CHAPTER II

MATERIAL AND METHODS

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- The histochemical analysis of proteoglycans and glycans is carried out using 33 different histochemical techniques which are necessary for coming to the relevant confirmatory conclusion.
- But it is not possible to provide every detailed procedure staining technique. Therefore a tabularized data of technique, their inferences and detail reference has been provided.

MATERIAL AND METHODS

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

Kingdom : Animalia
Phylum : Chordata
Class : Aves
Order : Galliformes
Family : Phasianidae
Genus : *Gallus*
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

Selected developmental stages:

Early development of brain is important for the animal/human development therefore doses were initiated at 24 hrs, 34 hrs, 40 hrs, 48 hrs , 72 hrs, 96 hrs and 120 hrs of development and further continuation at the same interval were used to show specified stages of embryo that explains state of brain development. Freshly fertilized

Gallus chick eggs of Giriraj strain were obtained from the government hatchery (Assistant Commissioner of Animal Husbandry Central Hatchery, Kolhapur).

Chemicals used:

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

Hydrogen peroxide:

At designed hrs of incubation H_2O_2 doses 0.05 mM, 0.5 mM, 1 mM and 1.5 mM were administered by window method in aseptic condition for the mortality study. The dose of 0.5 mM showed 50 % mortality which was further used to study effect of vitamin C. Hydrogen peroxide was purchased from local medical shop.

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.

Experimental protocols:

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 to seven different groups; I (24 hrs), II (34 hrs), III (40 hrs), IV (48 hrs), V (72 hrs), VI (96 hrs) and VII (120 hrs). The eggs were incubated in an aseptic egg incubator in vertical position such that the blunt end of egg always faced upward and was maintained at 37.5°C temperature and relative humidity 70-75%. The incubation was conducted to obtain embryos of 24 hrs, 34 hrs, 40 hrs, 48 hrs, 72 hrs, 96 hrs. and 120 hrs. These embryos were used further to continue the experiments.

Step II- Dose administration –

The embryos obtained from the step I at different hrs of incubation were used further for administration of dose. Group I, II and III each contains four subgroups (A, B, C and D) which are according to exposure period to different doses i.e. 24, 48, 72 and 96 hrs. Group IV and V contains three (A, B and C) and two (A and B) subgroups respectively i.e. exposed to 24, 48 and 72 hrs and 24 and 48 hrs respectively. Remaining two groups i.e. VI and VII contains only one subgroup, A which was

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given exposure of 24 hrs. In each of group six embryos were maintained. Embryos of each subgroup were administered with different doses of H_2O_2 and vitamin C accordingly, they were numbered (i, ii, iii, iv, v, vi, vii, viii, ix, x, xi and xii). Normal and control groups were maintained independently with each of the experimental groups studied.

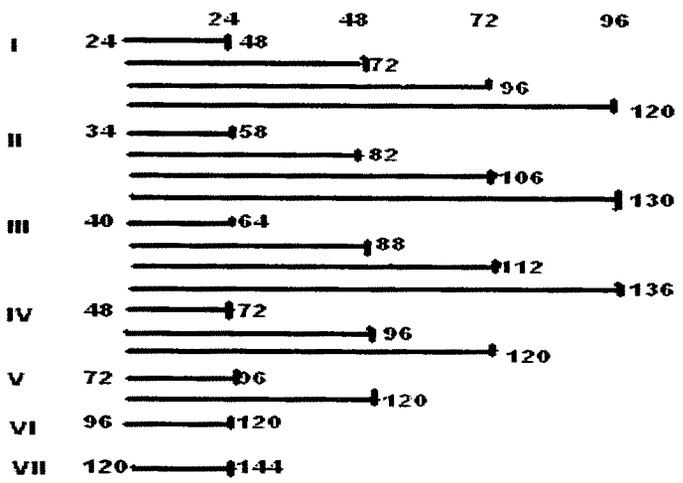
Method for dose administration (Window method):

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 Vitamin C doses were injected in final volume of Hanks Balanced Salt Solution of 1 ml. Different concentrations of vitamin C were adjusted during experimental work.. Here the precaution was taken that the 1 ml dose is spread on the embryonic plate uniformly at different stages of development as mentioned in the I-VII groups. 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 (group I to VII) 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. Embryos from each group were dissected, brain was removed quickly, weighed and chilled in ice cold 0.9% NaCl. Homogenates were prepared in chilled distilled water and were used to conduct the biochemical assays. From 72 hrs of development onward the brain can be distinguished therefore, therefore bioassays of earlier stages were performed with whole embryo. Experiments were performed with running two groups with 6 embryos in each group.

Table 1: Exposure time to H_2O_2 and vitamin C to different developmental stages of chick embryo (in hrs).

Groups According to Developmental stages (in hrs)	Groups according to Time of exposure to the Treatment (in hrs)				
	A	B	C	D	
Initiation of treatment (in hrs)					Final development (in hrs)

Group I, II and III each contains 4 sub groups(A, B, C and D) = 12 groups

Group IV contain 3 subgroups (A, B and C) groups = 3

Group V contains 2 sub groups (A and B) groups = 2

Group VI and VII contains 1 sub group (A) groups = 2

Total groups 19

Table 2: Time of exposure to H_2O_2 and vitamin C initiated at different developmental stages of chick embryo (in hrs).

Groups According to Developmental stages (in hrs)			Groups according to Time of exposure to the Treatment (in hrs)			
			A	B	C	D
			24	48	72	96
I	24	24+	✓	✓	✓	✓
II	34	34+	✓	✓	✓	✓
III	40	40+	✓	✓	✓	✓
IV	48	48+	✓	✓	✓	-
V	72	72+	✓	✓	-	-
VI	96	96+	✓	-	-	-
VII	120	120+	✓	-	-	-

Group I A: Eggs incubated at 37°C for 24 hrs (group I) and administered with following doses and the incubation was carried further for 24 hrs (group A).

Group-i): **Normal:** Eggs incubated for 24 hrs without any treatment and the incubation was carried further for 24 hrs (Group A).

Group-ii): **HBSS Control:** At 24 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 24 hrs .

Group-iii) **0.05 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 24 hrs.

Group -iv) **0.5 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -v) **1.0 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vi) **1.5 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -x) **3 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -xi) **4 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-xii) **5 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group I B: Eggs incubated at 37°C for 24 hrs (group I) and administered with following doses and the incubation was carried further for 48 hrs (group B).

Group-i): **Normal:** Eggs incubated for 24 hrs without any treatment and the incubation was carried further for 48 hrs.

Group-ii): **HBSS Control:** At 24 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 48 hrs.

Group-iii) **0.05 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 48 hrs.

Group -iv) **0.5 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -v) **1.0 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vi) **1.5 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 24 hrs of incubation,embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs (group A).

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -x) **3 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -xi) **4 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-xii) **5 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group IC: **Eggs incubated at 37°C for 24 hrs (group I) and administered with following doses and the incubation was carried further for 72 hrs (group C).**

Group-i): **Normal:** Eggs incubated for 24 hrs without any treatment and the incubation was carried further for 72 hrs.

Group-ii): **HBSS Control:** At 24 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 72 hrs.

Group-iii) **0.05 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 72 hrs.

Group -iv) **0.5 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -v) **1.0 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -vi) **1.5 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group -x) **3 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group -xi) **4 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-xii) **5 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group I D: **Eggs incubated at 37°C for 24 hrs (group I) and administered with following doses and the incubation was carried further for 96 hrs (group D).**

Group-i): **Normal:** Eggs incubated for 24 hrs without any treatment and the incubation was carried further for 96 hrs.

Group-ii): **HBSS Control:** At 24 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 96 hrs.

Group-iii) **0.05 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 96 hrs.

Group -iv) **0.5 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 96 hrs.

Group -v) **1.0 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 96 hrs.

Group -vi) **1.5 mM H_2O_2:** At 24 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 96 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and d development was continued for 96 hrs.

Group -x) **3 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group -xi) **4 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group-xii) **5 mg Vitamin C:** At 24 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group II A: **Eggs incubated at 37°C for 34 hrs (group II) and administered with following doses and the incubation was carried further for 24 hrs (group A).**

Group-i): **Normal:** Eggs incubated for 34 hrs without any treatment and the incubation was carried further for 24 hrs.

Group-ii): **HBSS Control:** At 34 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 24 hrs.

Group-iii) **0.05 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 24 hrs.

Group -iv) **0.5 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -v) **1.0 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vi) **1.5 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -x) **3 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -xi) **4 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-xii) **5 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group II B: **Eggs incubated at 37°C for 34 hrs (group II) and administered with following doses and the incubation was carried further for 48 hrs (group B).**

Group-i): **Normal:** Eggs incubated for 34 hrs without any treatment and the incubation was carried further for 48 hrs.

Group-ii): **HBSS Control:** At 34 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 48 hrs.

Group-iii) **0.05 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 48 hrs.

Group -iv) **0.5 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -v) **1.0 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vi) **1.5 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -x) **3 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -xi) **4 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-xii) **5 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group II C: Eggs incubated at 37°C for 34 hrs (group II) and administered with following doses and the incubation was carried further for 72 hrs (group C).

Group-i): **Normal:** Eggs incubated for 34 hrs without any treatment and the incubation was carried further for 72 hrs.

Group-ii): **HBSS Control:** At 34 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 72 hrs.

Group-iii) **0.05 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 72 hrs.

Group -iv) **0.5 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -v) **1.0 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -vi) **1.5 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group -x) **3 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group -xi) 4 mg Vitamin C: At 34 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-xii) **5 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group II D: **Eggs incubated at 37°C for 34 hrs (group II) and administered with following doses and the incubation was carried further for 96 hrs (group D).**

Group-i): **Normal:** Eggs incubated for 34 hrs without any treatment and the incubation was carried further for 96 hrs.

Group-ii): **HBSS Control:** At 34 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 96 hrs.

Group-iii) **0.05 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 00.05 mM of H_2O_2/ml HBSS and development was continued for 96 hrs.

Group -iv) **0.5 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 96 hrs.

Group -v) **1.0 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 96 hrs.

Group -vi) **1.5 mM H_2O_2:** At 34 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 96 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group -x) **3 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group -xi) **4 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group-xii) **5 mg Vitamin C:** At 34 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group III A: Eggs incubated at 37°C for 40 hrs (group III) and administered with following doses and the incubation was carried further for 24 hrs (group A).

Group-i): **Normal:** Eggs incubated for 40 hrs without any treatment and the incubation was carried further for 24 hrs.

Group-ii): **HBSS Control:** At 40 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 24 hrs.

Group-iii) **0.05 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 24 hrs.

Group -iv) **0.5 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -v) **1.0 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vi) **1.5 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -x) **3 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -xi) **4 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-xii) **5 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group III B: Eggs incubated at 37°C for 40 hrs (group III) and administered with following doses and the incubation was carried further for 48 hrs (group B).

Group-i): **Normal:** Eggs incubated for 40 hrs without any treatment and the incubation was carried further for 48 hrs.

Group-ii): **HBSS Control:** At 40 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 48 hrs.

Group-iii) **0.05 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 48 hrs.

Group -iv) **0.5 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -v) **1.0 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vi) **1.5 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -x) **3 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -xi) **4 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-xii) **5 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group III C: Eggs incubated at 37°C for 40 hrs (group III) and administered with following doses and the incubation was carried further for 72 hrs (group C).

Group-i): **Normal:** Eggs incubated for 40 hrs without any treatment and the incubation was carried further for 72 hrs.

Group-ii): **HBSS Control:** At 40 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 72 hrs.

Group-iii) **0.05 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 72 hrs.

Group -iv) **0.5 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -v) **1.0 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -vi) **1.5 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group -x) **3 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group -xi) **4 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-xii) **5 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group III D: Eggs incubated at 37°C for 40 hrs (group III) and administered with following doses and the incubation was carried further for 96 hrs (group D).

Group-i): **Normal:** Eggs incubated for 40 hrs without any treatment and the incubation was carried further for 96.

Group-ii): **HBSS Control:** At 40 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 96 hrs.

Group-iii) **0.05 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 96 hrs.

Group -iv) **0.5 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 96 hrs.

Group -v) **1.0 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 96 hrs.

Group -vi) **1.5 mM H_2O_2:** At 40 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 96 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group -x) **3 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group -xi) **4 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group-xii) **5 mg Vitamin C:** At 40 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 96 hrs.

Group IV A: Eggs incubated at 37°C for 48 hrs (group IV) and administered with following doses and the incubation was carried further for 24 hrs (group A).

Group-i): **Normal:** Eggs incubated for 48 hrs without any treatment and the incubation was carried further for 24 hrs.

Group-ii): **HBSS Control:** At 48 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 24 hrs.

Group-iii) **0.05 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 24 hrs.

Group -iv) **0.5 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -v) **1.0 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vi) **1.5 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -x) **3 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -xi) **4 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-xii) **5 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group IV B: Eggs incubated at 37°C for 48 hrs (group IV) and administered with following doses and the incubation was carried further for 48 hrs (group B).

Group-i): **Normal:** Eggs incubated for 48 hrs without any treatment and the incubation was carried further for 48 hrs.

Group-ii): **HBSS Control:** At 40 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 48 hrs.

Group-iii) **0.05 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 48 hrs.

Group -iv) **0.5 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -v) **1.0 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vi) **1.5 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -x) **3 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -xi) **4 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-xii) **5 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group IV C: Eggs incubated at 37°C for 48 hrs (group IV) and administered with following doses and the incubation was carried further for 72 hrs (group C).

Group-i): **Normal:** Eggs incubated for 48 hrs without any treatment and the incubation was carried further for 72 hrs.

Group-ii): **HBSS Control:** At 48 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 72 hrs (group A).

Group-iii) **0.05 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 72 hrs.

Group -iv) **0.5 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -v) **1.0 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -vi) **1.5 mM H_2O_2:** At 48 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 72 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group -x) **3 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group -xi) **4 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group-xii) **5 mg Vitamin C:** At 48 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 72 hrs.

Group VA: **Eggs incubated at 37°C for 72 hrs (group V) and administered with following doses and the incubation was carried further for 24 hrs (group A).**

Group-i): **Normal:** Eggs incubated for 72 hrs without any treatment and the incubation was carried further for 24 hrs.

Group-ii): **HBSS Control:** At 72 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 24 hrs.

Group-iii) **0.05 mM H_2O_2:** At 72 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 24 hrs.

Group -iv) **0.5 mM H_2O_2:** At 72 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -v) **1.0 mM H_2O_2:** At 72 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vi) **1.5 mM H_2O_2:** At v hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 72 hrs of incubation, embryos ere administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -x) **3 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -xi) **4 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-xii) **5 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group IV B: **Eggs incubated at 37°C for 72 hrs (group V) and administered with following doses and the incubation was carried further for 48 hrs (group B).**

Group-i): **Normal:** Eggs incubated for 72 hrs without any treatment and the incubation was carried further for 48 hrs.

Group-ii): **HBSS Control:** At 72 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 48 hrs.

Group-iii) **0.05 mM H_2O_2:** At 72 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 48 hrs.

Group -iv) **0.5 mM H_2O_2:** At 72 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -v) **1.0 mM H_2O_2:** At 72 hrs of incubation, embryos was administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vi) **1.5 mM H_2O_2:** At 72 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 48 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -x) **3 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group -xi) **4 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group-xii) **5 mg Vitamin C:** At 72 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 48 hrs.

Group VI A: Eggs incubated at 37°C for 96 hrs (group VI) and administered with following doses and the incubation was carried further for 24 hrs (group A).

Group-i): **Normal:** Eggs incubated for 96 hrs without any treatment and the incubation was carried further for 24 hrs.

Group-ii): **HBSS Control:** At 96 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 24 hrs.

Group-iii) **0.05 mM H_2O_2:** At 96 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 24 hrs.

Group -iv) **0.5 mM H_2O_2:** At 96 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -v) **1.0 mM H_2O_2:** At 96 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vi) **1.5 mM H_2O_2:** At 96 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 96 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 96 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 96 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -x) **3 mg Vitamin C:** At 96 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -xi) **4 mg Vitamin C:** At 96 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-xii) **5 mg Vitamin C:** At 96 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group VII A: Eggs incubated at 37°C for 120 hrs (group VII) and administered with following doses and the incubation was carried further for 24 hrs (group A).

Group-i): **Normal:** Eggs incubated for 120 hrs without any treatment and the incubation was carried further for 24 hrs.

Group-ii): **HBSS Control:** At 120 hrs of incubation, embryos were administered with HBSS (1 ml) and development was continued for 24 hrs.

Group-iii) **0.05 mM H_2O_2:** At 120 hrs of incubation, embryos were administered with 0.05 mM of H_2O_2/ml HBSS and development was continued for 24 hrs.

Group -iv) **0.5 mM H_2O_2:** At 120 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -v) **1.0 mM H_2O_2:** At 120 hrs of incubation, embryos were administered with 1.0 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vi) **1.5 mM H_2O_2:** At 120 hrs of incubation, embryos were administered with 1.5 mM of H_2O_2 /ml HBSS and development was continued for 24 hrs.

Group -vii) **0.5 mM H_2O_2+ 3 mg Vitamin C:** At 120 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-viii) **0.5 mM H_2O_2+ 4 mg Vitamin C:** At 120 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 + 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-ix) **0.5 mM H_2O_2+ 5 mg Vitamin C:** At 120 hrs of incubation, embryos were administered with 0.5 mM of H_2O_2 +5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -x) **3 mg Vitamin C:** At 120 hrs of incubation, embryos were administered with 3 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group -xi) **4 mg Vitamin C:** At 120 hrs of incubation, embryos were administered with 4 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

Group-xii) 5 mg Vitamin C: At 120 hrs of incubation, embryos were administered with 5 mg of Vitamin C/ml HBSS and development was continued for 24 hrs.

From the above experimental groups, brain was used for further processing. For the study of distribution of GAGs and SA histochemical techniques (>26) staining methods were used. All of them were used to get permanent stained preparations of brain regions. For this paraffin sections were utilized which were obtained by routine microtechnique details of which have been given below.

Preparation of tissue for histology-

The embryos were removed after designed incubation hrs. The whole embryos were immediately transferred to fixatives.

Fixation and microtechnique-

Whole embryos were fixed in Bouin's fixative, 10% buffered formaldehyde, 4% paraformaldehyde and Calcium Acetate Formalin (CAF). The fixatives were prepared as follows:

I) Bouin's fixative:

It was prepared as described by Thompson (1966).

Solution I-	Saturated picric acid (tri-nitrophenol)	20g
	Distilled water	1000ml

Dissolve picric acid in distilled water with the aid of heat, allow it to cool and decant the supernatant.

Solution II-	Bouin's stock solution	
	Solution I	750ml
	Paraformaldehyde(4%)	250ml

Bouin's fixative:

Solution II	95ml
Picric acid	5 ml

Glacial acetic acid was added to stock Bouin's fluid just before the fixative is to be used. The pH of this fixative should be appropriate 1.5 to 1.7.

II) Buffered 10% formaldehyde-

Phosphate buffer (pH 7.0 to 7.2):

Dibasic anhydrous sodium phosphate (NaHPO4)	6.5 gm

Monobasic acid potassium phosphate(KH2PO4)	4.0gm
Formaldehyde	100ml

III) Paraformaldehyde(4% pH 7.2):

Phosphate buffer (pH 7.2):

Dibasic anhydrous sodium phosphate (NaHPO4)	6.5 gm
Monobasic acid potassium phosphate(KH2PO4)	4.0gm
Paraformaldehyde	4.0gm

IV) Calcium acetate formalin:

Calcium acetate	2gm
Formaldehyde	10ml
Distilled water	90ml.

Tissues were processed and stained with hematoxylin and eosin for histology. For the histological studies Bouin's fixative was used. The whole embryos were fixed in different fixatives for appropriate time. Further tissues were processed for paraffin sectioning as per routine microtechnique procedure (Thompson 1966; Pearse, 1968). The wax sections were cut off 0.4 to 0.5 μ thickness. The paraffin sections were used for glycosaminoglycans, sialic acid and glycans hyluranan demonstration. The histochemical analysis of glycosaminoglycans and sialic acid were carried out by using >26 differential staining methods. To get an insight of the Glycosaminoglycans the battery of histochemical techniques was employed in the present investigation. Staining reactivities and their interpretation is appended as a Table 3 at the end of the present chapter for ready reference. Because large numbers of techniques are used, details of the techniques are not given but Table no. 3 includes details of reaction and references. The results of various techniques used in the project are of stained preparations of CAF fixed brains.

Table no 3: Histochemical Method employed for visualizing Glycosaminoglycans

Histochemical Method	Chemical reaction involved	Histochemical result	Reference
1) Periodic acid Schiff's reaction (PAS)	Oxidation of vicinal hydroxyls to dialdehydes by periodate and formation of coloured complex with Schiff's reagent	All polysaccharides and glycosaminoglycans colour pink to magenta	McManus J .F .A.(1946), Techniq of Histo- and Cytochemistry Nat (Lond), 158 Hotchkiss, R.D.(1948) Mocrochemi reaction resulting in the staining in tl staining of polysaccharides structure in fixed tissue preparations. Arch.Biochem.,16:131
2) Periodic acid phenylhydrazine Schiff's	Phenylhydrazine selectively blocks periodate engendered dialdehydes in glycosaminoglycans in periodate reactive glycosaminoglycans available to subsequent Schiff's reanebt	Periodate reactive acidic glycosaminoglycans stained red presumably are those in which acid groups are proximal to vicinal glycols	Spicer S.S., Leppi T.J., Stoward P.J. (1965). Suggestions for a histo-chemical terminology of carbohydra rich tissue components". J. Histocher Cytochem.,13:599 Spicer S.S., Horn R.G. and Leppi T.. (1967) "Histochemistry of connectiv tissue mucopolysaccharides, symposium on connective tissue research methods'Chapter 17
3) Amylase digestion PAS	Hydrolyses and removes glycogen	Loss of PAS reactivity in sites containing glycogen	Lillie R.D.(1954) "Histochemical techniques and practical Histochemistry" 3rd edition , Blakist Thompson S. W.(1966), " Selected Histochemical and Histopathologica methods" Charles C. Thomas

			Histochemical and Histopathological methods" Charles C. Thomas Publisher, Illinois. U.S.A. N.Y. Lison L (1960). *Histochimie et Cytochimie Animales, Principles et Methodes*. 3rd edn. Gauthier-Villards, Paris, 532.
4) Saponification –PAS	Ester+ water or base yield acid+ alcohol or phenol the hydrolysis of an ester under basic conditions to form an alcohol and the salt of a carboxylic acid. Which bindn with Shiff's reagent	Basophilia of carboxyls blocked by esterification with methanol is restored as a result of this de-esterification if the residue is not acid or alkali labile. Basophilia blocked by methanol removal of sulfate esters is not restored by saponification	Spicer S.S., Horn R.G. and Leppi T.J. (1967) "Histochemistry of connective tissue mucopolysaccharides, symposium on connective tissue research methods'Chapter 17
5)Acetylation PAS	introduces an acetyl functional group into an organic compound.	Adds acetyl group on neutral glycosaminoglycans. Acetylated-deacetylated prior to the PAS reaction, the activity of 1, 2-glycol and 1, 2-aminohydroxyl groups will be restored.	Thompson S. W. (1966), "Selected Histochemical and Histopathological methods" Charles C. Thomas Publisher, Illinois. U.S.A.
6)Acetylation-Deacetylation-PAS	Deacetylation is the removal of the acetyl group.	Acetylated-deacetylated prior to the PAS reaction, the activity of 1, 2-glycol and 1, 2-aminohydroxyl groups will be restored.	Spicer S S and Lillie R D. 1966. Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. J Histochem Cytochem7(2):123–125. Thompson S. W.(1966), " Selected Histochemical and

			Histopathological methods" Charles C. Thomas Publisher, Illinois. U.S.A.
7) Colloidal iron PAS	The results was more or less similar to that was observed fo AB 2.5-PAS staining.	Colloidal iron reactive periodates unreactive acid glycosaminoglycans stain blue. Colloidal iron and PAS reactive glycosaminoglycans colour purple-blue. Neutral glycosaminoglycans colour pink magenta.	Ritter HB, Oleson J J.1950 Combined Histochemical Staining of Acid Polysaccharides and 1,2 Glycol Groupings in Paraffin Sections of Rat Tissues. Am J Pathol. Jul;26(4):639–645. Mowry RW: The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins, with revised directions for the colloidal iron stain, the use of Alcian blue G8X and their combinations with the periodic acid-Schiff reaction. *Ann NY Acad Sci* 106:402– 423, 1963
8) Alcian blue pH2.5	Probably formation of alcian blue complexes with carboxyls and sulfate group	Sialyted glycoprotein's and weakly acidic sulfomucins stain; the most strongly acidic sulfomucins stain weakly or not at all.	Mowry R. W., (1956) Alcian blue technics for the histochemical study of acidic carbohydrates. J. Histochem. Cytochem. 4:407
10) Phenyl-Deacet-Sap-PAS	Phenylhydrazine selectively blocks periodate engendered dialdehydes in glycosaminoglycans in periodate reactive glycosaminoglycans	Periodate reactive acidic glycosaminoglycans stained red presumably are those in which acid groups are proximal to vicinal glycols	Thompson S. W.(1966), " Selected Histochemical and Histopathological methods" Charles C. Thomas Publisher, Illinois. U.S.A. N.Y.

	available to subsequent Schiff's- Deacetylation is the removal of the acetyl group- Ester+ water or base yield acid+ alcohol or phenol the hydrolysis of an ester under basic conditions to form an alcohol and the salt of a carboxylic acid. Which bindn with Shiff's reagent.		Spicer S S and Lillie R D. 1966. Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. J Histochem Cytochem7(2):123–125.
11) Sulfer induced metachromacia	Addition of sulfur group of GAGs	Metachromatic staining at induced sulfur residues.	Thompson S. W.(1966), " Selected Histochemical and Histopathological methods" Charles C. Thomas Publisher, Illinois. U.S.A. N.Y. Spicer S S and Lillie R D. 1966. Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. J Histochem Cytochem7(2):123–125.
12) PA*-Bh-Sap-PAS	Oxidation with the help of periodic acid aldehyde generated are reduced to generate Schiff;s unreactive alcohol with sodium borohydride followed by saponification	Sialic acid with O-acyl substituents at C_7, C_8 or C_9 are PAS positive	D. Volz, P.E. Reid, C. M. Park, D. A. Owen, W. L. Dunn, 1987. A new Histochemical method for the selective periodate oxidation of ttal tissue sialic acid. Histochemical journal 19, 311-318.
13) AB pH 2.5-PAS	Addition of results by single methods	Alcian blue reactive peroidate unreactive acid	Mowry, R. W. and C. H. Winkler: The coloration of acidic

		glycosaminoglycans stain blue Alcian blue and PAS reactive glycosaminoglycans colour purple bluc. Ncutral glycosaminoglycans colour pink magenta.	carbohydrates of bacteria and fungi in tissue sections with special reference to capsules of Cryptococcus neoformans, Pneumococci, and Staphylococci. Amer. J. Pathol. 32: 628-629 (1956).
14) AF-AB pH 2.5	Formation of salt complexes between cationic staining entity and sulfated and carboxyl groups.	Sulfomucins stain purple or blue-purple, sialomucins and other non sulfated acidic glycosaminoglycans stain blue	Spicer S S and Lillie R D. 1966. Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. J Histochem Cytochem7(2):123–125.
15) Acid hydrolysis	Removes sialic acid from glycosaminoglycans.	Complete or partial loss of alcinophilia or metachromasia indicates the probable presence of sialomucins.	Quintarelli G., Tsuiki S., Hashimoto Y. and Pigman W.(1961), Studies of sialic acid-containing mucins in bovine submaxillary and rat sublingual glands. J Histochem Cytochem. 1961 Mar;9:176–183.
16) Mild methylation AB pH 2.5 (37^0c).	Esterification of carboxyl groups.	Generally mild methylation abolishes the alcinophilia of carboxymucins	Fisher, E. R., Lillie, R. D.: The effect of methylation on basophilia. J. Histochem. Cytochem. **2**, 81–87 (1954). Spicer S S. 1960. A correlative study of the histochemical properties of rodent acid mucopolysaccharides. J Histochem Cytochem. Jan;8:18–35.
17) Mild methylation saponification AB pH 2.5 (37^0c).	Restoration of carboxyl groups.	Restoration of the alcinophilia after saponification of methylated sections, indicated the presence of carboxyl groups.	Spicer S S and Lillie R D. 1959. Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. *J*

			Histochem Cytochem 7(2):123–125. Spicer S S. A correlative study of the histochemical properties of rodent acid mucopolysaccharides. J Histochem Cytochem. 1960 Jan;8:18–35.
18)Active methylation AB pH 2.5 (60^0C)	Carboxyl groups are esterifird sulfomucins are desulfated.	Active methylation abolishes alcinophilia pf carboxymucins through esterification and of sulfomucins through hydrolytic removal of the sulfated groups.	Fisher and Lillie (1954),) Spicer S S. A correlative study of the histochemical properties of rodent acid mucopolysaccharides. J Histochem Cytochem. 1960 Jan;8:18–35..
19) Active methylation saponification AB pH 2.5 (60^0C)	Restoration of carboxyl groups. Sulfomucins are hydrolytically removed during active methylation are not restored following subsequent saponification .	Restoration of the alcinophilia after subsequent saponification indicates the presence of carboxyl groups and loss of alcinophilia indicates the presence of sulfated groups.	Spicer S S and Lillie R D. 1959. Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. *J* Histochem Cytochem 7(2):123–125. Spicer S S. A correlative study of the histochemical properties of rodent acid mucopolysaccharides. J Histochem Cytochem. 1960 Jan;8:18–35.
20)Saponification –AB pH 2.5	Ester+ water or base yield acid+ alcohol or phenol the hydrolysis of an ester under basic conditions to form an alcohol and the salt of a carboxylic acid. Which bindn with AB pH 2.5	Basophilia of carboxyls blocked by esterification with methanol is restored as a result of this de-esterification if the residue is not acid or alkali labile. Basophilia blocked by methanolytic removal of sulfate esters is not restored by saponification	Spicer S S and Lillie R D. 1959. Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. *J* Histochem Cytochem 7(2):123–125.

21) Colloidal iron	Probably formation of complexes between cationic colloidal ferric aggregates and carboxyls, sulfated and phosphates esters.	Non-sulfated acid glycosaminoglycans and some sulfated glycosaminoglycans colour blue	Hale, C. W.1946: Histochemical demonstration of acid mucopolysaccharides in animal tissues. Nature (Lond.) **157**, 802 Rinehart JF, Abul-Haj SK 1951 An improved method for histologic demonstration of acid mycopolysaccharides in tissues. AMA Arch Pathol 52:189–194 MOWRY, R. W. 1961 Minimizing non-selective and variable staining of acidic carbohydrates when (Krecke's) colloidal iron is used in Hale's reaction. J. Histochem. Cytochem. **9**, 609–610 (abst.). Mowry RW, 1963. The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. Ann NY Acad Sci 106:402–423
22)Alcian blue pH1	Probably fprmation of alcian blue complexes with sulfated groups.	Weakly and strongly acidic sulfpmucins are selectively stained,	Lev, R., Spicer, S. S. 1964. Specific staining of sulphate groups with alcian blue at low pH. J. Histochem. Cytochem. **12**, 309.
23) AB pH 1.0- PAS	Addition of results by single method.	Sulfomucins stain blue or blue-purple. Neutral and non-sulfated periodate reactive glycosaminoglycans stain pink magenta.	Spicer SS, Leppi TJ, Stoward PJ. 1965. Suggestions for a histochemical terminology of carbohydrate-rich tissue components. *J Histochem Cytochem.* 13(7):599–603.;

			Spicer S.S., Horn R.G. and Leppi T.J. 1967. "Histochemistry of connective tissue mucopolysaccharides, symposium on connective tissue research methods'Chapter 17 Spicer S.S., Horn R.G. and Leppi T.J. 1967. "Histochemistry of connective tissue mucopolysaccharides, symposium on connective tissue research methods'Chapter 17
24) Aldehyde fuschin (AF)	Formation of salt complexes between cationic staining entity and sulfated and carboxyl groups.	Sulfated glycosaminoglycans stain dark purple, sialumucins and hyaluronic acid colour light purple.	Gomori G.(1950), Aldehyde-fuchsin: a new stain for elastic tissue. Am. J. Clin.Pathl.,20,:665-6 Halmi N. S. and Davies, 1953. Comparison of aldehyde fuschin staining , metachromacia and periodic acid Sciff's reavtivity of various tissue. J. Histochem. Cytochem. 1953 1: 447-459
25) Azure A or touludine blue at controlled pH levels	Formation of blue orthometachromacia or purple or red metachromatic	Strongly sulfated glycosaminoglycans stain pyrple red at pH 0.5 to 1.5, sialomucins	Spicer S S. A correlative study of the histochemical properties of rodent acid mucopolysaccharides. J Histochem Cytochem. 1960

	sait complexes with the extinction values indicating degree of acidity of the polymer	stain purple –red at pH 2.5 to 3.5, hyaluronic acid and weakly acidic glycosaminoglycans stain purple at pH 4.5 to 5.0	Jan;8:18–35. Pearse A. C. E. (1968), Histochemistry. Edn. Vol.1(Little Brown and Co. Batson)
26) Alcian blue at pH 5.6 with graded concentration of $MgCl_2$.	Alcian blue complexes with sulfated groups. Different sulfomucins vary in the critical electrolyte concentration at which alcinophilia is lost.	Non-sulfated acidic glycosaminoglycans are not stained at and above 0.1 M Mg++ concentration. Sulfomucins stain selectively at and above 0.2 M Mg++ concentration.	Scott J E, Dorling J (1965) Differential staining of acid glycosaminoglycans (mucopolysaccharides) by Alcian blue in salt solutions. Histochemie 5:221–233