DISCUSSION

Animal studies are essential to understand MeOH toxicity as MeOH tends to affect almost all the physiological systems in a body. Rodents are less susceptible to MeOH toxicity (LD$_{50}$ 6–7 g/kg ip) (Smith and Taylor 1982) and have been shown to effectively oxidize formate to CO$_2$ through the folate-dependent one-carbon pool. Since the results need to be extrapolated to human situation, a modification in the folate system in the rodent model is necessary. Further, it has been reported that even a moderate folate deficiency did not lead to any significant change in the LD$_{50}$ of MeOH even though there was an increase in blood formate levels (Smith and Taylor, 1982). Smith and Taylor (1982) concluded that urinary excretion was a primary means by which mice and probably also rats eliminate high level of blood formate and thus the increased susceptibility of primates to MeOH is due to formate toxicity. Hence, rats were fed with a folate deficient diet (FDD) for 45 days and folate deficiency was confirmed by the FIGLU excretion in urine. FIGLU has been reported to accumulate in the urine of folic acid deficient rat (Rabinowitz and Tabor, 1958, Spray and Witts, 1959) and is conclusive that the animals fed with FDD, had definitely become a folate deficient model.

Oxygen is essential for life. It has been estimated that about 1-4% of consumed oxygen is converted to O$_2^-$ and H$_2$O$_2$ at the mitochondrial level due to electron leakage (Turrens et al., 1982, Konstantinov et al., 1987) which results in free radical generation. ROS can increase the permeability of blood brain barrier after tubulin formation, and inhibit the mitochondrial respiration. If unchecked, it can lead to a geometrically progressing lipid peroxidation (Gilman et al., 1993). This relay needs to have a control.
A free radical or ROS is a compound with one or more unpaired electrons in its outer orbital (Jesberger and Richardson, 1991). Such unpaired electrons make these species (ROS) very unstable and therefore quite reactive with other molecules, as they try to pair with electrons and generate a more stable compound. The generation of free radicals is considered a physiological process, still they do not cause any damage as there are regulatory mechanisms to control their action (Slater, 1981). Antioxidant enzymes in the brain include Cu/Zn superoxide dismutase (SOD-1) and Mn superoxide dismutase (SOD-2) both catalyzing the reaction of O$_2^·$ to H$_2$O$_2$. Catalase and glutathione peroxidase (GPx) subsequently catalyze the conversion of H$_2$O$_2$ to H$_2$O.

The brain is the most susceptible organ to oxidative damage due to its high oxygen demand (Commenges et al., 2000, Halliwell, 1992). Although the brain accounts for less than 2% of the body weight it consumes 20% of the basal oxygen uptake. High oxygen consumption is linked to leakage of electrons along the respiratory chain with subsequent radical formation. A second reason is the high amount of PUFA's present in neuronal membranes that take part in lipid peroxidation (Floyd, 1999). Free radicals like molecular, sigma and delta singlet oxygen, hydroxyl radical anion, superoxide anion radical, perhydroxide radical, hydrogen- and lipid peroxides are called reactive oxygen intermediates (ROI) or oxygen free radicals (OFR) (Fridovich, 1978). Concentration of vitamin A, E, reduced glutathione (GSH) and activities of antioxidant superoxide dismutase (SOD) and glutathione peroxidase (GPX) was found to be significantly higher in the embryonic liver tissue than in the brain, whereas concentration of ascorbic acid was higher in the latter organ (Sura et al., 1996, Gaal et al., 1995). Of all the body tissues, the brain contains the highest concentrations of ascorbic acid (Rice, 2000). Vitamin C is able to scavenge a broad spectrum of ROS and is therefore one of the most important exogenous antioxidants in the body. "Oxidative stress" is the general phenomenon of oxidant exposure and antioxidant depletion, or oxidant-
antioxidant imbalance (Bidlack et al., 1998, Erasmus, 1993) The CNS is vulnerable to free radical damage because of brain's high oxygen consumption, its abundant lipid content, and the relative paucity of antioxidant enzymes as compared with other tissues (Skaper et al., 1999) Moreover, brain has a high ratio of membrane surface area to cytoplasmic ratio, extended axonal morphology prone to injury, and non-replicating neuronal cells

Consequences of free radical excess

Oxygen radicals can also cause formation of the protein peroxides (Gebicki and Gebicki, 1993) Changes of the primary structure of proteins cause modification in their secondary and tertiary structures Free radicals generation also result in denaturation, aggregation and fragmentation of protein molecules (Da Vies, 1987) It has been reported that free radical-induced oxidative stress leads to increased amounts of oxidized proteins in cortical synaptosomal membranes (Hensley et al., 1995, Butterfield et al., 1996) Butterfield et al. (1996) suggested that the deficits in memory and learning may be related to altered membrane protein structure due to free radical oxidative stress Free radical-mediated damage to neuronal membrane components has been implicated in the etiology of many neurodegenerative disorders, especially Alzheimer's disease (Butterfield et al., 1994, Butterfield, 1996)

Possibility of MeOH exposures and its effect on CNS

In the atmosphere, MeOH is mainly present in the vapor phase MeOH has low ozone forming potential, especially when compared to constituents in gasoline like aromatic and olefinic hydrocarbons, and MeOH is generally less volatile than gasoline
Most information on MeOH toxicity in humans is related to acute exposures (HEI, 1987) Exposure to a toxic dose of MeOH results initially in a transient central nervous system depression, which is similar to ethanol, but much weaker than that produced by ethanol. The anesthetic effect is probably caused by MeOH itself (Li et al., 1999, Kinoshita et al., 1998). These information lead to the analysis of free radical changes in brain though earlier reports were focused on liver, which is a major organ for MeOH metabolism. Further, supplementation to reduce free radical-induced damages by MeOH is also found to be scanty.

**MeOH affects vision**

MeOH is recognized as a serious neurotoxin capable of producing severe visual impairment and blindness (Eells, 1992, Roe, 1955) Formic acid is the toxic MeOH metabolite, responsible for both the metabolic acidosis and the ocular toxicity characterizing human MeOH poisoning (Hayreh et al., 1980, Tephly and McMartin, 1984) Formic acid disrupts mitochondrial electron transport and energy production (Hayreh et al., 1980, Sharpe et al., 1982) by inhibiting cytochrome oxidase activity, the terminal electron acceptor of the electron transport chain (Nicholls, 1975, 1976) In the eye, the optic nerve (Baumbach et al., 1977) and the retina (Murray et al., 1991) are sensitive tissues. The Retinal Pigment Epithelium (RPE) undergoes oxidative stress because it is metabolically very active (Miceli et al., 1994, Kennedy et al., 1995), there are large oxygen fluxes across its boundaries (Wangsa-Wirawan and Linsenmeier, 2003). In MeOH-intoxicated rats, retinal ATP and ADP are depleted (Seme et al., 2001), and photoreceptors and the adjacent retinal pigment epithelium show functional and/or structural changes. With MeOH exposure of the animals, functional decrements are detected in photoreceptor cells by electroretinography (Seme et al., 1999) and both photoreceptors and retinal pigment epithelial cells show morphologic changes (Murray et al., 1991) Hence, in this study care was
taken to see the free radical status in optic nerve and retina by analyzing the scavenging system

**Antioxidant supplementation in alcohol intoxication**

Ethanol is involved in the generation of free radicals intercellularly as well as intracellularly (Repetto and Llesuy, 2002). Nordmann et al (1990) reported that an acute ethanol load enhanced the lipid peroxidation in the cerebellum. This is accompanied by an increase in the cytosolic concentration of low-molecular-weight iron derivatives that may contribute to the generation of aggressive free radicals. They further suggested that ethanol-induced decrease in the main antioxidant systems (superoxide dismutase, α-tocopherol, ascorbate and selenium) is likely to contribute to the cerebellar oxidative stress and most of these disturbances can be prevented by allopurinol administration. Sergent et al (2005) reported that vitamin E (a free radical chain-breaking antioxidant) prevented the ethanol-induced increase in membrane fluidity. Koyuturk et al., (2004) suggested that the combination of vitamin C, vitamin E and selenium has a protective effect on ethanol-induced duodenal mucosal injury. Both vitamin E and beta-carotene were found to protect rat neurons against oxidative stress induced by ethanol exposure (Copp et al., 1999). Ethanol-induced free radicals and hepatic DNA strand breaks are prevented *in vivo* by antioxidants (Navasumrnt et al., 2000) and reports are available that vitamin C, vitamin E or vitamin E and selenium exert a synergistic effect in the prevention of biological membranes from ethanol induced oxidations (Navasumrnt et al., 2000; Liu and Cho, 2000).

**Supplementation is essential**

The acceleration of free radical production or the accumulation of free radicals can lead to the exhaustion of the defence mechanisms. These defence mechanisms involve numerous molecules and enzymes. Generally, molecules that
can neutralize free radicals by accepting them are called scavengers (Slater et al., 1981) which forms the antioxidant category. A number of in vitro studies have shown that antioxidants – both endogenous and dietary can protect nervous tissue from damage by oxidative stress (Contestabile, 2001) It has been reported that the brain is deficient in oxidative defense mechanisms and hence is at a great risk of damage mediated by reactive oxygen species (ROS) resulting in molecular and cellular dysfunction (Gupta et al., 2003) It is clear that brain is vulnerable to oxidative stress, especially when a toxicant can modify the physiological balance between anti- and pro-oxidant mechanisms (Gonthier et al., 2004) Based on these reports, an effort is made to find an antioxidant supplement therapy during MeOH intoxication and the choice is α-LA.

**Actions of LA as an antioxidant**

LA and its reduced form, dihydrolipoic acid, are natural components of biologic membranes, acting as mitochondrial lipoamid co-factor of α-cetoacid de-hydrogenase (Reed and Hackert, 1990, Liu et al., 1995) The mitochondria are the main site of antioxidant action of LA Within the intracellular space, the dihydrolipoic acid is produced from LA and acts as an antioxidant in the extracellular space (Packer et al., 1995) The exogenous LA is rapidly reduced to dihydrolipoic acid, in mitochondria the enzyme lipoate de-hydrogenase induces reduction of LA using NADH as the co-factor (Brewenga et al., 1996), in the cytosol the enzyme glutathione reductase actively participates in this reduction using NADPH as the co-factor (Handelmann et al., 1994) Roy and colleagues (1997) suggested that LA could reduce NADH levels, using it as a co-factor during its reducing action of stress During oxidative stress, both LA and dihydrolipoic acid act as free radical scavengers and the final oxidative stress justifies the observed LA anti-oxidant activity.
Momi et al. (2002) emphasized the antioxidant and pro-oxidant activities of LA and dihydrolipoic acid. There are many evidences that both LA and dihydrolipoic acid exhibit direct free radical scavenging properties and that there are only in vitro evidence available for their pro-oxidant properties. Cardoso do Vale et al. (2003) reported that when LA is infused directly into the common carotid artery, it rapidly gained access to cerebral parenchyma without systemic enzymatic reduction. In this study, administration of LA to control animals showed an increase in the brain GSH level in all the regions except cerebellum and hypothalamus. The association between GSH and LA as well as its relationship with vitamin C had been documented. In vitro, human T-lymphocyte cells, bathed in various concentrations of LA, increased the intracellular level of GSH, which had a positive correlation to the concentration of LA added in the medium (Han et al., 1995). LA is rapidly converted to DHLA once inside the cell, it is then transported out of the cell and used as an antioxidant (Packer et al., 1995). Collectively, Han et al. (1995) suggested that DHLA is a contributing factor to the GSH increase after the addition of LA. Lipoic acid raises GSH values by increasing cysteine availability (Han et al., 1997), which is the rate-limiting factor in GSH biosynthesis. Lipoic acid also causes faster ascorbic acid recycling (Xu and Wells, 1996). One of the most important properties of LA is its ability to regenerate vitamin C, GSH and indirectly vitamin E. Old rats supplemented with LA showed a higher mitochondrial membrane potential, ambulatory activity, with higher GSH and ascorbate concentration (Hagen et al., 1999). This is well in agreement and correlates with the increase in GSH level observed in LA treated group, appears that it is quite natural after LA administration.

**Brain hydroxyl and superoxide radicals**

The superoxide radical is the most well known oxygen derived free radical (Yu, 1994) and leads to the formation of additional reactive species. Effective
antioxidant defence requires concerted actions of both superoxide dismutase (which produces hydrogen peroxide from superoxide) and glutathione peroxidase system that removes the hydrogen peroxide (Rabaud et al, 1997, Favier, 1997) Superoxide dismutase catalyze the reaction of superoxide radicals to yield hydrogen peroxide and oxygen (Fridovich, 1972, Mccord and Fridovich, 1969) H₂O₂ formed within cells may be the destructor of catecholamine nerve terminals through 6-hydroxydopamine or 6-aminodopamine (Heikkila and Cohen, 1971, 1972, Heikkila et al, 1973)

In the present study, it is clear that MeOH intoxication generates free radicals in the brain and it can result in brain injury as indicated by the increase in superoxide radical and hydroxyl radical in the folate deficient animals It was shown that MeOH increased superoxide anion radical production in liver microsomes (Skrzydlewskas, 1996, Skrzydlewskas and Farbiszewski, 1996, 1997, 1999) Superoxide anions and hydroxyl radicals formed during aldehyde oxidation can oxidatively modify amino acid residues of proteins, in particular, residues of aromatic and sulfhydryl amino acids (Da Vies, 1987, Kleinveld et al, 1989) Apart from its own toxicity, hydrogen peroxide in the presence of iron leads to the generation of toxic hydroxyl radicals (Blake et al, 1987). Hydrogen peroxide, the product of this reaction, is more toxic than the oxygen-derived free radicals and requires to be scavenged further by tissue thiols (glutathione redox pathway) and catalase (Bannister et al, 1987, Fridovich, 1995) Generated hydroxyl methyl derivatives may further react with nucleophilic groups and form methylene bridges and this formation participated mainly by lysine residues, (rarely) arginine and other residues (Tome et al, 1985)

The MeOH group in this study also showed normal superoxide and hydroxyl radical level Other groups receiving MeOH with normal diet did not deviate from controls as the rodents have the ability to metabolize MeOH faster
(Tephly and Mc Martin, 1984) FDD fed LA supplemented animals showed normal superoxide radicals whereas FM group showed marked elevation in superoxide radicals. However, in this study, the FLM group could only reduce the increased hydroxyl than the FM rats, which suggests that the dose requirement may probably be more

**SOD**

The first line of defense against ROS is SOD. The function of this enzyme is to convert two superoxide radical molecules into oxygen and hydrogen peroxide (Fridovich, 1975). Hydrogen peroxide is handled by several mechanisms: CAT causes direct breakdown of hydrogen peroxide to oxygen and water. Another mechanism of $H_2O_2$ detoxification is its reduction by reduced GSH catalyzed by the enzyme GPx. This enzyme not only protects against hydrogen peroxide, but also has the ability to react with lipid peroxides (Comporti et al, 1973).

In this study, no alteration was observed in any of the brain regions of the controls fed with LA as well as MeOH treated control rats. This indicates the ability of the animals to metabolize the dosage of MeOH used in this study. However, a uniform increase in the SOD was observed after acute MeOH intoxication in the folate deficient status in all the brain regions. This may be due to the uniform distribution of MeOH in all the brain regions as MeOH is freely permeable through membrane and blood brain barrier. This increase in SOD may be a protective measure to catalyze the conversion of superoxide anions to hydrogen peroxide and molecular oxygen. Some authors showed that oxidative stress could result in antioxidant enzyme induction (Goligorsky et al, 1993). Further, strengthened by the report that in rats intoxicated with MeOH, the activities of SOD and CAT were elevated probably due to the formation of superoxide anion (Skrzydlewska and Farbiszewski, 1997). It is clear that
consumption of MeOH provokes changes in the activity of antioxidant enzymes. In FLM group, though the SOD level in most of the regions was found to be normal, only in cerebral cortex and hippocampus it markedly decreased from the FM animals indicating that in these regions the free radical generated may be more or the enzyme activity might be insufficient. Manoli et al (2000) and Baek et al (1999) concluded in their study that the vulnerability to oxidative stress in the brain is region specific.

In this study, it was observed that one-day acute MeOH exposure leads to an increase in SOD activity in retina as well as in optic nerve. However, in the retina of rat intoxicated with MeOH for 3 weeks Sayed et al (2002) observed that MeOH drastically altered the antioxidant defense system and they could observe a highly significant depletion of glutathione levels and inhibition of superoxide dismutase activity, concurrent with significant increase in thioctic acid reactive substances. Moreover, they also reported that supplementation with vitamins E and C to MeOH intoxicated animals showed an effective relief. The histopathological and ultrastructural study of retina in rats showed preservation of the membranous structures of the retinal cells, especially mitochondria that assumed their normal shape. This may be attributed to the inhibition of free radical production and lipid peroxidation and subsequently minimum degree of tissue damage. It appears that supplementation of antioxidants during acute as well as prolonged exposure are beneficial.

The discrepancy in SOD activity between this report and the present study is due to difference in the duration of MeOH exposure. However, FLM group showed a marked decrease in SOD activity when compared to FM which also came to normal except in cerebral cortex and hippocampus. Even retina and optic nerve showed a normal SOD activity after supplementation with LA. Voloboueva et al (2005) suggests that the protective effect of LA involves multiple pathways.
and that LA could be effective against age-associated increase in oxidative stress and mitochondrial dysfunction in retinal epithelial cells and support the use of LA.

**GPx and CAT**

GPx is a major antioxidative enzyme in many tissues and speculated to play a major role in the antioxidative mechanism of the brain (Barlow-Walden *et al.*, 1995). The effectiveness of GPx activity is more due to its placement in both mitochondria and cytosol. CAT is much more important for clearance of H$_2$O$_2$ in neurons, whereas the glutathione system appears to play a more significant role in glial cells (Dringen *et al.*, 1999). CAT is the marker enzyme for peroxisomes and decomposes H$_2$O$_2$ produced by the peroxisomal oxidases (de Duve and Baudhuin, 1966). According to Dringen and Hamprecht (1997), catalase and GPx can substitute for each other, whereas according to Aebi (1984) the limitation of CAT activity is due to its location at microperoxisomes. In rats, ethanol metabolism occurs mainly due to alcohol dehydrogenase, while mainly CAT causes MeOH oxidation. Together with SOD and the glutathione system, it forms the cellular defense mechanism against oxidative stress (Halliwell and Gutteridge, 1989; Sies, 1991). The presence of CAT mRNA in the cytoplasm of neuronal cells in rat brain, corresponding to the distribution of CAT protein had been reported previously by Moreno *et al.* (1995).

Possible cause for the increased CAT activity in all the brain regions in folate deficient rat must be due to the following fact. It is essential to recollect that in MeOH metabolism, the H$_2$O$_2$ formed by increased SOD activity need to be catabolized by increased CAT peroxidative pathway leading to the accumulation of formate and generation of free radicals. Thus, the elevation in the CAT can be justified, indicating that the free radical protective system is getting activated after MeOH intoxication due to the slow and diverted pathway. However, the
supplementation of LA could normalize the CAT, indicating the effective role of LA in preventing the free radical accumulation.

Treichel et al. (2004) analyzed the retinal pigment epithelial (RPE) catalase and glutathione in the two retinal cell lines to determine the differences in these antioxidant systems contributing to cell-type-specific differences in cytotoxicity. In vitro, cultured cell lines from photoreceptors (661W) and the RPE (ARPE-19) revealed that treatment with the MeOH-derived toxin formic acid produces lower toxicity in ARPE-19 cells line than 661W cell line, due to high antioxidant levels in ARPE-19. Further, they suggested that this is especially true for CAT, as it was markedly higher after formic acid treatment and formic acid induced increased CAT activity in the cultured ARPE-19 cells. From this report, it is clear that increase in activity of CAT is for a better protection and it is due to the formic acid formed from MeOH.

In this study, in the retina, CAT activity significantly increased in FM group when compared to all other groups. But, in optic nerve, the CAT activity remained unaltered in FM compared to the entire group except MeOH group. Increase in CAT activity have been observed in liver homogenates of MeOH-treated rats (Skrzydlewksa and Farbiszewski, 1997) and in cultured retinal pigment epithelial cells subjected to other forms of oxidative stress resulting from treatment with H₂O₂ (Miceli et al., 1994; Tate et al., 1995). One possibility is an increase in the concentration of the enzyme's substrate hydrogen peroxide, which is likely to be produced during formate exposure and which has been speculated to regulate CAT activity (Makar and Mannering, 1968). Probably the increased rate of degradation of MeOH in normal rats when exposed to MeOH may be reason for such changes observed.

CAT acts as a preventive antioxidant and plays an important role in the protection against the deleterious effects of LPO. Inspite of its increased activity,
the accumulated free radicals resulted in an elevation of CAT activity indicating that it is not sufficient to meet the excessively produced free radicals. Parthasarathy et al. (2006) reported that lipid peroxidation and enzymatic and non-enzymatic antioxidants in the acute MeOH exposed group animals were found to be significantly increased in lymphoid organs such as spleen, thymus, lymph nodes and bone marrow of rats and this supports the present findings.

The activities of SOD (dismutation of superoxide to hydrogen peroxide and molecular oxygen) and glutathione peroxidase (an enzyme reducing hydrogen peroxide to water at the expense of reduced glutathione) increased in all the brain regions of FM group in this study, again reinforcing the production of free radicals. Relevantly, it is reported that H$_2$O$_2$ caused oxidative cleavage to Cu, Zn-SOD (Ookawara et al., 1992, Sato et al., 1992).

In this study, in control and MeOH groups as well as in LA group, CAT and GPx activities remained similar whereas both were elevated in all the brain regions in FM group with a uniform increase in LPO. Since LA alone treated group, did not show any variation in CAT and GPx activities from control indicating that LA did not have any role in increasing such enzyme activity. Hence, the increased enzyme activity observed in FM group must be due to the over production of H$_2$O$_2$ as a result of increased SOD activity. During MeOH intoxication, the supplementation of LA normalized the GPx levels in most of the brain regions, completely normalized the CAT activity, whereas in midbrain and hypothalamus it showed a marked increase in GPx increase from controls. It is appreciable that a marked decrease in GPx activity in the entire brain regions studied with reference to FLM group was observed. However, no alteration was observed in the GPx level in the retina as well as the optic nerve of these FM group. Glutathione is found in photoreceptor outer segments and in the retinal pigment epithelium (Beatty et al., 2000). Further, retinal pigment epithelial cells
have higher activity of glutathione peroxidase, an enzyme that uses glutathione as an electron donor to reduce hydroperoxides, than many other tissues including photoreceptors (Handelman and Dratz, 1986, Naash and Anderson, 1989). Probably due to the higher concentration of GPx naturally existing in these regions, no alteration could be observed.

Regarding ROS, relatively little is known about the sensitivity of photoreceptors to oxidative stress, but RPE cells are notably resistant, most likely because of high concentrations of antioxidants (Liles et al., 1991, Ohia et al., 1994). Among the antioxidants that are highly expressed in RPE cells, catalase (Beard et al., 1988) is an enzyme that not only scavenges ROS, but also participates in the catalase peroxidative pathway, which may help oxidize and thus detoxify formate together with the folate-dependent pathway (Palese and Tephly, 1975). This is the major cause behind the retinal damage reported during MeOH poisoning in human.

**Non enzymatic antioxidant**

**Protein thiols**

Proteins constitute the major 'working force' for all forms of biological work. Their exact conformation and pattern of folding are tightly connected to their activity and function. Protein thiols, this term generally indicates the formation of mixed disulphide between proteins and glutathione. Intra protein disulphide bonds are viewed, in classical text books, as part of the tertiary structure of the protein and their formation is an important step in protein folding. Similarly, many disulphide bonds are important in the quaternary structure of proteins, in the formation of homo- or hetero-multimers. Many enzymes have essential thiols and are thus inactivated during oxidative stress, including that induced by oxygen poisoning or radiation (Gerschman et al., 1954). The protein
thiols include the GSH. GSH is the most abundant intracellular non-protein thiol, and moreover, glutathionylation is the main form of S-thiolation. However, outside the cell, for instance in plasma, GSH is virtually absent and the predominant low-molecular-mass thiol is cysteine.

In this study, the protein thiols showed a marked decrease in the FM group. Decrease in brain protein thiols due to oxidative damage has also been reported by Patsoukis et al. (2004; 2005). FLM animals showed normal protein thiol levels as well as a normal GSH level. The role of GSH depletion and protein thiol modification in the development of oxidative cell damage has been explained by Orrenius (1995). Possibly, by correcting the GSH level, LA could have maintained the normal protein thiol level during MeOH intoxication.

**GSH and GSSG**

The tripeptide glutathione (GSH; g-L-glutamyl-L-cysteinylglycine) is the cellular thiol that can function both as reductant in the metabolism of hydrogen peroxide and organic hydro peroxides (Cotgreave and Gerdes, 1998). Reduced GSH is one of the most abundant intracellular thiols in the central nervous system and acts as a major cellular antioxidant by supporting GSH peroxidase-dependent reduction of hydrogen peroxide and organic peroxides (DiMonte et al., 1992; Garcia–Ruiz et al., 1995 ; Meister, 1995). GSH reacts directly with radicals in non-enzymatic reactions (Saez et al., 1990; Winterbourn and Metodiewa, 1994) and is also an electron donor in the reduction of peroxides catalyzed by GPx (Chance et al., 1979). During glutathione peroxidase-catalysed metabolism of hydro peroxides, GSH serves as an electron donor and GSSG formed in the reaction is subsequently reduced back to GSH by glutathione reductase. Due to this interrelation, it is always better to consider the GSH and GSSG together to draw a meaningful conclusion. In addition, GSSG itself can exert deleterious
effects through nonspecific reactions with the free sulphydryl groups of proteins to form mixed disulfides, reactions that can lead to inactivation of enzymes possessing sulphydryl groups at their active sites (Halliwell and Gutteridge, 1989). Hence, the maintenance of GSH levels is important.

It appears that the astroglial cells mostly provide this glutathione system. Even at a cellular ratio of 1 astroglial cell to 20 neurons a significant protection against H$_2$O$_2$ toxicity towards neurons has been observed (Desagher et al., 1996). The first metabolite (formaldehyde is rapidly oxidized by formaldehyde dehydrogenase) is the GSH-dependent enzyme. GSH homocostasis plays a central role in defending tissues against oxidative stress (Sen, 1995; 1997).

In this study, it is interesting to note that when LA administration to control led to an increase in the GSH level and when such animals are exposed to MeOH, no reduction in GSH was noticed in spite of free radical generation. The ability of LA to enhance intracellular content of the crucial endogenous antioxidant GSH has been reported (Han et al., 1995; Sen et al., 1997). Moreover DHLA, which is the reduced form of LA, is known to regenerate major physiological antioxidants of lipid and aqueous phases, such as vitamin E, ascorbate, and GSH (Packer et al., 1995).

Recent studies have uncovered the mechanism by which LA increases cellular GSH (Han et al., 1995; Sen, 1997; Sen et al., 1997). DHLA markedly improves cysteine availability within the cell, resulting in accelerated GSH synthesis (Sen, 1997). It has been reported that reduced LA is able to regenerate both vitamin C and GSH without the use of enzymes (Pfaffly, 2001). This allows the indirect regeneration of vitamin E, helping to prevent lipid peroxidation.
DHLA + GSSG  ➔  LA + 2 GSH

DHLA + DHAA  ➔  LA + vit C

From the foregoing, it is clear that the increased level of GSH observed after LA supplementation is quite possible. LA supplementation is reported to protect against oxidative lipid damage in the heart, liver and red gastrocnemius muscle (Khanna et al., 1999). Glutathione plays an important role in the detoxification of ROS in brain. It was also reported that stress reduces GSH levels and leads to increased levels of ROS (Liu, 1996). This implies that the supplementation of LA is beneficial even during toxic conditions.

In the present study, the GSH was decreased markedly in the FD animals. In FM animals, not only in the brain regions but also in retina and in optic nerve, the GSH levels decreased. In the phase of GSH deficiency, its level increases and usually a hyper compensation is observed. This phenomenon may be explained on the basis of regulatory mechanisms of GSH biosynthesis. γ-Glutamyl cysteine synthetase is down-regulated by cellular GSH levels, a decrease of GSH levels may therefore enhance its biosynthesis. Based on this, Parke and Piotrowski (1996) reported why GSH depletion is only seen following a single high dose of a chemical, repeated exposure studies usually fail to reveal GSH deficit. In this study also, acute exposure to MeOH was studied. Depletion of GSH increases the vulnerability to free radical-induced damage.

The decrease in GSH concentration seems to be clear because the MeOH metabolism also depends upon GSH (Pankow and Jagielski, 1993). Depletion of GSH by formate could result directly from formate-induced peroxidative stress, because inhibition of mitochondrial electron transport has been shown to profoundly increase the production of reactive oxygen species including
superoxide and hydrogen peroxide (DiMonte et al., 1992; Garcia–Ruiz et al., 1995; Meister, 1995). GSH is normally present in high concentration in the retina and has been shown to play a key role in antioxidant defenses (Schutte and Werner, 1998; Winkler and Giblin, 1983). Studies have shown that retinal GSH may be depleted during periods of oxidative stress (Schutte and Werner, 1998; Winkler and Giblin, 1983). GSH reduction can explain additionally a decreased concentration of vitamin C, which enters the cell mainly in the oxidized form where it is reduced by GSH (Briviba and Sies, 1994). Because of the critical involvement of GSH in cellular defence mechanisms, depletion of intracellular GSH under conditions of mitochondrial impairment may augment the susceptibility of the brain as well as the optic nerve and retina to oxidative stress through a perturbation of GSH status. It is essential to note that in FLM group any alteration in GSH level is inhibited. This is note worthy to prevent MeOH induced ocular toxicity.

When oxygen radicals are present in large amounts, GSSG formation exceeds its clearance and the ratio of reduced to oxidized glutathione (GSH/GSSG) decreases. Hence, this ratio is frequently used as an indicator of the level of oxidative stress in cells. Storey (1996) also suggested that the ratio of reduced/oxidized glutathione in the cell is a good indicator of the level of oxidative stress. It may be true because in the present study, the GSH was observed to be normal in FLM animals. However, the ratio still revealed the fact that inspite of LA supplementation the oxidative stress had been encountered as it is further supported by the LPO levels.

**Protein carbonyls**

A carbonyl group is a functional group composed of a carbon atom double-bonded to an oxygen atom. Reactive oxygen species (ROS) are known to convert
amino groups of protein to carbonyl moieties (Chavko and Harabin, 1996, Perry et al, 2000) Oxidative modification of protein leads to increased recognition and degradation by proteases and loss of enzymatic activity (Davies and Goldberg, 1987, Rivett and Levine, 1990) Several studies (Oliver et al, 1987, Stadtman and Oliver, 1991, Stadtman, 1992) reported that protein carbonyl modification primarily mount from iron catalyzed oxidation Parihar and Pandit (2003) suggested based on their data on protein carbonyl that all regions of brain are not equally susceptible for oxidative insults However based on the results of the present study, it may be that even distribution of MeOH may lead to uniform increase in protein carbonyl formation

In this study, the FM animals showed marked increase in the protein carbonyl content compared to the entire groups studied whereas the protein carbonyl content was markedly reduced after LA treatment in FLM group in all the regions while in hypothalamus, completely normalized. It appears that in MeOH intoxication the metabolite formed may be responsible for such increased protein carbonyl formation in brain rather than the free radicals accounted in this study. Formaldehyde is the most chemically reactive aldehyde, so it cannot be found as a free compound in MeOH intoxication (McMartin et al, 1979). It reacts most easily with cysteiny1, methionyl, lysyl, arginyl and tyrosyl residues, and to a smaller degree with other amino acid residues and peptide bonds of proteins (Skrzydlewska, 1994, Tome et al, 1979). Moreover, they added that the greater reactivity of formaldehyde than that of acetaldehyde is a reason for bigger changes in protein structure and proteolytic activity after MeOH than ethanol intoxication.

Accumulation of oxidative damage to antioxidant proteins, especially Cu, Zn-SOD may cause dysfunction of defense systems against oxidative stress. Increase in carbonyl modification of Cu,Zn-SOD was reported with increasing concentrations of H2O2. When Cu,Zn-SOD is incubated with relatively high levels
of H$_2$O$_2$, it may become inactivated and then releases copper which can, with the H$_2$O$_2$, constitute a Fenton system. H$_2$O$_2$ reacts with copper leading to form bound hydroxyl radical such as Cu(I)-hydroperoxo complex, which may release OH (Kawanishi et al., 2001). This type of damage caused to antioxidant system by MeOH and its metabolite could play a vital role for the imbalanced antioxidant defence system in the brain and fully supported by the elevated protein carbonyl level observed.

**LPO**

Oxidative damage is a continuously ongoing process, and low levels of reactive oxygen species and markers of oxidative stress are detectable in tissues of animals even at rest (Chance et al., 1979; Floyd, 1995). This is a fact as in this study also that the control itself had measurable LPO levels. LPO is initiated by the abstraction of a hydrogen atom from the side chain of polyunsaturated fatty acids in the membrane. To remove the continuously generated free radicals, the endogenous antioxidant enzymes increase, which prevent oxidative cell damage (Kalra et al., 1994). Hence, when the generation of reactive free radicals overwhelms the antioxidant defense, LPO of the cell membrane occurs. From the foregoing reports, it is clear and comprehensive that MeOH induces increased free radical load in the brain.

The increased free radical generation is indicated after MeOH exposure by the LPO level in spite of increased levels of enzymatic antioxidant and decrease in non enzymatic antioxidant levels in the entire brain regions in FM animals. LA treatment was found to reduce the LPO level but could not totally prevent the alteration in the LPO levels except in cerebellum while all the other regions showed increased LPO levels compared to control. LPO is measured as MDA. In different situations such as irradiation, maximal increase in MDA content was
observed in brain, testis, spleen, kidney and liver and protection by LA pretreatment in terms of MDA content was partial in brain (51.67%) (Manda et al., 2007). LA and its metabolites are mostly hydrophilic/hydrophobic dual antioxidants, strengthening the antioxidant defense network and potentiating the effects of vitamin E may be a more important mechanism by which LA may protect against lipid peroxidation, as reported previously (Packer et al., 1995; 1997). Agar et al. (2004) also reported that the cerebral hemispheres of adult rats are susceptible to the oxidative neurotoxic effects of ethanol, which may partly be antagonized with vitamin E treatment. All these reports point out that treatment with LA reduce the LPO level in all the samples studied.

The elevation of free radicals in the brain could not be ignored. In the presence of trace amounts of Fe or Cu, O$_2^-$ and H$_2$O$_2$ initiates reaction sequences which form highly reactive hydroxyl radicals (Thompson et al., 1987). The peroxidation of lipids can be initiated by free radicals (Girotti, 1985; Thompson et al., 1987). The hydroxyl radicals initiate self-propagating reactions leading to peroxidation of membrane lipids and destruction of proteins (Asada and Takahashi, 1987; Halliwell, 1987 ; Bowler et al., 1992). Free radicals-mediated LPO can result in membrane disorganization and subsequently the decrease in membrane fluidity had been reported (Fernandez and Videla, 1981; Jaya et al., 1993). Lipid peroxidation byproducts have been widely recognized to increase membrane fluidity either by interacting with membrane proteins (Buko et al., 1996; Subramaniam et al., 1997) or more directly by their own rearrangement (Jain et al., 1994; Gabbita et al., 1998). The LPO level is not only an indicator of oxidative stress but also membrane injury and alteration in the cellular functions. The membrane injury causes disruption of the tissue integrity (Bagchi et al., 1999). It causes the structural and functional changes of the membranes (Chen and Yu, 1984). ROS, by oxidizing tubulin, are also able to disrupt the microtubule cytoskeleton and thereby increase membrane fluidity (Yoon et al.,
1998; Remy-Kristensen et al., 2000). Oxidative stress leading to inactivation in various tissues, particularly in brain neurons has been reported (Lehotsky et al., 1999).

Such a change in proteins and lipid has been reported in alcohol intoxication. Free radicals generated during the metabolism of alcohols can react with proteins and lipids changing their structure and functions (Aust et al., 1993; Skrzydlewska and Farbiszewski, 1995). The volume of the lipid peroxidation marker, lipofuscin was significantly reduced in the brain of adult rats chronically exposed to ethanol (de Freitas et al., 2004).

**Expression of Heat Shock Protein (Hsp)**

Heat shock proteins represent several families of cellular stress-response proteins, some of which are expressed constitutively and others expressed largely under conditions of stress (Welch, 1992). Heat-shock proteins are so-called chaperones. This term is used to classify these proteins because they help the long chains of amino acids correctly fold into shape as functioning proteins so they do not clump together. Hsp is also reported to play a role in the assembly and/or disassembly of other proteins, as well as to facilitate the translocation and insertion of other proteins into membranes of cytoplasmic organelles (Chiroco et al., 1988; Deshaies et al., 1988; Cheng et al., 1989). The well-characterized program of gene expression, leading to the synthesis of Hsp's exerts cytoprotective functions (Sorger, 1991). In support of a protective function, the presence of Hsps has been correlated with reduced damage to hippocampal cells of the brain caused by ischemic insult (Nowak, 1990; Nowak and Jacewicz, 1994), reduced glutamate-precipitated cytotoxicity in cerebellar granule cells (Lowenstein et al., 1991). RNA in situ and immuno histochemical studies revealed that mortalin, a unique member of Hsp70 in rat tissues showed a higher expression in
neurons and nerve fibres than in surrounding glial cells in brain (Kaul et al., 1997).

In this study, the control animals showed very little expression which is well in agreement with many reports suggesting that the inducible 70 kDa heat shock protein Hsp70, is normally not present in the rat brain and it is induced in vulnerable brain cells in response to a number of different manipulations that induce cellular injury paradigms (Massa et al., 1996; Sharp and Sagar, 1994). The possible cause for the increased expression of these Hsp70 in FM group may be due to the elevated levels of free radicals as indicated by the scavenging enzyme levels and a decrease in the biological antioxidants. It appears that the role of Hsp is to protect the oxyradical-induced changes as oxygen radical induced synthesis of stress proteins leading to tolerance to oxidative stress has been reported (Marini et al., 1996).

In this study, LA treatment to normal animals decreased the expression of Hsp 70 protein compared to control, probably due to their free radical scavenging ability whereas it was similar to that of controls in western blot study indicating translation is reduced for the Hsp 70 protein by LA. Based on the experiments, few reported the fact that stress protein expression, in general is strongly regulated at the level of transcription level and the differences occur in the levels of translation levels into differences in the amounts of protein (Wu 1995, Morimoto et al., 1997) and this report supports the present observation.

FLM group showed marked decrease in Hsp70 expression from the FM group, but it was not normalized. This may be due to the excess production of free radical, which was still elevated in the FLM animals as indicated by the carbonyls and LPO levels. Several studies have demonstrated the induction of Hsp by oxidative stress (Polla et al., 1987; Jornot et al., 1991; Heufelder et al., 1992; Lu et al., 1993). Moreover ROS play an important role in the induction of the binding
activity of heat shock factor-1 and the accumulation of mRNA for Hsp70 and Hsp90 in ischemic reperfused heart (Nishizawa et al., 1999) had been reported.

It has been reported that LA is an antioxidant that exerts anti-inflammatory effects in monocytes by inhibiting tumor necrosis factor production (Kiemer et al., 2002) and adhesion molecule expression (Lee and Hughes, 2002) thereby indicating LA interference with protein expression. In this study, the FD animals showed more expression of the protein compared to control as well as LA supplemented group.

Free radicals generated in the brain are also reported to influence gene expression, subsequently effecting apoptosis and neuronal death (Gilgun-Sherki et al., 2002). The increased activity is caused by the induction of gene expression because increased dismutation of the superoxide anions accelerates the generation of hydrogen peroxide, and it is known that oxidants including hydrogen peroxide are able to induce the expression of genes encoding antioxidant system enzymes. Such an induction of antioxidant enzymes provoked by hydrogen peroxide has been observed in human fibroblast culture (Rushmore et al., 1991). Free radicals generated in the brain are also reported to influence gene expression, subsequently effecting apoptosis and neuronal death (Gilgun-Sherki et al., 2002). The ethanol-induced decrease in the main antioxidant systems is a likely contributor to the cerebellar oxidative stress (Nordmann et al., 1990).

Since overexpression of Hsp70 in a cultured neuronal cell model has been reported to reduce both aggregate formation and cell death (Takeuchi et al., 2002), the increased expression of Hsp70 in the FM animals than the LA supplemented group indicates that Hsp70 elevation may be due to increased free radical encountered. However, LM animals showed marked increase from control indicating that some metabolites other than free radical may also play a role.
During MeOH intoxication, the metabolites formed are more toxic. Brain samples of rats when stained for Hsp70 immuno-histochemically, after formaldehyde gas inhalation during the early postnatal period, Hsp70 neurons were found in the hippocampus of rats (Songur et al., 2004) confirm this view. Further, strengthened by Tephy’s (1991) suggestion, the importance of MeOH-metabolism products in poisoning was confirmed by alleviating manifestations of poisoning by using inhibitors that slow MeOH oxidation.

Formic acid apparently penetrates mitochondria where it exerts its toxic effect by inhibiting cytochrome oxidase (Nicholls, 1975). Formic acid is believed to be the species that binds to and inhibits cytochrome oxidase, and the apparent affinity of formate for cytochrome oxidase increases as pH decreases (Nicholls, 1976). Cell death from formate exposure is believed to result from inhibition of the mitochondrial electron transport chain which reduces ATP to levels too low to perform essential cell functions dependent upon aerobic metabolism, and which increases reactive oxygen species (ROS) secondary to electron transport chain blockade, subjecting cells to increased oxidative stress. Complicating the cell’s ability to survive the ROS increase is that antioxidants may become depleted as a consequence of inhibition of mitochondrial function (DiMonte et al., 1992). Some et al. (2001) showed that the antioxidant glutathione is decreased in retina after MeOH intoxication. Photoreceptors may be more sensitive than retinal pigment epithelial cells to both the increased ROS and the ATP depletion that are consequences of formate exposure.

From the foregoing collectively, one can presume that not all changes in LPO and protein carbonyl may be mediated exclusively by free radicals, rather the metabolites of MeOH may be held responsible, at least partially.
C-Jun N-terminal kinase 3 (JNK3)

JNKs, also known as stress-activated kinases (SAPK), are a family of three (JNK1, -2, -3) kinases, which are activated by numerous stimuli including cytokines, osmotic stress, and death inducing stimuli such as growth factor deprivation damage, dopaminergic toxins, and ischemic insult (Lin et al., 1995, Park et al., 1996; Morris et al., 2001, Hunot et al., 2003, Hayley et al., 2004). JNK3 is expressed predominantly in neurons but also in cardiac smooth muscle and testes, which has 2 isoforms. Several coexisting mechanisms guarantee the specificity of JNK in signal transduction (Chang and Karin, 2001, Pearson et al., 2001). It is important to emphasize that in some instances of neuronal injury, sustained c-Jun induction/activation does not necessarily lead to death (Herdegen et al., 1998, Raivich et al., 2004). JNK activation leads to induction of TNF-α expression (Reap et al., 1997) to commit cells to apoptosis. JNK3 is predominately expressed in the brain and is most consistently associated with neuronal death.

In this study, folate deficiency did not induce any expression of JNK3 indicating FD status didn’t have any influence in the expression of this molecule. However, FM animals showed marked increase in all the brain regions compared to control again pointing out that brain is highly affected by MeOH and its metabolites. The role of JNK activation in apoptosis has been extensively discussed (Xia et al., 1995). In neuronal systems, a large and growing body of evidence suggests that the JNK/c-Jun pathway can function in a pro-apoptotic manner. JNK3 is critical for c-Jun phosphorylation and death induced by focal ischemia in vivo, while JNK1 and 2 deficiencies do not appear to play a role (Kuan et al., 2003). It must be remembered that the animals that were exposed to MeOH only once (one third of LD₅₀) have such a consequence, sub lethal exposure of multiple doses to human by adulterant illicit liquor, may definitely have at least
some damage to neuronal cells. Correlatively reactive oxygen species (free radicals) functioning as an activating signal for the JNK pathway (Lo et al., 1996) is also reported. Since in these animals free radical accumulation and scavenging enzymatic and non enzymatic alterations were observed, the JNK expression in the brain regions can be possibly explained.

In LA group, a decrease was found in the JNK3 expression in cerebellum and pons medulla compared to unsupplemented group. FLM group showed an increase in the expression of JNK3 only in midbrain and hypothalamus from control. This indicates that the supplementation of LA is more beneficial.

**Histology**

MeOH induced changes after an acute exposure (24 hrs) were studied, which revealed that in kidney no marked changes was noticed. The normal liver showed liver parenchyma with central vein surrounded by normal hepatocytes whereas FD animals showed occasional hepatocytes with macronuclei and MeOH fed control showed occasional micro vesicular fat globules. Moreover, in FM animals the liver showed damage in the hepatocytes darkly staining macronuclei with increased acidophilia of the cytoplasm and further, focal hepatocytic necrosis with mononuclear collections were observed. Similar reports are available for MeOH acute toxicity. Rats that was given a single dose of MeOH (1.5 g/kg b.w.) into the stomach through a gastric tube, the liver showed visible changes. Only 6 hrs after intoxication, lobular peripheral hepatocytes presented characteristic features of vacuolar degradation persisting up to 48hrs (Kasacka and Skrzydlewska, 1999). It must be pointed out that these researchers used one half of the dose used in the present study, which is also fed via the oral route. In oral administration, the blood MeOH level reaches peak relatively slower than the intraperitoneal route. In spite of the lower dosage, they could observe changes in
the liver. Even with the same dosage as that of this study, the changes in liver were reported. Kurcer et al. (2007) reported that when received intra gastric, 3 g/kg MeOH as a 50% solution in isotonic saline once, showed after 6 and 24 hrs, liver lobular lytic necrosis, and portal inflammation. Further, they reported that melatonin has protective effects against MeOH-induced hepatic injury. In FLM animal's cords of hepatocytes showed occasional hepatocyte with macronuclei indicating LA supplementation could protect to a major extent but not completely.