CHAPTER – IV

GENERAL DISCUSSION

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Proteins were marginally increased in CAM by HBSS treatment at 96 hrs with Vitamin C treatment given at different hrs proteins was increased significantly. H_2O_2 treatment at different hrs reduced the protein significantly indicating retarded growth. Simultaneous treatment of H_2O_2 + 3 mg Vitamin C had protected the protein content at all hrs of treatment but contents were significantly increased over normal protein content of CAM but remained lower than only Vitamin C treated embryos.

HBSS increased TCA-TBA reactive substances marginally 66 hrs onward treatments. Treatment of Vitamin C had also shown marginal increase in almost all intervals studied. Hydrogen peroxide significantly TCA-TBA reactive substances. Which were increased with the increasing hrs of treatment indicating increase in drug metabolizing system response in CAM is increased with the advancement in development. Simultaneous treatment of H_2O_2 and Vitamin C reduced the production of TCA-TBA reactive substances but quantitatively levels remained above the corresponding normal levels. These levels were more near to the levels of TCA- TBA reactive substances noted in only Vitamin C treated embryos

Glutathione levels in HBSS treated embryos remained at all the intervals below normal levels (except at 72 hrs) but in per gm expression they were above the normal levels marginally. Vitamin C treatment had significantly decreased the levels which were bellow the levels of glutathione reported in HBSS treated embryos indicating influence of Vitamin C on glutathione production. Since Vitamin C is known antioxidant (Caderlas *etal;* 1996) and may be involved in direct consumption of free radicals which seems to inhibit glutathione production and trend remained same in per mg tissue protein and per gm tissue expression. Thus O and OH free radicals are managed directly by Vitamin as well as through glutathione production.

Treatment of H_2O_2 had significantly reduced glutathione content at all intervals in mg tissue protein expression or per gm tissue expression. Since simultaneously TCA-TBA reactive substances are also increased. The noted glutathione content is resultant content of glutathione content already present in embryos as a natural free radical scavenger against free radicals and the content which is sythesized against the free radical response. Vitamin C treatment along with H_2O_2 brought the glutathione content was marginal at 88 hrs but significantly low at 48, 66,

and 96 hrs of treatment while at 55 hrs and 72 hrs treatment the content of glutathione is significantly high.

At 88 hrs the marginally low glutathione is associated with marginally high content of TCA-TBA reactive substances. Similarly at 48 and 96 hrs high levels are coupled with high contents of TCA-TBA reactive substances but highly significant increase in glutathione is coupled with normal levels of TCA-TBA reactive substances indicating the close relation between free radical and free radicals scavenger contents. Some of the differences are indicators of overlapping situation of glutathione consumption and glutathione synthesis (induced and native) since the growth of the embryo was normal with 100% survival the alterations in the glutathione range are not influencing the embryonic development.

HBSS control increased HCHO content marginally at all intervals of its introduction in embryos except at 96 hrs of interval. Where it is marginally and at 55 hrs it was significantly high. The results indicate HBSS mediated production of HCHO seems to utilize free radicals which seems to be oxy and hydroxy which are known to stimulate HCHO synthesis (Thrasher 2005). Whenever it is marginally at these intervals TCA-TBA reactive substances are also marginally low which also confirm the relationship in present work.

Vitamin C alone given to the embryos indicated marginally low HCHO content along with marginally low TCA-TBA reactive substances. At 72 hrs both are high which reveals this relationship clearly. Treatment of H_2O_2 had elevated the levels of TCA-TBA reactive substances and as stated in section I 50% mortality was coupled with this at all the intervals studied and survivals were with the abnormalities in brain. This is also coupled with increased HCHO content indicating utility of H_2O_2 generated oxy and Hydroxy radicals generated by H_2O_2 introduced to embryos.

Vitamin C treated with H_2O_2 resulted in marginally low content of HCHO at 48 hrs and significantly high at 55,66, 72, and 88 hrs coupled with high content of TCA- TBA reactive substances indicating the metabolisms which are inducing HCHO synthesis with its clearance but still TCA-TBA reactive substances remained high indicating the other free radicals which do not generate HCHO and may utilizing other free radical scavengers or cleared slowly. At late hrs o treatment the time may have been and hence less generation of free radicals.

The turn over of HCHO and its clearance seem to be not affecting the growth of the embryo. Since the survival is 90% without any abnormality.

HBSS treatment improved the survival by 5%. 0.05 mM H_2O_2 /embryo increased the mortality by 10% in normal animals at all the hrs studied except at 88 and 96 hrs. Where it was not altered. The results indicate that after 88 and 96 hrs the dose of H_2O_2 may not have been metabolized up to 144 hrs. When embryos were observed or dose at these hrs may not be leading to death of the animals. Dose of 0.5 mM treatment showed at all intervals studied average 50% mortality. The 50% survivals showed abnormalities especially of nervous system along with retarded growth.(there fore this dose was used for further studies).the antioxidant used was Vitamin C which effects were also dose dependent. As stated earlier it was used against 50% mortality showed 0.5 mM H_2O_2 dose. Only 3 mg dose of Vitamin C showed survival as in HBSS treated embryos without any abnormality 4 mg Vitamin C dose showed mortality in early hours (77%) to late hrs (20%). But last two intervals showed mortality as in normal indicating less hrs of exposure had not influenced the mortality but 5 mg dose had shown 15% mortality even at this hrs. The survival in both the doses showed the abnormalities in development there fore only 3 mg dose was used for angiogenesis alterations and other studies. These results have shown that free radical generation in early hrs is fatal to the embryos. Vitamin C 3mg had protected the embryo from death as well as the abnormalities at all hrs. this was used further for angiogenic and other studies.

The dose of 0.5 mM H_2O_2 had influenced the diameter of CAM very significantly in early hrs than at late intervals. This indicated that the growth of CAM and its extension must be influencing the growth of the embryo leading to death, retarded growth and abnormalities in addition to the direct influence of free radicals. Vitamin C 3 mg had protected this effect showing marginal to significant increase in area of CAM up to 72 hrs while introduction at further hrs remained marginally increased.

The area in which primary veins are extended had increased in HBSS treated embryos but in H_2O_2 treated embryos their extension is reduced at all the hrs primarily affecting proliferation of primary veins. But simultaneous treatment of H_2O_2 and Vitamin C had protected this effect at all the hrs and 55% area was extended above the normal at all the hrs. the results indicate that Vitamin C mediated

scavenging of free radicals at early hrs influence more in extension of primary veins. At further hrs Vitamin C had influenced the extension because this effect is significantly observed in only Vitamin C treated embryos. Though area of the primary veins has been altered the number was not altered indicating that its divergence and bifurcations have not been influenced.

Secondary vitelline veins showed significant increase in area covered as well as number on left side in HBSS treated embryos. Number on right side was decreased but in this case area of its extension was not influenced indicating selective influence in bifurcation of secondary veins. H_2O_2 treatment has decreased both number and secondary vein extended area irrespective of right and left side in presence of HBSS. i.e. free radicals influenced these veins at all hrs of treatment intervals connecting the HBSS influence. In presence of Vitamin C area was increased above the normal embryos indicating stimulation of secondary vein extension at all hrs of treatment. But number of veins was decreased than normal at early hrs and marginally increased at left side at late hrs indicating differential response to Vitamin C protection at different developmental hrs but right side was significantly decreased up to 66 hrs. But at late hrs this difference was lowered. In presence of H_2O_2 + Vitamin C area was increased over normal but of secondary veins was influenced. In early hrs in left side number of vein decreased but increased in left side such response was not given by right hand side veins indicating selective response of the veins at different developmental hrs.

HBSS increased the left side number and also the area covered. But right side vein number was marginally low at 48, 72, and 88 hrs. At these intervals embryos seem to respond to these factors selectively. H_2O_2 irrespective left and right sides showed decrease in the number and area covered. Result of H_2O_2 + Vitamin C treatment showed significant protection on left side as compared to right side. The number was even high over normal. This influence indicates modified response of Vitamin C since Vitamin C alone had increased the right side capillaries significantly than that of left side except at 88 hrs. where the veins were increase on left side. This seems to be due to differential free radical response at these hrs since during development also. Number of tertiary veins on right side remained high over the number over the number noted on left side and also the area covered by them. HBSS stimulated the total number of tertiary veins significantly at 55 and 66 hrs and 96 hrs

of treatment indicating there is no relation between hrs of exposure to HBSS but it seems development specific. But in case if Vitamin C 66 hrs onwards significantly high number of tertiary veins was observed indicating except early hrs of development Vitamin C influenced the process of angiogenesis positively while at 55 hrs it showed marginal inhibition. These results indicate necessity of Vitamin C in development of angiogenesis increasing nutrient uptake in the developmental hrs stated. But in presence of H_2O_2 generated free radicals only at 66 hrs highly significant increase in number was observed which seems to be resultant of cumulative effect of HBSS and Vitamin C and inhibitory effects of free radicals.

Since irrespective of the total number observed in protective experimental schedule at the different levels of intervals the mortality at hatching was protected so also the normal growth. But the effect if these influence needed to be studied on further development whether these effect on post hatching growth.

In conclusion angiogenesis is influenced by H_2O_2 exposure and bent $O^{\bullet}$ and $OH^{\bullet}$ free radicals generated at CAM. Decrease in angiogenesis is highly significant evidently 48 and 55 hrs and 72 hrs as compared to remaining hrs. HBSS independently stimulates angiogenesis but in presence free radicals it has not influenced the inhibitory effect of $O^{\bullet}$ and $OH^{\bullet}$ radicals. But along with antioxidant Vitamin C exposure hrs specific synergic effect of Vitamin C and HBSS was observed. But Vitamin C stimulates angiogenesis independently as well as presence of $O^{\bullet}$ and $OH^{\bullet}$ radicals which is dose specific. The antiangiogenic effects of $O^{\bullet}$ and $OH^{\bullet}$ were associated with high levels of free radicals generated TCA-TBA reactive radicals significantly on both side at 55,66, 72,and 88hrs. but at 96 hrs of exposure angiogenesis on right side was significantly decreased but on left side angiogenesis was significantly stimulated in presence of high levels of TCA-TBA reactive substances. This is the stage where anterior, posterior and lateral vitelline veins are well developed and more defined (Mc Ewen, 1969; Patten ,1977; Carlson, 2007).the results indicate that same levels of TCA-TBA reactive substances give antagonistic effects on left and right vitelline angiogenesis in Cam but area covered by the tertiary veins are decreased on the both sides. The results indicate that the extension of tertiary veins is independently guarded from the branching of the tertiary veins and are inhibited by concentration of TCA-TBA reactive substances noted at the interval or some other factors may be guarding it and needs to be studied. Similarly these

results also coupled with high levels of HCHO while contents of free radicals scavenger glutathione remained decreased at all hrs except at 48 hrs where it levels are increased.

Influence of Vitamin C had shown protection of angiogenesis on both sides Vitamin C in presence of induced $O^{\bullet}$ and $OH^{\bullet}$ radicals stimulated the angiogenesis by differential manner. At 48 and 55 hrs the increase is marginally above marginally above the number of tertiary veins that are noted in normal animals on left side but on right side the number is decreased. At 66, 72, 88, and 96 hrs the number of tertiary veins are significantly increased on left side even more than are noted in normal but the features of alterations on right side are marginal decrease in number at 66 and 72 hrs and significant decrease in number at 88 and 96 hrs. the area covered by tertiary vitelline veins on left and right side show increase at all hrs except 96 hrs where it shows marginal decrease.

Thus the results indicate that left side tertiary veins are influenced positively showing stimulation of angiogenesis in late hrs of exposure. Whether this differential response is further reflected on post hatching development and breeding status of animals needed to be studied.