PART -I

LONG-TERM STUDIES OF CYCLOPHOSPHAMIDE
ON THE CHICK EMBRYO
a) EMBRYOTOXIC EFFECT OF CYCLOPHOSPHAMIDE IN CHICK EMBRYO

The embryotoxic effect of cyclophosphamide (CP) is achieved only after bioactivation. Two embryotoxic metabolites of CP namely phosphoramide mustard (PM) and acrolein (AC) have been identified (Mirkes et al., 1984; Slott and Hales, 1988). These two metabolites caused unique embryotoxic effects in 10-day rat embryos exposed to in vitro.

CP has been associated with development of lung fibrosis both in animals and humans (Batist and Andrews, 1981; Copper et al., 1986; Kanekal et al., 1992). Animal studies indicate that CP alters normal ovarian and uterine structure and functions in mice, and inhibit fertilization and embryo development (Plowchalk and Matison, 1992; Plowchalk and Meadows, 1992).

Many studies have demonstrated that CP and many other chemotherapeutic agents cause gene mutations, aneuploidy in somatic cells, as well as an increased frequency of secondary treatment related tumors in human and cancer survivors (Sandoval et al., 1993; Povirk and Shuker, 1994; Ben-Yeluda et al., 1996).

There are number of studies on the embryotoxic effect of cyclophosphamide on 10th or at later stages of embryonic development. The present investigation was undertaken to assess the embryotoxic effect of CP soon after fertilization in chick embryo and to specify the
malformation spectrum induced by CP with a view to establish time-course and dose-related effects.

Results

Chick embryos were exposed to different concentrations of cyclophosphamide (ranging from 0-1000 µg) at different stages of embryonic development (ED). Table 3 represents a dose-dependent effect of CP at various stages of development.

The embryotoxic effect at every stage showed a dose-dependent increase in the death of the embryos and was manifested as early (death of the embryo within 48 h of CP treatment) and late death (death of the embryo till the 12th day of incubation).

The CP on embryonic development day 0 (ED 0) resulted in the 100 per cent mortality with 500 and 1000 µg, whereas, 50,100,200 and 400 µg resulted in 18, 42, 60 and 84 per cent of mortality respectively. At this stage of ED 0, CP above 200 µg caused maximum fetal death within 48 h of drug treatment.

CP produced a concentration-dependent increase in the incidence of malformed embryos, ranging from 26 per cent with 100 µg to 100 per cent with 1000 µg. No malformations were observed with 50 µg. Thus this is non-toxic dose to chick embryo.

Treatment of different doses of CP on ED 2, ED 4, ED 7 and ED 9 showed a decreased rate of mortality and malformations when compared to CP treatment of same concentration on ED 0. Thus with increase in the age of the embryo higher concentration was required to cause the same per cent of effect.
Table 3: Dose-dependent effect of cyclophosphamide at various stages of embryonic development

<table>
<thead>
<tr>
<th>Embryonic Days (ED)</th>
<th>Con. (μg)</th>
<th>No. of embryos exposed</th>
<th>No. of dead embryos (Aprox)</th>
<th>Early Death (%)</th>
<th>Late Death (%)</th>
<th>% of mortality</th>
<th>Volume of AF (ml)</th>
<th>Weight of embryos (g)</th>
<th>% of malformation</th>
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<td>4.20±0.25**</td>
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<td>No. of embryos exposed</td>
<td>No. of dead embryos (Aprox)</td>
<td>(^A)Early Death (%)</td>
<td>(^B)Late Death (%)</td>
<td>(^A) % of mortality</td>
<td>(^B) % of mortality</td>
<td>Volume of AF (ml)</td>
<td>Weight of embryos (g)</td>
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<td>4.32±0.22(^*)</td>
<td>4.22±0.40(^\text{**})</td>
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</table>

\(^A\) early death indicates the death of the embryo within 48 h of drug treatment.
\(^B\) late death indicates the death of the embryo after 48 h and till the 12th day of incubation.
\(^t\)\(^P<0.02\), \(^*\)^\(^P<0.01\), \(^**\)^\(^P<0.001\) compared with respective controls.
NS: Not significant.
CP of 1000 μg caused almost 100 per cent mortality at all the stages of ED, whereas 50 μg was least effective.

The lethal dose (LD₅₀) was calculated directly from the graph drawn between percentage of mortality and concentration of CP in μg.

In embryos treated on ED 0, LD₅₀ was assessed as a dose close to 150 μg while dose of 210 μg corresponded to LD₅₀ value of ED 2, while the LD₅₀ values of ED 4, 7 and 9 were 310, 405 and 450 μgs, respectively.

Table 3 shows significant decrease in the weight of the embryo with increase in the concentration of CP at every stage of embryonic development. On ED 4 with 100 μg and 200 μg there is a slight decrease in the weight of the embryos when compared with the same concentration to the ED 2.

Table 3 also shows the volume of amniotic fluid remained unaltered at lower concentrations at each embryonic development. But at concentration 400 μg on ED1, 500 μg and 1000 μg at other stages showed a significant increase in the volume of amniotic fluid.

Anomalies noted were multiple and never single in any fetus. The malformations observed are, hemorrhagic brain, hydrocephalous, thickening of neck, displaced limbs, hemorrhagic and amniotic fluid. Growth retardation was the major defect observed with teratogenic dosage (Table 4).
Table 4: Cyclophosphamide-induced malformation in chick embryos

<table>
<thead>
<tr>
<th>Day of treatment (within 24 h of incubation)</th>
<th>CP dose (μg)</th>
<th>Affected structures</th>
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<td>Stunted growth (%)</td>
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<td>Thicken ing of the neck (%)</td>
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<tr>
<td></td>
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<td>Hemorrhagic brain (%)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>Short beak (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>Limbs defect (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>Hemorrhagic fluid (%)</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Day 0</td>
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<td>Hemorrhagic brain (%)</td>
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<td>Hemorrhagic fluid (%)</td>
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Embryos treated on ED 0 showed hemorrhagic brain, hemorrhagic fluid only at a concentration above 100 μg. In comparison to controls, the percentage of surviving embryos exhibited increased growth retardations with 100, 200, 400 μg, whereas 500 μg and 1000 μg caused indistinguishable damage (necrosis) to the developing fetus. Short beak and limb were observed only at a higher dose of CP i.e., 400 μg.
As seen from the Table 4, CP treatment affected number of structures at various embryonic stages. There was a decline in the percentage of individual structures affected with increase in the age of the embryos when compared to ED 0.

The dose, which was highly toxic at one particular stage of embryonic development, may not be toxic to other stage.

The plates (1 - 13) show the effect of different doses of CP within 24 and 48 h of embryonic development. The results clearly indicate the embryotoxic effect of CP at higher concentrations.

**Histological variations in Liver, Kidney and Brain**

The effect of CP was correlated with histopathological changes. CP caused mild dilatation of sinusoids in liver parenchyma and also caused ballooning degeneration and congestion of hepatocytes (plates 14-18). CP did not cause any damage to heart cells up to 500 μg. All the cells remained intact (plates 19-23). In the brain, CP caused disruption of cerebellar epithelium, aggregation of basophilic granules and mitotic figures (plates 24-28).

Protein profiles of AF, AM (plate 29) and WET are shown in plate 30.

**Discussion**

Single dose of CP was found to be teratogenic and embryotoxic at a concentration beyond 100 μg all the stages of development producing abnormal and growth retardation indistinguishable from embryos exposed in vivo to bioactivated CP (Fantel et al., 1979)
Embryotoxic effects of CP in our experiments showed a dose-dependent increase regardless of the age of the embryo at the time of CP administration (Claussen et al., 1980). CP caused most of the fetal deaths within 48 h of the drug administration during early stages of development i.e. on ED 0 and ED 2. This may be due to the fact that the enzymes required for CP bioactivation are present in small concentrations in the egg yolk, whereas, the other antineoplastic compounds exert embryotoxic effect in chick embryos after ED 5 when the functional activity of the embryonic liver starts (Heinrich-Hirsh et al., 1990) hence CP effects are observed within 48 h of its first injection. This confirms that CP affects the embryonic system soon after its fertilization.

Administration of low doses < 100 µg, didn’t cause any malformations at any stage of development when compared to controls. The characteristic defects produced by CP are stunted growth, limb defects, brain defects, thickened neck and hemorrhagic brain and amniotic fluid. Our data is in agreement with Mirkes (1985) and Novotna and Jelink (1990).

Our results indicate that CP of 100 and 200 µg to 4-day-old chick embryo caused high per cent of malformations than early days of treatment.

Growth retardation and internal hemorrhage represent the most frequent malformation. Altered properties of yolk sac or extra embryonic vascular network may reduce nutrient transfer and hence responsible for growth retardation of the embryo. In comparison to controls growth retardation was less apparent at the later stages of embryonic development suggesting partial compensation of cell loss during the later stages of development.
According to Cohlan (1954) swollen amniotic sac with excess fluid was due to hypervitaminosis-A. Alterations in amniotic fluid due to various teratogenic agents have been reported (Gullentetti et al., 1962). The excess amniotic fluid treated first with meclizine HCl has been described to vasodilatation of sac caused by drug resulting in elevation of capillary blood pressure favoring amniotic fluid production or inhibiting its reabsorption (Kendrick and Weaver, 1963).

The increase in amniotic fluid at higher concentration is not clear. The viscid nature of the fluid may be due to the rupture of blisters formed during the CP treatment on the surface of fetus. Blister formation has been correlated to lactic acid formation (Singh, 1971; Grabowski, 1961).

Bulk of evidence indicates that DNA is the primary target of CP-induced teratogenicity. CP metabolites are known to interact with cellular macromolecules. Little and Mirkes et al. (1992) demonstrated that the phosphoramidé mustard binds preferentially to DNA and, at embryotoxic concentrations, damages DNA by forming cross-links (Little and Mirkes, 1987). Acrolein, a toxic metabolite of CP, binds preferentially to embryonic proteins and does not cause DNA damage at embryotoxic concentrations (Little and Mirkes, 1990). This indicates DNA as a primary target of action for phosphoramidé mustard embryotoxicity and proteins as the primary target of action for acrolein embryotoxicity. Binding of phosphoramidé mustard and acrolein to macromolecules are one of the first pathway culminating in abnormal development.

The histopathological changes found in various embryonic organs were similar to those found in the treated pregnant mice (Mirkes et al, 1981;
Mirkes et al., 1983). As CP can cross the placenta it affects the same target organs as that of the adult.

Treated heart showed no major histopathological changes even at 500 μg. One explanation for the resistance of heart cells is that the concentration of CP metabolites found in the heart after exposure is insufficient to produce damage (Little and Mirkes, 1991).
Plate 1. Normal chick embryo treated with saline after 24 h

Plate 2. Effect of 100 µg of CP on chick embryo after 24 h
Plate 3. Effect of 200 μg of CP on chick embryo after 24 h

Plate 4. Effect of 400 μg of CP on chick embryo after 24 h
Plate 5. Effect of 500 µg of CP on chick embryo after 24 h

Plate 6. Overall view of different concentrations of CP on chick embryos after 24 h
Plate 7. Normal chick embryo treated with saline after 48 h

Plate 8. Effect of 100 μg of CP on chick embryo after 48 h
Plate 9. Effect of 200 µg of CP on chick embryo after 48 h

Plate 10. Effect of 400 µg of CP on chick embryo after 48 h
Plate 11. Effect of 500 µg of CP on chick embryo after 48 h

Plate 12. Overall view of different concentrations of CP on chick embryos after 48 h
Plate 13  Effect of different doses of CP on 9-day-old chick embryo after 24 h
$A = 500 \text{ mg of CP}$

$B = 400 \text{ mg of CP}$

$C = 200 \text{ mg of CP}$

$D = \text{ Control}$
Plate 14. Photomicrograph of normal chick embryonic liver. H&E. 10 X 10

Plate 15. Photomicrograph of chick embryonic liver treated with CP 100 ME. H&E. 10 X 10. No significant changes seen in the hepatocytes.
Plate 16. Photomicrograph of chick embryonic liver treated with CP 200 µg H&E. 10 X 40. No significant changes seen in the hepatocytes.

Plate 17. Photomicrograph of chick embryonic liver treated with CP 400 µg H&E. 10 X 40. Mild ballooning, degeneration of hepatocytes seen.
Plate 18. Photomicrograph of chick embryonic liver treated with CP 500 µg H&E. 10 X 40. Mild dilatation of sinusoids in liver parenchyma seen.

Plate 20. Photomicrograph of chick embryonic heart treated with CP 100 μg H&E. 10 X 10. No significant changes observed.

Plate 21. Photomicrograph of chick embryonic heart treated with CP 200 μg H&E. 10 X 10. No significant changes observed.
Plate 22. Photomicrograph of chick embryonic heart treated with CP 400µg H&E. 10 X 40. No significant changes observed.

Plate 23. Photomicrograph of chick embryonic heart treated with CP 500µg H&E. 10 X 40. No significant changes observed.

Plate 25. Photomicrograph of chick embryonic brain treated with CP 100 μg H&E. 10 X 40. Mitotic figures seen.

Plate 27. Photomicrograph of chick embryonic brain treated with CP 400 μg H&E. 10 X 40. Aggregation of basophilic granules observed.
Plate 29. Protein profile of chick amniotic fluid and amniotic membrane at different days of embryonic development.

Plate 30. Protein profile of whole embryonic tissues at different days of embryonic development.
b) **CYCLOPHOSPHAMIDE-INDUCED OXIDATIVE STRESS IN CHICK EMBRYO**

Cyclophosphamide (CP) is commonly used antitumor and immunosuppressive drug. Although CP has been proved to be a very effective chemotherapeutic agent, its use has been linked to numerous side effects in the animals and humans (Clem and Bickers, 1991; Paul and Bruce, 1991; Gould and Miller, 1975; Cooper et al., 1986; Kanekal et al., 1992). The cause of CP-induced tissue injury is, however, unclear and there is increasing evidence that reactive oxygen species (ROS) could be responsible for negative effects (Patel, 1990).

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. These are continuously produced during respiration and cell-mediated immune functions (Tiwari, 2001). They are also generated through environmental pollutants, cigarette smoke, automobile exhaust fumes, radiation, air pollutants, pesticides and various drugs (Halliwell and Chirico, 1993; Halliwell et al., 1992; Jacob, 1994; Li and Trush, 1994). Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to scavenge them and protect the body against their deleterious effect (Nose, 2000; Finkel and Holbroock, 2000). However, the amounts of these protective antioxidants present are sufficient only to cope with the physiological rate of free radical generation. Additional burden produced within the body can tip the free radical balance and may lead to oxidative stress leading to tissue injury and subsequent diseases (Dringen, 2000; Sies, 1991; de Groot and Rauen, 1998; Davies, 1995). Thus, Oxidative stress may be increased in
CP-treated patients owing to the hyper production of ROS such as $O_2^{-}$, H$_2$O$_2$ and hydroxide radicals (Brogaard and Clausen, 1997).

Most of the cancer patients undergoing chemotherapy using CP induce lipid peroxidation by inactivating the antioxidant enzymes, thereby rendering the system to free radical attack (Subramaniam et al., 1995). Acrolein and phosphoramide mustard are the metabolites of CP that are among the causative agent that reduce the activity of antioxidant enzymes.

Among the enzymatic scavengers, SOD causes the dismutation of $O_2^{-}$ to H$_2$O$_2$ while the other antioxidant enzymes, CAT and GPx are involved in the conversion of H$_2$O$_2$ to H$_2$O.

Lipid peroxidation of cellular structures, a consequence of increased free radical activity is thought to play an important role in ageing, atherosclerosis and other diseases (Nakata et al., 1996; Sopher et al., 1996; Jenner, 1996; Mantha et al., 1993).

Lipid peroxides are formed as a result of action of oxygen free radicals on polyunsaturated fatty acid (PUFA) and is known to occur in all the biological membranes (Tappel, 1973). As a result of free radical action on PUFA, various products are formed, which includes lipid peroxides, hydroperoxides, epoxy alcohols and short chain malondialdehydes (MDA), ethane, pentane, 4-OH alkenals. MDA and 4-OH alkenals are the most extensively studied lipid peroxidation products (Mead et al., 1980; Benedetti et al., 1980). MDA, a bifunctional aldehyde easily reacts with proteins and thus inactivates many enzymes. If the lipid bilayer of all is oxidized to sufficient degree then it loses its ability to maintain electrolyte and homeostasis. LPO reacts with various macromolecules inactivating them, and resulting in a cell death. MDA,
measured, as thiobarbituric acid reactive substances (TBARS) is the most commonly used marker of LPO.

Several studies have been shown elevated levels MDA in CP treated mice and rats (Berrigan et al., 1980). Venkatesan and Chandrakasan (1995) reported a significant increase in MDA levels in serum and lung of rats after CP treatment. Bhanumathi and Devi (1994) showed CP-induced depletion in liver GSH and high rate of lipid peroxidation. So there are several reports of CP-induced lipid peroxidation. There are no reports so far on the effects of CP on lipid peroxidation in chick amniotic fluid and embryonic tissues. The activities of antioxidant enzymes on CP treatment are limited.

Venkatesan and Chandrakasan (1994) evaluated early biochemical changes in lung lavage fluid alterations in lavage cell functions after CP treatment to detect tissue damage in rats. There are also reports of biochemical changes induced by CP in mice (Shah et al., 1996). We have made an attempt to study the biochemical changes induced by CP in chick amniotic fluid and embryonic tissues in an effort to elucidate the CP toxicity.

**Results**

Two doses of CP were selected on the basis of LD$_{50}$ values, namely 100 and 200 µg. These doses were injected to 7-day-old chick embryos through the air sac.

Effect of cyclophosphamide on lipid peroxidation in amniotic fluid is represented in Fig.8.
Fig. 8: Effect of cyclophosphamide on lipid peroxidation in amniotic fluid of 7-day-old chick embryo.

![Graph showing effect of cyclophosphamide on lipid peroxidation in amniotic fluid](image)

Values are average of six sets of separate experiments (Mean±SD)

Fig. 9: Effect of cyclophosphamide on lipid peroxidation in amniotic membrane of 7-day-old chick embryo.

![Graph showing effect of cyclophosphamide on lipid peroxidation in amniotic membrane](image)

Values are average of six sets of separate experiments (Mean±SD)
The concentration of lipid peroxidation product in AF after CP treatment significantly elevated in a dose-dependent manner from control groups till 48 h. CP of 100 μg was more effective in inducing lipid peroxides levels at 72 h In amniotic fluid than that of CP 200 μg. The maximum percentage of induction was seen at 24 h with CP 200 μg.

Lipid peroxidation in AM and whole embryonic tissue WET is shown in Fig. 9 & 10. In AM CP caused dose-dependent increase in LPO levels compared to controls. Maximum induction was seen at 24 h with CP 200 μg. In WET CP caused significant increase in dose-dependent manner till 48 h but at 72 h LPO levels reached to near normal values with CP 200 μg.

CP treatment significantly enhanced the LPO levels in brain and liver till 48 h compared to controls (Fig.11 & 12). In brain at 72 h CP 200 μg declined the LPO and reached to near normal levels.

The heart tissues were least affected with CP treatment (Fig.13).

Fig.14 shows the effect of CP on SOD in varying time interval. In normal conditions the SOD activity increased with duration. CP treatment caused decrease in enzymatic activity within 4 h treatment and continued to decline till 24 h in a dose-dependent manner. But with increase in age of the embryo (at 72 h) CP 200 μg elevated the SOD activity significantly when compared to the controls whereas with CP 100 μg enzymatic activity of SOD reached to near normal values.
Fig. 10: Effect of cyclophosphamide on lipid peroxidation in whole embryonic tissue of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)

Fig. 11: Effect of cyclophosphamide on lipid peroxidation in brain of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)
Fig. 12: Effect of cyclophosphamide on lipid peroxidation in liver of 7-day-old chick embryo.

Values are average of six sets of separate experiments (Mean±SD)

Fig. 13: Effect of cyclophosphamide on lipid peroxidation in heart of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)
Significant decrease in SOD activity was seen in AM, WET, brain, and liver (Fig.15-18). With duration a slight increase in the enzyme activity was observed in AM, brain and liver with CP 200 μg compared with CP treatment at 48 h. In the heart tissues, a slight change was observed with CP treatment (Fig.19). CP 200 μg was more effective during the first two days than CP 100 μg in decreasing the superoxide dismutase activity.

CP significantly diminished the catalase activity in chick AF, AM, WET, brain and liver in a dose-dependent manner till 48 h compared with controls (Fig.20-24). With CP 200 μg a slight increase in enzymatic activity was observed in AF, AM, WET, Brain and liver compared to CP treatment at 48 h indicating the recovery of the lost activity with time.

Catalase (CAT) activity in the embryonic heart was not changed much with CP (Fig.25).

CP significantly suppressed the glutathione (GSH) activity in AF, AM, WET, brain, and liver till 48 h in a dose-dependent manner compared to controls (Fig.26-30). As seen in the figures (27, 29 & 30) CP 100 μg declined the GSH activity significantly at 72 h, compared to same concentration at 48 h, whereas, an increase in GSH activity was reported with 200 μg when compared to CP 200 μg at 48 h in most of our studies (AF, WET, brain and liver). Thus the late death and early death can be corroborated with decrease and increase trend in the antioxidant activity.
Fig. 14: Effect of cyclophosphamide on superoxide dismutase activity in amniotic fluid of 7-day-old chick embryo

![Graph showing effect of cyclophosphamide on superoxide dismutase activity in amniotic fluid.](image)

Values are average of six sets of separate experiments (Mean±SD)

Fig. 15: Effect of cyclophosphamide on superoxide dismutase activity in amniotic membrane of 7-day-old chick embryo

![Graph showing effect of cyclophosphamide on superoxide dismutase activity in amniotic membrane.](image)

Values are average of six sets of separate experiments (Mean±SD)
Fig. 16: Effect of cyclophosphamide on superoxide dismutase activity in whole embryonic tissue of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean ± SD)

Fig. 17: Effect of cyclophosphamide on superoxide dismutase activity in brain of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean ± SD)
Fig. 18: Effect of cyclophosphamide on superoxide dismutase activity in liver of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)

Fig. 19: Effect of cyclophosphamide on superoxide dismutase activity in heart of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)
Fig. 20: Effect of cyclophosphamide on catalase activity in amniotic fluid of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean ± SD)

Fig. 21: Effect of cyclophosphamide on catalase activity in amniotic membrane of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean ± SD)
Fig. 22: Effect of cyclophosphamide on catalase activity in whole embryonic tissue of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)

Fig. 23: Effect of cyclophosphamide on catalase activity in brain of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)
Fig. 24: Effect of cyclophosphamide on catalase activity in liver of 7-day-old chick embryo

![Graph showing catalase activity in liver of 7-day-old chick embryo.]

0 in x-axis represents 4h after CP treatment

Values are average of six sets of separate experiments (Mean±SD)

Fig. 25: Effect of cyclophosphamide on catalase activity in heart of 7-day-old chick embryo

![Graph showing catalase activity in heart of 7-day-old chick embryo.]

0 in x-axis represents 4h after CP treatment

Values are average of six sets of separate experiments (Mean±SD)
Fig. 26: Effect of cyclophosphamide on reduced glutathione in amniotic fluid of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)

Fig. 27: Effect of cyclophosphamide on reduced glutathione in amniotic membrane of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)
Fig. 28: Effect of cyclophosphamide on reduced glutathione in whole embryonic tissue of 7-day-old chick embryo

![Graph showing Effect of cyclophosphamide on reduced glutathione in whole embryonic tissue of 7-day-old chick embryo]

Values are average of six sets of separate experiments (Mean±SD)

0 in x-axis represents 4h after CP treatment

Fig. 29: Effect of cyclophosphamide on glutathione in brain of 7-day-old chick embryo

![Graph showing Effect of cyclophosphamide on glutathione in brain of 7-day-old chick embryo]

Values are average of six sets of separate experiments (Mean±SD)

0 in x-axis represents 4h after CP treatment
CP decreased the GPx activity in a dose-dependent manner till 24 h in AF (Fig. 31). At 48 and 72 h CP 100 µg showed increase activity compared to controls. In AM and WET significant decrease in GPx activity was observed with both the concentrations till 48 h (Fig. 32 & 33). At 72 h in AF, WET and Liver with CP 100 µg showed increased enzymatic activity compared to controls (Fig. 31, Fig. 33 & Fig. 35). In brain GPx activity did not show any significant increase after 48 h of CP treatment (Fig. 34). Heart tissues remained unchanged with CP treatment throughout the experiment (Fig. 36).

The GST activity decreased at 24 h in a dose-dependent manner compared to controls in AF (Fig. 37). At 72 h CP 100 µg significantly increased GST activity compared to controls and CP 200 µg. No significant increase in GST activity was observed with CP treatment in WET (Fig. 38). In Brain decreased GST activity was observed with dose and duration (Fig. 39).

CP treatment on GR showed a varied behavior. CP decreased the GR activity significantly in liver (Fig. 40). GR activity in brain significantly increased within 4 h with CP 200 µg and continued till 72 h when compared to controls (Fig. 41). No significant change in GR activity was observed in heart tissue with CP treatment (Fig. 42).
Fig. 30: Effect of cyclophosphamide on glutathione in liver of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)

Fig. 31: Effect of cyclophosphamide on glutathione peroxidase activity in amniotic fluid of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)
Fig. 32: Effect of cyclophosphamide on glutathione peroxidase activity in amniotic membrane of 7-day-old chick embryo

![Bar chart showing the effect of cyclophosphamide on glutathione peroxidase activity in amniotic membrane of 7-day-old chick embryo.](image)

Values are average of six sets of separate experiments (Mean±SD)

Fig. 33: Effect of cyclophosphamide on glutathione peroxidase activity in whole embryonic tissue of 7-day-old chick embryo

![Bar chart showing the effect of cyclophosphamide on glutathione peroxidase activity in whole embryonic tissue of 7-day-old chick embryo.](image)

Values are average of six sets of separate experiments (Mean±SD)
Fig. 34: Effect of cyclophosphamide on glutathione peroxidase activity in brain of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)

Fig. 35: Effect of cyclophosphamide on glutathione peroxidase activity in liver of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)
Fig. 36: Effect of cyclophosphamide on glutathione peroxidase activity in heart of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)

Fig. 37: Effect of cyclophosphamide on glutathione-S-transferase activity in amniotic fluid of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)
Fig. 38: Effect of cyclophosphamide on glutathione-S-transferase activity in whole embryonic tissue of 7-day-old chick embryo

![Graph showing effect of cyclophosphamide on glutathione-S-transferase activity in whole embryonic tissue of 7-day-old chick embryo. The x-axis represents time (h) and the y-axis represents μmol of CDNB-GSH conjugate formed/mg protein. Control, CP 100, and CP 200 groups are compared. 4h after CP treatment is indicated. Values are average of six sets of separate experiments (Mean±SD).]

Fig. 39: Effect of cyclophosphamide on glutathione-S-transferase activity in brain of 7-day-old chick embryo

![Graph showing effect of cyclophosphamide on glutathione-S-transferase activity in brain of 7-day-old chick embryo. The x-axis represents time (h) and the y-axis represents μmol of CDNB-GSH conjugate formed/mg protein. Control, CP 100, and CP 200 groups are compared. 4h after CP treatment is indicated. Values are average of six sets of separate experiments (Mean±SD).]
Fig. 40: Effect of cyclophosphamide on glutathione reductase in liver of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)

Fig. 41: Effect of cyclophosphamide on glutathione reductase GR in brain of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)
Fig. 42 Effect of cyclophosphamide on glutathione reductase in heart of 7-day-old chick embryo

Values are average of six sets of separate experiments (Mean±SD)

Discussion

The objective of the present investigation was to evaluate and assess the long-term effects of cyclophosphamide in chick embryos. The two doses were selected on the basis of LD_{50} values calculated on 7-day-old chick embryo.

The formation of reactive oxygen species (ROS)/free radical is a naturally occurring intracellular metabolic process. These harmful species are known to cause oxidative damage to a number of molecules in the cell, including membrane lipids, proteins and nucleic acids (MaCord, 1993;
Halliwell and Gutteridge, 1990. The potential harmful effect of these species is controlled by cellular antioxidants defense system (Bondy and Orozco, 1994). Antioxidant enzymes such as SOD, CAT, and GSH-Px and GST are essential in both scavenging ROS/free radicals and maintaining cellular stability (Halliwell and Gutteridge, 1989). In a healthy organism, these pro-oxidants are kept in check by several antioxidant line of defense. When there is enhanced generation of ROS, as in the case of exposure to radiation, this delicately maintained balance is shifted in favor of prooxidants resulting in oxidative stress (Free, 1994). Many chemicals and drugs can increase the rate of ROS/free radical formations in specific organs of the body.

CP induces production of free radicals. The formation of MDA is considered as an index of lipid peroxidation.

Administration of CP to 7-day-old chick embryo resulted in significant increase in MDA levels in amniotic fluid, amniotic membrane, brain, liver and whole embryo. In this study we found dose-dependent increase in TBARS levels in all the tissues tested except in heart tissues.

Several authors in number of studies reported significant increase in lipid peroxides with CP treatment and our results on lipid peroxidation is in agreement with their results (Rekha et al., 2001; Joseph et al., 1999; Sulkowska et al., 1998; Berrigan et al., 1987; Kaya et al., 1999). Significant increase in the lipid peroxides within 48 h in chick embryonic tissues and AF is one the reasons for tissue damage/death/malformation in chick embryos with CP treatment.

The significant increase in LPO levels was also observed during early hours of treatment. These results provide evidence that CP
generate free radicals, which can interact with membrane lipids and consequently increase the production of lipid hydroperoxides.

A decrease in LPO levels at 72 h with CP 200 µg in AF, WET, brain and liver is due to increased SOD, CAT, GPx and GST activities.

Endogenous antioxidants provide a major protection to the cellular molecules by removing superoxide and hydroperoxide radicals. Superoxide radical may be reduced to hydrogen peroxide by SOD and catalase converts this hydrogen peroxide to water.

In the present study SOD activities were significantly decreased in a dose-dependent manner in most of the tested parameters except in the heart where no significant change was observed. Decrease in SOD activity was reported by Sulkowska et al (1998) with CP treatment.

In amniotic fluid increased activity of SOD was observed at 72 h with CP 200 µg. This may be due to the high activity of SOD in AF as reported by Sarala et al (1996).

SOD activity in response to drug toxicity could be a defensive adaptation of the system from an oxidative damage. Cells are capable of increasing the synthesis of SOD in response to hyper-oxidant stress (Abe et al., 1984).

Catalase related studies are very limited. In this study, decreased CAT activities were reported in AF, AM, WET, Brain and Liver which are in accord with Subramaniam et al (1995) and Rekha et al (2001).

GSH is essential to maintain structural and functional integrity of the cells. GSH depletion altered levels of intracellular calcium, lowered threshold oxidative stress, caused enhancement of DNA repair (Coleman ···
et al., 1998; Eric and Nathan, 1997; Carmichael et al., 1998). GSH is known to protect against CP-induced bladder damage. Our findings agree well with this and the activities of GPx.

Antioxidant tripeptide GSH is a critical endogenous antioxidant that acts directly as a free radical scavenger and in conjunction with the detoxification enzyme GPx and GST. Studies in human and animal models showed decreased activity of GSH after chemotherapeutic agents. Our results showed decreased GSH activity in AF, AM, brain and liver, in a dose-dependent manner. This is in agreement with the findings of Gurtoo et al. (1981). The maximum reduction was observed at 24 and 48 h with CP treatment in most of the tested parameters. This may be due to increased rate of GSH oxidation than the regeneration of GSH. Our authors in number of studies reported significant decrease in GSH levels with CP treatment. (Rekha et al., 2001; al-Harbi et al., 1996; Subramaniam et al., 1995; Bhanumathi and Devi, 1994).

GPx is considered to be a most important \( \text{H}_2\text{O}_2 \) removing enzymes in mammalian cells and is more important in catalase (Gaetani et al., 1989). The activity of GPx is dependent on the availability of GSH, which in turn, maintained by de novo synthesis, via, GR and the levels of NADPH via glutathione reductase.

Our results show a dose dependent decrease in GPx activity till 48 h in AF, AM, WET and liver. The decrease may be due to the increase in LPO levels observed in the above. The hydroperoxides formed were metabolized by GPx, preventing their breakdown to free radical products which could perpetuate lipid peroxidation (Caramagnoli et al., 1983).

GST is one of the groups of selenium dependent enzymes that inhibit GPx activities with fatty acid peroxides. GST is an important
detoxifying enzyme and plays a physiological role in inhibiting the detoxification of potential alkylating agents (Trakshel and Maines, 1988). Our results showed a varied activity. There was no significant change in GST levels with increase in dose in WET. In AF CP 100 μg caused increased GST activity at later stages when compared to controls. In brain, GST activity decreased with dose and duration.

The induction of this enzyme could be due to biochemical basis and adaptation of the system towards the oxidative damage in the tissue (Chung and Maines, 1981).

In this study, decreased activities of SOD, CAT, and GPx when compared to controls in CP treated embryos could be due to increased production of lipid peroxides and subsequent GSH depletion.

Our findings suggest that CP is a toxic agent to the developing fetus and shows its toxicity by increasing the levels of free radicals. To counter this, the system has a self-defense mechanism against the reactive oxygen species by increasing the activity of endogenous antioxidant enzymes. When the capacity of the system to self-defense decline it leads to increased oxidative stress leading ultimately to tissue damage or cell death.

The use of AF as a sensitive indicator for drug toxicity is a possibility in future, which requires extensive study.
c) BIOCHEMICAL CHANGES IN CHICK EMBRYO WITH CYCLOPHOSPHAMIDE TREATMENT

Results

Table 5 represents the biochemical parameters in chick amniotic fluid in controls and cyclophosphamide (CP) treated embryos. CP treatment was given on the 7th day of incubation and bio-chemical analysis was done after 48 h of treatment for long-term study.

Significant increase (P<0.001) in the levels of glucose, proteins, urea, uric acid, creatinine and inorganic phosphorous were observed with 200 µg of CP treatment, whereas, CP of 100 µg caused no significant increase in these biochemical parameters except for uric acid which showed an increased trend (p<0.01) when compared to controls.

Ascorbic acid levels were significantly decreased (P<0.001) in a dose-dependent manner with CP treatment in AF.

The enzymatic activity of alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were increased considerably (P<0.001) with CP treatment in AF compared with to controls.

No significant change in the ionic balance was observed in amniotic fluid, though a slight increase in the K+ with CP 200 µg was observed.

Table 6 shows the effect of CP on biochemical parameters in chick embryonic liver.

Liver protein was found to decrease with drug treatment. CP 200 µg was more damaging than CP 100 µg.
Table 5. Biochemical studies in chick amniotic fluid with cyclophosphamide treatment

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>CP-100 μg</th>
<th>CP-200 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>36.36±2.12</td>
<td>38.19±2.50 NS</td>
<td>42.91±2.01*</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>17.08±1.56</td>
<td>15.33±1.75 NS</td>
<td>21.16±2.13**</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>9.88±0.62</td>
<td>10.84±1.02 NS</td>
<td>14.83±1.47*</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>8.16±2.25</td>
<td>12.16±1.60**</td>
<td>17.08±2.15*</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.45±0.10</td>
<td>0.40±0.17 NS</td>
<td>1.31±0.56*</td>
</tr>
<tr>
<td>Inorganic Phosphorous (mg/dL)</td>
<td>0.72±0.01</td>
<td>0.75±0.05 NS</td>
<td>0.88±0.23 NS</td>
</tr>
<tr>
<td>Potassium mEq/L</td>
<td>1.51±0.35</td>
<td>1.87±0.34 NS</td>
<td>2.27±0.16*</td>
</tr>
<tr>
<td>Sodium mEq/L</td>
<td>150.00±9.48</td>
<td>155.0±7.07 NS</td>
<td>158±13.6 NS</td>
</tr>
<tr>
<td>Ascorbic acid (mg/dL)</td>
<td>2.31±0.14</td>
<td>1.31±0.16*</td>
<td>0.67±0.17*</td>
</tr>
<tr>
<td>Alkaline phosphatase&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.92±0.22</td>
<td>4.7±0.27*</td>
<td>5.65±0.2*</td>
</tr>
<tr>
<td>Aspartate aminotransferase&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3.26±0.22</td>
<td>4.96±0.27*</td>
<td>6.07±0.30*</td>
</tr>
<tr>
<td>Alanine aminotransferase&lt;sup&gt;3&lt;/sup&gt;</td>
<td>8.70±0.56</td>
<td>9.68±0.51*</td>
<td>11.21±1.15*</td>
</tr>
</tbody>
</table>

NS: Not significant, * P<0.001, **P<0.01

Units of:

1. Alkaline phosphatase expressed as μmol phenol liberated/min/mg protein
2. Aspartate aminotransferase as μmol pyruvate liberated/min/mg protein
3. Alanine aminotransferase as μmol pyruvate liberated/min/mg protein
Table 6. Biochemical studies in chick liver with cyclophosphamide treatment

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>CP-100 µg</th>
<th>CP-200 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (mg /100 mg dry wt)</td>
<td>13.16±0.33</td>
<td>12.62±0.20**</td>
<td>10.50±0.85*</td>
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<tr>
<td>Inorganic phosphorous (mg/100 mg dry wt)</td>
<td>1.35±0.31</td>
<td>0.54±0.04*</td>
<td>0.58±0.11*</td>
</tr>
<tr>
<td>Ascorbic acid (µg/100 mg, dry wt)</td>
<td>101.16±1.47</td>
<td>78.18±2.15*</td>
<td>83.00±7.73*</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>7.51±0.29</td>
<td>8.80±0.63*</td>
<td>12.96±1.39*</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>4.27±0.31</td>
<td>5.55±0.19*</td>
<td>7.95±0.55*</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>9.79±0.73</td>
<td>11.12±0.73*</td>
<td>17.50±2.34*</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>5.62±0.19</td>
<td>6.59±0.24*</td>
<td>7.70±0.70*</td>
</tr>
<tr>
<td>Succinate dehydrogenase(^a)</td>
<td>1.45±0.10</td>
<td>1.50±0.27(^{NS})</td>
<td>1.86±0.21(^{**})</td>
</tr>
<tr>
<td>Lactate dehydrogenase(^b)</td>
<td>66.30±1.67</td>
<td>65.10±0.65(^{NS})</td>
<td>86.70±2.90*</td>
</tr>
<tr>
<td>Malate dehydrogenase(^c)</td>
<td>74.30±0.04</td>
<td>80.33±3.2*</td>
<td>94.0±3.60*</td>
</tr>
</tbody>
</table>

NS Not significant, * P<0.001, **P<0.01, ^P<0.05

Units of

Acid phosphatase: µmol phenol liberated/min/mg protein
Alkaline phosphatase: µmol phenol liberated/min/mg protein
Aspartate aminotransferase are expressed as µmol pyruvate liberated/min/mg protein
Alanine aminotransferase: µmol pyruvate liberated/min/mg protein
\(^a\)Succinate dehydrogenase: µmol of succinate oxidized/min/mg protein
\(^b\)Lactate dehydrogenase: µmol of pyruvate formed/mg protein.
\(^c\)Malate dehydrogenase: nmol of NADPH oxidized/min/mg protein
Increased activity of acid phosphatase (ACP), ALP, AST and ALT was observed in chick embryonic liver with drug treatment when compared to controls.

Ascorbic acid (ASA) level in embryonic liver was significantly decreased (P<0.001) with CP 100 and CP 200 µg.

Lactate dehydrogenase (LDH), malate dehydrogenase (MDH), and succinate dehydrogenase (SDH) activities were studied with CP administration in liver (Fig.6). A significant increase in LDH activity was observed only with CP 200 µg. MDH activity was significantly increased by CP treatment (P<0.001) whereas SDH showed no response to CP treatment.

Table 7 & 8 represents the effect of CP on whole embryonic tissue (WET) and heart.

CP declined protein content significantly in WET whereas in heart no significant change was observed with CP. Creatinine levels were not influenced by either concentration of CP in WET.

The ASA levels were significantly reduced (P<0.001) in WET whereas in the embryonic heart slight increase in ASA levels were detected.

LDH activity was significantly enhanced in both WET and heart with CP 200 µg. MDH and SDH activity showed no significant increase with CP treatment in WET and heart tissues.
Table 7. Biochemical studies in chick whole embryonic tissue with cyclophosphamide treatment

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>CP-100 µg</th>
<th>CP-200 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (mg /100 mg dry wt)</td>
<td>65.66±2.80</td>
<td>64.16±3.48</td>
<td>52.16±3.54*</td>
</tr>
<tr>
<td>Creatinine (mg /100 mg dry wt)</td>
<td>0.17±0.21</td>
<td>0.14±0.20</td>
<td>0.14±0.30</td>
</tr>
<tr>
<td>Ascorbic acid (µg/100 mg. dry wt)</td>
<td>0.89±0.30</td>
<td>0.81±0.17*</td>
<td>0.22±0.08*</td>
</tr>
<tr>
<td>Lactate dehydrogenase b</td>
<td>7.62±0.19</td>
<td>8.34±0.22</td>
<td>10.07±0.06*</td>
</tr>
<tr>
<td>Malate dehydrogenase c</td>
<td>7.50±0.50</td>
<td>7.08±0.15</td>
<td>7.43±0.02</td>
</tr>
</tbody>
</table>

NS Not significant, * P<0.001, **P<0.01, †P<0.05

bLactate dehydrogenase: µmol of pyruvate formed/mg protein.

cMalate dehydrogenase: nmol of NADPH oxidized/min/mg protein

Table 8. Biochemical studies in chick embryonic heart with cyclophosphamide treatment

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>CP-100 µg</th>
<th>CP-200 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (mg /100 mg dry wt)</td>
<td>61.81±1.50</td>
<td>60.15±0.50</td>
<td>60.60±1.15</td>
</tr>
<tr>
<td>Ascorbic acid (µg/100 mg. dry wt)</td>
<td>84.35±1.84</td>
<td>86.3±2.21</td>
<td>95.0±3.37*</td>
</tr>
<tr>
<td>Succinate dehydrogenase a</td>
<td>0.63±0.21</td>
<td>0.84±0.04*</td>
<td>0.74±0.016</td>
</tr>
<tr>
<td>Lactate dehydrogenase b</td>
<td>16.37±1.47</td>
<td>21.66±1.55*</td>
<td>22.6±1.54*</td>
</tr>
<tr>
<td>Malate dehydrogenase c</td>
<td>30.56±0.38</td>
<td>32.00±2.0</td>
<td>28.6±2.51</td>
</tr>
</tbody>
</table>

NS Not significant, * P<0.001, **P<0.01, †P<0.05

aSuccinate dehydrogenase: µmol of succinate oxidized/min/mg protein

bLactate dehydrogenase: µmol of pyruvate formed/mg protein.

cMalate dehydrogenase: nmol of NADPH oxidized/min/mg protein
Discussion

In the present study the administration of two different doses of CP (100 and 200 μg) resulted in significant biochemical changes in AF and other embryonic tissues. The biochemical changes in amniotic fluid and tissues would be well used to extrapolate the findings similar to the CP induced changes in higher model system and even with humans, provided the mechanism by which the damage induced is clearly understood.

The result, which we observed following different doses of CP administration in chick embryo, reveals alterations in several marker enzymes.

The level of glucose was significantly increased with higher concentration in CP in amniotic fluid compared to controls. The change in glucose level is an indicator of alterations in carbohydrate metabolism. It is also a major source of energy for the nervous system and erythrocytes. There are very few studies on the effect of CP on glucose. Few studies have indicated the use of CP in inducing diabetes in NOD mice (Maruyama et al., 1991). The increase levels of glucose in amniotic fluid could be due to alteration in the membrane permeability and diffusion of embryonic glucose into amniotic fluid or may have a direct effect on glucose metabolism in AF, for more confirmed reports further studies are needed. The change in glucose level is an indication of toxicity of the drug.
Increased levels of urea, uric acid and creatinine may be due to the damages incurred by high dose of CP on the function of embryonic kidneys.

CP administration caused significant elevation in marker enzymes of amniotic fluid as well as in embryonic tissues. The significant increase in the activities of enzyme in liver and AF are due to the effect of CP on hepatocytes and leakage of the enzyme to AF.

Our studies are in agreement with reports of increased activities of alkaline phosphatase with CP administration (Venkatesan and Chandrakasan, 1994, 1995) in lavage fluid and tissues. Our study also showed an increased liver acid phosphatase activity with CP.

AST and ALT are the most sensitive markers, which are considered as the indices for the diagnosis of liver and other related disease. These are important class of enzymes linking carbohydrate and amino acid metabolism and establish a relationship between the intermediates of TCA cycle and amino acid.

There is an increased activity of ALT and AST in AF and liver. These findings are in an agreement with the results of Gosh et al. (1999), which showed increased activities of ALT, AST, ALP and ACP in cyclophosphamide, treated albino rats. Latha and Pannikar (1999) also showed an increased levels of ALT in CP treated mice.

The increased protein content in amniotic fluid may be due to the leakage of RBC cells into amniotic fluid. The decreased levels in the tissues are due to the acrolein, a toxic metabolite of CP formed after biotransformation. This metabolite preferentially binds to the embryonic
proteins and thus damaging the proteins in the tissues. The results indicate that CP 200 μg could be more toxic than CP 100 μg.

In the present study the enzymatic activities of LDH, MDH and SDH were significantly increased with higher dose of CP in AF and embryonic tissues. This is in agreement with the results published by (Shalani et al., 2001; Venkatesan and Chandrakasan, 1994).

It is clear from our data that CP caused significant alterations in biochemical parameters of amniotic fluid as well as embryonic tissues with dose and duration. The precise mechanism by which CP caused most of the changes in the biochemical parameters still remains unclear. Hence, further studies are needed to clarify this issue.

Our data further strengthens the use of amniotic fluid as an indicator of drug toxicity.