

# Dominant lethal mutations of topoisomerase II inhibitors etoposide and merbarone in male mice: a mechanistic study

Sabry M. Attia

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**Abstract** Two topoisomerase II inhibitors, etoposide and merbarone, were tested for the induction of dominant lethal mutations in male mice. Etoposide was administered at a dosage of 30 or 60 mg/kg. Merbarone was administered at a dosage of 40 or 80 mg/kg. These males were mated at weekly intervals to virgin females for 6 weeks. In the present experiments, regardless of the agent, spermatids appeared to be the most sensitive germ-cell stage to dominant lethal induction. Etoposide and merbarone clearly induced dominant lethal mutations in the early spermatid stage only with the highest tested doses. The mutagenic effects were also directly correlated with reactive oxygen species accumulation as an obvious increase in 2',7'-dichlorofluorescein fluorescence level was noted in the sperm of animals treated with higher doses of etoposide and merbarone. Treatment of male mice with *N*-acetylcysteine significantly protected mice from etoposide- and merbarone-induced dominant lethality. Moreover, *N*-acetylcysteine treatment had no antagonizing effect on etoposide- and merbarone-induced topoisomerase II inhibition. Overall, this study provides for the first time that etoposide and merbarone induce dominant lethal mutations in the early spermatid stage through a mechanism that involves increases in oxidative stress. The demonstrated mutagenicity profile of etoposide and merbarone may support further development of effective chemotherapy with less mutagenicity.

**Keywords** Chemotherapy · Mutagenicity · Topoisomerase II · Reactive oxygen species

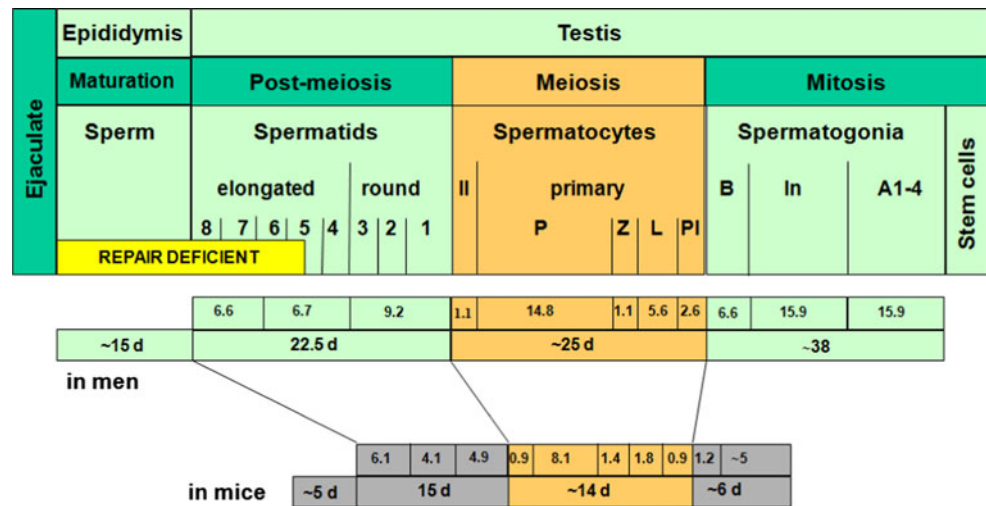
## Introduction

The nuclear enzyme topoisomerase II is a ubiquitous enzyme that relaxes supercoiled DNA and decatenates intertwined DNA by breakage and reunion of DNA double strands (Burden and Osheroff 1998). Thus, topoisomerase II has been predicted to be involved in several cellular events during the cell cycle such as DNA replication, transcription, DNA recombination, and sister chromatid segregation. Since topoisomerase II plays an important role in many cellular processes, topoisomerase II inhibitors are among the most useful anticancer drugs for many types of cancer (Larsen et al. 2003). While several well-characterized topoisomerase II inhibitors including etoposide appear to stabilize enzyme–DNA cleavable complexes leading more directly to DNA breaks, other groups of drugs including merbarone have been reported to inhibit topoisomerase II activity by acting at other stages in the catalytic cycle of the enzyme, where both DNA strands are believed to be intact (Drake et al. 1989; Chen and Beck 1995; Skoufias et al. 2004). Nevertheless, several studies from our laboratory and others have demonstrated that merbarone, a topoisomerase II inhibitor that does not stabilize the cleavable complex, induced strong dose-dependent genotoxic effects in mammalian cells similar to those seen with etoposide (Wang and Eastmond 2002; Attia et al. 2002, 2003; Attia 2008). These data indicate that in contrast to numerous published reports (Drake et al. 1989; Chen and Beck 1995; Skoufias et al. 2004), catalytic inhibitors of topoisomerase II can be potent DNA-damaging agents in cellular systems.

Etoposide has been reported to cause both structural chromosome aberrations and aneuploidy in primary oocytes of the mouse (Mailhes et al. 1996) and the Chinese hamster (Tateno and Kamiguchi 2001). Similar chromosomal effects of the inhibitor were found in mouse primary

S. M. Attia (✉)  
Department of Pharmacology, College of Pharmacy,  
King Saud University, PO Box 11451, Riyadh, Saudi Arabia  
e-mail: attiasm@yahoo.com; attiasm@ksu.edu.sa

**Fig. 1** Duration of male germ cell development in mice and humans (days). *In*, intermediate; *Pl*, preleptotene; *L*, leptotene; *Z*, zygotene; *P*, pachytene; *II*, meiosis II. Adapted from Adler (1996)



spermatocytes (Kallio and Lähdetie 1996; Attia et al. 2002; Marchetti et al. 2006). Etoposide also induced specific locus mutations in primary spermatocytes of mice (Russell et al. 1998). The specific locus mutations were predominantly deletions caused by the effects on the recombination events during pachytene. In a report on a series of compounds, Albertini et al. (1995) listed merbarone as a compound that induced a large increase in structural chromosome aberrations in CHO-K5 cells. Kallio and Lähdetie (1997) studied the effects of merbarone in male mammalian meiosis in vivo using two different cytogenetic approaches: C banding of chromosomes and analysis of spermatid micronuclei using CREST staining. They observed that merbarone injection increased the frequencies of polyploid and hypoploid metaphase II spermatocytes and induced significant levels of micronuclei in spermatids with approximately 80% of the micronuclei containing kinetochore signals, indicating the formation from chromosome loss. Moreover, molecular cytogenetic approaches using multicolor sperm fluorescence in situ hybridization assay with centromeric DNA probes showed that merbarone-induced aneuploidy during meiosis in spermatocytes of male mice that resulted in hyperhaploid sperm and caused meiotic arrest that results in diploid sperm (Attia et al. 2002).

So far, there are no published dominant lethal mutations studies for etoposide and merbarone. Therefore, the aim of the present study was to investigate the ability of etoposide and merbarone to induce dominant lethal mutations in male mouse germ cells. Oxidative stress marker such as intracellular reactive oxygen species generation was assessed as a possible mechanism underlying this dominant lethality. In addition, inhibition of the catalytic activity of topoisomerase II by etoposide and merbarone was evaluated in the presence and absence of the free radical scavenger *N*-acetylcysteine (NAC). Dominant lethal mutation assays help in

the identification of agents that present a risk of transmissible genetic damage (Jha and Bharti 2002; Chamorro et al. 2003). In this test, different stages of gametogenesis may be scored for mutations depending upon the interval between treatment and fertilization. In the present investigation, the time schedule chosen for mating represents the pre-meiotic (35–41 days), meiotic (21–35 days), and post-meiotic (1–21 days) germ cells. As shown in Fig. 1, weeks 1, 2, 3, 4, 5, and 6 post-treatment sperm represent the spermatozoa of epididymis, late spermatids, early spermatids, meiotic germ cells, and B-spermatogonial stages, respectively, at the time of treatment (Adler 1996).

## Materials and methods

### Animals

Adult male and female white Swiss albino mice aged 10–14 weeks and weighing 25–30 g were obtained from Experimental Animal Care Center at our university. The animals were maintained in an air-conditioned animal house at a temperature of 25–28°C, relative humidity of ~50%, and photocycle of 12:12-h light and dark periods. The animals were provided with standard diet pellets and water ad libitum. All experiments were carried out according to the Guidelines of the Animal Care and Use Committee at our university.

### Chemicals

Etoposide and merbarone (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD, USA) were dissolved in dimethyl sulfoxide (DMSO) in sterile distilled H<sub>2</sub>O, mixed on a magnetic stirrer for at least 30 min prior to the administration, and administered by

intraperitoneal injection within 1 h following preparation. Controls mice received equivalent volumes of DMSO.

#### Dominant lethal test

Two separate dominant lethal studies were carried out. In the first experiment, males were treated intraperitoneally with single doses of 30 and 60 mg/kg etoposide. These doses were chosen as the maximum tolerated dose of etoposide in this strain. A concurrent control group of males was injected intraperitoneally with DMSO. Each group consisted of 30 males. They were mated 4 h after treatment at a ratio of 1:1 in the first 3 weeks or 1:2 in the rest 3 weeks to untreated virgin females. Every week, the females were replaced by fresh batch, and the system of caging was continued for 6 weeks to cover the entire spermatogenic cycle. This 42-day mating scheme provided data for the analysis of all stages of spermatogenesis except stem spermatogonia. Every morning, mating was confirmed by checking the presence of vaginal plug representing congealed contents of the seminal vesicle. In the second experiment, males were treated intraperitoneally with single doses of 40 and 80 mg/kg merbarone. A concurrent control group of males was intraperitoneally injected with DMSO. Each group consisted of 30 males. They were mated to untreated virgin females 4 h after treatment as in the first experiment.

Females with plugs were removed from the mating pans. All females were sacrificed for uterine evaluation between the 12th and 14th day following termination of the mating period. Each uterus was removed and examined for number and status of all implantation sites. The total number of implants, number of live implants, number of early resorptions or moles (dead implants), and late deaths were recorded at the time of each dissection (Ehling et al. 1978). Dominant lethality was expressed as % dominant lethality =  $[1 - (\text{live implants per female in the experimental group} / \text{live implants per female in the control group})] \times 100$ .

#### Reactive oxygen species production

To ascertain the involvement of free radicals in the mutagenicity of etoposide and merbarone, separate groups of five male mice were treated intraperitoneally with single doses of 30 and 60 mg/kg etoposide, or 40 and 80 mg/kg merbarone [without or post-treated orally by gavage with 200 mg/kg/day of NAC (Sigma-Aldrich, St Louis, MO) for 3 weeks] used. Control and NAC groups were also included. Two weeks after etoposide and merbarone treatment, animals treated with the highest doses of etoposide and merbarone in combination with NAC were also mated to untreated virgin females to determine the dominant lethality as described above. All males were killed 3 weeks after etoposide and merbarone treatment. Immediately after

cervical dislocation, both *caudae epididymes* of each animal were dissected. An aliquot of sperm suspension ( $\sim 2 \times 10^5$ ) was centrifuged, and the pellets were resuspended in 200  $\mu$ l PBS and then incubated with 200  $\mu$ l of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) (4  $\mu$ M) for 60 min at 37°C in dark (Bakheet and Attia 2011). The fluorescence intensity was monitored with a FLUOstar OMEGA microplate reader (BMG LABTECH Ltd., Germany) from an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Results were expressed as fold of control.

#### Topoisomerase II inhibition assay

To determine whether NAC treatment would have an antagonizing effect on etoposide- or merbarone-induced topoisomerase II inhibition, topoisomerase II- $\alpha$  activity was measured by inhibition of decatenation of kinetoplast DNA (kDNA) by 100  $\mu$ M etoposide, 200  $\mu$ M merbarone, and/or 500  $\mu$ M of NAC using a TopoGen (Columbus, OH, USA) assay kit. The reaction mixture (total volume, 20  $\mu$ l) contained 5 $\times$  complete assay buffer [0.5 M Tris-HCl (pH 8), 1.5 M NaCl, 100 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 300  $\mu$ g BSA/ml, and 20 mM ATP] and kDNA (200 ng) as a substrate. Reactions were incubated with 2 units of topoisomerase II- $\alpha$  in the presence of drugs for 30 min at 37°C. The total volume of the reaction mixture was adjusted to 20  $\mu$ l with dH<sub>2</sub>O. The reactions were terminated with 4  $\mu$ l of stop buffer/gel loading dye (5% Sarkosyl, 25% glycerol, 0.125% bromophenol blue), followed by proteinase K (50  $\mu$ g/ml) treatment for 30 min at 37°C. Samples were subjected to electrophoresis on a 1% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide in 1 $\times$  TAE buffer at 100 V for 1 h. Gels were destained with dH<sub>2</sub>O for 20 min at room temperature and photographed using UV transilluminator.

## Results and discussion

In vivo cytogenetic assays involving mammalian germ cells have been used extensively for genetic toxicological studies. Such studies are of immense importance since some of the effects produced by exposure to hazardous chemicals may be transmitted to next generation through gametes (Jha and Bharti 2002; Chamorro et al. 2003). Dominant lethal mutation is one occurring in a germ cell which does not cause dysfunction of the gamete, but which is lethal to the fertilized egg or developing embryo. Induction of dominant lethals by exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species and the importance of dominant lethal test to assess the genotoxic effect of chemicals is well established (Ehling et al. 1978; Ashby and Clapp 1995). In the present study,

**Table 1** Dominant lethal effects induced in male mice by etoposide

Mating interval (weeks)	Dose (mg/kg)	Pregnant ♀		Total implants		Live implants		Dead implants		% Dead implants per ♀	Dominant lethal <sup>a</sup> (%)
		<i>n</i>	%	<i>n</i>	per ♀	<i>n</i>	per ♀	<i>n</i>	per ♀		
1	0	29	96.66	316	10.89	285	9.82	31	1.06	9.81	0
	30	29	96.66	312	10.75	273	9.41	39	1.34	12.5	4.21
	60	28	93.33	315	11.25	280	10	35	1.25	11.11	−1.75
2	0	29	96.66	334	11.51	298	10.27	36	1.24	10.77	0
	30	30	100	338	11.26	296	9.86	42	1.4	12.42	3.98
	60	28	93.33	335	11.96	291	10.39	44	1.57	13.13	−1.13
3	0	29	96.66	330	11.37	300	10.34	30	1.03	9.09	0
	30	30	100	332	11.06	289	9.63	43	1.43	12.95	6.87
	60	28	93.33	319	11.39	266	9.5	53	1.89	16.61**	8.16
4	0	51	85	563	11.03	503	9.86	60	1.17	10.65	0
	30	47	78.33	509	10.82	454	9.65	55	1.17	10.80	2.05
	60	45	75	515	11.44	445	9.88	70	1.55	13.59	−0.26
5	0	53	88.33	583	11	529	9.98	54	1.01	9.26	0
	30	49	81.66	575	11.73	520	10.61	55	1.12	9.56	−6.32
	60	50	83.33	585	11.7	520	10.4	65	1.3	11.11	−4.19
6	0	52	86.66	567	10.90	496	9.53	71	1.36	12.52	0
	30	49	81.66	589	12.02	534	10.89	55	1.12	9.33	−14.25
	60	52	86.66	575	11.05	522	10.03	53	1.01	9.21	−5.24

\*\*  $P < 0.01$  versus corresponding control (chi-square test); ♀ = female

<sup>a</sup> Dominant lethality was expressed as % dominant lethal =  $[1 - (\text{live implants per female in the experimental group} / \text{live implants per female in the control group})] \times 100$

no significant difference was found in the percentage of post-implantation loss in the 30 mg/kg etoposide and 40 mg/kg merbarone-treated groups compared with untreated control animals. On the other hand, etoposide and merbarone were found to induce post-implantation loss in the third week with the highest doses (60 and 80 mg/kg, respectively), and these differed significantly from the control values (Tables 1 and 2). The frequency of dead implants induced by 60 mg/kg etoposide and 80 mg/kg merbarone was significantly increased by factors of 1.82 and 1.56, respectively, compared with the corresponding control values (Figs. 2 and 3).

Although etoposide and merbarone are the inhibitors of topoisomerase II, their metabolism may be associated with the production of free radicals (Haim et al. 1986; Muindi et al. 1993; Kagan et al. 1999). Accumulation of these free radicals may cause damage to cellular genome and other critical biomolecules, ultimately inducing mutagenesis and carcinogenesis (Attia 2010). The present study demonstrates that etoposide and merbarone are able to generate reactive oxygen species as determined by measuring the fluorescence of DCFH-DA-stained sperm cells. The 2',7'-dichlorofluorescein (DCF) fluorescence level in mice treated with etoposide and merbarone was significantly increased as compared to the control animals (Fig. 4). The mutagenic effects was also directly correlated with reactive oxygen species accu-

mulation as an obvious increase in DCF fluorescence level was noted in animals treated with higher doses of etoposide and merbarone. The frequency of DCF fluorescence level induced by 30 and 60 mg/kg etoposide was significantly increased by factors of 1.37 and 1.92, respectively, compared with the control value. Compared to the control group, the frequency of DCF fluorescence level was significantly increased by merbarone treatment only at the 80 mg/kg.

Treatment of male mice with 200 mg/kg NAC, a reactive oxygen species scavenger, for 3 weeks caused a clear significant decrease in reactive oxygen species generated by etoposide and merbarone as compared to etoposide and merbarone alone (Fig. 4). The frequency of DCF fluorescence in all animals treated with NAC was nearly similar to that in the control group (Fig. 4). Even in animals treated with highest doses of etoposide and merbarone, NAC post-treatment maintained the level of percent of dead implants per female that was lower than that observed in control animals that not treatment with NAC (data not shown), indicating that free radical intermediates formed during etoposide and merbarone metabolism are primarily responsible for their mutagenic effects.

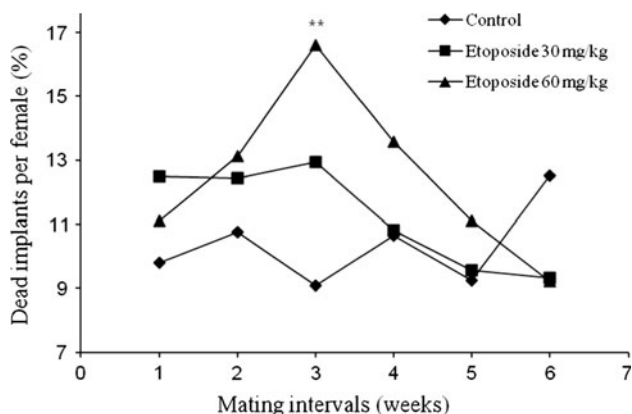
The intriguing question was whether NAC has influence on the inhibitory activities of etoposide and merbarone on topoisomerase II. The catalytic activity of topoisomerase II in vitro was examined using the kDNA decatenation assay.

**Table 2** Dominant lethal effects induced in male mice by merbarone

Mating interval (weeks)	Dose (mg/kg)	Pregnant ♀		Total implants		Live implants		Dead implants		% Dead implants per ♀	Dominant lethal <sup>a</sup> (%)
		<i>n</i>	%	<i>n</i>	per ♀	<i>n</i>	per ♀	<i>n</i>	per ♀		
1	0	28	93.33	295	10.53	260	9.28	35	1.25	11.86	0
	40	27	90	312	11.55	277	10.25	35	1.29	11.21	-10.48
	80	28	93.33	285	10.17	255	9.10	30	1.07	10.52	1.92
2	0	30	100	344	11.46	312	10.4	32	1.06	9.30	0
	40	29	96.66	339	11.68	306	10.55	33	1.13	9.73	-1.45
	80	26	86.66	315	12.11	275	10.57	40	1.53	12.69	-1.70
3	0	30	100	350	11.66	316	10.53	34	1.13	9.71	0
	40	29	96.66	339	11.68	302	10.41	37	1.27	10.91	1.13
	80	29	96.66	329	11.34	279	9.62	50	1.72	15.19*	8.66
4	0	53	88.33	573	10.81	513	9.67	60	1.13	10.47	0
	40	49	81.66	589	12.02	534	10.89	55	1.12	9.33	-12.59
	80	48	80	564	11.75	502	10.45	62	1.29	10.99	-8.04
5	0	51	85	563	11.03	509	9.98	54	1.05	9.59	0
	40	48	80	553	11.52	498	10.37	55	1.14	9.94	-3.95
	80	52	86.66	595	11.44	529	10.17	66	1.26	11.09	-1.93
6	0	51	85	547	10.72	488	9.56	59	1.15	10.78	0
	40	50	83.33	558	11.16	512	10.24	46	0.92	8.24	-7.01
	80	54	90	565	10.46	504	9.33	61	1.12	10.79	2.45

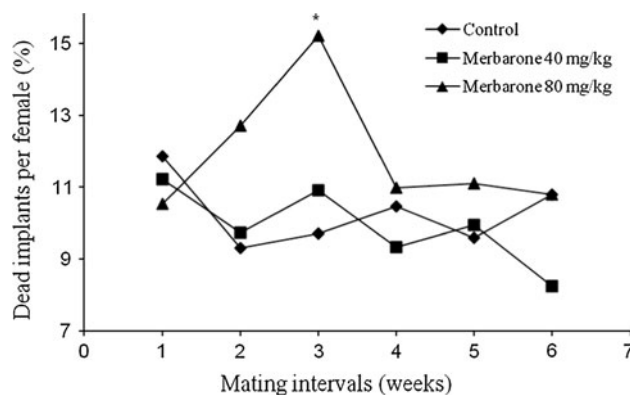
\*  $P < 0.05$  versus corresponding control (chi-square test); ♀ = female

<sup>a</sup> Dominant lethality was expressed as % dominant lethal =  $[1 - (\text{live implants per female in the experimental group} / \text{live implants per female in the control group})] \times 100$



**Fig. 2** Percentage of dead implants per female after etoposide treatment at different mating intervals. \*\* $P < 0.05$  versus corresponding control (chi-square test)

Fig. 5 shows that the fully catenated kDNA (Lane 1) cannot enter the gel due to its large size; however, following incubation with purified topoisomerase II- $\alpha$  (Lanes 2–7), two decatenation products are seen: nicked monomer kDNA (slower migrating band) and covalently closed circular DNA (relaxed). As shown in lanes 2 and 3, the control and NAC (500  $\mu\text{M}$ ) do not affect decatenation of kDNA, while 100  $\mu\text{M}$  etoposide and 200  $\mu\text{M}$  merbarone totally block the reaction (lanes 4 and 5). Importantly, the addition of NAC could not reverse etoposide-induced topoisomerase II inhi-

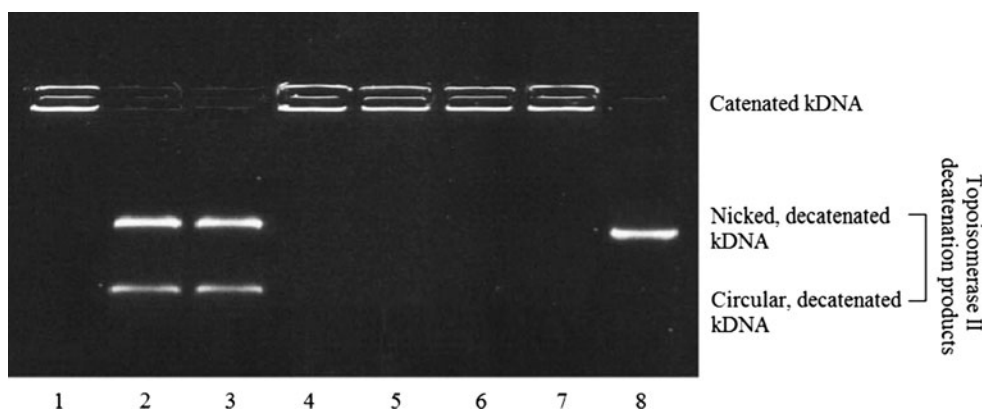
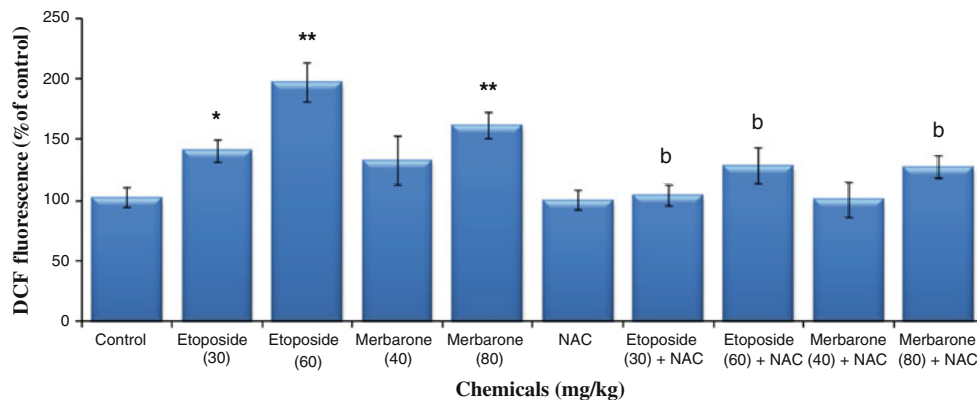


**Fig. 3** Percentage of dead implants per female after merbarone treatment at different mating intervals. \* $P < 0.05$  versus corresponding control (chi-square test)

biton. Similarly, NAC had no effect on merbarone-induced topoisomerase II inhibition. The present results confirm the literatures that have described the antioxidants as having no antagonizing activity on etoposide-induced topoisomerase II-DNA complex formation in human erythroleukemia K562 cells (Lozzio and Lozzio 1979). In addition, NAC diminished the extent of topoisomerase II inhibitor-induced mutagenicity in normal cells without compromise of chemotherapy efficacy (D'Agostini et al. 1998; Neuwelt et al. 2004). Consequently, NAC should not diminish the antitumor activity of etoposide and merbarone through its



**Fig. 4** Effects of *N*-acetylcysteine (NAC; 200 mg/kg/day) on etoposide and merbarone-induced generation of intracellular reactive oxygen species in the sperm cells of mice (mean  $\pm$  SD). \* $P < 0.05$ ; \*\* $P < 0.01$  versus control (Kruskal–Wallis test followed by Dunn’s multiple comparisons test). <sup>b</sup> $P < 0.01$  versus corresponding etoposide or merbarone (Mann–Whitney *U*-test)



**Fig. 5** *N*-acetylcysteine (NAC) did not reverse the inhibitory effects of etoposide and merbarone on kDNA decatenation by human topoisomerase II $\alpha$ . Lane 1 is a kDNA marker; lane 2, control; lane 3, NAC (500  $\mu$ M); lane 4, etoposide (100  $\mu$ M); lane 5, merbarone (200  $\mu$ M);

lane 6, etoposide + NAC; lane 7, merbarone + NAC; and lane 8 is linear kDNA marker produced by digestion of kDNA with Xho I. This gel is representative of at least three independent experiments

ability to prevent the formation of free radical intermediates. These results also suggest that etoposide- and merbarone-induced mutagenicity involves oxidative stress through the formation of reactive oxygen species. These reactive species could attack many cellular substrates, eventually inducing mutagenesis.

In conclusion, intraperitoneal administration of etoposide and merbarone induces germinal mutations in the post-meiotic phase of gametogenesis in male mice as expressed by a dominant lethal effect when acute treatment was used, which are in the clinically relevant dose range. Both compounds increased reactive oxygen species accumulation. These free radicals formed from etoposide and merbarone are primarily responsible for their dominant lethality. Thus, male cancer patients receiving chemotherapy with these drugs may stand a higher risk of siring chromosomally abnormal offspring and genetic counseling of cancer patients before having a child should take these results into consideration. The demonstrated mutagenicity profile of etoposide and merbarone may support further development of effective chemotherapy with less mutagenicity. Examples of strategies that could be followed in drug design to

minimize the metabolic liability associated with reactive metabolite formation are the following: (a) replacement of the structural alert with substituents that are resistant to metabolism or can be metabolized to nonreactive species; (b) blocking the functional groups that are known to undergo bioactivation by a functional group that does not undergo activation; and (c) incorporating a bulky substituent close to the site of metabolism so that metabolism could not occur at the site of metabolic activation. Of course elimination of reactive metabolite formation will be of no benefit if it also eliminates the antitumor effects of the drug, and therefore, it is essential that the antitumor effects of drug candidates be tested at each step in the optimization of the structure (Attia 2010). Moreover, the simultaneous use of antioxidants with chemotherapy is advisable to reduce the mutagenic risk of reactive oxygen species generated by these drugs (Attia et al. 2009; Bakheet et al. 2011).

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**Conflict of interest** The author declares that no competing interests exist.

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