in initial cycles are not detected, which is called a linear ground phase of the reaction (Stephenson, 2016). After this phase, the fluorescence starts to rise exponentially, followed by a log phase where the curve becomes more or less linear. The last phase observed during the cycle is a plateau phase showing flat curve without undulations (Stephenson, 2016). The material provided for the reaction has to be sufficient to avoid the early plateau in the presence of enough substrate of interest. LightCycler96 (Roche diagnostics, Switzerland) was used for performing this experiment. The essential components of PCR such as enzyme, buffer, $MgCl_2$, and detection-dye was all contained in a single SYBR Green master-mix (Takara Bio, Japan). The mixture in a 96-well plate (Genaxy, USA) was prepared as follows.

Material	**Volume per vial (µL)**
SYBR Green master-mix (2x)	05.0
Forward Primer (5 µM)	00.5
Reverse Primer (5 µM)	00.5
Substrate (cDNA sample)	01.0
Nuclease-free H_2O	03.0
Total system volume	10.0

The observation of excess dimer formation often led to further dilution of both forward and reverse primers to be used in the abovementioned system. The protocol ran for the reaction in light cycler was as described below. The fluorescence was acquired at step 3.

Parameters	Step 1 Initial denaturation	Step 2 Denaturation	Step 3 Annealing	Step 4 Final extension
Temperature (ºC)	95	95	60	72
Time (seconds)	100	10	30	30
cycles		40 cycles		

Amplification of the cDNA templates using specific primers was done as per the abovementioned protocol, which was followed by the following procedure of melting curve evaluation.

Parameters	Step 1	Step 2	Step 3
Temperature (ºC)	95	65	97
Time (seconds)	10	60	1

The data derived from this experiment was first annotated fully in the application provided with the instrument – LightCycler96 version 1.1. The annotation of the plate components such as standard solution, samples, negative controls, etc derived the Cq values in the analysis section of this software. The Cq values were then used for calculation of fold change using several steps of calculation using Microsoft Excel. The method used for this kind of analysis was derived from Livak and Schmittgen (2001), which is known as ΔΔCq method. Following are the steps used to calculate it till the derivation of fold change of each gene in experimental group as compared to control group.

Step 1: Calculation of ΔCq: This step omits the experimental errors such as variation in initial amount of mRNA used for cDNA preparation of each sample, pipetting variations in different wells.

ΔCq = Cq of the target gene – Cq of the housekeeping (reference) gene

Step 2: Calculation of ΔΔCq: This step derives the actual difference between the ΔCq values of control and experimental group samples. Usually sample 2 here is the experimental group and sample 1 is the control group.

ΔΔCq = ΔCq of the target gene in sample 2 – ΔCq of the target gene in sample 1

Step 3: Calculation of fold change: $2^{-\Delta\Delta Cq}$

The fold change values for each gene was plotted in logarithmic scale. The data was analysed using the one-way analysis of variance (ANOVA) for understanding of significance. Confidence interval was set as 95 % for all the experiments. Dunnett's post-hoc test was used after ANOVA.

PROTEIN ANALYSIS

Evaluation of proteins in the tissue was done with the help of western blotting technique. The technique is divided into the steps like polyacrylamide gel electrophoresis (PAGE), protein transfer by semi-dry western blot, and immunolabeling the protein of interest.

I. Isolation of Protein

Protein was isolated from samples using the lysis buffer. An mL of lysis buffer was added in about 50 mg tissue, which was freshly collected. The sample was collected and prepared at 4 °C by keeping all the reagent vials in crushed ice. Wherever possible, pre-cooled reagents were used to avoid the degradation of protein structure. The samples were homogenised in pre-chilled mortar-pestles. The homogenate was incubated at 4 – 8 °C for 2 hours. Supernatant was collected after centrifugation of these samples was carried out at 4 °C and 8000 *g* for 20 minutes. Isolated protein was immediately used for sample preparation step, after which it was stored at -20 °C until use. For the step of sample preparation, quantification of protein was mandatory. This was done using the Bradford assay (Bradford, 1976). Sample (5 µL) was added to the 200 µL Bradford reagent in the well of 96-well plate. Each sample was loaded to three wells to avoid technical variation in reading. The concentration was derived using the standard graph, which was prepared with the help of various concentrations of BSA. The sample buffer (5x) was added at the final working concentration (1x) to the samples as per their quantities required for loading in PAGE system. After the addition of sample buffer, water was used to make the volume up to the final one. This mixture was heated at 80 °C for 10 minutes. The prepared samples were either stored at -20 °C for later use or were used for PAGE.

II. Polyacrylamide Gel Electrophoresis

The assembly of PAGE contained the gel prepared between glass plates bearing the wells to accommodate the prepared samples, tank buffer, and electric supply. The gel was casted after

addition of 2 % agar at the bottom of the casting unit sealed with the Cellophane tape from two horizontal sides. Resolving gel was prepared and poured first followed by distilled water. Water kept the gel away from atmospheric gases. Half an hour later when this gel was polymerized fully, water was removed by placing tissue papers at the edge, tilting the casting assembly. The stacking gel was added with APS and TEMED, then immediately poured over the solid resolving gel using micropipette. Immediately after this step, the comb was inserted first at 45 ° angle, to avoid air bubbles. It was then fixed straight in its position and the gel was kept undisturbed for 45 minutes till it is properly polymerized. The casting unit was placed and fixed in the gel unit pre-added with tank buffer. The samples were loaded along with the dual stained marker (BioRad, India). Electric current was set at 50 volts until the samples cross stacking gel. Later, it was set to 70 volts for the rest of the run. The gel was fixed using fixation buffer until the preparation for semi-dry transfer was completed.

III. Semi-dry transfer

The fixed gel was submerged in the Bjerrum-Schafer-Nielsen buffer for 30 minutes at 4 °C. The semi-dry transfer unit was added with the drops of the same buffer on both bottom and roof of the assembly. The stack was prepared such as four Whatman filter papers sized exactly as the gel, flanked the PVDF membrane placed beneath the gel. The air bubbles were removed with the help of test tubes by rolling it on the stack in a single direction twice or thrice. The transfer of proteins was allowed by setting the electric current of 100 mA for 22 minutes. The membrane was then immediately submerged in a ponceau stain, washed with distilled water a couple of times once the colour developed. The quality of transfer was checked with the help of this stain.

IV. Immunolabelling of desired protein

The membrane was blocked with the blocking buffer for 1 hour at room temperature before submerging it in a primary monoclonal antibody overnight. The antibody was used as per the antigen of interest. On the second day, four washes of the membrane were carried out keeping it on a rocker for 10 minutes each. Secondary antibody of the isotype of primary antibody was added to the membrane after this step. The membrane incubated in secondary antibody for 1 hour was then washed four times as earlier. Later, the membrane was submerged in the solution of Streptavidin, conjugated with ALP for 15 minutes. Followed by four subsequent washes, the ALP substrate p-Nitrophenyl Phosphate (pNPP) was added so as to develop a colour as an indirect indication of antigen quantity. Distilled water was added as

soon as the colour developed. The membrane was photographed and used for band intensity analysis via Doc-ItLs software. Relative band intensities were calculated as earlier suggested. The statistical operations were carried using GraphPad PRISM software and ANOVA test.

V. Immunohistochemistry

This technique was used to specify the placement of particular antigen in a tissue architecture. Majority of the times, the tissues were sectioned in a transverse, longitudinal, or sagittal plane before performing immunolocalization. However, in case of extremely thin tissues, lacking intercellular collagenous depositions – such as the embryonic days 0, 1, and 2 – were isolated and freshly used for immunolocalization followed by a fixation. The tissues required to section were immediately embedded in the Tissue-Tek OCT compound (Cryo-embedding medium, Sakura, USA). The embedded material was either stored in -80 °C or was directly used for sectioning. Other than cryo-sectioning, some tissues were outsourced (to Unipath Laboratory, Vadodara) for sectioning at room temperature using the microtome instrument. These were first collected in 10 % neutral buffered formalin, later embedded in paraffin wax. The sections prepared using cryostat instrument were handled in a different manner than the microtome-made sections.

i. Sample preparation for cryo-sections

Sections derived from cryo-sectioning procedure do not need elaborate sample preparation because of the character of OCT compound, which easily gets washed away with solvents. These sections were therefore, washed with pre-chilled PBS for several times gently to avoid their disintegration. The samples were now ready for the immunolocalization procedure.

ii. Sample preparation for microtome-sections

The sections derived using microtome were placed in the coupling jar filled with xylene to wash off the wax. Slides were kept in xylene for 15 minutes and the step was repeated for four times. After the fourth time, ethanol gradients were applied to the sections using coupling jars again. Slides were immersed in 100 % ethanol for 10 minutes twice. Then they were subsequently immersed in 95 %, 70 %, 50 % and 30 % ethanol for 5 minutes each. Lastly, they were rinsed with deionized water, and rehydrated in TBST for 10 minutes. PBS could have been used for washing instead of TBS in the protocols of western blot as well as immunocytochemistry, which was not for a reason. The colorimetric reagent used in this procedure was ALP based. The usage of PBS could have added to the nonspecific coloration

due to incorporation of PBS-liberated phosphates, which was avoided with the help of an alternate buffer TBS. Finally, these slides were used for the immunolocalization process as described below.

iii. Immunolocalization steps

The localization was carried out using specific monoclonal primary antibody to the antigen of interest. These antibodies were prepared by diluting them for 1000 times in the antibody dilution buffer. The first step to this procedure was the *antigen retrieval* step. The samples were added with the retrieval buffer for 15 minutes. This step was followed by a permeabilization step. This was carried out using a brief exposure to cold acetone for less than a minute. The next step was *blocking*, which was performed by immersing the slides of sections or whole embryos in the blocking buffer for 30 minutes at room temperature. The primary antibody was added after removal of blocking buffer. The sections or embryos were not washed before addition of primary antibody. An overnight incubation in primary antibody at 4 °C was followed by four TBST washes for 10 minutes each at room temperature on the following day. The samples were then incubated for 2 hours in biotinylated secondary antibody. This and all the further steps were carried out at room temperature. Excess of secondary antibody was washed off using the TBST washes thrice – 15 minutes each. Streptavidin – ALP conjugate was added for further process, wherein the streptavidin was allowed to react with the biotin resting on primary antibody-attached secondary antibody for 1 hour. Lastly, ALP substrate was added for colour development, which was replaced by water as soon as the slightest of the colour was visible. Each time while conducting this experiment, the verification of the developed colour being specific, was done using the antibody controls. These samples were collected and processed in the same manner as the other samples were, except for the step of primary antibody addition. Here, they were added with TBS overnight. The colour development in such samples was conclusive of non-specificity of either of the antibodies used in that particular experiment. Such samples were not used for further evaluation or inference-making. In-vitro studies included immunocytochemistry procedure, wherein, FITC-labelled secondary antibodies were used instread of biotinylated. Cells were counterstained with DAPI nuclear stain.

CELL CULTURE

cESMC were isolated from chick embryo at HH 37 (Hamburger and Hamilton, 1951). All the procedures were carried out under laminar air flow hood. The embryo was isolated and taken into PBS (calcium magnesium-free; autoclaved). All the four limbs were separated and washed so as to get rid of RBCs. Digits were removed. The limbs were deskinned with the help of blunt scalpel. The tissues were then put in 10 mL Dulbecco's Modified Eagle Medium (DMEM) with low glucose and pyruvate (Life Technologies, USA) in centrifuge tube. 10 % trypsin – EDTA (Life Technologies, USA) was added and tissue was minced with the help of autoclaved sterile scissors. This tube was incubated at 37 ºC in water-bath for 10 minutes. Post-incubation, 10 % trypsin neutralising solution (Life Technologies, USA) was added to the tube. The content was centrifuged at 1000 rpm at room temperature for 10 minutes to pellet down the cells and remove trypsin. Pellet was re-suspended in 10 mL media mix. Media mix contained DMEM-low glucose with 10 % FBS (Life Technologies, USA) and 1x antibiotics (Life Technologies, USA). This prepared cell suspension was seeded onto a non-coated sterile culture vessel for two hours allowing fibroblast cells to attach. Later, inspecting the attached fibroblast cells, media was isolated which contained majorly the muscle cells. These cells were counted and seeded in 60 mm tissue-culture grade dishes (Tarsons, India).

CHEMICAL COMPOSITIONS OF REAGENTS AND BUFFERS

Commonly used material

Phosphate Buffered Saline (PBS)

Material	Molecular weight
NaCl	137.0 mM
KCl	002.7 mM
Na_2HPO_4	010.0 mM
NaH_2PO_4	01.8 mM

Prepared in distilled water; pH was adjusted to 7.4.

Gene expression analysis

DEPC water

An mL of diethyl pyrocarbonate (DEPC) was added to 1 L of autoclaved distilled water. It was stirred on magnetic stirrer for 4 to 5 hours in a loosely closed vessel, which was used

after autoclaving it and was stored in 8 – 12 °C. All the reagents used in RNA isolation and cDNA preparation were prepared in DEPC water to prevent RNA from RNase. This was stored at 8 – 12 °C.

TBE (Tris/Borate/EDTA) buffer (1x)

Material	**Volume / weight**
Tris	10.8 g
Borate	05.5 g
0.5 M Na_2EDTA	04.0 mL
DEPC water	adjusted up to 1 L
Total volume	01.0 L

Stored at room temperature, reused for less than ten times as agarose gel electrophoresis tank buffer.

RNA loading dye (5x)

Material	**Volume / weight**
EDTA 0.5 µM	0008 µL
Formalin	0072 µL
Glycerol	0200 µL
Formamide	0030 µL
Bromophenol blue	0025 mg
TBE buffer	0430 µL
DEPC water	Adjusted p to 1 L
Total volume (µL)	1000 µL

Stored at 8 – 12 °C.

EtBr

25 mM: 7.885 mg in 1 mL deionized water. Stored at 8 – 12 °C.

DNA loading dye (6x)

Material	**Volume / weight**
Glycerol	300.0 µL
Bromophenol blue	002.5 mg
Deionized water	700.0 µL
Total volume	001.0 mL

Stored at 8 – 12 °C.

Protein analysis

Lysis buffer

Material	Concentration
Tris base	050.0 mM
NaCl	200.0 mM
$CaCl_2$	010.0 mM
Triton X-100	000.1 %

pH was adjusted to 7.5; 1 % protease inhibitor (Sigma-aldrich, USA) was added freshly before addition of the lysis buffer to the samples; Stored at 8 – 12 °C.

Bradford reagent

Material	Weight / volume
Coomassie™ brilliant blue	100 mg
Ethanol 95 %	050 mL
Above mixture was allowed to stand for 2 hours at room temperature	
Ortho-phosphoric acid 85 %	100 mL
Above mixture was allowed to stand overnight at room temperature	
Deionized water	Up to 1000 mL
The mixture was filtered through Whatman filter paper of grade 1	

Stored at room temperature in amber bottle until the colour changed.

Sample buffer

Material	Concentration
Tris base	250.0 mM
SDS	010.0 %
Glycerol	050.0 %
Bromophenol blue	000.1 %

pH was adjusted to 6.5; At the time of use, β-mercaptoethanol was added at the final concentration of 100 mM; Stored at 8 – 12 °C.

Gel stock (30 %)

Material	Concentration
Acrylamide	0290 g
Bis–acrylamide	0010 g
Deionized water	1000 mL
Total volume	1000 mL

Incubated at room temperature overnight, filtered through Whatman paper of grade 1; Stored at 8 – 12 °C.

Resolving Gel (12 %)

Material	Volume
Gel Stock 30 %	2000 µL
Deionized water	1600 µL
Tris Cl (pH 8.8, 1.5 M)	1300 µL
SDS 10 %	0050 µL
APS 10 % (freshly prepared)	0040 µL
TEMED	0003 µL
Total volume	5000 µL

Stacking Gel (4 %)

Material	Volume
Gel Stock 30 %	0400 µL
Deionized water	1798 µL
Tris Cl (pH 6.8, 1 M)	0750 µL
SDS 10 %	0030 µL
APS 10 % (freshly prepared)	0020 µL
TEMED	0002 µL
Total volume	3000 µL

Both APS and TEMED were added lastly just before pouring the gel in a gel plate system.

Fixative for polyacrylamide gels

Material	Volume
Methanol	050 mL
Acetic acid	010 mL
Deionized water	040 mL
Total volume	100 mL

Prepared freshly.

Tris Buffered Saline (TBS)

Material	Concentration
Tris	050 mM
NaCl	150 mM

Prepared in distilled water; pH was adjusted to 7.4; Stored at 8 – 12 °C.

Bjerrum-Schafer-Nielsen buffer for Semi-dry transfer

Material	Concentration
Tris base	048 mM
Glycine	039 mM

Deionized water was used as a solvent; pH was adjusted to 9.2; Buffer was not stored, was prepared freshly, and cooled before use.

TBST

% Triton X-100 in TBS; Stored at 8 – 12 °C.

Blocking buffer (TBS-MT)

5 % skimmed milk powder in TBST; prepared freshly.

Antibody dilution buffer

Material	Volume
BSA	002.00 g
Sodium azide	000.02 g
TBST	100.00 mL

Stored at 8 – 12 °C.

BCIP-NBT (50x)

Material	Weight / Volume
BCIP	09.40 mg
NBT	18.75 mg
DMSO 67 % (in water)	01.00 mL

Stored at 2 – 8 °C; diluted in ALP substrate buffer freshly at the time of use.

ALP substrate buffer

Material	Concentration
Tris base	0.10 M
NaCl	0.10 M
$MgCl_2$	0.05 M

pH was adjusted to 9.5; stored at 8 – 12 °C.

1% Acid-alcohol

Material	Volume
HCl	001 mL
Ethanol	070 mL
Deionized water	029 mL
Total volume	100 mL

Stored at room temperature.

Retrieval buffer

Material	Concentration
Tris-HCl	10 mM
Disodium EDTA	01 mM

This solution was prepared in deionized water; pH was adjusted to 8; stored at 8 – 12 °C.

List of Antibodies

Name	Clonality	Host	Molecular weight (kDa)	Catalogue number
COX-2	Polyclonal	Rabbit	70	SAB4200576
COX-1	Polyclonal	Rabbit	68	SAB4502490
GAPDH	Polyclonal	Rabbit	36	G9545
Cl. CASP-3	Polyclonal	Rabbit	17	C9585
A-dystroglycan	Monoclonal	Rabbit	156	IIH6C4
MyoD1	Monoclonal	Mouse	34.5	PCRP-MYOD1-2A5

Table 4: *List of antibodies along with their specifications.*

Primer List

Gene name	Forward primer	Reverse primer
18s rRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC
BMP2	ATGTTGGACCTCTATCGCCTG	CCAAAACTTCTTCGTGGTGG
BMP4	TGGAAGAACGTGTCCATCGC	AGACTGGCATGGTTGCTCTC
BMP7	ATCTGCCTACAAAATTGGTTCTC	TACTCACAGCGCATTCTCACTT
CASP3	AAAGATGGACCACGCTCAGG	TGACAGTCCGGTATCTCGGT
COX-1	CGGAGCTGCCTATCGCC	CACGGTTAACGGTGCC
COX-2	AACACAACTGCCCTACTCCG	CTGGTCCTTTGCGGTGATCT
FGF8	GAGACCGACACCTTTGGGAG	TTGCCGTTACTCTTGCCGAT
FGF9	GGTCACGGACTTGGACCATT	GCTGTCTACTCCTCGGATGC
GLI3	TCTCGTAGCAGTTCGTCAGC	TCAGAGCAGGGCTT
GREM1	GCGCTGTGTTTCTTCTGACG	GGGATAGCGCCTTGAGATCC
LAMA2	TCCCCTCTTGATTCGTGTGC	AAGCCAGAGTCAGCCATTGT
MYF5	CCAGGAGCTCTTGAGGGAAC	AGTCCGCCATCACATCGGAG
MYOD1	CGGAATCACCAAATGACCCAA	ATCTGGGCTCCACTGTCACT
MYOG	CATCCAGTACATCGAGCGCC	GCTCAGGAGGTGATCTGCG
NOGGIN	GCACCCGGACCCTATCTTTG	TAGGGTCGAAGTGTCCTCCC
SHH	TGCTAGGGATCGGTGGATAG	ACAAGTCAGCCCAGAGGAGA
SMO	TCCATCAAGAGCAACCACCC	AGAACCCAAAGATGCCCAGC
TGF-β1	TCGACTTCCGCAAGGATCTG	CCCGGGTTGTGTTGGTTGTA
TIMP3	ACTCAGGACACCAAGCGAAG	AAGGAAGGTACAGCGTGCTC
WNT3A	TCGGAAACTCCCCTTTCAGC	TGCTCATCTTGCCTGGAGTG

Table 5: *Primer sequences as derived from ncbi.*