C H A P T E R - IV

LIPID PEROXIDATION

LIPID PEROXIDATION

In recent years Mehendale (1985) has reviewed the process of lipid peroxidation in hepatotoxicity with particular attention to CCl_4 toxicity. One of the most clearly defined hypotheses relating to the action of hepatotoxic agents is the lipid peroxidative damage of cellular membranes. Lipid peroxidation may be occurring in two steps. Some toxic events initiate lipid peroxidation and organic free-radicals generated by the initiation process serve to propogate the reactions. The concept of lipid peroxidative damage was advanced by Slater, (1978) as the principal mechanism of CCl_4 induced liver injury and has found ample experimental support (Recknagel and Glende, 1978). This theory holds that CCl_4 is homolytically cleaved by a cytochrome P-450 monoxygenase system to produce CCl_3 free radical. In the aerobic environment, the CCl_3 free radical enters an hydrogen abstraction reaction to form an organic free radical of the fatty acid and chloroform. The cytochrome P-450 system is encased in a phospholipid membrane rich in polyenoic fatty acids. Hence these polyenoic fatty acid are the most likely immediate targets for the initial lipid peeroxidative attack to occur. In the aerobic environment of the hepatocyte, the organic fatty acid radicals rearrange yielding organic peroxy and hydroperoxy radicals. These radicals destroy the cytochrome P-450 hemoprotein, thus compromising the mixed function oxygenase activity. The rapid decomposition of the endoplasmic reticulum and its function is a direct result of this

lipid peroxidative process.

The lipid peroxides and hydroperoxides attack the lipid and possibly the proteins especially the functional groups such as sulfhydryl groups compromising their function. Generation of organic radicals by the CCl_3 free radical cause another chain of reactions. The organic radicals themselves interact with other lipids either in same membrane or in other membranes after being diffused from the original site of formation. This interaction results in the formation of other organic free radicals, peroxy and hydroperoxy radicals. The entire process is propagated to continue the autocatalytic reactions until the membranes are destroyed and many cell functions are disrupted culminating in the demise of the hepatocyte.

Destruction of cytochrome P-450 has been demonstrated in animals treated with CCl_4 and <u>in vitro</u> incubation containing CCl_4. Two hypotheses have been put forward concerning the mechanisms by which cytochrome P-450 system is destroyed (Recknagel and Glende, 1978). One holds that the CCl_3 free radical formed at the cytochrome P-450 catalytic site directly attacks the phospholipid casing of the membrane bound P-450 complex. An alternative hypothesis holds that the CCl_3 free radical is liberated from the catalytic site of cytochrome P-450 and interacts with cellular membranes to initiate lipid peroxidation. Resultant organic fatty acid radicals, peroxy and hydroperoxy radicals attack and destroy the

cytochrome P-450 complex. In any case, destruction of cytochrome P-450 hemoproteins does occur in CCl_4 induced liver injury. This is in fact the basis for autoprotection afforded in experimental animals by prior exposure to a small dose of CCl_4 against a subsequently administered larger dose of CCl_4. Actual decrease in cytochrome P-450 levels in the liver tissue of animals treated with CCl_4 as well as in vitro microsomal incubations with CCl_4 have been demonstrated by a number of investigators. Loss of glucose-6-phosphatase activity and representative monooxygenase activities, protein synthesis, capacity to form and secrete low density lipoproteins are some of the other consequences of CCl_4 induced liver injury on the function of endoplasmic reticulum.

It may be that many free radicals initiate lipid peroxidation process for instance many auto-oxidations, certain oxidase, and leakage of electrons from normal electron transport chains results in the generation of superoxide anions. These anions may give rise to other radical forms of oxygen such as singlet oxygen, OH radical and H_2O_2 which may initiate autocatalytic lipid peroxidative processes. Evidence for the involvement of superoxide anion radical in the initiation of such processes in the presence of superoxide dismutase, an enzyme responsible for removing the superoxide anions. Antidotes for lipid peroxidative damage are not available. Antioxidant compounds can protect against lipid peroxidation but prior presence is required for their protective action, CCl_4 induced

lipid peroxidation can be obtained by antioxidants such as Vitamin E, pyrogallol and other polyols and sulfhydryl compounds such as cysteamine. The protection from lipid peroxidation by use of antioxidants is based on the presence of the biological antioxidants viz - SH-compounds, glutathion, SH proteins, cysteine, methionin which protect the oxygen labile components such as polyunsaturated and sulfhydryl enzymes (Miquel et al.,1977). However, these agents have to be administered prior to, simultaneously or immediately after the administration of CCl_4 in order for their protective action to be manifested.

Another defence mechanism against peroxidation in vivo involves the structural separation i.e. the catalysts for peroxidation are presumed to be structurally isolated from the unsaturated lipids and the lipids appear to be protected by the masking of the double bonds with proteins (Barber and Bernheim, 1967) Philpoff et al. (1974) also postulated that low oxygen tension in most tissues would be protective since the molecular O_2 plays a key role in free-radical reaction.

When lipid peroxidation occurs the concurrent destruction of enzyme activity also occurs in isolated microsomes. NADPH dependent lipid peroxidation, cytochrome P-450 destruction and loss of monooxygenase are closely related as well as loss of glucose-6-phosphatase activity is also closely related. Since it is membrane bound in endoplasmic reticulum (ER) microsomes (Lehninger, 1982;

Nardlie et al. 1982).

Since the lipid peroxidation is specific and dominating feature of the CCl_4 poisoning; it was felt that along with histological alterations, changes in in vitro lipid peroxidation activity in liver and kidney would also give confirmation to the histologically observed activities. Lipid peroxidation leads to damage of microsomal membrane system thus affecting or decreasing glucose-6-phosphatase enzyme activity which is membrane bound and taken as the marker enzyme of microsomal fraction (Nordlie, 1982; Burchell and Waddell, 1991). Therefore to confirm the membrane damage if any, during alterations, glucose-6-phosphatase activity is also estimated as per Burchell et al.(1988). Lipids estimated gravametrically while proteins were assayed as per Lowry (1951).

During CCl_4 poisoning lipids are accumulated in liver, in present project the vehicle used for CCl_4 is liquid paraffin which is also hydrophobic and we hardly know about the metobolisms by which it is handled by the cells. But to have a basis of comparison in evaluating the lipid peroxidation; contents of lipids and contents of proteins are used in discussion and are given in Table.4

Thus the histological recovery of liver can be evaluated with probable metabolic activities taking place in liver and also in kidney.

Alterations occurring in lipid peroxidation in liver :

Liver :

Observations :

The curative effects of the mandur bhasma are studied after the 7 days' treatment since 15 days are required to bring total histological picture of liver to normal.

The aim to select the treatment of 7 days is to study the transitory metabolism of curation as it is explained in Chapter I - Introduction.

The alterations in lipid peroxidation in vitro conditions are expressed as p moles of malondialdehyde production per mg protein. The resuults are given in Table. 4

Group I

Normal Rat liver :

In vitro activity of lipid peroxidation in liver of normal rat is 158.00 p mole/mg protein.

Group II :

CCl_4 treated rat liver :

The lipid peroxidation was 225.75 p mole/mg protein.

Group III

Liquid paraffin treated rat liver :

Table 4 - Lipid Peroxidation in Liver and Kidney

MDA released (P mole/mg Protein)

Group	Liver	Kidney
Normal	158.00 ± 10.74	145.00 ± 6.68
CCl_4	225.71 ± 12.50	241.67 ± 18.00
Liquid Paraffin	180.57 ± 8.33	290.00 ± 21.14
Liquid Paraffin + CCl_4	316.00 ± 22.75	241.67 ± 11.49
Mandur bhasma	45.15 ± 2.90	192.85 ± 10.50
CCl_4 + Mandur bhasma	270.86 ± 16.67	241.67 ± 17.75
Liquid Paraffin + Mandur bhasma	135.43 ± 9.34	145.00 ± 7.72
Liquid paraffin + Mandur bhasma + CCl_4	45.15 ± 2.16	192.85 ± 8.40

In vitro lipid peroxidation calculated were 180.57 p moles/mg protein.

Group IV

CCl_4 + Liquid paraffin treated rat liver :

The malondialdehyde liberated in vitro was 316.00 p moles/mg protein.

Group V

Mandur Bhasma treated rat liver :

In vitro production of malondialdehyde estimated was 45.15 p moles/mg protein.

Group VI

CCl_4 + Mandur Bhasma treated rat liver :

The lipid peroxidation that was estimated showed 270.86 p moles/mg protein.

Group VII

Liquid Paraffin + Mandur bhasma treated rat :

In vitro production of malondialdehyde estimated was 135.43 p moles/mg protein.

Group VIII

CCl_4 + Liquid paraffin + Mandur Bhasma treated rat liver :

The lipid peroxidation was estimated as per malondialdehyde production was 45.15 p moles/mg protein.

Alterations occurring in lipid peroxidation in Kidney :

Kidney :

Group I :

Normal rat :

The kidney of normal rat showed 145 p moles/mg protein malondialdehyde formation.

Group II

CCl_4 treated rat :

In kidney on CCl_4 treatment showed 241.67 p moles/mg protein malondialdehyde production.

Group III

Liquid Paraffin treated rat :

290.00 p moles/mg proteins malondialdehyde production was observed in kidney.

Group IV

Liquid Paraffin + CCl_4 treated rat :

The lipid peroxidation estimated in kidney was 241.67 p moles/mg protein.

Group V

Mandur Bhasma treated rat :

In kidney the estimated lipid peroxidation was 192.85 p moles/mg protein.

Group VI

CCl_4 + Mandur bhasma treated rat :

The estimated lipid peroxidation activity in kidney was 241.67 P moles/mg protein.

Group VII

Liquid paraffin + Mandur bhasma treated rat :

In the kidney the estimated lipid peroxidation was 145. 00 P moles/mg protein.

Group VIII

CCl_4 + Liquid paraffin + Mandur bhasma treated rat :

The liberated malondialdehyde in kidney in vitro condition were calculated. They showed 192.85 P moles/mg protein.

Discussion :

Normal liver showed 158.00 P moles/mg protein lipid peroxidation and 2.73 units of glucose-6-phosphatase/mg protein activity. With the administration of CCl_4 the lipid peroxidation was increased by 1.42 folds but glucose-6-phosphatase activity was decreased by 5.46 folds. 0.87 fold increase was observed over normal in only liquid paraffin treated rat liver in lipid peroxidation and 5.0 fold decrease was observed in glucose-6-phosphatase activity. Similarly CCl_4 was, when administered with the liquid paraffin vehicle the lipid peroxidation was increased by 0.5 fold

and 2.70 fold decrease was observed in glucose-6-phosphatase activity. Only mandur bhasma treatment decreased the lipid peroxidation by 3.5 folds but 0.63 fold decrease in glucose-6-phosphatase activity was observed. 1.70 fold increase was observed in lipid peroxidation in liver in CCl_4 + mandur bhasma treated rat. 0.03 fold increase was observed in glucose-6-phosphatase activity. In liquid paraffin + Mandur bhasma treated rats 1.17 fold decrease was observed in lipid peroxidation but glucose-6-phosphatase activity was also decreased by 2.35 folds. Liquid paraffin + Mandur bhasma + CCl_4 treated rats 3.5 fold decrease in lipid peroxidation was observed but there was no alteration in glucose-6-phosphatase activity.

The alterations when considered along with the histological alterations we can discuss some probable mode of recovery. The lipid peroxidation and glucose-6-phosphatase enzyme activities are at equilibrium in normal rat liver. In CCl_4 treated liver lipid peroxidation was increased but not significantly, since the interval of 7 days without CCl_4 treatment may have evoked some selfcuring processes or the free radicals form that may have generated during lipid peroxidation, 7 day discontinuity of drug may lead to ceasation of free radical formation and the membrane damage which is already set in may be getting through self recovery processes. Since glucose-6-phosphatase activity is not recovered it seems that membrane components of RER are not recovered yet. The periarterial

necrotic zones and centrolobular necrotic zones support this probability.

Liquid paraffin treatment has not increased the lipid peroxidation significantly but has decreased the enzyme activity of glucose-6-phosphatase. These results may be indicative that liquid paraffin may have settled on the membranes since glucose-6-phosphatase activity is decreased significantly. Simultaneously increase in lipid contet is also observed.

Liquid paraffin + CCl_4 treatment increased the lipid peroxidation significantly along with very conspicuous decrease in glucose-6-phosphatase activity with marked increase in lipid content. Thus even 7 days of ceasation of treatment has not initiated the signiificant spontaneous recovery of the cells. The histology in both the regions indicate the persisting necrotic condition.

Only mandur bhasma treatment suppressed lipid peroxidation even below the normal levels of lipid peroxidation, without causing any change in the lipid content, but brought the glucose-6-phosphatase activity below the levels those were observed in normal liver. The healthy conditions of hepatocytes in histological architecture goes well with lowered lipid peroxidation. The low levels of glucose-6-phosphatase activity may be showing lowered metabolism rather than membrane destruction since protein synthesis seems to be increased as it is observed in case of estimated total

Table 5 - Comparison of lipid Peroxidation, Glucose-6-Phosphatase activities and Protein and lipid content from Liver and Kidney.

	Liver				Kidney			
	Lipid Peroxidation (P mole/ mg Protein)	Glucose-6-phosphatase (P mg Protein)	Protein (P gm tissue)	Lipid. (P gm tissue)	Lipid Peroxidation (P mole/ mg Protein)	Glucose-6-phosphatase (P mg Protein)	Protein (P mg tissue)	Lipid (P gm tissue)
Normal	158.00 ± 10.74	2.73 ± 0.16	365.00 ± 22.60	125.45 ± 4.50	145.00 ± 6.68	3.16 ± 0.17	395.00 ± 18.30	233.33 ± 14.67
CCl_4	225.71 ± 12.50	0.50 ± 0.02	497.50 ± 33.33	157.89 ± 16.67	241.67 ± 18.00	0.50 ± 0.01	497.50 ± 23.67	239.41 ± 17.70
Liquid Paraffin	180.57 ± 8.33	0.54 ± 0.02	462.50 ± 37.60	245.85 ± 22.00	290.00 ± 21.14	0.83 ± 0.03	300.00 ± 17.86	700.00 ± 46.33
Liquid Paraffin + CCl_4	316.00 ± 22.75	1.01 ± 0.01	497.50 ± 41.26	213.33 ± 19.10	241.67 ± 11.49	1.16 ± 0.40	430.00 ± 27.54	360.00 ± 19.92
Mandur bhasma	045.15 ± 2.90	1.71 ± 0.01	497.50 ± 34.91	129.41 ± 5.63	192.85 ± 10.50	3.80 ± 0.14	395.00 ± 21.11	83.33 ± 4.00
CCl_4 + Mandur Bhasma	270.86 ± 16.67	2.83 ± 0.01	300.00 ± 20.15	431.82 ± 30.78	241.67 ± 17.75	3.24 ± 0.17	262.50 ± 19.32	222.22 ± 19.11
Liquid Paraffin + Mandur bhasm	135.43 ± 9.34	1.16 ± 0.01	430.00 ± 29.40	275.85 ± 19.25	145.00 ± 7.72	3.03 ± 0.20	330.00 ± 20.00	325.00 ± 22.45
Liquid Paraffin + Mandur bhasma + CCl_4	045.15 ± 2.16	2.71 ± 0.01	462.50 ± 21.23	160.00 ± 8.42	192.85 ± 8.40	3.33 ± 0.19	300.00 ± 18.69	333.33 ± 19.00

proteins of liver as well as heavy basophilia in hepatocytes.

CCl_4 + mandur bhasma showed increased lipid peroxidation, marked increase in lipids and simultaneously increased glucose-6-phosphatase activity. When these observations are evaluated with the histological architecture it seems about 60 % of the liver area is partially recovered or in the process of recovery and hence this may be the metabolic condition where lipid peroxidation and glucose-6-phosphatase dependent metabolisms are at equilibrium or cells may be adapting the protective mechanisms those are possibly adapted *in vivo* by cells e.g. 'structural isolation from the unsaturated lipids making double bond with proteins' as stated by Barber and Bernheim (1967) or manipulating the presence of biological oxidants viz. SH compounds, glutathion, SH proteins, cysteine, methionin as suggested by Fridivich 1975 /and Tappel (1968) or may be maintaining low levels in internal oxygen tension (Philpoff *et al.*, 1974). But conclusive proof is needed to point out any one of the above suggestions.

In liquid paraffin + Mandur bhasma treated rats lipid peroxidation was below the normal levels with increased lipids and low levels of glucose-6-phosphatase activity this may be indicative of low levels of all the microsomal (ER) activities which can be supported by the histological picture of hepatocytes which shows coagulated type of necrosis as described in Chapter III.

In CCl_4 + liquid paraffin + Mandur bhasma treated rats, lipid peroxidatioon was decreased with decrease in lipid content and with normal levels of glucose-6-phosphatase activities. These observations when evaluated with the histological picture of the liver more than 75 % of the recovery of hepatocytes indicate almost cured picture of liver.

Thus the results indicate that some intrinsic methods which are suggested above of curing or recovery which are induced by mandur bhasma in the liver and are assessed on the basis of histology, lipid peroxidation and recovery of membrane of microsomes as expressed by glucose-6-phosphatase activity.

The levels of peroxidation in normal rat kidney were 145.00 p moles/mg protein. CCl_4 treatment increased 1.66 fold lipid peroxidation in kidney while liquid paraffin treatment also increased 2 fold lipid peroxidation in kidney. CCl_4 administration along with the vehicle liquid paraffin has not altered the value expressed in kidney of CCl_4 kidney rats. Mandur bhasma alone had increased the lipid peroxidation 1.33 fold. CCl_4 + mandur bhasma treatment had not altered the levels expressed of lipid peroxidation in kidney of CCl_4 treated rat. Liquid paraffin + Mandur bhasma treatment brought the levels of lipid peroxidation to normal. CCl_4 + liquid paraffin + Mandur bhasma treatment increased the lipid peroxidation by 1.33 fold.

The comparison of Lipid peroxidation, glucose-6-phosphatase and enzyme activities /lipid content and protein content is given in Table.5

In CCl_4 treated rats just like liver; kidneys also showed increased lipid peroxidation and decreased glucose-6-phosphatase activities with no change in lipid content.

The evaluation with the histology indicates foggyness in kidney proximal tubules.

The treatment with liquid paraffin only increased lipid peroxidation with the tremendous increase in lipid content and marginal increase in glucose-6-phosphatase activity. The lipids or liquid paraffin may be accumulated in kidney with partial maintenance of glucose-6-phosphatase activity in kidney. In histological picture of the kidney the cloudyness of proximal tubules was very much evident showing some deposits may be lipid and/or liquid paraffin or metabolized product of liquid paraffin. The decrease in enzyme activity reveals suppression of enzyme related metabolisms.

Liquid paraffin + CCl_4 together decreased the lipid peroxidation as compared to only CCl_4 treated rat along with two fold increase in glucose-6-phosphatase activity showing that some relief with altered metabolism. From histological architecture it seems that some deposits which remained unstain may be removed. It may have been lipid since lipid content dropped to the half the value.

Mandur bhasma alone elevated the lipid peroxidation values marginally with conspicuous drop in lipid content while significant increase in glucose-6-phosphatase activity showing mandur bhasma induced increased activity of metabolisms related to glucose-6-phosphatase enzyme.

Mandur bhasma treatment to CCl_4 treated rats has not altered the levels of lipid peroxidation those were observed in kidneys or any alteration in lipids, but with significant rise in glucose-6-phosphatase activity. When these observations are evaluated considering the histological observations it gives partial recovery of prooximal tubules from the foggyness which may be the result of increased enzyme activity and lipid peroxidation thus maintaining both the activities at equilibrium and protecting the membranous part of ER or in in vivo condition biological anti-oxidants, isolation of the structure or low oxygen tension may be cause of high levels of lipid peroxidation along with high levels of Glucose-6-phosphatase activity (Fridivich, 1975, Tappel, 1968; Barber and Bernheim, 1967; Philpoff et al 1974).

Liquid paraffin + Mandur bhasma treatment depleted the high levels of lipid peroxidation showed in kidneys in only liquid paraffin treated rats with signifiicant increase in glucose-6-phosphatase activity. Thus repeating the situation that was observed in CCl_4 + mandur bhasma treated rats keeping both peroxidative activities and membrane bound enzyme activities at

equilibrium or offering *in vivo* protection suggested earlier (Fridivich, 1975; Tapel, 1968; Barber and Bernheim,1967; Philoff *et al.*,1974).

Similar are the alterations taking place in CCl_4 + liquid paraffin + mandur bhasma treated rat kidneys; and may be mimicking similar type of equilibriums of the peroxidative events and glucose-6-phosphatase activity related events may be through *in vivo* protections suggested above. The histological picture shows recovery or curing kidney out of stress.

Thus mandur bhasma exhibits curative effect on liver from CCl_4 treated induced injury. Recovery of kidney follows the liver recovery.

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