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RESEARCH ARTICLE

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HISTOPATHOLOGICAL AND BIOCHEMICAL STUDIES OF ETOPOSIDE TREATED LIVER OF RAT

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ABSTRACT

Etoposide is a known anticancer drug used for treatment of various tumors. Studies were performed on rats treated with etoposide for 8 weeks. Histopathological alterations like parenchymal ruptures and vacuolation of hepatocyte were observed. The blood sinusoids were dilated and the central blood vessels were also congested with amorphous material. Transmission electron microscopic (TEM) observations showed vesicuolation of smooth and rough endoplasmic, electron dense mitochondria with intact matrix and presence of active golgi apparatus with its characteristic secretory granules and lipid vacuoles. Biochemical findings indicated a significant change in glutathione-s-transferase (G-ST), glutathione reductase (GR), catalase, cytochrome P450 (CYP^{450}) and cytochrome b5 (CYP b_5). However glutathione (GSH), glutathione peroxidase (GPx) and gamma glutamyl transferase (GGT) showed non-significant change. No significant increase was manifested in lipid peroxidation (Lpx) levels in the liver. The metabolic activation of drug by CYP^{450} displayed significant increase and significant decrease of CYP b_5.These changes demonstrate that etoposide at given dose level is not involved in exerting cytotoxic action on the liver.

KEYWORDS

Etoposide, Mitochondria, Endoplasmic Reticulum, Glutathione, Lipid peroxidation.

INTRODUCTION

Etoposide is a semisynthetic epipodophillotoxin usually used in combination with other anticancer agents in the treatment of tumors of testis, lung cancers and other neoplasm's. It is also used to treat leukemia's either alone or in combination with other antitumor drugs, or radiation therapy [1]. Etoposide shares one of the common lipophilic properties along with various other naturally derived anticancer agents due to its complex ring structure. Drug resistance in case of etoposide has been associated to the involvement of MDR 1 gene, which encodes a protein named P-glycoprotein (P-gp). This protein is also involved in efflux of substrate out of cell which is an energy dependent process. P-gp also has a physiological role in normal tissues which involves excretion and protection of tissues from naturally occurring xenobiotics. The metabolic activation of etoposide can be carried out using etoposide glucuronide[2]. Studies by Li.X (2007) have reported that morin administered orally serves as inhibitor of CYP isozymes and P-gp[29].

The mechanism of action involves one-electron oxidation to form the etoposide-phenoxyl radical that can be redox-cycled by intracellular thiols such as GSH and, to a lesser extent by protein thiols [3]. Anti cancer drugs including etoposide have also been found to exert apoptotic response in cells which may activate unique signaling molecules including members of the caspase family. A very similar mechanism has been proposed for apoptosis induction by anticancer drugs. The site of initial caspase activation is the mitochondrial membrane which releases Cytochrome-C. Cytochrome C combines with Apaf-1 caspase complex (located at the mitochondria membrane) and induces apoptosis by the drugs [4]. Cytotoxic action of etoposide and related epipodophyllototoxins is considered to be dependent on dual mechanisms, mediated through cleavage of DNA strand via direct inhibition of DNA topoisomerase II (topo II) as well as direct or indirect DNA modification [5].

The efficacy is associated with increased risk of secondary acute myelogenous leukemia's. Thus it is excluded in the drug regimens [3]. The interaction of etoposide with antioxidants and their combined effect on hepatic damage is seldom documented in clinical oncology.

Current investigations were therefore carried out with an aim to determine the effect of long-term treatment of etoposide drug regimens on histopathological and ultra structural findings of liver tissue followed with studies on GSH, GSH-dependent enzyme and CAT in rat. Studies were also supported further by analyzing hepatic Lpx and microsomal drug metabolizing enzymes namely CYP^{450} and CYP b_5 in rats after etoposide treatment.

MATERIALS AND METHODS

Adult male albino rats of Wistar strain weighing 220-250g obtained from Rajudyog biotechnology division Maharashtra, India were used. The institutional animal ethical committee approved the study. Etoposide was procured from Dabur India Ltd. Bovine serum albumin, reduced and oxidized GSH, thio barbituric acid (TBA), butylated hydroxytoluene (BHT), L-gammaglutamyl-p-nitro-anilidehydrochloride, sodium azide, Dinitro thio benzoic acid (DNTB), NADPH, sodium dithionite, sodium formate were purchased from SRL India. All chemicals used were of analytical grade.

Rats were maintained under identical conditions and were fed with the standard pellet and water ad libitum. The animals were acclimatized for a period of two-weeks and were then treated. They were coded in groups of two per cage and then were subsequently

examined for further study. Experimental rats were injected with 1.0 mg of etoposide per kg i.p daily for a period of 8 weeks. Control group received 0.5 ml of saline daily along with the treated set of the rats. The change in the body weight was monitored per week. Five rats each per cage were maintained in both the groups. At the end of the treatment, rats were sacrificed under ether anesthesia. The liver was dissected out, washed in ice-cold saline, blotted and a homogenate was prepared in 0.1M sodium phosphate buffer (pH 8.0). The homogenate was further centrifuged and the supernatant fractions obtained were utilized for the assay of GSH- related enzymes like GR [6], Gpx [7] GGT [8], GST [9] and CAT [10]. Trichloroacetic acid (TCA) treated samples were utilized for the estimation of reduced GSH [11] and Lpx [12]. The resulting supernatant fractions were recentrifuged for an additional 60 min at 105,000g. The microsomal pellets obtained were collected and used for the estimation of CYP^{450} and b_5 [13]. Total protein content in etoposide treated samples were analyzed and compared with controls [14]. All spectrophotometric readings were observed on Shimadzu UV-160 double beam spectrophotometer. The significance of difference between the means was calculated by student's t-test and results were expressed as mean ± SEM.

Histopathological Studies:

Light Microscopy

The histological changes of liver by light microscopy were studied using paraffin method as described earlier [15]. Liver tissue was cut into pieces of desired size and fixed into Bouin's fixative (standard aqueous picric acid 75 ml + conc. Formalin 25ml + glacial acetic acid 5 ml) for 24-48 h. Tissues were removed from fixative solution and washed thoroughly with distilled water for few hours to remove extra Bouin's fixative. After fixation, samples were washed with 70% alcohol to remove excess of picric acid from the tissues and dehydrated in graded series of ethanol. Excess of alcohol was washed with water from the tissues and clear tissues were kept in filtered molten paraffin which was then sectioned to 3.0µm thick series of section using rotary microtome (Microme, Model No. HM 310). The sections were stained with hematoxylin followed by eosin as secondary stain. The slides were mounted by using DPX mountant as an adhesive agent. The (x20) magnification was used to observation.

Transmission Electron Microscopy:

Liver tissue was removed from the animals after decapitation, sliced into one mm pieces in a drop of 3% gluteraldehyde. Tissue was then immersed in fresh ice cold fixative for two hours and then in 0.1 M cacodylate buffer for next 4 hours. The tissues were then rinsed in buffer and post-osmicated in 1% osmic acid for one to two hours. The tissue was dehydrated in an ascending series of alcohol, followed by propylene oxide and finally embedded in resin that was polymerized at 600°C. Subsequently the blocks were prepared in araldite and 1 m sections were cut with a glass knife on LKB-2000S, ultramicrotome mounted on glass slides and stained with buffered toluidine blue. Appropriate areas were selected with the light microscope. Ultrathin sections of selected blocks were cut with a diamond knife, picked up on copper grids and stained with uranil acetate and lead citrate for final viewing. These sections were scanned and photographed on JEM-JEOL 100s electron microscope. Magnification used to observe the sectioned tissue was (x10, 000).

RESULTS

Light Microscopy of Liver:

Light Microscopic (LM) studies of liver after etoposide treatment showed little changes on the tissue. Parenchyma of hepatic lobules appeared almost normal with occasional ruptures of some cells (Figure. 1) and vacuolations in few of them. Most of the cells had normal morphology. The drug also caused narrowing of portal spaces. Accumulation of amorphous material in the lumen of central biliary duct (CBD) was observed. This was due to significant effect of Etoposide and was indicated by biliary obstruction (Figure 2).

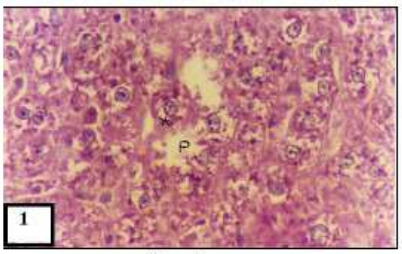

Figure 1
Light Micrographs of rat liver treated with Etoposide for 8 weeks shows the prescence occassional ruptures in parenchymal (P) tissue with vacuolations (*) in few of them. Magnification (x20).

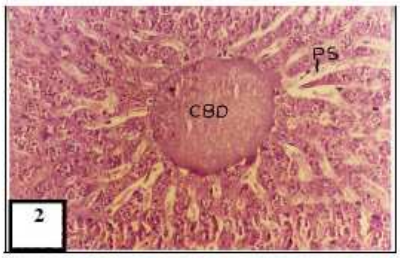

Figure 2
Light Micrographs of rat liver treated with Etoposide for 8 weeks shows narrow portal spaces (PS). Central biliary duct (CBD) shows amorphous material in the lumen.Magnification (x20).

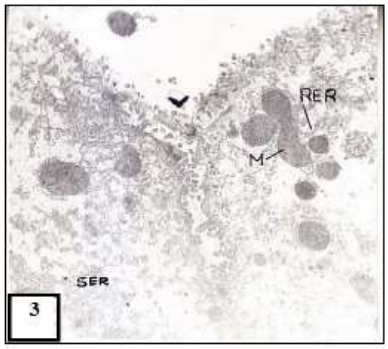

Figure 3:
Electron micrographs of rat liver treated with Etoposide for 8 weeks shows following features of highly dilated cisternae of smooth endoplasmic reticulum (SER) with the presence of amorphous material. Many intact mitochondria (M), free arrays of rough endoplasmic reticulum (RER) and sinusoidal spaces (arrow head) are seen. (Maginification x 12,000).

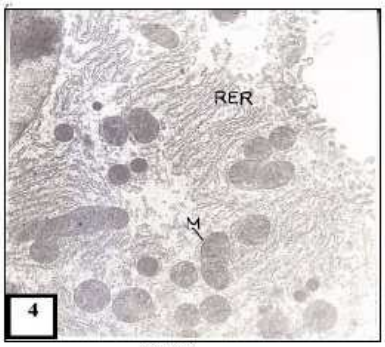

Figure 4
Electron micrographs of rat liver treated with Etoposide for 8 weeks shows noticeable features the cytoplasm shows accumulation of large mitochondria (M) with dense matrix and with intact and tubular crystae. Note the numerous tubular and laminar cisternae of rough endopalsmic reticulum (RER). (Magnification x 12,000).

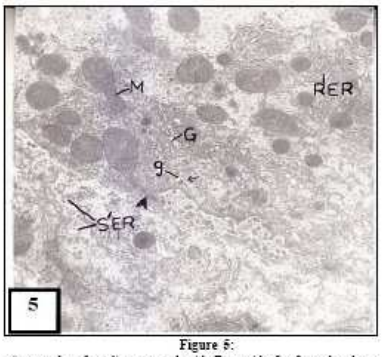

Figure 5:
Electron micrographs of rat liver treated with Etoposide for 8 weeks shows following features note large distribution of mitochondria (M) with dense matrix. Cells with small but active golgi apparatus (G) and occassional presence of secretory granules (g) as well as infrequent endocytosis (thin arrow) free cisternae of rough endoplasmic reticulum (RER), increased smooth endoplasmic reticulum (SER) and sinusoidal spaces (arrow head) are also visible. (Maginification x 12,000).

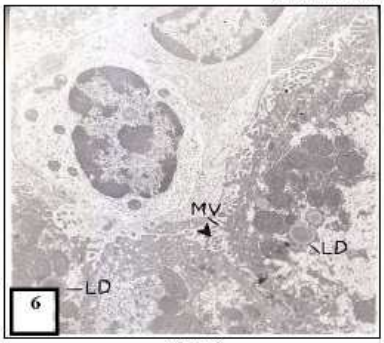

Figure 6:
Electron micrographs of rat liver treated with Etoposide for 8 weeks shows following drug effect note the prescence of lipid droplets (LD) of variable density and intact microvilli (Mv) in the very long sinusoidal spaces (arrow head). (Maginification x 8,000).

Electron Microscopy of Liver:

Electron Microscopy (EM) of liver after etoposide treatment showed significant changes. Nucleus of almost all the cells showed heterochromatic regions and a large nucleolus, which was suggestive of nuclear condensation (Figure. 6). Cytoplasm was dense with heavy accumulation of several types of organelles. Mitochondria with dense matrix but intact and tubular crystae in ample indicated stress related response on the cell (Figure 4 & 5) endoplasmic reticulum exhibited the dynamics of its arrangements and profiles. Many tubular and laminar cisternae of rough endoplasmic reticulum was also seen (Figure 4). Highly dilated cisterna of SER with some amorphous material filled was also observed (Figure 3). Few lipid droplets were observed randomly scattered in the cytoplasm (Figure 6). Small Golgi apparatus, almost obscure in the dense accumulation of SER was noticed along with few vesicles filled with secretion. Occasional endocytosis was also observed (Figure 5). Moderately dilated sinusoidal spaces with intact microvilli were observed.

General Health

In addition to histopathological and ultrastructural studies, a significant decrease in weight was observed in etoposide treated rats (Table 1). The drug did not cause any irritation at the site of the injection. The organ weights of treated rats are shown in Table 1. The weight of the liver in etoposide treated group showed non-significant increase as compared to controls. Total protein content and LPx values showed a non- significant change in etoposide treated rats as compared to controls (Table 1).

Non-significant change was observed on GSH levels as well as Gpx and GGT activity of etoposide treated group as compared to the controls (Table 2). The activity of GST and CAT was significantly higher in etoposide treated rats as compared to the controls (Table 2). The mean values of GR activity in drug treated group were significantly lowered as compared to controls. The decrease in activity was significant in treated animals (Table 2). The level of drug metabolizing enzyme CYP^{450} in liver showed significantly elevated values whereas; CYP b_5 level was significantly reduced after drug treatment as compared to controls (Table2).

Table 1

General and stress-induced parameter in Etoposide–treated and control rats (mean ± SEM)

PARAMETER	GROUPS	
	CONTROL (n=5)	ETOPOSIDE (n=5)
Body weight (g)	367±12.308	304±14.352*
Organ weigh (g)	11.46±0.04	12.826±0.797**
Protein (mg/g tissue)	371.06±14.92	375.86±10.23**
Lipid peroxidation (nmol of Malondialdehyde formed per mg protein)	0.2±0.02	0.239±0.021**

Etoposide rats versus control rats: *p<0.05, ** non- significant

Table 2

Glutathione and antioxidant enzyme activity in hepatic tissue from Etoposide treated and control rats (mean ± SEM)

PARAMETER	GROUPS	
	CONTROL (n=5)	ETOPOSIDE (n=5)
Glutathione (µmol/mg protein)	13.8 ± 1.3	13.818±0.91**
Glutathione-s-transferase (ηmol of cDinitrobenzene bound/mg protein)	6.3±0.4	20.66±1.186*
Glutathione reductase (ηmoles/mg protein)	5±0.4	2.879±0.191*
Glutathione peroxidase (µgm utilized/min/mg protein)	269.1±24.06	267.76±26.8**
Gammaglutamyltransferase (µmol of p-nitroaniline/ min/mgprotein)	2.0881±0.03	2.38±0.1312**
Catalase (unit/mg protein)	0.02±0.00008	0.07±0.005*
Cytochrome p450 (nmol/mg protein)	444±37.46	656.99±31.7*
Cytochrome b5 (nmol/mg protein)	615±37.0	475.53±42.4*

Etoposide rats versus control rats: *p<0.05, ** non-significant

DISCUSSION

Histological studies showed that parenchyma of hepatic lobules appeared almost normal with occasional rupture of some of the cells and vacuolations in some of them. The major findings of the study were that the lumen of the CBD showed accumulation of amorphous material, which indicated biliary obstruction. Similar results were also observed in case of CCl_4 treated liver of rat [16]. In comparison to histological studies, the ultrastructural changes of liver after etoposide treatment revealed slightly enhanced changes. The changes include increased heterochromatin and presence of a small nucleolus in the nucleus of almost all the cells, which is suggestive of nuclear condensation [17]. Dense cytoplasm along with heavy accumulation of several types of organelles indicates that few cells may have undergone apoptosis. Etoposide treatment at given dose level increased the number of mitochondria with dense matrix, and intact and tubular crystae which was ample in number, indicative of drug effect. Furthermore, endoplasmic reticulum exhibited dynamic arrangement and profiles of its cisternae. Numerous tubular and laminar cisternae of RER and highly dilated cisternae of SER with amorphous material were an indication of detoxifying enzymatic mechanisms, which is known to occur due to long term administration of drug [18]. There were also few lipid droplets, randomly scattered in the cytoplasm of hepatocytes of the drug treated animals. Decrease in hepatic GSH levels as compared to control of etoposide treated rats was insignificant. Similar values of LPx in drug treated and control rats were found, which perhaps indicates that cell membranes are less prone to oxidative damage by the free radicals. Non-significantly altered levels of MDA by LPx and other antioxidants suggests that P-glycoprotein pathway might be efficiently involved in effluxing the drug out of the cells thereby lowering the oxidative stress on the tissue. In contrast to our report Limoli et al., (1998) in their study showed etoposide-induced formation of lipid peroxyl radicals and accumulation of LPx products in Chinese hamster ovary cells[19]. The variations in the results are probably due to the difference in the tissues. The increase in LPx products as seen by the authors can also be accounted for their in vitro studies of ovarian cell line. Significant increase in the activity of hepatic GST indicates that the enzyme plays an important physiological role in protecting the tissue against oxidative stress induced by endogenous lipid peroxides. Thus, our results are in congruence with the results presented by Mittal et al., (2001), who said that preirradiation treatment with P. hexandrum enhanced liver GST[20]. The GR activity was significantly depleted in etoposide treated rats as compared to controls. This change in the activity is contradictory to the findings of Katki et al., (1987) which were able to show that

etoposide elevates GR activity in liver[21]. However, other investigators reported that inactivation of enzyme may result due to increased levels of free radicals or due to the metabolites of etoposide [22]. Documentation is not available on the clinical significance of Gpx and CAT in liver tissue after etoposide treatment. Therefore, an attempt has been made to study the role of these enzymes in liver damage after etoposide treatment. Our findings demonstrate a non-significantly decreased activity of Gpx and significantly increased activity of CAT. A non-significant decrease in the value of GSH could be one of the reasons for such a non-significant decrease in Gpx. GSH is used as a co-substrate to metabolize H2O2 that results in formation of water and oxidized GSH (GSSG), which is controlled by Gpx[23]. Thus, in the present investigation when GSH itself was on the decrease, lower activity of Gpx was a logical finding. Previous studies have reported that higher activity of Gpx is required for decomposition of H_2O_2 and various hydro and lipid peroxides [24].

In present investigation, although we have not checked the levels of reactive oxygen species (ROS) and free radicals, it is quite evident from our studies that their levels in the hepatic tissue of treated rat were perhaps higher than the normal because in the hepatic tissue of treated group the activity of CAT was found significantly higher than the control [25]. From these observations it can also be said that the attack by free radicals and (ROS) in treated samples were high enough to significantly increase the activity of CAT but low enough to keep the activity of Gpx significantly decreased.

Effects of higher doses of etoposide on hepatic GGT activity with reference to its toxicity and therapeutic efficacy in regard to its antitumor property are not reported in literature. GGT is required for the metabolic activation of a series of drugs and quinines to a toxin [26]. In our study we have noticed a non-significant decrease in the hepatic GGT activity. Therefore, we propose that decrease in activity of the enzyme is responsible for lowering the effect of drug on the tissue. This by reducing the utilization of GSH and sulfhydryl groups of proteins might in further cascade of reaction lead to reactive thiol radicals ($RS^{\cdot}$) [27]. The oxidative activation of etoposide can be brought about by the array of enzymes, which include CYP^{450} monooxygenases, prostaglandin synthetase and tyrosinase, which ultimately manifest into cytotoxicity [28].

In the present study, when multiple doses of etoposide were administered to rats, effect on oxidative enzyme activity was found to be maximum and was significant than controls, which was suggestive of metabolic activation of etoposide to a phenoxyl radical. The results suggest that efficient phenolic scavengers of peroxyl radicals such as etoposide which are commonly considered as potent antioxidants may themselves produce oxidative stress due to secondary reactions of their phenoxyl radicals with thiols. The later part of the reaction with thiols is found to be contradictory to the present report as GSH levels in the study showed almost similar levels to that of control. Moderate histological changes and slightly enhanced ultrastructural changes in the tissue corresponded with the unaltered levels of GSH. However, CYP b_5 in the liver of etoposide treated rat showed depletion in their levels. As the role of CYP b_5 is complex, the decreased value probably indicates the decreased interaction of the enzyme with various CYP^{450} species.

CONCLUSION

From the present study, it is observed that etoposide can act as an antioxidant towards the membrane phospholipids and in certain cases act as pro-oxidant towards drug-metabolizing enzymes. The redox status etoposide may also contribute towards the antitumor potential. The pathological and biochemical changes demonstrate that etoposide at given dose level is not involved in exerting cytotoxic action on the liver.

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Abbreviations

MDR 1: Multi Drug Resistance 1
P-gp: P-glycoprotein
TBA: Thio barbituric acid
BHT: Butylated Hydroxytoluene
DNTB: Dinitro thio benzoic acid
NADPH: Reduced Nicotinamide Adenine Dinucleotide Phosphate
TCA: Trichloroacetic Acid
CBD: Central Biliary Duct
TEM: Transmission electron microscopic
SER: Smooth endoplasmic reticulum
RER: Rough endoplasmic reticulum
G-S-T: Glutathione-s-transferase
GR: Glutathione reductase,
CAT: Catalase,
CYP^{450}: Cytochrome P450
CYP b_5: Cytochrome b5
GSH: Glutathione
GPx: Glutathione peroxidase
GGT: Gamma Glutamyl Transferase
Lpx: **L**ipid Peroxidation

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