CHAPTER 4

MATERIALS AND METHODS

4.1. EXPERIMENTAL DESIGN 1: STUDY OF EXPOSURE TO MERCUY AND CADMIUM SINGLY ON FISH INTESTINAL MACROPHAGES (*IN VIVO*)

4.1.1. Biological material

The live fresh water teleost *C. punctatus* Bloch. of average length 14.0±1.5 cm and average body weight 25±5g, were purchased from a local fishery; upon arrival at the laboratory, the animals were acclimatized to the laboratory condition in glass aquarium with continuously aerated and dechlorinated tap water at least 5 to 6 days prior to the experiment. Only healthy fish that were not diseased, as determined by general appearance (colour, skin luster, eyes and behaviour), were used for the studies. Water quality characteristics were determined and the mean values for test water qualities were as follows: temperature 27.8 ± 1.5 ^{0}C, pH 7.4 ± 0.003, dissolved oxygen 6.5 ± 0.2 mg/L, alkalinity 251 ± 2.8 mg/L as $CaCO_3$, total hardness 456± 3.5 mg/L and salinity (%) 30.8 ± 3.5. The fishes were fed twice daily with pelleted diet (prawn powder, fish powder and minced liver in 2:2:1) and were maintained on a photoperiod with 12h light and 12h dark. *C. punctatus* Bloch. was considered suitable for immunomodulatory studies and was thus utilized as the model organism in this work, because it has well documented general biology, short developmental time, easy culturing and year round reproduction.

4.1.2. Exposure to mercury and cadmium

After acclimatization, fish were divided into three groups; one served as control and the other two served as mercuric chloride ($HgCl_2$) treated and cadmium chloride ($CdCl_2$) treated group respectively. Sub-lethal toxicity of $HgCl_2$ (0.3 mg/L), {a sub-lethal concentration of LD_{50} (1.8 mg/L)} (Sastry and Gupta 1978) and $CdCl_2$ (1.96 mg/L), {a sub-lethal concentration of LD_{50} Value 11.8 mg/L)} (Vineeta. *et al*, 2007) on the intestinal macrophages of *Channa punctatus* was analyzed after an exposure of 4 days

and 7 days respectively. After 4 days and 7 days of exposure five fish from each chamber were dissected by cutting the ventral aorta.

4.1.3. Separation of macrophages

Clove oil was used to produce unconsciousness followed by exsanguination, to sacrifice the animals. It was ensured that the animals did not regain consciousness before death occurred by observing opercular movement. Unconscious animals were decapitated before aseptic retrieval of tissues and cells. Animal carcasses and blood were exhumed in an incinerator. The fish were dissected; whole gut of the fish was isolated, immediately placed in Leibovitz (L-15) medium supplemented with heparin (10 IU/ml) and fetal bovine serum (FBS) (2%), and then homogenized in ice cold condition. Cell suspension is then transferred to tubes and kept in ice for cell debris to settle. The supernatant is then layered over Ficoll (45%) and subjected to density- gradient centrifugation (Chung and Secombes, 1998). The band of macrophage- enriched fraction at the interface is collected, washed and resuspended in L-15 medium containing FBS and allowed to adhere on plastic surface. The adherent cells were collected and tested for viability as determined by Trypan Blue dye exclusion technique.

4.1.4. Bioaccumulation, Morphological Alteration and Ultra structural Analysis

4.1.4.1. Heavy metal bioaccumulation by Atomic Absorption Spectrophotometer

The different organs were allowed to dry at 120ºC until reaching a constant weight, concentrated nitric acid and hydrogen peroxide (1:1 v/v) (SD fine chemicals) was added. The digestion flasks were heated to 1300ºC until all the materials were dissolved and diluted with double distilled water appropriately. The elements Hg and Cd were

assayed using Shimadzu AA 6200 Atomic Absorption Spectrophotometer at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya. The results were expressed as ppm/ g tissue.

4.1.4.2. Morphological alteration of macrophages

The cell suspension fixed in an equal volume of 2.5% glutaraldehyde in Hanks Balanced Salt Solution (HBSS) and centrifuged at 2000 rpm for 5 min. The pellet was resuspended in HBSS. Smears of cells were drawn on glass slides, air-dried, fixed in methanol and stained with Giemsa. Cells were observed under oil immersion microscope. Any cell deviating from spherical outline was scored as polarised and this was expressed as a percentage of the total number of cells counted (Ou *et al*., 2009; Mantovani *et al*., 2005).

4.1.4.3. Ultrastructural analysis of tissue by Scanning Electron Microscope

The intestine was excised and rinsed in heparinized saline, rinsed in 0.1 M cacodylate buffer at pH 7.5, infiltered with 2.5% glutaraldehyde for 24 hr fixation at 4ºC, rinsed in buffer, trimmed into 8.0 mm squares and subjected to post-fixation in 1% OsO4 in 0.1 M cacodylate buffer at pH 7.5 for 2 h and dehydrated through graded acetone. The mucosal surface of each tissue was mounted on metal stubs, coated with gold using a JFC-1100 (Jeol) ion sputter. Finally, the tissues were scanned with a JSM-6360 (Jeol) Scanning Electron Microscope at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya.

4.1.4.4. Ultra structural analysis of tissue by Transmission Electron Microscope

The anterior portion of the intestine was excised immediately after perfusion, cut into small blocks of 1.0- 1.5 mm cube size and incubated in perfusion fixative for at least 30

min at 40C. The fixation was continued in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.6) containing 4 % PVP and 0.05 % calcium chloride for 20 min at 4^0C. After rinsing in cacodylate buffer, tissue block was post fixed at 40C for 1 h with 1% osmium ferrocyanide (Karnovsky 1971). After repeated rinsing in 0.1 M cacodylate and 0.05 M maleate buffers (pH 5.2), the tissue was stained en bloc with 1 % uranyl acetate in maleate buffer for at least 1 h at 4^0C. Specimens were dehydrated in a graded series of ethanol and embedded in Spurr's medium (Spurr 1969). Ultrathin sections of 60 to 80 nm thickness were stained with alkaline lead citrate for 1 min and examined in a JEM-2100, 200 Kv (Jeol) transmission electron microscopes at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya.

4.1.5. Study of effect of mercuric chloride and cadmium chloride (*in vivo*) singly on oxidative stress and antioxidant defenses in fish *Channa punctatus* Bloch.

4.1.5.1. Respiratory Burst Activity

Respiratory burst activity of intestinal phagocytes of control and treated fish was measured by the method of Fujiki and Yano (1997). The respiratory burst activity was expressed as A630 nm per 106 cells.

4.1.5.2. Estimation of Lipid Peroxidation (LPO)

The LPO activity was determined by the procedure of Utley *et al*. (1967). The rate of lipid peroxidation was expressed as nanomoles of thiobarbituric acid reactive substance (TBARS) formed per hour per milligram of protein using a molar extinction coefficient of 1.56×105 M-1cm-1. Protein content of each sample was determined using method of Lowry *et al*. (1951).

4.1.5.3. Catalase (CAT) activity

The pellet was dissolved in 1 ml of 0.1 M phosphate buffer. 10-30µl of Triton X-100 was added to the sample and allowed to stand for 5 min.100µl of the sample was then added to a cuvette containing 400µl of 0.1 M phosphate buffer (pH 7.4) and the reaction was initiated by addition of 10µl of 60mmol H_2O_2. The decomposition rate of H_2O_2 was measured at 240nm for 1min using a spectrophotometer. One unit (U) of Catalase activity is defined as the amount of enzyme catalyzing 1µmol of H_2O_2 per min at 25^0C (Claiborne, 1985).

4.1.5.4. Superoxide Dismutase (SOD) activity

A volume of 100 µl of the sample was mixed with 100 µl of Tris-HCl buffer (ph 8.5), then 50 µl of 1mmol ethylene diamine tetraacetic acid (EDTA) and 50 µl of 2.64mmol pyrogallol was added. The absorbance of the reaction mixture was recorded at 420 nm. One unit of SOD activity was determined as the amount of enzyme that inhibited the auto- oxidation of pyrogallol by 50%. The activity was expressed as U/mg protein (Marklund and Marklund, 1974).

4.1.5.5. Glutathione S- Transferase (GST) Activity

GST activity was measured by the method described by Mannervik and Gutenberg (1981). Enzyme activity was calculated using a molar extinction coefficient of 9.6 × 103M-1cm-1.

4.1.5.6. Glutathione Peroxidase (GPx) Activity

Total cellular GPx activity was determined by measuring the decrease in absorbance (340 nm) due to the decline in NADPH at 23–25ºC (Lorentzen *et al.*, 1994). The

activity of GPx was expressed as U/mg protein and 1mU was defined as 1 nmol of NADPH consumed/min/mL of sample.

4.1.5.7. Glutathione Reductase (GR) Activity

The principle of the method is the reduction of oxidized glutathione by glutathione reductase in the presence of NADPH (Carlberg and Mannervik 1975). One Unit was defined as an amount of the enzyme which will reduce 1 μM of oxidized glutathione per minute at pH 7.6 at 25 °C, using a molar extinction coefficient of 6.22 x 103 for NADPH.

4.1.5.8. Reduced Glutathione (GSH) Assay

Non-enzymatic antioxidant, reduced glutathione, was assayed by the method of Ellman (1959). The amount of glutathione was calculated using a GSH standard curve and expressed as micrograms of GSH formed/mg protein

4.1.6. Study of effect of mercuric chloride and cadmium chloride (*in vivo)* singly on innate immune responses in fish *Channa punctatus* Bloch.

4.1.6.1. Phagocytosis assay

A volume of 100 µl of cells from both control and exposed groups were allowed to adhere separately on glass slides for 1 h whereas non-adherent cells were washed out with Dulbecco's phosphate buffered saline (DPBS) (1x). To the glass slides containing the adhered macrophages 10% heat killed *Staphylococcus aureus* was added and incubated for 3 h at 37 0 C which were then washed with DPBS (1x) and dried. The cells were at last fixed in 50% methanol, stained with Giemsa, observed under oil immersion microscope and counted for number of bacterial cells ingested (Czuprynski *et al*, 1984).

Phagocytosis is expressed as Phagocytic index (P.I.):

P.I. (%)= (percentage of macrophages containing bacteria)×(mean number of bacteria per macrophage).

4.1.6.2 Preparation of bacteria *(Staphylococcus aureus* MC524) for intracellular killing assay

To obtain bacteria in the mid-logarithmic phase 100 µl of an overnight culture made in nutrient broth was added to 10 ml of nutrient broth and incubated for 2 - 5 h at 37°C with orbital shaking. The bacteria were washed in 10 mM sodium phosphate buffer (pH 7.4) and their concentration was estimated by spectrophotometry at A620 on the basis of the relationship: A620 0.2 = 5 × 107/ml (Yao *et al.*,1997)

4.1.6.3. Intracellular killing assay

Bacteria were incubated with macrophages in L-15 FBS for 20 min at 37^0C. After various time intervals, samples were plated onto nutrient agar to determine the number of viable intracellular bacteria (Leigh *et al*., 1986). Intracellular killing is expressed as the percentage decrease in the initial number of viable intracellular bacteria.

4.1.6.4. Cell adhesion assay

Cells were seeded separately for treated and control group in 96 well microtiter plates and allowed to adhere for different times. In time, wells were washed with Hank's balanced salt solution (HBSS), and then 100 µl of 0.5% crystal violet in 12% neutral formaldehyde, and 10% ethanol was added to each well and incubated for 4 h to fix and stain the cells. Wells were washed and air dried for 30 min. Crystal violet was extracted from the macrophage adhered in the wells by lysing with 0.1% sodium dodecyl sulphate

(SDS) in HBSS. Absorbance was measured spectrophotometrically at 570 nm. Cell adhesion was expressed as increased absorbance at 570 nm (Lin *et al*, 1997).

4.1.6.5. Nitric oxide (NO) release assay

Cells (10^6 cells/ml) were suspended in DPBS and were stimulated with lipopolysaccharide or LPS (100 ng/ml). The cell free supernatants were used for nitric oxide release assay using Griess reagent. Readings were taken in a UV spectrophotometer at 550 nm. The absorbance was plotted on a nitrate standard curve and NO release was expressed as µM (Sasaki *et al*., 2000).

4.1.6.6. Myeloperoxidase (MPO) release assay

100 µl of cells from control and treated group were taken into microcentrifuge tubes and stimulated with LPS (100 ng/ml) for 1 h at 37^0C and centrifuged at 13,000 rpm for 10 min. The supernatants thus obtained from both the sets was recovered separately and kept at -20^0 C until further use. The cell free supernatant was used for assay of the partial MPO release for both groups. The pellet that recovered from the two groups were lysed in 0.01% SDS and then centrifuged again. The supernatant was recovered as before for total MPO release assay. Subsequently 100 µl of cell free supernatant as well as from lysis of cells were reacted with 100 µl of substrate buffer and kept at 37^0C for 20 min, then the reaction was stopped by adding 100 µl of 2(N) H_2SO_4 and absorbance was measured at 492 nm (Bos *et al*, 1990).

4.1.6.7. Cytokine assays

ELISA was used for detecting fish TNF-α and IL-6 like molecules. The 96 well microtitre plates (Iwaki Glass, Japan) were coated with 100 µl of fish serum or cell supernatant diluted 10 times in phosphate buffer saline (PBS) and incubated in a moist

chamber overnight at 4ºC. TNF- α and IL-6 rabbit polyclonal antibody was used as the primary antibody (Sigma, USA). Vectastain ABC- PO kit (Vector Lab., USA) was applied for ELISA and peroxidase ABTS substrate kit (Vector Lab., USA) was used for coloration. The plate was read at 490 nm on a microplate reader (BioRad).

4.1.6.8. Statistical analysis

All the values were expressed as mean ± standard error of mean (SEM). The data were compared by using the 'Student t' test (two-sample assuming unequal variances). All the differences were considered significant at $P<0.05$. All treatments were assayed in triplicate for each fish. Statistical comparisons were done between control and exposure data from same species.

4.2. EXPERIMENTAL DESIGN 2: STUDY OF EXPOSURE TO MERCURY AND CADMIUM SIMULTANEOUSLY ON FISH INTESTINAL MACROPHAGES *(IN VIVO)* AFTER 4 DAYS AND 7 DAYS.

4.2.1. Biological material

The live fresh water teleost *C. punctatus* Bloch. of average length 14.0±1.5 cm and average body weight 25±5g, were purchased from a local fishery; upon arrival at the laboratory, the animals were acclimatized to the laboratory condition in glass aquarium with continuously aerated and dechlorinated tap water at least 5 to 6 days prior to the experiment. Only healthy fish that were not diseased, as determined by general appearance (colour, skin luster, eyes and behaviour), were used for the studies. Water quality characteristics were determined and the mean values for test water qualities were as follows: temperature 27.8 ± 1.5 ^{0}C, pH 7.4 ± 0.003, dissolved oxygen 6.5 ± 0.2 mg/L, alkalinity 251 ± 2.8 mg/L as $CaCO_3$, total hardness 456± 3.5 mg/L and salinity

(%) 30.8 ± 3.5. The fishes were fed twice daily with pelleted diet (prawn powder, fish powder and minced liver in 2:2:1) and were maintained on a photoperiod with 12h light and 12h dark. *C. punctatus* Bloch. was considered suitable for immunomodulatory studies and was thus utilized as the model organism in this work, because it has well documented general biology, short developmental time, easy culturing and year round reproduction.

4.2.2. Exposure to mercury and cadmium simultaneously (multimetal challenge)

After acclimatization, fish were divided into four groups: Group I- mercury treated, Group II- cadmium treated, Group III- multi-metal, mercury and cadmium simultaneously treated and Group IV- control. Sub-lethal toxicity of $HgCl_2$ (0.3 mg/L), {a sub-lethal concentration of LD50 (1.8 mg/L)} (Sastry and Gupta 1978) and $CdCl_2$ (1.96 mg/L), {a sub-lethal concentration of LD50 Value 11.8 mg/L)} (Vineeta. *et al*, 2007) on the intestinal macrophages of Channa punctatus was analyzed after an exposure of 4 days and 7 days respectively. After 4 days and 7 days of exposure five fish from each chamber were dissected by cutting the ventral aorta.

4.2.3. Separation of macrophages

The fish was dissected, whole intestine of the fish was isolated, immediately placed in Leibovitz medium (L-15) supplemented with heparin (10 IU/ml) and fetal bovine serum (2%), and then homogenized in ice cold condition. Cell suspension is then transferred to tubes and kept in ice for cell debris to settle. The supernatant is then layered over Ficoll (45%) and subjected to density- gradient centrifugation (Chung and Secombes, 1998).The band of macrophage- enriched fraction at the interface is collected, washed and resuspended in L-15 medium containing FBS and allowed to adhere on plastic

surface. The adherent cells were collected and tested for viability as determined by Trypan Blue dye exclusion technique.

4.2.4. Study of effects of mercuric chloride and cadmium chloride *(in vivo)* simultaneously on bioaccumulation, morphological alteration and ultrastructural damage:

4.2.4.1. Heavy metal bioaccumulation by Atomic Absorption Spectrophotometer

4.2.4.2. Morphological alteration of macrophages

4.2.4.3. Ultrastructural analysis of tissue by Scanning Electron Microscope

4.2.4.4. Ultra structural analysis of tissue by Transmission Electron Microscope

4.2.5. Study of effects of mercuric chloride and cadmium chloride *(in vivo)* simultaneously on oxidative stress and antioxidant defenses in *Channa punctatus* Bloch.

4.2.5.1. Respiratory Burst Activity (Fujiki and Yano 1997)

4.2.5.2. Estimation of Lipid Peroxidation (LPO) (Utley *et al*., 1967)

4.2.5.3. Catalase (CAT) Activity (Claiborne, 1985)

4.2.5.4. Superoxide Dismutase (SOD) Activity (Marklund and Marklund, 1974)

4.2.5.5. Glutathione S- Transferase (GST) Activity (Mannervik and Gutenberg 1981)

4.2.5.6. Glutathione Peroxidase (GPx) Activity (Lorentzen *et al*., 1994)

4.2.5.7. Glutathione Reductase (GR) Activity (Carlberg and Mannervik 1975)

4.2.5.8. Reduced Glutathione (GSH) Assay (Ellman 1959)

4.2.6. Study of effects of mercuric chloride and cadmium chloride *(in vivo)* simultaneously on innate immune functions in *Channa punctatus* Bloch.

4.2.6.1. Phagocytosis assay (Czuprynski *et al*, 1984)

4.2.6.2. Preparation of bacteria (*Staphylococcus aureus* MC524) for intracellular killing assay (Yao *et al*., 1997)

4.2.6.3. Intracellular killing assay (Leigh *et al*., 1986)

4.2.6.4. Cell adhesion assay (Lin *et al,* 1997)

4.2.6.5. Nitric oxide (NO) release assay (Sasaki *et al*., 2000)

4.2.6.6. Myeloperoxidase (MPO) release assay (Bos *et al*., 1990)

4.2.6.7. Cytokine assays

4.2.6.8. Statistical analysis

The data are expressed as mean± S.E. Data were analyzed using Student's t-test (two-sample assuming unequal variances) for determining the significant change over control values. The significance level was set at $P<0.05$.

In case of multi metal exposure, besides one- tailed Student's t test, a statistical approach applying an additivity response surface methodology (Suhnel, 1992) was used to test the validity of the synergy concept for the multi-metals mercury and cadmium. A multivariate- modified ANOVA has been applied to search for evidences of interaction between the heavy metals mercury and cadmium, when administered simultaneously. However, as F tests do not provide conclusive evidences for proving heavy metal synergy, an isobologram has been plotted for TNF-α release (pg/ml). For this purpose an ordinary least squares regression was run between effects (TNF-α release) and dose

levels of metals (single and multi-metal) in the log- linear form. Assuming additivity of effects, TNF-α release was fixed at 400 units and the minimum dose levels of mercury plotted for each dose level of cadmium to get the isobol for 400 units TNF-α release. An isobologram is a two-dimensional graph with the doses of agents A and B (mercury and cadmium, in the study) as coordinate axes, in which one or several lines, the isobols, are shown connecting different dose combinations which produce the same magnitude of effect (Loewe, 1953).

4.3. EXPERIMENTAL DESIGN 3: STUDY OF RELATIVE GENE EXPRESSION QUANTIFICATION OF TUMOR NECROSIS FACTOR (TNF-α) AND INTERLEUKIN-6 GENE USING 18S GENE AS ENDOGENOUS CONTROL IN THE INTESTINE OF *CHANNA PUNCTATUS* AFTER THE SIMULTANEOUS EXPOSURE OF MERCURY AND CADMIUM FOR 7 DAYS.

4.3.1. Biological material

The live fresh water teleost *C. punctatus* Bloch. of average length 14.0±1.5 cm and average body weight 25±5g, were purchased from a local fishery; upon arrival at the laboratory, the animals were acclimatized to the laboratory condition in glass aquarium with continuously aerated and dechlorinated tap water at least 5 to 6 days prior to the experiment. Only healthy fish that were not diseased, as determined by general appearance (colour, skin luster, eyes and behaviour), were used for the studies. Water quality characteristics were determined and the mean values for test water qualities were as follows: temperature 27.8 ± 1.5 0C, pH 7.4 ± 0.003, dissolved oxygen 6.5 ± 0.2 mg/L, alkalinity 251 ± 2.8 mg/L as $CaCO_3$, total hardness 456± 3.5 mg/L and salinity (%) 30.8 ± 3.5. The fishes were fed twice daily with pelleted diet (prawn powder, fish powder and minced liver in 2:2:1) and were maintained on a photoperiod with 12h light

and 12h dark. *C. punctatus* Bloch. was considered suitable for immunomodulatory studies and was thus utilized as the model organism in this work, because it has well documented general biology, short developmental time, easy culturing and year round reproduction.

4.3.2. Exposure to mercury and cadmium simultaneously (multimetal challenge)

After acclimatization, fish were divided into four groups: Group I- mercury treated, Group II- cadmium treated, Group III- multi-metal, mercury and cadmium simultaneously treated and Group IV- control. Relative gene expression of tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) gene were studied in the fish intestine after the exposure of $HgCl_2$ (0.3 mg/L), {a sub-lethal concentration of LD_{50} (1.8 mg/L)} (Sastry and Gupta 1978) and $CdCl_2$ (1.96 mg/L), {a sub-lethal concentration of LD_{50} Value 11.8 mg/L)} (Vineeta *et al*, 2007) for 7 days. After 7 days of exposure five fish from each chamber were dissected by cutting the ventral aorta. After dissection, whole intestine of the fish was isolated.

4.3.3. RNA isolation

Total RNA was isolated from all the 4 samples using TRIZOL reagent (total RNA isolation reagent, Life Technologies) as per manufacturer's protocol.

4.3.4. Quantitative and Qualitative analysis of total RNA:

Quantification of total RNA was done using NanoDrop 8000 spectrophotometer. Further, the quality of total RNA was analyzed on 1% denaturing agarose gel as per following method.

- 1 gm of agarose was dissolved in 36.25 ml DEPC treated water, heated and then cooled to 60OC. 5 ml of 10 X MOPS buffer was added followed by 8.75 ml of 37% formaldehyde, this was then mixed thoroughly and casted in the AGE unit.
- RNA sample (2 µl) was mixed with 2 µl of 2X RNA Loading dye, heated at 70OC for 5 minutes, chilled on ice for 5 minutes and mixed with 0.5 µl of 1% EtBr and loaded on the gel.
- The electrophoresis was carried out using 1X MOPS buffer. The image was captured using the ChemiDoc XRS instrument (Bio-Rad)

4.3.5. First cDNA synthesis using RevertAid H Minus First Strand cDNA Synthesis Kit

Complementary DNA (cDNA) was prepared from total RNA samples using H minus First Strand cDNA synthesis kit (Thermo Scientific) using random hexamer primer as per manufacturer's protocol. Steps are given below:

Table 4.1: The following components were added in 0.2 ml PCR micro centrifuge tube in the order given below and kept on ice.

Components	Quantity/Conc.
Total RNA Template	1 µg
Primer (Random Hexamers)	1 µl
DEPC -treated water	To make up remaining volume
5x Reaction Buffer	1x
RiboLock RNase Inhibitor	1 ul
10 mM dNTP mix	10 pico-mole
M-MuLV Rev. Transcriptase	50 unit
Total Volume	**20**

- After mixing gently, centrifuged and incubated for 5 minutes at 25^0C followed by 60 minutes at 42^0 C.
- The reaction was terminated by heating at 70^0 C for 5 minutes and keep hold at 4^oC.

4.3.6. Real Time PCR

In the present assay, sample S-1 was used as calibrator and sample S-2, S-3 & S-4 used as test sample for relative quantification of gene expression Real Time PCR reaction setup for TNF & IL-6 gene by taking 18S as housekeeping gene. Reactions was performed in triplicate using Light Cycler 480 SYBR Green l Master (2X concentrated). Real time PCR setup was done on the following manner:

Table 4.2: Light Cycler reaction set-up

Components	Quantity
Nuclease free water	9.5µl
cDNA(diluted 1:10)	1.0 µl
Forward Primer (10pmole /ml)	1.0µl
Reverse Primer (10pmole /ml)	1.0µl
LightCycler 480 SYBR Green I Master	12.5µl
Total Volume	**25µl**

Table 4.3: Light Cycler reaction condition

Programs

Program Name	pre-incubation		
Cycles	1	Analysis Mode	None

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:05:00	4.80		0	0	0

Program Name	amplification		
Cycles	45	Analysis Mode	Quantification

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:00:10	4.80		0	0	0
60	None	00:00:10	2.50		0	0	0
72	Single	00:00:20	4.80		0	0	0

Program Name	melting curve		
Cycles	1	Analysis Mode	Melting Curves

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:00:05	4.80		0	0	0
65	None	00:01:00	2.50		0	0	0
95	Continuous		0.19	3	0	0	0

Program Name	cooling		
Cycles	1	Analysis Mode	None

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
40	None	00:00:30	2.50		0	0	0