ORIGINAL ARTICLE JGEB Vol. 7, No. 2, 2009 ISSN:1687-157X

The Protective Role of Folic Acid, Vitamin B12 and Vitamin C on the Mutagenicity of the Anticancer Drug Ifosfamide

Souria M. Donya, Fawzia A. Aly and Mona A. M. Abo-Zeid

Department of Genetics and Cytology, National Research Centre, Dokki, Cairo, Egypt

ABSTRACT

Ifosfamide (Holoxan, IFO) is an oxazaphosphorine alkylating agent with a broad spectrum of antineoplastic activity. IFO can damage DNA during any phase of the cell cycle and therefore, is not phase-specific. Thus the objective of this investigation is to measure the potential cytotoxicity of IFO alone and in combination with vitamins (FA, VB12 and VC) The genotoxic potential of IFO was evaluated *in vivo* using different mutagenic end points. Male Swiss mice were injected with different doses of IFO intraperitoneally to investigate the genotoxicity in somatic and germ cells. The doses were 8, 16 and 24 mg IFO/kg body wt. as single doses and 8 mg IFO/kg body wt. as a repeated dose for three consecutive days. Samples were collected after 24 h, 7 and 14 days after treatments. IFO induced chromosomal aberrations (in somatic and germ cells), SCEs and sperm shape abnormalities, which were highly significant in a dose dependent manner 24 h after treatments. Chromosomal aberrations were declined with increasing the time of recovery. However, the tetraploid cells in mouse bone marrow were increased. IFO increased the percentage of DNA fragmentation in mouse spleen cells as measured by diphenylamine (DPA) assay and confirmed by agarose gel-electrophoresis. Oral administration of folic acid (10 mg/kg body wt.), vitamin B12 (0.3 mg/kg) and vitamin C (50 mg/kg body wt.) declined the chromosomal aberrations in somatic and germ cells 24 h after concurrent treatment with IFO. The used doses of vitamins reduced the percentage of DNA fragmentation induced by 24 mg IFO/kg body wt. with DPA assay. In conclusion, the study indicates that the anticancer drug IFO is a mutagenic agent in mouse somatic and germ cells. Vitamins (FA, VB12 and VC) play a beneficial role against the mutagenicity of this drug.

Key Words: IFO, FA, VB12, VC, chromosomal aberrations, SCEs, sperm shape abnormalities, DNA damages.

Corresponding Author: Mona A. M. Abo-Zeid
E-mail: monaabozeid@yahoo.com

Journal of Genetic Engineering and Biotechnology, 2009, 7(2): 41-50

INTRODUCTION

Ifosfamide is an oxazaphosphorine alkylating agent with a broad spectrum of antineoplastic activity. It is used alone or in combination regimens for the treatment of a variety of haematological malignancies such as lymphomas and multiple myeloma and solid tumors including sarcoma, ovarian, testicular, cervical, breast, lung cancer and bone tumors *(Dechant et al., 1991; Zhang et al., 2005 and Goto et al., 2007)* and is also used as systemic anticancer therapy in gynecological cancer patients with renal dysfunction *(Li et al., 2007)*. IFO destroys tumor cells through apoptosis initiated by DNA damage, modulation of cell cycle and other antiproliferative effects. IFO is used concurrently with the uroprotective mesna to avoid hemorrhagic cystitis *(Siu and Moore, 1998)*. According to many authors who studied the action of vitamins *in vivo*, the treatment protocols that yield the best result in terms of reduction of chromosomal damage were those in which vitamins (A, B12, C, E and FA) were administered as pre-treatment or in simultaneous treatment with the clastogenic agent *(Ghaskadbi et al., 1992; Aly et al., 2002 and Costa and Nepomuceno, 2006)*.

The present study was undertaken to assess the cytogenetic effect of IFO at different doses. The protective roles of FA, VB12, VC on the induced chromosomal aberrations was also studies.

MATERIALS AND METHODS

Test subastances:

Ifosfamide (Figure 1) was purchased from Holoxan, Baxter Frankfurt am Main, Germany. Based on *Paget and Barnes (1964)* evaluation of drug activities, it was used as reference to convert the human therapeutic dose to mice therapeutic dose.

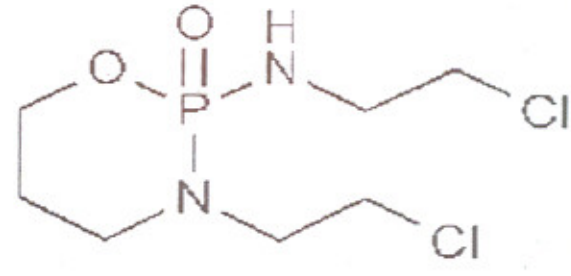

Figure 1: Ifosfamide

Folic acid (FA): Nile Co. for Pharm. and Chem. Ind. Cairo, Egypt.

Vitamin B12 (B12): Amriya Pharm. Ind., Alexandria, Egypt.

Vitamin C (VC): S.D.Fine-Chem.Ltd., Mumbai, India.

Cytogenetic studies:

Animals:

Laboratory-bred strain Swiss albino male mice of 8-10 weeks old with an average weight of 27.5+2.5g obtained from the National Research Center, Cairo, Egypt, were

used.Animals were housed in groups (5 animals/ group) and maintained under standard food and water ad libitium.

Doses and treatments:
IFO doses were i.p. administered at 8, 16, 24 mg/kg b.wt. as a single doses and repeated treatment for three consecutive days were conducted using the lowest dose 8 mg IFO /kg b. wt. Oral treatment of vitamins at 10 mg FA/kg b. wt., 0.3 mg VB12 /kg b.wt. and 50 VC /kg b. wt. were taken concurrently with 24 mg IFO/ kg. The anticancer drug IFO and vitamins were dissolved in distilled water.

Control groups of animals received distilled water and others received vitamins alone were collected concurrently with the treated groups.

Chromosomal aberrations in somatic and germ cells:
Samples were harvested after 24 h, 7 and 14 days of treatment with the different doses of IFO. The groups of animals received the concurrent administration of 24 mg IFO/kg b. wt. with each of the three vitamins separately were sacrificed after 24 h.

Sister chromatid exchanges (SCEs):
Samples were harvested 24 h after treatment with the different doses of IFO.

Sperm-shaped abnormalities:
Mice were treated once i.p. with each of the three doses of IFO. Samples were collected after 35 days from the treatments

Cytogenetic parameters:
For chromosomal aberrations in somatic and germ cells, bone-marrow metaphases were prepared according to *Yosida and Amano (1965)*. The diakinase-metaphase I cells collected from the spermatocytes were made following the air-drying technique of *Evans, et al. (1964)*. Slides were stained with 7 % Giemsa stain in phosphate buffer (pH 6.8). 100 well spread metaphases per animal were analyzed for chromosomal aberrations. The types of aberrations in bone-marrow cells included gaps breaks, deletions, fragments, centric fusions, centromeric attenuations. The types of aberrations in spermatocytes were XY univalents, autosomal univalents, fragments and breaks.

For sister-chromatid exchanges, the method described by *Allen (1982)* was adopted with some modifications. Bone-marrow cells were fixed and stained with fluorescence plus Giemsa method of *Perry and Wolff (1974)*. The frequency of SCE's was recorded for each animal in at least 30 metaphases.

For sperm- shape abnormalities, the epididymides were excised and minced in isotonic sodium citrate solution (2.2%). Smears were prepared and sperms were stained with Eosin Y. *(Wyrobek and Bruce, 1978)*. At least 1000 sperm per animal (5000/group) were assessed for morphological abnormalities of the sperm shape.

DNA Fragmentation Assay:
The groups of animals treated with different doses 8, 16 and 24 of IFO were collected 24h after treatments and repeated dose 8 mg IFO/kg b.wt. for three days. The other groups of animals received concurrently 24 mg IFO/kg b.wt. with each of the vitamins doses and were sacrificed 24 h after treatment.

The method of DNA fragmentation assay was carried out according to *Perandones, et al. (1993)*. Mouse spleen was mechanically dissociated in hypotonic lysis buffer. The cell lysate was centrifuged at 13.000xg for 15 min. then, the supernatant containing small DNA fragments was separated immediately and half the supernatant was used for gel-electrophoresis. The other half, as well as the pellet containing large pieces of DNA were used for the colorimetric determination by Diphenylamine (DPA) assay.

Statistical Analysis:
The significance of the results from the control data was calculated using ($2X^2$ contingency Table) for chromomosal aberrations in somatic and germ cells and t- test for SCE's, sperm- shape abnormalities and DNA fragmentation assays.

RESULTS

Cytogenetic effect of IFO:
Effect of IFO on somatic cells:
Chromosomal aberrations in bone marrow cells:
Table (1) illustrates a detailed study of single and repeated treatments with IFO for 24 h, 7 and 14 days on the induction of chromosomal aberrations in mouse bone marrow. The percentage of induced aberrations increased by increasing the dose of IFO. It was found to be statistically highly significant ($p<0.001$) after excluding gaps.The percentage of chromosomal aberrations decreased with increasing the time of recovery (Figure 2).

Sister chromatid exchanges (SCEs):
All the tested doses induced a statistically significant increase in the frequency of SCEs ($p<0.001$) over that of the control (Table 2) (Figure 3)

Effect of IFO on germ cells:
Chromosomal aberrations in diakinase metaphase I (spermatocytes):
Aberration rates of control animals showed no variation. The significant effect of IFO on the induction of chromosome aberrations in spermatocytes was observed after single and repeated treatment. The percentage of chromosomal aberrations was dose dependent and decreased as the time after treatment increased (Table 3) (Figure 4)

Sperm-shape abnormalities:
IFO induced a dose dependent and statistically significant increase in the percentage of sperm shape abnormalities. (Table 4) shows the different types of observed abnormalities (Figure 5).

The protective effect of vitamins:
Tables (5 and 6) demonstrated the protective effect of FA, VB12 and VC on the induction of the chromosomal aberrations in somatic and germ cells after concurrent treatment with IFO. The results showed that FA, VB12

and VC exerted a significant reduction in the percentage of chromosome aberration induced by 24 mg IFO/kg b. wt. as a single dose and 8 mg IFO/kg b. wt. for 3 consecutive days as a repeated dose.

DNA fragmentation assay:

Effect of IFO:

DPA assay:

Mean percentage of DNA fragmentation in mouse spleen cells was markedly increased ($p<0.001$) after treatment with the single and repeated doses of IFO (Table 7).

Agarose gel-electrophoresis:

DNA fragmentation assessed by agarose gel-electrophoresis was increased in a dose dependent manner with the increasing of IFO (Figure 6)

Protective effect of vitamins:

Table (8) illustrates the mean percentage of DNA fragmentation induced in mouse spleen cells after i.p. treatment with 24 mg IFO /kg b. wt. and oral concurrent treatment with 10, 0.3 and 50 mg/kg b. wt. FA, VB12 and VC, respectively. The percentage of DNA fragmentation decreased to 12.68%, 10.64% and 11.15% after treatment with FA, VB12 and VC, respectively, compared with 12.86% for IFO alone. (Figure 4) shows the DNA fragmentation assessed by agarose gel- electrophoresis, which was decreased after treatment with vitamins compared to that with 24 mgIFO / kg b.wt. (Figure 7)

Table 1: Mean percentage of different types of chromosomal aberrations induced in mouse bone marrow cells 24 h., 7 and 14 days after treatment with different doses of Ifosfamide.

Dose	Duration of treatment	% of cells with different types of structural aberrations							% of cells with different types of numerical aberrations		Total chromosomal aberrations	
		Gap	Br or F.	Del.	C. F.	C. A.	G+Br. and/ or F.	Br.+ F.	41 Ch.	Tetrap.	Including gaps	Excluding Gapsg
I. Control	24h.	2.00	1.00	0.00	0.20	0.00	0.00	0.00	0.00	0.20	3.40	1.40
	7 Days	2.00	0.80	0.00	0.20	0.20	0.00	0.00	0.00	0.20	3.40	1.40
	14 Days	0.40	0.80	0.00	0.00	0.20	0.20	0.20	0.00	0.40	3.20	1.80
II. Single dose 8 mg/kg	24 h.	13.8	7.40	0.00	0.20	0.40	0.40	6.60	0.00	1.40	30.2***	16.4***
	7 days	9.60	6.60	0.00	0.40	0.00	1.80	0.20	0.00	2.60	21.2***	11.6***
	14 days	9.60	4.20	0.00	0.40	0.60	0.80	0.40	0.00	3.60	18.4***	10.0***
16 mg/kg	24 h.	15.0	9.80	0.00	0.40	0.60	2.00	16.40	0.20	2.00	46.4***	31.4***
	7 days	13.0	10.0	0.00	0.00	2.40	1.00	2.60	0.00	2.80	31.8***	18.8***
	14 days	9.4	6.00	0.00	0.00	0.00	1.80	1.20	0.00	4.00	22.4***	13.0***
24 mg/kg	24 h.	18.2	7.00	0.60	0.20	2.00	1.80	26.0	0.00	2.60	58.4***	40.2***
	7 Days	13.4	11.6	0.40	0.20	0.20	2.20	3.40	0.00	3.20	34.6***	21.2***
	14 Days	13.4	8.00	0.20	0.00	0.60	2.20	1.20	0.20	4.00	29.6***	16.4***
III. Repeated dose 8 mg/kg X 3 days	24 h.	14.2	12.8	0.40	1.00	0.20	0.80	6.60	0.00	2.40	38.4	24.2***
	7 Days	13.0	8.60	0.00	0.20	0.00	1.00	1.40	0.00	2.60	26.8	13.8***
	14 Days	8.00	5.20	0.00	0.40	0.60	1.20	0.40	0.00	4.00	19.8	11.8***

The total number of scored cells is 500 (5 animals/group) ***p<0.001 G.: Gaps, Br.: Breaks, F.: Fragments, Del.: Deletions, C. F.: Centric fusions, C. A.: Centromeric attenuations, Tetrap.: Tetraploidy.

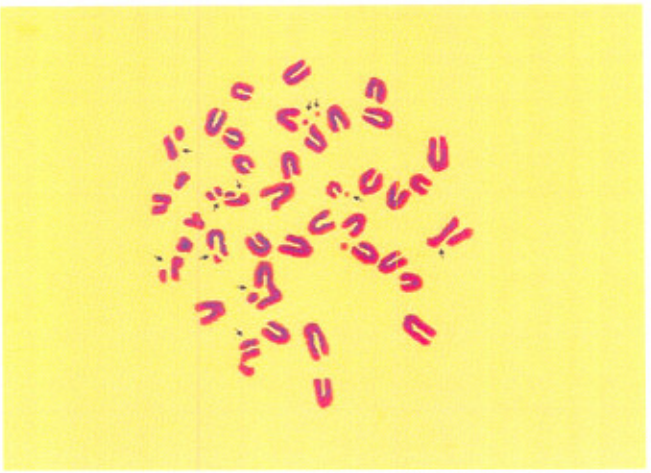

Figure 2: A metaphase with chromosomal aberrations breaks and fragments in in mouse bone marrow cells after i.p. treatment with IFO.

Table 2: Frequency of sister chromatid exchanges in mouse bone marrow cells induced by different doses of Ifosfamide.

Dose	No. of scored cells	Total no. of SCE's	Mean± SE
I. Control	150	583	3.89±0.058
II. Single dose			
8 mg/kg	161	2183	*** 13.56 +0.31
16 mg/kg	160	3842	*** 24.01+0.81
24 mg/kg	156	4030	*** 25.83+0.56
III. Repeated dose 8 mg/kg X 3 days	159	2638	*** 16.59+0.54

***P<0.001.

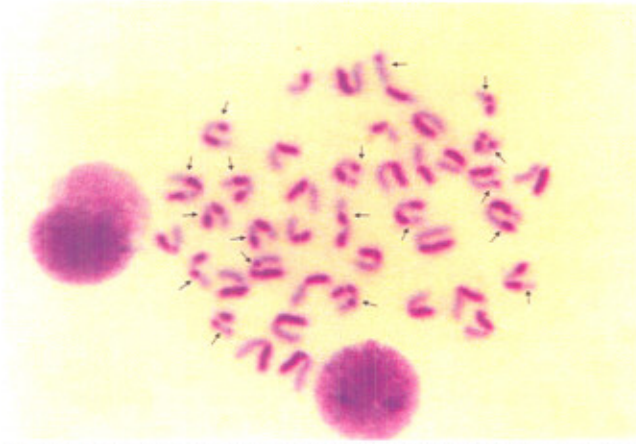

Figure 3: A metaphase with sister chromatid exchanges in mouse bone-marrow cells after i.p. treatment with IFO.

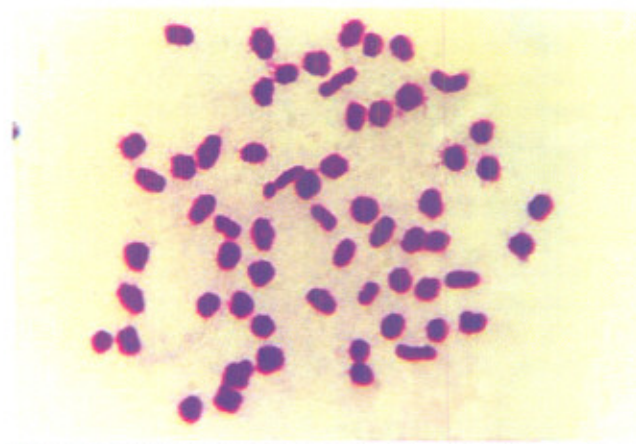

Figure 4: A diakinesis-metaphase I cell with triploid number of chromosomes (3n) in mouse spermatocyte after i.p. treatment with IFO.

Table 3: Mean percentage of different types of diakinase metaphase I cells with chromosomal aberrations induced in mouse spermatocytes 24 h., 7 and 14 days after treatment with different doses of Ifosfamide.

Dose	Duration of treatments	% of cells with different types of chromosomal aberrations					Total chromosomal aberrations
		XY un.	Auto. un.	XY+ Auto. un.	Br. or F.	3n	
I. Control	24 h.	1.60	0.60	0.00	0.00	0.00	2.20
	7 Days	1.40	0.80	0.00	0.20	0.00	2.40
	14 Days	1.20	0.60	0.00	0.00	0.00	1.80
II. Single dose 8 mg/kg	24 h.	3.20	2.40	0.60	0.60	0.20	7.00 ***
	7 Days	1.80	1.80	0.20	0.60	0.00	4.40 n.s.
	14 Day	2.80	0.20	0.20	0.20	0.00	3.4 n.s.
16 mg/kg	24 h.	5.80	4.40	1.40	1.40	0.00	13.0***
	7 Days	6.20	1.20	0.00	1.40	0.20	9.00***
	14 Day	3.00	1.20	0.60	0.60	0.20	5.60**
24 mg/kg	24 h.	5.40	3.40	2.80	1.80	0.00	13.40***
	7 Days	4.80	3.00	0.40	1.60	0.00	9.80***
	14 Days	4.20	1.60	0.60	0.80	0.20	7.40***
III. Repeated dose 8 mg/kg X 3days	24 h.	3.00	4.60	1.20	0.40	0.00	9.20***
	7 Days	3.20	2.40	0.40	1.00	0.00	7.00***
	14 Days	3.00	1.20	0.20	0.60	0.00	5.00**

The total number of scored cells is 500 (5 animals/ group);
XY un.: XY univalents, Auto. un.: Autosomal univalents, XY+ Auto. un.: XY univalents plus Autosomal univalents, Br.: Breaks, F.: Fragments. ** $p<0.01$, *** $p<0.001$ n.s.= not significant.

Table 4: Mean percentage of different types of sperm shape abnormalities in mouse sperms induced by different doses of Ifosfamide.

Dose	No. of scored sperms	No. of abnormal sperms	Mean (%) ± SE	No. and % of Different types of abnormal sperms								
				Amor. %	Triang. %	W. Hook %	Ban. shape %	Big head %	Small head %	Forked head %	Coiled tail %	Head and tail %
I. Control	5099	99	1.94±0.23	1.02	0.33	0.24	0.04	0.04	0.04	0.00	0.22	0.02
II. Single dose												
8 mg/kg	5146	146	* 2.84+0.19 ***	1.52	0.82	0.25	0.09	0.00	0.02	0.02	0.09	0.0
16 mg/kg	5331	331	6.21+0.09 ***	1.99	3.08	0.49	0.09	0.06	0.04	0.02	0.41	0.0
24 mg/kg	5430	430	7.92+0.21	2.71	3.52	0.76	0.07	0.04	0.07	0.11	0.58	0.06
III. Repeated dose 8 mg/kg X 3 days	5212	212	*** 4.07+0.22	1.78	1.06	0.49	0.13	0.00	0.04	0.02	0.49	0.0

*** $p<0.001$, * $p<0.05$
Amor.: Amorphous, Triang.: Triangular, W. hook : Without hook, Ban. shape: Banana shape.

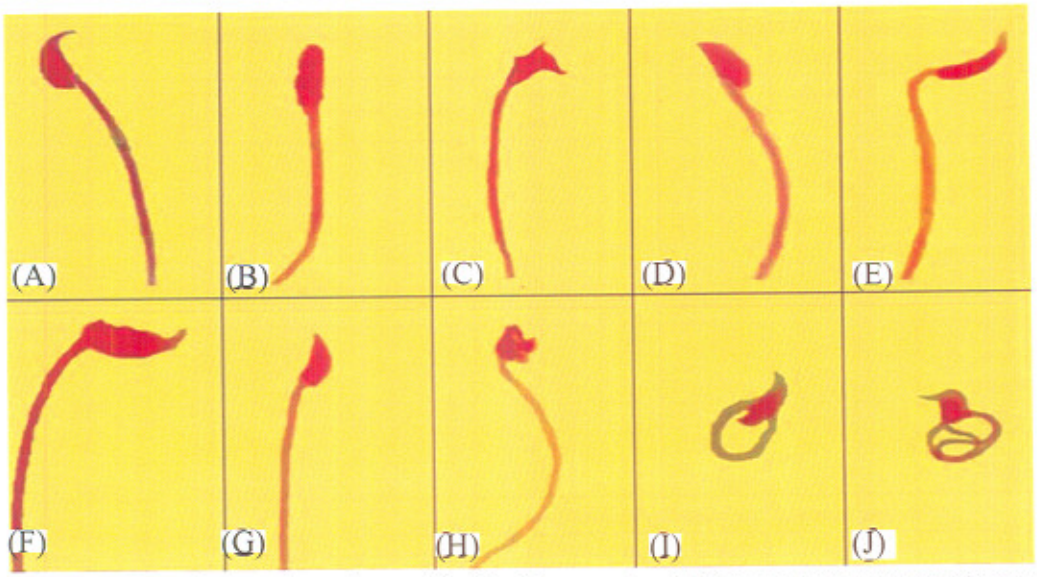

Figure 5: Types of sperm-shape abnormalities found in normal and IFO treated mice (A) normal sperm with a definite head by a marked hook and tail, (B and C) amorphous- shape, (D) without hook, (E) banana –shape, (F) big head,(G) small head,(H) triangular, (I and J) coiled tail.

Table 5: Number and mean percentage of chromosomal aberrations in mouse bone marrow cells induced by different doses of Ifosfamide plus different doses of vit. FA, VB12, VC.

Treatment and Doses	No. of cells with different types of structural aberrations							No. of cells with numerical aberration		Total chromosomal aberrations				Inhibition % of aberrant cells excluding gaps
	G.	Br. or F.	Del	C.F.	C.A.	G+Br and/ or F	Br.+ F	41 Ch.	Tetrap.	Includ gaps No.	%	Exclud. gaps No.	%	
I. Control	10	5	0	1	0	0	0	0	1	17	3.40	7	1.40	-
FA (10 mg/kg)	11	9	0	0	0	2	0	0	3	25	5.00	14	2.80	-
VB12 (0.3 mg/kg)	12	8	0	0	0	1	0	0	2	23	4.60	11	2.20	-
VC (50 mg/kg)	10	8	0	2	0	0	0	0	4	24	4.80	14	2.80	-
II. Treatment and Protection for Single Dose														
IFO (24 mg/kg)	91	35	3	1	10	9	130	0	13	*** 292	58.4	*** 201	40.2	-
IFO+ FA (24 +10)	36	25	0	1	5	9	80	0	9	*** 165	33.0	*** 129	25.8	35.82
IFO+ VB12(24+0.3)	36	29	0	0	0	7	95	1	13	*** 181	36.2	*** 145	29.0	27.86
IFO+ VC(24+50)	32	22	1	0	3	2	101	0	17	*** 178	35.6	*** 146	29.2	27.36
III. Treatment and Protection for Repeated Dose														
IFO (8 mg/ kgX3days)	71	64	2	5	1	4	33	0	12	*** 192	38.4	*** 121	24.2	-
IFO+ FA(8+10)	38	24	0	0	4	3	15	0	11	*** 95	19.0	*** 57	11.4	52.89
IFO+ VB12 (8+0.3)	35	30	0	0	1	2	16	0	16	*** 100	20.0	*** 65	13.0	46.28
IFO+ VC(8+50)	40	37	1	0	5	3	28	1	13	*** 128	25.6	* 88	17.6	27.27

The total number of scored cells is 500 (5 animals/ group); (*** p<0.001: Significance compared to Control)
(* p<0.05 *** p<0.001: Significance compared to treatment with IFO).
G.: Gaps, Br.: Breaks, F.: Fragments, Del.: Deletions, C. F.: Centric fusions, C. A.: Centromeric attenuations, 41 Ch.: 41 Chromosomes, Tetrap.: Tetraploidy, IFO: Ifosfamide, FA: Folic acid, VB12: Vitamin B12, C: Vitamin C.

Table 6: Number and mean percentage of diakinase metaphase I cells with chromosomal aberrations in mouse spermatocytes induced by different doses of Ifosfamide plus different doses of vit. FA, VB12, VC.

Treatment and Doses	Normal Cells	XY un.	Auto. un.	XY+ Auto. un.	Br. or Frag.	3n	Total Aberr. No.	Total Aberr. %	Inhibition % of aberrant Cells
I. Control	489	8	3	0	0	0	11	2.20	-
FA (10 mg/kg)	487	7	6	0	0	0	13	2.60	-
VB12 (0.3 mg/kg)	488	8	1	0	3	0	12	2.40	-
VC (50 mg/kg)	490	8	2	0	0	0	10	2.00	-
II. Treatment and Protection for Single Dose									
IFO (24 mg/kg)	433	27	17	14	9	0	67***	13.4	-
IFO+ FA (24 +10 mg/kg)	472	15	8	2	3	0	28•••	5.60	58.21
IFO+VB12(24 +0.3 mg/kg)	474	13	8	2	3	0	26•••	5.20	61.19
IFO+ VC(24 +50 mg/kg)	471	17	8	2	3	0	30•••	6.00	55.22
III. Treatment and Protection for Repeated Dose									
IFO (8 mg/kgX3 days)	454	15	23	6	2	0	46***	9.20	-
IFO+ FA (8 +10 mg/kg)	478	11	6	2	2	1	22••	4.40	52.17
IFO+ VB12 (8 +0.3 mg/kg)	477	11	8	0	3	1	23••	4.60	50.00
IFO+ VC (8 +50 mg/kg)	480	14	5	1	0	0	20•••	4.00	56.52

The total number of scored cells is 500 (5 animals/ group) (*** p<0.001: Significance compared to Control) (•• p<0.01 ••• p<0.001: Significance compared to treatment with IFO)
Br.: Breaks, F.: Fragments, , IFO: Ifosfamide FA: Folic acid, VB12:Vitamin B12, VC: Vitamin C.

Table 7: Mean percentage of DNA fragmentation induced in mouse spleen cells 24 h after treatment with different doses of ifosfamide. DPA assay.

Drug	Dose (mg/kg)	DNA Fragmentation Mean%±SE
I. Control	-	2.950±0.621
II. Single dose		
	8	20.73±1.441***
	16	21.03±1.511***
	24	21.77±1.076***
III. Repeated dose	8 X 3 days	21.25±0.898***

(5 animals/ group)
(*** p<0.001: Significance compared to Control).

Table 8: Mean percentage of DNA fragmentation induced in mouse spleen cells 24 h. after concurrent treatment with ifosfamide plus folic acid, vitamin B12 and vitamin C. DPA assay.

Drug	Dose (mg/kg)	DNA Fragmentation Mean%±SE	DNA Fragmentation Inhibition %
I. Control	-	2.95±0.621	-
FA	10	2.56±0.869	-
VB12	0.3	3.17±0.787	-
VC	50	2.87±0.716	-
III. IFO	24	21.77±1.076***	-
IFO+FA	24+10	12.68±1.123•••	41.74
IFO+VB12	24+0.3	10.64±1.116•••	51.12
IFO+VC	24+50	11.15±0.944•••	48.76

(5 animals/ group)(*** p<0.001: Significance compared to Control) (•• p<0.01 ••• p<0.001: Significance compared to treatment with DNR and IFO).

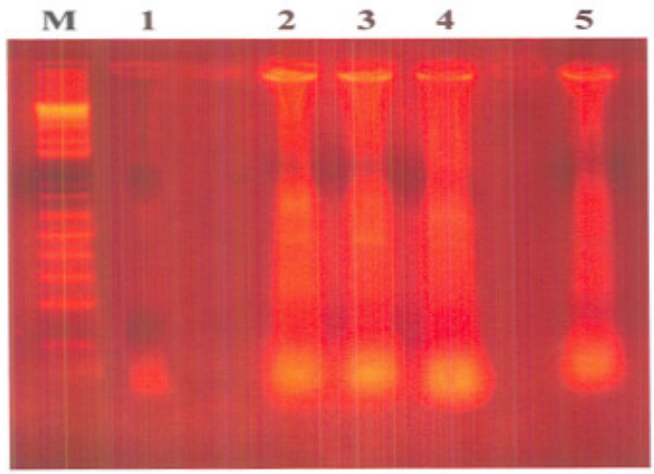

Figure 6: Effect of IFO on DNA fragmentation .Lane M: 1K base DNA ladder. Lane 1: Control. Lanes 24-: DNA of mice treated with 8, 16 and 24 mg IFO/ kg b. wt. respectively. Lane 5: DNA of mice treated with 8 mg IFO /kg repeatedly for 3 days .

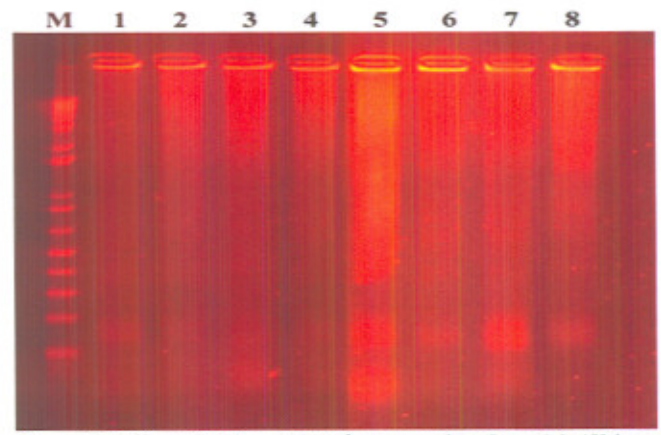

Figure 7: Effect of IFO on DNA fragmentation. Lane M: 1K base DNA ladder. Lane 1: Control. Lane2-4: DNA of mice administered FA, VB12 and VC respectively. Lane 5: DNA of mice treated with 24 mg IFO /kg b. wt. Lanes 6-8: DNA of mice treated concurrently with IFO plus FA, VB12 and VC respectively. (FA: 10mg/kg b. wt., VB 12: 0.3mg/kg b.wt. and VC: 50 mg/kg b. wt.)

DISCUSSION

Ifosfamide, as all other alkylating agents, destroy tumor cells through apoptosis initiated by DNA damage, modulation of cell cycle and other anti-proliferative effects. Thus it can damage DNA during any phase of cell cycle and therefore, it is not phase specific. The main mechanism is inhibition of DNA replication, as the interlinked strands cannot separate *(Zhang et al., 2005).*

IFO induced highly significant percentage of structural chromosomal aberrations in mouse bone marrow cells and diakinesis metaphase I cells (spermatocytes) which increased with dose increasing. Adler and El-Tarras *(1990)* demonstrated that cisplatine, an alkylating anticancer drug, induced chromosomal aberrations in primary spermatocytes and spermatogonial stem cells of male mice at 5, 7.5 and 10 mg/kg b.wt. Alvarez-Gonzalez, et al. *(2001)* found that IFO induced micronuclei in muose bone marrow cells. Also this was reported when injected i.p. tumor bearing mice at 90 mg/ kg for 1-3 days led to loss of IP+/-19q *(Leuraud et al., 2004).* Although the structural aberrations in mouse cells decreased with increasing the time of recovery, the numerical aberrations increased and reached their maximum after 14 days of recovery. The positive correlation between tetraploid cells and long duration of treatment may lead to induction of secondary carcinoma. This hypothesis supported with the study of *Kubota, et al. (1997)* who found that induction of secondary carcinoma appeared in patient treated with therapeutic regimens containing daunorubicin and cyclophosphamide. Also, they observed a third malignancy caused by alkylating agents (therapeutic regimens) containing IFO.

Separation of chromosomes forming XY and autosomal univalents was the most common type of aberrations in mouse spermatocytes in the present study. Concerning numerical aberrations, it is worth to mention that a very low frequency of triploid diakinasis metaphase I cells were observed after treatment with different doses of IFO. This phenomenon may be attributed to the effect of the anticancer on the spindle apparatus *(Temtamy et al., 1982 and Hemavathy and Krishnamurthy, 1988).* Such results agree with *Amer et al. (2002)* who observed triploid spermatocytes in mice treated with 1 mg Mitomycine C /kg b.wt.

IFO at single and repeated doses induced a highly significant and a dose dependent increase in SCE's frequencies in mouse bone marrow cells. The mean values of SCE's/cell were higher than three folds of the control indicating that IFO is a strong inducer of SCE. Induction of SCE's was observed in cultured V79 chinese hamster cells after treatment with IFO and cyclophosphamide *(Sirianni and Huang, 1980).* Aly, et al. *(2003)* demonstrated that both cisplatin and gemcitabine separately induced SCE's in mouse bone marrow cells in a dose dependent manner.

Sperm-head abnormalities are usually taken as a characteristic criterion and as an applied test for monitoring the mutagenic potential for many chemicals *(Brusick, 1980).* Tail deformities were reported to reduce fertility in human and animals *(Topham, 1983).*The mean percentage of sperm shape abnormalities were dose dependent with IFO. The maximum percentage was 7.92+0.21 ($p<0.001$) 24 h after treatment with 24 mg IFO/kg b. wt., such results coincide with the results obtained by cisplatin *(Giri et al., 1998),* Mitomycin C 1 mg/kg b.wt *(Farghaly and Ibrahim, 2003)* and cyclophosphamide at 20, 60 mg/kg b. wt. *(El-Nahas et al., 1989; Kumar et al., 2004 and Hassan et al., 2006)* which induced highly significant sperm shape abnormalities in mice.

Apoptosis is a form of programmed cell death shown to play a key role in normal development and oncogenesis. Its hall mark biochemical feature of endonuclease activation, give rise to internucleosomal DNA fragmentation *(Perandones et al., 1993).* The present study indicated the apoptotic changes induced by IFO in mouse spleen cells (*in vivo*) revealed a significant increase in the percentage of DNA fragmentation with (DPA) assay and was confirmed by agarose gel electrophoresis. The observed increase in DNA fragmentation might be due to the induction of DNA strand breaks by this compound. Many studies demonstrated that IFO have the potential to induce DNA fragmentation and apoptosis in various tissues *in vivo* and *in vitro*. Latz, et al. *(1997)* demonstrated that 1ug/ml IFO for 2 h induced DNA fragmentation in different cell lines *in vitro* such as V79 chines hamster,caski-(squamous ca.), widr-(colon ca.) and MRI-221 (melanoma) cells. Hartley, et al. *(1999)* observed the presence of DNA cross linking in the lymphocytes of patients treated with IFO at 3.09/m^2 /day by continous intravenous infusion over 3-5 days or as a 3h infusion daily for 3 days. Ypsilantis, et al. *(2004)* demonstrated that IFO induced enterocyte apoptosis and DNA fragmentation in the rabbit small and large intestine in a dose and intestinal site-dependent manner and it had a dose related apoptotic, but steady anti-mitotic effect on intestinal crypt cells, which led to mucosal atrophy in the small intestine of the rabbit.

The inhibition of DNA synthesis, specially the cellular DNA may be induced by cross links between the anticancer drugs and the DNA molecules. IFO generates bifunctional alkylating nitrogen mustards which are converted to chemically reactive carbonium ions at neutral pH and react with the 7-nitrogen atom of purine bases in DNA, especially when they are flanked by adjacent guanines. The second arm in phosphoramide mustard can react with a second guanine moiety in an opposite DNA strand or in the same strand to form cross links. The O^6 atom of guanine may also be a target for oxazaphosphorines *(Zhang et al., 2005)*

In a trial to minimize the genotoxicity effect of IFO. FA, VB12 and VC were administered simultaneously with single and repeat doses. The results showed that the maximum effect of FA appeared after repeated treatment in mouse bone marrow cells and spermatocytes. The possible mechanism of FA action is connected with thymidylate synthetase activity and through DNA synthesis *(Glover, 1982)* and with modifying cellular nucleotide pools *(Kunz, 1988).* Also, FA is involved in both methyl metabolism and in DNA synthesis and repair *(Duthie and Hawdon, 1998).* Donya and Aly *(2003)* found that FA caused a highly significant inhibition in the percentage of aberrant metaphases induced in mice somatic and germ cells after treatment with methotrexate (anticancer drug).

VB12 is required for the synthesis of methionine and S-adenosyl methionine, the common methyl donor required for the maintenance of DNA conformation *(Zingg and Jones, 1997)*. It is essential for one-carbon metabolism and cell division thus its synthesis is very complex and restricted to microorganisms *(Afman et al., 2001 and Ulleland et al., 2002)*. The percentage of inhibition reached 61.12% and 50.00% in mouse spermatocytes after single and repeated treatments with IFO plus VB12. The present results agreed with the studies carried out by *Joksic, et al. (2006)*, who demonstrated that VB12 reduced the incidence of micronuclei induced by ribavirin, a synthetic purine nucleoside analogue with a broad spectrum of antiviral activity, in phytohemaglutinine-stimulated human lymphocytes.

Oral administration of VC to mice treated with IFO (concurrent administration) minimized the percentage of chromosomal aberration induced in somatic and germ cells after both single and repeated treatments. VC is a powerful reducing agent (antioxidant) and plays a part in intracellular oxidation/reduction system and binding oxidants (free radicals) produced endogenously. Besides, VC can promote the removal of oxidative DNA damage from the DNA and/or nucleotide pool, through the upregulation of repair enzymes, perhaps induced by the vitamin's proxidative properties *(Cooke et al., 1998)*. Ghaskadbi, et al. *(1992) and Vijayalaxmi and Venu (1999)* reported that the modifying effect of ascorbic acid at low doses on cyclophosphamide induced micronuclei in mice. Also, Giri, et al. *(1998)* found that the frequency of all mutagenic parameters in Swiss albino mice treated with VC plus cisplatin were significantly less than those treated with cisplatin alone suggesting a protective role of ascorbic acid against cisplatin.

The concurrent administration of IFO at 8 mg/kg b. wt. with vitamins FA, VB12, VC reduced the percentage of DNA fragmentation in mouse spleen cells as measured by DPA and agarose gel electrophorasis. Simultaneous treatment of human peripheral blood mononuclear cells (in vitro) with cisplatin and melatonin (free radical scavenger and general antioxidant pineal hormone) decreased cisplatin induction of DNA fragmentation from 45% to 28% *(Hassan et al., 1999)*. VC diminished the extent of DNA damage evoked by selenium-cisplatin conjugate but had no effect on the kinetics of DNA repair in human lymphocytes *(Blasiak and Kowalik, 2001)*. But post treatment of VC for mice treated with cyclophosphamide did not affect DNA damage level using comet assay in peripheral white blood cells *(Franke et al., 2005)*.

The present study indicates that the anticancer drug IFO is a mutagenic agent in mouse somatic and germ cells. Vitamins (FA, VB12 and VC) play a beneficial role against the mutagenicity of this drug.

REFERENCES

Adler, I. D. and El-Tarras, A. 1990. Clastogenic effects of cis-diamminedichloroplatinum. II. Induction of chromosomal aberrations in primary spermatocytes and spermatogonial stem cells of mice. Mutation Research **243**(3):173-178.

Afman, L. A., Van Der Put, N. M., Thomas, C. M., et al. 2001. Reduced vitamin B_{12} binding by transcobalamin II increases the risk of neural tube defects. QJM: Monthly Journal of the Association of Physicians **94**(3):159-166.

Allen, J. W. 1982. A method for conducting in vivo SCE induction analysis in mice. Rep. no. 27711; Genetic Toxicology Division, U.S. Environ. Protection Agency, Research Triangle Park, North Carolina.

Alvarez-Gonzalez, I., Madrigal-Bujaidar, E., Dorado, V. and Espinosa-Aguirre, J. J. 2001. Inhibitory effect of naringin on the micronuclei induced by ifosfamide in mouse and evaluation of its modulatory effect on the Cyp3a subfamily. Mutation Research **480-481**:171-178.

Aly, F. A. E., Donya, S. M. and Aly, K. M. 2002. Protective effects of the folic acid and vitamin B_{12} against chromosome damage induced by manganese sulfate in cultured mouse spleen cells. Cytologia **67**(3):221-228.

Aly, M. S., Ashour, M. B., El-Nahas, S. M. and Abo-Zeid, M. A. F. 2003. Genotoxicity and cytotoxicity of the anticancer drugs gemcitabine and cisplatin, separately and in combination: *In vivo* studies. Journal of Biological Sciences **3**(11):961-972.

Amer, S. M., Fahmy, M. A., Aly, F. A. and Farghaly, A. A. 2002. Cytogenetic studies on the effect of feeding mice with stored wheat grains treated with malathion. Mutation Research **513**(1-2):1-10.

Blasiak, J. and Kowalik, J. 2001. Protective action of vitamin C against DNA damage induced by selenium-cisplatin conjugate. Acta Biochimica Polonica **48**(1):233-240.

Brusick, D. 1980. Fundamentals of genetic toxicology. In Principles of genetic toxicology. New York: Plenum Press. PP. 11-44.

Cooke, M. S., Evans, M. D., Podmore, I. D., et al. 1998. Novel repair action of vitamin C upon *in vivo* oxidative DNA damage. FEBS Letters **439**(3):363-367.

Costa, W. F. and Nepomuceno, J. C. 2006. Protective effects of a mixture of antioxidant vitamins and minerals on the genotoxicity of doxorubicin in somatic cells of Drosophila melanogaster. Environmental and Molecular Mutagenesis **47**(1):18-24.

Dechant, K. L., Brogden, R. N., Pilkington, T. and Faulds, D. 1991. Ifosfamide/mesna. A review of its antineoplastic activity, pharmacokinetic properties and therapeutic efficacy in cancer. Drugs **42**(3):428-467.

Donya, S. M. and Aly, K. M. 2003. Protective effects of vitamin C folic acid and vitamin B_{12} on the mutagenic effect of methotrexate. Scientific Medical Journal **15**(4):1-15.

Duthie, S. J. and Hawdon, A. 1998. DNA instability (strand breakage, uracil misincorporation and defective repair) is increased by folic acid depletion in human lymphocytes *in vitro*. The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology **12**(14):1491-1497.

El-Nahas, S. M., de Hondt, H. A. and Abdou, H. E. 1989. Chromosome aberrations in spermatogonia and sperm abnormalities in Curacron-treated mice. Mutation Research **222**(4):409-414.

Evans, E. P., Breckon, G. and Ford, C. E. 1964. An air-drying method for meiotic preparation from mammalian testis. Cytogenetics *3:289*-294.

Farghaly, A. A. and Ibrahim, A. A. E. 2003. The protective role of folic acid on the mutagenicity induced by sodium sulfite in different tissues of male mice. Bulletin of the National Research Center, Egypt **28**(6):749-760.

Franke, S. I., Pra, D., da Silva, J., et al. 2005. Possible repair action of vitamin C on DNA damage induced by methyl methanesulfonate, cyclophosphamide, FeSO4 and CuSO4 in mouse blood cells *in vivo*. Mutation Research **583**(1):75-84.

Ghaskadbi, S., Rajmachikar, S., Agate, C., et al. 1992. Modulation of cyclophosphamide mutagenicity by vitamin C in the in vivo rodent micronucleus assay. Teratogenesis, Carcinogenesis and Mutagenesis **12**(1):11-17.

Giri, A., Khynriam, D. and Prasad, S. B. 1998. Vitamin C mediated protection on cisplatin induced mutagenicity in mice. Mutation Research **421**(2):139-148.

Glover, T. W. 1981. FUdR induction of the X chromosome fragile site: Evidence for the mechanism of folic acid and thymidine inhibition. American Journal of Human Genetics **33**(2):234-242.

Goto, T., Okuma, T., Nakada, I., et al. 2007. Preoperative adjuvant therapy for primary malignant bone tumors. Gan To Kagaku Ryoho. Cancer and Chemotherapy **34**(11):1750-1754.

Hartley, J. M., Spanswick, V. J., Gander, M., et al. 1999. Measurement of DNA cross-linking in patients on ifosfamide therapy using the single cell gel electrophoresis (comet) assay. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research **5**(3):507-512.

Hassan, N. H. A., Fahmy, M. A., Farghaly, A. A. and Hassan, E. E. S. 2006. Antimutagenic effect of selenium and vitamins against the genotoxicity induced by cobalt chloride in mice. Cytologia **71**(3):213-222.

Hassan, M. I., Ahmed, M. I., Kassim, S. K., et al. 1999. Cis-platinum-induced immunosuppression: Relationship to melatonin in human peripheral blood mononuclear cells. Clinical Biochemistry **32**(8):621-626.

Hemavathy, K. C. and Krishnamurthy, N. B. 1988. Cytogenetic effects of Cuman L, a dithiocarbamate fungicide. Mutation Research **208**(1):57-60.

Joksic, I., Leskovac, A., Petrovic, S. and Joksic, G. 2006. Vitamin B_{12} reduces ribavirin-induced genotoxicity in phytohemaglutinin-stimulated human lymphocytes. The Tohoku Journal of Experimental Medicine **209**(4):347-354.

Kubota, M., Sawada, M., Watanabe, K., et al. 1997. Myelodysplastic syndrome presenting as third malignancy after non-Hodgkin's lymphoma and osteosarcoma. Annals of Hematology **74**(2):95-97.

Kumar, S., Gautam, A. K., Agarwal, K. R., et al. 2004. Demonstration of sperm head shape abnormality and clastogenic potential of cypermethrin. Journal of Environmental Biology **25**(2):187-190.

Kunz, B. A. 1988. Mutagenesis and deoxyribonucleotide pool imbalance. Mutation Research **200**(1-2):133-147.

Latz, D., Schulze, T., Schraube, P., et al. 1997. Combined effects of ionizing radiation and 4-hydroxy-ifosfamide (IFO) in different cell lines. Clinical Radiology S27:104.

Leuraud, P., Taillandier, L., Medioni, J., et al. 2004. Distinct responses of xenografted gliomas to different alkylating agents are related to histology and genetic alterations. Cancer Research **64**(13):4648-4653.

Li, Y. F., Fu, S., Hu, W., et al. 2007. Systemic anticancer therapy in gynecological cancer patients with renal dysfunction. International Journal of Gynecological Cancer **17**(4):739-763.

Paget, G. E. and Barnes, J. M. 1964. Evaluation of drug activities. In Pharmacometrics, edited by D. R. Laurence and A. L. Bacharach. London: Academic Press. PP. 50.

Perandones, C. E., Illera, V. A., Peckham, D., et al. 1993. Regulation of apoptosis in vitro in mature murine spleen T cells. Journal of Immunology (Baltimore, Md.: 1950) **151**(7):3521-3529.

Perry, P. and Wolff, S. 1974. New Giemsa method for the differential staining of sister chromatids. Nature **251**(5471):156-158.

Sirianni, S. R. and Huang, C. C. 1980. Comparison of induction of sister chromatid exchange, 8-azaguanine- and ouabain-resistant mutants by cyclophosphamide, ifosfamide and 1-(pyridyl-3)-3,3-dimethyltriazene in Chinese hamster cells cultured in diffusion chambers in mice. Carcinogenesis **1**(4):353-355.

Siu, L. L. and Moore, M. J. 1998. Use of mesna to prevent ifosfamide-induced urotoxicity. Supportive Care in Cancer: Official Journal of the Multinational Association of Supportive Care in Cancer **6**(2):144-154.

Temtamy, G. A., de Hondt, H. A. and El-Ghor, M. A. 1982. Effect of novalgin on chromosomes of Rattus norvegicus. The Egyptian Journal of Genetics and Cytology **11**(1):105-111.

Topham, J. C. 1983. Chemically-induced changes in sperm in animals and humans. Chemical Mutagens 8:201-234.

Ulleland, M., Eilertsen, I., Quadros, E. V., et al. 2002. Direct assay for cobalamin bound to transcobalamin (holo-transcobalamin) in serum. Clinical Chemistry **48**(3):526-532.

Vijayalaxmi, K. K. and Venu, R. 1999. *In vivo* anticlastogenic effects of L-ascorbic acid in mice. Mutation Research **438**(1):47-51.

Wyrobek, A. J. and Bruce, W. R. 1978. The induction of sperm-shape abnormalities in mice and humans. In Chemical mutagens: Principles and methods of their detection, edited by A. Hollaender and F. J. de Serres. New York: Plenum Press. PP. 257-285.

Yosida, T. H. and Amano, K. 1965. Autosomal polymorphism in laboratory bred and wild Norway rats, Rattus norvegicus, found in Misima. Chromosoma **16**(6):658-667.

Ypsilantis, P., Tentes, L., Assimakopoulos, S. F., et al. 2004. Dose related effects of ifosfamide on enterocyte apoptosis in different sites of the rabbit intestine. Toxicology **200**(2-3):135-143.

Zhang, J., Tian, Q., Chan, S. Y., et al. 2005. Insights into oxazaphosphorine resistance and possible approaches to its circumvention. Drug Resistance Updates **8**(5):271-297.

Zingg, J. M. and Jones, P. A. 1997. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. Carcinogenesis **18**(5):869-882.