CHAPTER IV

GENERAL DISCUSSION

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On the basis of the mortality data and abnormalities on hatching, selection of H_2O_2 dose for introduction/induction of free radicals was done (1 ml of 0.5 mM H_2O_2 /embryo).

Vitamin C dose (3 mg /ml/embryo) was selected as per the survival of H_2O_2 treated embryo and associated abnormalities on hatching.

The different intervals at which initiation of these experimental conditions were done to embryo was decided on the basis of brain development stages. So that H_2O_2 induced free radicals sensitive and vitamin C protective stages of brain development could be identified.

The parameters used for studies include lipid peroxidation which is free radical associated effect judging parameter (Esterbauer *et al.*, 1991), while glutathione is natural free radical scavenger which is inherently present in cells (Sips and Gandolfi, 1991) and protects the cellular activities against free radicals. Similarly formaldehyde is the metabolite that involves protein metabolism at different levels (Paulsen *et al.,* 1974) and is known to produce in toxicological and stressed conditions in tissue. Since proteins are used as the reference parameters for growth and expression of other parameters it had also been studied.

Vitamin C is externally implemented because it is also known to function as free radical scavenger but with critical concentration (Catley and Anderson, 2006). Since brain could be distinguished 72 hrs onwards of development earlier stages were performed with whole embryo.

The results of H_2O_2 treatment indicated that 0.5 mM H_2O_2 given at different hrs showed 50% mortality. Presence of HBSS alone improved the survival rate of normal embryos. Three mg dose of vitamin C given simultaneously also protected survival rate at different time hrs of developmental schedule used in protocol.

The different parameters used to evaluate the alterations provided the free radical management potencies with stressful conditions evaluated.

Dose of 0.5 mM H_2O_2 (1 ml/embryo) exposure initiated at 24, 34, 40, 48, 72, 96 and 120 hrs and was continued until the 144 hrs showed average 50% mortality.

Thus it seems that free radicals that were inherently generated and H_2O_2 treatment induced cumulatively resulted in approximately 50% mortality.

Thus neural tube closure stage (24 hrs), brain vesicle formative stage (34 hrs), five neuromere formation of hind brain stage (40 hrs), flexion and torsion stage (48 hrs), differentiation and initiation of different brain regions stage (120 hrs) were equally sensitive to cumulative resultant free radicals impact that resulted in 50% mortality.

Thus prolong treatments to early hrs of embryo and short treatments to advanced stage embryos seems to have in total 50% mortality effect may be producing more free radicals to lead 50% mortality.

Vitamin C 3 mg /ml/embryo simultaneous treatment with 0.5 mM H_2O_2 1 ml/embryo protected the mortality effect on embryos without generating any abnormality effect on hatching in control vitamin C treated embryo also. This dose is important because 4 mg and 5 mg vitamin C doses have shown abnormalities in neck regions and bulky bodied animal with difficulties in movements in experimental and control categories. Critical concentration of vitamin C required for particular metabolism or free radical scavenging is also observed in cultured cells and neurons. Thus, this critical vitamin C concentration demand for free radical scavenging so that the normalization of the developmental conditions to protect the mortality.

The inherent presence of free radicals during normal development showed development dependant increase.

HBSS presence had not influenced this significantly, but since 5% improvement has observed in mortality it seems free radicals are not related with the mortality improvement in any other metabolism may have involved in it.

H_2O_2 (0.5 mM) + Vitamin C treatment though normalized H_2O_2 induced free radicals which were TBA-reactive, 96 hrs embryo (treatment from 24-72 hrs) showed no TBA-reactive free radicals which was also true in case of vitamin C control embryo. Thus these values were below the normal TBA-reactive free radical value. This may be the threshold concentration of vitamin C as free radical scavenging role deciding state. This lowered free radical concentrations seemed to have not affected the development

adversely, but larger concentrations of vitamin C may be consuming free radicals which may have been required for normal development, but simultaneously induce the associated abnormality in hatched animals. Though other experimental schedules had dropped values significantly below normal with vitamin C treatment alone, initiation of dose (0.5 mM H_2O_2 + vitamin C) at 40, 72, and 96 hrs and continuation for 48 and 72 hrs; 48 hrs; 24 hrs respectively also showed marginal drop in TBA-reactive free radical species.

These results also indicated that vitamin C actively plays free radical scavenger role in experimental and normal embryos with equal potency.

The alterations in glutathione content are very significant. HBSS control glutathione values were not altered significantly, which was also true in case of vitamin C. Thus both have not influenced normal glutathione generation which seems to be necessary for normal development.

H_2O_2 treatment increased the glutathione content in 24 and 34 hrs of initiation and 24 hrs exposure.

But at remaining especially late hrs, glutathione content was increased. Less hrs of exposure in late hrs also depleted glutathione content the reductions may be due to delay in new synthesis of glutathione which was already consumed by free radicals. At some hrs the free radical production seems to be high and hence glutathione content must have been immediately dropped.

Vitamin C treatment in control seems to induce glutathione as most of the experimental schedules showed glutathione high values. The perception of theses results may be considered as that vitamin C may have been involved in direct scavenging of free radicals and *in situ* glutathione may remained unaffected.

Thus it seems glutathione content is resultant of direct effect of vitamin C mediated free radical scavenging and inherent response of embryonic cells to produce glutathione for management of cumulative free radical pool in embryo.

HBSS control had not influenced in early hrs formaldehyde production. But in late hrs of treatment formaldehyde content remained low. This is indicator of stress relief and may be responsible for 5% improved mortality than normal.

All hrs of treatment initiation of vitamin C including 24, 40, 48 and 72 hrs of to normal embryo, induced formaldehyde production indicating adverse effect of vitamin C, which seems to be managed at present concentration used and experimental conditions involved as the developmental consequences were normal. Embryo at early neural tube closure to brain enlargement stages do not posses formaldehyde productions potency.

H_2O_2 (0.5 mM) induced formaldehyde production at all hrs of initiation and at all treatment intervals used indicating more prominent consistent effect of H_2O_2 induced stress. Increased O^- and $-OH^-$ radicals at different stages of development ought to induce N-demethylase activities end product of which is formaldehyde (Clejan and Cederbaum, 1991). Oxidation of glycerol to formaldehyde is also the pathway of formaldehyde production (Clejan and Cederbaum, 1991). Thus H_2O_2 had not only induced formaldehyde production through either of the or both the both the pathways indicating pathophysiological stress over the embryo. Vitamin C treatment given simultaneously have though controlled the major production of formaldehyde still it remained significantly high over the normal but under the presence of 3 mg vitamin C its adverse effects not seem to have affected the final developmental status and mortality. Therefore, pool of formaldehyde seems to be buffered by vitamin C mediated other metabolic reactions. As it is observed earlier vitamin C concentration specific control seems to be effective.

All these results indicate that the alterations in brain as consequences of increased $-OH^-$ and O^- even in normal conditions of growth have mortality consequences and/or long term effects on embryo survival and development. The external use of free radical scavengers especially that of vitamin C has limited effects on survival and protection.

Subtle stress of free radicals in development results in HCHO production leading to retarded growth.

Glutathione dependant HCHO dehydrogenase enzyme converts HCHO to formate (Tayebeh *et al.,* 1989) and formate is further utilized in glucogenic amino acid purine which enters further in carbohydrate or acetoacetate and choline synthesis (Annison and White (1962) or leads to the formation of Co_2 through catalase activity. If it increased in amount .

But in present observations since vitamin C given simultaneously with H_2O_2 had increase the formaldehyde content further i.e. vitamin C not influencing any of the HCHO clearance pathway stated above and HCHO must have been utilizing the normal metabolism for clearance. Since embryonic growth is normal in animals i.e. the formaldehyde concentration may be in tolerable limits or may be cleared speedily by using any of the pathways in successive developmental hrs.