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Evaluation of the Protective Effects of Omega-3 Fatty Acids against Methotrexate Induced Testicular Toxicity in Male Albino Mice

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ABSTRACT

Antimetabolite drug Methotrexate is the mainstay for the treatment of many neoplastic disorders. However, it should be used with extreme caution because of its likeliness to cause testicular impairment due to the intracellular accumulation and subsequent enhancement of enzyme inhibition. Therefore the present study to evaluate the protective effects of Omega-3 fatty acids against methotrexate induced testicular toxicity in male albino mice. The male mice were divided in 5 groups each consisting of 6 mice. The total study is about 20 days. The entire group was treated with single dose of methotrexate inj 20mg/kg, i.p except group I. Group-III, IV & V treated with Omega -3 fatty acids at different doses of 125, 250 & 375mg/kg, p.o. The mating behaviours were monitored, including: mounting frequency (MF), intromission frequency (IF), mounting latency (ML), intromission latency (IL), ejaculatory latency (EL). Testosterone was estimated after blood collected from retro orbital venous plexus of all animals at the 10^{th} and 20^{th} day of the experiment. Increase in the sexual vigour of MF, IF, ML, IL & EL were observed in all dosed groups in a dose dependent manner. Hormonal analysis revealed that the levels of testosterone increased gradually in all the experimental groups. Histopathological results showed the lumen of the seminiferous tubule, with the presence of more spermatozoa compare to that of control animal. In conclusion, the methotrexate-induced testicular toxicity was effectively protected by Omega-3 fatty acids. There was almost complete recovery of the testes in the Omega-3 fatty acids included treatment group. This effect was found to be, the protective effects of Omega-3 fatty acids an action that may be due to its antioxidant properties.

Key words: Omega-3 fatty acids, Methotrexate, testicular toxicity, mating behaviours.

INTRODUCTION

Methotrexate is the mainstay for the treatment of many neoplastic disorders. It is an antimetabolite that acts by inhibiting the dihydrofolate reductase enzyme thus inhibiting the synthesis of DNA (Rosowsky *et al.,* 1986). Owing to the enormous therapeutic effects it is used as part of combination chemotherapy regimens to treat many kinds of cancers.

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However, it should be used with extreme caution because of its likeliness to cause testicular impairment due to the intracellular accumulation and subsequent enhancement of enzyme inhibition. Testicular toxicity is aggravated by the oxidative stress due to generation of free radicles. This has lead to depressed Spermatogenesis, testicular atrophy, premeiotic spermatocytes, hypogonadal levels of testosterone, virilizing effects on penis of male children, impaired production of sperm, atrophy of the seminiferous tubules, reduction in sperm count, and/or increased incidence of sperm with abnormal morphology or reduced motility, testicular degeneration and altered antioxidant activities.

Ingestion of fatty fish or fish oil containing antioxidants can be utilized for scavenging the free radicles and normalize the toxicity (Liangli yu 2001). Fish oil is rich in n-3 poly unsaturated fatty acids such as omega-3- fatty acids which includes eicosapentanoic acid (EPA), docosahexanoic acid (DHA) and Alpha linoleic acid.

Owing to the anti oxidant properties of omega-3 fatty acids, it normalized the weight reductions induced in prostate, seminal vesicles and testes, and protected against the decrease in sperm count, motility and viability as well as, the increase in sperm abnormalities. In addition omega-3 fatty acids also restored the antioxidant activities (reduced the malondialdehyde level, increased the reduced glutathione, superoxide dismutase and glutathione peroxidase levels) that were harmfully affected by methotrexate administration.

MATERIALS AND METHODS

Experimental design

Healthy male albino mice showing brisk sexual activity were selected for the study. The male mice were divided in 5 groups each consisting of 6 mice. The total study is about 20 days. The entire group was treated with single dose of methotrexate inj 20mg/kg, i.p except group I.

Group- I - Control Normal saline (10ml/kg/p.o)
Group-II - Negative Control methotrexate inj 20mg/kg, i.p
Group-III - Omega -3 fatty acids 125mg/kg, p.o
Group-IV - Omega -3 fatty acids 250mg/kg, p.o
Group-V - Omega -3 fatty acids 375mg/kg, p.o

Mounting behavior

Healthy male albino mice and Female mice showing non-oestrus cycle were used for mating behaviour analysis. Female mice with maximum receptivity with male mice were selected for the experiment. The tests for sexual desire were carried out on 10th and 20th day after treatment of Omega -3 fatty acids. All sexual behaviours studies were carried out between 13:00 and 16:00 at room temperature 26 ºC–28 ºC. Sexual behavior studies were monitored in a separate room for 2 h following the administration and were given 20 min adaptation periods, after which a female mice was placed in the same cage with the male in 1:1 ratio. The male and receptive female mice were introduced into the mating cages, with 1:1 ratio. The mating behaviours were monitored, including: number of mounts without Intromission until ejaculation or mounting frequency (MF), number of intromission from the time of introduction of the female until ejaculation or intromission frequency (IF), the time interval between the introduction of the female time to the first mount by the male or mounting latency (ML), the interval from the time of introduction of the female to the first intromission by the male or intromission latency (IL), time from the first intromission of a series up to the ejaculation or ejaculatory latency (EL). The values of the observed parameters for control, methotrexate and Omega -3- fatty acids treated groups were recorded (Ratnasooriya, 2000).

Hormonal analysis

The blood was collected from retro orbital venous plexus of all animals at the 10th and 20th day of the experiment. Testosterone was estimated after separation of serum by using Radio Immunoassay (RIA). The RIA was carried out in diagnostic Endocrinology and clinical Biochemistry service, No. 56/64, First Avenu, Indra Nagar, Adyar, Chennai-20.

Histological Study

For histological work, tissues were fixed in Bouin's fluid and processed for routine microtomy. 6µm thick paraffin sections were made stained with Cason's trichrome and Haematoxylin-Eosin procedures. From the well-stained sections of the testes and the epididymis of various groups, observations were made and photomicrographs taken. Well stained sections were mainly used for the cytometric measurements. The quantitative data were recorded properly for analysis.

Statistical analysis

The data were expressed as mean ± standard error mean (S.E.M). The Significance of differences among the groups was assessed using one way and multiple way analyses of variance (ANOVA). The test followed by Dunnet's test *P* values less than 0.05 were considered as significance.

RESULTS

Male sexual behavior

Increase in the sexual vigour of MF and IF table (1) were observed in all dosed groups (namely 125, 250 mg/kg and 375 mg/kg body weight) in a dose dependent manner that was statistically significant ($P < 0.05$) when compared with the negative control. By the 20th day of the experimental period in the highest dosed group (375mg/kg), both MF and IF had increased to 2 times of their respective control values. In contrast, the mount latency and intromission latency decreased significantly with the doses and as the experimental period increased ($P < 0.05$). There was also statistically significant prolongation of ejaculatory latency ($P < 0.05$) following the administration of various doses of Omega-3 fatty acids.

Hormonal analysis

Hormonal analysis revealed that the levels of testosterone increased gradually in all the experimental groups. Particularly on the 20th day, the levels of both hormones increased in a significant manner. However, the increase in testosterone (Groups III–V) was lower when compared to the Methotrexate treated group (Group II) was shown in fig 1.

Histopathological studies of testis

Control – Seminiferous tubule of the control mice testis showing different germ cell types with abundant spermatids and mature sperms.

Negative control – Negative control mice testis showing different germ cell types with abnormal spermatids and immature sperms.

Omega-3 fatty acids 125mg/kg – Testicular section of the male albino mice showing production of spermatogenesis after oral administration of the Omega 3 fatty acids 125mg/kg. Note the more number of mature spermatid.

Omega-3 fatty acids 250mg/kg – Testicular section of the male albino mice after oral administration of Omega-3 fatty acids 250mg/kg. The regressed seminiferous tubules show the presence of secondary spermatocyte, spermatid and spermatozoa. Note the presence of spermatids within the tubule.

Omega-3 fatty acids 375mg/kg - A necrotic structure of testicular section after oral administration of Omega-3 fatty acids 375mg/kg. Sertoli cells, spermatogonial cells and primary spermatocytes are present within the tubules. Note the lumen of the seminiferous tubule, with the presence of more spermatozoa compare to that of control animal.

Table 1. Protective Effect of Omega 3 fatty acids on mating behavior in methotrexate treated male mice

Group I (Control normal saline 10 ml/kg, p.o)			Group II (methotrexate 20mg/kg, i.p)		Group III (Omega 3 fatty acids 125mg/kg, p.o)		Group IV (Omega 3 fatty acids 250mg/kg, p.o)		Group V (Omega 3 fatty acids 375mg/kg, p.o)	
Mating behavior	10^{th} day	20^{th} day	10^{th} day	20^{th} day	10^{th} day	20^{th} day	10^{th} day	20^{th} day	10^{th} day	20^{th} day
ML	12.16 ±0.26	11.05±0.18	13.21±0.43	12.84±0.21	10.21±0.19	9.45±0.70**	4.27±0.23*	5.35±0.11*	2.35±0.40**	2.07±0.12**
IL	11.01±0.58	10.02±1.41	12.27±0.53	13.25±1.21	5.26±0.49	6.11±0.18*	2.85±0.19*	2.20±0.13*	0.78±0.13**	1.84±0.24**
EL	224±1.44	256±1.28	228±1.23	207±2.18	270±1.15	285±2.14	376±3.55*	1204±4.18**	374±2.25**	1514±2.78**
MF	68.01±2.70	67.05±3.06	70.30±1.44	63±2.14	88.40±1.44	139.32±1.18**	92.40±1.43*	153±1.26**	134±2.14**	168.52±1.24**
IF	73.05±2.05	71.67±2.43	78.01±2.15	65±2.15	96.50±3.01	133±1.48**	95.04±1.45*	163±2.17**	117.44±1.07**	174±2.29**

ML: mounting latency, **IL**: intromission latency, **EL**: ejaculation latency, **MF**: mounting frequency and **IF:** intromission frequency.
Statistical significant test for comparison was done by ANOVA, followed by Dunnet's test *p<0.05; ** p<0.01.
Mating behaviour scores of various groups in 10th, 20th days of experiment. Values are expressed as mean ± SEM of six observations. Comparison between: Group I &II Vs group III, IV&V.

Fig 1. Protective Effect of Omega -3- fatty acids on testosterone level in methotrexate treated male mice

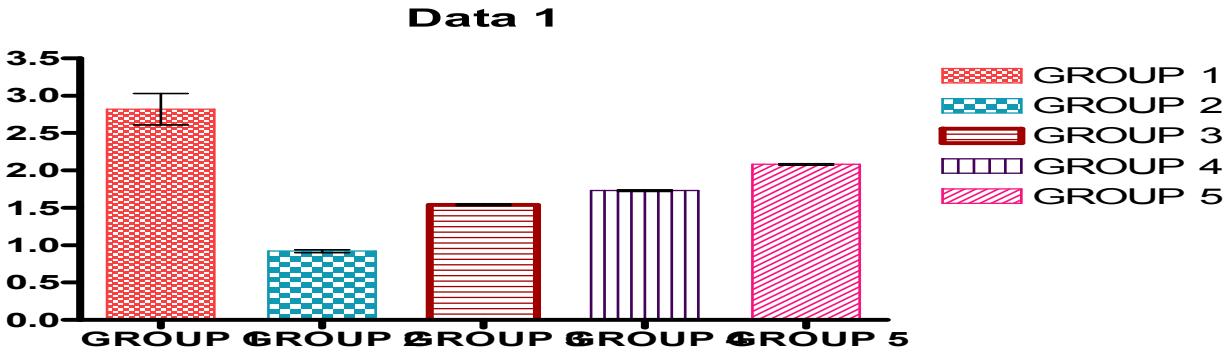
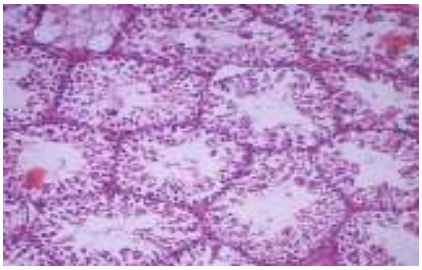
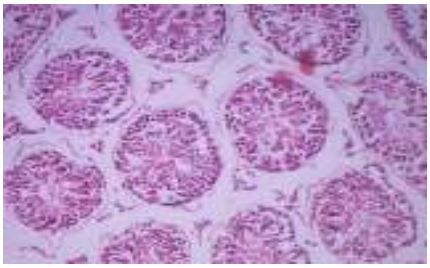


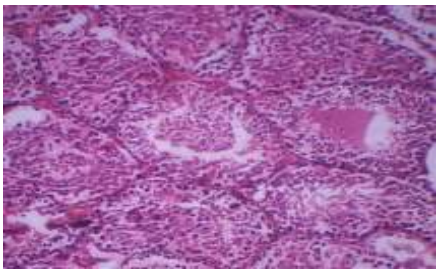
Fig 2:Histopathological studies of testis in male mice (10X)



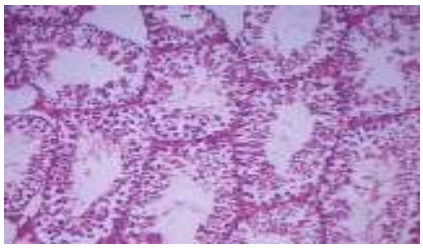
Group I
(Control-normal saline 10 ml/kg, p.o)



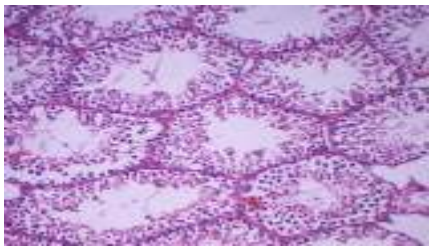
Group II
(methotrexate inj 20mg/kg, i.p)



Group III
(Omega-3 fatty acids 125mg/kg, p.o)



Group IV
(Omega-3 fatty acids 250mg/kg, p.o)



Group V
(Omega-3 fatty acids 375mg/kg, p.o)

DISCUSSION & CONCLUSION

The use of antioxidants as adjuncts for the treatment and/or amelioration of toxicities of chemotherapeutic regimes have been attracting focus in recent times. From our results, methotrexate treatment induces a decrease in plasma antioxidant levels which may be caused from the consumption of antioxidants due to the induced oxidative stress and is ameliorated by Omega-3 fatty acids administration. Omega-3 fatty acids have also been seen not to be deleterious on testicular epithelium on long term use.

Although methotrexate has been studied in a variety of animal models, toxicities observed upon methotrexate injection varied depending on the dose, duration of exposure and species of the animal. It has been reported in past publications that male mice exposed to methotrexate have also been implicated in causing similar adverse effects in the testes of several other animals, such as weight loss of testicles, seminal vesicle and prostate gland, and changes in the form of testes. In addition, it also implicates the fact that epididymis increases upon occurrence of sperm granuloma, reduction in fertility and occurrence of reproductive toxicity.

It is clear that methotrexate reaches the testes. In this study, testicular function in the single methotrexate treatment group was significantly lower than that of the vehicle control group. In addition, it was observed that serum testosterone had been significantly decreased by methotrexate in contrast to that of the vehicle control group. However, testosterone of the blood plasma was not affected in mice exposed to methotrexate.

The methotrexate is an anticancer drug that possesses testicular toxicities. This finding is in agreement with previous works *(*Lim J & Miller MG.*1997*). The use of Omega-3 fatty acids, in this study, as an antioxidant reference drug, has resulted in significant protection against methotrexate testiculartoxicities.

Omega-3 fatty acids treated group's mice, such increase in the frequency of mount and intromission suggests that libido, sexual vigour and sexual performance were unimpaired (Ratnasooriya *et al.,* 2000). The prolonged ejaculatory latency indicates enhancement of sexual function and suggests an aphrodisiac action. It has been documented previously that sexual behavior and erection

are dependent on an androgen that may be acting both centrally and peripherally. Testosterone supplementation has previously been shown to improve sexual function and libido, in addition to the intensity of orgasm and ejaculations which might also be expected to improve. The continued administration of the Omega-3 fatty acids at various doses which led to the significant increase in serum testosterone may be responsible for the marked effect on sexual behavior indices of the male mice.

On the other hand, a significant finding was that concomitant Omega-3 fatty acids administration has successfully restored the serum testosterone level reduction caused by methotrexate. It was reported that Omega-3 fatty acids reduces the lipid peroxide level caused by ferrous sulphate plus ascorbic acid, and improves the oxidative stress in vitro induced impairment in sperm motility and availability (Bansal AK & Bilaspuri GS, 2009).

More recently (Davitashvili DT *et al.,* 2009), it was found that addition of Omega-3 fatty acids into the nutrient medium of rat brain cortical cells could prevent the H_2O_2 oxidative stress induced cytotoxicity. Also, Omega-3 fatty acid was reported to preserve high aerobic capacity, and to reduce the high oxidative damage and susceptibility to oxidants elicited in the skeletal muscle as a response to cold stress (Venditti P *et al.,* 2009*)*.

Moreover, Omega-3 fatty acids was proven to have beneficial effect against cardiovascular diseases, lipid peroxidation, protein cross linking; DNA mutation and homeostasis are all due to its antioxidant activity (Prasad K., *2009)*]. Also, it was clearly indicated that the protective effect of Omega-3 fatty acids against methotrexate - induced testicular toxicity is due to its antioxidant effects *(*Bhatia AL *et al.,* 2006).

Histopathological and morphometric results from this experiment indicate a significant reduction in the tubular and epithelial areas in the testis of methotrexate treated mice in comparison to controls and Omega-3 fatty acids -treated rats. These results also mirror other testicular parameters that were measured and further alludes that methotrexate induced damage may be via Sertoli cell destruction since the nutritional and structural support of germ cells are maintained by Sertoli cells.

This observation is at variance with the histological observations Omega-3 fatty acids is expected to protect testicular tissues to the ravages of methotrexate-induced injuries due to its antioxidant properties.

In conclusion, the methotrexate-induced testicular toxicity was effectively protected by Omega-3 fatty acids. There was almost complete recovery of the testes in the Omega-3 fatty acids included treatment group. Therefore, Omega-3 fatty acids may be useful in the prevention and treatment of methotrexate-induced testicular damage. This effect was found to be, the protective effects of Omega-3 fatty acids an action that may be due to its antioxidant properties. The ability of Omega-3 fatty acids in protecting a wide range of endogenous endpoints presumably occurred through protective membrane stabilization. Further study on the toxico kinetics of methotrexate after feeding Omega-3 fatty acids is needed.

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