Research Article

Protective Effect of Vitamin D$_3$ Against Flutamide Induced Genotoxicity

Asmaa S. Salman[1]*, Lamiaa M. Salem[2], Ayman A. Farghaly[1], Karima F. Mahrous[2]
[1]Genetics and Cytology Department, Genetic Engineering and Biotechnology Division, National Research Center, 12622 Dokki, Giza, Egypt.
[2]Cell Biology Department, Genetic Engineering and Biotechnology Division, National Research Center, 12622 Dokki, Giza, Egypt.
*Corresponding author's E-mail: dr_salman99@yahoo.com

ABSTRACT

Flutamide (FM) is a nonsteroidal anti-androgen used for treatment of prostate cancer. Vitamin D$_3$ (VD$_3$) is a significant factor in detoxification and protection against different toxins. The present study was undertaken to investigate the protective effect of VD$_3$ against FM-induced genotoxicity. Sperm shape abnormalities, sister chromatid exchanges (SCE's) and gene expression were used to assess FM-induced genotoxicity and to evaluate the protective effects of VD$_3$. Mice were orally treated with FM at doses 26, 52 and 78 mg/kg b.wt./day for 28 days. VD$_3$ was simultaneously administered at a dose of 0.13 μg/kg b.wt. with the highest dose of FM. The percentages of sperm abnormalities were statistically and dose-dependent increased in 7, 14, 21 and 35 days after treatment with FM. Concurrent administration of VD$_3$ significantly reduced this percentage. These findings provide evidence that FM significantly affect different stages of spermatogenesis and that the most affected stage is the late spermatide. Treatment with FM significantly and dose-dependently increased the percentage of SCE's and the co-administration of VD$_3$ with FM significantly reduced this percent by 36.72. The results show that there was a significant decrease in the expression level of AR, GR and LDH-C mRNA in FM groups. However, treatment with VD$_3$ resulted in a significant ($p < 0.05$) increase in the expression level of the examined genes. As well as, treatment of VD$_3$ with FM significantly ($p < 0.05$) up regulated the expression of the three genes.

Keywords: Flutamide, Gene expression, Mice, VD$_3$, SCEs, Sperm abnormalities.

INTRODUCTION

For more than 10 years there has been increasing scientific interest on environmental chemicals with androgenic and/or anti-androgenic activities capable of interfering with the endocrine systems of wildlife species and humans.[1-3] Suspected androgenic chemicals or anti-androgens can bind to the androgen receptor (AR) and induce androgen-dependent gene expression or block the androgen action through the AR.

Antiandrogens represent a group of potentially important environmental endocrine-disrupting chemicals.[4] The antiandrogen flutamide (FM) is a nonsteroidal orally active AR antagonist that interferes with endogenous androgen binding to ARs in target organs.[5] It has been used for treatment of prostate cancer in human.[6] However, the efficacy of this drug is somewhat overshadowed by the occurrence of toxic or fatal liver complications.[7-9]

Several studies have addressed *in vitro* and *in vivo* effects of FM on steroidogenesis in testes and ovaries.[10-11] In mammals, FM administration has also been found to affect the initial step of spermatogenesis and cause a reduced sperm count due to inhibition of differentiation of spermatogonia to spermatocytes.[12-14] Ohsako *et al.*, observed a statistically reduction in the daily sperm production on day 36 after the first dose of FM in rats.[15] Late embryonic and early postnatal exposure to FM also can cause gonadal and reproductive tract abnormalities.[16] Antiandrogens affect aspects of sexual differentiation and development, causing feminization and demasculinization

of male offspring dosed in utero during sensitive developmental stages.[17-19] There are indications that anti androgenic chemicals also can affect sexual differentiation in some species of fish exposed during early life stages.[20-21]

Vitamin D$_3$ (VD$_3$) is a well-known potent regulator of cell growth and differentiation and there is recent evidence of an effect on cell death, tumor invasion and angiogenesis, which makes it a candidate agent for cancer regulation.[22] Furthermore, VD$_3$ was found to induce mechanisms of detoxification of endo- and xenobiotics and protection against environmental toxins.[23-24]

Very few foods naturally contain vitamin D. Typically oily fish including salmon, mackerel and herring; cod-liver oil and sun-dried mushrooms, contain this vitamin.[25] The action of sunlight on the skin resulting in the production of VD$_3$ is responsible for most (90-95%) of peoples' VD requirements.[26-27]

The biologically active form of VD$_3$ (1, 25(OH)$_2$D$_3$) is known to play a major role in mammalian calcium and phosphorus homeostasis and bone health. It may play an important role in human cancer. Increased risk of breast, prostate and colon cancer have been associated with reduced serum concentration of 1, 25(OH)$_2$D$_3$.[28] Cultured breast, colon, prostate, skin, lung and a variety of other cell lines, when exposed to 1,25(OH)$_2$D$_3$, had marked inhibition of cellular growth and induction of terminal differentiation.[26,29-32]

The purpose of the study was to determine the influence of administration of FM on cytogenetic and molecular

genetics and to assess whether 1alpha-OH vitamin D_3 (1alpha-OHD$_3$) supplementation is able to prevent its side effects.

MATERIALS AND METHODS

Animals

Laboratory-bred strain Swiss albino male mice of 9- 12 weeks old with an average weight of 27.5±2.5 g obtained from the National Research Center, Cairo, Egypt, were used. Animals were housed in polycarbonate boxes with steel-wire tops in groups (5 animals/ group). Ambient temperature was controlled at 22±3 °C with a relative humidity of 50±15% and a 12-h light/dark photoperiod. The animals were given standard food and water *ad libitum* and sacrificed after treatment by cervical dislocation.

Chemicals

FM was purchased from Alwatanya Co., Egypt and VD$_3$ was purchased from LEO Pharmaceutical Co., Denmark. All other chemicals used were of analytical grade.

Experimental design

A. Animal treatment

For sperm-shape abnormalities assay 24 groups (five animals for each) were orally treated with the examined doses for five consecutive days. 12 groups were treated with FM at dose levels of 26, 52 and 78 mg/kg b.wt. 4 groups of mice were given 0.13 µg VD$_3$/kg b.wt. Simultaneously with the highest dose of FM. 4 groups of mice were given 0.13 µg/kg b.wt. VD$_3$. 4 groups of mice were used as negative untreated control. Animal groups were sacrificed 7, 14, 21 and 35 days after the first treatment. Sperm from negative (non-treated) was tested for each time interval.

For sister chromatid exchanges (SCE's) and molecular genetics assays mice were orally treated with FM at doses 26, 52 and 78 mg/kg b.wt./day for 28 days. VD3 was simultaneously administered at a dose of 0.13 µg/kg b.wt. with the highest dose of FM.

B. Cytogenetic assays

I: Sperm-shape abnormalities

Sperm were prepared according to the recommended method of Wyrobek and Bruce.[33] The epididymides were excised and minced in 2ml physiological saline, dispersed and filtered to remove large tissue fragments. Smears were prepared and stained with 1% Eosin Y. For each animal at least 1000 sperm were analyzed to determine the different sperm abnormalities.

II: Sister chromatid exchanges

For analysis of SCE's, all samples were collected after 24 h. after the last treatment. The method described by Allen, for conducting *in vivo* SCE's induction analysis in mice was applied with some modifications. Approximately 55 mg 5'-Bromodeoxyuridine tablets were inserted in mice subcutaneously 21-23 h. before sacrifice. Mice were injected intraperitoneally with colchicine at a final concentration of 3 mg/kg b.w 2 h. before sacrifice. Bone-marrow cells from both femurs were collected and the fluorescence-photolysis Giemsa technique was used.[35] Forty well spread metaphases were analyzed per mouse to determine the frequency of SCE's/cell.

C. Molecular genetics assays

I: RNA isolation and reverse transcription (RT)

Total RNA was isolated from testes tissues using Trizol reagent (Invitrogen, Paisley, UK). RNA samples were subjected to DNase1 treatment to remove genomic DNA contamination in the presence of RNase inhibitor. The purity and integrity of the total RNA was determined by spectrophotometry and agarose gel electrophoresis.[36] The first-strand cDNA was prepared from the 5 µg of total RNA using Fermentas kits (Sigma, St. Louis, MO) as per the manufacturer's instructions. The RT program used was: 60 min at 420C (cDNA synthesis); 5 min at 940C (denaturation). Afterwards the reaction tubes containing RT preparations were ash-cooled in an ice chamber until used for DNA amplification through polymerase chain reaction (PCR).

II: Polymerase chain reaction (PCR)

The first-strand cDNA from different mice samples was used as the template for amplification by the PCR with the following pairs of specific primers (from **5' to 3'**): androgen receptor (AR), glucocorticoid receptor (GR), lactose dehydrogenase-c (LDHC) and β-actin that is taken from the literature[37] (Table 1).

Table 1: Primers used for semi-quantitative RT-PCR

Gene	Primer sequence Forward primer (5' to 3')	Reverse primer (5' to 3')	Cycle Used (C)	Anneal Temp	GenBank accession
AR	TGCTGCCTTGTTATCTAGTCTCA	ACCATATGGGACTTGATTAGCAG	26	60	M20133
GR	GGAGAATTATGACCACACTCAAC	GCAGTAGGTAAGGAGATTCTCAA	26	60	M14053
LDH-C	CAAGGAGCAGCTAATTCAGAACC	CTTCTCTCCAATCAGGTAACGGA	22	60	NM017266
β-actin	CTGTGCCCATCTATGAGGGTTAC	AATCCACACAGAGTACTTGCGCT	22	60	V01217

Table 2: Number and percentage of different types of sperm shape abnormalities in male mice after treatment with flutamide and/or vitamin D_3

Treatment and doses (mg/kg b.wt.)	Days after treatment	Examined sperm No.	No. and % ( ) of sperms with							Abnormal sperm No.	Abnormal sperms Mean % ± S.E.	Inhibition %
			Head abnormalities						Tail abnormalities			
			Amorphous	Triangle	Without hook	Banana	Small	Big	Coiled			
Control (Non-treated)	7	5035	20 (0.39)	58 (1.15)	13 (0.25)	-	-	-	11 (0.21)	102	2.0 ± 0.13	46.7
FM												
26		5031	87 (1.7)	48 (0.95)	34 (0.67)	3 (0.05)	-	-	25 (0.49)	197	3.9 ± 0.45**	
52		5108	107 (2.0)	51 (0.99)	47 (0.92)	4 (0.07)	-	-	32 (0.6)	241	4.7 ± 0.51**	
78		5075	111 (2.1))	69 (1.3)	48 (0.94)	4 (0.07)	-	-	35 (0.68)	267	5.26 ± 0.48**	
FM 78 + VD$_3$(0.13 µg/kg)		5024	59 (1.1)	40 (0.79)	18 (0.35)	-	-	2(0.03)	21 (0.4)	140	2.8 ± 0.38♦♦	
VD$_3$ (0.13 µg/kg)		5032	27 (0.53)	50 (0.99)	18 (0.35)	-	-	1(0.01)	20 (0.39)	116	2.3 ± 0.16	
Control (Non-treated)	14	5012	21(0.4)	50 (0.99)	9 (0.17)	-	-	-	16 (0.3)	96	1.9 ± 0.18	34.8
FM												
26		5000	133 (2.6)	85 (1.7)	73 (1.4)	2(0.04)	-	1(0.02)	63 (1.2)	357	7.14± 0.51**	
52		5023	141 (2.8)	98 (1.9)	97 (1.9)	2(0.03)	1(0.01)	1(0.01)	92 (1.8)	432	8.64 ± 0.58**	
78		5009	120 (2.3)	165 (3.2)	113 (2.2)	5(0.09)	1(0.01)	2(0.03)	125 (2.4)	531	10.62 ± 0.53**	
FM 78 + VD$_3$(0.13 µg/kg)		5014	139 (2.7)	81 (1.6)	65 (1.2)	1(0.01)	-	-	60 (1.2)	346	6.92 ± 0.55♦♦	
VD$_3$ (0.13 µg/kg)		5009	23(0.45)	66 (1.3)	11 (0.2)	-	-	-	16 (0.3)	116	2.3 ± 0.3	
Control (Non-treated)	21	5018	25 (0.49)	57 (1.1)	9 (0.17)	-	-	-	15 (0.29)	106	2.1 ± 0.2	36.49
FM												
26		5030	53 (1.0)	54 (1.0)	10 (0.19)	-	1(0.01)	1(0.01)	28 (0.55)	147	2.9 ± 0.28	
52		5026	55 (1.0)	71 (1.4)	18 (0.35)	-	2(0.03)	3(0.05)	33 (0.65)	182	3.6 ± 0.41 *	
78		5032	65 (1.29)	67 (1.3)	15 (0.29)	-	1(0.01)	8(0.15)	51 (1.0)	207	4.11 ± 0.22**	
FM 78 + VD$_3$(0.13 µg/kg)		5022	40 (0.79)	48 (0.95)	12 (0.23)	-	-	1(0.01)	30 (0.59)	131	2.6 ± 0.18♦♦	
VD$_3$ (0.13 µg/kg)		5007	27 (0.53)	60 (1.19)	9 (0.17)	-	-	1(0.01)	17 (0.3)	114	2.27 ± 0.22	
Control (Non-treated)	35	5021	23 (0.45)	65 (1.3)	10 (0.2)	-	-	-	13 (25)	111	2.21± 0.19	27.7
FM												
26		5003	31(0.6)	68 (1.35)	11 (0.2)	-	-	1(0.02)	20 (0.4)	131	2.6 ± 0.2	
52		5011	36 (0.7)	67 (1.3)	13 (0.25)	-	-	-	26 (0.5)	142	2.8 ± 0.25	
78		5016	50 (0.99)	79 (1.57)	19 (0.37)	-	-	1(0.02)	27 (0.53)	176	3.5 ± 0.34 *	
FM 78 + VD$_3$(0.13 µg/kg)		5030	33 (0.65)	63 (1.25)	11 (0.2)	-	-	-	21 (0.4)	128	2.5 ± 0.18	
VD$_3$ (0.13 µg/kg)		5008	24 (0.47)	57 (1.1)	8 (0.15)	-	-	-	12 (0.23)	101	2.0 ± 0.2	

*Significant at 0.05 level (t-test) comparing to control (non-treated); ** Significant at 0.01 level (t-test) comparing to control (non-treated); ♦♦ Significant at 0.01 level (t-test) comparing to treatment

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Table 3: Frequency of sister chromatid exchanges in mouse bone marrow cells treated with flutamide and/or vitamin D_3

Treatment and doses (mg/kg b.wt.)	Chrom. No.	No. of scored metaphases	No. and % ( ) of different types of SCE's/chromosome				Total No. of SCE's	SCE's/cells Mean ± S.E.	Inhibition %
			Single	Double	Triple	Quadruple			
Control (Non-treated)	8000	200	901 (11.26)	18 (0.22)	1 (0.01)	-	940	4.7 ± 0.45	
FM									
26	8000	200	1321 (16.5)	73 (0.9)	30 (0.37)	-	1557	7.78 ± 0.41**	
52	8000	200	1459 (18.2)	201 (2.5)	27 (0.33)	-	1942	9.71 ± 0.32**	36.72
78	8000	200	1775 (22.1)	375 (4.6)	51 (0.63)	3 (0.03)	2690	13.45 ± 0.52 **	
FM 78 + VD$_3$(0.13 µg/kg)	8000	200	1391 (17.3)	134 (1.67)	13 (0.16)	1 (0.01)	1702	8.51 ± 0.1♦♦	
VD$_3$(0.13 µg/kg)	8000	200	915 (11.4)	20 (0.25)	2 (0.02)	-	961	4.8 ± 0.17	

**Significant at 0.01 level (t-test) comparing to control (non-treated); ♦♦ Significant at 0.01 level (t-test) comparing to treatment

β-actin, a house-keeping gene, was used for normalizing mRNA levels of the target genes. The PCR cycling parameters were one cycle of 94 0C for 5min, 35 cycles of 94 0C for 30 s, 60C (Cu-Zn SOD and GPx gene) for 30 s, 70 0C for 40 s, and 72 0C for 5 min. The PCR products were electrophoresed onto ethidium bromide stained a 2.0% agarose gels. The ethidium bromide-stained gel bands were scanned and the signal intensities were quantified by the computerized Gel-Pro program.

Statistical analysis.

The significance of the difference between groups and negative control and between FM with VD$_3$ against FM alone was calculated using the t-test.

Evaluation of the activity of VD$_3$ to reduce sperm shape abnormalities and SCE's induced by FM was carried out according to the formula : Inhibition percent = [(FM - VD$_3$ and FM) / (FM)] X100.[38]

RESULTS

Sperm shape abnormalities

As shown in table (2), the three tested doses of FM induced a statistically highly significant percentage of sperm abnormalities in male mice. Such percentage was found to be dose-dependent. Within seven days of treatment, it reached 3.9, 4.7 and 5.26% (p<0.01) after treatment with 26, 52 and 78 mg FM/kg b.wt. respectively, which increased to 7.14, 8.64 and 10.62% (p<0.01) after 14 days of treatment. Such percentage decreased after 21 and 35 days of treatment to reach 4.1 (p<0.01) and 3.5% (p<0.05) respectively with the higher dose of the drug. The dominant abnormalities found were amorphous, triangular, without hook heads and coiled tail.

Simultaneous treatment of mice with VD$_3$ and the higher dose of FM reduced the percentage of sperm abnormalities. It reached 2.8, 6.92 and 2.6% (p<0.01) within 7, 14 and 21 days respectively. The percentage of reduction reached 36.49% within 21 days of treatment. Fig (1) illustrates the different types of abnormalities.
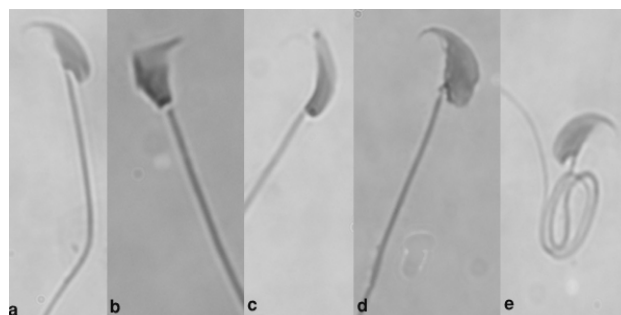

Figure 1: Sperm shape abnormalities induced in male mice treated with FM showing (a) normal, (b) triangular (c) banana (d) big head and (e) coiled tail

Sister chromatid exchanges

All the tested doses of FM induced a statistically highly significant (p<0.01) percentage of SCE's. Such percentage increased with increasing the dose of the drug reaching 7.78 ± 0.41, 9.71 ±0.32 and 13.45 ±0.52 /cell after treatment with the three tested doses respectively.

Concurrent administration of VD$_3$ and the higher dose of FM decreased the percentage of the induced SCE's. The percentage of reduction reached to 36.72% Table (3) and Fig (2).
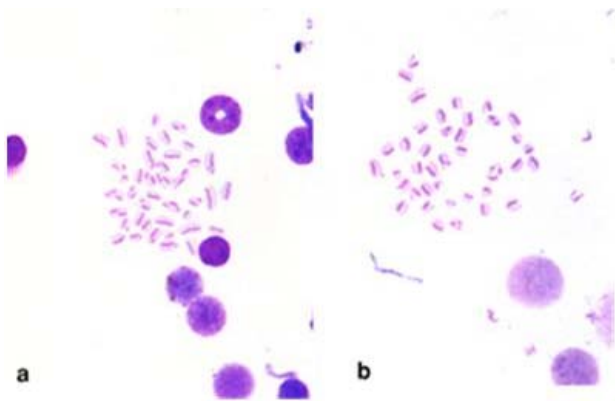
Figure 2: Sister Chromatid exchanges in bone marrow cells of mouse treated with FM (a) or FM plus vitamin D_3 (b)

Evaluation of gene expression

Bands produced from amplifying cDNA of AR, GR, LDH-C and the house keeping gene β-actin as a control were analyzed and the results of gene expression was based on quantifying the signal intensities in each band. Results were expressed as the ratio between maximum optical density (max OD) for each band of the target amplification product and the corresponding max OD of β-actin. Expression of AR, GR and LDH-C mRNA in testes of the different groups of mice are summarized in Figs. (3-5). The results show that there was a significant decrease in the expression level of the examined genes in the FM group of mice as compared to the other groups. However, treatment with Vitamin D resulted in a significant increase in the expression level of AR, GR and LDH-C mRNA ($P < 0.05$) versus the FM groups. As well as treatment of VD_3 prior to FM treatment significantly ($P < 0.05$) up regulated the expression of three genes
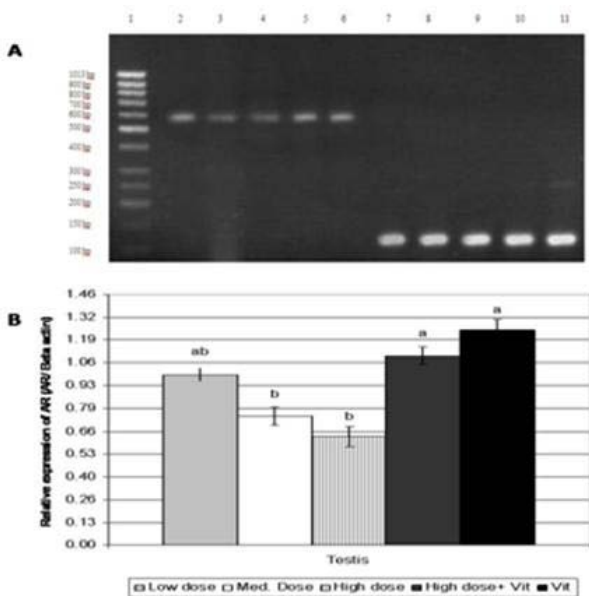

Figure 3: A) RT- PCR confirmation of AR gene expressed in testis tissues of male mice. RT- PCR was performed with total RNAs isolated from testis tissues. Lane 1 represents DNA marker, lane 2 to 4 represent Low, medium and high

doses of FM, respectively. Lane 5 represents high dsoe of FM+ VD_3. Lane 6 represents VD_3 only. Lanes 7 to 11 represent β- actin gene. All samples were normalized on the basis of β- actin expression. **B)** Expression of AR gene by semi-quantitative RT-PCR. The RNA recovery rate was estimated as the ratio between the intensity of AR gene and the β-actin gene.[a,b] within each column, means superscripts with different letters are significantly different ($P ≤ 0.05$).

DISCUSSION

In recent years, there has been an increasing awareness of the genotoxic potential of a wide variety of drugs and chemicals to which human population is exposed either environmentally or occupationally. This awareness is paralleled by the recent development of appropriate, sensitive and practical methods for detecting and estimating the effects of these substances.
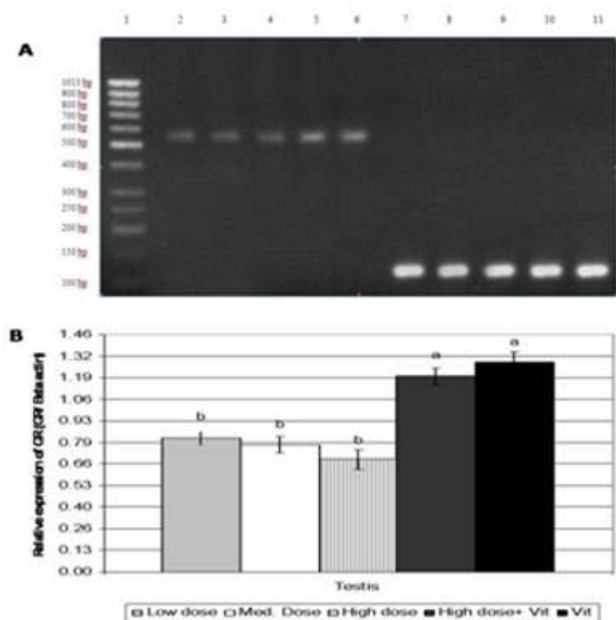

Figure 4: A) RT- PCR confirmation of GR gene expressed in testis tissues of male mice RT- PCR was performed with total RNAs isolated from testis tissues. Lane 1 represents DNA marker, lane 2 to 4 represent Low, medium and high doses of FM, respectively. Lane 5 represents high dsoe of FM+ VD_3, Lane 6 represents VD_3 only. Lanes 7 to 11 represent β- actin gene. All samples were normalized on the basis of β- actin expression. **B)** Expression of GR gene by semi-quantitative RT-PCR. The RNA recovery rate was estimated as the ratio between the intensity of GR gene and the β-actin gene. [a, b,] within each column, means superscripts with different letters are significantly different ($P ≤ 0.05$).

Studying the sperm shape abnormalities was used to investigate the genotoxic effects of FM. The head shape abnormalities reflect changes in the DNA content which in turn disrupts the process of differentiation of spermatozoa.[33] Also, sperm head abnormalities are usually taken as characteristic criteria and as an applied test for monitoring the mutagenic potential for many

chemicals.[39] Tail deformities were reported to reduce fertility in human and animals.[40]

In the present study, the mean percentages of sperm shape abnormalities significantly and dose dependently increased with FM dose in all time schedules. It reached 5.26 (P<0.01) 7 days post-treatment and increased to its maximum frequency reaching 10.62 (P<0.01) 14 days post-treatment with 78 mg FM/kg b.wt. These results provide evidence that FM significantly affects different stages of spermatogenesis and that the most affected stage is the late spermatide.[41] This finding support those obtained by Viguier-Martinez *et al.*, and Chandolia *et al.*,.[21-14] They emphasized that FM administration caused a reduced sperm count due to an inhibition of differentiation of spermatogonia to spermatocytes. Spermatids with deformed nuclei and/or acrosomal caps were observed in the seminiferous epithelium of FM-treated mice. In addition, complete or partial deletion in the ectoplasmic specialization between the Sertoli cell and spermatids was observed.[11] Administration of FM alters sperm ultrastructure, sperm plasma membrane integrity and its ability, and sperm mitochondrial oxidative capability in the boar.[42]
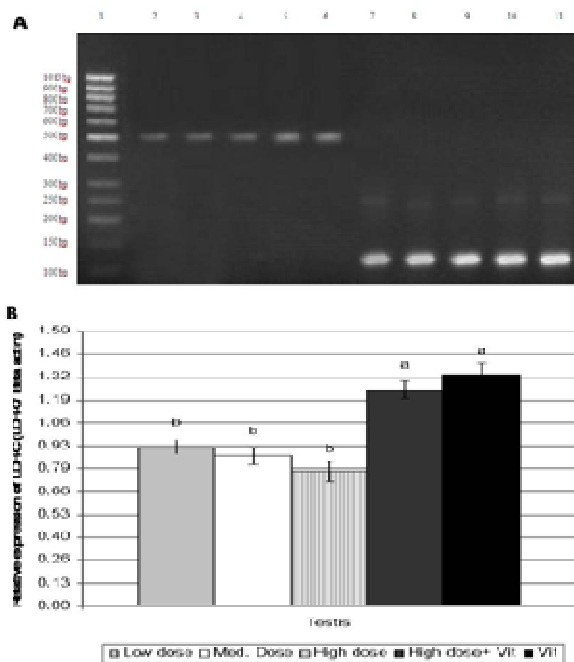

Figure 5: A) RT- PCR confirmation of LDH-C gene expressed in testis tissues of male mice. RT- PCR was performed with total RNAs isolated from testis tissues. Lane 1 represents DNA marker, lane 2 to 4 represent Low, medium and high doses of FM, respectively. Lane 5 represents high dsoe of FM+ VD$_3$. Lane 6 represents VD$_3$ only. Lanes 7 to 11 represent β- actin gene. All samples were normalized on the basis of β- actin expression. **B)** Expression of LDH-C gene by semi-quantitative RT-PCR. The RNA recovery rate was estimated as the ratio between the intensity of LDH-C gene and the β-actin gene. **a, b,** within each column, means superscripts with different letters are significantly different (*P ≤ 0.05*).

SCE's frequency is a sensitive marker of mutagenesis which occurs after exposure to genotoxic agents.[43] Sister chromatid exchanges is widely used as a reliable and sensitive indicator of chromosome or DNA instability, since the SCE patterns can reveal a general genome instability.[44-45] Thus, SCE's in mouse bone marrow cells were used in the present studies as cytogenetic end point in genetic risk assessment of FM.

It was observed that administration of FM induced a highly significant and dose dependent elevation of SCE's in mouse bone marrow cells. Its frequency reached 13.45 ± 0.52 (P<0.01) after treatment with the higher dose of FM compared to 4.7 ± 0.45 for non-treated control, which may occur due to the formation of electrophilic metabolites. FM is oxidatively metabolized into electrophilic metabolites by microsomal cytochrome P-450 which may lead to its genotoxicity.[46] Studies performed by Nu´n˜ez-Vergara *et al.* demonstrated that the nitro group in the FM molecule and its one-electron reduction product participate in the toxic events exerted by the drug.[47]

So far the majority of reproductive toxicity studies on FM have been concerned on the *in vivo* effects of FM on the reproductive organs.[48] Several studies also addressed *in vivo* effects of FM on steroidogenesis in testis such as Leydig cell proliferation in the testes and increase of circulating androgen by inhibitory action of FM on the negative feedback effects of testosterone on the hypothalamus and pituitary, and subsequent enhancement of the luteinizing hormone or gonadotropin releasing hormone pulse.[13-14,49-50]

Androgens, mainly testosterone, acting via androgen receptors (ARs) govern expression of genes determining male sexual differentiation, development of sex accessory glands and maintenance of spermatogenesis. Thus, the presence of functional ARs is an absolute requirement for normal male and female reproduction.[51-53]

FM is a synthetic anti-androgenic compound shown to bind the androgen receptor and block androgen actions.[16] Androgens have a critical role in male sex differentiation and the development and function of the testis.[54] Several experiments in intact male rats have shown that FM increases serum gonadotropin and testosterone concentrations and intratesticular testosterone levels shortly after acute administration and a 4-fold increase in serum testosterone concentration was reported in male hamster.[13-14,55-57]

In the adult rat testis, AR protein has been localized to Sertoli cells, peritubular myoid cells, and Leydig cells.[58] An *in situ* hybridization study showed that the signal for AR mRNA was most intense in the Sertoli cells at stage VII-VIII of the seminiferous tubule.[59] The AR gene itself is a target for androgen, and AR mRNA levels in the ventral prostate of adult rats have been shown to be down regulated by androgens, suggesting the presence of an auto regulation system on the AR gene.[60-61]

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In the testis, GR has been reported to be localized to Leydig cells.[62] Although there have been no reports on the regulation of GR gene expression by androgen in the testis, a glucocorticoid and GR complex has been reported to inhibit cAMP dependent up-regulation of P450scc gene expression,[63] suggesting that GR is one of the regulatory factors for T production in the testis. Therefore, down-regulation of GR mRNA by FM to the same level as AR mRNA seems to be implicated in the markedly higher level of T production after FM treatment. Burnstein and coworkers are trying to identify the AR gene transcription machinery on a molecular basis.[64-65] Using a prostate cancer cell line PC3, they identified two distinct AR responsive elements in exon D and exon E of the AR gene itself, that were found to be involved in the up-regulation of AR gene transcription by androgen.[64] They subsequently discovered that the elements contain specific DNA sequences bound to the protooncogene: immediate early gene products Myc and Max, suggesting that these proteins might be involved in the AR gene autoregulation system.[65] The significant up-regulation of c-myc mRNA by FM in the present study might also implicate Myc in the AR gene down-regulation.

In order to reduce the genotoxic damage caused by exposure to free radicals due to chemical compounds, therapeutic drugs, air pollutants, and metabolic procedures, the use of antimutagens has been studied as a possible alternative.[66-67] Vitamins are complex organic substances needed in a very small amount for many of the processes carried out in the body and play an important role in the regulation of a vast array of physiological and pathological events. The mechanisms underlying the action of vitamins include enhancement of the immune system, modification of carcinogen metabolism, alteration of cell proliferation, stimulation of the repair of carcinogen-induced DNA damage and scavenging free radicals or electrophiles, which damage DNA and other cell targets.[68-69] From this viewpoint, the protective role of VD_3 against the genotoxic effects of FM was studied.

In the present study, simultaneous administration of VD_3 caused a highly significant reduction in the percentage of aberrant cells induced by FM in germ and somatic cells. This vitamin suppressed the sperm shape abnormalities induced by FM by 27.7-46.7%. Likewise, the percentage of inhibition of SCE's reached 36.7 when VD_3 was administered with the higher dose of FM. VD_3 was found to be a significant factor in detoxification and protection against environmental toxins by inducing mechanisms of detoxification of endo- and xenobiotics.[23-24] Sarkar et al., demonstrated that vitamin D_3 effectively suppressed the frequencies of structural chromosomal aberrations and sister chromatid exchanges in lymphoma-bearing mice during the entire phase of tumor growth that significantly coupled with almost two fold increase in survival time.[70]

Bonaccorsi et al., concluded that VD analog was able to reduce proliferation of the prostatic cancer cell lines in vitro.[71] Seubwai et al., demonstrated that treatment with $1,25(OH)_2D_3$ in the cholangiocarcinoma cell lines with high expression of VD receptor significantly reduced cell proliferation in a dose-dependent manner.[72] These data indicates an active role for these receptors in mediating the antiproliferative effects of this vitamin. Pleiotropic anticancer effects of $1,25(OH)_2D_3$ on normal and cancerous prostate cells were described by numerous laboratories. The mechanisms for these effects in the prostate are not completely characterized but include marked inhibition of: cell proliferation; invasion; migration; metastasis and angiogenesis.[73-78]

CONCLUSION

Present evidence concluded that supplementation with VD_3 in mice protects against FM-induced genotoxicity and suggest the possibility of synergistic effects of the combination of these compounds. More in vivo and in vitro studies could shed more light on the anticancer activity of this combination.

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