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# Biochemical Toxicology of Argemone Oil. Role of Reactive Oxygen Species in Iron Catalyzed Lipid Peroxidation

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Contamination of mustard oil with the resembling seed oil of the weed Argemone mexicana L., is quite common in India and other tropical countries (Tandon et al., 1975; Khanna and Singh, 1983). Consumption of such contaminated oil even for short duration causes a clinical condition popularly termed as Epidemic Dropsy which include erythema, oedema, glaucoma, breathlessness, hepatomegaly and tachycardia (Tandon et al. 1975; Khanna and Singh 1983; Shanbhag et al. 1968; Mason-Bahr and Apted 1982; Rathore 1982). The toxicity of argemone oil has been attributed to its physiologically active alkaloids, sanguinarine and dihdyrosanguinarine (Sarkar 1948). Histopathological studies in argemone alkaloids intoxicated animals showed focal necrosis in the liver along with vascular proliferation and fatty changes (Chandra et al. 1972). Our recent studies have indicated that intake of argemone oil causes destruction of hepatic microsomal cytochrome P-450 content and its dependent monooxygenases (Upreti et al. 1990). It has also led to a significant depletion of hepatic glutathione content and a concomitant increase in lipid peroxidation in homogenate (Upreti et al. 1990).

The importance of inorganic iron as a catalyst or as a part of a catalytic system for the peroxidation of unsaturated lipid both in vivo and in vitro has been indicated (Wills 1969a; Searle and Wilson 1983; Shedlofsky et al. 1983). It is known that iron, incorporated in enzymes or acting non-enzymatically, can catalyze lipid peroxidation (Wills 1969a; Wilson 1970). Since lipid peroxidation is thought to be an essential marker for membrane damage by various toxicants, we studied the effect of argemone oil on $Fe^{+2}$ catalyzed microsomal lipid peroxidation, and the role of reactive oxygen species in this process.

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## MATERIALS AND METHODS

Catalase, 2,5-dimethylfuran (DMF), histidine and superoxide dismutase (SOD) were obtained from Sigma Chemical Company (St. Louis, MO). $\beta$-Carotene, mannitol and sodium benzoate were obtained from BDH (Poole, England). Thiobarbituric acid (TBA) was a product of E. Merck (Darmstadt, Germany). Ethanol was purchased from Spectrochem (India). Ferrous sulphate and magnesium chloride were procured from BDH, Glaxo (India). Riboflavin was obtained from Sisco Research Laboratory (India). All the other chemicals used were of highest purity commercially available.

Adult male wistar albino rats (150 ± 10 g), derived from Industrial Toxicology Research Centre, animal breeding colony, were raised on commercial pellet diet (Hindustan Lever Ltd., Bombay) and water *ad libitum* under standard laboratory conditions. The experimental group was given intraperitoneal injections of argemone oil (5 ml/kg body weight) daily for three successive days. Control group received the same volume of mustard oil. Animals were ensanguinated 24 hours after the last injection. Livers were removed, washed in chilled solution of 0.1 M phosphate buffer, pH 7.4 and weighed.

The procedure used for preparation of microsomal fraction from liver homogenates was essentially the same as described by Das and Ray (1988). The microsomal fractions were either used on the same day or kept under liquid nitrogen at -80°C.

Lipid peroxidation in hepatic microsomes prepared from control and argemone oil treated rats was assessed by measuring the formation of malonaldehyde (MDA) as described by Wright *et al*. (1981) with the following modifications.

The tubes having 0.5-0.7 mg microsomal protein in 0.6 ml of $Ca^{+2}$ free 0.1 M phosphate buffer, pH 7.4, containing $10^{-4}$ M $MgCl_2$ and $FeSO_4$ (1 mM) were incubated for 15 minutes at 37°C in a metabolic shaker with constant shaking. Whenever needed scavengers of reactive oxygen species were also added in the microsomal suspension prior to incubation. The reaction was terminated by the addition of 0.6 ml of 10% (w/v) trichloroacetic acid. One ml of 0.5% (w/v) TBA was added to the reaction mixture which was then heated for 10 minutes in a boiling water bath. Samples were cooled under running tap water and centrifuged, and levels of TBA reactive material (MDA) were measured at 535 nm. The concentration of MDA was calculated by using a molar extinction coefficient of $1.5 \times 10^{-5}$ $M^{-1}$ $cm^{-1}$ and

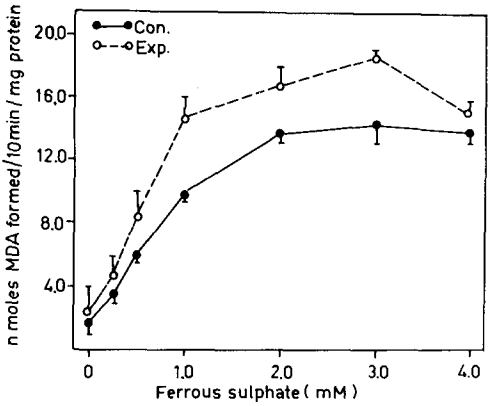


Figure 1. Effect of argemone oil on $FeSO_4$ concentration supported hepatic microsomal lipid peroxidation. Data represent mean ± S.E. of 4 values.

expressed in terms of nmoles MDA formed/10 minutes/mg protein.

The protein content in trichloroacetic acid precipitate was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

## RESULTS AND DISCUSSION

The rate of lipid peroxide formation following incubation of microsomal suspensions prepared from control and argemone oil treated rats, at different concentration of $FeSO_4$ is shown in Figure 1. The addition of low concentrations of $FeSO_4$ enhanced the peroxidative activity, in both control and argemone oil administered groups. The rate of formation of MDA was found to be directly proportional to $FeSO_4$ upto 1 mM showing first order kinetics. At higher concentration of $FeSO_4$ a saturation kinetics of zero order reaction was evident (Figure 1).

Time dependent rate of microsomal lipid peroxide formation, in the presence of $FeSO_4$ (1 mM) is shown in Figure 2. The rate of lipid peroxidation was dependent on the time of incubation of $FeSO_4$ and was found to increase linearly upto 20 minutes and showed a steady state thereafter. At all the time intervals, lipid peroxide formation was, however, higher in microsomes prepared form argemone oil treated rats as compared to control (Figure 2). An optimum reaction time of 15 minutes was, therefore, choosen for subsequent experiments.

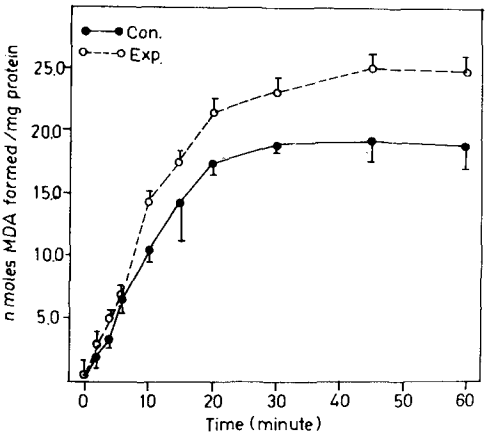


Figure 2. Time dependent effect of $FeSO_4$ on argemone oil induced hepatic microsomal lipid peroxidation. Data represent mean ± S.E. of 4 values.

The effect of varying concentrations of hepatic microsomal protein on $FeSO_4$ dependent lipid peroxidation is depicted in Figure 3. In the presence of $FeSO_4$, hepatic microsomal lipid peroxidation was found to be linear upto a protein concentration of around 0.86 mg. The maximum difference in lipid peroxidation in microsomes prepared from control and argemone oil treated rats was observed between 0.60-0.86 mg of microsomal protein in the presence of $FeSO_4$. Thus, 0.60-0.86 mg of microsomal protein was used for all the subsequent experiments.

The effect of scavengers of reactive oxygen species on $FeSO_4$ catalyzed lipid peroxidation in hepatic microsomes prepared from control and argemone oil treated rats is shown in Table 1. DMF, histidine, riboflavin and $\beta$-carotene produced a respective inhibition of 66.6, 65.1, 82.5 and 63.8% in argemone oil induced microsomes, while a less degree of inhibition is observed in control microsomes.The inhibition of SOD and catalase in argemone induced microsomes is almost the same as compared to control microsomes (Table 1). Addition of ethanol, mannitol or sodium benzoate produced an inhibition of 38.9, 31.4, 47.9% in control and 52.4, 45.9, 60.5% in argemone induced microsomes, respectively (Table 1).

Optimal microsomal lipid peroxidation was observed at 1 mM concentration of $FeSO_4$. The time course study revealed that 20 minutes of incubation gives maximal lipid peroxidation. Also, a low protein content in the

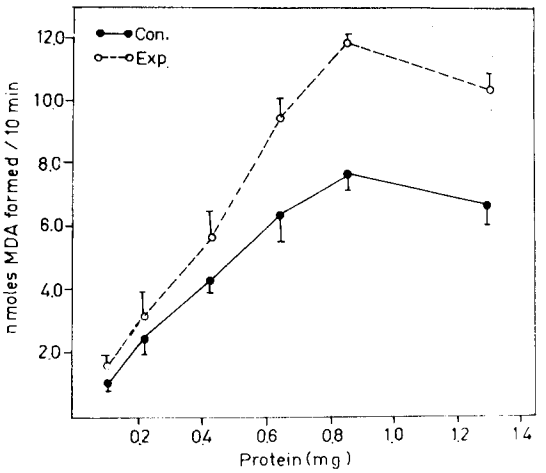


Figure 3. Effect of argemone oil on protein dependent $FeSO_4$ supported hepatic microsomal lipid peroxidation. Data represent mean ± S.E. of 4 values.

microsomal incubates was found to yield more lipid peroxides in terms of MDA per mg protein. This is consistent with the findings of Wills (1969b) who showed that a better dispersal of endoplasmic reticulum stimulated lipid peroxidation as a result of increased proximity of membraneous unsaturated lipid radicals. At the same time dilution of microsomal suspensions might result in lower production of some unknown lipid peroxidation inhibitors (Wills, 1969b).

Microsomal lipids has been found to be a typical radical chain reaction (Sivengen et al. 1979). A large number of peroxidants and catalytic inhibitors, viz., iron, ascorbic acid, oxygen, and chelated iron are known to catalyze the peroxidation of unsaturated lipids associated with biomembranes (Mead, 1976). Ferrous ion stimulate lipid peroxidation through the generation of either hydroxyl radical or superoxide anion or hydrogen peroxide (George, 1954). It has been suggested that the ferryl or perferryl species may be involved in iron stimulated lipid peroxidation (Tien et al. 1982). Thus, the studies with ferrous ion suggest that on in vivo administration of argemone oil,less amounts of lipoperoxides or lipohydroperoxides could be decomposed by iron compounds.

There are several lines of evidence to suggest that the singlet oxygen ($^1O_2$) is a likely mediator of microsomal lipid peroxidation (Pederson and Aust, 1975). Our results indicate that DMF, histidine, and $\beta$-carotene, all of which are known quenchers of singlet oxygen

Table 1. Effect of in vitro addition of scavengers on $FeSO_4$ dependent hepatic microsomal lipid peroxidation in control and argemone treated rats

| Scavengers | Conc. | nmoles MDA formed/10 min/mg protein | | | |
|---|---|---|---|---|---|
| | | Control | % inhibition | Experimental | % inhibition |
| None (microsomes only) | - | 10.22±0.03 | - | 14.43±0.03 | - |
| DMF | 5.0 mM | 4.32±0.01* | 57.6 | 4.82±0.02* | 66.6 |
| Histidine | 5.0 mM | 4.63±0.02* | 54.7 | 5.04±0.02* | 65.1 |
| Riboflavin | 10.0 mM | 3.13±0.04* | 69.4 | 2.53±0.03* | 82.5 |
| β-Carotene | 0.1 mM | 4.84±0.02* | 52.7 | 5.23±0.03* | 63.8 |
| SOD | 25 µg/ml | 7.36±0.03* | 28.0 | 9.75±0.03* | 32.5 |
| Catalase | 50 µg/ml | 6.56±0.01* | 35.8 | 9.54±0.02* | 38.9 |
| Ethanol | 10.0 mM | 6.24±0.02* | 38.9 | 6.87±0.04* | 52.4 |
| Mannitol | 10.0 mM | 7.01±0.03* | 31.4 | 7.80±0.03* | 45.9 |
| Sodium benzoate | 10.0 mM | 5.32±0.02* | 47.9 | 5.70±0.04* | 60.5 |

Data represent mean ± S.E. of four values.
*$P < 0.05$ when compared to microsomal incubates (Student's 't' test).

(Kellogg and Fridovich, 1975), substantially abrogated ferrous dependent hepatic microsomal lipid peroxidation evoked by argemone oil *in vivo*. The effect of these scavengers in affording protection against argemone oil induced enhancement of lipid peroxidation was concentration dependent (Data not shown). From these results it can be concluded that the generation of singlet oxygen accompanies the lipid peroxidation in hepatic microsomes that occurred during argemone oil administration. Furthermore, our studies show that scavengers of hydroxyl radical provide appreciable protection against argemone oil induced $Fe^{+2}$ dependent microsomal lipid peroxidation. SOD and catalase, scavengers of superoxide anion and hydrogen peroxide, respectively (McCord et al., 1971) had no protective effect against argemone oil mediated enhancement of lipid peroxidation. It is thus possible that reactive oxygen species are generated endogenously by argemone oil in endoplasmic reticulum and lipid peroxidative damage is caused by these reactive oxygen species.

Incidences of *in vivo* lipid peroxidation resulting in cellular, pathological and physiological disturbances in various biomedical disorder such as beta lipoproteinemia, erythropoetic protoporphyria, atherosclerosis and inflammation are of common occurrence (Tappel, 1975). Reactive oxygen species such as singlet oxygen only propagate an initial attack upon lipid rich membrane to cause lipid peroxidation. Additional mediators such as alkoxy radical could be involved in this process and could be quenched by scavengers used in our studies.

Thus, it can be concluded that $^1O_2$ and OH radicals are involved during argemone oil induced lipid peroxidation which may be a possible mechanism where by it exerts hepatotoxic effect.

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