MATERIAL AND METHODS

ANIMAL MAINTENANCE AND ETHIC STATEMENT

All Sailfin Mollies, *Poecilia latipinna* (Lesueur, 1821), were obtained from a commercial supplier. At start of the experiment, all were less than one year of age with an average total body length of 4-5cm and weighing between 4-5g. All animals were quarantined, screened for parasitic infections and then healthy ones were placed in glass aquaria containing dechlorinated tap water and acclimatized to laboratory conditions for two weeks with constant aeration at the animal facility of Department of Zoology, The M.S. University of Baroda (827/ac/04/CPCSEA). The daily photoperiod was 14hr of light and 10hr of darkness, and the water temperature was maintained in the range of 25-29°C. About 10% of the aquarium water was changed every day and replaced with fresh tap water. Animals were fed once a day with a commercial food (White *et al*., 1994; Santos-Ruiz *et al*., 2002). All animal utilization protocols were carried out agreeing to the ethical principles (Drugs and Cosmetics Rules, 2005) approved by the Institutional Animal Ethics Committee (IAEC) [Form B No. ZL/IAEC/15-2010] constituted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

DRUG DOSAGE AND ROUTE OF INJECTION

A 10mM stock solution of LDN193189 in 1% Dimethyl sulfoxide (DMSO) was prepared and stored at -20ºC. A primary screening for dose selection was done using three doses, based on literature survey. A low dose of 2.0mg/kg body weight, mid dose of 2.5mg/kg body weight and a high dose of 3.0mg/kg body weight were tested for its effect on fin regeneration.

The dose was prepared fresh from the stock solution for each of the experiments. Healthy animals of both the sexes were randomly allocated into groups named control and treated. Intraperitoneal method was chosen for injecting doses. Out of selected three doses, based on morphological observations, a dose of 2.5mg/kg body weight turned out to be more appropriate in hampering the BMP signalling and henceforth, that dose was eventually finalized for further studies. Control animals were injected with 1% DMSO and treated ones with 2.5mg/kg body weight of LDN193189 at a maximum volume of 25μl/animal by microsyringe (Hamilton Bonaduz AG, Switzerland). The treatment was started in each group a day before amputation and continued till desired stage is achieved.

CAUDAL FIN AMPUTATION AND MORPHOMETRIC MEASUREMENTS

Fishes were anesthetized by hypothermia before amputations. Fin amputations were made with sterile surgical stainless steel blade (Size 11, Kehr Surgical Private Limited, India). The fins reaching the sequential stages i.e. wound healing (24hpa), blastema (60hpa) and differentiation (5dpa) were collected as per requirement of each of the experiments done. The lengths of fin regenerates were measured using digital calliper (Mitutoyo, Japan).

HISTOLOGY

Hematoxylin and Eosin Staining

Hematoxylin and eosin stain (H&E) (Carson, 1990) involves application of hemalum, which is a complex formed from aluminium ions and oxidized Hematoxylin. This stains nuclei of cell blue in colour. The nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colours other eosinophilic structures in various shades of red, pink or orange. For histology, decalcification of the regenerate along with the fixation of the caudal fin was performed. The formalin fixed tissues were washed and subjected to dehydration in ascending grades of alcohol. The tissues were embedded in paraffin wax, sectioned into ribbon and fixed on glass slides. After staining and destaining with hematoxylin and eosin, they were permanently mounted with DPX mount for histopathological studies.

Reagents

4% Paraformaldehyde (fixative)

Xylene

Different grades of alcohol (70%, 80%, 95% and 100%)

Acid Ethanol (1ml concentrated HCl + 400ml 70% ethanol)

Harris's hematoxylin

Eosin

DPX (mounting medium)

Procedure

The area of interest was dissected and was fixed in 10% formalin was washed thoroughly in running water. For caudal sections proper decalcification was followed by using strong acid in the 10% constitution. The fixed tissue samples were dehydrated by placing them in increasing concentrations of alcohol (50, 70, 90 and 100%) for clearing and subjected to cold infiltration at room temperature in a mixture containing paraffin wax dissolved in Xylene for 30min. The tissues were hot infiltrated in oven at 58°C to 60°C in molten paraffin wax for 1hour. The hot infiltrated tissues were quickly removed and blocked quickly using metal blocks. The solidified blocks were trimmed to 8 to 10 micron thickness using microtome (Leica RM 2155). The ribboned sections were placed on glass microscopic slides coated with egg albumin.

The microscopic slides were then exposed to decreasing grades of alcohol (100, 90, 70, 50 and 30%) for 5min. The sections were immersed in Hematoxylin stain for 5min and washed again in running tap water to remove the excess stain. The slides were dried and dehydrated in increasing concentration of alcohol (30, 70, and 90 for a few seconds). After dehydrating, the slides were cleared in Xylene twice for 10min. These cleared tissue sections were permanently mounted with DPX. It was then observed and captured under the microscope (Leica DM2500).

IMMUNOHISTOCHEMICAL STUDY

Immunohistochemistry of Caspase-3

For whole mount immunohistochemical (IHC) staining, regenerating caudal fins were dissected at different stages of regeneration and the zone of interest was fixed for 4hr, in 4% PFA prepared in PBS. Tissue was then air dried for 10min and fixed in ice-cold acetone for 15min. After fixation, tissue was washed with PBS thrice for 5min each and blocked in 10% bovine serum albumin in PBS for 60min at RT. In the next step, tissue was incubated with appropriate primary antibody (1:1000 dilution of Rabbit Anti-Caspase3) overnight inside a moist chamber at 4ºC. Next day, tissue was washed with PBS and incubated with corresponding ALP-conjugated secondary antibodies (1:300 dilution of Goat Anti-rabbit ALP conjugate) for 2hr at room temperature. Tissue was washed and incubated with 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT) substrate chromogen solution (sigma) for 5min. Then washed thrice with PBS, for 5min each and mounted temporarily in PBS and photographed under Leica DM2500 microscope.

IMMUNOHISTOFLUORESCENCE STUDY

In vivo DNA replication labelling

To observe cell proliferation in the regenerating fins of the Control and Treated animals at Blastema stage of regeneration, BrdU staining was performed.

Principle

Bromodeoxyuridine (BrdU) is an analog of the DNA precursor thymidine. In proliferating cells the DNA has to be replicated before division can take place, which occurs during the S phase. If BrdU is injected at this stage, the cells would incorporate it into their DNA just like they would incorporate thymidine. The amount of BrdU in the DNA of proliferating cells can be detected using specific anti-BrdU antibodies immunocytochemically.

Materials

BrdU stock

4% PFA

2N HCl

0.1M Borate buffer

Citrate buffer (10mM Citric acid, 0.05% Tween 20, pH 6)

Primary antibody (mouse anti-BrdU, dilution of 1:100)

FITC tagged Secondary antibody (goat anti-mouse, dilution of 1:50)

Method

BrdU powder (Sigma, St Louis, USA) was dissolved in aquarium tank at concentration of 25µg/L; animals were incubated in tank for 24hr. After incubation in BrdU, tail fin was excised and the zone of interest was fixed in 4% PFA. Later, tissue was embedded in OCT medium and 8-10µm thick sections were cut using cryostat and taken on slides. The sections were subjected to antigen retrieval using citrate buffer method for 20min at 80ºC temperature in oven. Sections were allowed to cool down for 20min at RT, rinsed thrice in PBS for 5min each and permeabilised in 2N HCl for 60min at room temperature for denaturation of DNA. Again, the sections were rinsed thrice in PBS for 5min each. The acid was neutralised by immersing sections in 0.1M Borate buffer for 15min, rinsed in PBS for 5min and were blocked in 10% BSA prepared in PBS for 60min at RT. The sections were then incubated in primary antibody (1:100 dilution of Mouse Anti-BrdU in 5% (BSA) (Sigma-Aldrich, USA) overnight in moist chamber at 4°C in dark. The sections were then washed in PBS for 15min. Next day, sections were washed with PBS (3 times, each for 5min) and incubated with Fluorescein isothiocyanate conjugated secondary antibody (1:50 dilution of Goat Anti-Mouse IgG-FITC in 5%BSA) (Genei, Merck, USA) for 90min at room temperature. The sections were washed again in PBS (3times, each for 5min) and mounted with PBS. Images were acquired using a fluorescent light by a Leica DM 2500 fluorescent microscope.

SKELETAL PREPARATION

Alcian blue and Alizarin red staining

Alcian blue is a dye that stains the proteoglycan components of the extracellular matrix associated with chondrocytes and those are used to visualize cartilage patterns in larvae and in adults, whereas Alizarin red stains calcified matrix associated with bone.

Reagents

4% Paraformaldehyde (PFA)

PBS

Ethanol

0.1% Alcian blue 8GX (A5268, Sigma)

0.1% Alizarin red S (A5533, Sigma)

0.5% Potassium hydroxide (KOH)

2% Sodium borate

Glycerol

Method

For visualizing the skeletal development, portions of caudal fins from treatment and control groups were amputated during differentiation stage and were fixed in 4% PFA and later washed in PBS buffer solution for 2-3 days, then stained for 6hr in dark at room temperature with a filtered 0.1% Alcian blue (in 30% acetic acid and 70% ethanol), and dehydrated in a graded water:ethanol (70%, 90% and 100%) series. The tissues were then stored in 100% ethanol overnight at 4°C to fix the alcian blue in the fin rays and also to de-stain the surrounding soft tissues. Next day, fins were rehydrated in water:ethanol series, macerated in a fresh solution of 0.5% trypsin in 2% sodium borate for 15min at 37°C, and extensively rinsed with frequent water changes. Bones were stained by placing the fins in a fresh solution of 0.1% Alizarin red S in 0.5% KOH for 5hr in dark at room temperature. Fins were destained in graded sequence of glycerol and 0.5 % KOH and finally stored in 100% glycerol at 4°C (Ferretti and Géraudie, 1995; Laforest *et al*., 1998).

HYDROXYPROLINE ASSAY

Collagen is one of the few proteins which contains the amino acid hydroxyproline. The hydroxyproline assay was performed as described by Edwards and O'Brien (1980).

Reagents

Chloramine-T reagent

1.41g Chloramine-T (sigma) in 100 ml distilled water.

Aldehyde-perchloric acid reagent

15g p-dimethyl-amino-benzaldehyde (Sigma)

60 ml n-propanol

26ml perchloric acid (70%, reagent grade)

Make up the volume to 100 ml with distilled water

Buffer solution

133 g citric acid monohydrate

32 ml glacial acetic acid

320 g sodium acetate trihydrate

91 g sodium hydroxide

800ml n-propanol

The buffer solution is made up to a volume of 3000 ml with distilled water. The pH was adjusted between 6.0 - 6.5 with 0.2 mol/l NaOH solution and brought to a volume of 4000ml with distilled water (1-2 ml of toluene is added as a preservative).

Hydroxyproline standard preparation

Concentrations of hydroxyproline standards were prepared as follows:

Primary Standard - 0.5 g stock hydroxyproline (Calbiochem) was dissolved in 1000 ml of the buffer (500 µg hydroxyproline per ml buffer)

Secondary standard- 20 ml primary standard brought to a final volume of 100ml with the buffer (100µg/ml)

4µg/ml- 4 ml of secondary to a final volume of 100 ml

2 µg/ml -2 ml of secondary to a final volume of 100 ml

0µg/ml - pure buffer.

Method

The regenerated caudal tissue was excised and acid hydrolysed (10mg tissue/ml of 6M HCl) at 120°C in a pressure vessel for 4hr. Upon completion of hydrolysis, hydrolysates and standards tubes were kept in oven (60°C) for 8hr for complete evaporation of water. Later citric/acetate buffer (citric acid, sodium acetate, sodium hydroxide, glacial acetic acid and n-propanol, pH 6.0) and chloramine T solution (chloramine T dissolved in Milli-Q water) were added. The tubes were incubated for 20min at room temperature and Ehrlich's solution [aldehyde-perchloric acid reagent (p-dimethyl-amino-benzaldehyde) perchloric acid and n-propanol] was added to the tubes, which were then incubated at 60°C in water bath for 15min. The absorbance of the reaction product was read at 550 nm. Sample concentrations were determined from the standard curve.

PROTEIN ESTIMATION (Bradford, 1976)

Bradford reagent

Coomasie Brilliant Blue G-250 (100mg) was dissolved in 50 ml of 95% ethanol. To the above, 100ml of 85% (w/v) ortho-phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre (Final concentration: 0.01 % w/v coomassie brilliant blue G-250, 4.7 % (w/v) ethanol and 8.5 % (w/v) phosphoric acid).

Method

Fin regenerates from each group (n=10) of each stage of epimorphic regeneration were harvested and 10% homogenate was made using PBS and lysis buffer (1:1); cold centrifuged at 8000 rpm for 15min and the supernatant was then used for the estimation of protein content by the Bradford assay. To a suitable amount of tissue supernatant (20µL), 200µL of Bradford reagent was added and mixed well in a 96 well plate. Blank contained 20µL of water. After 5min, the absorbance was read at 595nm. The standard curve was used to determine the protein in unknown samples (Bovine Serum Albumin was used as standard).

SDS-PAGE

To determine alterations in protein expression pattern and the stage specific effect of LDN193189 during regeneration, SDS-PAGE profiling of the fin regenerates for control and treated groups at all the three defined stages was done. 10µg of protein was loaded in each well for the SDS-PAGE procedure.

Principle

The molecular weight of protein may be estimated if they are subjected to electrophoresis in the presence of a detergent Sodium Dodecyl Sulfate (SDS). SDS disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. If the proteins are denatured and put into an electric field, they will all move towards the positive pole at the same rate, with no separation by size. Hence, the proteins have to be placed into a matrix that will allow different sized proteins to move at different rates. The matrix of choice is polyacrylamide, which is a polymer of acrylamide monomers. When this polymer is formed, it turns into a gel and the electric potential is used to pull the proteins through the gel. So the entire process is called Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Materials

1.0M Tris-HCl, pH 6.8 (for stacking gel)

1.5M Tris-HCl , pH 8.8 (for resolving gel)

10% SDS (Electrophoresis grade)

Resolving gel (amounts for 12% gels, 5ml)

1.64ml ddH_2O

2.00ml 30% (29:1) Acrylamide/Bis-Acrylamide

1.26ml 1.5M tris-HCl, pH 8.8

50µl 10% (w/v) SDS

50µl 10% (w/v) Ammonium persulphate (APS), made fresh on the day of use

2µl TEMED (N,N,N′,N′-Tetramethylethylenediamine)

Stacking gel (amounts for 5% gels, 2ml)

1.4ml ddH_2O

0.332ml 30% (29:1) Acrylamide/Bis-Acrylamide

0.252ml 1M tris-HCl, pH 6.8

20µl 10% (w/v) SDS

20µl 10% (w/v) Ammonium persulphate (APS), made fresh on the day of use

2µl TEMED (N,N,N′,N′-Tetramethylethylenediamine)

1X SDS Gel sample loading buffer

50mM tris HCl (pH6.8)

100mM dithiothreitol

2% SDS

0.1% Bromophenol blue

10% glycerol

5X Gel running buffer, pH 8.3

125mM Tris base

1250mM glycine

10% SDS

Gel staining (Silver staining)

Fixative Solution: 30% methanol and 10% glacial acetic acid

2% $AgNO_3$

2.1% Na_2CO_3

40% formaldehyde

Method

The glass plates were assembled properly as per instructions in the manual, and the whole clamp was adjusted on the casting stand. Then the Resolving gel was casted allowing the gel to polymerize for 45min to one hour, followed by the casting of Stacking gel. Meanwhile the samples were prepared. After the gel was polymerized completely, samples and the molecular weight markers were loaded in the wells and the gel was allowed to run at a constant current of ~100V. The run is considered completed after the tracking dye reaches the bottom. The gel was then removed from the unit, the glass plates were separated carefully and the gel was taken into a container containing fixative solution and was kept overnight. The gels were then stained with 2% $AgNO_3$ and developed using ice cold Na_2CO_3 and 40% formaldehyde. After the bands appear reaction was stopped using 7% glacial acetic acid. Gels were analyzed by Gel Doc (GeNei, Doc-ItLs software).

GELATIN ZYMOGRAPHY

This technique involves the electrophoresis of secreted protease enzyme through discontinuous polyacrylamide gels containing substrate. After electrophoresis removal of SDS from the gel by washing in 2.5% Triton X-100 solution, followed by incubation of 18hr in renaturing buffer which allows enzymes to renature and digest the protein substrate. Staining of the gel with Coomassie Brilliant Blue allows the bands of proteolytic activity to be detected as clear bands of lysis against a blue background.

Principle

The separation occurs in a polyacrylamide gel containing a specific substrate that is co-polymerized with the acrylamide (Heussen and Dowdle, 1980; Fernandez-Resa *et al*., 1995). During electrophoresis, the SDS causes the MMPs to denature and become inactive. The activation of latent MMPs during zymography is believed to involve the "cysteine switch" because the dissociation of Cys73 from the zinc molecule is caused by SDS. After electrophoresis, the gel is washed, which causes the exchange of the SDS with Triton X-100, after which the enzymes partially renature and recover their activity (Heussen and Dowdle, 1980; Woessner, 1995). Additionally, the latent MMPs are auto activated without cleavage (Oliver *et al*., 1997). Subsequently, the gel is incubated in an appropriate activation buffer. During this incubation, the concentrated, renatured MMPs in the gel will digest the substrate (Fernandez-Resa *et al*., 1995; Hawkes *et al*., 2001). After incubation, the gel is stained with Coomassie blue, and the MMPs are detected as clear bands against a blue background of undegraded substrate (Fernandez-Resa *et al*., 1995; Hawkes *et al*., 2001). The clear bands in the gel can be quantified by densitometry (Woessner, 1995). The zymography is based on the following principles: (i) During electrophoresis, gelatin is retained in the gel; (ii) MMP activity is reversibly inhibited by SDS during electrophoresis; and (iii) The SDS causes the separation of MMP-TIMP complexes during electrophoresis. This enables the detection of MMPs and TIMPs independently of one another (Hawkes *et al*., 2001).

Materials

Gelatin stock solution (10mg/ml in ddH_2O)

Zymogram Renaturing Buffer

Triton X-100, 25% (v/v) in ddH_2O

Zymogram Developing Buffer

50mM Tris Base

Tris HCl

0.2M NaCl

5mM $CaCl_2$

0.02% Brij 35

Double distilled H_2O

Coomassie blue R250 staining solution (100ml)

0.25g Coomassie brilliant blue R250

90ml Methanol: H_2O (1:1v/v)

10ml Glacial acetic acid

Coomassie brilliant blue R250 destaining solution (100ml)

Methanol: Glacial acetic acid: ddH_2O (50:10:40)

Method

12% gel was made according to standard procedures of SDS-PAGE as explained above. When preparing the running gel, gelatin stock solution (10mg/ml in ddH_2O) was added to get the gelatin concentration of 0.1% (1mg/ml). Samples were applied and the gel was run with Tris-Glycine SDS Running Buffer according to the standard running conditions (~100V, constant voltage). The run is complete when the bromophenol blue tracking dye reaches the bottom of the gel. After running, the gel is incubated with zymogram renaturing buffer with gentle agitation for 30min at room temperature. The gel was then incubated with zymogram developing buffer for approximately 4hr. The gel was then stained with Coomassie blue R250 for 30min, followed by destaining with an appropriate Coomassie R250 destaining solution. Areas of protease activity appear as clear bands against a dark blue background where the protease has digested the substrate.

GENE EXPRESSION STUDY

Fin amputations for each stage of epimorphic regeneration were made as mentioned above. Total 10 animals were used in each group for every stage for gene expression studies.

Total RNA isolation

Total RNA isolation was done using TRIzol method. Tissue was collected and homogenised in TRIzol (0.5ml TRIzol per 14-18mg tissue) and left for 5min at room temperature. Tissue was centrifuged at 12000g for 20min at 4^{o}C. Supernatant was collected and incubated with 200µl Chloroform and was mixed gently for 2-3min followed by a spin at 12000g (15min, 4^{o}C). The upper aqueous layer was carefully aspirated without contamination from lower layers into a new microcentrifuge tube. 500µl isopropanol was added and incubated for at least 30min at 4^{o}C. It was spun at 12,000g (10min, 4^{o}C) and supernatant was discarded. Pellet left at the bottom was washed with 1ml of 70% ethanol and was gently mixed and spun at 7500g (5min, 4^{o}C). Ethanol washes were given thrice to the pellet. Pellet was Air-dried for 5 to 10min (Over Air drying was avoided, else pellet would not re-dissolve). Finally, pellet was dissolved in minimum amount of DEPC-treated water.

Quantification of isolated RNA

After dissolving RNA into DEPC treated water, it was estimated at 260nm and 280nm by UV spectrophotometer. $O.D._{260}/O.D._{280}$ was taken as a measure of purity check.

Synthesis of cDNA using kit-based protocol

cDNA was synthesized after estimating RNA of each sample using the High-Capacity cDNA Reverse Transcription Kit of Applied Biosystems. Following is the procedure for cDNA synthesis.

Procedure

(A) 2X reverse transcription Master mix was prepared

(B) RNA was added to reverse transcription reactions

(C) Reverse transcription was performed in a thermal cycler.

(A) 2X Master Mix preparation (per 20µL reaction):

- The kit components were thawed on ice.
- The volume of the components needed to prepare the required number of reactions were calculated.

Components	Volume (µL)
10X RT buffer	2
25X dNTP mix	0.8
10X RT random primers	2
RT enzyme	1
Water	4.2
Total volume	10

- Place the 2X master mix on ice and mix gently.

(B) Preparation of the cDNA Reverse Transcription reactions:

- 10µL of 2X master mix was pipetted into each of an individual tube.
- 10 µL of RNA sample (1µg/µL) was taken into each tube, pipetting up and down two times to mix.
- The tubes were sealed.
- The tubes were briefly centrifuged to spin down the contents.
- The tubes were then placed in the thermal cycler.

(C) Thermal cycler Program for Reverse transcription

Conditions	Step1	Step2	Step3	Step4
Temperature (^{o}C)	25	37	85	4
Time (min)	10	120	5	α

Semi-quantitative Reverse Transcription Polymerase Chain reaction

After the preparation of cDNA, semi-quantitative PCR was performed in BioRad T100 thermal cycler. All the components were kept at 4^{o}C while making the reaction mixture. 2X Master-mix was obtained from Sisco Research Laboratories, Mumbai. All the primer sequences were obtained from NCBI. The primer sequences are listed in table i. PCR reaction system and program were as follows:

Table-i: Sequences of Primers

Gene	Forward primer	Reverse primer	T_a (^{o}C)	Product size(bp)
dlx5	GGAATGCGGATGGGGGATTT	CCACAGCTGAGCCGAAAAAC	60	173
runx2	CTGGTGCCTTTTGGGTTGTG	TCGACACGTCTTGCTTAGCG	60	165
mmp2	GTCTCCTGGCTCATGCCTTT	TTTCACCACTTGGCCCTCTC	61	102
mmp9	GCCTTCAAGGTGTGGAGTGA	ATCCCCGTGGTCAGCTTTTC	61	102
18srRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC	57-63	144

Reaction System:

Components	Volume (µL)
2X Master mix	5.0
Forward primer (5pmoles/µL)	1.0
Reverse primer (5pmoles/µL)	1.0
cDNA Sample	1.0
Water	2.0
Total volume	10

PCR Program:

Steps	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Temperature(^{o}C)	95	95	58-63	72	72
Time (Sec)	180	15	15	15	180
		35 Cycles			

Agarose gel electrophoresis

PCR products were detected by 2% agarose gel electrophoresis. Gel was prepared in 1X TBE buffer. PCR products were mixed with 1X DNA loading dye before loading into gel.

The gel was allowed to run at 100V for 30min. After that, the image was taken by GeNei Imaging System in ultraviolet light. 100bp ladder was used as marker for confirmation of correct size of PCR products.

Quantitative Real time Reverse Transcription PCR

After standardizing PCR program in semi-quantitative, quantitative real-time PCR was performed in a Light Cycler 96 (Roche Diagnostics, Switzerland). Table ii shows the PCR program. After completion of the last step of the thermal cycler, melting curve analysis was performed as mentioned in table iii. Melt curve analyses were conducted to assess uniformity of product formation, primer-dimer formation and amplification of non specific products. The PCR product was monitored in real time, using the SYBR Green 1 fluorescence. Threshold for detection of PCR product was set in the log-linear phase of amplification. The comparative cycle threshold (Cq) method was used for relative quantification of gene expression. The amount of mRNA, normalized to the endogenous control (18srRNA) and relative to a calibrator, was given by $2^{-\Delta\Delta Cq}$, with Cq indicating the cycle number at which the fluorescence signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR. The fold change in the target gene relative to the 18srRNA endogenous control gene was calculated using Livak method ($2^{-\Delta\Delta Cq}$) (Livak and Schmittgen, 2001).

ΔCq = Cq (target) - Cq (18SrRNA)

$\Delta\Delta Cq = \Delta Cq$ (test sample)/ ΔCq (calibrator)

Table-ii: Thermal cycler program for Real-time RT-PCR

Steps	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Temperature(^{0}C)	95	95	58-63	72	72
Time (Sec)	180	10	10	10	180
		42 Cycles			

Table-iii: Thermal cycler Program for Melting curve analysis

Temperature (^{0}C)	95	63-68	97
Time (Sec)	10	60	Continuous

STATISTICAL ANALYSIS

The data were subjected to Bartlett test for homogeneity and the significance level of the treatment groups with control group was evaluated through Student's t-test with 95% confidence limit. The values are expressed as mean ± standard error of mean. For multiple group comparison the data were subjected to One Way Analysis of Variance (ANOVA) followed by Bonferroni post-hoc test.