CHAPTER-V

SUMMARY AND CONCLUSION
The major problem in cancer chemotherapy still is the lack of selectivity of available drugs. The need to explore the relevant toxicity imposed by cytotoxic agents at the cellular level is due to the fact that the changes and/or toxicity in the normal tissues can be alleviated by co-administration of protective agents and controlled diet without interfering the cytotoxic effect. The antineoplastic drugs can be classified in to various classes depending upon their mechanism of action and origin like alkylating agents, antimetabolites, nitrosoureas and antibiotics etc.

The alkylating agent cyclophosphamide is the most commonly prescribed cytotoxic agent in clinical medication. It is also used as an immunosuppressive agent in the preparation of organ transplantation procedures and in the treatment of disease states thought to be of autoimmune aetiology. Cyclophosphamide has to undergo biochemical activation in liver by cytochrome P-450 for its activity. In liver it is converted in to phosphoramide and acrolein which are active cytotoxic metabolites. After conversion it goes into circulation for its antitumor activity. This study was therefore undertaken with a view to find histopathological and histochemical changes due to deposition of these two metabolites in different organs. In addition to this its clinical use has been compromised by the dose dependent/dose limiting toxicity to various organs.

The main difference between normal tissue and malignancy is not in the rate of cell replication because, in most of the normal tissues, the rate of proliferation is equivalent to the rate
of cell death, whereas in the malignancy, proliferation exceeds the death rate of cells. This is because the neoplasm lacks the autoregulation of proliferation and tumor cells become detectable, when the population reaches about $10^9$ to $10^{10}$ cells. This requires 30 to 33 doubling times. The neoplasm becomes lethal when population reaches about $5 \times 10^{11}$ to $5 \times 10^{12}$ cells after 39 to 42 doubling times. The cells cycle in the neoplasm exists in two stages (i) $G_0$, and (ii) $G_1+S+G_2+M$. The second one comprises all phases committed to cellular division. This proliferation is devoid of autoregulation and requires the administration of cytotoxic or antineoplastic drug.

One of the main properties of neoplastic cells is having composition and activities similar to those of the host cells. This is the basic problem in designing antineoplastic drug to make it possible to attack only the tumor cells. At the same time it also imparts severe deleterious effects on normal cells. This in turn causes the toxicity in various organs. This emphasizes the necessity to undertake the detailed toxicity associated with drug. The present study has been undertaken with view to study various histochemical and histopathological toxicity on albino rats, as a model animal. The outcome of this study will prove useful during the treatment of various types of cancer by monitoring histochemical and histopathological changes.

Apart from this, the conventional drug delivery system plays a marginal role in providing efficient treatment of neoplastic diseases. The archaic manner of administering drugs is obviously inefficient and often results in severe toxic side effects. The encapsulation of pharmacologically active substances in
microvesicles offers potential possibilities. Therefore the present work has been undertaken keeping in mind the relevant toxicity of cyclophosphamide through conventional delivery system and comparing with emerging novel drug delivery system by encapsulating cyclophosphamide in erythrocytes. Encapsulated drug in circulating vesicles may by acting as a slow release system providing sustained drug level in the blood at higher levels and for longer periods.

LD$_{50}$ of free cyclophosphamide and cyclophosphamide loaded gluteraldehyde treated erythrocytes for 96 hours were 105 mg/kg and 26.5 mg/kg body weight respectively.

**Preparation of cyclophosphamide solution and carrier erythrocytes**

(i) Calculated quantity of cyclophosphamide was dissolved in 0.9 % NaCl solution to achieve desired concentration of cyclophosphamide for acute and chronic study.

(ii) For preparation of carrier erythrocytes hypertonic preswell method reported by Field et al. (1989) was adopted. In short, washed RBCs (Red Blood Cells) were suspended at 40 % hematocrit in phosphate buffer saline (pH 7.4). After this the cells were loaded with cyclophosphamide involving three sequential mechanism i. e. hypotonic lysis, resealing and reannealing. These cells were then crosslinked with diluted gluteraldehyde solution to enhance stability.
The optimized parameters in terms of volume of aqueous drug solution added, drug concentration and temperature were 300μl, 5 mg/ml and 4°C respectively at which highest encapsulation efficiency (0.76 mg/ml of packed cells) was observed. The amount of drug entrapped in cells was determined by lysing the cells in distilled water followed by deproteinization with acetonitrile and to the supernatants collected was added with 20 % NaNO₂ and 20 % HCl and absorbance was determined at 350nm.

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\text{Percent encapsulation} = \frac{\text{Amount encapsulated}}{\text{Amount of drug added}}
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**Experimental Procedure**

**Dose schedule**

For **acute study**: 25-mg/kg body weight daily up to 96 hours (Free cyclophosphamide).

6.5 mg/kg body weight daily up to 96 hours (cyclophosphamide loaded gluteraldehyde treated erythrocytes).

For **chronic study**: 10-mg/kg body weight twice a week up to 3 months.

**Acute study:**

Acute study was determined by dividing 32 rats into four equal groups of 8 rats each. **Group I** received only 0.9 % saline solution and served as a control. **Group II** received 25-mg/Kg body weight cyclophosphamide (dissolved in 0.9 %
saline solution) intraperitoneally daily up to 96 hours. Albino rats of Group III and IV were administered with cyclophosphamide loaded gluteraldehyde treated erythrocytes (equivalent to 6.5 mg/Kg body weight) and same amount of plain gluteraldehyde treated erythrocytes respectively through the same route and for same duration. The rats from different groups were humanely sacrificed simultaneously daily up to 4 days (96 hours).

*Chronic Study*

For chronic study 32 albino rats were divided into 4 equal groups having 8 rats in each group. Group I and III were treated as control and administered with 0.9 % plain saline solution, Group II and IV were administered with 10-mg/kg body weight cyclophosphamide (dissolved in 0.9 % saline solution) intraperitoneally twice a week up to 3 months. The rats comprising from different groups were humanely sacrificed after 30, 60 and 90 days.

*Processing of tissues*

After acute and chronic exposure of drug the abdomen of animal was opened and liver, lung, kidney and intestine were taken out and fixed in Bouins fluid for 24 hours. Then the organs were washed in running water for further 24 hours. The dehydration was carried out with different grades of alcohol. Organs were cleared using xylene. After embedding in paraffin wax the sections (5 to 6 μm) were cut with the help of microtome and stained with standard prescribed methods for respective histochemical and histopathological study. After staining
the tissue sections of control and treated groups, were examined under microscope.

_Histochemical study_

The histochemical study was conducted in terms of qualitative study of proteins, carbohydrates, lipids and nucleic acid contents in the experimental tissues. The following methods have been used.

(a) Proteins : Mercuric Bromophenol Blue (HgBB) method.

(b) Carbohydrates : Periodic acid/ Schiff (PAS) method.

(c) Lipids : Sudan black B method.

(d) Nucleic acid : Methyl Green Pyronin Y (PMG) method.

_Histopathological study : _Haematoxin-Eosin method_

**RESULTS AND DISCUSSION**

The present study deals with the histochemical and histopathological changes in liver, lungs, kidney and intestine of albino rats under the influence of acute and chronic exposure of cyclophosphamide. The histopathological study in liver, lungs kidney and intestine has also been performed on acute administration of cyclophosphamide loaded erythrocytes. The observations of the study have been mentioned in the form of microphotographs.
Histochemical study

Experimental tissues liver, lung, kidney and intestine of albino rat were selected and processed accordingly to monitor the qualitative changes in protein, carbohydrate, lipid and nucleic acid contents induced by cyclophosphamide administration. Protein, carbohydrate, lipid and nucleic acid contents in liver, lung, kidney and intestine of the control rats have been observed similar to that of initial control rat in both acute and chronic study.

(i) Liver

(a) Protein

In the case of acute study, no remarkable change in protein contents has been observed in liver. In case of chronic study slight decrease in protein contents has been observed which may be due to disturbance in protein metabolism.

(b) Carbohydrate

No remarkable change has been observed in carbohydrate contents of liver during acute study. After 3 months post treatment slight increase in glycogen contents in hepatic cells of liver has been observed which could be accounted for due to reduction in the glycogen phosphorylase and glucose phosphatase.

(c) Lipid

No significant change in lipid content of liver has been observed during acute exposure (96 hours) of cyclophosphamide. During chronic study (3 months) liver
showed slight increase in lipid content. This is due to interference of the protein synthesis which depresses the rate of beta lipoprotein synthesis that leads to increased lipid contents.

(d) Nucleic acids

No remarkable change has been observed in nucleic acid contents of liver during acute study. After 3 months post treatment slight decrease in nucleic acid content in liver has been observed which may be due to inhibition of RNA and DNA synthesis.

(ii) Lungs

(a) Protein

During acute study, no significant change has been observed in protein content of lungs. In case of chronic exposure, slight decrease has been observed which may be due to damage of alveolar wall.

(b) Carbohydrate

No remarkable change has been observed in carbohydrate contents of lung during acute (96 hr) and chronic study (90 days).

(c) Lipid

During acute study lung showed no change in lipid contents qualitatively while in the case of chronic exposure, lung showed slight increase in lipid
contents. This may be due to interference of the protein synthesis which depresses
the rate of beta lipoprotein synthesis that leads to increased lipid contents.

(d) **Nucleic acids**

No remarkable change has been observed in nucleic acid content of lung. In
the case of chronic study (90 days) slight decrease in nucleic acid contents has
been observed which is due to the same reason as discussed earlier.

(iii) **Kidney**

(a) **Protein**

No change in protein contents has been observed in acute study while in case
of chronic study decrease in protein content was found due to the reason quoted
earlier.

(b) **Carbohydrate**

Carbohydrate contents in kidney have not been altered after acute and chronic
exposure of the drug.

(c) **Lipid**

During acute study no change has not been observed while slight increase
in lipid content has been observed during chronic exposure of the drug.
(d) **Nucleic acids**

No remarkable change has been observed in nucleic acid contents after acute exposure (96 hr) while slight decrease in nucleic acid contents has been found after chronic exposure (90 days) which is due to inhibition of RNA and DNA synthesis.

(iv) **Intestine**

(a) **Protein**

No change has been observed during acute study while slight decrease was found after chronic treatment due to the reason as discussed earlier.

(b) **Carbohydrate**

Carbohydrate contents in intestine has not been altered after acute and chronic exposure, which is due the reason as discussed above.

(c) **Lipid**

During acute study no change in lipid content has been observed while slight increase in lipid content was observed during chronic treatment.

(d) **Nucleic acids**

During acute study no remarkable change in nucleic acid contents has been observed while slight decrease has been observed during chronic exposure which is due to the fact as discussed earlier.
Histopathological study

The histopathological study has been undertaken employing both free cyclophosphamide (acute and chronic exposure) and cyclophosphamide loaded gluteraldehyde treated erythrocytes (acute exposure).

(a) Employing free cyclophosphamide

The liver, lungs, kidney and intestine showed different degree of variation in histopathological pattern as compared to organs belonging to control group.

Acute study

(i) Liver

At acute stage there was no significant change in histopathological features except for 96 hours, when necrosis in liver cells has been observed.

(ii) Lungs

No changes have been detailed in early phases of acute study while compact alveoli have been observed after 96 hours.

(iii) Kidney

In kidney degeneration in uriniferous tubules has been observed after acute exposure of the drug.

(iv) Intestine

No structural changes were observed in intestine during acute study.
**Chronic study**

(i) **Liver**

The chronic exposure of drug imparted various changes in liver like dilatation of hepatic cells with acentric nuclei.

(ii) **Lungs**

The chronic exposure of cyclophosphamide caused damaged alveolar walls in lungs.

(iii) **Kidney**

Necrosis and massive lymphoid infiltration have been observed during chronic exposure of the drug.

(iv) **Intestine**

During chronic exposure ruptured wall of villi and fused Brunner’s glands have been observed.

(b) **Employing cyclophosphamide-loaded gluteraldehyde treated erythrocytes**

The administration of cyclophosphamide loaded gluteraldehyde treated erythrocytes showed dramatic histopathological changes during acute study. The administration of gluteraldehyde treated unloaded erythrocytes showed no toxicity in liver, lung, kidney and intestine which indicates that gluteraldehyde is not toxic to the tissues at given concentration.

Though the liver showed equal degree of toxicity on acute administration of drug loaded gluteraldehyde treated erythrocytes bearing 6.5mg/kg body weight of
cyclophosphamide compared to administration of 25 mg/kg body weight of free
cyclophosphamide after 96 hours, in both cases slight necrosis in liver cells has
been observed) which indicates that same amount of cyclophosphamide is
reaching in the liver in both the cases. It means that proportion of drug reaching to
liver is substantially higher through drug loaded gluteraldehyde treated
erthrocytes (which diverts the access of drug to other organs) compared to free
drug administration. Therefore it can be anticipated that negligible amount of drug
is reaching to other organs which is revealed through same histopathological
features in lung, kidney and intestine in control set of experiment. The observed
degree of toxicity in liver can be overcome by partial regeneration through natural
remediation mechanism. Therefore it can be concluded that cyclophosphamide
loaded gluteraldehyde treated erythrocytes can be of immense potential which
could be exploited for hepatic solid tumors, simultaneously reducing toxicity in
other organs.

CONCLUSION

Though the cyclophosphamide is potent and a drug of choice at various
levels for the treatment of several neoplastic diseases, it shows toxicity of various
degree (both histochemical and histopathological) in different organs after acute
and chronic exposure. Apart from this the use of drug loaded gluteraldehyde
treated erythrocytes can be of potential application as organ specific delivery
coupled with reduction in toxicity in other vital organs could be achieved.