CHAPTER - II

MATERIAL AND METHOD

MATERIAL AND METHODS

II.1 Experimental animal:

The Chicken (*Gallus gallus*, sometimes *G. gallus domesticus*) is a domesticated fowl likely descended from the wild Indian and southeast Asian Red Junglefowl (*Gallus gallus*) and the related Grey Junglefowl (*G. sonneratii*). *Gallus gallus murghi* is subspecies derived from the Red Junglefowl of India. *Gallus gallus murghi* which has high immune power and is sustainable to the village envirement, along with its high quality flesh and egg laying capacity. For this purpose various hybrid strains were produced. Giriraj is one of the hybrid strain of *Gallus gallus murghi*.

Classification:

Scientific name: *Gallus gallus*

Common name: murghi, Domestic chicken

1.Kingdom : Animalia

2. Phylum : Chordata

3.Class : Aves

4. Order : Galliformes

5. Family : Phasianidae

6. Genus : *Gallus*

7. Species : *G. gallus*

Special features of Giriraj strain-

i) Available with variuos attractive feathers.

ii) Long life span

iii) Large sized light brown eggs

iv) High immune power

v) High hatchabilty rate

II.1..1 Selected developmental stages:

Selected hours to study angiogenesis were 48, 55, 66, 72, 88, and 96 hrs. These hours are according to the development of CAM and vitelline veins of CAM. There fore doses were initiated at hours explained above and development was

continued for 144 hrs .The reasons for the same are given in Chapter I Introduction Freshly fertilized Gallus chick eggs of Girriraj strain were obtained from the government hatchery (Assistant commissioner of animal husbandry central hatchery kolhapur).

II.1..2 Chemicals used:

II.1..2.A Hanks Balanced Salt Solution (HBSS): HBSS Purchased from HiMedia Laboratories Pvt. Ltd. 23 Vadhani ind. Est., LBS Marg, Mumbai-4000 086, India.

II.1..2.B Hydrogen peroxide:

At designed hrs of incubation H_2O_2 doses 0.05 m M, 0.5 m M, 1 m M and 1.5 m M were administered by window method in aseptic condition for the mortality study. The dose of 0.5 m M showed 50 % mortality which was further used to study effect of vitamin C. Hydrogen peroxide was purchased from local medical shop. The dose used for the study are is 0.5 m M ; reasons for the same are given in Chapter I Introduction.

II.1..2.C Vitamin C:

Three mg/egg dose of vitamin C was selected which had shown the highest hatchability in normal chick (Ipek *et al.*, 2004). Different doses of vitamin C viz. 3, 4 and 5 mg vitamin C/egg were used to test the free radical management potency against H_2O_2 damage induced eggs. Vitamin C used was purchased from S.d. fine chem Ltd. Mumbai. Vitamin C dose (3 mg) was used for reasons given in Chapter I introduction .

II.1..3 Experimental protocol:

Experimental work was carried out in three steps as follows

Step I : Incubation of eggs

The shells of fertilized eggs were disinfected with 70% alcohol. The eggs were divided in six groups. 48 hrs 55 hrs, 66hrs, 72 hrs, 88 hrs, 96 hrs. the eggs were incubated in an aseptic incubator in vertical position such that the blunt end of egg always faced upward and was maintained at 37. 5 0 c temperature and relative humidity at 70 to 75 % The incubation was conducted to obtained embryos of 48 hrs

55hrs 66hrs 72 hrs 88 hrs and 96 hrs . These embryos were used further to continue experiments.

Step II : Dose administration

The embryos obtained from step I at different hours of incubation were used further for administration of dose . At different developmental stages . (48 hrs 55 hrs , 66hrs, 72 hrs ,88 hrs , 96 hrs) . dose was initiated (as described above) and development was continued up to 144 hrs . Embryos of each group were administered with different doses of H2O2 and vitamin C accordingly they were numbered as(I,II,III,IV,V,VI,VII,VIII,IX,X,XI,) . Normal and control groups were maintained independently with each of the experimental group studied.

Method for dose administration (Window method):

After designed period of incubation (described above) the eggs were cleaned with 70% alcohol. A small window was made at the blunt end of each of the egg, under aseptic conditions and H_2O_2 and H_2O_2+ Vitamin C doses were injected in final volume of 1 ml Hanks Balanced Salt Solution . Different concentrations of vitamin C were adjusted during experimental work.. Here the precaution was taken that the 1 ml dose was spread on the embryonic plate uniformly at different stages of development as mentioned in the groups I-XI . All the treatments were given in final volume of HBSS. as 1 ml with retaining pH and HBSS composition. The windows were sealed with sterilized adhesive tape. All embryo exposures were conducted in proper sterilized conditions prescribed by window method (Korn and Cramer, 2007).

Step III:

Hydrogen peroxide treatment was initiated as a single dose at different hrs of development (groups I to XI) and the embryos were observed for mortality in the following interval of development as shown in Table 1 and Table 2. Total weights and survival on hatchability and abnormalities were noted if any. Eggs were opened in plate having 0.9% buffered NaCl. Saline to view the chorioallontoic membrane for

study. Homogenates were prepared in chilled distilled water and were used to conduc the biochemical assays.

Table 1 Exposure time H_2O_2 and Vitamin C to different development stages of chick embryos in hrs .

Groups according to development stages	Groups according to time of exposure to the treatment						Final development in hrs
	A	B	C	D	E	F	
Initiation of treatment in hrs							
I	48						
II		55					
III			66				144Hrs
IV				72			
V					88		
VI						96	

Table 2. Exposure Schedule of H2O2 and Vitamin C at different development stages of chick embryo in hrs.

Groups according to developmental stages in hrs	Groups according to time of exposure to treatment						Final development in hrs
I	48	56	72	78	89	96	
II 48	-	-	-	-	-	✓	
III 55	-	-	-	-	✓	-	144 HRS
IV 66	-	-	-	✓	-	-	
V 72	-	-	✓	-	-	-	
VI 88	-	✓	-	-	-	-	
VII 96	✓	--	-	-	-	-	

Eggs incubated at 37^0 C for 48 hrs were administered with following doses and the incubation was carried further for 144hrs.

Group I : Normal – Eggs were incubated for 144 hrs without any treatment.

Group II : HBSS control- At48, hrs of incubation embryos were administered with 1 ml HBSS and development was continued up to 144 hrs .

Group III : 0. 05 H_2O_2 - Treatment of 0.05 m M H_2O_2 was initiated at 48 hrs of incubation and incubation was continued up to 144 hrs .

Group IV : 0.5 m M H_2O_2 .Treatment of 0.5 m M H2O2 was initiated, at 48 hrs Incubation and continued up to 144hr.

Group V : 1mM H_2O_2 . At 48 of incubation 1 m M H_2O_2 was initiated and continued up to 144hrs.

Group V 1.5mM H_2O_2 . Treatment of 1.5 m M H_2O_2 was initiated at 48, hrs of Incubation and continued up to 144hrs..

Group VI : 0.5 m M H_2O_2 + 3 mg Vitamin C- Treatment of Vitamin C3mg and 0.5 H_2O_2 was initiated at 48,hrs Of Incubation and continued up to 144hrs of incubation

Group VII : 0.5 m M H_2O_2 + 4 mg Vitamin C- Treatment of Vitamin C 4 mg and 0.5 H_2O_2 was initiated at 48,hrs Of Incubation and continued up to 144hrs of incubation

Group VIII : 0.5 m M H_2O_{2+} 5 mg Vitamin C-

Treatment of 5 mg Vitamin C + 0.5 H_2O_2 mM was initiated at 48, hrs Of Incubation and continued up to 144hrs.

Group IX : 3 mg Vitamin C- Treatment of Vitamin C 3 mg was initiated at 48, hrs of Incubation and continued up to 144hrs

Group X : 4 mg Vitamin C- Treatment of Vitamin C 4 mg was initiated at 48, hrs of Incubation and continued up to 144hrs

Group XI : 5 mg Vitamin C Treatment of Vitamin C 5mg was initiated at 48, hrs of Incubation and continued up to 144 hrs.

Eggs incubated at 37^0 C for 55 hrs were administered with following doses and the incubation was carried further for 144hrs.

Group I : Normal – Eggs were incubated for 144 hrs without any treatment.

Group II : HBSS control- At 55, hrs of incubation embryos were administered with 1 ml HBSS and development was continued up to 144 hrs .

Group III : 0. 05 m M H_2O_2 - Treatment of 0.05 mM H_2O_2 was initiated at 55hrs of and incubation was continued up to 144 hrs .

Group IV : 0.5 m M H_2O_2 . Treatment of 0.5 mM H2O2 was initiated, at 55 hrs Incubation and continued up to 144 hrs.

Group V : 1 m M H_2O_2 At 55 hrs of incubation 1mM H_2O_2 was initiated and continued up to 144 hrs.

Group V :1.5 m M H_2O_2 . Treatment of 1.5 mM H_2O_2 was initiated at t55, hrs of Incubation and continued up to 144 hrs..

Group VI : 0.5 m M H_2O_2 + 3 mg Vitamin C - Treatment of Vitamin C3mg and 0.5 m M H_2O_2 was initiated at 55,hrs Of Incubation and continued up to 144 hrs.

Group VII : 0.5 m M H_2O_2 + 4 mg Vitamin C - Treatment of Vitamin C 4mg and 0.5 m M H_2O_2 was initiated at 55,hrs Of Incubation and continued up to 144 hrs.

Group VIII : 0.5 m M H_2O_{2+} 5 mg Vitamin C - Treatment of 5mg Vitamin+0.5$H_2O_2$was initiated at 55, hrs Of Incubation and continued up to 144 hrs.

Group IX : 3 mg Vitamin C - Treatment of Vitamin C3mg was initiated at 55, hrs of Incubation and continued up to 144 hrs

Group X : 4 mg Vitamin C - Treatment of Vitamin C 4mg was initiated at 55, hrs of Incubation and continued up to 144 hrs

Group XI: 5 mg Vitamin C - Treatment of 5mg Vitamin C was initiated at t48, hrs of Incubation and was continued up to 144hr

.

Eggs incubated at 37^0 C for 66 hrs were administered with following doses and the incubation was carried further for 144 hrs.

Group I : Normal – Eggs were incubated for 144 hrs without any treatment.

Group II : HBSS control- At 66, hrs of incubation embryos were administered with 1 ml HBSS and development was continued up to 144 hrs .

Group III : 0. 05 m M H_2O_2 - Treatment of 0.05mM H_2O_2 was initiated at 66hrs of incubation and incubation was continued up to 144 hrs .

Group IV : 0.5 m M H_2O_2 Treatment of 0.5 mMH2O2 was initiated, at 48 hrs Incubation and continued up to 144 hrs.

Group V : 1m M H_2O_2 At 66hrs of incubation 1mM H_2O_2 was initiated and continued up to 144 hrs.

Group V 1.5 m M : H_2O_2 Treatment of 1.5m M H_2O_2 was initiated at 66, hrs of Incubation and continued up to 144 hrs..

Group VI : 0.5 m M H_2O_2 + 3mg Vitamin C Treatment of Vitamin C3mg and 0.5 H_2O_2 was initiated at 66,hrs Of Incubation and continued up to 144hrs .

Group VII : 0.5 m M H_2O_2 + 4mg Vitamin C Treatment of Vitamin C 4mg and 0.5 H_2O_2 was initiated at 66,hrs Of Incubation and continued up to 144hrs .

Group VIII : 0.5 m M H_2O_{2+} 5mg Vitamin C- Treatment of 5mg Vitamin C and 0.5 H_2O_2 m M was initiated at 66, hrs of Incubation and was continued up to 144 hrs.

Group IX : 3mg Vitamin C- Treatment of Vitamin C 3mg was initiated at 66, hrs of Incubation and continued up to 144 hrs

Group X : 4 mg Vitamin C- Treatment of Vitamin C 4mg was initiated at 66, hrs of Incubation and continued up to 144 hrs

Group XI : 5mg Vitamin C - Treatment of 5mg Vitamin C was initiated at 66, hrs of Incubation and was continued up to 144 hrs.

Eggs incubated at 37^0 C for 72 hrs were administered with following doses and the incubation was carried further for 144hrs.

Group I :: Normal – Eggs were incubated for 144 hrs without any treatment.

Group II : HBSS control- At 72, hrs of incubation embryos were administered with 1 ml HBSS and development was continued up to 144 hrs .

Group III : : 0. 05 m M H_2O_2 - Treatment of 0.05 mM H_2O_2 was initiated at 72 hrs of and incubation was continued up to 144 hrs of incubation.

Group IV :; 0.5 m M H_2O_2 . Treatment of 0.5 mM H2O2 was initiated, at 72 hrs Incubation and continued up to 144 hrs.

Group V : 1 m M H_2O_2 At 72, hrs of incubation 1mM H_2O_2 was initiated and continued up to 144 hrs.

Group VI :1.5 m M H_2O_2 . Treatment of 1.5 mM H_2O_2 was initiated at 55, hrs of incubation

Incubation and continued up to 144 hrs..

Group VI : 0.5 m M H_2O_2 + 3 mg Vitamin C - Treatment of 3mg Vitamin C+ 0.5 m M H_2O_2 was initiated at 72,hrs Of Incubation and continued up to 144 hrs.

Group VII : 0.5 m M H_2O_2 + 4 mg Vitamin C - Treatment of 4 mg Vitamin C+ 0.5 m M H_2O_2 was initiated at 72,hrs Of Incubation and continued up to 144 hrs.

Group VIII : 0.5 m M H_2O_{2+} 5 mg Vitamin C - Treatment of 5mg Vitamin C + $0.5H_2O_2$ mM was initiated at 72, hrs Of Incubation and continued up to 144 hrs.

Group IX : 3mg Vitamin C - Treatment of 3mg Vitamin C was initiated at 72, hrs of Incubation and continued up to 144 hrs

Group X : 4mg Vitamin C - Treatment of 4 mg Vitamin C was initiated at 72, hrs of Incubation and continued up to 144 hrs

Group XI : 5mg Vitamin C - Treatment of 5mg Vitamin C was initiated at 72 , hrs of Incubation and continued up to 144 hrs

Eggs incubated at 37^0 C for 88, hrs were administered with following doses and the incubation was carried further for 144hrs.

Group I : Normal – Eggs were incubated for 144 hrs without any treatment.

Group II : HBSS control- At 88, hrs of incubation embryos were administered with 1 ml HBSS and development was continued up to 144 hrs .

Group III : 0. 05 m M H_2O_2 - Treatment of 0.05mM H_2O_2 was initiated at 88,hrs of incubation and incubation was continued up to 144 hrs .

Group IV : 0.5 m M H_2O_2. Treatment of 0.5 mMH2O2 was initiated, at 88, hrs Incubation and continued up to 144 hrs.

Group V : 1 m M H_2O_2 At 88,hrs of incubation 1mM H_2O_2 was initiated and continued up to 144 hrs.

Group V : 1.5 m M H_2O_2 Treatment of 1.5m M H_2O_2 was initiated at 88, hrs of incubation

Incubation and continued up to 144 hrs..

Group VI : 0.5 m M H_2O_2 + 3mg Vitamin C -Treatment of 3 mg Vitamin C and 0.5 H_2O_2 was initiated at 88,hrs Of Incubation and continued up to 144 hrs of incubation.

Group VII : 0.5 m M H_2O_2 + 4 mg Vitamin C -Treatment of 4 mg Vitamin C and 0.5 H_2O_2 was initiated at 88,hrs Of Incubation and continued up to 144 hrs of incubation

Group VIII : 0.5 mM H_2O_2+ 5mg Vitamin C - Treatment of 5mg Vitamin C and 0.5 H_2O_2 m M was initiated at 88, hrs Of Incubation and was continued up to 144 hrs.

Group IX : 3mg Vitamin C - Treatment of Vitamin C 3 mg was initiated at 88, hrs of Incubation and continued up to 144 hrs .

Group IX : 4 mg Vitamin C - Treatment of Vitamin C 4 mg was initiated at 88, hrs of Incubation and continued up to 144 hrs

Group XI : 5mg Vitamin C - Treatment of 5mg Vitamin C was initiated at 88, hrs of Incubation and was continued up to 144 hrs

Eggs incubated at 37^0 C for 96, hrs were administered with following doses and the incubation was carried further for 144hrs.

Group I : Normal – Eggs were incubated for 144 hrs without any treatment.

Group II : HBSS control- At 96, hrs of incubation embryos were administered with 1 ml HBSS and development was continued up to 144 hrs .

Group III : 0. 05 m M H_2O_2 - Treatment of 0.05m M H_2O_2 was initiated at 96, hrs of and incubation was continued up to 144 hrs .

Group IV : 0.5 m M H_2O_2 . Treatment of 0.5m MH2O2 was initiated, at 96, hrs Incubation and continued up to 144 hrs.

Group V : 1 m M H_2O_2 At 96, hrs of incubation 1m MH_2O_2 was initiated and continued up to 144hrs.

Group V 1.5 m M H_2O_2 . Treatment of 1.5 m M H_2O_2 was initiated at 96, hrs of incubation and continued up to 144 hrs

Group VI : 0.5 m M H_2O_2 + 3mg Vitamin C - Treatment of 3mg Vitamin C+ 0.5 m M H_2O_2 was initiated at 96,hrs Of Incubation and continued up to 144hrs.

Group VII : 0.5 m M H_2O_2 + 4 mg Vitamin C - Treatment of 4 mg Vitamin C+ 0.5 m M H_2O_2 was initiated at 96,hrs Of Incubation and continued up to 144hrs

Group VIII : 0.5 m M H_2O_2+ 5mg Vitamin C - Treatment of 5mg Vitamin C $0.5H_2O_2$was initiated at 96, hrs Of Incubation and continued up to 144hrs.

Group IX : 3mg Vitamin C - Treatment of 3mg Vitamin C was initiated at 96, hr

Group X : 4 mg Vitamin C - Treatment of 4 mg Vitamin C was initiated at 96, hrs of Incubation and continued up to 144 hrs

Group XI : 5mg Vitamin C - Treatment of 5mg Vitamin C was initiated at 96, hrs of Incubation and continued up to 144 hrs.

Study of Angiogenesis:

Angiogenesis of the CAM was studied. For the quantification of the angiogenesis of CAM the embryos were first opened and floated in the PBS containing glycerin. The embryos with the intact CAM were transferred to the bottom of the inverted glass Petri dish with the help of the brush. Folds were removed by PBS containing glycerin and thus CAM was fully spread for observation.

Diameter of CAM was measured in 6 different planes using divider and mean was noted. From diameter area was deduced mathematically. Results were confirmed by graph transparencies.

Stereoscopic microscope was used to count the Primary, Secondary and Tertiary Vitelline veins For measurement of area covered by different veins bifurcation points were used as initiation and termination markers. Area was measured on microscope and was confirmed by using graph transparencies.

The alterations are presented in Table 2. (7- 24).

II.1...4 Bioassays:

II.1..4. A <u>Total Proteins:-</u>

Total proteins were estimated using Folin-Ciocalteu-Phenol reagent (Lowry *et al*, 1951).

Preparation of Chemicals:-

i) Lowry's A – 25sodium carbonate in 0.1n NaOH.

ii) Lowry's B – 0.5% Copper Sulfate in 1% Sodium tartarate

iii) Lowry's C – 50 ml Lowry's A+ 1 ml Lowry's B. Prepare fresh just prior to use.

iv) Folin-Ciocalteu-Phenol reagent:-

Sodium tungstate ($Na_2WO_4.2H_2O$)	100 gms
Sodium molybdate ($Na_2MO_4.2H_2O$	25 gms
Distilled water	700 ml
Conc. HCL	100 ml
Phosphoric acid (85%)	50 ml

Reflux the above mixture for 10 hours in glass apparatus.

Then add to this mixture.

Lithium Sulphate	150 gms
Distilled water	50 ml

Add 5 drops of bromine water and boil this to remove excess bromine water.

Dilute to 1N and use.

Assay of protein was conducted as follows .

Assay of Protein:-	Blank	Sample
Distilled water	1.5 ml	1.4 ml
Homogenate	0.0 ml	0.1 ml
Lowry's C	3.0 ml	3.0 ml
	Wait for 15 minutes	
Folin-Ciocalteu-Phenol reagent		
	0.5 ml	0.5 ml

wait for 60 minutes. Then take reading at 660 nm.

Calculation:- Optical density was converted into protein value from the standard graph plotted as optical density versus concentration of protein. Calculate the amount of protein per gm tissue.

II.1..4.B <u>Lipid Peroxidation(LPO):</u>

The assay of lipid peroxidation was performed by method described by Buege and Aust (1978).

Chemicals:-

Stock TCA-TBA-HCL Reagent

15% w/v TCA

0.375% w/v TBA

0.25 N Hydrochloric Acid (HCl)

This solution may need mildly heating to assist in the dissolution of TBA.

Bioassay of Lipid Peroxidation(LPO):

Lipid Peroxidation was conducted in different tubes as follows .

	Blank	Sample
Distilled water	2.0 ml	0.0 ml
Homogenate	0.0 ml	2.0 ml
TCA-TBA-HCL Reagent	4.0 ml	4.0 ml

The tubes were heated for 15 minutes in a water bath at 80°C. The tubes were cooled under tap water and were centrifuged at 1000 ×g for 10 minutes. The supernatants were taken out and optical density was measured at 535 nm against blank.

Calculation:- Concentration of Malonaldehyde is calculated using an extinction coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$

II.1..4.C <u>Glutathione:-</u>

Biochemical assay for glutathione was performed by to method proposed by Grunnert and Phillips (1951).

Chemicals:-

1) 0.067 M - Sodium Nitroprusside

2) 0.067 M – Sodium Cynide in 1.5 M Na_2CO_3 (Sodium Carbonate)

3) Saturated Sodium Chloride (NaCl)

Bioassay for Glutathione:-

Bioassay was conducted in different tubes as follows .

	Sample	Blank
Distilled Water	0.0 ml	1. 0 ml
Homogenate	1.0 ml	0.0 ml
Sodium Nitroprusside	1.0 ml	1.0 ml
Sodium Cyanide	0.5 ml	0.5 ml
Sodium Chloride	3.0 ml	3.0 ml

All the tubes were shake for uniform solution and optical density were measured at 520 nm against blank.Glutathione content was calculated from standard graph .

II.1..4.D Formaldehyde: (HCHO)

The assay of formaldehyde were performed by method described by Werringloer (1978).

i) Protein precipitation: Trichloroacetic acid, 12.5%, barium hydroxide, 0.3 N saturated

,

ii) Nash reagent (final concentration in parenthesis): 12.5g of ammonium acetate (6 M), 1.5 ml acetyle acetone (60 mM) and 2.25 ml of acetic acid (0.15 M) are dissolved in distilled water to give final volume of 250 ml with a pH of about 6.7.

Bioassay of formaldehyde: It was conducted as follows

	Blank	Sample
Homogenizing buffer	1.0 ml	0.0 ml
Microsomes	0.0 ml	1.0 ml

12.5% TCA	1.5 ml	1.5 ml
centrifuge at 40,000 x g for 10 minutes		
Supernatant	2.0 ml	2.0 ml
Nash reagent	1.0 ml	1.0 ml

Calculations: Formaldehyde concentrations were determined using molar extinction coefficient 7950 X^{-1} cm^{-1}.

II.1..5 Statistical analysis:

The results were the mean values of 6 embryos in each experimental conditions. The statistical calculations were carried out with the help of XLSTAT 7.5 computer programme.

Significance- The probability *p* obtained 't' and 'Z' values at least as calculated for a given number of degree of freedom is given in Fischer's table (Fischer, 1938). The *p* values are signified according to the following conventions.

$P > 0.05$ the difference is said to insignificant

$P < 0.05$ the difference is said to be almost significant

$P < 0.02$ the difference is said to be significant

$P < 0.001$ the difference is said to be highly significant