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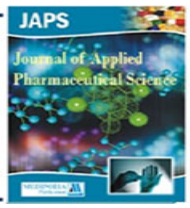

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Genotoxicity of anticancer drug Azathioprine (Imuran): role of Omega-3 (ω-3) oil as protective agent

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ABSTRACT

Omega-3 (ω-3), is long-chain, polyunsaturated fatty acids (PUFAs) of plant and marine origin. The present study was conducted to evaluate the protective effect of omega-3 against cytotoxicity and genotoxicity of anticancer drug; Azathioprine (Imuran): Male albino mice were administrated two levels; therapeutic (5mg|kg) and double therapeutic (10mg|kg) doses. Azathioprine was intraperitoneally injected for 3 times at 48 hour interval. Omega-3 was orally administered with 2 ml/kg for ten consecutive days either before or after Azathioprine treatments. At the end of experimentation period, samples of bone marrow were collected from five mice within each group for micronucleus assay. The liver and testis tissue samples were removed and stored at – 80 °C until use for DNA extraction, and determination of glutathione contents. Another animal group was treated at the same regimen and were used for the determination of sperm abnormalities and sacrificed after 35 days. The results indicated that oral administration of omega-3 either before or after treatment of Azathioprine was effective in reduction of the frequencies of Mn-PCEs, decreased the DNA fragmentation, total sperm abnormalities and significantly increased sperm count, percentage of PCEs, and enhanced the ratio of PCEs to NCEs. However, random amplified polymorphism of DNA (RAPD) showed distinct differences in animal groups intoxicated with Azathioprine before and after omega-3 treatment, which reflected DNA protective effect of omega-3. Depletion in glutathione content in testis was also observed in Azathioprine treated mice, which was improved by oral administration of Omega-3 either before or after treatment with Azathioprine.

Keywords: Omega-3, micronucleus test, sperm abnormalities, RAPD, Azathioprine Genotoxicity

INTRODUCTION

Omega-3 (ω-3) fatty acids are long-chain, polyunsaturated fatty acids (PUFAs) of plant and marine origin (Holub, 2002). Because these essential fatty acids cannot be synthesized by the human body, they must be derived from dietary sources (Holub, 2002). Flaxseed, hemp, canola and walnut oils are rich sources of the parent omega-3, alpha linolenic acid (ALA) (Holub, 2002).

Most ω-3 PUFAs are derived from ALA, found mainly in the chloroplasts of green vegetables and grass (Gurr *et al.*, 2002). Also diets enriched in polyunsaturated fatty acids (PUFA), particularly diets containing supplements of omega 3 components are found in abundance in fish oils (Decsi *et al.*, 2005 & Krauss-Etschmann *et al.*, 2007), as ω-3 PUFAs eicosapentaneoic acid (EPA) and docosahexaneoic acid (DHA) may be supplied directly from fish oils (Simopoulos, 2002). In addition to general dietary consumption, many women of reproductive age take PUFA supplements for various health reasons such as rheumatoid arthritis (Simopoulos, 2002) and menstrual dysfunction (Dickerson *et al.*, 2003).

Polyunsaturated fatty acids are transferred from the mother to the fetus across the placenta. Recent studies have suggested that ω-3 fatty acid intake has a significant impact on growth, vision, brain, and vital organ development in breast-fed infants (Cheatham *et al.*, 2006; Mitmesser and Jensen 2007 and Ozias *et al.*, 2007). Therefore, maternal PUFA deficiency could adversely affect fetal development.

Current research suggests that the anti-inflammatory activity of long-chain omega−3 fatty acids may translate into clinical effects (Wall *et al.*, 2010). For example, there is evidence that rheumatoid arthritis sufferers taking long-chain omega−3 fatty acids from sources such as fish have reduced pain compared to those receiving standard nonsteroidal Anti-inflammatory medicines (NSAIMs) (Ruggiero *et al.*, 2009). Sixty-four healthy Danish infants from nine to twelve months of age received either cow's milk or infant formula alone or with fish oil. Those infants supplemented with fish oil were found to have improvement in immune function maturation, with no apparent reduction in immune activation (Damsgaard *et al.*, 2007). Moreover omega-3 fatty acids play a crucial role in all systems of the body to function normally, including; skin, respiratory system, circulatory system, brain and organs as well as normal growth and development (Berbert *et al.*, 2005 and Boskou 2000).

Azathioprine and 6-thioguanine (6-TG); are examples of thiopurine; anti-metabolites which are widely used in the treatment of cancer and inflammatory conditions (Aarbakke *et al.*, 1997), including rheumatoid arthritis, systemic lupus erythromatosis, psoriasis and inflammatory bowel disease (Van Scoik *et al.*, 1985). Azathioprine is typically administered in tablet form; it is also used in the therapy of organ transplant patients (Sachin *et al.*, 2005) to prevent rejection following transplantation (Englwood, 2001). Azathioprine is a pro-drug which is cleaved chemically to produce 6-mercaptopurine, in red blood cells (Sachin *et al.*, 2005) by the enzyme called glutathione-S-transferase (Van Scoik *et al.*, 1985). More than 80% of Azathioprine is converted to 6-MP (Sandborn, 1998 and Cuffari *et al.*, 2000). Azathioprine may also be metabolized by the enzyme called aldehyde oxidase to 8-hydroxy-Azathioprine, which in turn is converted to an inactive metabolite, 6-thiouric acid, by xanthine oxidase (Zimm *et al.*, 1983).

Azathioprine and its active metabolite, 6-mercaptopurine (6-MP), are purine analogues that interfere with the synthesis of adenine and guanine ribonucleosides. These ribonucleosides are important precursors of DNA and RNA. Azathioprine and 6-MP act predominantly on rapidly dividing cells such as the T lymphocytes, these drugs are not only cytotoxic but also immunosuppressive and anti-inflammatory (Goldstein, 1987). The effects are dose-related, small doses of either drugs have anti-inflammatory action, but larger doses have immunosuppressive and cytotoxic action (Goldstein, 1987), leading to a variety of DNA modifications, such as chromatid damage (Fairchild *et al.*, 1986), DNA strand breaks and DNA–protein cross links. Also it is required as an active DNA mis-match repair system, and whilst the effects of purine starvation have been suggested, DNA damage seems to be the main mechanism for the cytotoxic effects of thiopurines (Karran and Attard, 2008).

The aim of the present study was to assess the genotoxicity induced by anticancer drug; Azathioprine (Imuran) at both doses therapeutic and 2×therapeutic doses on both somatic and germ cells in mice. The present study was extended to assess the protective and curative role of the omega-3 in attenuating the Azathioprine cytotoxicity and genotoxicity.

MATERIALS & METHODS

Drugs and Chemicals

Imuran (Azathioprine)

Imuran (Azathioprine) which manufactured by RPG Life Science Ltd., India, was injected intraperitoneal (*i.p.*) with volume 0.1ml, in two doses the first one represent the therapeutic dose (5 mg/kg b. wt.). The second dose represents the 2×therapeautic dose (10 mg/kg b. wt.). Each dose was injected 3 times with 48 hour interval.

Omega-3

Omega-3 was purchased from SEDICO pharmaceutical Company, Egypt. It was applied to the animal by oral gavages with volume 2 ml/kg for ten consecutive days either before or after Azathioprine treatments.

Experimental animal

90 Adult male mice (10 - 12 week-old, with an average weight 25±5 g) of Swiss strain were randomly drawn from the stock colony of "National Research Center" Dokki- Egypt. Mice were held in polypropylene cages and were housed in a controlled atmosphere with a temperature range of 25±5 °C and mean relative humidity of 50 ± 5%. The animals were maintained on commercial mouse pellets ad libitum and had free access to water during a week of acclimatization and throughout the studies. All experiments inclusive of animal handling and sacrifice were conducted strictly in conformation with standard guidelines of the “Institutional Ethics Committee”.

Experimental design

Male mice were divided into 9 groups (10 animals/ group) as follows: Group 1, untreated control; group 2, solvent group (0.1 ml of 0.9% NaCl); group 3 & 4 injected intraperitoneally (*i.p.*) with Azathioprine (5 & 10 mg/kg b.wt.) for 3 times with 48 hour

interval, group 5, treated orally with 2 ml/kg of Omega-3 for ten consecutive days; groups 6 & 7 treated orally with 2 ml/kg of Omega-3 for ten consecutive days either before or after Azathioprine treatments at 5 mg/kg respectively; groups 8 & 9, treated orally with 2 ml/kg of Omega-3 for ten consecutive days either before or after Azathioprine treatments at 10 mg/kg respectively. At the end of experimentation period, samples of bone marrow were collected from five mice within each group for micronucleus assay. The liver and testis tissues were collected, and stored at –80 °C until use for DNA extraction, and determination of glutathione contents. The other 5 animals from each group were used for the determination of sperm abnormality and sacrificed after 35 days and epididymis were removed for the sperm abnormality study.

Micronucleus test

Bone marrow smears were prepared following the extraction of marrow from one femur was done according to the method described by Schmid, (1975). This assay was performed with some modifications adopted by Salamone *et al*., (1980).

Sperm abnormality test

The cauda epididymidis (free of fats, vas deferens and other tissues) of either control or treated mice was removed from each side and minced in 2 ml of saline solution (0.9% NaCl) and processed according to the method described by (Muralidhara and Narasimhamurthy, 1991). Spermatozoa were counted using heamocytometer and a drop of a homogenate smeared on a cleaned slide, allowed to air dry and stained by Eosin Yellow to determine the head and tail abnormalities of sperms.

DNA fragmentation (Apoptosis) test

Apoptotic changes in animals were evaluated colorimetrically by DNA stained with Diphenylamine (DPA) and by monitoring fragmented DNA using agarose gel electrophoresis according to the procedure of (Perandones et al., 1993) with some modifications, the excitation wavelength was 600 nm.

Extraction of DNA and performing RAPD- PCR

The extraction of DNA from liver tissue cells was performed according to the procedure of extraction of DNA from tissue by (Sambrook and Russel 2001) with some modifications. The random amplified polymorphic DNA (RAPD) technique for DNA isolated from liver tissue cells was performed according to the method of Singh and Roy, (2006). Six primers previously selected because of their good RAPD profiles and their good *in vivo/ in vitro* correlation were used in this study. Their sequences are, A04: 5-AAT CGG GCT G-3, A09: 5-GGG TAA CGC C-3, A19: 5-CAA ACG TCG G-3, A20: 5-GTT GCG ATC C-3, B02: 5-TGA TCC CTGG-3 and B03: 5-CAT CCC CCT G-3. The amplification products separated by gels were visualized and documented using the Gel Documentation system, Gel-Pro Analyzer (Media Cybernetics, USA).

Determination of reduced glutathione

In the testis, tissue homogenate content of reduced glutathione (GSH) was measured according to the method described by (Ellman, 1959). The assay is based on the reduction of 5, 5 – dithiobis – (2 – nitrobenzoic acid) (DTNB) by SH groups of glutathione to form 2 – nitro-S-mercaptobenzoic acid per mole of glutathione. The product was measured spectrophotometically at 412 nm.

Statistical analysis

For statistical analysis the SPSS computer program was used. The statistical analysis was carried out by one-way ANOVA setting the probability level to $P<0.05$, followed by Waller–Duncan k-ratio (Walter and Duncan, 1969). The treated groups were compared both with each other and with untreated control groups.

RESULTS

Micronucleus test

Table (1) Showed that the frequencies of micronucleated PCE as resulted from administration of omega-3 has no significant increases in Mn-PCEs. The frequency of Mn-PCEs in omega-3 treated group (8.6 ± 0.60) was within the accepted spontaneous range for control (7.2 ± 0.66). Azathioprine treatment induced significant *(P< 0.05)* increase in the frequencies of Mn-PCEs at therapeutic dose (23.4 ± 0.87) compared to control group. Moreover, high statistically significant increase in the incidence of Mn-PCEs over the control value was observed following treatment with 2 × therapeutic dose of Azathioprine treated group (41.4 ± 1.60) compared to control group Fig (1). Oral administration of omega-3 either before or after treatment of Azathioprine (at therapeutic or 2× therapeutic doses) was effective in reducing the frequencies of Mn-PCEs however the post-treatment of omega-3 was more effective than pre-treatment. In spite of these reductions, the frequencies of Mn-PCEs were still significantly higher than the control value.

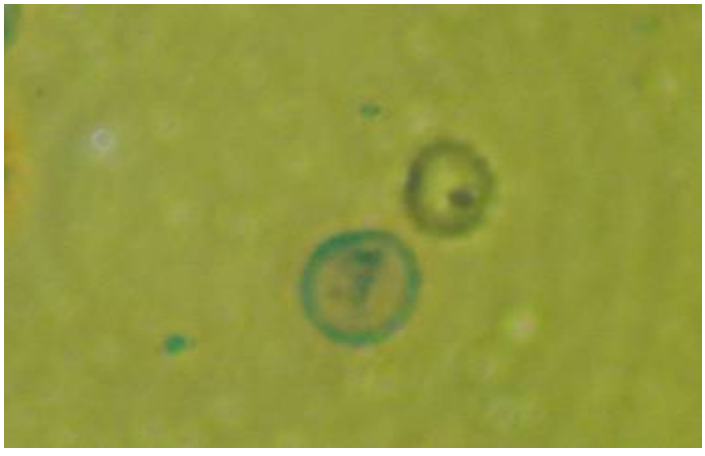

Fig. 1: Showing a micronucleated polychromatic erythrocyte (Mn-PCE) stained with 5% Giemsa stain, with magnification power 1000 X.

Table. 1: The frequencies of micronucleated polychromatic erythrocytes (Mn-PCEs) in the bone marrow following omega-3 oil treatment to mice subjected to Azathioprine.

Treatments	No Mn-PCEs /5000	Mn-PCEs M ± SE /1000	% of MnPCEs	% of inhibitions
Control	36	7.2 ± 0.66 [e]	0.72	--
Solvent	45	9.0 ± 0.71 [e]	0.90	--
Aza	117	23.4 ± 0.87 [c]	2.34	--
2×Aza	207	41.4 ± 1.60 [a]	4.14	--
Omega-3	43	8.6 ± 0.60 [e]	0.86	--
Omega+Aza	92	18.4 ± 0.75 [d]	1.84	30.9
Aza+Omega	79	15.8 ± 0.80 [d]	1.58	46.9
Omega+2×Aza	137	27.4 ± 1.78 [b]	2.74	40.9
2×Aza+Omega	104	20.8 ± 1.02 [d]	2.1	60.2

Number of animals/group = 5, Data are expressed as: mean ± standard error (SE). Means assigned with the same letter show insignificant differences between these values. Means with different superscripts (a, b, c, d and e) between groups in the same column are significantly different at $P< 0.05$. Aza means Azathioprine (therapeutic dose) and 2 × Aza means Azathioprine (2 × therapeutic dose).

When Azathioprine treated at therapeutic dose (5 mg/Kg), a significant decrease in the percentage of PCEs (42.12 ± 1.28) was induced when compared to the control group (56.8 ± 1.85) Table (2). Animals that had received 10 mg/Kg of Azathioprine (2× therapeutic dose) exhibited highly significant bone marrow cytotoxicity and a serve reduction in percentage of PCEs was detected, (36.18 ± 1.06) as well as the ratio of PCEs / NCEs (0.58 ± 0.02) when compared to the control group (1.33 ± 0.09) indicating severe bone marrow toxicity. On the other hand, omega-3 pre-/post-treatment to Azathioprine significantly increased the percentage of PCEs and the ratios of PCEs to NCEs were significantly enhanced when compared to Azathioprine at therapeutic dose treated group.

Table. 2: The ratio of polychromatic erythrocytes (PCEs) to the normochromatic erythrocytes (NCEs) in bone marrow following omega-3 oil treatment to mice subjected to Azathioprine.

Treatments	PCEs % (mean ± SE)	NCEs % (mean ± SE)	Ratio PCEs / NCEs
Control	56.8 ± 1.85 [a]	43.20 ± 1.85 [d]	1.33 ± 0.09 [b]
Solvent	59.78 ± 1.41 [a]	40.22 ± 1.41 [d]	1.50 ± 0.08 [a]
Aza	42.12 ± 1.28 [c]	57.88 ± 1.28 [b]	0.73 ± 0.04 [d]
2× Aza	36.18 ± 1.06 [d]	63.82 ± 1.06 [a]	0.58 ± 0.02 [e]
Omega-3	60.70 ± 0.49 [a]	39.30 ± 0.49 [d]	1.55 ± 0.03 [a]
Omega+Aza	44.02 ± 1.62 [b]	55.98 ± 1.62 [c]	0.79 ± 0.05 [c]
Aza+Omega	47.52 ± 1.19 [b]	52.48 ± 1.19 [c]	0.91 ± 0.04 [c]
Omega+2×Aza	44.42 ± 1.15 [b]	55.58 ± 1.15 [c]	0.80 ± 0.04 [c]
2×Aza+Omega	46.52 ± 1.66 [b]	53.48 ± 1.66 [c]	0.88 ± 0.06 [c]

Number of animals/group = 5, Data are expressed as: mean ± standard error (SE). Means assigned with the same letter show insignificant differences between these values. Means with different superscripts (a, b, c, d and e) between groups in the same column are significantly different at $P< 0.05$. Aza means Azathioprine (therapeutic dose) and 2 × Aza means Azathioprine (2 × therapeutic dose).

Sperm characteristics

The sperm count in Azathioprine treated groups (at therapeutic and 2× therapeutic doses) showed a significant *(P<0.05)* decline (26.0 ± 1.58 and 22.2 ± 1.83 respectively) compared to control group (30.6 ± 1.91). On contrary, animals treated with omega-3 alone showed insignificant change in sperm count (34.9 ± 1.54) in comparison with the control group (30.6 ± 1.91) Table 3. The current results clearly indicated that, animals treated with Azathioprine at both doses showed a significant increase *(P<0.05)* in the frequency of abnormal sperm (3.6 % and 3.5 % at 5 and 10 mg/kg b. wt. respectively) compared to the control group. Treatment with omega-3 either before or after treatment of Azathioprine at both doses resulted in a considerable reduction in sperm abnormalities. Pre-/post-treatment with omega-3 was completely inhibited the percentage of sperm abnormalities induced by Azathioprine at both doses. Various morphological sperm abnormalities in head and tail were recorded in Table (4).

Table. 3: Epididymal sperm count and their abnormalities following omega-3 oil treatment to mice subjected to Azathioprine.

Treatments	Sperm count × 10^6/ml (Mean ± SE) × 10^6/ml	Total morphological sperm abnormalities (%)
Control	30.6 ± 1.91[b]	1.68[b]
Solvent	31.4 ± 1.31[b]	1.9[b]
Azathioprine	26.0 ± 1.58[c]	3.6[a]
2×Azathioprine	22.2 ± 1.83[d]	3.5[a]
Omega-3	34.9 ± 1.54[a]	1.58[c]
Omega +aza	22.0 ± 0.63[d]	1.14[d]
Aza+ Omega	22.6 ± 0.75[d]	1.16[d]
Omega +2×aza	24.2 ± 1.07[c]	1.54[c]
2×aza+ Omega	23.4 ± 0.75[c]	1.02[d]

Number of animals/group = 5, Data are expressed as: mean ± standard error (SE). Means assigned with the same letter show insignificant differences between these values. Means with different superscripts (a, b, c, d and e) between groups in the same column are significantly different at $P< 0.05$. Aza means Azathioprine (therapeutic dose) and 2 × Aza means Azathioprine (2 × therapeutic dose).

DNA laddering and fragmentation

DNA fragmentation and laddering was performed on the DNA extracted from the liver tissue of mice to determine the percentage of DNA fragmentation induced by Azathioprine and the possible protective role of omega-3. DNA damage was evaluated by measuring the level of fragmented DNA colorimetrically using Diphenylamine (DPA) and by comparing DNA profiles on agarose gel electrophoresis Fig (2). The results indicate that the percentage of DNA fragmentation were significantly increased in groups treated with Azathioprine either at therapeutic dose (22.75%) or 2× therapeutic dose (31.53%) when compared to the control group (12.29%) as indicated by DPA assay Table (5). While treatment with omega-3 alone showed insignificant change in the percentage of DNA fragmentation (11.07%) compared to the control group (12.29%). Omega-3 post-treatment to Azathioprine significantly resulted in partial or complete recovery in the DNA.

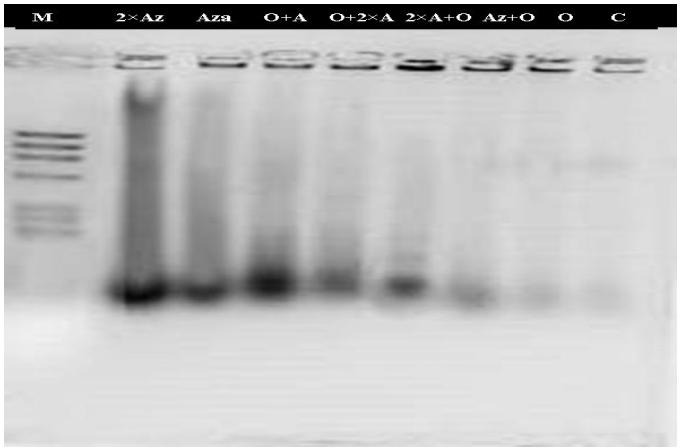

Fig. 2: 1.2% agarose gel electrophoresis of DNA extracted from mice liver treated with, Azathioprine at two doses and omega-3. *Lane M:* DNA molecular weight marker. *Lane 2:* 2×Aza: 2×Azathiprine (10 mg/kg). *Lane 3*: Aza: Azathioprine (5 mg/ kg), *Lane 4*: O+Aza: Omega-3 +Azathioprine, *Lane 5*: O + 2×Aza: Omega-3+ 2×Azathioprine, *Lane 6:* 2×Aza +O: 2×Azathioprine + Omega-3, *Lane 7*: Aza +O: Azathioprine+ Omega-3, *Lane 8:* O: Omega-3, *Lane 9:* C: Control.

Table. 4: Morphological sperm abnormalities following omega-3 oil treatment to mice subjected to Azathioprine.

Treatments	Sperm tail abnormalities	Sperm head abnormalities			Total sperm abnormalities / 1000 sperm	
	Coiled	Amorphous	Without-hock	Banana	Mean ± SE	Range
Control	3.0 ± 0.63[b]	4.6 ±0.93[b]	5.4± 1.03[c]	3.8±1.5[a]	16.8 ± 1.88[bc]	12 – 23
Solvent	2.8 ± 0.58[b]	4.4 ±0.68[b]	6.6 ±1.29[b]	5.2±0.8[a]	19.0 ± 1.14[b]	15 – 21
Azathioprine	14.2 ±3.63[a]	10.2 ± 2.58[a]	10.6 ±2.73[a]	1.0±0.63[bc]	36.0 ± 4.06[a]	25 – 46
2×Azathioprine	7.8 ± 1.86[a]	14.2 ± 4.59[a]	11.8 ±2.85[a]	1.2±0.49[b]	35.0 ± 3.63[a]	25 – 48
Omega-3	2.2 ± 1.11[b]	4.4 ± 1.69[b]	8.0 ± 2.26[ab]	1.2±0.37[b]	15.8 ± 2.78[c]	9 – 24
Omega+aza	1.0 ±0.32[c]	3.0 ± 1.0[c]	7.6 ±1.21[b]	0.4±0.4[d]	11.4 ± 1.12[d]	9 – 15
Aza+Omega	1.4 ± 0.51[c]	3.2 ± 1.02[c]	6.4 ±0.68[b]	0.6±0.25[c]	11.6 ± 1.69[d]	6 – 16
Omega+2×aza	0.4 ± 0.4[d]	8.8 ±1.07[a]	5.4 ±0.60[c]	0.8±0.37[c]	15.4 ± 0.75[c]	14 – 18
2×aza+omega	1.2 ± 0.97[c]	5.2 ±2.01[b]	3.4 ±1.03[d]	0.4±0.25[d]	10.2 ± 2.13[d]	6 – 17

Number of animals/group = 5, Data are expressed as: mean ± standard error (SE). Means assigned with the same letter show insignificant differences between these values. Means with different superscripts (a, b, c, d and e) between groups in the same column are significantly different at $P< 0.05$. Aza means Azathioprine (therapeutic dose) and 2 × Aza means Azathioprine (2 × therapeutic dose).

Table. 5: DNA fragmentation percentage following omega-3 oil treatment to mice subjected to Azathioprine.

Treatment	DNA Fragmentation %		Inhibition %
	Mean ± SE	Change	
Control	12.29 ± 1.0 [c]	0	--
Solvent	12.73 ± 0.86 [c]	+ 0.44	--
Aza	22.75 ± 2.18 [b]	+ 10.46	--
2×Aza	31.53 ± 2.85 [a]	+ 19.24	--
Omega-3	11.07 ± 0.34 [c]	- 1.22	--
Omega + Aza	18.69 ± 1.35 [b]	+ 6.4	38.81
Aza + Omega	12.11 ± 1.34 [c]	- 0.18	101.72
Omega + 2×Aza	23.41 ± 1.85 [b]	+ 11.12	42.20
2×Aza + Omega	19.84 ± 1.43 [b]	+ 7.55	60.76

Number of animals/group = 5, Data are expressed as: mean ± standard error (SE). Means assigned with the same letter show insignificant differences between these values. Means with different superscripts (a, b, c, d and e) between groups in the same column are significantly different at $P< 0.05$. Aza means Azathioprine (therapeutic dose) and 2 × Aza means Azathioprine (2 × therapeutic dose).

Table. 6: Testis glutathione depletion following omega-3 oil treatment to mice subjected to Azathioprine.

Treatments	Glutathione content in the testis tissues µ mol / g tissue			
	After 24 hours		After 35 days	
	M ± SE	changes	M ± SE	changes
Control	3.37 ± 0.99 [b]	0	3.94 ± 0.77[ab]	0
Solvent	3.24 ± 0.78 [b]	- 0.13	3.32 ± 0.38 [b]	- 0.62
Aza (5mg/kg)	2.35 ± 0.42 [c]	- 1.02	2.19 ± 0.33 [bc]	- 1.75
2×Aza (10mg/kg)	1.86 ± 0.16 [c]	- 1.51	2.29 ± 0.34 [bc]	- 1.65
Omega-3	5.08 ± 0.20 [a]	+1.71	3.77 ± 0.23 [b]	+ 0.17
Omega-3+Aza	4.46 ± 0.43 [a]	+ 1.09	3.46 ± 0.47 [b]	- 0.48
Aza+Omega-3	5.27 ± 0.82 [a]	+ 1.90	3.36 ± 0.35 [b]	- 0.58
Omega+2×Aza	2.84 ± 0.85 [b]	- 0.53	3.61 ± 0.42 [b]	- 0.33
2×Aza+Omega	2.86 ± 0.40 [b]	- 0.51	2.93 ± 0.60 [b]	- 1.01

Number of animals/group = 5, Data are expressed as: mean ± standard error (SE). Means assigned with the same letter show insignificant differences between these values. Means with different superscripts (a, b, c, d and e) between groups in the same column are significantly different at $P< 0.05$. Aza means Azathioprine (therapeutic dose) and 2 × Aza means Azathioprine (2 × therapeutic dose).

RAPD Profiling

Six random 10-mer primers were used to analyze instability in the genome of mice liver using RAPD-PCR fingerprinting and the presence of changes in the RAPD profiles obtained from the exposed population depending on the primer used. Of the Six primers, four produced reproducible and scorable amplification fingerprints. Two of the four primers (Operon (OP) A04 and A09) produced similar RAPD fingerprints for controls and treatment groups rendering it uninformative in revealing alterations in liver DNA. The remaining two primers, B02 and B03, detected changes in the RAPD profiles of the hepatic DNA compared to untreated controls. A total of 14 loci of different bands were amplified by the two primers, with an average about seven loci per primer of the 14 loci that were amplified, 6 bands were polymorphic giving (42.8%) polymorphism, the sizes of bands ranged from 245 - 2315 bp. DNA profiles presented in Fig (3) were generated using B02 primer and a mixture of four individuals from each replicate. Profiles generated by these primers

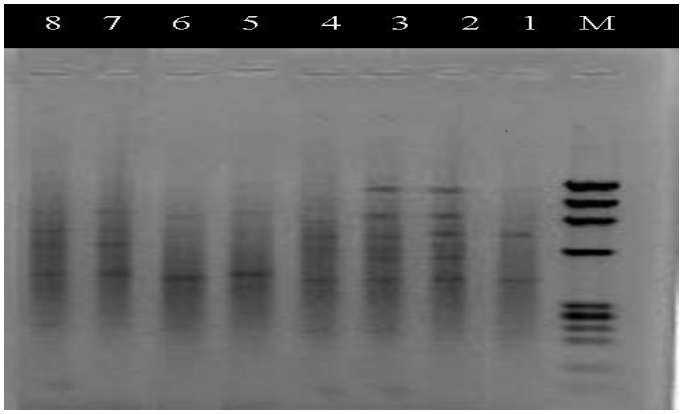

Fig (3): RAPD fingerprinting of hepatic genomic DNA, Photo represent PCR product with primer B02 with omega-3 treated groups. The DNA marker is in lane M, lane 1: represents untreated mice (control), lane 2: mice treated with Aza, lane 3 mice treated with 2× Aza, lane 4: mice treated with omega-3, lane 5: mice treated with O+Aza, lane 6: mice treated with Aza+O, lane 7: mice treated with O+2× Aza and lane 8: mice treated with 2× Aza+O.

revealed differences between control and exposed individuals, with visible changes in the number and size of amplified DNA fragments. The most obvious result was the appearance of new bands in DNA samples of the group treated with Azathioprine alone at either therapeutic or 2 × therapeutic doses. The new bands resulted from the genetic alteration in DNA in Azathioprine-treated groups. Where as none of these new bands were found in the genomic DNA of the samples collected from the animals belong to the control group or the animals that received omega-3.

Biochemical study

The biochemical parameter determine the alteration in the intracellular glutathione concentration induced by Azathioprine at both doses (5 and 10 mg/kg b. wt.) as well as the improving effects of omega-3; when the GSH was measured either after 24 hours from the last treatment or 35 days from the beginning of the treatments, the results revealed that, Azathioprine induced a significant (*P<0.05)* depletion in testis GSH content after 24 hr. either at therapeutic dose (2.35 ± 0.42) or 2× therapeutic dose (1.86 ± 0.16) when compared to that of the control value (3.37 ± 0.99) Table (6). While after 35 days Azathioprine insignificantly decreased GSH content either at therapeutic (2.19 ± 0.33) or 2× therapeutic doses (2.29 ± 0.34) when compared to the control group (3.94 ± 0.77). Omega-3 treatment significantly enhanced the testis GSH content in comparison with the control group. Moreover, omega-3 pre-/post-treatment to Azathioprine significantly increased the testis GSH content when compared to that of the Azathioprine subjected groups.

DISCUSSION

In the current study, we evaluated the possible protective effect of Omega-3 oil as natural antioxidant against genotoxicity and cytotoxicity induced by Azathioprine. Azathioprine is widely used in the treatment of cancer and inflammatory conditions (Aarbakke *et al.,* 1997), as well as in the therapy of organ transplant patients (Weinshilboum, 2001). Azathioprine or Imuran is an indirect acting agent as it requires metabolic activation by cellular enzymes to form its genotoxic and cytotoxic effects in both somatic and germ cells. The dosing schedule and route can markedly affect the toxicity of Azathioprine in mice (Elion and Hitchings, 1975).

In the present study, the results of the micronucleus test revealed that animals treated with Azathioprine at both, 5 and 10 mg/kg b. wt. which represent therapeutic and 2×therapeautic doses exhibited a significant (*P*< 0.05) increase cytotoxicity in bone marrow as indicated by a marked reduction in the average number of PCEs when compared to the control. Moreover, the ratio of PCEs/NCEs proved such toxicity. The common side effect of Azathioprine treatment in both animals and humans is bone-marrow depression and lymphocyte depletion a finding that is not surprising for a drug that is an immunosuppressive agent (Bendre *et al.,* 2005). However, its active metabolite, 6-mercaptopurine (6-MP) inflicts damage on rapidly dividing cells, such as bone marrow, intestinal epithelium and the reproductive organs in adults (Polifka and Friedman, 2002). The previous reports confirmed our results. In the same line, cytopenia and severe bone marrow suppression have been reported in both the human and veterinary literature, and may occur weeks to months after initiating treatment of Azathioprine (Wise and Callen, 2007). Humans may suffer from bone marrow suppression years after initiating therapy, as steady state levels of Azathioprine in the blood may take month to years to achieve (Petit *et al.,* 2008). While neutropenia is the most common cytopenia noted, anemia and thrombocytopenia may also occur (Petit *et al.,* 2008).

From results showed in the present study, there is no statistically significant difference in Mn-PCEs developed from treatment of mice with omega-3 oil in comparison with the control group. In this concern Das and Rao (2006) demonstrated that PUFAs induced insignificantly change in the number of Mn-PCEs compared to those of the control. Furthermore, all of the PUFAs (linoleicacid, α-linolenic acid, gamma-linolenic acid, arachidonic acid, dihomo-gamma linolenic acid and eicosapentaenoic acid) studied except eicosapentaenoic acid showed significant decrease in Diphenylhydantoin (DPH) induced genetic damages assessed by micronucleus (MN) test. However, gamma-linolenicacid (GLA) was found to be the most effective in reducing the number of MN containing lymphocytes both *in vitro* and *in vivo* to control values (Shivani *et al.,* 2009).

In our study, omega-3 oil post-treatment to Azathioprine in therapeutic dose showed a significant reduction in developing Mn-PCEs in bone marrow cells of mice. Our results indicated that using omega-3 as a post treatment is preferable than using it as a pretreatment with Azathioprine in both doses (5 and 10 mg/kg b. wt.). Our results are in consistent with, Das and Rao (2006) and other earlier investigations (Koratkar *et al.,* 1993; Devi *et al*., 1984 & Das *et al.,* 1985) which reported that, PUFAs such as GLA and prostaglandin products; PGE1 and PGE2 have anti-mutagenic and anti-carcinogenic actions. PUFAs when tested alone produced insignificant increase in the amount of genetic damage but when tested in combination with Diphenylhydantoin the number of micronuclei containing lymphocytes was reduced (Das and Rao, 2006; Shivani *et al.,* 2009).

In the present study Azathioprine (Imuran) at both doses 5 and 10 mg/kg showed a significant decrease in the count of sperm comparing to control group. Furthermore, it had a significant (*P <0.05*) increase in the total morphological sperm abnormalities. Similarly, Bendre *et al.,* (2005) demonstrated that Azathioprine dose of 50 mg/kg for 4 weeks significantly lowered sperm viability and a dose of 25 mg/kg Azathioprine for 16 weeks reduced sperm motility. These doses were also lethal for the mice (Sachin *et al.,* 2005). Testicular weight was decreased in mice treated with 10 mg/kg of Azathioprine for 16 and 23 weeks, a dose that was well-tolerated by the mice (Sachin *et al.,* 2005). However sperm counts, morphology and cauda epididymes weights were not influenced by Azathioprine treatment (Sachin *et al.,* 2005). Sachin *et al.,* (2005) reported that a mouse model for Azathioprine mutant selection was developed and Azathioprine toxicity was evaluated in germ cells of treated mice. Groups of 20 male C57BL/6 mice were treated by

oral gavage 3 times/week 0, 5, 10, 25, 50 and 100 mg/kg b. wt. and 3-8 mice from each group were sacrificed at various times for up to 23 weeks (Sachin *et al.,* 2005). Mice treated with 25-100 mg/kg of Azathioprine were all dead by 14 weeks of treatment (Sachin *et al.,* 2005). Also this survey showed that Azathioprine dose of 50 mg/kg for 4 weeks significantly lowered sperm viability and a dose of 25 mg/kg Azathioprine for 16 weeks reduced sperm motility (Sachin *et al.,* 2005).

Linoleic acid, a polyunsaturated fatty acid present in omega-3 oil, is known to increase membrane fluidity and allows for osmosis, intracellular and extra cellular gaseous exchange (Lovejoy, 2002). It is easily susceptible to lipid peroxidation. Also, the presence of oleic acid, a monounsaturated fatty acid also reduces the susceptibility of the testis to lipid peroxidation (Bourre *et al*., 2004; Lovejoy, 2002). This probably explains the better sperm count at groups treated with omega-3 oil. As omega-3 oil has a significant effect in increasing the count of sperm in comparison with Azathioprine in doses of 5 and 10 mg/kg b. wt. Omega-3 oil pre/post-treatment to Azathioprine showed decrease in the percent of total morphological abnormalities in sperms. The results of this study about omega-3 were in agreement with the results reported by (Farias *et al.,* 1975; Eynard *et al.,* 1992; Fanani and Maggio, 1997; Calderon and Eynard, 2000; Garcia *et al.,* 2002; Pike and Casey, 2002 & Sanchez and Perillo, 2002).

However omega-3 oil has a great significant effect on decreasing the total sperm abnormality in morphology in comparison with Azathioprine treated groups. There is no significance effect of omega-3 on sperm abnormality in morphology compared to control group. Using omega-3 oil either before or after Azathioprine treatment in both doses showed a significant decreasing effect on total sperm abnormality in morphology induced by Azathioprine as indicated in Table (4). According to (Claire *et al.,* 2007), omega-3 PUFA showed protective effect on sperms as declared in the present study, (particularly omega-3 PUFAs in fish oil) are promoted for general health reasons. Medina *et al.,* (2006) confirmed that the severe deficiency of EFAs arrest spermatogenesis therefore, decreased sperm numbers and development of abnormal spermatazoal morphology. In our study, omega-3 oil as post-treatment to Azathioprine in 2 × therapeutic dose is preferable than using it as a pre-treatment.

Fish oils may also benefit fertility in cattle and reduce the risk of preterm labor in women, but in both cases current evidence to support this is inconclusive (Claire *et al.,* 2007). Spermatozoa require a high PUFA content to provide the plasma membrane with the fluidity essential at fertilization (Claire *et al.,* 2007). However, this makes spermatozoa particularly vulnerable to attack by reactive oxygen species, and lifestyle factors promoting oxidative stress have clear associations with reduced fertility. Adequately powered trials that control for the ratios of different PUFAs consumed are required to determine the extent to which this aspect of our diets does influence our fertility (Medina *et al.,* 2006).

The inactive pro-drugs, 6-TG and Azathioprine, are metabolized to DNA precursors, such as 6-thio-2-deoxyguanosine tri-phosphate (6TdGTP), in the 2-deoxyribonucleotide pool where they are substrates for DNA polymerases (Lepage, 1963). 6-TG and Azathioprine incorporation into DNA appears to be in part necessary for their cytotoxic action (Lepage, 1963 & Lee and Sartorelli, 1981), leading to a variety of DNA modifications, such as chromatid damage, DNA strand breaks and DNA–protein cross links (Fairchild *et al.,* 1986). Azathioprine causes 6-TG to accumulate in patients' DNA (Warren *et al*, 1995; Cuffari *et al*, 1996 & O'Donovan *et al*, 2005). DNA 6-TG interacts with ultraviolet A (UVA) to generate Reactive oxygen species (ROS), which oxidize the 6-TG to guanine-6-sulphonate; (O'Donovan *et al*, 2005), a powerful block to DNA polymerases *in vitro* (O'Donovan *et al*, 2005; Zhang *et al*, 2006). Montaner *et al.,* (2007) indicated that, combined 6-TG–UVA also causes a new oxidative modification of the DNA replication and repair protein, proliferating cell nuclear antigen. In the same line, O'Donovan *et al.,* (2005) showed that incorporation of 6-TG into DNA of cultured cells and subsequent exposure to UVA generates ROS, with implied oxidatively generated modification of DNA. Moreover, it was reported that DNA damage seems to be the main mechanism for the cytotoxic effects of thiopurines (Karran and Attard, 2008).

In the present study there was a significant increase in the percentage of DNA fragmentation with Azathioprine in both doses 5 and 10 mg/kg b. wt. when compared to control group. As well as, Azathioprine increased DNA instability in the genome of mice liver using RAPD-PCR fingerprinting and the presence of changes in the RAPD profiles obtained from the exposed population depending on the primers used. Zhang *et al.,* (2007) reported that, aqueous solutions of both 6-TG and its corresponding 2-deoxyribonucleoside can auto-oxidize, following UVA irradiation and subsequent generation of O_2. There is precedent for an agent, which is normally thought to target DNA, to also exert a similar effect via modification of free 2-deoxyribonucleotides and incorporation into DNA and damaged it (Tsuzuki *et al.,* 2007). Combined, the data presented here, along with other reports emerging in the literature, strongly suggest that Azathioprine derived oxidative stress, and damage to DNA specifically, occurred with a dose of 10 mg/kg b. wt. of Azathioprine.

Fortunately, our results demonstrated that omega-3 oil pre-/post-treatment to Azathioprine showed high significance in reducing the percentage of DNA fragmentation compared to Azathioprine treated mice at both doses (5 and 10 mg/kg). These results were in consistence with those obtained by Shivani *et al.,* (2009) who reported that EFAs like α-linolenic acid and gama-linolenic acid can substantially reduce the number of DNA damage containing cells induced by Diphenylhydantoin (DPH) that commonly used for the treatment of epilepsy.

Our data demonstrated that, when the GSH was measured either after 24 hours from the last treatment or 35 days from the beginning of the treatments, the results revealed that, Azathioprine induced a significant ($P<0.05$) depletion in testis GSH content after 24 hr. either at therapeutic dose or 2× therapeutic dose when compared to that of the control. Glutathione depletion might be

happened due to free radicals and reactive oxygen species generation produced from the interaction of Azathioprine with DNA (Montaner *et al.*, 2007). Assesment of the protective effect of omega-3 pre-/post-treatment to Azathioprine at two doses 5 and 10 mg/kg during two different times (after 24 hr. from last treatment and 35 day from the beginning of the treatment) on GSH content in testis tissue of mice as a biochemical parameter was measured. From previous studies, GSH is a well known endogenous antioxidant (Meister and Anderson 1983; Deneke and Fanburg 1989 & Meister, 1992). There is a study on rotenone, which reduced GSH levels (Seyfried *et al.*, 2000 and Sherer *et al.*, 2002). GSH normally acts through a combination of various reduction and conjugation reactions to protect cells against both exogenous toxicants and the reaction of endogenous compounds (Krzywanski *et al.*, 2004). From data obtained in Table (6) there is a significant increase on GSH content in testis tissue of mice among omega-3 oil treated mice and control group. Moreover, our data illustrated that omega-3 oil pre-/post-treatment to Azathioprine significantly increased the testis GSH content when compared to that of the Azathioprine subjected groups, this reveals that omega-3 oil has a great protective effect in elevating GSH content in mice treated with Azathioprine. Supplementation with omega-3 fatty acids has beneficial effects on serum triglycerides, HDL-cholesterol, lipid peroxidation and antioxidant enzymes, which may lead to decreased rate of occurrence of vascular complications in diabetes (Kesavulu *et al.*, 2002). As well as Iraz *et al.*, (2005) reported that dietary supplementation with omega-3 fatty acids might possibly protect tissues from oxygen free radical injury in the various diseases in which the oxidant/antioxidant defense mechanisms are disturbed. Our data supported an idea suggested by Barbosa *et al.*, (2003) who reported that omega-3 EFA supplementation may have free radical scavenger activity. Administration of omega-3 EFA may stimulate vitamin E incorporation into membranes to avoid lipid peroxidation resulting from increased membrane omega-3 EFA content (Chautan *et al.*, 1990). Treatment with omega-3 EFA has been reported to decrease lipid peroxidation in the corpus striatum and to increase antioxidant enzyme activities in the hippocampus and corpus striatum of rats (Sarsilmaz *et al.*, 2003 a, b). In conclusion, our findings suggested possible protective and curative effects of omega-3 oil against the cytotoxicity and genotoxicity induced by anticancer drug, Azathioprine.

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