4.0 METHODOLOGY

4.1 ANIMAL MODEL FOR RF-EMF RADIATION IMPACT STUDY

The experimental animal chosen for the *in vivo* study were guinea pigs *(Cavia porcellus)* of Duncan-Hartley strain. They were exposed to RF-EMF radiation from a cell phone tower at two distances, while maintaining control (C) animals away from RF-EMF radiation exposure. The experimental animals were maintained at these two distances for a period of six months. Appropriate permission was sought from Institutional Animal Ethics Committee (IAEC) for animal research and appropriate care for the animals was undertaken as per guidelines of Committee for the Purpose and Control and Supervision of Experiments on Animals (CPCSEA),India, for laboratory animal facilities (Reg. No.833/a/04/CPCSEA). A temperature of20-26℃ (68-75°F) with a relative humidity of 30-70% is recommended for guinea pig housing (**Mark A.*et al*., 2012).**

4.1.1 CARE OF GUINEA PIGS

The guinea pigs were maintained for a period of six months at two distances from the cell phone tower (RF-EMF sources) in Cage C1 at 5 m and Cage C2 at 20 m. The third cage was the sham-exposure control Cage C. The control group animals were in a similar environment, but in the absence of RF-EMF exposure, as measured by the radiation detection instruments. At the end of the six-month period, the animals were 185 days old (6.5 months). The difference in weight was significant at 5% level for the guinea pigs in Cage C2 at 20 m exposure distance. The mean weight of female guinea pigs was more than male guinea pigs. Diet consisted of carrots, cucumber, Bengal gram, tomato, cabbages, Lucerne grass, and greens, fortified with vitamin C. Water was given *ad libitum* in clay water pans. The cages were cleaned every day and sterilized fresh husk was supplied as bedding **(Figure 4.1)**.

Figure 4.1: Feeding activity of the guinea pig in Cages C, C1 and C2

4.1.2 RF-EMF RADIATION MEASUREMENTS OF THE CAGES

The RF-EMF radiation measurements were carried out using the radiation detector everyday for six months. The measurements were taken inside and outside the cages of the control animals, animals at a distance of 5 m and animals at a distance of 20 m from the raidation source. Four healthy Duncan-Hartley strains of guinea pigs were maintained for a period of six months in each cage. The power density was determined for cumulative radiation **(Figure 4.1)**.

Required equipment

- Radiation measuring digital detector (Sanlar Imex Services Private Limited, Mumbai, Maharashtra)
- Radiation detector showing colours (Saiurja Systems, Mumbai) such as red, yellow and green.

Figure 4.2: Radiation dedector showing color changes in control (green) and experimental cage C2 (yellow)

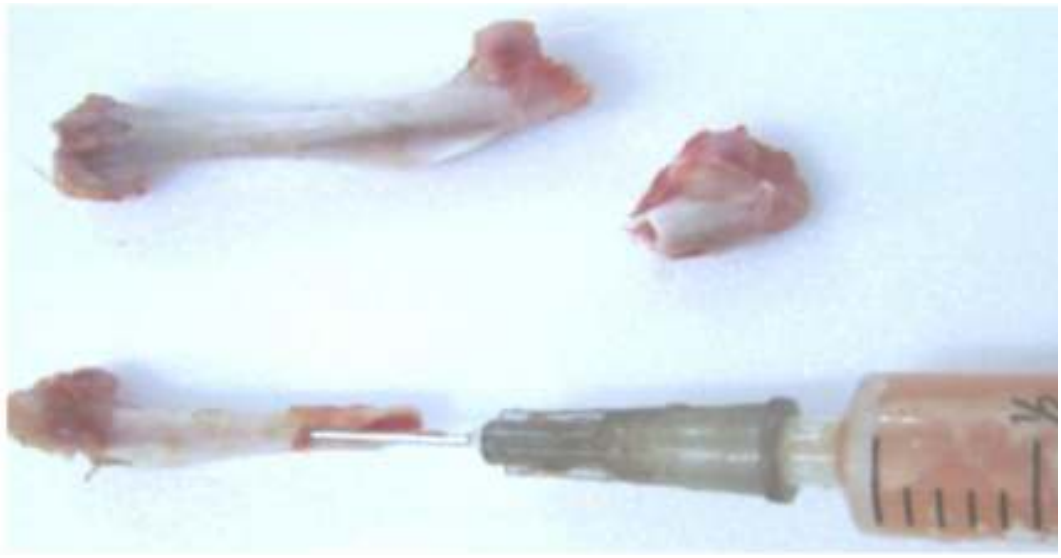
4.2 STEPS OF SAMPLE PREPARATION AND STEM CELL CULTURE

The samples were taken from experimental and control groups of guinea pigs (*Cavia porcellus*) and the mesenchymal stem cells were isolated.

4.2.1 ASPIRATION OF SAMPLE FROM BONE MARROW

The guinea pigs were sacrificed and femur was removed after dissection under laminar airflow chamber in fumigated lab conditions. The femur bone was removed from animal and using steril technique, the distal end of the syringe was screwed into the proximal hub of the needle. The syringe was filled with 5 ml heparin or normal saline during aspiration, quickly inserted and marrow sample was aspirated. The aspiration process was repeated and the required quantity was obtained. The needle was take out collected in a tube. This collection protocol was reviewed and approved by ethical committee of Entomology Research Institute, Loyola College. Exactly 10 ml of bone marrow samples were collected from each group of guinea pigs (experimental period=6 months, no.animals=2 male, 3 female in each cage). The collected samples were processed within two hours from the time of collection (**Figure 4.3)**.

Figure 4.3: Bone marrow aspiration from adult guinea pig



Cell Culture Laboratory

Cell culture equipment was accessed from Frontier Lifeline Research Institute, Gumidipoondi, Chennai, and the method of culturing MSCs was clearly established. Stem cell culture laboratory, maintained in a sterile condition, was accessed and included a laminar flow, biological safety cabinet, 5% CO2incubator maintained at 37°C, inverted microscope with interference phase optics for observing cultured cells and a haemocytometer (glass) for counting cells with the microscope.

Growth Media for MSCs

The media used for MSC culture was Dulbecco's Modified Eagles Media (DMEM), containing 10% bovine serum. It was used in two groups of stem cell cultures. It was isolated from adult guinea pig bone marrow. The DMEM media was added in the culture plate at every passage (2-3ml) and kept at 4˚C to maintain the viability of the media. Media status contained 445 ml DMEM low glucose and 10% bovine serum (HIMEDIA).

4.2.2 ISOLATION OF MONONUCLEAR CELLS FROM GUINEA PIG BONE MARROW

Materials and Reagents Required

- Heparin Injection IP (Palani Pharma)

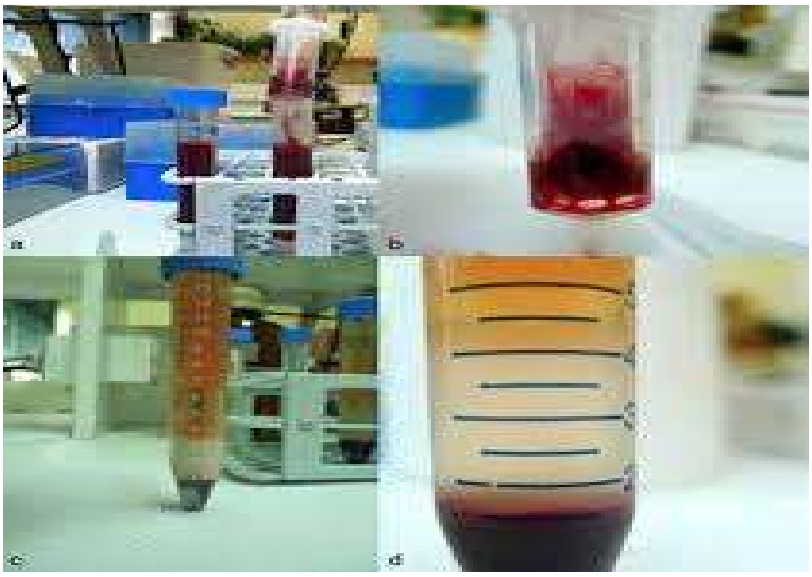
- Ficoll solution
- RBC buffer solution (0.7% ammonium chloride solution)
- Sodium chloride solution
- PBS and normal saline

Procedure

The bone marrow samples (5 to 10ml) collected from control and exposed guinea pigs (6 months) were transferred immediately into a 50 ml eppendorf tube containing preservative-free sodium heparin or normal saline (Sodium chloride 0.9%). The bone marrow sample was then diluted with 1×DPBS in the ratio of 1:2 in a sterile conical tube. 10 ml of Ficoll solution was taken in a separate conical tube and the diluted bone marrow sample was carefully overlayed along the side of the centrifuged tube. The tube was then centrifuged at 500 rpm for 30 minutes at 20˚C. Using the disposable plastic pasteur pipet, the band containing MNCs was carefully collected and transferred into a fresh centrifuge tube.

These MNCs were then diluted with PBS and centrifuged at 450g for 10 minutes at 4˚C. The buffer was aspirated and discarded, and then, the pellet was treated with 0.7% of freshly prepared sterile ammonium chloride solutionto lyse RBCs. These MNCs were then incubated at room temperature for 2 minutes and then 0.9% cold sodium chloride was added to stop the lysing action. The cells were then mixed well and centrifuged at 300g for 5 minutes at 4˚C. this pellet obtained was re-suspended and counted using a haemocytometer.

Figure 4.4: Steps of Mononuclear cell isolation from bone marrow of Guinea pig



4.2.3 COUNTING OF CELL SAMPLES

Heamocytometer (Enumerator)

Materials and Required

Inverted phase contrast microscope with 10x objective

Pipetman, 1-20µl

Pipetman, 1-200µl

Pipette tips, 10-200µl

0.4% Trypan blue stain

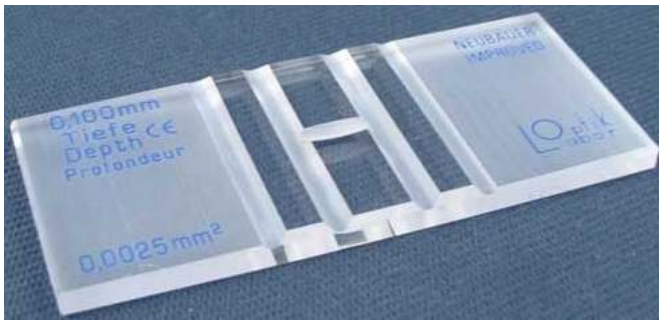
Haemocytometer with cover slip (Neubauer chamber)

1×DPBS

Procedure

The cell suspension to be enumerated and assessed for viability was diluted in PBS and trypan blue solution (0.4% Trypan in DPBS). 100µl (0.1 ml) of trypan blue (0.4% w/v) was mixed with 100 µl cell suspension and made up to 1:2 dilution of the cells. In Figure 4.2.2 haemocytometer was cleaned using 70% ethonal. The cover slip was gently affixed on the haemocytometer to avoid moisture bubbles. A single drop of the trypan blue cell mixture (approximately 10µ) was then allowed to run under the cover slip. The chamber was allowed to fill by capillary action. The haematometer grid was then visualized under the inverted phase contrast microscope (10X objective). Trypan blue was a “vita stain” and hence it was excluded from live cells. Live cells appear colourless and bright (refractile) under inverted phase contrast microscope. Dead cells stain blue due to membrane integrity and are non-squares and cell counts were recorded.

Figure 4.5: Stem Cell Counted using haemocytometer



4.2.4 CELL COUNTING FORMULA AND CALCULATION OF CELL VIABILITY

Formula for cell counting

$$\text{Number of cell: Total no. of cells counted in Cell suspension/ml} = \frac{\text{Total cells counted in corner squares} \times \text{Dilution factor} \times \text{Depth factor}}{\text{Number of squares}}$$

Calculation of cell viability

$$\%\ \text{viability} = \frac{\text{Total No. of Viable cells}}{\text{Total cells counted (Viable + Dead)}} \times 100$$

4.2.5 STEM CELL CULTURE

Required reagents and chemicals

- T-25 culture flask (Bio-Gene)
- T- 75 culture flask (Bio-Gene)
- FBS (Bio-Gene)
- Antibiotic and antimycotic solution (Bio-Gene)
- Trysin EDTA 1X (Bio-Gene)
- 1X DPBS (Bio-Gene)
- DMEM-LG(Bio-Gene)
- Syringe fiters (Bio-Gene)

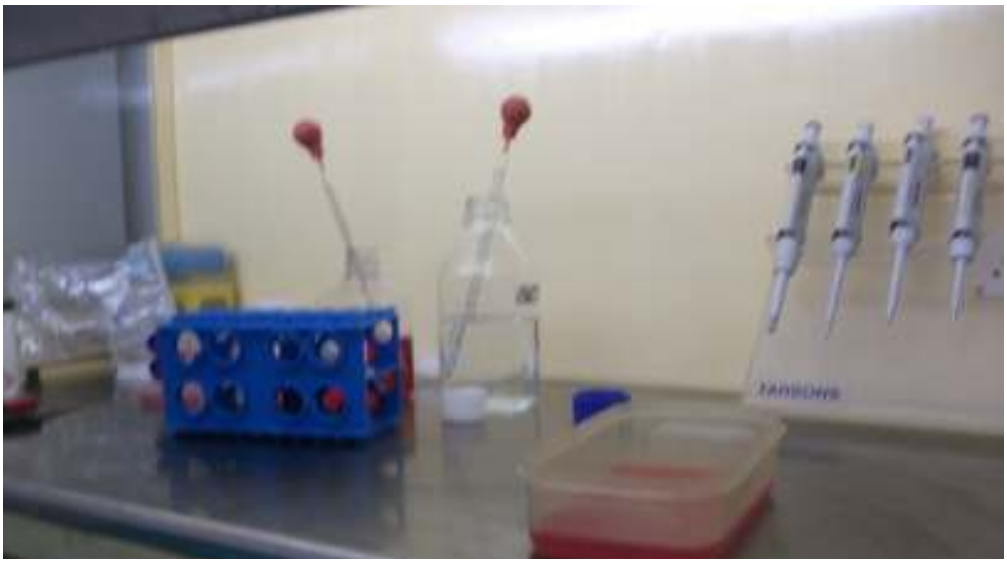
4.2.5.1 MEDIA PREPARATION FOR BONE MARROW STEM CELL CULTURE

DMEM-LG solution was added to 10% FBS and 1% antibiotic solution (100 µml-ascorbate-2 phosphate, 2mM L-glutamine, 50 µ/ml penicillin and 50 µg/ml streptomycin) and filter sterilized with 0.2µm filter.

Protocol

The isolated mononuclear cells were counted and then seeded on to T25 and T75 cell culture flasks with the seeding density of 1×10^4 cells/cm^2. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with low glucose. The flasks were then incubated at 37˚C in 5% relative humidity and with a pressure of 60 psi for 48- 72 hrs to allow adherent cells to attach. The spent media and non-adherent cells were removed and washed with 10 ml PBS. The cell culture flask was then replaced with fresh cell cultured and continued to incubate. The medium was changed, once every three heterogenous cells appeared to diminish quickly through serial passage, particular, cells expressing haematopoietic and endothelial markers were lost in passage 2 **(Figure 4.5)**.

Figure 4.6: Media preparation for stem cell culture



4.2.5.2 SUBCULTURE OF MESECHYMAL STEM CELL FOR EXPANDING

The primary cell culture was sbcultured once they attained 80-90% confluency. To harvest the cultures, the media was removed and the adherent cells were rinsed with 20-30 ml of PBS and aspirated. 10 ml of 0.5% trypsin-EDTA solution was added to the flask so as to distribute trypsin across the surface area of the flaks. The flask was then incubated for 2-5 minutes at 37˚ CO_2 incubator. The cells were then examined by inverted phase-contrast microscopy. After 80-90% of the cells were rounded up or detached, and the sides of the flask were gently tapped to dislodge any remaining attached cells. The trypsin was inactivated by adding 10 ml of cell culture media into the flask. The flask was transferred into a clean 50 ml conical tube. The complete detachment of the adhered cells was confirmed by viewing the flask under microscope. The flask was then rinsed with 20 ml of 1×DPBS and combined with the cell suspension. The cell suspension containing trypsin and meduim was centrifuged at 300g for 5 minutes at 20˚C. The cell concentration was determined using haematocytometer and distributed into tissue culture flask (T75 flask) according to its split ratio. The harvested cells were reseeded at a density of 5×10^5 cells/cm^2 in an appropriate culture flask. After sub culturing, these cells were designated as passage 1. The resultant MSC cultures were successfully expanded up to passage 3, without significant loss of the stem cell phenotype (**Figure 4.6)**.

Figure 4.7: Stem cell subculture process

4.3 FACS ANALYSIS

The Flow cytometry is a quantitative technique that permits visualization of cells by multiple parameters using fluorescence. In comparison to spectrophotometer, it was able to measure fluorescence emission per cell and allowed accurate counting of single cells. All Flow cytometric experiments were performed on a BD FACS caliber. The MSCs were characterized for various haematopoitic, mesenchymal and subpopulation cells with its surface markers using Flow cytometry before and after sorting. The cell counts were performed on a Becton Dickinson FACS Aria (http://www. Bd.com/)using a 488 – nm argon – ion LASER and 632 nm red laser for excitation; fluorescence emission was collected using its corresponding detectors.

4.3.1 MARKERS REQUIRED

- CD 34-PE (BD Biosciencies)
- CD 45 – APC-CY7 (BD Biosciencies)
- CD29- PE (BD Biosciencies)
- CD 90- PERCP (BD Biosciencies)
- CD 105 – APC (BD Biosciencies)

4.3.2 PREPARATION OF SAMPLES AND MARKERS

Samples were prepared as follows:

- Tube 1: CD 45 APC CY7
- Tube 2: CD 34 PE
- Tube 3: CD 29 PE
- Tube 4: CD 105 APC
- Tube 5: CD 90 PERCP
- Tube 6: Unstained sample for instrument setup
- Tube 7 HLADR PERCP

4.3.3 PROTOCOL OF THE ANALYSIS

To confirm the immunophenotypic characteristics of MSCs, they were subjected to flow cytometry. 1×10^5 cells were harvested at the end of passage 2 after incubation with 0.25% trypsin – EDTA for 5 minutes. The cell suspension was washed with PBS before conjugation with the antibodies. 10µl of respective antibodies were added in the respective tubes containing

100µl of suspension. All the tubes were then incubated at room temperature for 20 minutes in dark. After incubation, cells were washed using BD FACS wash buffer to remove the unbound antibodies. After centrifugation, the supernatant was discarded and the pellet was further re-suspended in 500µl of PBS and vortexed. The labeled cells were then analyzed by FACS cytometer using BD FACS diva software (Becton Dickinson).

4.3.4 EXAMINATION OF SAMPLE

The Flow cytometry instruments were set using unstained cells. The plots for sample analysis were created as following; the first was created with forward scatter (FSC) and side scatter (SSC) in all experiment sets to identify the different cell populations and to eliminate debris. The subsequent plots were created using the respective flourochrome (X axis) along with SSC (Y axis) and unstained sample (tube 7) was run and recorded. The results from this tube were sent to the gates and the analysis regions. Using this as a control, the readings of cells staining positive for the given marker was determined by the percentage of cells present within an established gate. A minimum of 10,000 events were collected for each experiment and recorded (**Figure 4.7)**.

Figure 4.8: Flow cytometric analysis for characterization of MSCs

4.3.5 DIFFERENTIATION OF MSCs

The guinea pig bone marrow MSCs were differentiated into other cells, like heamatopoitic stem cell. The cultured and confluent stem cells were mixed with some negative and positive markers for differentiating the same cells. Morpho-phenotypic alternations were noted and were further confirmed using respective staining techniques.

Required materials

- Confocol microscope
- MSCs suspension
- Cell culture plate or Flash (T25,T75)
- Control cell model (for identify the cell)

These materials were used to differentiate the cells from one another, such as non-adherent stem cells and adherent stem cells; mesechymal stem cells being an adherent, attaches on the plastic cell culture plate. Those tests were used for cell morphological size and structure determining.

4.4 REAL TIME POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

The Real Time Polymerase Chain Reaction (RT-PCR) was applied to find the gene expression of genes of interest, such as Fos, TNF-alpha, GFAP, HSB1, VEGF and TGF-beta, implicated in RF-EMF radiation exposure. RT-PCR is a method in which an RNA strand was reverse transcribed into its complementary DNA or cDNA using the enzyme reverse transcriptase, and the resulting cDNA was amplified using RT-PCR. It was performed using a Mastercycler ep realplex 2.2 system (Eppendorf, Hamburg, Made in Germany).

Sample preparation and tissue homogenate: At the termination of exposure, animals were sacrificed immediately and the bone was dissected out. The bone marrow samples were collected in sodium chloride (Nirlife). The bone marrow was isolated from the guinea pig, centrifuged and collected MNCs. The isolated MNCs were for seeded stem cell culture. After cultured stem cell was centrifuged and supernatant was collected for estimation. RT-PCR was performed to validate the expression of RF-EMF sensitive genes. In this analysis, target gene expression was standardized to the housekeeping gene (HKG), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chen C, 2014).

4.4.1 PRIMER DESIGNING

A primer is a short synthetic oligonucleotide, which is used in many molecular techniques from PCR to DNA sequencing. Primers were designed by choosing the FASTA sequence of the gene of interest in genome browser, NCBI. Using Primer3 software, with the helpof the FASTA sequence, the left and right primers, with product size ranging from 90-120 were designed. Optimal length of PCR primers was 18-22 bp. Primers with melting temperatures in the range of 52-58oC generally produced the best results and hence were chosen. Primer melting temperature

was the estimate of the DNA-DNA hybrid stability and was critical in determining the annealing temperature. The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer was between 40-60%. Primers with secondary structures and hairpins were avoided.

Isolation of RNA by QIAGEN Kit Method: The stem cells were disrupted and lysate was homogenized in Buffer QRL1. 0.5 ml of Buffer QRV1 was added and mixed thoroughly by vortexing and centrifuged at 15,000 x g for 20 minutes at 4°C. The supernatant was carefully transferred into an RNase-free 2 ml collection tube. 0.8 ml of ice-cold isopropanol was added and mixed thoroughly"58 by vortexing and incubated for 5 minutes on ice; centrifuged at 15,000 x g for 30 minutes at 4°C;nucleic acids pelleted. 1 ml of Buffer QRE was pipetted into the QIAGEN-tip to equilibrate. The buffer was allowed to enter the column by gravity flow. The supernatant was discarded. 0.15 ml Buffer QRL1 was added to the nucleic acid pellets. The nucleic acid pellets were dissolved by heating the tube for 3 minutes at 60°C followed by vortexing for 5 seconds and sharply flicking the tube, repeating twice. 1.35 ml of Buffer QRV2 was added and mixed thoroughly by vortexing and centrifuged at 5000 x g for 5 minutes at 4°C. Dilution with Buffer QRV2 created optimal conditions for binding RNA to QIAGEN Resin. Sample was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The flow-through was collected for later DNA isolation and placed at room temperature. 2 ml of Buffer QRW was pipetted onto the QIAGEN-tip and allowed to enter the resin by gravity flow. 1 ml of preheated (45°C) Buffer QRUR was pipette into the QIAGEN-tip and the RNA was eluted by gravity flow into a 2-ml collection tube. One volume of ice-cold isopropanol was added and mixed thoroughly by vortexing and placed on ice. It was incubated on ice for 10 minutes and centrifuged at 15,000 x g for 30 minutes at 4°C to precipitate the RNA. The supernatant was carefully removed. 0.5 ml of 70% ethanol was added to the RNA pellet, vortexed, centrifuged at 15,000 x g for 15 minutes at 4°C, and the supernatant was carefully removed. This step was repeated. The RNA pellet was air-dried for approximately 10minutes at room temperature with the tube resting upside down on a paper towel. The RNA was dissolved in a small volume of RNase-free water by heating the tube for 3 minutes at 60°C followed by vortexing for 5 seconds and sharply flicking the tube. This step was repeated twice.

Reconstitution of the primers: The vials were centrifuged at 8000 g for 2 minute. The cap wasopened carefully and required amount of RNAse- and DNAse-free sterile water was added. Thevials were kept at room temperature for 10 minutes and then mixed by vortexing for 15-20 seconds.The vials were centrifuged at 8000 g for 2 minutes. The vials were used as stock. A 1:10 dilutionof the working solution was prepared.

cDNA conversion: RNA was converted into cDNA as shown in step 1 (Table 4.1). The vials were incubated at 70˚C for 10 minutes. Then the vials were transferred to ice for 5 minutes. After step 1, the components of step 2 were added, incubated and mixed for 10 minutes. Then the vials were maintained at 37˚C for 50 minutes and finally 95˚cfor 10 minutes.

4.4.2 PROCESS OF RT- PCR

The RT-PCR process was done two steps; first, the reverse transcription and then the PCR. This method is more sensitive than the one-step method. Kit method was used to isolate RNA and cDNA from the pure, and attention was given to quality, to get the best results.

Step1

Template RNA, primer, dNTP mix and nuclease-free water were mixed in a PCR tube. RNase inhibitor and reverse transcriptase were added to the PCR tube and the PCR tube was placed in the thermal cycler for one cycle that included annealing, extending and then inactivating the reverse transcriptase; proceeded directly to PCR and the rest stored on ice until PCR could be performed.

Step 2

In second step, added master mix (containing buffer, dNTP mix, $MgCl_2$, Mgcl2, Taq polymerase and nuclease-free water) to each PCR tube. The appropriate primer was added, and PCR tubes placed in thermal cycles for 40 cycles of the amplification program, which included three-process denaturation, annealing, elongation. The RT-PCR products could then be analyzed with gel electrophoresis (Agarose gel, buffer solution). The steps of cDNA conversion are given in (**Table 4.1.)**

Table 4.1 Steps of cDNA conversion

Step 1		**Step 2**	
Component	**Amount**	**Component**	**Amount**
dTNPs	1 µl	RT Buffer	2 µl
Oligodt	1 µl	RT Enzyme	1 µl
RNA Template	8 µl	RNase free H2O	7 µl
Total	10. µl	Previous RNA	10 µl
		Total	20 µl

Annealing temperature for RT-PCR analysis: The annealing temperature for each gene was found by performing Gradient PCR. Primer Sequences was shown in **(Table 4.2).**

Table 4.2 Primer Sequences

S.NO	Gene	Annealing Temperature	Primer Sequence 5'to 3'
1	GAPDH	64	5-CATCCACTGGTGCTGCCAAG-3′ 5′-GTCCTCGGTGTAG CCCAAGA-3′
2	TNF-alpha	53	5-GAGCTCGCAGAGGAG-3' 5- CACCAGCTGGTTGTCGCTCAGGCC-3'
3	Fos	61	5'-AGAAACACGTCTTCCCTCGA-3' 5'-TTGTGGATGCTTGCAAGTCC-3'
4	TGF-beta	64	5'-CGGGGCCTGGACACCAACTATTGC-3' 5'- CTGCTCCACCTTGGCTTTGCGGCCCAC-3'
5	HSB1	58	5'-GCAAGCACGAAGAGAGACAG-3' 5'-TCTCCTCCTCCCTGTCTCC-3'
6	GFAP	53	5'-CGAGGAAGTGACTGGTCTGA-3' 5'-CCGCAGTTGGAAGAGACATG-3'
7	VEGF	58	5'-TTTCGGGAAGCAGACCTCTT-3' 5'-GTCGAAGTGATGGTGTGTGG-3'

Procedure

The cDNA obtained as described above was subjected to Real-Time PCR analysis using SYBR green. The RT-PCR was performed to determine the expression of genes of interest using primers (Table 4.2). The Master Mix conditions were as shown in **(Table 4.3).**

Table 4.3: Master Mix for RT-PCR

Components	**1X(µl)**
Master mix	5.0
Forward primer	1.0
Reverse primer	1.0
Template	2.0
Nuclease free H2O	1.0

RT-PCR Cycling condition for TGF beta, GAPDH and VEGF: PCR cycling condition for TGF beta, GAPDH and VEGF genes were as shown in **(Table 4.4).**

Table 4.4: PCR cycling conditions for TGF beta, GAPDH and VEGF genes

Initial Denaturation	95	2 mins	
Denaturation	95	15 sec	40 cycles (40X)
Annealing	64	15 sec	
Extension	72	30 sec	
Final Extension	72	5 mins	

RT-PCR Cycling Condition of TNF alpha: PCR cycling condition for GAPDH and TGF-beta genes were as shown in **(Table 4.5)**

Table 4.5: PCR cycling conditions for TNF alpha genes

Initial Denaturation	95	2 mins	
Denaturation	95	15 sec	40 cycles
Annealing	64	15 sec	
Extension	72	30 sec	
Final Extension	72	5 mins	

RT-PCR Cycling Condition of HSB1 and GFAP: PCR cycling condition for of GFAP and TNF-alpha genes were as shown in **(Table 4.6).**

Table 4.6RT-PCR Cycling Condition of HSB1 and GFAP

Initial Denaturation	95	2 mins	
Denaturation	95	15 sec	40 cycles
Annealing	55	15 sec	
Extension	72	30 sec	
Final Extension	72	5 mins	

RT-PCR Cycling Condition of FOS gene: PCR cycling condition for of VEGF, HSB1 and FOS genes were as shown in **(Table 4.7).**

Table 4.7 RT-PCR Cycling Condition of FOS gene

Initial Denaturation	95	2 mins	
Denaturation	95	15 sec	40 cycles
Annealing	61	15 sec	
Extension	72	30 sec	
Final Extension	72	5 mins	

Data Analysis

Data were analyzed, with GAPDH as reference gene, using Realplex 2.2 software (Eppendorf). Threshold cycle (CT/Ct) and melting curve (Tm) values were determined by software. The foldchangein gene expression was calculated by Livak Method (2-ΔΔCT) (Livak KJ and Schmittgen TD.,2001) and was normalised to housekeeping gene, GAPDH. Subsequently, the relative geneexpression levels were calculated in reference to the control. This method assumes that both targetand reference genes are amplified with efficiencies near 100% and within 5% of each other.

Fold change calculation: First, the CT of the target gene was normalised to that of the reference:

(ref) gene, for both the test sample and the calibrator sample as follows:

$$\Delta CT \text{ (test)} = CT \text{ (target, test)} - CT \text{ (ref, test)}$$

$$\Delta CT \text{ (calibrator)} = CT \text{ (target, calibrator)} - CT \text{ (ref, calibrator)}$$

Second, the ΔCT of the test sample was normalised to the ΔCT of the calibrator, as follows:

$$\Delta\Delta CT = \Delta CT(\text{test}) - \Delta CT(\text{calibrator})$$

Finally, the expression ratio was calculated as follows:

$$2^{-\Delta\Delta CT} = \text{Normalized expression ratio}$$

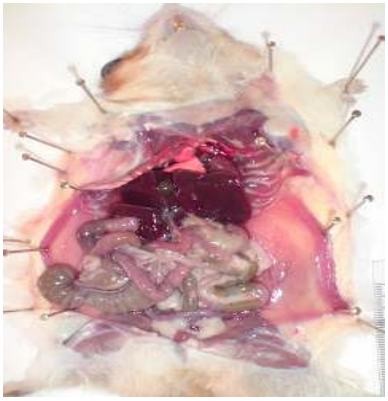
4.5 HISTOPATHOLOGY

Tissue of brain, heart, kidney, lung, liver, muscle and skin from both experimental C2 group and control C group of guinea pigs were dissected, taken out, immediately fixed in Bouin's solution and embed in paraffin. Tissues were sectioned at 5µ, processed and stained in Haematoxilin and Eosin for histopathological study. The following are the steps used for the guinea pig histopathological study.

4.5.1 DISSECTION OF GUINEA PIG, *CAVIA PORCELLUS*

Common anesthesia was used for the guinea pigs. Two different groups of guinea pigs were anesthetized. Measured doses of ketamine and xylazine 20mg/Kg, 2mg/Kg, according to the weight of guinea pigs were injected intramuscularly. The animals were placed on a woodboard, as the dissections were carried out. The organs were then placed in 10% Bouin's solution (**Figure 4.8)**.

Figure 4.9: After 24 hours fixating in Bouins fluid 10%, formaldehyde with 4% picric acid



4.5.2 HISTOPATHOLOGY OF GUINEA PIG EXPOSURE GROUP (C2) AND CONTROL GROUPS (C)

The organs were dissected out of the guinea pigs from experimental group C2 and control group C and fixed in Bouin's solution. After fixation for 24 hours, the organs were processed for the histopathological study in Billroth Hospital, Aminjikarai, Chennai, Tamil Nadu, South India. The following procedure and chemical reagents were used.

4.5.3 FIXATION

The tissue samples were received in Bouin's solution with 10 % formalin, fixed for 24 hours. From the samples, selective bits were chosen from the experimental and control specimens. The selected bits were placed in the labeled capsules and then put in room temperature for a few minutes. The experimental tissue samples were transferred to cleaned bottles with chemicals and were kept in hot air oven.

REQUIRED CHEMICAL REAGENTS

The following chemicals were used for the histopathological study and kept in hot air oven for 30 minutes before using the samples.

- Isopropyl alcohol - I
- Acetone - I
- Acetone - II
- Xylene - I
- Xylene – II
- Dewax with xylene I & II
- Hematoxylin& Eosin
- 1% Acid with alcohol
- Alcohol
- HCl - Hydro chloric Acid
- Ammonia water
- Liquid ammonia

4.5.4 SECTIONING

After the completion of the hot air oven process, all the capsules were taken out of processing bottle and dried to free them from xylene. After the drying process, the dry capsules were put in liquid paraffin wax for 1 hour.

Embedding

After the completion of one hour of processing in the liquid paraffin wax, the samples were embedded and labeled on the embedded paraffin wax. The embedded tissue was made into a paraffin block. Each and every tissue samples were labeled clearly. For cutting the embedded tissue samples, a microtome was used and the block was cut to a thickness of 4-5µ sections. This was followed by staining of the samples. The embedded block was incubated for the removal of unwanted wax. It was incubated at 65˚C temperature for 30 minutes. The embedded wax is shown in (**Figure 4.9).**

Figure 4.10: Embedded wax for staining

4.5.5 STAINING (HAEMATOXYLIN & EOSIN)

Required materials and chemicals

- Dewax with xylene I & II
- Isopropyl alcohol
- Distilled water
- Haematoxylin& Eosin
- Acid alcohol
- Concentrated HCL
- Ammonia water
- Liquid ammonia

The Haematoxylin and Eosin staining were carried out in the following steps: Dewax with xylene I &II were processed for 5 minutes. This process continued for all the slides for 5 minutes each. After 10 minutes in isopropyl alcohol, it was washed in distilled water for 1 minute. The slides were processed in Haematoxylin stain for 5 minutes. At the end of the process, it was washed in distilled water for 1 minute. Then with 1% acid alcohol (99ml alcohol in 1 ml acid), it was washed for 1 minute. 30ml of distilled water was taken and the slides were washed thoroughly.

This was repeated and washed in water mixed with HCl for 2 minutes. Finally, washed in ammonia water (liquid ammonia) for 1 minute and 3 drops Eosin was added and left for 1 minute. At the end of the process, it was washed once in water and dried in room temperature. The sample was then mounted (**Figure 4.10)**.

Figure: 4.11 Stained slides of guinea pig's organs from group C2 and group C