Chapter 2
Role of Cholesterol in embryonic development
3.1 Introduction:

Cholesterol is an essential lipid found in all mammalian cells, as a major lipid component of the membrane. In addition to its role as a structural component of cell membranes, it also act as a precursor molecule for sterol based compounds including bile acids, oxysterols, neurosteroids, glucocorticoids, mineralocorticoids and sex steroids such as esterogen and testosterone (Correa-Cerro et al., 2005).

Cholesterol is emerging as important molecule in many aspects of processes involved in both cell and developmental biology including genetic disorders arising out of defects in its biosynthetic enzymes. Lipid rafts of cell membranes, rich in cholesterol and sphingolipids, control cell signaling via receptor tyrosine kinases and G protein-coupled receptors (Pike, 2003). The findings that signal transduction is often altered when lipid rafts are disrupted by depletion of cellular cholesterol suggest that cholesterol-rich domains participate in the control of cell signaling (Pike, 2005). Cholesterol is thought to serve as a spacer between the hydrocarbon chains of sphingolipids and acts as a glue that keeps the raft assembly together (Simons and Ehehalt, 2002).

Cholesterol is a remarkably versatile molecule and determines the biophysical properties of cellular membranes (Yeagle, 1985). It serves as a precursor for steroid hormones and regulates the function of signaling molecules like hedgehog (Mann and Beachy, 2000). Given this multitude of functions, it is not surprising that acquired or genetic defects in cholesterol metabolism cause severe diseases (Farese and Herz, 1998; Moebius et al., 2000; Roux et al., 2000; Kelly and Herman, 2001; Nwokoro et al., 2001) including arteriosclerosis (Sacks, 1998); McNamara, (2000) Smith–Lemli–Opitz syndrome (Opitz et al., 2002) and Niemann–Pick type C disease (Vanier, 1999).

Cells are provided with several compounds that are essential for cell growth and division by cholesterol biosynthesis pathway, such as mevalonic acid (Quesney—Huneeus et al., 1983; Cuthbert and Lipsky, 1990; Martínez-Botas et al., 2001), farnesyl pyrophosphate (Chakrabarti and Engleman, 1991), geranylgeranyl pyrophosphate (Tatsuno et al., 1997) and cholesterol (Chen et al., 1975; Kandutsch and Chen, 1977; Fernández et al., 2004).

It has been shown that cultured human cells when treated with distal inhibitors of cholesterol synthesis inhibit cell proliferation and selectively arrest cell
cycle in G2/M phase (Fernández et al., 2005). Inhibitors of cholesterol biosynthesis induce holoprosencephaly and this observation points out the essential role for cholesterol in mediating ligand-receptor interactions that activate the hedgehog signaling pathway (Ingham, 2001). Rat embryos treated with distal inhibitors of cholesterol cause anomalies of brain, limb and genitalia implying a posttranslational function attributed to cholesterol (Roux et al., 2000). The inhibitor induced patterning defects of limbs reveal that malformations originate from imbalance of Indian hedgehog expression (Gofflot et al., 2003) and that inhibition of cholesterol synthesis results in reduced cholesterol levels in the growth plate suppressing chondrogenesis (Wu and De Luca, 2004).

Compactin, a potent inhibitor of HMG-CoA reductase which is a rate limiting enzyme in cholesterol biosynthesis has been shown to induce abnormal gastrulation during sea urchin development (Carson and Lennarz, 1979). It is also known that cholesterol has a role in neuronal differentiation mediated through neuron-glial interactions during development (Goritz et al., 2005). Massive synaptogenesis depends on cholesterol production by glial cells that is delivered to neurons by apolipoprotein E-containing lipoproteins (Jüttner and Rathjen, 2005). More recently, it is revealed that the mitochondria-dependent death cascade induced by blocking intracellular cholesterol trafficking is caspase dependent (Huang et al., 2006).

The importance of sterols in normal embryonic development has been underscored by the discovery that inborn errors of cholesterol synthesis cause human and murine malformation syndromes (Porter, 2002). Five disorders involving enzyme defects in post-squalene cholesterol biosynthesis have been identified since 1998 (Herman, 2003). Besides, Smith-Lemli-Optiz Syndrome is a multiple congenital anomaly mental retardation syndrome due to an inborn error of cholesterol biosynthesis (Correa-Cerra et al., 2005). Several such reports point out that cholesterol has multiple essential roles in embryonic development.

Cholesterol is a major lipid component of plasma membrane of animal cells and is essential for cell proliferation and other processes in addition to the structural requirement. When HL-60 (human promyelocytic) cells were cultured in cholesterol free medium and treated with different distal inhibitors of cholesterol biosynthesis which blocks synthesis at different steps allowing synthesis of isoprenoid derivatives and different sets of sterol intermediates. Inhibition of only Δ7-reductase allow cell
proliferation and blocking upstream of this enzyme results in inhibition of cell cycle arrest in G2/M phase (Fernández et al., 2005).

In humans, congenital defects of sterol Δ7-reductase cause Smith-Lemli-Opitz (SLO) syndrome characterized by antenatal growth retardation, malformations, and multiple organ anomalies (Porter, 2003). This syndrome is reproduced in sterol Δ7-reductase deficient mice (Wassif et al., 2001; Fitzky et al., 2001) and in animals treated which were with sterol Δ7-reductase enzyme inhibitors (Repetto et al., 1990; Kolf-Clauw et al., 1996; 1997), and it has been reported that the resultant abnormalities may be prevented at least in part by cholesterol feeding (Roux et al., 1979b; Gaoua et al., 2000).

Study on rat shows that nearly all (93%) of the cholesterol during development was synthesized by embryo itself and neither the maternal circulation nor the placenta/yolk sac contribute significant amount of cholesterol to the fetus. It was found that fetal brain synthesize all of its cholesterol on its own whereas liver supplies about half of the cholesterol for development of heart, lung and kidney by circulation (Jurevics et al., 1997).

Accumulating data provides evidence for a pivotal role played by cholesterol in development and pattern formation, mainly through its involvement in the maturation and transduction of the Hedgehog signal (Porter et al., 1996; Kuwabara and Labouesse, 2002; McCarthy et al., 2002; McCarthy and Argraves, 2003). Spatially controlled cholesterol synthesis is important from the very early stages of Xenopus development, where it is mainly required for the proper development of the central nervous system (Tadjuidje and Hollemann, 2006).

Defects in the genes of cholesterol biosynthesis, lipid transfer and lipoprotein assembly, and in receptors that mediate cellular uptake of cholesterol and lipids affect the formation of function of the nervous system, which seems to be critically dependent on the supply of cholesterol especially during the early stages of development (Farese and Herz, 1998). Genetically defective or pharmacologically inhibited enzymes which are known to cause defective cholesterol biosynthesis and give rise to developmental or postnatal abnormalities are β-hydroxyl-β-methyl glutaryl (HMG)-CoA reductase, mevalonate kinase, Δ7-reductase and Δ24-reductase (Herz and Farese, 1999).
The prototypical example of post-squalene cholesterol biosynthetic defects is SLOS. Other disorders are autosomal dominant chondrodysplasia type 2, HEM dysplasia and some cases of Antley-Bixler syndrome (Correa-Cerro et al., 2005).

Based on the literature survey, it has been decided to use cholesterol biosynthesis inhibitors AY9944 and Mevinolin to check the effect(s) of cholesterol inhibition on early development of frog and chick embryos. AY9944 is the distal inhibitor of cholesterol biosynthesis pathway which blocks cholesterol synthesis at the last step involving Δ7 dehydroreductase enzyme. This enzyme is responsible for conversion of 7-dehydrocholesterol into cholesterol. Mevinolin is another such inhibitor which blocks the enzyme HMG-CoA reductase responsible for the conversion of HMG-CoA into mevinolic acid which is the rate limiting step of cholesterol biosynthesis. By blocking cholesterol biosynthesis at this site blocks not only the synthesis of cholesterol but also the synthesis of other intermediate sterols, which are required by various other pathways (Fig3.1).

**Figure3.1:** Pathway of Cholesterol biosynthesis showing blockage site by inhibitors

Methyl-β-cyclodextrin, another compound which acts as a cholesterol depletor was also been used. It has cholesterol sequestering capacity which allows the depletion of pre-existing cholesterol present in the embryo thus, leaving the embryo with minimum available cholesterol (Fig 3.2).
Figure 3.2: Structure of Methyl β cyclodextrin (MBCD) showing cholesterol binding site of glucose oligomer

3.2 Material and Methods:

This section describes the materials and methods used for the collection of frog embryos (*Microhyla ornata*), dejellying of frog spawns and collection of chick embryos.

Also, this section describes the materials and methods used for treating of frog and chick embryos with cholesterol biosynthesis inhibitor and/or depletor to observe the effect on embryonic development.

The present section, in addition, also describes the material and methods used for preparation of whole mount and the histology of frog and chick embryos.

3.2.1. Materials:

3.2.1.1. Animal model used:

Gastrulating embryos (stage 8/9, formation of blastopore lip) of *Microhyla ornata* were used after dejellying of spawns with 2% cysteine and removing their vitelline membrane with the help of a pair of watchmaker’s forceps under stereo zoom binocular microscope. Gastrulating embryos (stage 4, primitive streak) of *Gallus domesticus* were cultured *in vitro* by New’s ring technique (1955) and used as animal models to find out the effect of inhibition of cholesterol biosynthesis and depletion of cholesterol on embryonic development.
3.2.1.2. Lipid biosynthesis Inhibitors and depletor

**Cholesterol biosynthesis inhibitors-**
AY9944 and Mevinolin (Sigma, USA).

**Cholesterol depletor-**
Methyl-β-Cyclodextrin (Sigma, USA).

**Standard used in inhibitor study:**
Water soluble cholesterol was used for treatment to observe the effects of cholesterol biosynthesis inhibitors in presence of externally available cholesterol (Sigma, USA).

3.2.1.3. Glassware for inhibitor studies:
Flasks (100ml, 250ml and 500ml), Small Petri plates (50mm dia.), Beakers (10ml, 50ml, 250ml and 500 ml), Measuring cylinders (50ml, 100ml and 1000 ml) and Screw cap glass vials (15ml), Bake dish (1500ml.), Big Petri dishes (90mm diameter), Watch glasses (75mm diameter), (all from Borosil, India), Cavity blocks and Pasteur Pipettes (Micro-Aid, India.), and Bent pipettes and Glass rings (20mm diameter) were made to order by a local glass blower.

3.2.1.4. Plasticware and other materials for inhibitor studies:
Watchmaker’s fine forceps -#5 (Sigma, USA), Accu-pipettes (T10, T20, T100 and T1000 micropipette), Micro tips, disposable (2µl -200µl and 200µl -1000µl), Beaker PP Autoclavable(250ml, 500ml and 1000 ml), Wash Bottle- LDPE material (500ml.), Aspirator bottle with stopcock (5lit., 10lit. and 20lit.) PP Autoclavable, Rack for micro tube PC Autoclavable, Micro tip Box PP Autoclavable, Wide mouth bottles PP Autoclavable (250ml.), Beaker PPAutoclavable(250ml and 500ml) (all from Tarson, India.), Rubber bulb, pointed end Forceps and Fine hair brush No. 1, Blunt end Forceps, Small size scissors, Medium size scissors, Enamel bowl, Black plastic sheet, Plastic bags (for waste disposal) and Absorbent Cotton were locally purchased.
3.2.1.5. Chemicals for inhibitor studies:

The following chemicals used for preparation of frog ringer solution and for Pannett and Compton (PC) Saline were of either analytical or ExcelR grade.

Agar agar (Qualigens, India), CaCl₂, MgCl₂, NaCl, KCl, Glucose, Na₂HPO₄, NaH₂ PO₄, CaCl₂.2H₂O, MgCl₂.6H₂O, Na₂HPO₄.12H₂O, NaH₂PO₄.2H₂O and NaOH (all from Merck, Germany).

3.2.1.6. Fixative used:

The control and their respective treated frog embryos were fixed in freshly prepared Bouin’s fixative for the histological studies.

The treated chick embryos along with their respective controls were fixed in aceto-ethanol fixative for the whole mount preparation and in Bouin’s fixative for the histological preparations to check the abnormalities caused at the morphological level.

3.2.1.7. Glassware and other material for histology of embryos:

Glass Slides (75mm x 25mm), Microscopic cover glass (22mm x 40mm), Microscopic cover glass (22mm x 22 mm), Cavity blocks with lid, Glass Coplin jars, Pasteur pipette (all from Micro-Aid, India), Microtome Blades and stainless steel razor (Feather Safety Razor Co. Ltd., Germany).

Desiccator Vacuum, Slide Boxes (50 places) and Coplin jars (Tarson, India). Aluminum spatula and wooden blocks (1” x 1cm) were made to order by local vendor. Fine hair brush (No. 1), forceps and spirit lamp were locally purchased.

3.2.1.8. Chemicals for the histological studies:

DPX mountant, Xylene, Paraffin wax, Picric acid, Eosin 2% w/v, Hematoxylin (Delafield’s), NaHCO₃, Ammonium alum [Al NH₄(SO₄)₂.12H₂O] (all from Qualigens, India.), Glycerol, Eosin (M.S) spirit soluble, Sodium salicylate (all from S.D. Fine Chemicals Ltd., India), Glacial acetic acid (BDH Glaxo Laboratories, India.), Formaldehyde (Fisher scientific, India.), Hematoxylin (Sigma, USA), Sulphuric acid 98% GR (Merck, India) and Ethnaol (locally purchased).
### 3.2.1.9. Instruments used for the study:

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<td>ProgRes C₃</td>
<td>Jenoptik, Germany</td>
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<tr>
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<td>Tempo instruments, India</td>
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<td>Drying oven (50ºC)</td>
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<td>Fine weighing balance</td>
<td>Sartorius, USA</td>
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<td>Lab Hosp, India</td>
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3.2.2. **Preparation of solutions:**

3.2.2.1. **Preparation of L-Cysteine:**

Refer materials and methods; section 2.2.2.1 of chapter 2: Embryonic profiles of Cholesterol and Phospholipids by HPLC and their significance in embryonic development.

3.2.2.2. **Stock solution of Inhibitors:**

(i) **AY9944** [trans-1, 4-bis (2-chlorobenzylaminoethyl) cyclohexane dihydrochloride]: Purity 99%.

Stock solution was prepared by dissolving AYA9944 in DMSO and was stored at -20ºC. Different concentrations of working solutions were prepared by diluting the stock solution in either frog ringer solution or PC saline and were prepared fresh before use.

(ii) **Mevinolin (6-α-methylcompactin):** Purity ≥ 98%

Stock solution was prepared by dissolving Mevinolin DMSO and was stored at -20ºC. Different concentrations of working solution were prepared by diluting the stock solution in either frog ringer solution or PC saline and were prepared fresh before use.

(iii) **Methyl-β-cyclodextrin (M.W. 1316.3383):**

Different mM concentrations of the depletor were prepared by dissolving methyl-β-cyclodextrin in either frog ringer solution or PC saline and were prepared fresh before use.

3.2.2.3. **Frog Ringer Solution (1 Liter):**

For treatment of frog embryos with lipid biosynthesis inhibitor and / depletor, the dejellied and vitelline membrane removed embryos were kept in frog ringer solution to prevent them from osmotic shock. The treatment solution was also prepared by diluting the stocks in frog ringer solution. Stock solutions of ringer were prepared a day in advance and working solution was freshly prepared before use. The frog ringer solution was prepared according to Sullivan, (1968).
1. **Solution A:**
   - NaCl: 2.92 gm.
   - KCl: 0.18 gm. (500 ml)
   - CaCl₂: 0.26 gm.
   - MgCl₂: 0.20 gm.

2. **Solution B:**
   - NaH₂PO₄: 0.62 gm. (100 ml)

3. **Solution C:**
   - Na₂HPO₄: 0.28 gm. (100 ml)

4. **Glucose**
   - 0.39 gm. (300 ml)

**Working solution (100 ml):**

50 ml of solution A + 10 ml of solution B + 10 ml of solution C + 30 ml of glucose solution. pH of the solution was adjusted to 7.4 with 1M NaOH.

**3.2.2.4. Pannett and Compton (PC) Saline:**

PC saline was used for *in vitro* chick embryo culture to prevent the embryo from osmotic shock during culturing. The PC saline solution was prepared according to Pannett and Compton, (1924).

1. **Glucose Solution (1%):**
   - Dissolve 6.75 gm. of glucose in 675ml. of distilled water.

2. **Solution A:**
   - Chemicals were dissolved in 100 ml of distilled water and volume of the solution was made up to 200 ml with distilled water.
   - NaCl: 24.22 gm.
   - KCL: 3.10 gm.
   - CaCl₂·2H₂O: 3.08 gm.
   - MgCl₂·6H₂O: 2.54 gm.

3. **Solution B1:**
   - Na₂HPO₄·12H₂O: 1.879 gm. in 360ml. distilled water
4. Solution B2:
NaH$_2$PO$_4$.2H$_2$O 0.16 gm. in 80 ml. distilled water
The levels of liquid in all flasks were marked and the loss of solution during autoclaving was compensated with autoclaved DW.

Working solutionB:
Mix 44ml. of solution B1 and 4ml. of solution B2. 45ml of this solution was taken for preparation of PC saline.

Working PC Saline:
1% Glucose solution (675ml of solution 1) + 30ml of Solution A + 45 ml of Solution B

3.2.2.5. Bouin’s Fixative:
Frog embryos were fixed in Bouin’s fixative for histological sectioning as described by Humason, G. L, (1962).
No damage was observed with fixation for overnight or longer for several weeks causes.

Composition:
Picric Acid, saturated aqueous 75 ml
Formaldehyde (40%) 25 ml
Glacial Acetic Acid 5 ml

3.2.2.6. Aceto-ethanol Fixative:
Fixation was done overnight. (Humason,1962)
For 100 ml of fixative,
Ethanol 75 ml
Acetic acid 25 ml

3.2.2.7. Meyer’s Albumin:
Meyer’s albumin was used to adhere the histological sections on glass slides.

Composition:
Egg white 50 ml
Glycerol     50 ml  
Sodium salicylate or thymol crystals  1 gm.

**Preparation method:**

Egg white was beaten (only until well broken up, but not stiff) with egg beater and poured into a tall cylinder. This was allowed to stand until the air brings suspended material to the top (overnight). The liquid was poured off from bottom and to it an equal volume of glycerol was added. Sodium salicylate (Thymol, Merthiolate, or commercially available formaldehyde can also be used) in proportion of 1:100 (w/v) was added as preservative to prevent the growth of molds.

The solution was filtered through filter paper and was kept at 4°C in refrigerator. Meyer’s albumin can be used for months (Humason, 1962).

3.2.2.8. Delafield’s Hematoxylin stain preparation:

4 gm. of hematoxylin powder was dissolved in 25 ml absolute ethyl alcohol. This solution was mixed gradually into 400 ml saturated aqueous ammonium alum (approximately 1 part alum to 11 parts of distilled water).

The solution was left exposed to light for oxidation by keeping the flask with a cotton plug near window for 3 - 5 days and then filtered through filter paper. To this, 100ml of glycerol and 100 ml of methyl alcohol was added.

It was allowed to ripen for at least 6 weeks in dark. The ripened hematoxylin stain can be kept for years in a stoppered bottle in dark. The hematoxylin stain can be diluted according to the need (Humason, 1962).

3.2.2.9. Alcoholic Eosin stain:

1 gm of spirit soluble eosin was dissolved in 100ml of 70% ethanol (stock solution). Working solution of eosin stain was prepared by diluting it 1:1 (v/v) proportion with 70% ethanol (Humason, 1962).

3.2.2.10. Acid and Alkali water:

For the proper bluing of the stained histological sections, 0.1 N HCl was used as acid water to remove the unbound hematoxylin stain from the
sections and 1% NaHCO₃ was used as alkali water for fastening of blue color (Humason, 1962).

### 3.2.2.11. Chromic Acid:

Chromic acid was used for cleaning the glassware (Humason, 1962).

- Potassium dichromate: 20.0 gm
- Water: 200 ml

Dissolve dichromate in water, when cool add very slowly:
- Sulfuric acid, concentrated: 20.0 ml

### 3.2.3. Methods:

#### 3.2.3.1. Sample collection:

**(I) Collection of frog spawn:**

For procedure of spawn collection, refer materials and methods; section 2.2.3.1 of chapter 2: Embryonic profiles of Cholesterol and Phospholipids by HPLC and their significance in embryonic development.

For inhibitor study, frog embryos were collected at 6:00 am so as to get embryos before gastrulation. The embryos were used after removal of jelly and vitelline membrane. Vitelline membrane was removed mechanically by using pointed watchmaker forceps (#5) under stereo zoom binocular microscope. These vitelline membrane denuded embryos were kept in frog ringer solution to avoid osmotic shock.

**(II) Collection of chick embryos:**

Disease free freshly laid, chick eggs were purchased either from Venkateshwara Hatcheries, Pune or from Institute for veterinary and biological products, Pune. Once in the laboratory, the eggs were immediately washed with tap water to remove any adhered dirt, air dried and disinfected by wiping the surface with 70% alcohol. These eggs were then either kept in incubator at 37°C for incubation or at 15°C if to be used next day. Even it is best to use eggs as soon as possible still chick eggs can be kept at 15°C for 3-4 days, but chances of developmental abnormalities are more with the extended cold storage.
3.2.3.2. Inhibitor treatment in frog:

For treatment of the frog embryos with lipid biosynthesis inhibitors and / depletor the vitelline membrane denuded embryos were immersed in 2ml of Sullivan’s Frog Ringer solution in a pair of small Petri-plates containing slabs of 1% agar to avoid sticking of embryos to the glass surface. The embryos were treated at early gastrulation stage (stage 8). The batches of the vitelline membrane denuded embryos were maintained in separate Petri-plates receiving treatment of either specific inhibitor or with a combination of inhibitors.

In solvent control category, the vitelline membrane denuded embryos were maintained only in the Ringer solution containing 0.01% DMSO. For the Master control category, the embryos were maintained with the intact vitelline membrane in the Ringer solution. At least 10 embryos were used in each category in all the sets of experiments performed. The embryos in each category received treatment for 24 hours and were observed under binocular microscope to check the morphological abnormality.

3.2.3.3. In vitro culturing of chick embryo:

The glassware required for culturing was sterilized before use by baking them at 200ºC for 2-3 hours in a dry sterilizer. For sterilization of all the dissection instruments, solutions and petri-plates containing cotton rings; autoclaving at 15 lbs pressure for 20 minutes was carried out.

Chick embryos were cultured in vitro using New’s single ring technique (New, 1955 and 1966). Freshly laid eggs of chick (G. domesticus) were incubated for 18 hours at 37.5ºC with 95% humidity to get Hamburger and Hamilton stage 4 (Hamburger and Hamilton, 1951).

After incubation, eggs were allowed to cool to room temperature for 30 minutes and egg shell was cleaned by wiping with 70% ethanol. The shell was broken with a fine stroke of blunt forceps at the broad end of the egg and shell was removed carefully to make a window big enough to take out the yolk ball. Thick albumin was discarded and thin albumin was collected in a beaker with the help of Pasteur pipette. The intact yolk ball was taken out in a sterile cake dish containing enough PC saline to submerge the yolk ball. The yolk ball was freed of adhering thick albumin and chalazae.
The vitelline membrane was cut equatorially keeping the blastoderm in the center. The vitelline membrane was carefully peeled off along with the blastoderm and transferred onto a watch glass which was placed over a moistened cotton ring in a 90 mm petri-plate. The vitelline membrane was carefully cleaned to remove as much yolk as possible. A glass ring was placed on the membrane in such a way that the blastoderm lies in the center. Extra vitelline membrane was stretched and folded over the ring to allow the blastoderm to stay flat and stretched. After the membrane is firmly attached to the ring, extra membrane was cut with the help of pointed scissors. Yolk particles present below the membrane were cleaned by flushing PC saline with the help of Pasteur pipette.

After cleaning, 1 ml-2ml of thin albumin was placed around the glass ring and with the help of forceps. The glass ring along with the membrane was lifted slightly to allow the albumin to go under the membrane and then released to give the vitelline membrane the desired bulge. This gives the required curvature of the membrane and nutrition to the embryo.

The embryo cultures were then treated with either/ or biosynthesis inhibitors of cholesterol and phospholipids. After the required treatment, the embryos were kept at 37°C for 24 hours in the incubator.

3.2.3.4. Inhibitor treatment in chick:

The chick embryos were cultured as described in above and observed under binocular microscope. Embryos with normal morphology of stage HH4 (Hamburger and Hamilton stage 4) were then selected for further study. The embryos were treated with 100µl of inhibitor solution by placing it drop by drop on the blastoderm. The solvent control (0.01% DMSO in PC saline) and master control (PC saline) were also maintained. The cultures were then kept at room temperature for half an hour so as to allow the diffusion of inhibitor to the embryo. The cultures were incubated at 37°C and observed after 24 hours for any morphological abnormality. Treated embryos showing abnormalities with their respective control embryos were fixed in either Bouin’s or Cornoy’s fixative and processed further for whole mount staining and histology.
3.2.3.5. Tissue fixation in Bouin’s fixative:

After treatment, frog embryos along with their respective control embryos were directly put into fixative and kept in screw tight bottles for overnight.

Treated chick embryos along with their respective control embryos were taken out from petri-plate and their vitelline membrane was removed slowly from the glass ring and was transferred to PC saline. The blastoderm was then gently removed from the vitelline membrane and transferred on a glass slide with the help of forceps. Few drops of the fixative were placed drop by drop on the blastoderm with Pasteur pipette, to spread the blastoderm. The extra blastoderm was cut around the embryo in a rectangular shape with the help of sharp blade. The embryo was then transferred to screw cap bottles for overnight fixation.

The upgraded series of alcohol was used to remove the water content from the embryo and was stored in 70% alcohol until processed for whole mount or histology.

3.2.3.6. Tissue fixation in Aceto-ethanol fixative:

Chick embryos were trimmed as described above and fixed in Aceto-ethanol fixative in screw cap bottles for overnight.

After overnight fixation, embryos were directly transferred in 50% ethanol (thrice for 20 min. each wash) to remove the traces of acetic acid. The embryos were then transferred to 70% ethanol and stored in it till further processing.

3.2.3.7. Protocol for histology:

Frog and chick embryos treated with cholesterol biosynthesis inhibitors and/ or depletor with their respective control embryos were processed for the histological studies to observe the effect of treatment at the tissue level.

Bouin’s fixed embryos were gradually dehydrated in graded series of ethanol and cleared in with graded concentrations in ethanol.

Cold infiltration was carried out for three times with xylene: paraffin wax (1:1) for 30 minutes at room temperature followed by three changes of hot infiltration with molten paraffin wax (60°C). Finally the embryos were embedded individually in fresh molten paraffin wax at 60°C. Blocks were prepared and sections of 7µ thickness were cut.
Sections were de-paraffinized in xylene and gradually hydrated in graded series of ethanol. After a quick wash with distilled water, they were stained with hematoxylin for 2 minutes until the colour appears purple and differentiated with acid water and alkali water. These were then gradually dehydrated in alcohol series, stained with 1% eosin and differentiated in 90% ethanol. After total dehydration, sections were cleared in xylene and mounted in DPX (Humason, 1962).

3.2.3.8. Protocol of Whole mount:

Chick embryos fixed in Cornoy’s fixative were used for whole mount preparation to check the morphological features of the treated embryos along with their respective control embryos.

For the whole mount preparation, the embryos were first rehydrated from 70% ethanol in down grade series of ethanol till water and then stained with hematoxylin. The embryos were then passed through acid water to remove the excess and unbound stain from the tissue and then from alkali water to fasten the stain. The embryos were then again dehydrated in increasing grades of alcohol till 70% ethanol, counter stained with eosin in 70% ethanol and transferred to 100% ethanol. Embryos were then passed through alcohol: xylene (50:50) and Xylene before mounting them on the slide with DPX.

3.3. Results:

3.3.1. Effect of cholesterol biosynthesis inhibitors/ or depletor on embryos of *M. ornata*:

**Dose determination of Cholesterol biosynthesis inhibitors (AY9944 and Mevinolin) and cholesterol depletor MBCD:**

The effective dose for AY9944, Mevinolin and MBCD was determined by using different doses of inhibitors and depletor treat Frog gastrulating embryos (stage 8). Since the molecular size of MBCD (Molecular Weight 3141KD) is quite large and could not pass through the pores of vitelline membrane, it was decided to use vitelline membrane denuded embryos for the treatment in all categories so as to maintain the uniformity. For this purpose spawns were collected from wild and dejellied with 2% cysteine. The vitelline membrane was removed with the help of watchmaker forceps (#5) under stereo zoom binocular microscope. Different doses of inhibitors and depletor were selected and *M. ornata* embryos of developmental stages.
around 7-8 were treated with the inhibitors and/or depletor. Effective treatment concentration of each of the specific inhibitor was determined basing on maximum number of surviving embryos with maximum number of abnormal embryos during experimental span of 24 hrs. The embryos were treated with 2 ml of inhibitor solution in Sullivan’s Frog Ringer.

**Dose determination of AY9944:**

Five different doses of AY9944 ranging from 30 µg/ml - 70 µg/ml with an increment of 10 µg/ml were selected for treatment. It was observed that after 24 hour treatment with 60 µg/ml concentration, only 85% embryos were survived. Therefore, two more doses were introduced i.e. 55 µg/ml and 65 µg/ml in order to check the LD50 value and near 100% survival with maximum no. of abnormal embryos. As evident from the results in table 2, at 55 µg/ml concentration around 95% embryos were abnormal with 100% survival rate and at 65 µg/ml concentration only 55% embryos were survived. Therefore LD50 was determined around 65µg/ml and 55 µg/ml was selected as treatment dose (Table 3.1 and Figure 3.3).

<table>
<thead>
<tr>
<th>AY9944 Dose (µg/ml)</th>
<th>% Abnormal</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td><strong>55</strong></td>
<td><strong>97</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td><strong>65</strong></td>
<td><strong>100</strong></td>
<td><strong>55</strong></td>
</tr>
<tr>
<td>70</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 3.1:** Dose determination of AY9944 in *M. ornata*
Dose determination of Mevinolin:

Similarly, five different doses of Mevinolin were selected for treatment ranging from 1.5 µg/ml – 3.5 µg/ml with an increment of 0.5 µg/ml. Out of these five doses, at 2.5 µg/ml, 100% embryos were abnormal with 100% survival rate. The LD50 was determined around 3.0 µg/ml. For further experiments, 2.5 µg/ml was selected as treatment dose (Table 3.2 and Figure 3.4).

Table 3.2: Dose determination of Mevinolin in M. ornata

<table>
<thead>
<tr>
<th>Mevinolin Dose (µg/ml)</th>
<th>% Abnormal</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>2.0</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td><strong>2.5</strong></td>
<td><strong>100</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>3.0</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>3.5</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 3.3: Histogram of AY9944 for M. ornata
Dose determination of MBCD:

It was known from the literature (Sadler and Jacob, 2004) that MBCD at 50mM concentration depletes nearly 50% of cholesterol from the cells. Therefore, dose range from 30mM to 70mM with an increment of 10 mM was selected for MBCD. Out of these five selected doses 40mM was selected as the treatment dose as it induces maximum no. of abnormal embryos with maximum survival rate. This is evident from the results shown in table 3.3 and figure 3.5.

Table 3.3: Dose determination of MBCD in M. ornata

<table>
<thead>
<tr>
<th>MBCD Dose (mM)</th>
<th>% Abnormal</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td><strong>40</strong></td>
<td><strong>95</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>70</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 3.4: Histogram of Mevinolin for M. ornata
Dose determination of MBCD + AY9944:

In combined treatment of MBCD and AY9944, MBCD concentration was kept constant at 40mM, as below that concentration the cholesterol depletion was very less as evident from graph 3.10. Therefore, different concentrations of AY9944 were used ranging from 10 µg/ml to 50 µg/ml with an increment of 10 µg/ml. The results show that at 30 µg/ml concentration around 55% embryos survived after 24 hour of treatment. Therefore, a lower concentration of 25 µg/ml was used and the treated embryos showed 100% abnormality with 98% survival rate at this concentration. For further experiments 25 µg/ml was decided as the treatment dose (Table 3.4 and Figure 3.6).

Table 3.4: Dose determination of MBCD + AY9944 in M. ornata

<table>
<thead>
<tr>
<th>MBCD(40mM) + AY9944 (µg/ml)</th>
<th>% Abnormal</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td><strong>25</strong></td>
<td><strong>100</strong></td>
<td><strong>98</strong></td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Effect of cholesterol biosynthesis inhibitors (AY9944 and Mevinolin), cholesterol depletor (MBCD) and combined treatment (MBCD + AY9944) on early embryonic development of *M. ornata*

The effects of cholesterol biosynthesis inhibitor AY9944 (55 μg/ml), Mevinolin (2.5 μg/ml) and cholesterol depletor MBCD (40 mM) were studied using gastrulating embryos of *M. ornata*.

The effect of blocking cholesterol biosynthesis simultaneously with depleting the preexisting cholesterol in the frog embryos was also studied by combined treatment of AY9944 (25 μg/ml) and cholesterol depletor MBCD (40 mM).

The detailed treatment procedure is explained in materials and method chapter, sections 2.2.1.3.1 and 2.2.1.3.2. At least 10 embryos were used in each category of treatment during all the sets of experiments performed. The experiment for each set was repeated at least 10 times. The embryos of each category received treatment for 24 hrs. The results are shown in figure 3.6 and the data for abnormalities is given in table 3.5.

Figure 3.6: Histogram of MBCD + AY9944 for *M. ornata*
Fig 3.7: Effect of treatment with cholesterol inhibitors (AY9944 and Mevinolin), depletor (MBCD) and combined treatment (MBCD + AY9944) on early embryos of M. ornata [C- Control; AY-AY9944; Me-Mevinolin; M- MBCD; MA- MBCD + AY9944] (Scale bar =200µ).

Both the treated and control embryos were observed for morphological changes under binocular microscope at the time interval of one hour. No apparent effect was noticed in treated groups as compared to control till embryos reached stage 12 (circular blastopore).

The Mevinolin treated embryos showed shortening of anterio-posterior (AP) body axis from stage 12 onwards. AY9944 treated embryos also showed slightly reduced AP body axis at around stage 22-23 (neural tube closure; differentiation of head trunk and tail), as compared to the control embryos. This morphological variation continued till stage 25 (eye development).

After twenty-four hours treatment at stage 26, it was observed that the embryos treated with AY9944, Mevinolin and those treated with combination of AY9944 plus MBCD have developed abnormalities of AP axis, tail shape and body
pigmentation. Control embryos did not show any abnormalities whereas in master-control category few embryos were naturally abnormal (growth retardation).

Table 3.5: Effects of cholesterol inhibitors and / depletor on embryos of frog

<table>
<thead>
<tr>
<th>Number of Embryos</th>
<th>Master Control</th>
<th>Ringer Control</th>
<th>AY9944(55 µg/ml)</th>
<th>Mevinolin(2.5 µg/ml)</th>
<th>MβCD(40mM)</th>
<th>AY9944 + MβCD (40mM + 25 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>273</td>
<td>273</td>
<td>155</td>
<td>100</td>
<td>119</td>
<td>183</td>
</tr>
<tr>
<td>Normal</td>
<td>273</td>
<td>260</td>
<td>69</td>
<td>-</td>
<td>39</td>
<td>86</td>
</tr>
<tr>
<td>Abnormal</td>
<td>-</td>
<td>13</td>
<td>86</td>
<td>100</td>
<td>80</td>
<td>97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Categories of Abnormalities*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short axis</td>
</tr>
<tr>
<td>Bent tail</td>
</tr>
<tr>
<td>Less body pigmentation</td>
</tr>
<tr>
<td>Completely abnormal</td>
</tr>
<tr>
<td>Abnormal tail fin</td>
</tr>
</tbody>
</table>

* More than one category of abnormalities may be seen in single embryo

On the basis of the results obtained, it was concluded that the treatment of early gastrulating embryos of *M. ornata* by cholesterol biosynthetic inhibitors (AY9944 and Mevinolin) results in the formation of abnormal embryos. In most of the cases the resulted abnormalities involved reduction in AP axis.

AY9944 in combined treatment with MBCD shows reduction in number of embryos with short axis. Besides, in the combined treatment, the effect on pigmentation is less pronounced. Formation of abnormal tail-fin is an exclusive abnormality in the MBCD treatment and this may be an outcome of MBCD influence, also effect on tail bending is more pronounced in treatment with MBCD. Mevinolin has more profound effect in inducing abnormalities than the AY9944.
Therefore, it can be said that cholesterol biosynthetic inhibition at intermediate step of the pathway perturbs embryonic development more severely as compared to the inhibition at the terminal step of the pathway.

Length Measurement of frog tadpoles treated with cholesterol biosynthetic inhibitor and/depletor:

The embryos treated with cholesterol biosynthesis inhibitor and / depletor shows morphological abnormalities mainly related to AP axis. The degree of effect was checked by measuring the AP axis of control and treated embryos (using axiovision software). About 50 embryos were used in each category and data obtained by these measurements with standard deviation is shown in table 3.6.

**Table 3.6:** Length of AP axis of control and treated embryos in M. ornata

<table>
<thead>
<tr>
<th></th>
<th>Control (mm ± S.D.)</th>
<th>AY9944 55µg/ml (mm± S.D.)</th>
<th>Mevinolin 2.5µg/ml (mm± S.D.)</th>
<th>MBCD(40 mM) (mm± S.D.)</th>
<th>MBCD+AY9944 40mM + 25µg/ml(mm± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.8180± 0.35</td>
<td>2.5367± 0.36*</td>
<td>2.2600± 0.16*</td>
<td>2.9023± 0.29*</td>
<td>3.2638 ± 0.18*</td>
</tr>
</tbody>
</table>

(n=50); * shows statistical significance p<0.001

The results obtained by measurements of tadpole length clearly indicate that there is considerable reduction in the anterio-posterior axis of tadpoles after 24 hours of treatment with cholesterol biosynthesis inhibitors and depletor.

The average length of control embryos was 3.81 mm, whereas the length of AY9944 treated embryos was 2.53 mm, embryos treated with Mevinolin had almost half the reduction in length i.e. 2.26 mm and embryos treated with MBCD had average length of 2.90 mm as compared to that of the control embryos. In combined treatment of MBCD and AY9944, the average length of embryos was 3.26 mm, which is less than that of the control one, but is more as compared to that found in embryos treated with the individual treatment with AY9944 and MBCD. Statistical significance was calculated by student’s paired t- test using SPSS software (version 16.0).
Statistical analysis was carried out using Student’s t-test with the values representing the mean ± standard deviation of the total number of samples indicated in each legend.

**Hourly treatment of cholesterol biosynthetic inhibitor and/ depletor on *M. ornata* embryos:**

The aforementioned results indicated that there is a link between embryonic development and cholesterol requirement. Further it was planned to treat the stage 8 gastrulating embryos with the inhibitor and/ depletor. This was to check at which stage the requirement of cholesterol is crucial and the effect of inhibitors is irreversible. The treatment solution was changed with ringer at different time intervals, to study the effect of cholesterol biosynthesis inhibitors and/ depletor on embryo after 24 hours.

For this purpose 6 different experimental sets were prepared containing treatment solution and 10 embryos in each category. Treatment solution was changed with ringer solution at 2, 4, 6, 8, 10 hour interval and one set was kept overnight to compare with the previous results. The length was measured in each category after 24 hour of treatment respectively and the results are shown in table 3.7.

**Table 3.7: Length of AP axis of *M. ornata* embryos after treatment for different time interval**

<table>
<thead>
<tr>
<th>Time (Hour)</th>
<th>Control (mm ± S.D.)</th>
<th>AY9944 55µg/ml (mm ± S.D.)</th>
<th>Mevinolin 2.5µg/ml (mm ± S.D.)</th>
<th>MBCD (40 mM) (mm ± S.D.)</th>
<th>MBCD+AY9944 (40mM + 25 µg) (mm ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Hour</td>
<td>3.78 ± 0.12</td>
<td>2.75 ± 0.28*</td>
<td>3.62 ± 0.10*</td>
<td>3.48 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td>4 Hour</td>
<td>3.85 ± 0.22</td>
<td>2.84 ± 0.21*</td>
<td>3.56 ± 0.12*</td>
<td>3.28 ± 0.13*</td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>3.69 ± 0.25</td>
<td>2.82 ± 0.18*</td>
<td>3.50 ± 0.08*</td>
<td>3.20 ± 0.12*</td>
<td></td>
</tr>
<tr>
<td>8 Hour</td>
<td>3.88 ± 0.10</td>
<td>2.64 ± 0.14*</td>
<td>3.23 ± 0.13*</td>
<td>3.02 ± 0.21*</td>
<td></td>
</tr>
<tr>
<td>10 Hour</td>
<td>3.79 ± 0.11</td>
<td>2.56 ± 0.20*</td>
<td><em>3.01 ± 0.10</em></td>
<td><em>2.80 ± 0.23</em></td>
<td></td>
</tr>
<tr>
<td>24 Hour</td>
<td>3.80 ± 0.12</td>
<td><em>2.68 ± 0.24</em></td>
<td><em>2.10 ± 0.37</em></td>
<td><em>2.94 ± 0.13</em></td>
<td></td>
</tr>
</tbody>
</table>

(n=30); * shows statistical significance p<0.001

The results show that, in AY9944 treatment, the effect on embryos was prominent from 2 hour treatment category and there is consistent reduction in the AP
axis length in further time interval categories. This indicates, that AY9944 affects cholesterol biosynthesis even if it is present for short time span i.e. it has major effect on gastrulating embryos.

In Mevinolin treatment the effect was visible from 2 hour of treatment, as it blocks cholesterol biosynthesis at very early step and thus the embryos were not able to develop normally thereafter, showing considerable reduction in AP length.

However, after treatment with MBCD (after two hours), there was almost no difference in the AP length of tadpoles in comparison with that of control.

Embryos with combined treatment of MBCD and AY9944 show slight reduction in AP length. In combined treatment of MBCD and AY9944 from 2-6 hour the reduction in length may be due to the effect of AY9944. After which the reduction is more prominent, because of combined effects of MBCD and AY9944.

There was no major difference in the length of embryos treated with inhibitors and/depletor for 4 hours and 6 hours. After 8 hours of treatment, there was considerable reduction in the AP length of tadpoles in each category. And after 10 hour of treatment, there was further reduction in AP length. Overnight treated embryos were found to be most affected ones in this respect. There was no further reduction in AP axis length of treated embryos even if the treatment was given for 28 hours.

The abovementioned results indicate that the treatment with MBCD for 2 to 6 hour category the cholesterol is trapped initially, but it is replaced by ringer solution. It gave embryo enough cholesterol from its de novo biosynthesis to overcome from major deformity.

But in categories from 8 hour treatment onwards MBCD has more time for action for trapping most of the available cholesterol. Thus reduction in length was more prominent in later categories.

Treatment of frog embryo with cholesterol biosynthesis inhibitor and Cholesterol:
It was clear from the data of cholesterol biosynthesis inhibitors treatment that inhibition of cholesterol biosynthesis affects normal embryonic development. This is however, was unclear that the results obtained were because of unavailability of cholesterol. Therefore, it was decided to do experiment with inhibitor and to supply cholesterol externally to the embryo. By this way even though the biosynthesis is blocked; cholesterol will be available to the embryo.

The experiments were planned to use different doses of water soluble cholesterol in combination with set doses of AY9944 and Mevinolin. Cholesterol alone was also used in different doses to check its adverse effect on embryo, if any. The embryos were observed after 24 hours of treatment for abnormalities and their lengths were measured.

The results (table 3.8) show that there was no adverse effect of cholesterol alone on embryonic development of *M. ornata* and the AP length of embryos in all concentration was almost up to the normal length.

When cholesterol is supplied in 20 µg concentration with AY9944, embryos slightly recovered from effects of inhibitor with respect to the AP length. When concentration of cholesterol is increased, the AP length of the embryos was also increased.

Maximum effect of cholesterol was seen at 80 µg and 100 µg concentration. This indicates that if cholesterol is supplied in more concentration than the inhibitor, then the effects of inhibitor can be reversed. That means if abundant supply of external cholesterol is present, embryo can use it successfully for its development.

When cholesterol is supplied with Mevinolin, there was no evident effect at lower concentrations. At higher concentration of cholesterol there was very slight increase in AP length.

This indicates that when cholesterol synthesis is blocked at earlier step and intermediate sterols are also not available, external cholesterol alone is not sufficient for maintaining normal embryonic development.

*Table 3.8:* Length of AP axis of *M. ornata* embryos after treatment with cholesterol and cholesterol + inhibitors
Histology of embryos treated with cholesterol biosynthesis inhibitors (AY9944 and Mevinolin), cholesterol depletor (MBCD) and combination (MBCD + AY9944):

The effect of cholesterol biosynthesis inhibitor, AY9944 (67.5 µg/ml), Mevinolin (2.5 µg/ml), cholesterol depletor, methyl-β-cyclodextrin (MBCD, 50mM) and combined treatment of AY9944 with MBCD (25 µg/ml + 50mM) was explored on early embryonic development of frog embryos.

Histological studies of the treated embryos of *M. ornata* were performed (Material and Methods wide section 2.2.2.3.2) along with their respective control embryos to observe the effect of inhibitor at the tissue level. At least 10 embryos were used for this study in each category of treatment. The stained preparations were studied under stereo-zoom binocular microscope and results were recorded photographically.

The sagittal and transverse sections of AY9944 inhibitor treated embryos with respect to their control embryos are shown in Fig. 3.7. The AP axis of the embryos was found to be reduced and the tail was bent (bold arrow).

The transverse sections in panel 2 clearly showed narrow prosencephalon cavity (prosocoel) (shown by arrow head). In panel 3, the transverse sections passing through the treated embryo showed underdeveloped optic cups and eye lenses (bold arrow). Further, the treated sections also showed rhombencephalon cavity (rhombocoel), which is smaller and its ends are joined anteriorly (star) as compared to that observed in the sections of the control embryos.
From the results it has been observed that AY9944 inhibitor perturbs with the normal development of brain, axis and eyes.

**Figure 3.8: Effect of AY9944 (67.5 µg/ml) at tissue level**

1: Sagittal section of AY treated embryo showing short axis and bent tail (bold arrow) as compared to C (bar=200µm) 2: Transverse section showing reduced prosencephalon cavity region (arrowhead) 3: Transverse section showing underdeveloped lens and optic cups (bold arrow); abnormal rhombencephalon (star) in AY treated as compared to C (Bar-100 µm) [C-Control; AY- AY9944]

Sagittal sections in fig 3.8, panel 1 show that Mevinolin treated embryo show extremely reduced AP axis (arrow) and abnormality in the development of the pharynx (shown by bold arrow).

Transverse section passing through the embryo in panel 2 shows that brain and eye development were severely affected (star and bold arrow respectively). The development of optic cup and eye lenses was also retarded.

Transverse sections passing through the embryo as mentioned in Panel 3; further confirms abnormal rhombencephalon region (future metencephalon and myelencephalon region of the brain) shown by arrow head and abnormal
infundibulum region (shown by arrow). The section also confirms the undeveloped pharynx (star) and stunted eye development.

In panel 4, the transverse sections passing through the embryo showed that the spinal cord and notochord of the embryos were situated closely as compared to those of the control embryos. The section shows abnormal tail fin (shown by bold arrow).

Figure 3.9: Effect of Mevinolin at tissue level 1: Sagittal section of Mevinolin treated embryo showing short axis and bent tail (arrow); underdeveloped pharynx (bold arrow) as compared to C (bar=200µm) 2: Transverse section showing abnormal brain (star) and eyes (bold arrow) 3: Transverse section showing abnormal rhombencephalon (arrow head) and abnormal infundibulum region (arrow); undeveloped pharynx and stunted eye development (star) 4: Transverse section showing closely situated spinal cord and notochord (arrow); abnormal tail fin (bold arrow) in Mevinolin treated as compared to C (Bar-100 µm) [C-Control; Me-Mevinolin]

The sagittal sections of frog embryos treated with MBCD showed formation of bent tail and abnormal tail fin (arrow head) (fig. 3.9, Panel 1). The sagittal sections also show formation of shrank pharynx, giving it a collapsed appearance (arrow). In
panel 2 and 3, the transverse section passing through the treated embryos showed development of abnormal prosencephalon and rhombencephalon region of brain (star and bold arrow respectively). Panel 4 shows abnormal dorsal and ventral tail fin (shown by arrows) in transverse section passing through the respective region of the treated embryo.

**Figure 3.10:** Effect of MBCD at tissue level 1: Sagittal section of MBCD treated embryo showing bent tail and abnormal tail fin (arrow head); shrunken pharynx (arrow) as compared to C (bar=200µm) 2: Transverse section showing abnormal prosencephalon (star) 3: Transverse section showing abnormal rhombencephalon (bold arrow) and abnormal infundibulum region (arrow); undeveloped pharynx and stunted eye development (star) 4: Transverse section showing abnormal dorsal and ventral tail fin (arrows) in MBCD treated as compared to C (Bar-100 µm) [C-Control; MB- MBCD]

Sagittal sections passing through the frog embryos treated with combination of MBCD + AY9944 show collapsed pharynx as reported in fig 3.10, panel 1.
Transverse sections in panel 2 show that the inner walls of prosencephalic region came close to each other as the prosocoel was reduced (star). In panel 3 the transverse section show that the abnormality in brain region further continued to rhombencephalon region. The cells in eye lenses were underdeveloped (bold arrow head) and the infundibulum was also not developed properly (bold arrow).

**Figure 3.11:** Effect of MBCD + AY9944 at tissue level 1: Sagittal section of MBCD + AY994 treated embryo showing collapsed pharynx (arrow) as compared to C (bar=200µm) 2: Transverse section showing reduced prosocoel (star) 3: Transverse section showing abnormal rhombencephalon (arrow head); abnormal infundibulum region (bold arrow) and underdeveloped eyes and lens (bold arrowhead) in MBCD + AY994 treated as compared to C (Bar-100 µm) [C-Control; MA- MBCD + AY9944]

3.3.2. Study on cholesterol biosynthesis inhibitors/ or depletor in embryos of *Gallus domesticus*:
Dose determination of Cholesterol biosynthesis inhibitors (AY9944 and Mevinolin) and depletor MBCD in embryos of *Gallus domesticus*:

The effective dose of AY9944, Mevinolin and MBCD was determined by using different doses of inhibitors and depletor to treat *Gallus domesticus* gastrulating embryos (HH stage 4).

Different doses of inhibitors and depletor were selected and implemented on chick embryos. Effective treatment concentration of each of the specific inhibitor was determined based on maximum number of surviving embryos with maximum number of abnormal embryos during experimental span of 24 hrs. The embryos were treated with 100 µl of inhibitor solution in PC saline (Panett and Compton).

**Dose determination of AY9944:**

Seven different doses of AY9944 ranging from 30 µg/ml - 60 µg/ml with an increment of 5 µg/ml were selected for treatment in order to check the LD50 value and near 100% survival with maximum no. of abnormal embryos. As evident from the results in table 3.12, at 50 µg/ml concentration, around 97% embryos were abnormal with 100% survival rate and at 60 µg/ml concentration, only 35% embryos were survived. Therefore LD50 was determined in between 55- 60 µg/ml and 50 µg/ml was selected as treatment dose (Table 3.9 and Figure 3.11).

**Table 3.9:** Dose determination of AY9944 in *G. domesticus*

<table>
<thead>
<tr>
<th>AY9944 (µg/ml)</th>
<th>% Abnormal</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>35</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>59</td>
<td>100</td>
</tr>
<tr>
<td>45</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td><strong>50</strong></td>
<td><strong>97</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>55</td>
<td>100</td>
<td>68</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>35</td>
</tr>
</tbody>
</table>
Dose determination of Mevinolin:

Similarly, seven different doses of Mevinolin were selected for treatment ranging from 4 µg/ml – 16 µg/ml with a gradual increment of 2 µg/ml. Out of these seven doses, at the concentration of 10 µg/ml, 100% embryos were abnormal with 100% survival rate. The LD50 was determined around 14 µg/ml. For further experiments, 10 µg/ml was selected as treatment dose (Table 3.10 and Figure 3.12).

Table 3.10: Dose determination of Mevinolin in G. domesticus

<table>
<thead>
<tr>
<th>Mevinolin (µg/ml)</th>
<th>% Abnormal</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td><strong>10</strong></td>
<td><strong>100</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>14</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>16</td>
<td>100</td>
<td>30</td>
</tr>
</tbody>
</table>
Dose determination of MBCD:

Seven different doses ranging from 4 mM to 16 mM with an increment of 2 mM was selected for MBCD. Out of these seven selected doses, 12 mM was selected as the treatment dose as it induced maximum no. of abnormal embryos with maximum survival rate. The LD50 is determined to be around 14 mM. This is evident from the results shown in table 3.11 and figure 3.13.

**Table 3.11: Dose determination of MBCD in G. domesticus**

<table>
<thead>
<tr>
<th>MBCD Dose (mM)</th>
<th>% Abnormal</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td><strong>12</strong></td>
<td><strong>95</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>14</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>16</td>
<td>100</td>
<td>60</td>
</tr>
</tbody>
</table>
**Figure 3.14:** Histogram of MBCD for *G. domesticus*

**Dose determination of MBCD + AY9944:**

In combined treatment of MBCD and AY9944, MBCD concentration was kept constant at 12 mM, as mentioned in Table 3.12 and the abnormality developed in the chick embryo was very less as evident from graph 3.18.

Therefore, different concentrations of AY9944 were used ranging from 10 µg/ml to 60 µg/ml with an increment of 10 µg/ml. The results show that at 40 µg/ml concentration 100% embryos survived after 24 hour treatment and the treated embryos showed 98% abnormality at this concentration. For further experiments 40 µg/ml AY9944 and 12 mM MBCD was decided as the treatment dose (Table 3.12 and Figure 3.14).

**Table 3.12: Dose determination of MBCD + AY9944 in *G. domesticus***

<table>
<thead>
<tr>
<th>MBCD (12mM) + AY9944 (µg/ml)</th>
<th>% Abnormal</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td><strong>40</strong></td>
<td><strong>98</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>60</td>
</tr>
</tbody>
</table>
Effect of cholesterol biosynthesis inhibitors (AY9944 and Mevinolin), cholesterol depletor (MBCD) and combined treatment (MBCD + AY9944) on early embryonic development of the chick *Gallus domesticus*

The effects of cholesterol biosynthesis inhibitor AY9944 (50 µg/ml), Mevinolin (10 µg/ml) and cholesterol depletor MBCD (12 mM) were studied using gastrulating embryos of *G. domesticus*.

The effect of blocking cholesterol biosynthesis simultaneously with depleting the preexisting cholesterol in the chick embryos was studied by combined treatment of AY9944 (40 µg/ml) and cholesterol depletor MBCD (12 mM). The detailed treatment procedure is explained in materials and methods, sections 2.3.1.3.1 and 2.3.1.3.2.

At least 10 embryos were used in each category of treatment during all the sets of experiments performed. The experiment for each set was repeated at least 10 times. The embryos of each category received treatment for 24 hrs and the data is given in table 3.13.
Table 3.13: Effects of cholesterol inhibitors and / depletor on Gallus domesticus

<table>
<thead>
<tr>
<th>No. of Embryos</th>
<th>Master Control (Ringer)</th>
<th>Control (0.01% DMSO)</th>
<th>AY9944 (50 µg/ ml)</th>
<th>Mevinolin (10µg/ ml)</th>
<th>MBCD (12mM)</th>
<th>AY9944 + MBCD (40µg/ ml +12mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Normal</td>
<td>100</td>
<td>90</td>
<td>22</td>
<td>0</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>Abnormal</td>
<td>-</td>
<td>-</td>
<td>78*</td>
<td>100*</td>
<td>80*</td>
<td>79</td>
</tr>
</tbody>
</table>

Categories of Abnormalities*:

- Abnormal brain - (35) - (40) (50)
- Elongated axis - - (10) - (10) -
- Fused somites - - (55) (95) (65) (20)
- Reduced axis - - (35) (90) (35) (60)
- Enlarged left/right - - (11) - - -

* More than one category of abnormalities may be seen in single embryo.

From the results in table 3.13, it is evident that cholesterol biosynthesis inhibitor AY9944 has major impact on the length of AP axis as well as development of brain and somites of the chick embryos. It also has some degree of effect on enlargement of right optic vesicle. The effect of AY9944 is more pronounced on somitogenesis; as evident from highest number of somatic abnormalities induced (55/100). Induced abnormalities of embryonic axis (45/100) are second severely affected phenotype, which is clearly under regulation of cholesterol biosynthesis.

Neural tube and brain abnormalities (35/133) are equally vulnerable sites getting perturbed by inhibition of cholesterol synthesis. Cholesterol biosynthesis inhibitor, Mevinolin also has great impact on the development of axis and somites of the embryos. The effect on somitogenesis and the length of AP axis is evident by formation of the from highest number of somatic abnormalities (95/100) and reduced length of AP axis (90/100) induced.

In treatment with cholesterol depletor MBCD brain (40/100), AP axis (45/100) and somites (65/100) are vulnerable sites getting perturbed by inhibition of cholesterol synthesis. Combined treatment of chick embryos with cholesterol biosynthesis inhibitor and depletor (AY9944 and MBCD respectively) also
showed abnormalities related mainly to the development of the brain (50/100) and length of AP axis (60/100).

The results of treating chick embryos in vitro with treatment of cholesterol biosynthetic inhibitor clearly reveals that lack of cholesterol in early embryos leads to the formation of abnormalities involving structures along AP or and DV axis.

These findings fit well in current understanding of cholesterol modification of hedgehog protein, inborn disorders of cholesterol deficiency and possible involvement of cholesterol signaling in embryonic development.

**Length measurement of chick embryos treated with cholesterol biosynthesis inhibitors and depletor (n=50)**

The embryos treated with cholesterol biosynthesis inhibitor and / depletor shows morphological abnormalities mainly related to the length of AP axis. The degree of effect was checked by measuring the AP axis of control and treated embryos by using, a software axiovision. About 50 embryos were used in each category and data obtained is shown in table 3.14.

<table>
<thead>
<tr>
<th>Control (mm ± S.D.)</th>
<th>AY9944 (50µg/ ml) (mm ± S.D.)</th>
<th>Mevinolin (10µg/ ml) (mm ± S.D.)</th>
<th>MBCD (12mM ) (mm ± S.D.)</th>
<th>MBCD + AY9944 (12mM + 40 µg/ ml) (mm ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.62 ± 0.27</td>
<td>3.48 ± 0.52*</td>
<td>2.73 ± 0.16*</td>
<td>3.43 ± 0.23*</td>
<td>3.23 ± 0.25*</td>
</tr>
</tbody>
</table>

(n=50)(p<0.001)

The results obtained by measurement of chick embryos length clearly indicated that there is considerable reduction in the antero-posterior axis of tadpoles after 24 hours of treatment with cholesterol inhibitors and depletor. The average length of control embryos was 5.62 mm, whereas the length of AY9944 treated embryos was 3.48 mm. The embryos treated with MBCD had average length of 3.43 mm and in the embryos treated with Mevinolin, the axis was reduced severely i.e. 2.73 mm in length as compared to that of control embryos. In the embryos with combined treatment of MBCD and AY9944, the average length of embryos was 3.23 mm.
Hourly treatment of cholesterol biosynthetic inhibitor and/ depletor on chick embryos (n=30)

The results have indicated a link between the embryonic development and cholesterol requirement. Therefore, to check at which stage the requirement of cholesterol is crucial and the effect of inhibitors is irreversible, stage 4 gastrulating chick embryos were treated with the cholesterol biosynthesis inhibitor and/ depletor. The treatment solution was changed with PC saline at different time interval to see the effect on embryos after 24 hours.

For this purpose, 4 different experimental sets were prepared containing treatment solution and 10 embryos in each category. Treatment solution was changed with ringer solution at 4, 8, 12 hour interval and one set was kept overnight (24 hours) for comparison with the previous results. The length was measured in each category after 24 hour of treatment and represented in table 3.15.

Table 3.15: Length of AP axis of chick embryos after treatment for different time interval

<table>
<thead>
<tr>
<th></th>
<th>Control (mm ± S.D.)</th>
<th>AY9944 50 µg/ml (mm ± S.D.)</th>
<th>Mevinolin 10 µg/ml (mm ± S.D.)</th>
<th>MBCD (12 mM) (mm ± S.D.)</th>
<th>MBCD+AY9944 (12 mM + 40 µg/ml) (mm ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Hour</td>
<td>5.5837± 0.14</td>
<td>4.8527 ± 0.36*</td>
<td>3.0093 ± 0.14*</td>
<td>4.1833 ± 0.36*</td>
<td>3.5580 ± 0.06*</td>
</tr>
<tr>
<td>8 Hour</td>
<td>5.5127± 0.11</td>
<td>4.5367 ± 0.44*</td>
<td>2.9087 ± 0.19*</td>
<td>3.3173 ± 0.24*</td>
<td>3.2527± 0.15*</td>
</tr>
<tr>
<td>12 Hour</td>
<td>5.4680± 0.31</td>
<td><strong>3.3853 ± 0.48</strong></td>
<td>2.6173 ± 0.26*</td>
<td><strong>3.2007 ± 0.20</strong></td>
<td><strong>3.2207 ± 0.14</strong></td>
</tr>
<tr>
<td>24 Hour</td>
<td>5.5740 ± 0.17</td>
<td>3.2400 ± 0.19*</td>
<td><strong>2.4720± 0.24</strong></td>
<td>3.1567 ± 0.23*</td>
<td>3.1140 ± 0.15*</td>
</tr>
</tbody>
</table>

(n=30) (p<0.001)

The results show that, in AY9944 treated embryos, the effect was prominent from 4 hour treatment category onwards and there was consistent reduction in the AP length in further time interval categories. But maximum effect was observed after 12 hours category, after which there was only slight reduction in length, which was evident even after 24 hour of treatment.
Mevinolin also had the similar effect, which is visible from 4 hour of treatment; as it blocks cholesterol biosynthesis at very early step, and hence the embryos were not able to develop normally.

The embryos treated with Mevinolin inhibitor showed considerable reduction in AP length in 4 hours; after which there was slow but consistent decrease. 12 hour category showed maximum effect on AP length, after which there was only slight reduction in length.

In treatment with MBCD, after 8 hours there was less difference in length of tadpoles. In further time categories, there was only slight reduction in length.

Embryos with combined treatment of MBCD and AY9944 also showed significant reduction of length in 4 hours as compared to that reported in the control embryos. There was no major difference in the AP length of embryos after 8 hours. Overnight treated embryos were the most affected in all treatments.

The results indicates that, in treatment with MBCD for 4 hours, it trapped cholesterol initially, but replacing it by ringer solution gave embryos time enough to synthesize cholesterol by de novo mechanism to overcome from major deformities. But after 8 hours MBCD has more time for action by trapping most of the available cholesterol. Thus reduction in AP length was more prominent in later categories.

**Treatment of chick embryo with cholesterol biosynthesis inhibitor and Cholesterol:**

It was clear from the data of cholesterol biosynthesis inhibitors treatment that inhibition of cholesterol biosynthesis affects normal embryonic development. Therefore, it was decided to use both inhibitor and to supply cholesterol externally to the embryo. By this way even though the biosynthesis is blocked, cholesterol will be available to the embryo.

Different doses of water soluble cholesterol were used in combination with set doses of AY9944 and Mevinolin. Cholesterol alone was also used in different doses to check its adverse effect on embryo, if any. The embryos were observed after 24 hours of treatment for the study of the development of abnormalities. The results are shown in table 3.16.
The results showed that there was no adverse effect of cholesterol alone on chick embryonic development and the length of embryos in all concentrations was around normal length.

When cholesterol is supplied in 20 µg concentration with AY9944, the embryos could not recover from effects of inhibitor on AP length. When the concentration of cholesterol is increased, the length is also increased. Maximum effect of cholesterol was seen at 80 µg and 100 µg.

This indicates that, if cholesterol is supplied in more concentrations than the inhibitor, then the effects of inhibitor could be reversed. That means if abundant supply of external cholesterol is present, embryo can use it successfully for its development.

When cholesterol was supplied with Mevinolin, there was no evident effect at lower concentrations. Even at higher concentrations of cholesterol, there was no effect on the AP length.

This indicates that when cholesterol synthesis is blocked at early steps and intermediate sterols are also not available, external cholesterol alone is not sufficient for maintaining normal embryonic development of *G. domesticus*, which suggests the

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control (mm)</th>
<th>Cholesterol(mm)</th>
<th>Cholesterol + AY9944 (55µg)(mm)</th>
<th>Cholesterol + Mevinolin (2.5µg)(mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20µg</td>
<td>5.5837± 0.14</td>
<td>5.4420 ± 0.03</td>
<td>3.1200 ± 0.11</td>
<td>2.6960 ± 0.03</td>
</tr>
<tr>
<td>40µg</td>
<td>5.5127± 0.11</td>
<td>5.3480 ± 0.18</td>
<td>3.2400 ± 0.05</td>
<td>2.7860 ± 0.03</td>
</tr>
<tr>
<td>60µg</td>
<td>5.4680± 0.31</td>
<td>5.3900 ± 0.08</td>
<td>4.0160 ± 0.40</td>
<td>2.8180 ± 0.03</td>
</tr>
<tr>
<td>80µg</td>
<td>5.5740 ± 0.17</td>
<td>5.2540 ± 0.21</td>
<td>4.0660 ± 0.39</td>
<td>2.7420 ± 0.07</td>
</tr>
<tr>
<td>100µg</td>
<td>5.3944± 0.17</td>
<td>5.3040 ± 0.17</td>
<td>4.7240 ± 0.20</td>
<td>2.7560 ± 0.05</td>
</tr>
</tbody>
</table>

*(n=30)*(p<0.001)
requirement of intermediate sterols in addition to the cholesterol during early embryonic development.

**Whole mount and histology of chick embryos treated with cholesterol biosynthesis inhibitors (AY9944 and Mevinolin), cholesterol depletor (MBCD) and combination (MBCD + AY9944):**

Treatment with cholesterol biosynthesis inhibitor, AY9944 (50 µg/ml), Mevinolin (10 µg/ml), cholesterol depletor, methyl-β-cyclodextrin (MBCD, 12 mM) and combined treatment of AY9944 with MBCD (40 µg/ml + 12 mM) was explored on early embryonic development of chick embryos.

Whole mount of treated embryos along with their respective control embryos were prepared to observe the morphological abnormalities. Histological study of the treated embryos along with their respective control embryos was done to observe the effect of inhibitor at the tissue level. At least 10 embryos were used for histology in each category of treatment.

After treatment of chick embryos, whole mount of embryos were prepared and histology was done. The whole mount and histological sections were observed under stereo-zoom binocular microscope and recorded photographically.

**Whole mount and histology of AY9944 treated embryos:**

The whole mount and transverse sections of AY9944 inhibitor treated embryos with respect to their control embryos are shown in Fig. 3.15 and 3.16 respectively.

In panel 1 of figure 3.15 the whole mount preparation of AY9944 treated embryos showed that the anterio-posterior axis of embryo was smaller than that of the control embryo. The whole mount also showed holoprosencephaly, a condition in which the whole brain becomes prosencephalon, the neural folds of the brain failed to close; thus giving it characteristic appearance of an open brain. The somites in AY9944 treated embryos were found fused with each other (shown by arrow). Some embryos treated with AY9944 showed asymmetric optic vesicle (shown by arrow in panel 2 of figure 3.15).
Figure 3.16: Panel 1-Embryo showing holoprosencephaly (shown by bold arrow) and fused somites (arrow) (Scale bar =200µ). Panel 2- Asymmetric optic vesicle (arrow) (scale bar=50 µ) [C- Control; AY-AY9944]

Figure 3.17: Effect of AY9944 at tissue level. 1: Transverse sections showing open prosocoel (bold arrow) 2: Transverse sections showing asymmetric optic vesicle (arrow) 3: Transverse sections showing diffused somatic cells (star) 4: Transverse sections showing posteriorly open neural tube (arrowhead) (Scale Bar-50µm) [C- Control; AY- AY9944]
The transverse sections passing through the treated embryos (figure 3.16, panel 1) showed open prosencephalon cavity (bold arrow). Panel 2 shows transverse sections reporting asymmetric optic vesicle (arrow) and indicates clearly that the right optic stalk is constricted at the base and the right optic vesicle is smaller than the left one. Panel 3 shows section passing through the somitic region of the embryo. It shows that the cells in the somitic region of the treated embryos are loosely arranged (star) and are less differentiated than those seen in the control embryo. Panel 4 shows section passing through the posterior end of the embryo. In the treated embryo, the posterior part of the neural tube, and the neural folds are failed to come closer (arrow head) and thus appear different from those reported in the control embryo.

**Whole mount and histology of Mevinolin treated embryos:**

The whole mount and transverse sections of Mevinolin treated embryo is shown in figure 3.17 and 3.18 respectively.

*Figure 3.18: Whole mount of Mevinolin treated embryo showing abnormal bain (arrow), reduced axis (bold arrow) and diffused somites (star) [C- Control; Me- Mevinolin] (Scale bar =200µ).*
The whole mount preparation of Mevinolin treated embryos showed that the anterio-posterior axis of the embryo was severely reduced (bold arrow). The brain of treated embryo failed to differentiate into prosencephalon, mesencephalon and telencephalon (arrow). The somites also failed to develop normally as compared to those reported in control embryo (star).

The transverse section in figure 3.18 panel 1 shows that part of the roof of prosencephalon region invaginated internally; forming a knob like structure (shown by arrow head). Panel 2 shows mesencephalon region open anteriorly (arrow). In addition, the cells in this region are not well differentiated as compared to those of the control embryos. Panel 3 shows that the neural folds of neural tube are failed to close, and remained open (star). The somites in this region are not properly developed. Cells of somitic region are not compactly arranged unlike those reported in control embryo.
Panel 4 shows that the neural folds of posterior end of the neural tube failed to come close (bold arrow).

**Whole mount and histology of Methyl-β-cyclodextrin (MBCD) treated embryos:**

The whole mount of MBCD (12 mM) treated embryos showed that the anterior neuropore of the prosencephalon region of brain was still open. The brain region was less developed than that of the control embryo (arrow). The somites of the treated embryo failed to develop normally (arrowhead) as shown in figure 3.18.

![Image](image.png)

**Figure 3.20:** Whole mount of MBCD treated embryo showing less developed brain and open anterior neuropore (arrow); diffused somites (arrowhead) [C- Control; MB- MBCD] (Scale bar = 200µ).

The transverse section in figure 3.19 panel 1 shows abnormally developed brain region of MBCD treated embryos. The roof and floor of the prosencephalon of the treated embryo were invaginated internally (bold arrow). Panel 2 shows less differentiated cells of the mesencephalon region, which were loosely arranged (arrowhead). Transverse section in panel 3 shows underdeveloped somites (star) of the MBCD treated embryo. Transverse section in panel 4 clearly shows enlarged heart of treated embryos (arrow). The effects of the treatment on the embryonic tissue appear prominently and clearly in comparison with the histological details observed in the sections of similar regions of the control embryos.
Figure 3.21: Effect of MBCD (12mM) at tissue level 1: Transverse section showing abnormal prosencephalon (bold arrow) 2: Transverse section showing less differentiated cells of mesencephalon (arrow head) 3: Transverse sections showing diffused somites (star) 4: Transverse sections showing enlarged heart(arrow) (Scale Bar-50 µm) [C-Control; MB-MBCD]

Whole mount and histology of Methyl-β-cyclodextrin (MBCD) + AY9944 treated embryos:

Whole mount of MBCD + AY9944 showed shorter AP axis of the treated embryo than those of the control embryos (bold arrow). The brain of treated embryos was not well differentiated into prosencephalon, mesencephalon and telencephalon (arrow) and somites of the treated embryos showed diffused appearance (arrow head) as shown in figure 3.20.
**Figure 3.22:** Whole mount of MBCD + AY9944 treated embryos showing short axis (bold arrow), abnormal brain (arrow) and diffused somites (arrow head) [C-Control; MB-MBCD] (Scale bar =200µ).

**Figure 3.23:** Effect of MBCD + AY9944 at tissue level 1: Transverse section showing abnormal prosencephalon (arrow) 2: Transverse sections showing abnormal mesencephalon (arrow head) 3: Transverse sections showing diffused somites (star) 4: Transverse sections showing posteriorly open neural tube (bold arrowhead) (Scale Bar-50µm) [C-Control; MB-MBCD]
The transverse section in figure 3.21 panel 1 shows that the prosencephalon region of the brain is abnormal in the treated embryo as compared to that of the control one (arrow). Panel 2 shows an invagination in mesencephalon region (arrow head). Transverse sections in panel 3 shows that the cells of somites are not developed properly and are loosely arranged (star). Transverse section in panel 4 shows that the neural tube failed to close and the cells in this region are also underdeveloped (bold arrow head). The effects of the treatment on the embryonic tissue appeared prominently and clearly while comparing the histological details in the sections of similar regions of the control embryos.

3.4. Discussion:

To find out the effect of cholesterol inhibition on early embryonic development, gastrulating embryos of *Microhyla ornata* and *Gallus domesticus* were treated with cholesterol biosynthesis inhibitors and cholesterol depletor. It has been shown that administration of AY9944 to pregnant rats resulted in decreased maternal serum cholesterol level and increased Δ7-dehydrocholesterol and byproducts (Barbu et al., 1988; Wolf et al., 1996). Fetuses of such dams showed holoprosencephalic type malformation or less severely pituitary agenesis (Roux et al., 1979a).

The occurrence of malformations was related to the timing and to degree of maternal hypocholesterolemia (Roux et al., 1980; Barbu et al., 1984). Key role of cholesterol in normal embryogenesis is to modify embryonic signaling proteins. It has been shown recently that chondrocyte differentiation at least in part is dependent on hedgehog- family protein (Kolf-Clauw et al., 1998).

In the present study, from the results of dose determination, it was clear that initially as the dose increases, the percent abnormalities in both frog and chick embryos increase. In frog embryos, AY9944 treatment with dose of 55 µg/ml results in 97% abnormal embryos (Table 3.4; Fig 3.8), whereas in chick embryos 50 µg/ml of dose resulted in 97% abnormal embryos (Table 3.12; Fig 3.16). For Mevinolin 2.5 µg/ml dose in frog (Table 3.5; Fig 3.9) and 10µg/ml dose in chick (Table 3.13; Fig 3.17) resulted in 100% abnormal embryos. For MBCD 40 mM dose resulted in 95% abnormal embryos (Table 3.6; Fig 3.10) in frog and 12 mM resulted in 95% abnormal embryos in chick (Table 3.14; Fig 3.18). For MBCD+AY9944 40 mM with
25µg/ml dose resulted in 100% abnormal embryos (Table 3.7; Fig 3.11) in frog and 12 mM with 40µg/ml resulted in 98% abnormal embryos in chick (Table 3.15; Fig 3.19).

The results indicated that in frog and chick embryos, similar concentration of AY9944 was needed for the effect. Higher concentration of Mevinolin was needed in chick embryos than that required for the frog embryos to show its effects. And lower dose of MBCD was required for chick embryos as compared to that required for the frog embryos to visualize their adverse effects on their development.

After treatment of *M. ornata* embryos with cholesterol biosynthesis inhibitors (AY9944 and Mevinolin) and depletor (MBCD), it was observed morphologically that the effect was more pronounced on axis in all categories, which is evident from the numbers of short axis induced (65/100) in AY9944 treatment, (100/100) in Mevinolin treatment, (39/100) in MBCD treatment and (17/100) in combined treatment of MBCD with AY9944 treatment.

The second category most influenced by the treatment was the formation of the bent tail as seen in AY9944 treatment (20/100), in Mevinolin treatment (30/100), in MBCD treatment (50/100) and in combined treatment of MBCD + AY9944 (12/100). The data indicates that in case of combined treatment, the effect on bent tail was less on frog embryos. There were few embryos (12/100) having bent tail in ringer control category. This could be because of the effect of manual removal of vitelline membrane, which has led to the bending of tails in embryos. Thus it can be said that Mevinolin and MBCD had effect on the bending of tail of embryos.

The effect of treatment was also seen on body pigmentation of treated embryos. AY9944 (43/100) and MBCD (32/100) treated embryos were more affected, whereas Mevinolin (16/100) and MBCD + AY9944 (18/100) had less effect on body pigmentation. A very few number of embryos were completely abnormal, which could be naturally abnormal or they might have failed to thrive because of the treatment. Abnormal tail fin was the only category of abnormality found exclusively in embryos treated with MBCD.

In chick embryos, it was observed that the treatment of cholesterol biosynthesis inhibitors (AY9944 and Mevinolin) and depletor (MBCD) had effect on the morphological features such as axis, brain and somites of embryos. This is evident from the number of reduced axis in AY9944 treatment (35/100), in Mevinolin
treatment (90/100), in MBCD treatment (35/100) and in MBCD (60/100) with AY9944 treatment.

Secondly, most affected category was fused somites in treatment with AY9944 (55/100), Mevinolin (95/100), (65/100) MBCD and (20/100) MBCD plus AY9944.

Formation of the abnormal brain was the third category to be affected (35/100) in AY9944 treatment, (40/100) in MBCD treatment and (50/100) in treatment of MBCD with AY9944. Mevinolin had no external morphological effect on the embryo. Enlarged left or right optic vesicle was the exclusive category found in embryos treated with AY9944.

AY9944 in addition to the inhibition of the embryonic cholesterol, also interferes with the compensatory influx of exogenous lipoprotein cholesterol. When given to pregnant rats, it induces fetal malformation of holoprosencephalic type and the abnormalities were mainly related to the growth and differentiation retardation (Repetto et al., 1990). Administration of AY9944 to rats mainly shows decreased fetal weights, reduced ossification and skeleton malformations. Inhibition of Δ7 dehydrocholesterol reductase in animal model of SLOS induces skeletal malformations, suggesting that this biochemical defect could be responsible for other developmental defects besides holoprosencephaly (Kolf-Clauw et al., 1998).

The endoplasmic reticulum (ER) enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase converts HMG-CoA to mevalonate and catalyzes the rate limiting step in cholesterol biosynthesis. The mevalonate pathway also produces several non-sterol isoprenoid compounds; the level of HMG-CoA reductase activity may coordinate many cellular processes and functions (Ohashi et al., 2003). HMG-CoA reductase plays a major role in cholesterol biosynthesis. Inhibitors of HMG-CoA reductase, statins are potent hypo-cholesterolemic agents that exhibit some cholesterol-independent or so called pleitropic, effects, that involve improving or restoring endothelial function, enhancing the stability of atherosclerotic plaque and decreasing oxidative stress and vascular inflammation (Takemoto and Liao, 2001).

Tozawa and co-workers (1999) disrupted gene for squalene synthase, the first committed enzyme of sterol synthesis that results in embryonic death at mid-gestation with growth retardation and defective neural tube closure demonstrating that HMG-
CoA reductase is essential for the early development of the embryos. It is well known that cholesterol plays an essential role in mammalian embryonic development, including the covalent modification of the morphogenic sonic hedgehog signal pathway during early gestation (Porter et al., 1996).

Apart from loss of de novo cholesterol biosynthesis, inhibition of mevalonate pathway results in loss of non-sterol isoprenoids essential for protein isoprenylation modifications and potential perturbation on N-linked glycosylation through inhibition of dolichol synthesis (Goldstein and Brown, 1990). Non-isoprenoids serves as lipid attachment for a variety of intracellular signaling molecules, including small GTP-binding proteins, such as Rho, Ras and Rac, whose proper membrane localization and function are dependent on isoprenylation (Van-Aelst and D’Souza-Schorey, 1997). The role these protein play in pathways regulating cell survival, proliferation, differentiation and cytoskeletal organization, it is likely that the altered expression will results in abnormal function during embryogenesis (Marek et al., 1999).

Compactin, which is also a classic HMG-CoA reductase inhibitor similar to Mevinolin interrupts pre-implantation development of cultured embryos after 32 cell stage in vitro; the effect can be reversed by supplementation of mevalonate (Surani et al., 1983). Ultrastructural examination of these growth arrested embryos revealed presence of nuclei with highly condensed chromatin indicating apoptosis. However, in vivo studies with various statins exhibited neither teratogenic nor embryotoxic effect (Wise et al., 1990; Hrab et al., 1994).

Some other studies with Mevinolin (Lovastatin) showed that administration of this compound to pregnant rat resulted in fetal malformation of the vertebrae and ribs, as well as gastroschisis (failure of abdominal wall to close) demonstrating that HMG-CoA reductase is crucial in very early steps of embryogenesis (Minsker et al., 1983). It indicates that many of the intermediates of the cholesterol biosynthetic pathway are essential for fundamental cellular processes and the loss of these important pathways would result in cell death and early embryonic lethality. Similarly in our results embryos show severely reduced axis and brain abnormality which could be because of the altered or defective cell signaling or differentiation, cell apoptosis and growth retardation leading to defective neural structure formation and defective embryogenesis.
Lipid rafts which are rich in cholesterol and sphingomyelin play a role in signal transduction, acting as organizing centers or platforms for proteins which are involved in the initiation and propagation of signaling events (Simons and Toomre, 2000). Functional data from experiments with supported the concept of a critical role for lipid rafts in the development. Accumulating evidence supports a role for low-density membrane (LDM) microdomains (rafts and caveolae) in cell signaling and in trans-membrane and trans-cellular processing (reviewed by Anderson and Jacobson, 2002). MBCD has been extensively used for removing cholesterol from the food as well as from the cells in vitro, but there are very few reports of its effect on the early embryonic development (Moss et al., 2012; Takeo et al., 2008).

Cholesterol which is essential for viability and cell proliferation is a major sterol of mammalian cells. More than 90% of cellular cholesterol is located at the plasma membrane (Martínez-Botas, et al., 1999). Removal of cholesterol by MBCD from the cell membrane of mouse embryo (in vitro) slowed the rate of cleavage in culture and ultimately inhibited progression to the blastocyst stage (Comiskey and Warner, 2007). Clustering of cholesterol rich lipid rafts on the cell membrane, results in enhancement of cell binding and cell migration. Whereas, disruption of raft with β cyclodextrin diminishes cell-cell interactions and hampers cell migratory properties (Stokes, et al., 1990). Cell-cell interaction and cell migration are the two important phenomena occurring during the stage of gastrulation and are responsible for normal embryonic development. Any disturbance in these activities by depletion of cholesterol should lead to abnormal development in early embryonic stage. The occurrence abnormal tailfin in our results by treatment of MBCD can be explained by the above facts.

The length of control as well as treated embryos was measured after 24 hours of treatment in both M. ornata as well as G. domesticus. In case of M. ornata, the average length of control embryos was found to be 3.81 mm, whereas the length of AY9944 treated embryos was 2.53 mm, the length in embryos treated with Mevinolin had reduced almost to half in length i.e. 2.26 mm and embryos treated with MBCD had average length of 2.90 mm. In combined treatment of MBCD and AY9944, the average length of embryos was 3.26 mm, which is less than that of the control embryos. But is more as compared to that developed after the individual treatment with AY9944 and MBCD. Statistical analysis by student’s paired t-test showed that
the difference in lengths of treated and control embryos was statistically significant (p<0.001). This indicates that the treatment of cholesterol biosynthesis inhibitors (AY9944 and Mevinolin) and depletor (MBCD) induce developmental abnormalities related to AP axis in frog embryos.

In *G. domesticus* embryos, measurement of length after 24 hours of treatment showed that the average length of control embryos was 5.62 mm, whereas the length of AY9944 treated embryos was 3.48 mm, Mevinolin treated embryos had length of 2.73 mm, average length of MBCD treated chick embryos was 3.43 mm and the length of combined treatment of MBCD with AY9944 was 3.23 mm. Statistical analysis by student’s paired t-test showed that the difference in length of cholesterol biosynthesis inhibitor and depletor treated embryos was statistically significant (p<0.001). This indicated that the treatment of cholesterol biosynthesis inhibitors (AY9944 and Mevinolin) and depletor (MBCD) induces reduced axis, similar to the frog embryos. Thus, it can be said that cholesterol inhibition induce developmental abnormalities related to AP axis in developing embryos.

To check that at what stage requirement of cholesterol is crucial, frog embryos were treated at different time intervals. Statistical analysis by student’s t-test of the results shows that in case of *M. ornata*, for all treatments (AY9944, Mevinolin, MBCD and MBCD + AY9944) the AP axis length of embryos in 2 hour category is significantly different from the length of control embryos. If the treatment is given for longer period the effect on reduction of AP axis length is also enhanced. This indicated that as the treatment time was increased the inhibitors and depletor were able to deplete more cholesterol form the embryos. Therefore, more reduction in cholesterol leads to more reduction in length of embryos. However, in AY9944 and Mevinolin treatments, although the effect was prominent from 2 hour category, the reduction in length was slow but consistent and the major difference from control embryos was observed in 10 hour and overnight category, where the reduction in length was comparatively more. In MBCD and MBCD + AY9944 treatments the reduction in length was consistent throughout the time intervals and there was no major difference in the length in 10 hour and overnight category.

In *G. domesticus* embryos, it was observed that in AY9944 treated embryos statistical analysis by student’s paired t-test showed that the difference in length of 4 hour treatment was statistically significant (p<0.001). But the maximum effect of
treatment on length was observed in 12 hour category; after which there was only slight reduction in length in 24 hour treatment category. Embryos treated with Mevinolin inhibitor showed considerable reduction in the length in 4 hours; after which there was slow but consistent decrease. 12 hour category showed maximum effect on the length; after which there was only slight reduction in length.

In MBCD treatment, 4 hour category indicated that initially MBCD had trapped cholesterol but replacing MBCD with ring solution rescued the embryos and these embryos could utilize cholesterol from its de novo biosynthesis and therefore in this category the effect was not prominent. In 8 hour treatment category onwards MBCD had more time for trapping most of the available cholesterol which leads to more cholesterol deficit and irreversible damage. After this time period category the difference in length was not considerable indicating that cholesterol depletion by MBCD during gastrulation had more effect on embryonic development and once the embryo had progressed to neurulation the effect remained constant and there was no further significant decrease in the AP length. In MBCD with AY9944 treatment category, the effect was prominent from 4 hour treatment onwards and after 8 hour treatment there was no further significant decrease in length, indicating that depletion of cholesterol with inhibition of biosynthesis had more effect on embryos.

The length of embryos treated with cholesterol and cholesterol in combination with biosynthesis inhibitors was measured. Statistical analysis by student’s paired t-test of the results showed that the length of embryos treated with cholesterol alone was not significantly different from that of the control embryos. This indicates that treatment of cholesterol alone had no adverse effect on development of embryos and they were successful to develop normally.

When cholesterol was supplied with combination of AY9944 the embryos were able to recover in terms of AP length. The statistical analysis showed that though the length of frog embryos treated with AY9944 and cholesterol (3.44 ± 0.09 mm) was significantly different from the length of control embryos (3.79 ± 0.11 mm; \( p<0.001 \)). It was also significantly different (2.53± 0.36 mm; \( p<0.001 \)) in AY9944 treated embryos even at higher concentration of cholesterol. Similarly in chick, the length of embryos treated with AY9944 and cholesterol (3.12 ± 0.11 mm) was significantly different from the length of control embryos (5.5837± 0.14 mm; \( p<0.001 \)), it was also significantly different from the length observed in AY9944
treated embryos (3.2400 ± 0.19; \(p<0.001\)). This indicated that when cholesterol is supplied externally, the embryos were able to recover from the effect of AY9944, but they were not able to become completely normal because of the effect of treatment. It can further also be concluded that externally supplied cholesterol can rescue the embryo from the effect of AY9944.

In Mevinolin treatment externally supplied cholesterol had no rescue effect on the embryo even at higher concentrations of cholesterol. Statistical analysis showed that the length of frog embryos treated with Mevinolin and cholesterol (2.6481 ± 0.1063 mm) was significantly different from the length of control embryos (3.79 ± 0.1136 mm; \(p<0.001\)). Similarly in the chick embryos, the length of Mevinolin and cholesterol treated embryos (2.6960 ± 0.03) was significantly different from the length of control embryos (5.3944± 0.17; \(p<0.001\)); even when cholesterol was supplied in higher concentration. This indicated that when cholesterol synthesis is blocked at the earlier step and intermediate sterols are also not available, external cholesterol alone is not sufficient for maintaining normal embryonic development.

The fact that exogenous cholesterol can enter the embryonic tissues suggests that the teratogenic activity of inhibitors results from an imbalanced inhibition of fetal cholesterol biosynthesis by the compensatory cholesterol influx from the mother. Added cholesterol in culture medium corrects the metabolic abnormalities, not only by increasing the cholesterol, but also by decreasing the amount of aberrant sterols. Aberrant sterols are putative competitors of the physiological activities assumed by cholesterol and their decrease participates in the normal development of treated embryo. Exogenous cholesterol complements the cholesterol supply to the embryo tissues and down-regulates the biosynthesis of aberrant sterols (Llirbat et al., 1997).

Feeding with cholesterol rich diet was shown to restore the normal phenotype efficiently in rats treated with AY9944, which led to the suggestion that the lack of cholesterol is the principal cause of the SLO syndrome (Roux et al., 1979b; Gaoua et al., 2000). The oral administration of cholesterol in association with AY9944 completely prevents the characteristic malformations normally induced by AY9944 (Roux et al., 1979b).

Histological sections of cholesterol inhibitor and cholesterol depletor treated frog and chick embryos further confirmed the development of abnormalities at the
tissue level. Sagittal sections of AY9944 and Mevinolin treated embryos showed reduced axis, MBCD treated embryos showed abnormal tailfin and MBCD with AY9944 treated embryos showed collapsed pharynx. Transverse section of AY9944 treated embryos showed that the prosocoel and rhombocoel were constricted and optic lens and optic vesicle were underdeveloped. In Mevinolin treated embryos the complete brain became abnormal. MBCD treated embryos showed abnormal brain and tailfin. MBCD with AY9944 treated embryos also showed abnormal development of the brain and optic lens.

In chick, the whole mount of AY9944 treated embryos showed holoprosencephaly and asymmetric optic vesicle, whereas Mevinolin, MBCD and MBCD with AY9944 treated embryos showed abnormal brain and fused somites. The transverse sections of AY9944 treated embryos confirmed the abnormal development of the brain exhibiting widely open regions. Transverse sections through optic vesicles showed that right optic vesicle was smaller and constricted at the base. Section through somites showed less differentiated somitic cells and neural tube was open posteriorly. In Mevinolin treated embryos, the transverse sections also showed abnormal development of the brain tissues, open mesencephalon, stunted somites and open neural tube. Sections of MBCD treated embryos showed abnormal brain, less differentiated cells in mesencephalon and somites and enlarged heart. Transverse section of embryos treated with MBCD and AY9944 showed abnormal prosencephalon and mesencephalon, less differentiated cells in somites and open neural tube. Thus the histological studies also confirmed that inhibition and/or depletion of cholesterol biosynthesis results in formation of abnormal brain and neural organs.

In a similar study with rat it was shown that cholesterol depletion causes vertebral abnormalities in the embryos, which occur because of somite pattern perturbations (Kolf-Clauwet al., 1998). Similarly in our results somite formation was affected in chick embryos treated with cholesterol inhibitors and/or depletor. In another study by Roux and co-worker, it was shown that treatment with cholesterol inhibitor in rat results in forebrain hypoplasia, pituitary agenesis, irregularities and narrowness of mesencephalon and structure of the mesencephalon-rhombencephalon junction (Roux et al., 1979a). These results are in accordance with results observed by us.
Whole mount in situ hybridization (WMISH) analysis reveals first expression of \textit{Xdhcr7} (gene coding for \(\Delta 7\) dehydroreductase enzyme) during early gastrulation (NF stage 10.5) demarcating the dorsal blastopore. During gastrulation \textit{Xdhcr7} transcripts are found in the dorsal midline and were localized exclusively to the notochord. Furthermore, in late neurula stage embryos, a strong expression of \textit{Xdhcr7} is revealed in a punctuated pattern, which demarcates ciliated skin cells, reminiscent of the expression of the components found in the Notch signaling pathway (Deblandre \textit{et al.}, 1999). Enhanced \textit{Xdhcr7} expression is later detected in neural-derived structures including the brain, the epibranchial placodes and the optic vesicles. In a later stage of development (NF stage 38), the expression of \textit{Xdhcr7} is mainly restricted to head structures, and demarcates neural tissues and derivatives.

Interestingly, the expression of \textit{Xshh} starts with the increasing expression of \textit{Xdhcr7} at around stage 10, and is maintained throughout gastrulation to early organogenesis. Expression of \textit{Xdhcr7} is negatively regulated by hedgehog signaling in the developing central nervous system, but not in the notochord where \textit{Xdhcr7} and \textit{Xshh} transcripts co-localize. This suggests an early need for \textit{de novo} cholesterol biosynthesis in organizing centers, whereas the late demand is mainly restricted to neural tissues and derivatives, close to, or including \textit{Shh} expression territories.

The \textit{Xdhcr7} expression pattern provides the first evidence that \textit{de novo} cholesterol biosynthesis is spatially restricted to the organizing centers and neural structures during development. Importantly, loss- and gain-of-function experiments showed that a strict control of \textit{de novo} cholesterol biosynthesis is necessary for proper development of the central nervous system. Thus we can say that in our results the resultant abnormalities in embryos could be because of the deficiency of cholesterol.

3.5. Conclusion:

Our findings confirmed that unavailability of cholesterol during early embryonic development perturbed the normal lay down of AP body plan. These findings are well in concordance with the current understanding of cholesterol modification of hedgehog protein, inborn disorders of cholesterol deficiency and possible involvement of cholesterol signaling in the embryonic development.

The overall results clearly indicated the influence of cholesterol biosynthetic inhibition on axial structures, which is known to be under the regulation of Hedgehog.
gene expression and Wnt protein gradient. Somite formation is known to be under control of segmental patterning genes, somitic oscillator genes, \textit{Pax-Six-Eya-Dach} network, Mesp2 gene and maternal effect gene \textit{misty somites}. It would be of immense interest to know which of these genes are deregulated under deficiency of essential levels of cholesterol.