

Administration of Three Natural Products as Protective Agents Against The Genotoxic And Cytotoxic Effect of Methotrexate in Mice (*Mus Musculus*).

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Abstract

Background: Methotrexate is an antimetabolite and antifolate drug. It is used in treatment of cancer, autoimmune diseases.

Material and methods: 210 healthy adult male Swiss albino mice (*Mus musculus*), were allotted among three groups. The animal were given daily (**0.065mg/ml**) of methotrexate intraperitoneally followed by injection of Omega-3 plus at dose (**8.58 mg/20g mice**), Apple at dose (**1.43 mg/20 g mice**) and Psyllium at dose (**18.2mg /20 g mice**) for five days.

Results: Various Structural and numerical chromosomal aberrations in bone marrow cells; mitotic activity and sperm head abnormality were recorded, quantitated, and statistically analyzed. Also DNA extraction and apoptosis detection in liver was done. The intensity of apoptotic bands located at 200 b p ; 400b p ; 600 b p;800 b p and intact DNA measured by software Gel Pro program as maximum optical density values.

Conclusion:, Methotrexate had adverse effect on chromosomal and sperm head structure, also it induce apoptosis, necrosis and decrease total DNA in mice liver. Omega-3 plus, Apple, and Psyllium attenuate the methotrexate-related toxic effects.

Key words: **Methotrexate** – Omega-3 plus- Apple- Psyllium-chromosomal aberrations- sperm- DNA- liver.

Introduction

Methotrexate (MTX) is known as amethopterin. It is an antimetabolite and antifolate drug. Methotrexate (MTX), an antineoplastic drug, which is widely prescribed for the treatment of acute lymphoblastic leukaemia, non-Hodgkin's lymphoma, various types of sarcomas, carcinomas, breast and testicular cancers [1]. The drug was developed by Yellapragada Subbarao.

Patients with cancer take numerous alternative products to protect themselves from cancer. The

review article of Muriel [2] provides information about 47 herbs and natural products that have the potential to protect humans against cancer. The majority of these herbs and natural products are fruits, vegetables, animal or fish products, grains, and molecular components of plants or herbs that are found in human diets. Several grains such as barley, rice bran, and wheat bran protect against cancer. Various vegetables, fruits, and plants also show promise as protection against cancer: apple, asparagus,

blueberry, cabbage, cranberry, green tea, lavender tea, olive oil, peanut oil, and spinach [2].

Omega-3 Plus is composed of 1000gm Fish oil (EPA/ DHA 30%) and 100 gm wheat germ oil. Fish oil have scientific names of N-3 fatty acid, N-3polyunsaturated fatty acids and others. Salmon, Albacore, Tuna and Mackerel are just a few of the fish with high concentrations of omega three in their system. These fatty acids seem to decrease rates of prostate cancer [3] and prolong cancer remissions [4], also found that fish oils decrease production of lactic acid in tumor cells.

Wheat germ oil Obtained from the embryo or kernel of the wheat grain, wheat germ oil is a light yellow, fat soluble natural oil. The germ is the most nutritious portion of the wheat and it makes up about 2.5 % of the weight. Wheat germ oil also contains alpha- and gamma-tocotrienols [5,6]

Wheat germ oil Protects material in the cell nucleus and DNA from damaging free radicals. Studies have shown that vitamin E protects guanosine amino acid, which is a component of DNA, from damaged by hydroxyl and superoxide radicals. On the other hand, it destroys peroxynitrite, which is a substance similar to the nitrogen dioxide compounds present in cigarette smoke. Wheat germ oil not only prevents autoxidation of unsaturated fatty acids but also generates DNA protective properties [7].

Apple has a scientific name of *Malus sylvestris*. Individuals use apples for many conditions from cleaning their teeth to treating diarrhea, constipation, fever, and cancer. The antioxidant flavonoid quercetin in apples seems to have a protective property against lung cancer. Apple's antioxidant power is not simply due to its content of Vitamin C. In fact, the vitamin C content of apples contributes just 0.4% to their total antioxidant activity [8]. Fresh apples have been reported to suppress mammary carcinogenesis and proliferative activity and induce apoptosis (programmed cell death) in mammary tumours in rats [9].

Psyllium is a herbaceous low-growing annual plant native to India and Iran, and is also referred to as Ispaghul [10]. The seed husks of this plant, *Plantago ovata*, are commonly referred to as ispaghula husks [11] or psyllium [12]. Psyllium husk is a gel-forming, water-soluble fiber [13]. Psyllium is an effective blood cholesterol-lowering agent in human studies [14]. Adding high fiber foods (such as psyllium enriched cereals) to your diet may help lower heart disease risk. In fact, studies show that a diet high in water soluble fiber is associated with lower triglyceride levels, and a lower risk of cardiovascular disease [15]. Psyllium, may have a protective role for helping lower blood pressure, by adding fiber (12 g of soluble fiber per day) in diet [15].

The aim of the present work is to investigate the side effect of Methotrexate on chromosomes and sperms head morphology by cytogenetic

methods. Also, detection of its side effect on DNA in liver by method of molecular biology and the protective role of Omega -3Plus, Apple and Psyllium against Methotrexate effects.

MATERIAL AND METHODS

In this study 210 healthy adult male Swiss albino mice (*Mus musculus*), approximately three months old and weighting (± 20 g) were used in the present study. These mice were obtained from the National Research Center in Dukki, Cairo (N.R.C).The animals were kept in individual special rodents cages in the laboratory under constant condition of temperature ($25 \pm 3^\circ\text{C}$) with a reverses natural dark – light cycle 12 / 12 hrs. Animals were maintained on a standard rodent diet, obtained from Egyptian company of Oils and Soap Kafr-Elzayat Egypt, manufactured especially for laboratory purposes. The diet composed of 20% casein, 15% corn oil, 55% corn starch 5% salt mixture and 5% vitaminized starch. Water was available ad libitum.

Maintenance of animals and experimental procedures was approved by the animal ethical committee in accordance with the guide for care and use of laboratory animals of BenhaUniversity, Egypt. Methotrexate is available as Methotrexate Mylan 50mg/2ml, solution for injection was produced by Haupt pharma GmbH, France. Methotrexate was injected intraperitoneally at dose level 0.065mg/ml calculated according to Paget and Barnes [16] daily for 5 days.

Apple available as Apple-lite tablets, each tablet contains; 500 mg of apple fibers (apple

cuticle) and 50 mg of pure apple gel pectin (from apple pulp), produced by Arab Co. for pharmaceuticals& Medicinal plants (MEPACO-MEDIFOOD) Enshas El Raml-Shakeya, Egypt. The tablet was ground and dissolved in 100ml distilled water. It is used as a dose level of **(1.43 mg/20 g mice)** modified according to therapeutic dose of human [16] and each animal was injected intraperiotenally 0.26 ml daily for 5 days.

Omega-3-plus available as soft gelatin capsules, each capsule contains; 1000 mg Fish Oil and 100mg wheat germ oil.Omega-3-plus produced by South Egypt Drug industries Co.(SEDICO),6 October City, Egypt. It is used as a dose level of **(8.58 mg/20 g mice)** modified according to therapeutic dose of human [16] and each animal was injected intraperiotenally 0.1 ml daily for 5 days.

Psyllium available as Regumucil Powder, each 100 gm contains 49gm psyllium husk powder, produced by Kahira Pharm.&Chem.Ind.Co.for multipharma, Egypt. Each package contains 7 gm regumucil powder, dissolved in 250 ml distilled water. It is used as dose level **(18.2mg /20 g mice)** modified according to therapeutic dose of human [16] and each animal was injected intraperitonally 0.65 daily for 5 days.

The animals divided into three groups, group A, B and C; each group contains 70 male mice. Group A used to study chromosomal abnormalities. Group B used to study sperm head morphology by classical methods of

cytogenetic, while group C contain 60 male mice used to molecular studies. Each group divided into 12 sub group, 5 mice were allotted to each sub group.

Sub group (1): Control Group: - 5 mice were injected intraperitoneally with 0.9% sterile saline solution. **Sub group (2): Methotrexate Group:** - 5 mice were injected intraperitoneally with methotrexate at dose (0 .065mg/ml) daily for 5 days. **Sub group (3): Omega-3-plus Group:** - 5 mice were injected intraperitoneally with Omega-3-plus at dose (8.58 mg/20g mice) daily for 5 days. **Sub group (4): Apple Group:** - 5 mice were injected intraperitoneally with Apple-lite at dose (1.43 mg/20g mice) daily for 5 days. **Sub group (5): Psyllium Group:** - 5 mice were injected intraperitoneally with Regumucil at dose (18.2mg/20g mice) daily for 5 days. **Sub group (6): (MTX+Omega) Group:**- 5 mice were injected intraperitoneally with Methotrexate and omega-3-plus at dose ((0 .065mg/ml) + (8.58 mg/20g mice), respectively) daily for 5 days. **Sub group (7): (MTX+Apple) Group:** - 5 mice were injected intraperitoneally with Methotrexate and Apple at dose ((0 .065mg/ml) + (1.43 mg/20g mice), respectively) daily for 5 days. **Sub group (8): (MTX+Psyllium) Group:**-5 mice were injected intraperitoneally with Methotrexate and Psyllium at dose ((0 .065mg/ml) + (18.2mg/20g mice), respectively) daily for 5 days. **Sub group (9): (MTX after omega) Group:**- 5 mice were injected intraperitoneally with omega-3-plus

followed by Methotrexate at dose((8.58 mg/20g mice) then (0 .065mg/ml) , respectively) daily for 5 days. **Sub group (10): (MTX after Apple) Group:**-5 mice were injected intraperitoneally with Apple followed by Methotrexate at dose ((1.43 mg/20g mice) then (0 .065mg/ml), respectively) daily for 5 days. **Sub group (11): (MTX after Psyllium) Group:** - 5 mice were injected intraperitoneally with psyllium followed by Methotrexate at dose ((18.2mg/20g mice) then (0 .065mg/ml), respectively) daily for 5 days. **Sub group (12): (MTX before Omega) Group:** - 5 mice were injected intraperitoneally with Methotrexate followed by Omega -3-Plus at dose((0 .065mg/ml) then (8.58 mg/20g mice), respectively) daily for 5 days. **Sub group (13): (MTX before Apple) Group:**-5 mice were injected intraperitoneally with Methotrexate followed by Apple at dose ((0 .065mg/ml) then (1.43 mg/20g mice), respectively) daily for 5 days. **Sub group (14): (MTX before Psyllium) Group:**-5 mice were injected intraperitoneally with Methotrexate followed by Psyllium at dose ((0 .065mg/ml) then (18.2mg/20g mice), respectively) daily for 5 days.

Metaphase spreads were prepared according to Yosida and Amano [17]. Fifty well metaphase spreads were examined / each animal. The type and frequency of chromosomal aberrations were recorded and photographed. Mitotic activity of the cells was calculated as the number of dividing cells including prophase and metaphase

per 1000 cells. Cells with stickiness were considered as dividing cells.

Smears for sperm morphology were prepared and stained with eosin according to Mukherjee, et al [18]. One thousand sperms were counted for each animal, and the abnormal shape involving the head was recorded.

Statistical analysis was carried out using the student (t) test [19].

DNA extraction and apoptosis detection in tissues (liver) was done according to " salting out extraction [20] and modification [21]. DNA damage was detected in lysate tissue [22]. Gel was prepared using 1.8% electrophoretic grade agarose. All the gels of DNA were photographed with digital camera while the DNA were visualized at 312 nm UV light under a transilluminator.

RESULTS

various chromosomal aberrations were observed in the bone marrow cells of male mice treated with Methotrexate and protected with Omega-3 plus, Apple or psyllium. Both Structural and numerical types of aberrations and chromosomal stickiness were recorded.

The Structural aberrations included chromatid deletion (Fig.1a), fragmentation (Fig.1b), centromeric attenuation (Fig.1c), Centric fusion (Fig.1d), Ring (Fig.1e), end to end association (Fig.1f), break (Fig.1g) and gap (Fig.1h).The numerical aberrations included monosomy, trisomy and polyploidy(Fig.1i). Stickiness may give arise to sticky adhesions between two or

more chromosomes, and formation of sticky bridges at metaphase (Fig. 1j).

Table (1), fig. (2) showed the average of chromosomal abnormalities of bone marrow cells of mice treated with Methotrexate and protected with Omega-3plus, Apple and psyllium. It represented a very high significant increase in treated group with methotrexate (141.40 ± 17.76) than control group (7.40 ± 6.28), on the other hand it indicated that omega-3 plus made a high protective role against Methotrexate especially when injected with methotrexate (40.2 ± 9.97) or when injected after administration of methotrexate (27.8 ± 12.02). As well as, administration of Apple, and Psyllium with MTX showed a protective effect, when compared with animal groups treated with MTX only. The result indicated that pre-treatment with Apple and Psyllium is more protective than post-treatment. Marked decrease in mean values of structural chromosome aberrations in bone marrow cells (27.40 ± 11.97) and (36.8 ± 11.83) when injected with apple and psyllium respectively before methotrexate. Data represented a considerable increase in average of numerical aberration of chromosomes when animals treated with Methotrexate by (21.6 ± 4.30) in comparison with control. Omega -3-plus exhibited a noticeable reduction of the mean values of numerical aberration appeared in animal groups administrated with Omega-3- plus and its effect is the greatest especially before the anticancer drug administration. On the other side, the Apple and Psyllium revealed a sharp

decline in numerical aberration of chromosomes when treated with anticancer drug. Moreover, the Apple exhibited a great role to repair the aberration in mice bone marrow cells especially when administrated before methotrexate. [Table (2) fig. (3)].

There were a very highly significant reduction in the mitotic index in group injected with MTX in compare with control group. These results indicated that treatment with methotrexate reduced the mean values of mitotic index in compare with control group. While the three natural products (Apple, Omega-3-plus and Psyllium) repair a decrease in mitotic index caused by MTX.

Table (3), fig. (5) represented the incidence of abnormality in the shape of sperms per 1000 for each mice treated with methotrexate and protected with Omega-3-plus, Apple or psyllium, which include without hook shape (fig. 4a), banana shape (fig.4b) and amorphous shape (fig.4c).Methotrexate group showed a very high significance increase in the average of total abnormality compared with control group. The highest incidence of abnormality in the shape of sperms of mice treated with methotrexate was amorphous while without hook shape was the lowest frequency. On the other hand, the three natural products showing a protective effect when administrated with, after and before methotrexate. Omega-3-plus is the best protective natural product when administrated before anticancer drugs. Damage and optical density of DNA in mice treated with

Methotrexate and protected with Omega-3-plus, Apple and Psyllium were observed in liver (Fig. 6& 7 and table 4), it represents that: Damage in DNA of mice treated with methotrexate was observed when compared with control so the optical density of apoptotic bands of DNA at 200; 400; 600 and 800 bp showed a very significant increase than control. On the other hand, intact DNA decreased sharply than control. The obtained results showed that the natural products (Omega-3 plus, Apple, and Psyllium) reduce DNA damage induced by Methotrexate.

Table (5, 6, 7) showing the optical density of intact and apoptic fragments of DNA at 200, 400, 600, 800 bp in liver of mice treated with Methotrexate and the natural products together; Methotrexate after natural products and Methotrexate before natural products. It is clear that the intact DNA increase sharply in the liver of mice treated with Methotrexate and the natural products (Omega-3 plus; Apple and Psyllium) together than the mice treated with just methotrexate as demonstrated in figure (8 and 9). Besides, there is a protection action of natural products when treated after and before Methorexate as shown in figure (10, 11, 12, 13). There is a great protection action of Apple and psyllium when mice injected with Methotrexate after Apple or Psyllium. On the other hand, Omega- 3 plus has the best protection action when mice treated with Methotrexate before Omega-3 plus.

Discussion

The present study aimed to investigate the side effects of Methotrexate as anticancer drug on the chromosomes; sperm head abnormalities and the DNA damage in male mice (*Mus Musculus*), and protection by Omega-3Plus, Apple and Psyllium.

In the present study, Methotrexate was target because it is used as an antitumor drug used for treatment of various kinds of cancer [23]. At the same time, using Methotrexate in cancer chemotherapy has various side effects, including vomiting, diarrhea, mucositis, and decrease of nutrient absorption [24]. The toxic effects are not considered to be a result of direct action on the gastrointestinal tract tissues but rather to be the consequence of an inhibition in dihydrofolate reductase synthesis [25]. It affects not only tumor cells but also rapidly dividing host cells such as crypt cells of the gastrointestinal mucosa. It is characterized histologically by villus atrophy or crypt loss [26]. Methotrexate is under class of Antimetabolites drugs that interfere with DNA and RNA growth by substituting for the normal building blocks of RNA and DNA. These agents damage cells during the S phase [27].

The use of plants for the prevention of diseases is an ancient practice. Recently, there is a direction to use plants, herbs, composition of fruits, vegetables and grains in medicinal manufactures for their medicinal and protection abilities against many illnesses especially in case of cancer patients. Lifestyle and especially diet

can also serve as important sources of antioxidants. In vitro studies demonstrated that certain micronutrients may reduce DNA oxidation [28] as well as mutagenicity as [29].

The aim of the present study using three different natural products as a try to minimize the side effects in patients undergoing chemotherapy. In the present study we used Omega -3 Plus, Apple and Psyllium as trials testing the effectiveness of antioxidants as cancer prevention agents.

The fatty acids of fish oil seem to decrease rates of prostate cancer [3]. The omega-3 fatty acids in fish oil seem to be able to expand blood vessels, and this brings blood pressure down [30, 31]. Wheat germ oil obtained from the embryo or kernel of the wheat grain. Wheat germ is a rich source of B complex vitamins, with wheat germ oil being the richest source of tocopherols [32, 33].

The second natural product that we used in the present study is Apple. Apples are a rich source of nutrient as well as non-nutrient components and contain high levels of polyphenols and other phytochemicals [34]. There is some evidence for effects of flavonoid intake on reduced risk of lung cancer in some populations [35].

Psyllium as kind of prebiotics was associated with a reduced incidence of colon cancer in various populations [36]. Prebiotics have been shown to deactivate genotoxic carcinogens. DNA damage had been prevented and

chemopreventive systems may be stimulated in vivo in colon tissues [37].

In the present study, the increases in chromosomal aberrations were observed in the male *Mus musculus* (significant and highly significant difference) after treatment with Methotrexate. These aberrations are structural (include deletion, fragmentation, centric fusion, chromatid break, centromeric attenuation, gape, end to end association and stickiness) and numerical (included monosomy, trisomy and polyploidy). Mitotic index also affected after treatment with methotrexate.

It is obvious from the present study that the statistical analysis of chromosomal fragmentation, deletion, break, gap and stickiness of chromosomes of bone marrow cells of mice treated with methotrexate showed a very highly significant increase in comparison with control. These results agreed with Mondello et al.[38] who analyzed the effect of Methotrexate on chromosome morphology in cultured lymphocytes, and a high percentage of aberrant mitoses was found that; chromosome aberrations, such as gaps and breaks on all the chromosomes, but are preferentially located on chromosome n.3 at band p14. When the cells were continuously exposed to the Methotrexate, the chromosome damage appeared to be particularly severe. Also Choudhury et al. [39] results agreed with our result as they investigated cytogenetic toxicity of methotrexate in mouse bone marrow. They studied chromosomal aberrations and mitotic

index at 24-h post-treatment and micronucleus (MN) test at 30-h post-treatment. The induction of statistically significant number of chromosomal aberrations, percentage of aberrant metaphases and highly significant number of MN per thousand polychromatic erythrocytes by all the doses of MTX indicated that it has highly clastogenic effect. Significantly increased of gap was observed in this study in treated animals with methotrexate in comparison with control and this was due to the local loss of both DNA and chromosomal basic protein, this loss occur on a chromatid in the locus and doesn't represent real discontinuities in the chromosome [40]. It was occurred as a result of primary lesions which disrepair to give aberrations [41].

In the present study the numerical aberrations showed a significant difference in group treated with methotrexate in comparison with control. Our data represented considerable increase in monosomy, trisomy and polyploidy of chromosomes in groups injected with methotrexate. Aneuploidy in general arise as a non disjunction of homologous chromosomes at meiosis, or by non disjunction of sister chromatids at mitosis. The failure of disjoin or separate accurately can occur at any nuclear division in which the event occurs at the time occurrence. Non disjunction at meiosis gives rise to gametes with one more or less chromosome than usual. If such gametes are viable and fuse to produce a zygote, the zygote will be trisomic or monosomic for non disjunction chromosomes [42].

The value of chromosome stickiness increased in bone marrow cells of mice treated with methotrexate than control. Stickiness is due to the process of depolymerization of DNA, thus making the chromosome surface becoming sticky. Stickiness has been regarded later as physiological and unspecific disturbance attributed to the action of proteins on chromosome or form improper folding of the chromosome fibers into chromatids and thus chromosome become attached to each other by means of sub chromatid bridges [43].

The mean values of mitotic index decreased in mice after treated with methotrexate. Mitotic index is a measure for the proliferation status of a cell population. The apparent lower values for mitotic index reported in the present study may be due to decrease in cell number arrested in metaphase [44].

The results of the present study showed that Methotrexate induced genotoxic effects including DNA damage (apoptosis and necrosis) in the treated mice. Our result agreed with Martin et al. [45] who observed that Methotrexate induces oxidative DNA damage and is selectively lethal to tumour cells with defects in the DNA mismatch repair gene MSH2. Genotoxic mechanism of Methotrexate was explained by Frouin et al. [46] who explained that Methotrexate exert their antiproliferative activity through the inhibition of dihydrofolate reductase and, as a consequence, of thymidylate synthesis, thereby inducing nucleotide misincorporation and

impairment of DNA synthesis. As well as investigated a clear signs of apoptosis such as nuclear shrinkage, chromatin condensation and degradation. Remarkably, cell viability was lower and the apoptotic process was triggered more efficiently in the cells [46].

According to Ddhania et al. [47] who investigated that MTX-induced intestinal toxicity was evaluated at the dose of 2.5 mg/kg for short-term (5 days treatment) and 1 mg/kg for long-term (5 days in a week for four consecutive weeks treatment) study. They added that MTX treatment induced diarrhoea and mortality in rats, which indicating its severe toxicity in the target organ of investigation, i.e., intestine. Further, the intestinal toxicity of MTX was assessed by evaluating different parameters of oxidative stress, DNA damage, cytotoxicity as well as histological changes. Apoptosis is generally considered as an energy-dependent process that requires active participation of many proteins and other cellular macromolecules. Apoptosis seems to be induced by mild genotoxic stimuli; the strength of stimuli increases the cell death mode shifting it towards necrosis. This seems to be due to the fact that most the intense genotoxic stimuli damage the proteins or genes that make these proteins and other cellular macromolecules which may be required for apoptosis [48]. Apoptosis is mediated by members of the caspase family of proteases, and eventually causes the degradation of chromosomal DNA.

In the present study Omega-3 Plus acts as highly protective agent so aberrations decreased in all protected groups. As well as the result showed the best and very highly significant protective effect of Omega-3- Plus before Methotrexate drug administration than after administration of both anticancer drugs.

Hong et al. [49] who observed the protective effect of fish oil against oxidatively induced colon cancer. Whereas their study confirmed that fish oil protects against colon cancer by decreasing oxidative DNA damage at the initiation stage of colon tumorigenesis, oxidative DNA damage, proliferation, and apoptosis. On the other hand, Manna et al. [50] reported that Fish oil regulates cell proliferation, protect DNA damages and decrease HER-2/neu and c-Myc protein expression in rat mammary carcinogenesis.

Our Data indicated that Apple with MTX showing a protective effect, when compared with animal groups treated with just MTX only. As well as, the results indicated that the protective effect of Apple after MTX is more useful than before.

These result agreed with Liu et al. [51] who indicated that, Apples are one of the very few individual foods specifically identified in population studies as having the capacity to reduce cancer risk and more specifically lung cancer. Besides, Adhami et al.[52], stated that Fisetin which is commonly found in many fruits and vegetables such as apples, is more effective

against melanoma and cancers of the prostate, pancreas and the lungs.

There is a significant decrease in the chromosome aberration and DNA damage due to protective effect of Psyllium in our data. Moreover, the protective effect of psyllium was the best when Psyllium was given after MTX and is more useful than pre-treatment.

These result agreed with Segal et al. [36] who explained that Psyllium as kind of prebiotics was associated with a reduced incidence of colon cancer in various populations . As well as Cui et al. [53] indicated that whole-grain intake is protective against cancer, cardiovascular disease, diabetes and obesity.

Three types of sperm head abnormalities were recorded. Amorphous, banana like and without hook shape. Amorphous and banana like were the highest incidence of aberration in groups treated with Methorexate , while banana shape was the lowest frequency . On the other hand, the three natural products which used in the experiment (Omega -3 plus, Apple and Psyllium) showing a protective effect in the mean values of the sperm head abnormalities of animals *Mus musculus*. These result agreed with Choudhury et al. [54] who investigated that cytogenetic toxic effects of methotrexate, a widely prescribed antineoplastic drug, in the male germline cells of Swiss mice, was highly clastogenic to the spermatogonial cells at 24-h post-treatment after a single intraperitoneal exposure.

Methotrexate-induced cytotoxicity and genotoxicity in germ cells of mice indicated by Padmanabhan et al. [55]. As they observed the cytotoxic effect of MTX as sperm head abnormalities, seminiferous tubule damage, sperm DNA damage. As well as, they observed that MTX treatment showed a significantly reduction in the sperm count and increased the occurrence of sperm head abnormalities in a dose dependent manner. It induced the testicular toxicity as evident from the histology of testis. As well as Morris et al. [56] observed the significance of impaired spermatogenesis in a young man undergoing methotrexate treatment for severe psoriasis with associated arthritis.

In an attempt to explain the different mechanisms involved in the induction of the abnormal morphology of the sperm heads, Kaczmarek [57] stated that incomplete condensation of chromatin and the presence of large vacuoles and canals containing remnants of cytoplasm in various regions of the head is the cause of failure of sperm to pass through the final steps of maturation occurring normally during spermatogenesis. Moreover, Topham [58] mentioned the agents which accumulate in the testis can cause alterations in testicular DNA and disrupt the process of differentiation of spermatozoa directly.

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Table (1): Average of structural abnormalities observed in bone marrow cells of mice treated with MTX and protected with Omega, Ap or Ps.

	Groups	Mean ± SD															
		Deletion		Fragmentation		Centromeric attenuation		Centric Fusion		Chromosomal Ring		End to end association		Breaks		Gaps	
1	Control	2.2 ± 1.48	2 ± 1.22	0.8 ± 0.84	0 ± 0.55	1 ± 0.55	0.4 ± 0.55	1 ± 0.55	0 ± 0.55	7.40 ± 6.28							
2	MTX	23 ± 3.36 ***	49 ± 2.48 ***	8.8 ± 2.39 **	9 ± 2.35 **	6 ± 1.52 **	18.6 ± 1.95 ***	8 ± 1.3 ***	19 ± 2.41 ***	141.40 ± 17.76							
3	Omega	1 ± 1.00	3 ± 0.89	0.8 ± 0.84	0 ± 0.55	0 ± 0.55	0.6 ± 0.55	1 ± 0.55	0 ± 0.55	7.60 ± 5.47							
4	Apple	1.6 ± 0.55	3 ± 1.92	1.8 ± 0.45	1 ± 0.00	1 ± 0.84	1.8 ± 0.84	1 ± 0.84	1 ± 0.55	11.60 ± 5.98							
5	Psyllium	2.4 ± 0.55	4 ± 2.59	2.6 ± 0.55	1 ± 0.00	1 ± 0.84	2.8 ± 0.84	1 ± 0.71	1 ± 1.10	16.00 ± 7.16							
6	MTX +Omega	8.4 ± 1.14 **	10 ± 1.58 ***	6 ± 0.71 ***	4 ± 0.84 **	3 ± 1.1 **	2.2 ± 1.79	2 ± 1.52	4 ± 1.30 **	40.20 ± 9.97							
7	MTX+Apple	9 ± 1.22 **	8 ± 1.14 **	6.2 ± 0.84 ***	4 ± 1 **	4 ± 2.3 *	3.8 ± 0.84 **	3 ± 0.89 **	3 ± 1.30 *	41.20 ± 9.54							
8	MTX+Psyllium	8.6 ± 2.19 **	11 ± 1.14 ***	4.2 ± 0.84 **	4 ± 1.3 **	4 ± 0.84 **	3.8 ± 0.84 **	5 ± 1.3 **	4 ± 0.55 ***	44.40 ± 9.00							
9	MTX after Omega	18 ± 2.7 ***	38 ± 2.92 ***	5 ± 1.58 **	5 ± 1.14 **	5 ± 1.14 **	9 ± 1.58 ***	5 ± 0.84 **	8 ± 1.92 ***	93.40 ± 13.82							
10	MTX after Apple	7.6 ± 1.14 **	8 ± 1.30 **	2.6 ± 1.82	2 ± 1.92	2 ± 1.30	1.6 ± 1.14	1 ± 1.67 ***	2 ± 1.67	27.40 ± 11.97							
11	MTX after Psyllium	9.8 ± 1.48 **	10 ± 2.07 **	5.2 ± 1.79 **	4 ± 1.14 **	2 ± 1.22	1.8 ± 1.48	2 ± 1.22	2 ± 1.41 *	36.80 ± 11.83							
12	MTX before Omega	7.4 ± 1.14 **	7 ± 1.58 **	1.8 ± 1.79	3 ± 1.14 *	2 ± 2.07 *	3.4 ± 1.82 *	2 ± 1.48	1 ± 1.00	27.80 ± 12.02							
13	MTX before Apple	19 ± 2.3 ***	40 ± 3.11 ***	4.2 ± 1.92 *	4 ± 1.58 **	4 ± 1.58	11.8 ± 2.39 ***	4 ± 1.58	4 ± 3.16	91.60 ± 17.63							
14	MTX before Psyllium	18 ± 3.49 ***	40 ± 2.59 ***	3.8 ± 1.48 *	3 ± 1.14 **	4 ± 0.84 *	11.8 ± 2.59 ***	4 ± 2.49	4 ± 1.14	89.40 ± 15.76							

* Significant (p<=0.05), ** Highly Significant (p<=0.01), *** Very Highly Significant (p<=0.001)

MTX = Methotrexate, Omega = Omega-3-Plus, Ps = Psyllium, Ap = Apple

Table (2): Average of numerical chromosomal abnormalities and mitotic index observed in bone marrow cells of mice treated with MTX and protected with Omega, Ap or Ps.

	Groups	Mean \pm SD												Mitotic Index		
		Monosomy			Trisomy			Polyoidy			Total					
1	Control	0	\pm	0.00	0.2	\pm	0.45	0	\pm	0.00	0.20	\pm	0.45	570	\pm	23.72
2	MTX	5.6	\pm	0.89***	6.2	\pm	1.92**	9.8	\pm	1.48***	21.60	\pm	4.30	221	\pm	5.87
3	Omega	0	\pm	0.00	0	\pm	0.00	0	\pm	0.00	0.00	\pm	0.00	567.4	\pm	24.06
4	Apple	0	\pm	0.00	0	\pm	0.00	0	\pm	0.00	0.00	\pm	0.00	588.8	\pm	25.84
5	Psyllium	0	\pm	0.00	0	\pm	0.00	0	\pm	0.00	0.00	\pm	0.00	599	\pm	31.88
6	MTX +Omega	2.8	\pm	0.84**	1.8	\pm	1.1*	4.6	\pm	1.67**	9.20	\pm	3.61	579.2	\pm	6.80
7	MTX+Apple	4.2	\pm	1.30**	1.2	\pm	1.30	5	\pm	1.58**	10.40	\pm	4.19	562.4	\pm	3.97
8	MTX+Psyllium	2.6	\pm	1.5*	2	\pm	1.22	7.2	\pm	0.84***	11.80	\pm	3.58	553.4	\pm	4.39
9	MTX after Omega	3.8	\pm	0.84***	4.6	\pm	1.14	8	\pm	1.58***	16.40	\pm	3.56	250.2	\pm	2.39
10	MTX after Apple	1.4	\pm	0.89*	1	\pm	1.00	4.4	\pm	1.14***	6.80	\pm	3.03	406	\pm	8.63
11	MTX after Psyllium	1.4	\pm	1.14*	1.2	\pm	1.30	6.2	\pm	1.92**	8.80	\pm	4.37	350.2	\pm	3.70
12	MTX before Omega	0.8	\pm	0.84	0.8	\pm	0.84	4	\pm	1***	5.60	\pm	2.67	550.2	\pm	6.02
13	MTX before Apple	2.6	\pm	1.52*	3.2	\pm	1.48*	5	\pm	1.58**	10.80	\pm	4.58	220.8	\pm	3.42
14	MTX before Psyllium	3	\pm	2.23*	3	\pm	1.58*	4.8	\pm	0.84*	10.80	\pm	4.65	222.6	\pm	3.36

* Significant ($p \leq 0.05$),** Highly Significant ($p \leq 0.01$),*** Very Highly Significant ($p \leq 0.001$)

MTX = Methotrexate,

Omega = Omega-3-Plus,

Ps = Psyllium,

Ap = Apple

Table (3): Average of sperm head abnormalities observed in bone marrow cells of mice treated with MTX and protected with Ap, Omega or Ps.

	Groups	Sperm head abnormalities											
		Without hook			Banana			Amorphous			Total		
1	Control	4.4	±	0.55	3	±	1.00	3.6	±	2.07***	11	±	1.55
2	MTX	17	±	2.65***	26.6	±	3.21***	86	±	7.91	129.6	±	7.91
3	Omega	3	±	1.00	2.8	±	1.48	2.8	±	1.30	8.6	±	3.79
4	Apple	3	±	1.87	2.8	±	1.10	4.2	±	0.45	10	±	3.41
5	Psyllium	4.2	±	1.64	4.2	±	1.10	3.6	±	0.89***	12	±	2.74
6	MTX +Omega	3.4	±	1.14	7	±	1.58**	47.8	±	3.03**	58.2	±	1.14
7	MTX+Apple	2.2	±	0.84**	7.8	±	1.92**	58	±	1.58**	68	±	0.00
8	MTX+Psyllium	6.2	±	1.64	7.8	±	1.92**	58.2	±	2.38**	72.2	±	1.64
9	MTX after Omega	1.6	±	0.89**	3.6	±	0.89	42	±	2.12**	47.2	±	0.89
10	MTX after Apple	2.6	±	1.67	1.6	±	1.34	46.4	±	3.05***	50.6	±	3.01
11	MTX after Psyllium	5.4	±	1.14	3.8	±	0.84	54	±	3.08***	63.2	±	1.98
12	MTX before Omega	4	±	1.58	7.2	±	1.92*	50.4	±	2.70**	61.6	±	1.58
13	MTX before Apple	13.4	±	2.41**	23.6	±	2.70***	68.4	±	2.4**	105.4	±	0.00
14	MTX before Psyllium	13.8	±	1.30***	28.6	±	2.61***	71.2	±	4.81***	113.6	±	0.00

* Significant ($p<=0.05$),
MTX = Methotrexate,

** Highly Significant ($p<=0.01$),
Omega = Omega-3-Plus,

*** Very Highly Significant ($p<=0.001$)
Ap = Apple

Table (4) Optical density of intact and apoptotic fragments of DNA at 200, 400, 600 and 800 bp in liver of mice treated with Methotrexate and protected with Omega Ap or Ps.

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
	Control	MTX	Omega	Ap	Ps
Intact DNA	152.22	23.575	218.15	189.2	175.3
DNA at 800 bp	20.2	90.908	25.294	50.294	64.77
DNA at 600 bp	8.37	142.99	13.247	37.541	40.77
DNA at 400 bp	6.45	141.02	5.152	20.153	32.851
DNA at 200 bp	4.23	65.08	0.58824	6.988	12.184
Sum of apoptotic fragments	39.3	439.998	44.2	115	150.6

Table (5): Optical density of intact and apoptotic fragments of DNA at 200,400,600,800bp in liver of mice treated and protected together with MTX and with Omega, Ap or Ps.

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
	Control	MTX	MTX+Omega	MTX+Ap	MTX+PS
Intact DNA	152.22	23.575	134.86	132.02	114.96
DNA at 800 bp	20.2	90.908	78.011	111.79	90.96
DNA at 600 bp	8.37	142.99	47.241	79.188	120.46
DNA at 400 bp	6.45	141.02	33.126	55.129	98.99
DNA at 200 bp	4.23	65.08	18.563	44.812	30.388
Sum of apoptotic fragments	39.3	439.998	176.941	290.919	340.798

Table (6): Optical density of intact and apoptotic fragments of DNA at 200,400,600,800bp in liver of mice treated with MTX after Omega, Ap or Ps.

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
	Control	MTX	MTX after omega	MTX after Ap	MTX after Ps
Intact DNA	152.22	23.575	33.988	106.06	63.753
DNA at 800 bp	20.2	90.908	31.576	66.8	33
DNA at 600 bp	8.37	142.99	41.282	90.02	48.8
DNA at 400 bp	6.45	141.02	79.012	120.46	77.8
DNA at 200 bp	4.23	65.08	167.45	25.329	155.39
Sum of apoptotic fragments	39.3	439.998	319.32	302.609	314.99

Table (7): Optical density of intact and apoptotic fragments of DNA at 200,400,600,800bp in liver of mice treated with MTX before Omega, Ap or Ps.

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
	Control	MTX	MTX before omega	MTX before Ap	MTX before Ps
Intact DNA	152.22	23.575	103.04	23.575	21.034
DNA at 800 bp	20.2	90.908	45.282	90.908	51.724
DNA at 600 bp	8.37	142.99	65.541	92.99	116.08
DNA at 400 bp	6.45	141.02	90.25	91.02	116.01
DNA at 200 bp	4.23	65.08	109.19	45.08	28.931
Sum of apoptotic fragments	39.3	439.998	310.3	320	312.745

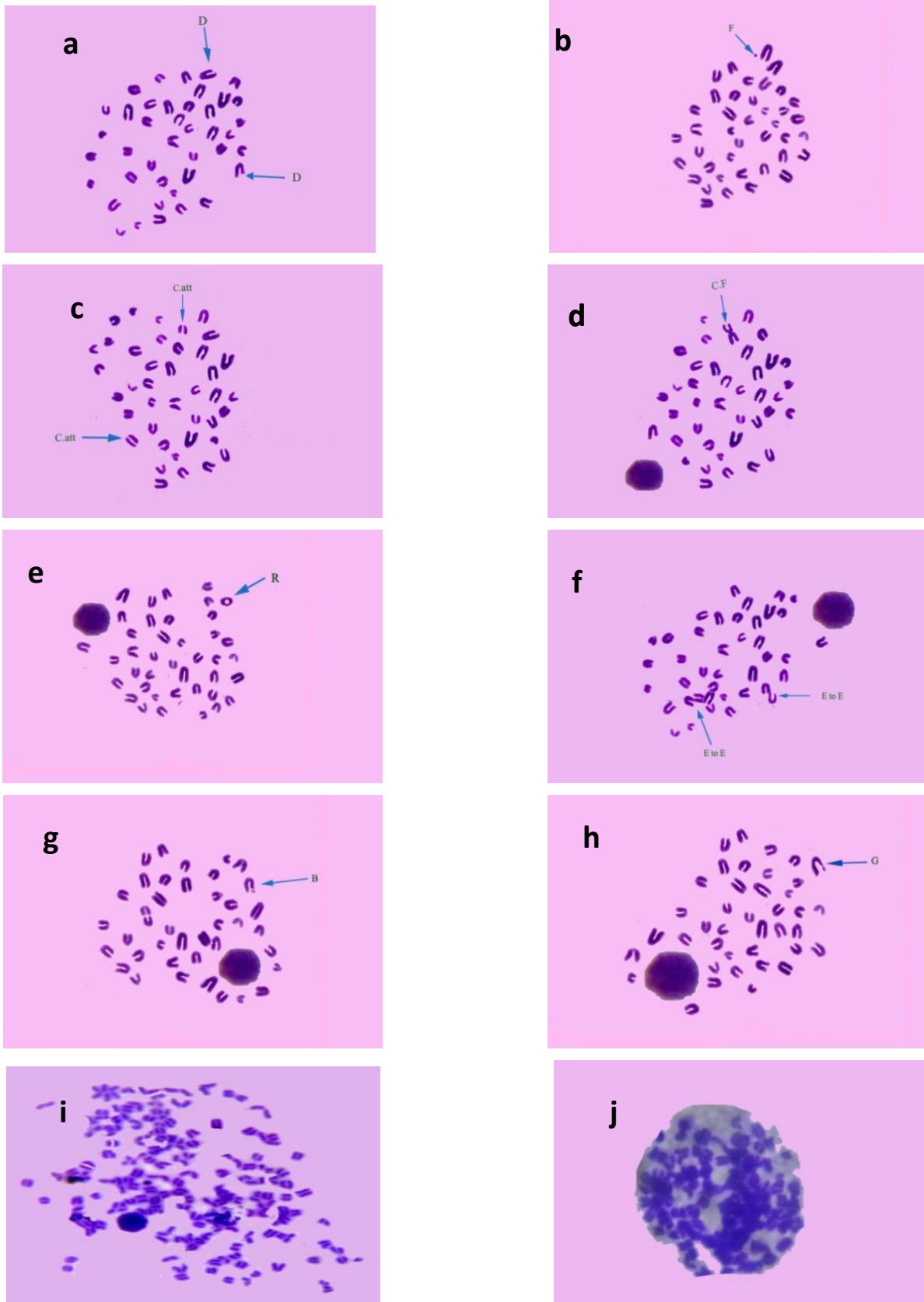


Figure (1): Types of chromosomal aberrations in rat bone marrow cells treated with Methotrexate. a: deletion (D); b: fragmentation (F); c: centromeric attenuation (C att); d: centric fusion (CF); e: chromosomal ring (R); f: end to end association (E to E); g: break (B); h: gap (G); i: Polyploidy and j: Sticky

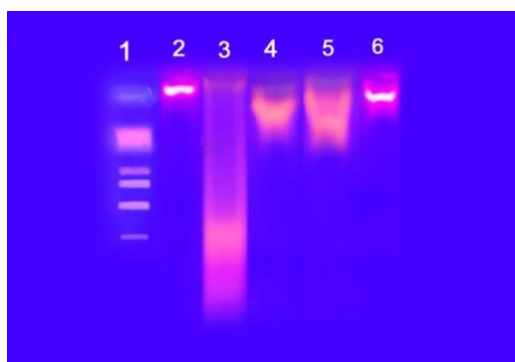


Figure (6): DNA damage in liver of mice treated with MTX and protected with Omega, Ap and Ps.lane1: DNA ladder; lane 2: Control; Lane 3: MTX; lane 4: Omega; lane 5: Ap; lane 6:Ps.

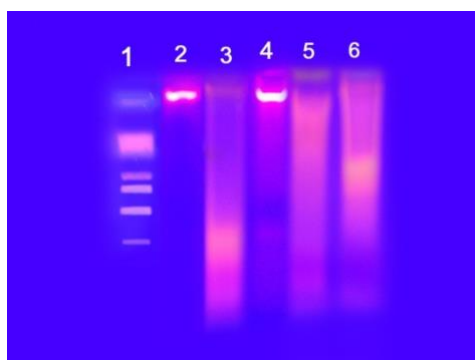


Figure (8): DNA damage in liver of mice treated and protected together with MTX and with Omega, Ap and Ps. lane1: DNA ladder; lane 2: Control; Lane 3: MTX; lane 4: MTX+Omega; lane 5: MTX+Ap; lane 6:MTX+Ps.

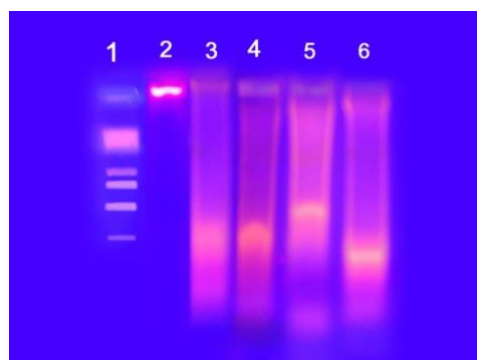


Figure (10): DNA damage in liver of mice treated with MTX after Omega, Ap and Ps. lane1: DNA ladder; lane 2: Control; Lane 3: MTX; lane 4: MTX after Omega; lane 5: MTX after Ap; lane 6:MTX after Ps.

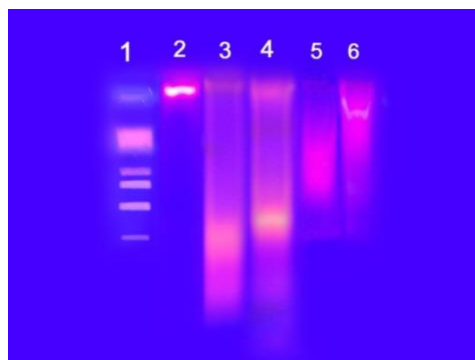


Figure (12): DNA damage in liver of mice treated with MTX before Omega, Ap and Ps. lane1: DNA ladder; lane 2: Control; Lane 3: MTX; lane 4: MTX before Omega; lane 5: MTX before Ap; lane 6:MTX before Ps.

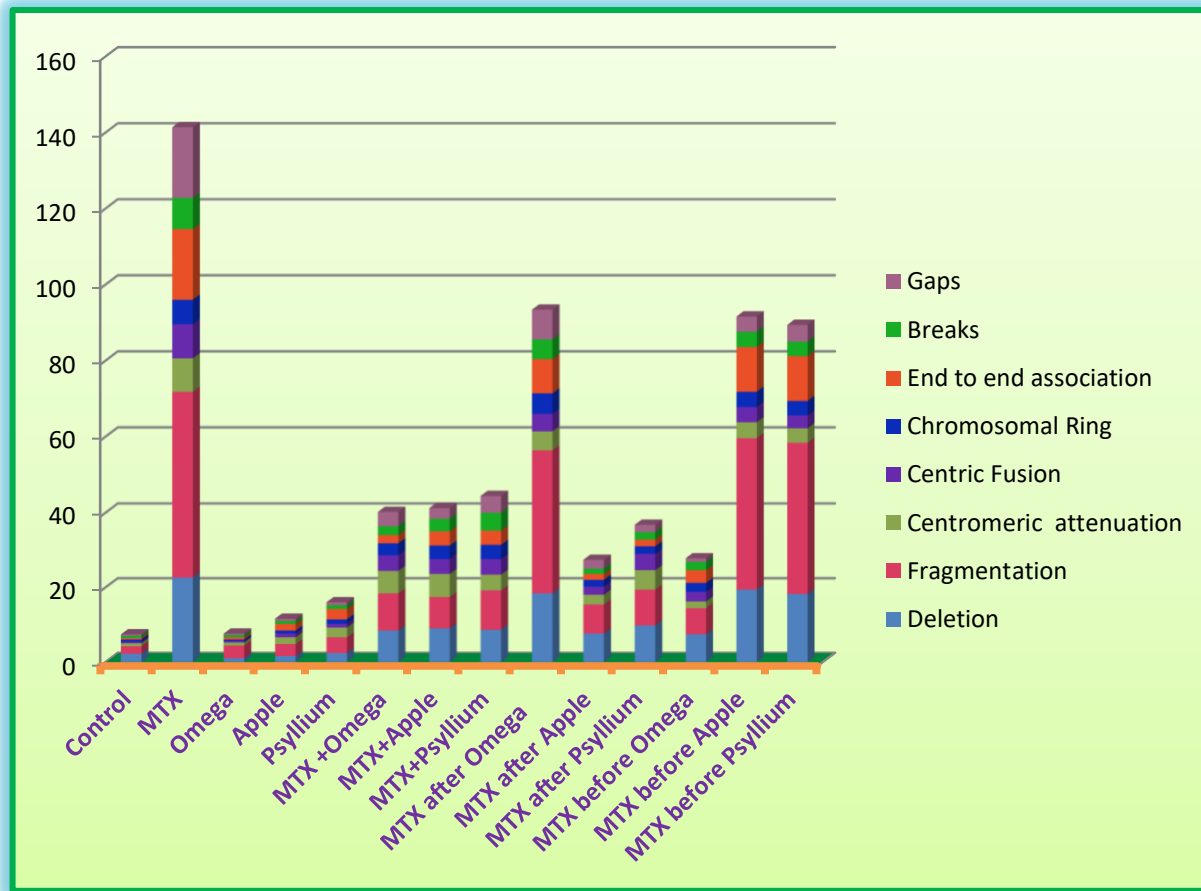
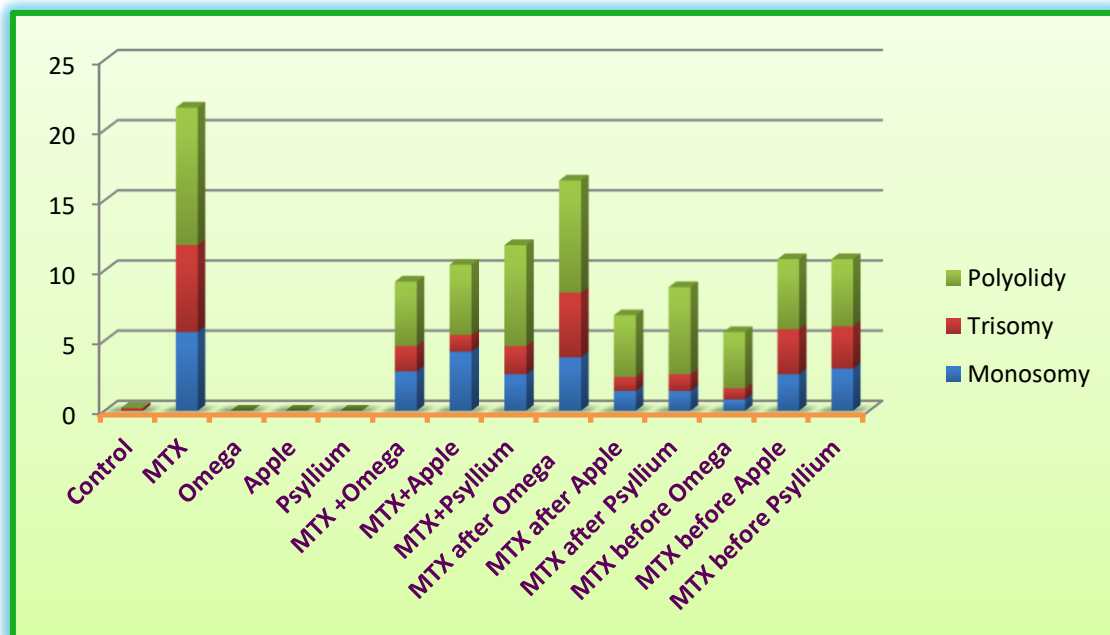


Figure (2): Average of structural abnormalities observed in bone marrow cells of mice treated with MTX and protected with Omega, Ap or Ps.



Figure(3): Average of numerical chromosomal abnormalities observed in bone marrow cells of mice treated with MTX and protected with Ap, Omega and Ps.

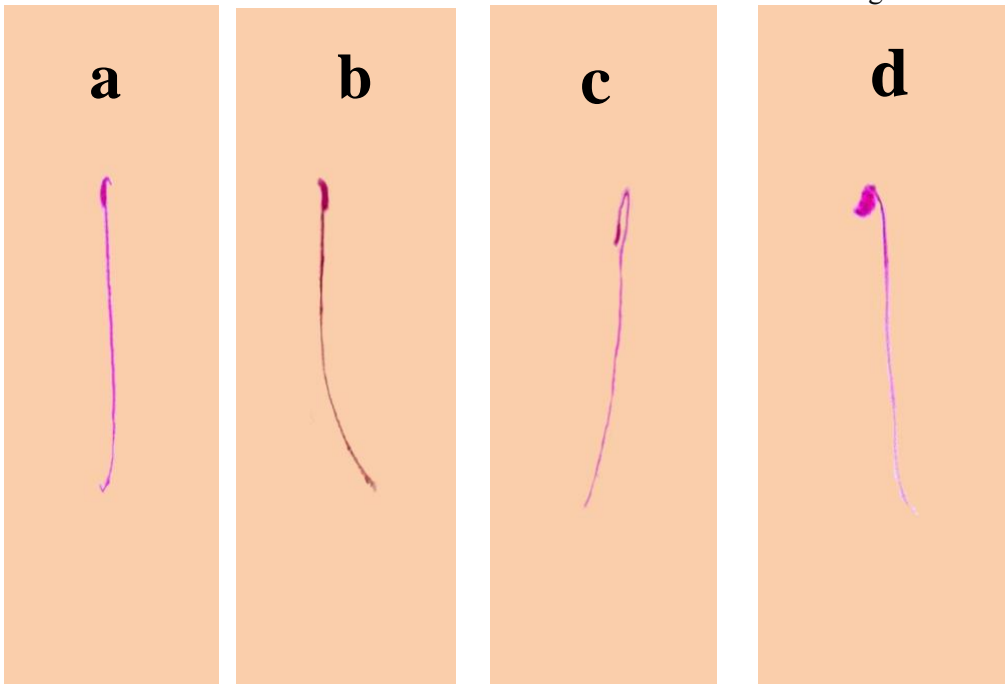


Figure (4): Average of sperm head abnormalities observed in bone marrow cells of mice treated with MTX and protected with Omega Ap or Ps. a: normal sperm; b: without hook; c: banana shape and d: amorphous.

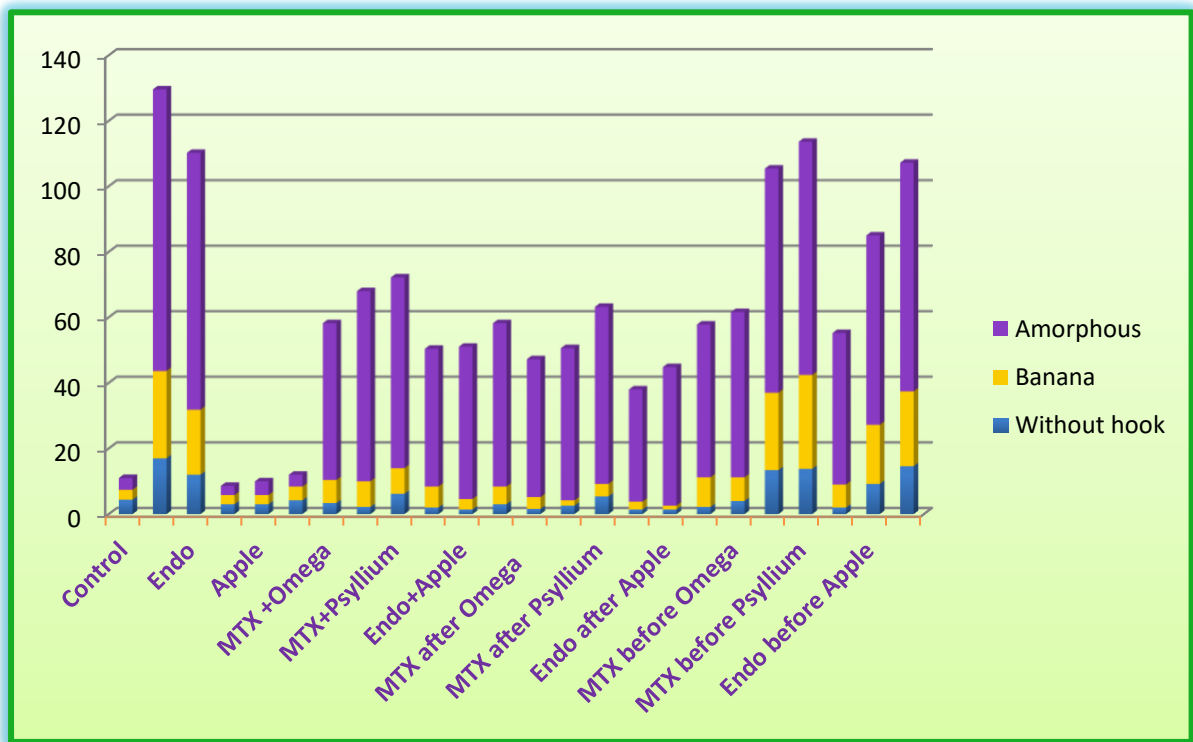
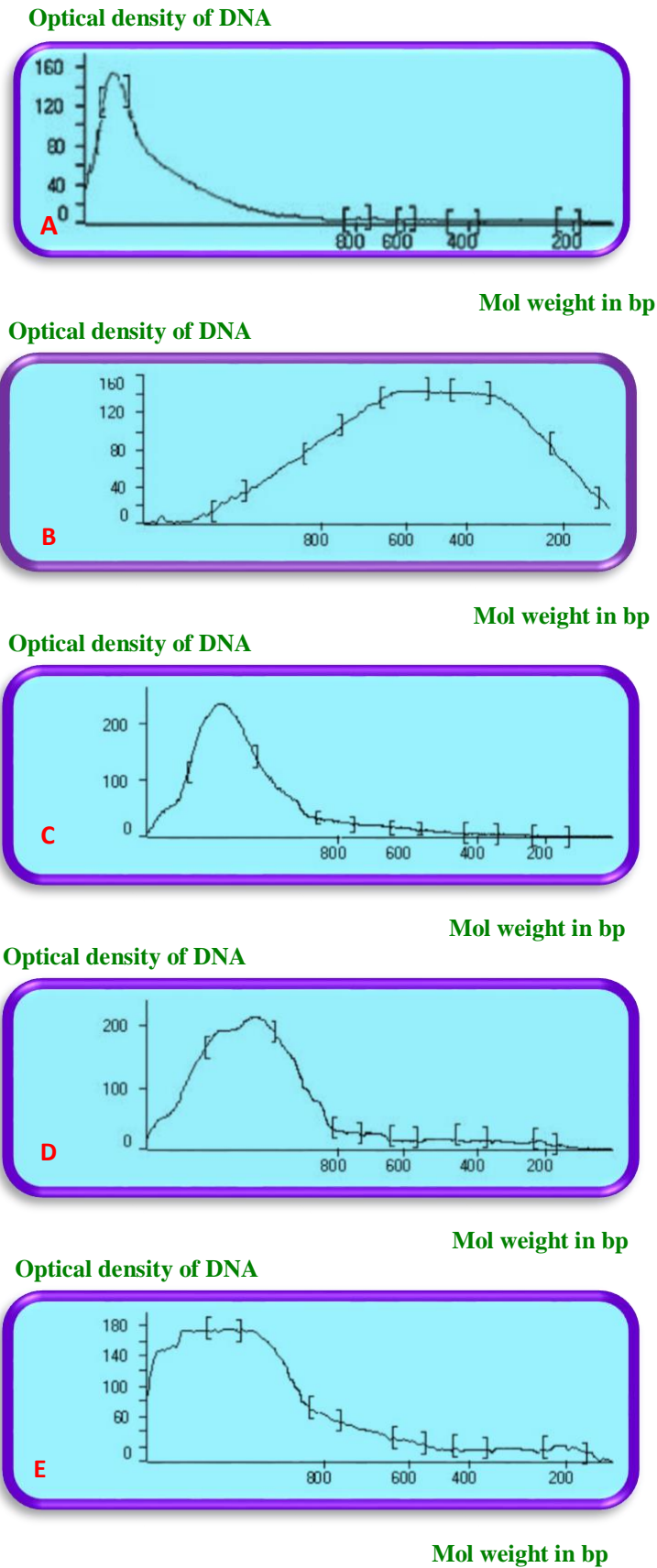
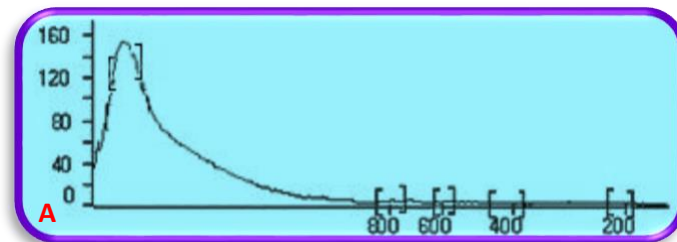


Figure (5): Average of sperm head abnormalities observed in bone marrow cells of mice treated with MTX and protected with Omega, Ap or Ps.



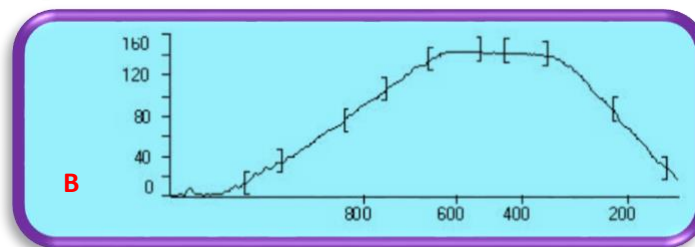
Figure(7): Histograms of optical density of intact and apoptic fragments of DNA at 200, 400, 600 and 800 bp in liver of mice treated with MTX and protected with Omega, Ap and Ps.(A): Control; (B): MTX; (C):Omega-3 Plus; (D): Apple; (E): Psyllium.

Optical density of DNA



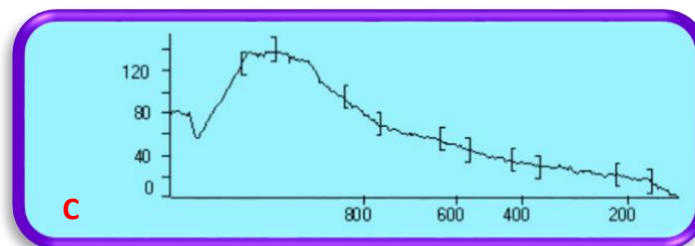
Mol weight in bp

Optical density of DNA



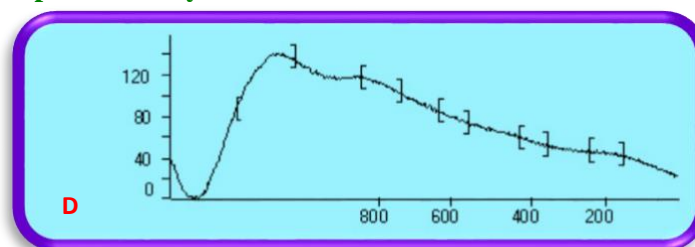
Mol weight in bp

Optical density of DNA



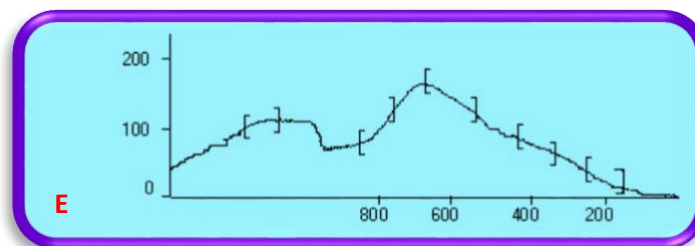
Mol weight in bp

Optical density of DNA



Mol weight in bp

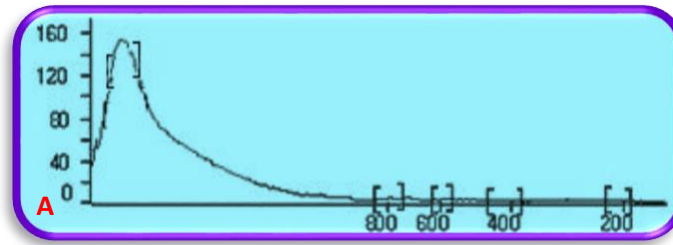
Optical density of DNA



Mol weight in bp

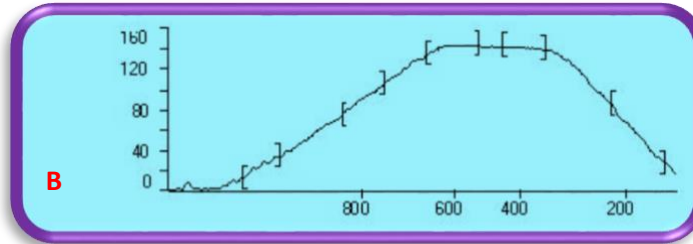
Figure(9): Histograms of optical density of intact and apoptic fragments of DNA at 200, 400, 600 and 800 bp in liver of mice treated and protected together with MTX and with Omega, Ap and Ps. (A): Control; (B): MTX; (C):MTX+Omega; (D):MTX+ Apple; (E):MTX+ Psyllium.

Optical density of DNA



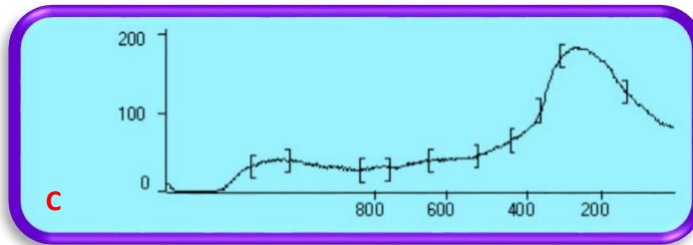
Mol weight in bp

Optical density of DNA



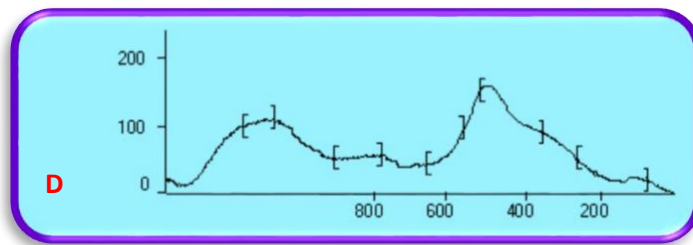
Mol weight in bp

Optical density of DNA



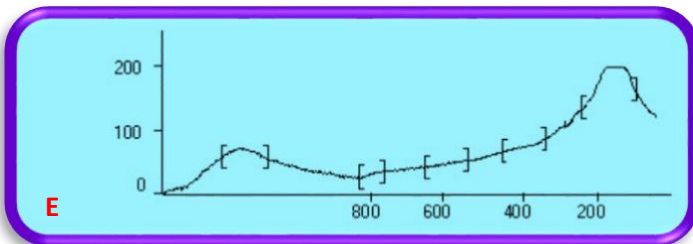
Mol weight in bp

Optical density of DNA



Mol weight in bp

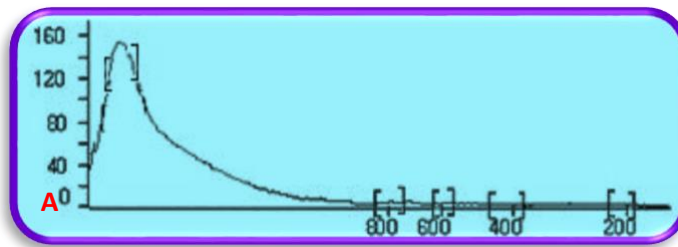
Optical density of DNA



Mol weight in bp

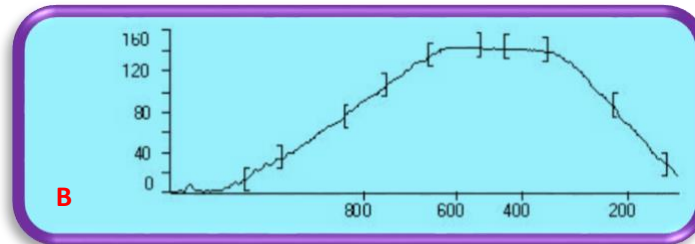
Figure(11): Histograms of optical density of intact and apoptic fragments of DNA at 200, 400, 600 and 800 bp in liver of mice treated with MTX after Omega, Ap and Ps. (A): Control; (B): MTX; (C):MTX afterOmega; (D):MTXafter Apple; (E):MTX after Psyllium.

Optical density of DNA



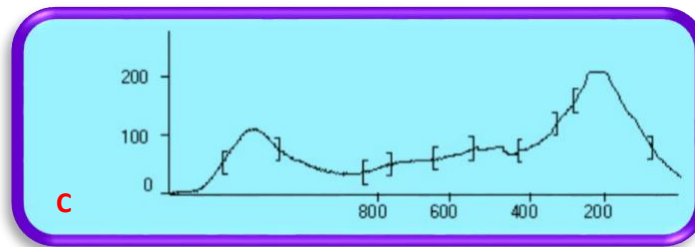
Mol weight in bp

Optical density of DNA



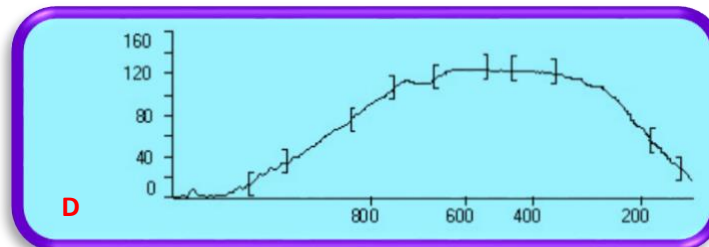
Mol weight in bp

Optical density of DNA



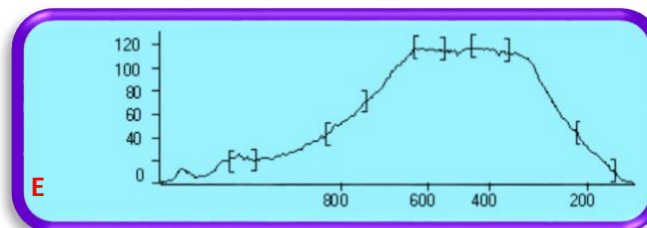
Mol weight in bp

Optical density of DNA



Mol weight in bp

Optical density of DNA



Mol weight in bp,

Figure(13): Histograms of optical density of intact and apoptotic fragments of DNA at 200, 400, 600 and 800 bp in liver of mice treated with MTX before Omega, Ap and Ps. (A): Control; (B): MTX; (C):MTX before Omega; (D):MTX before Apple; (E):MTX before Psyllium.