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Vitamin C & E Antagonistic Effect for Tamoxifen-Induced Rat Liver Damage: A Histopathological, Histochemical and Ultrastructural Studies.

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Abstract

Background: Tamoxifen is widely used to treat oestrogen dependent carcinoma of the breast. Previous long term studies have shown that oral administration of tamoxifen induces hepatoproliferative lesions and hepatocellular tumors in rats. This study was designed to evaluate the effects of tamoxifen on liver of rats and the possible protective effects of vitamin C and/ or vitamin E in amelioration of these effects.

Material and methods: A total of 70 adult female albino rats were used in this study. The animals were divided into seven groups. Each group contained 10 rats. The rats of the first group were kept as control. Animals of the second group were daily dosed orally with tamoxifen 20 mg/kg b. w. by stomach tube for two weeks. The third group was given vitamin C at dose level of 0.01 g/100 g b w by stomach tube, 15 min before daily administration of tamoxifen. The fourth group was given vitamin E at dose level of 100 mg/Kg b.w, 15 min prior to daily administration of tamoxifen through out the whole period. The fifth group was given combination of the two vitamins (vitamin C & vitamin E) at dose level of 0.01g/100 g b.w. and 100 mg/kg b.w. respectively, 15 min before daily administration of tamoxifen for two weeks. Each of the remaining two groups was daily given vitamin C (0.01 g/100 g b.w.) and/or vitamin E (100 mg/kg b.w.) only for two weeks. Paraffin sections were used for the histopathological study. For the histochemical investigations, sections were stained to demonstrate DNA, mucopolysaccharides and protein content.

Results: Histopathological effects of tamoxifen were demonstrated in liver as vacuolar degeneration, fatty changes and hydropic degeneration. Signs of degeneration in the form of karyolysis and karyorrhexis were also seen. Moderate dilatation of blood sinusoids, some displastic cells and chromatin clumping could be observed. Quantitative DNA image analysis (Lecia image) showed a decrease in DNA content (hypoploidy) in liver of rats treated with tamoxifen only. Tamoxifen induced histochemical changes consisted of marked diminution of protein and mucopolysaccharides content. No histopathological, histochemical and ultra structural changes could be noticed in rats treated with vitamin C and, or vitamin E only.

Conclusion: The treatment of rats with vitamin C and/or vitamin E prior to tamoxifen resulted in amelioration of the histopathological changes of liver as well as histochemical and ultrastructural changes.

Key words: Tamoxifen – liver damage – rat – Vitamin C – Vitamin E – ultrastructural changes.

Introduction

Breast cancer remains the most common malignancy in women world wide. Estrogen levels appear to be associated with an increased risk for the development of breast cancer (Lo. and Vogel, 2004). . In 1998 the National Surgical Adjuvant Breast and Bowel Project (NSABP) demonstrated that tamoxifen treatment reduced the incidence of both invasive and non -invasive breast cancer in population at high risk for disease (Tan- Chiu *et al.*, 2003).

Tamoxifen (TAM), a non steroidal antiestrogen is used as a chemotherapeutic and chemopreventive agent for breast cancer (Goss and Stresses-Weipple, 2004). Tamoxifen is a nonsteroidal triphenylethyl compound that belongs to a class of selective estrogen receptor modulators (SERMs), binds to estrogen receptors (ERs)

and elicits estrogen agonist or antagonistic responses, depending on the target tissue. Its estrogen antagonistic properties have made tamoxifen an important treatment modality for patients with breast cancer, especially those whose tumors are positive for ERs.

Dray *et al.*, (2000) reported a case of non-alcoholic steatohepatitis with cirrhosis in a woman receiving tamoxifen as adjuvant treatment for breast cancer. Tamoxifen (TAM) has been used as an agent for the treatment and prevention of breast cancer. However, long-term treatment of TAM in women increases the risk of the developing endometrial cancer. The secondary cancer may be due to the genotoxicity of TAM (Kim *et al.*, 2006). Tamoxifen-induced non-alcoholic steatohepatitis (NASH), may increase the demand on oncologists, not only with regard to screening for diabetes, but also for the suggested link of NASH with high incidence of coronary heart disease (Osman *et al.*, 2007). The incidence of toremifene-induced fatty liver was significantly lower than that induced by tamoxifen. Accordingly, in terms of fatty liver and NASH, toremifene is considered to be more appropriate agent than tamoxifen. (Hamada *et al.*, 2000).

TAM is liver carcinogenic in rats and has been associated with an increased risk of endometrial cancer in women (Curtis *et al.*, 2004). Furthermore TAM use has been associated with a 35% decrease in incidence of osteoporotic bone fractures (Decensi *et al.*, 1998). In mice, TAM produced proliferative lesions in the oviduct and uterus (Srinivas *et al.*, 2004) followed by uterine carcinoma (Newboid *et al.*, 1997).

TAM affects some types of visual pathway (Eisner *et al.*, 2004). Woman taking tamoxifen suffer from damage of retina and corneal opacities. These changes may have no immediate effect on visual acuity, but may predispose the eye to latter problems including cataracts (Epstein *et al.*, 1997). Tamoxifen induces menstrual irregularities in premenopasual woman. Amenorrhae (absence of menstrual cycle) often results and can be permanent (Sellman, 1998). Tamoxifen can induce multinucleated giant cells and germinal epithelial sloughing, seminiferous tubules distortion and these changes are detrimental to male fertility (Dsouza, 2003).

TAM and its metabolites, 4-hydroxytamoxifen (4OH-TAM), *N*-desmethyltamoxifen (DMT) and 4-OH-*N*-desmethyltamoxifen (endoxifen) exhibit antiestrogenic activities by competitively inhibiting the binding of potent agonists to the estrogen receptor (ER), thus antagonizing their proliferative effects (Johnson *et al.*, 2004). Despite the high therapeutic index of TAM for breast cancer, there are concerns regarding the increased occurence of uterine cancer as early as 2 years after initiating treatment (Fisher, 1994). TAM is classified as a selective estrogen receptor modulator (SERM) as a result of its differential effects in breast and uterine tissues. A number of factors influence the specificity and efficacy of SERM-bound, ER-mediated gene expression, and the subsequent physiological effects (Fong *et al.*, 2007).

TAM has demonstrated genotoxic activity in the rat liver causing DNA adducts (Divi *et al.*, 2001) unscheduled DNA synthesis, hepatic aneuploidy and mitotic spindle disruption (Phillips, 2001). For the formation of DNA adducts, metabolic activation of tamoxifen is indispensable; the metabolites α-hydroxytamoxifen (Beland *et al.*, 1999) and its *O*-sulfate (Shibutani *et a.l*, 1998) are characterized as proximate and ultimate carcinogens, respectively. On the other hand, major metabolites such as *N*-desmethyltamoxifen, tamoxifen *N*-oxide and 4-hydroxytamoxifen are generally characterized as detoxification forms, although the further metabolites, α- hydroxyl forms of the *N*-desmethyltamoxifen and tamoxifen *N*-oxide, are able to produce the DNA adducts (Umemoto *et al*, 2000). Long term administration of tamoxifen induced hepatoproliferative lesions and hepatocellular tumors in rats (Hirsimaki *et al.*, 1993). In the stage before the formation of hyperplastic nodules in the liver, the genes of several hepatic enzymes responsible for not only detoxification but also activation of tamoxifen were activated and that in the later stage (in the nodules), the gene activation of detoxification enzymes was selectively maintained, while that of

activation enzymes was suppressed. Thus, the overall change in the gene expression of the tamoxifen-metabolizing enzymes by tamoxifen treatment appears to be reasonable for the formation and growth of the hepatic hyperplastic nodules, because the increase in detoxification enzymes in the later stage would be expected to confer tamoxifen resistance to the induced nodules (Kasahara *et al.,* 2002). One of the proposed pathways for the metabolic activation of tamoxifen involves oxidation to 4-hydroxy tamoxifen, which may further oxidize to electrophilic Quinone methide (Costa *et al.,* 2001). Tamoxifen is well tolerated but causes steatosis in 43% of recipients (Nishino *et al.,* 2003). Steatohepatitis can develop, particularly in overweight women (Bruno *et al.,* 2005), and can lead to cirrhosis (Oien *et al.,* 1999). Tamoxifen administration decreases fatty acid synthase (FAS) expression in rat liver (Lelliott *et al.,* 2005), and tamoxifen both uncouples and inhibits mitochondrial respiration (Larosche *et al.*, 2007)

Antioxidants have been reported to play a significant role in protection against lipid peroxidation (Steenvooden and Henegouwen, 1999). Some investigators reported that antioxidants inhibit chemical carcinogenesis when the antioxidants are administered either prior or with carcinogen (Ames, 1983).

Vitamin C (ascorbic acid) has a considerable antioxidant activity: it scavenges reactive oxygen species and may, thereby, prevent oxidative damage to the important biological macromolecules, such as DNA, proteins, and lipids (Konopacka, 2004). Ascorbic acid (vitamin C) exerts protective role against acute ultraviolet B-rays (Sunburn cell formation) (Meves *et al.,* 2002), organophosphorous pesticides (Kurata *et al.,* 1993), and could reduce aflatoxin induced liver cancer (Yu *et al.,* 1994). Moreover vitamin C abolishes chromosome damage resulted from the effect of toxic substances (Trommer *et al.,* 2002), and help to protect the body against pollutants (Masaki *et al.,* 2000). Because vitamin C is a biological reducing agent, it is also linked to preventive of degenerative diseases such as cataracts, certain cancer and cardiovascular disease (Barros *et al.,* 2004 and Wang & sun, 2004). Increased vitamin C intake could possibly reduce and prevent nephrotoxic effect (Nagyova *et al.,* 1994). It assists in the prevention of blood clotting and bruising; it strengthens the walls of the capillaries (Tousoulis *et al.,* 2003) and it is also needed for healthy gum (Ambros *et al.,* 1998). Vitamin C helps to reduce cholesterol levels, high blood pressure and preventing atherosclerosis (Napoli *et al.,* 2004 and Zureik *et al.,* 2004). It protects susceptible cells from genotoxicity associated with antiestrogen metabolite-4- hydroxy tamoxifen (4-OH tom) (Sharma and Slocum, 1999), and inhibit DNA adduct induced by tamoxifen (Sierens *et al.,* 2001 and Sharma *et al.,* 2003).

Vitamin E (alpha tocopherol) is the naturally occurring lipid soluble antioxidant (Butterfield *et al.,* 1999). It is a powerful antioxidant that combats damaging free radical. It is important for reproduction, prevention of various diseases (Biri *et al.,* 1998). It appeared significant for reduction of hot flash (Barton *et al.,* 1998), toxicity of doxorubicin in tissue of rats (Geetha *et al.,* 1990). Alpha tocopherol prevents oxidation of LDL cholesterol and help to protect against atherosclerosis. Vitamin E exerted protective effects against cyanide induced tissue lesions in rabbits (Okolie and Iroanya, 2003) and protected neurons against oxidative cell death in vitro (Behl, 2000). Vitamin E maintained bone mineral density in ovariectomized rats and caused conflicting effect on bone calcium content (Norazlina *et al.,* 2000), and could enhance the proliferative status of prostate gland (Yao *et al.,* 1996). Moreover the increased level of enzyme in fibrosarcoma in rats was reduced by vitamin E administration (Vinitha *et al.,* 1995). Vitamin E is used in the treatment of Elzheimer's disease through preventing brain cell damage by destroying toxic free radicals (Klatte *et al.,* 2003 and Zandi *et al.,* 2004) and in the treatment of disorders in the central nervous system (Vatassery *et al.,* 1999).

Co administration of vitamin C and vitamin E reduces the tamoxifen induced hypertriglyceridemia (Babu *et al.,* 2000). Vitamin C and alpha tocopherol alone

reduce the growth of human melanoma (sk-30) cells in culture (Prasad *et al.*, 1994).

The aim of the present study is to investigate the possible protective effect of vitamin C and vitamin E to ameliorate tamoxifen induced histopathological, histochemical and ultrastructure changes in liver of rats.

Material And Methods

70 female albino rats weighting 130-160g were used in this study. The animals were divided into seven groups. Each group contained 10 rats.

Group (1): was kept as control.

Group (2): was given tamoxifen daily for two weeks at dose level of 20mg/kg b.w.

Group (3): was given vitamin C only at dose level of 0.01g/100g b.w. by stomach tube for two weeks.

Group (4) was given vitamin E only 100 mg/kg b.w. by stomach tube daily fro two weeks.

Group (5) was given vitamin C at dose level of 0.01g/100g b.w. (**Padget and Barnes, 1964**) by stomach tube, 15 min before tamoxifen administration daily for two weeks.

Group (6) was given vitamin E at dose level of 100 mg/kg b.w., 15 min before tamoxifen administration.

Group (7) was given combination of vitamin C at dose level of 0.01g/100g b.w. and vitamin E at dose level of 100mg/kg b.w., 15 min before tamoxifen administration daily for two weeks.

Histopathological and histochemcial studies:

The liver of different groups were removed and fixed in 10% formal saline. Paraffin sections 5 μm thick were stained with haematoxylin and eosin (Drury and Wallington, 1980) and Masson trichrome stain to demonstrate the collagen fibers (Masson, 1929). All sections were investigated by the light microscope.

Further sections were stained for DNA (Feulgen and Rosenbeck, 1942) and counterstained with Light Green. DNA analysis was performed by lecia Qwin 500 image cytomery in the department of pathology, National Research Center. For each section (100-120 cells) were randomly measured. The threshold values were defined by measuring control cells. The results are presented as histograms and tables which demonstrate the percentage of the diploid cells (2C), the triploid cells (3C), the tetraploid cells (4C) and the aneuploid cells (>5C). The DNA histogram classified according to Danque *et al.*, (1993). Protein stain (Mazia *et al.*, 1953) and mucopolysaccharids stain (Mac-Manus and Cason, 1950) were also performed.

The ultrastructural studies:

Sample processing for electron microscopy together with examining the thin sections and getting the electron micrographs was done in the Electron Microscope Unit, Institute of Ophthalmology. Small pieces of liver, about $1mm^3$ in size were prepared.

Thin sections 60-90 nm thick were prepared by using ultra cats/ FCS; the thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate (Watson, 1958) and finally examined with transmission JEM- 100x IL electron microscope. Photographs using Kodak films and photographic paper were taken and examined.

Results

1- Histopathological results:

The liver of control rats revealed the characteristic hepatic architecture (Fig. 1, A).

No pathological changes could be noticed in the liver of rats treated with either vitamin C or vitamin E.

The liver of rats treated with tamoxifen only showed hydropic degeneration, nuclei with variable sizes and dysplastic cells (Fig. 1,B). Fatty changes, vacuolar degeneration, mitotic figure and fibrosis were seen (Fig.1,C). Dilation, congestion of blood sinusoid and peripheral chromatin clumping were also observed (Fig.1, D).

Concerning rats treated with vitamin C and tamoxifen in combination for two

weeks, examination of liver sections showed marked diminution of hydropic degeneration, fatty changes and mitotic figure. No fibrosis and no chromatin clumping were noticed. While some hepatocytes still showed hypertrophy, others showed signs of degeneration in the form of karyolysis and karyorrhexis. The kupffer cells showed mild hypertrophy (Fig.1, E).

The rats treated with vitamin E and tamoxifen in combination, showed some protective effects as compared to the group of rats subjected to tamoxifen only. Examination of liver sections showed moderate hypertrophy of kupffer cells. Red blood cells were seen in the blood sinusoids. Focal area of necrosis was also noticed.

The liver of rats subjected to combination of vitamin C and vitamin E prior to administration of tamoxifen showed some histological changes, but these changes were somewhat less than those of rats treated with tamoxifen only. Examination of liver sections showed focal necrosis and a number of binucleated cells (Fig. 1, F).

Examination of control liver sections showed normal distribution of collagen, which showed small amount of wavy fibrils (Fig. 2,A). Treated group with tamoxifen showed collagen fibrils that occurred as wavy fibrils either singly or fused together in dense bundles (Fig. 2, B). The liver of rats subjected to vitamin C and / or vitamin E prior to administration of tamoxifen showed improvement in collagen deposition and connective tissue fibers as compared to liver of rats treated with tamoxifen only (Fig. 2, C).

Histochemical results:

DNA content in all the studied groups:

In the present study, the Qwine 500 image analyzer was used to evaluate the DNA content. The image analysis system automatically express the DNA content of each individual cell measured then gave the percentage of each cell out of the total number of cells examined. Also, it classifies the cells into four groups; diploid (2C), proliferating cells (3C), tetraploid (4C) and aneuploid cells (>5C). The proliferating cells were further classified according to Lee *et al.* (1999) into; (<10%) low proliferation index, (10-20%) medium proliferation index and (>20%) high proliferation index.

Normal distribution of DNA content in the liver of the control group showed that 20.18 % of the examined cells contained DNA (<1.5C), 65.13% of the examined cells contained diploid DNA value (2C), 12.84% of the examined cells contained (3C) DNA value (medium Proliferation Index) and 1.83% of the examined cells at (4C) area (table 1 & histogram 1). The group treated with tamoxifen showed that 93.54% of the examined cells contained DNA (<1.5C) this means decrease in DNA content (hypoploidy) compared to the control. (Table 2 & histogram 2).

In the present work the treatment of rats with tamoxifen along with vitamin C showed that 31.63% of the examined cells contained DNA (<1.5C), 61.22% of the examined cells contained diploid DNA value (2C), 6.12% of the examined cells contained (3C) DNA value (low Proliferation Index) and 1.02% of the examined cells at (4C) area (Table 3 & histogram 3).

The group treated with tamoxifen along with vitamin E showed that 43% of the examined cells contained DNA (<1.5C), 51% of the examined cells contained diploid DNA value (2C), 6% of the examined cells contained (3C) DNA value (low Proliferation Index) (Table 4 & histogram 4).

The group treated with tamoxifen along with combination of vitamin C and E showed that 9.90% of the examined cells contained DNA (<1.5C), 86.13% of the examined cells contained diploid DNA value. (2C), 3.96% of the examined cells contained (3C) DNA value (low Proliferation Index) (Table 5 & histogram 5).

These results indicate that treatment with vitamin C & E showed DNA values comparable to the control values (Fig. 3, A, C, D), while, the group treated with tamoxifen showed decreased DNA values (hypoploidy) (Fig. 3,B).

Examination of control liver sections showed moderate protein content in the cytoplasm of hepatocytes. Some nuclei showed deep protein content (Fig. 4, A).

After daily treatment of rats with an oral dose of tamoxifen for two weeks, the protein inclusions showed marked diminution in the cytoplasm of hepatocytes and staniability was mostly diffused (Fig. 4, B). Slight increase in protein content was noticed in the case of rats subjected to vitamin C in combination with tamoxifen as compared to liver of rats subjected to tamoxifen only. Moderate increase in protein content in the cytoplasm of hepatocytes was also recorded in the case of rats treated with vitamin E in combination with tamoxifen as compared to rats subjected to tamoxifen only (Fig. 4, C). The pretreatment of rats with combination of vitamins (vitamin C and vitamin E) along with tamoxifen showed marked increase in protein content in the cytoplasm of hepatocytes (Fig. 4, D).

Examination of control liver sections stained with periodic acid schiff's (PAS) showed mucopolysaccharide granules in the cytoplasm of hepatocytes; the peripheral zonal cells showed higher mucopolysaccharide content than the central zonal cells (Fig. 5, A).

Daily treatment of rats with tamoxifen only for two weeks induced marked decrease in stainability of PAS +ve materials (Fig.5, B).

Daily administration of vitamin E in combination with tamoxifen showed moderate increase in mucopolysaccharides content in the cytoplasm of hepatocytes and mild increase in mucopolysaccharides content could be noticed in the case of rats subjected to vitamin E and tamoxifen as compared to rats subjected to tamoxfien only (Fig. 5, C). Co administration of vitamins (vitamin C and vitamin E) in combination with tamoxifen showed marked generalized increase in mucopolysaccharides content in the cytoplasm of hepatocytes (Fig. 5, D).

Electron microscopic results:

Figure (6, A) showed electron micrograph of control liver cells. Hepatocytes of rats treated with tamoxifen only show areas of cytoplasmic dissolution, partial clumping of nuclear chromatin and corugated nuclear membranes. Mitochondria were swollen with dense matrix (Fig.6, B). Endoplasmic reticulum showed dilated cisternae with no obvious attached ribosomes (Fig.6, C). The treatment of rats with vitamin C or vitamin E showed improvement in the ultrastructural changes in the form of diminution of cytoplasmic dissolution and restoration of nuclear normal shape (Fig. 6, D).

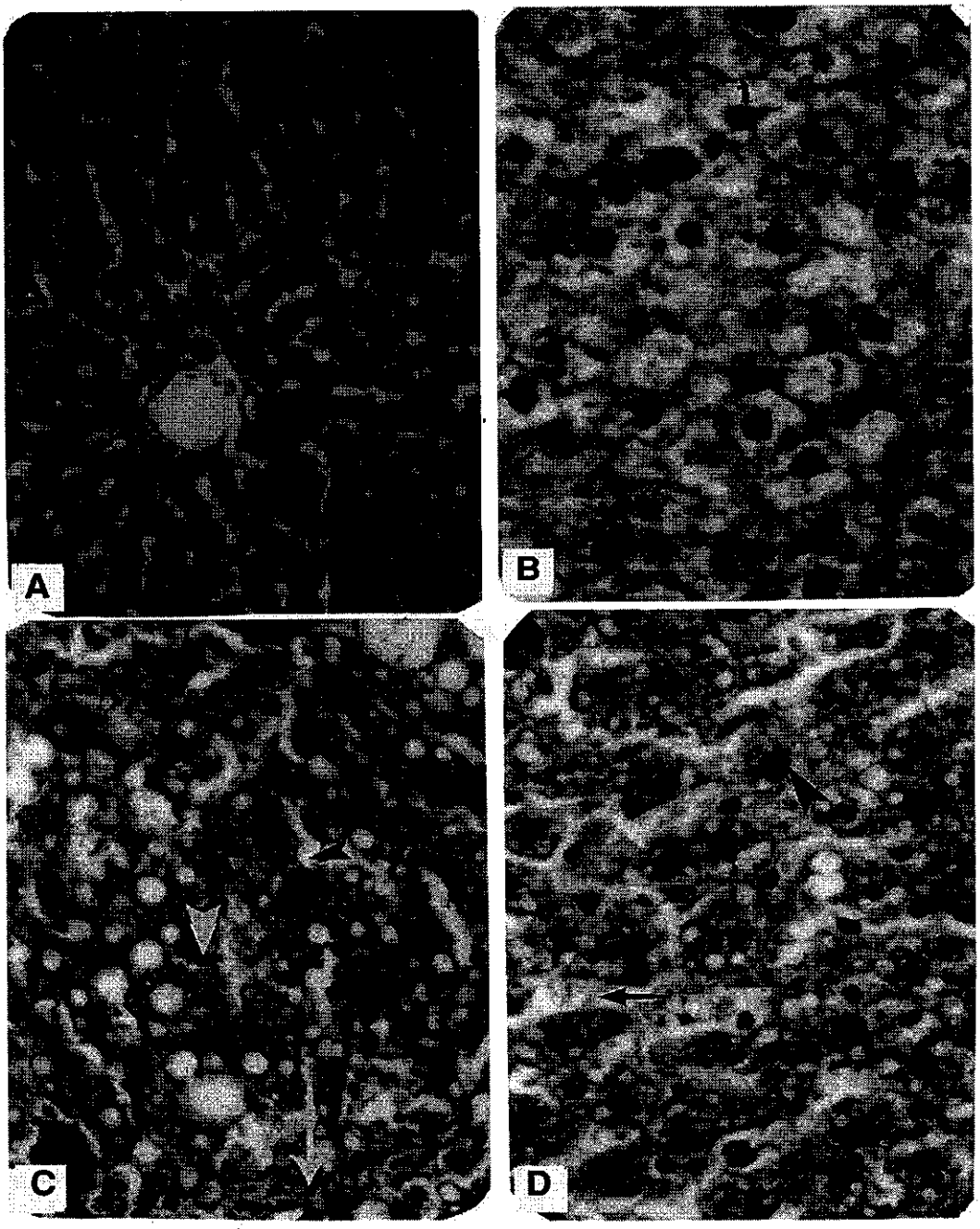


Fig. (1): (A) Section in the liver of control rat showing normal histological structure of hepatic lobules and central vein (Hx:&E. X 200). (B) Section of the liver of a rat treated with tamoxifen showing hydropic degeneration (1), variable sized nuclei (2). Also seen, many pyknotic nuclei and some hepatocytes are devoid of nuclei (Hx.&E. X400). (C) Section of the liver of the same group showing lymphocytic infiltration (arrow), fatty changes, vacuolar degeneration (arrow head) and mitotic figures (yellow arrow head) (Hx.&E. X 200). (D) Section of the liver of a rat treated with tamoxifen showing dilation and congestion of blood sinusoids (arrow) and peripheral chromatin clumping (arrow head) (Hx.&E. X400).

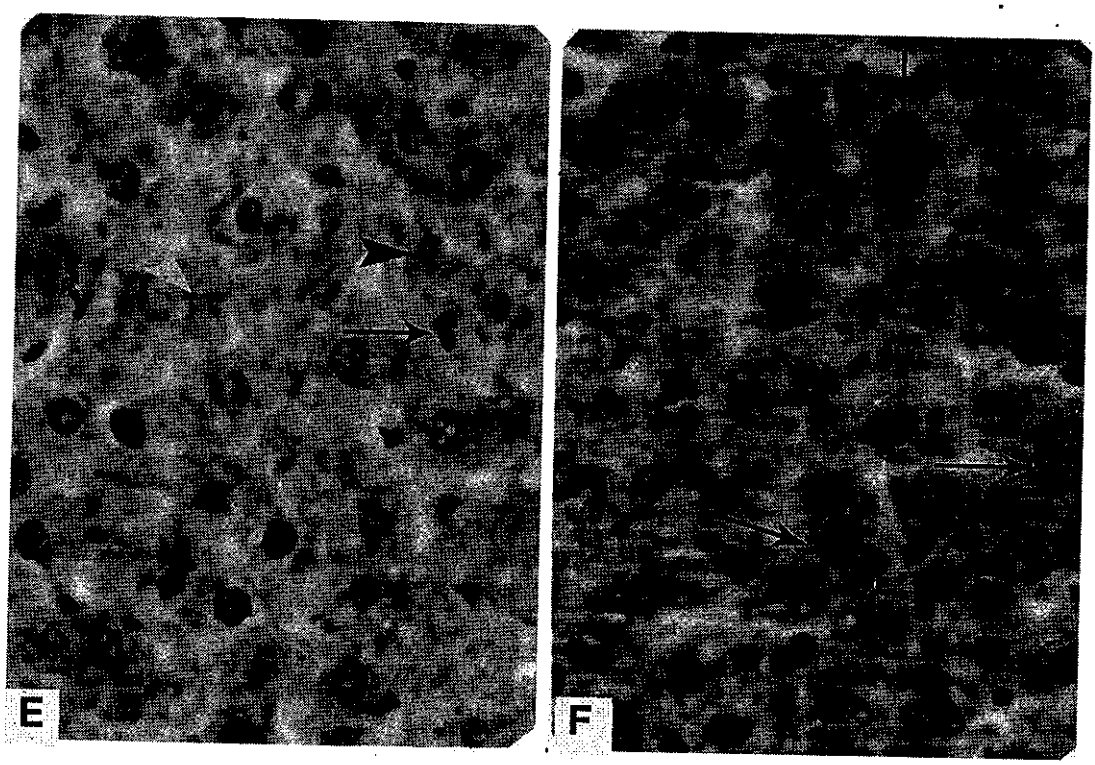


Fig. (1): (E) Section in the liver of a rat treated with vitamin C along with tamoxifen showing no fibrosis, no fatty changes and no vacuolar degeneration. Karyolysis (black arrow head), karyorrhexis (yellow arrow head) & mild hypertrophy of Kuppfer cells (arrow) were noticed. The same results were obtained with Vitamin E along with tamoxifen (Hx.&E. X400). (F) Section of the liver of a rat treated with tamoxifen along with vitamin C and vitamin E showing scattered binucleated cells (arrows). (Hx & E X 400)

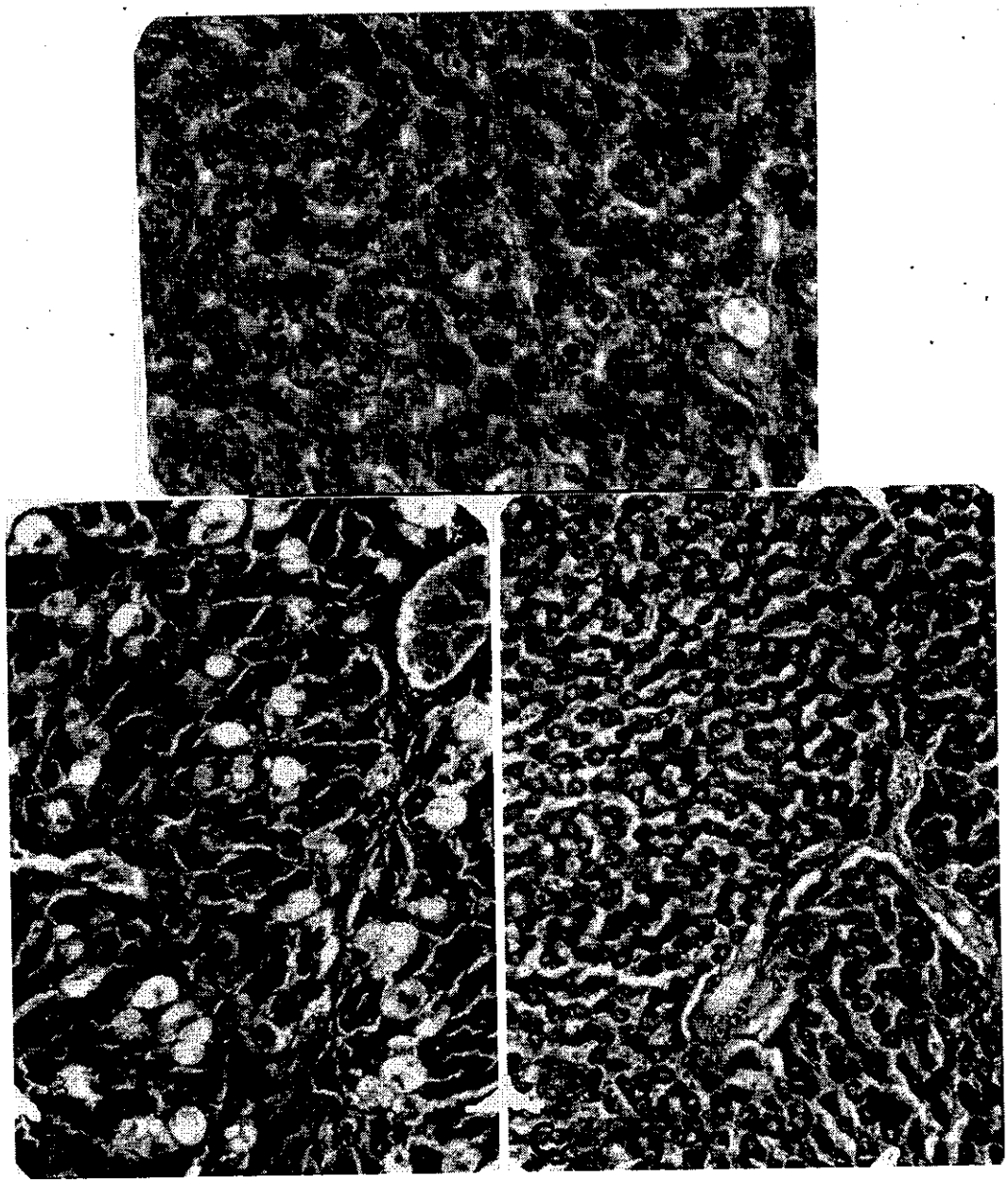


Fig. 2: Section of the liver of a rat showing collagen (A): control. (B): treated group with tamoxifen showing collagen fibrils occurred as wavy fibrils either singly or fused together in dense bundles especially in and around the portal area, around the central vein and in-between hepatocytes. (C): treated group with tamoxifen along with vitamin C showing mild amount of fibrous tissue in the portal area.

(Masson trichrome stain x 200)

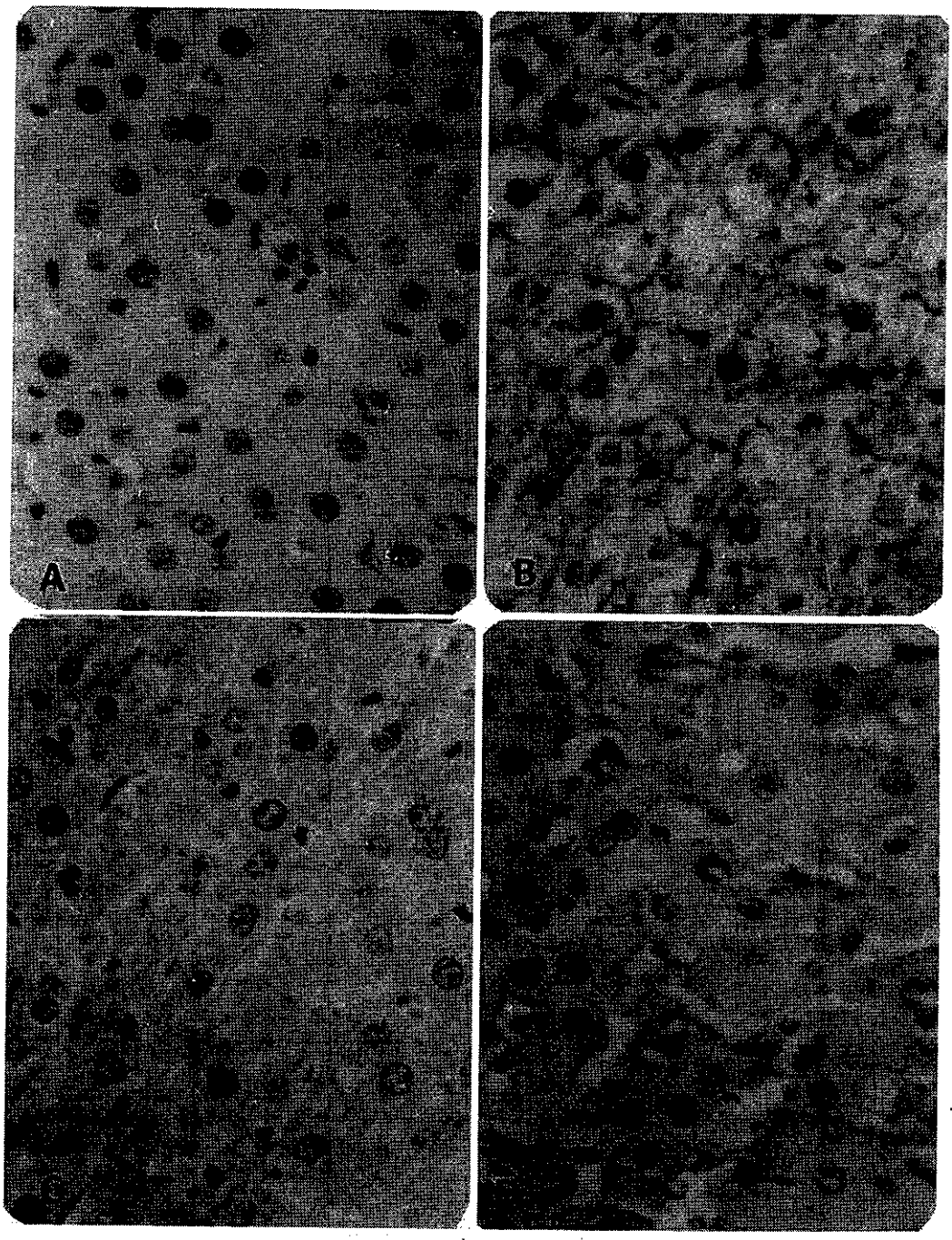
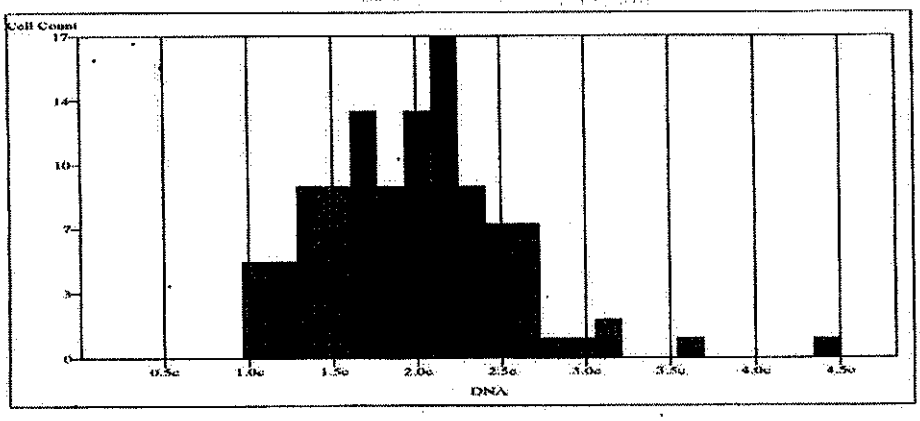


Fig. (3): Section of the liver of a rat showing DNA in hepatocytes. (A): cotnrol. (B): Treated with Tamoxifen: showing a decrease in DNA content (C): Treated with vitamin C along with Tamoxifen showing mild improvement in DNA content. (D): Treated with vitamin E in combination with tamoxifen showing moderate improvement in DNA content.

(Feulgen reaction x 400)

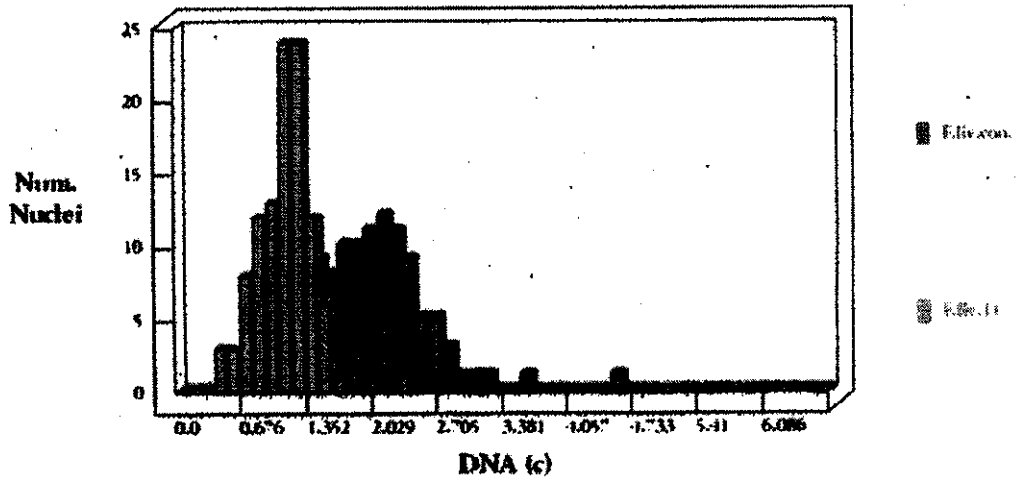
DNA cytometry

Histogram (1) & Table (1): DNA Ploidy of the control liver.



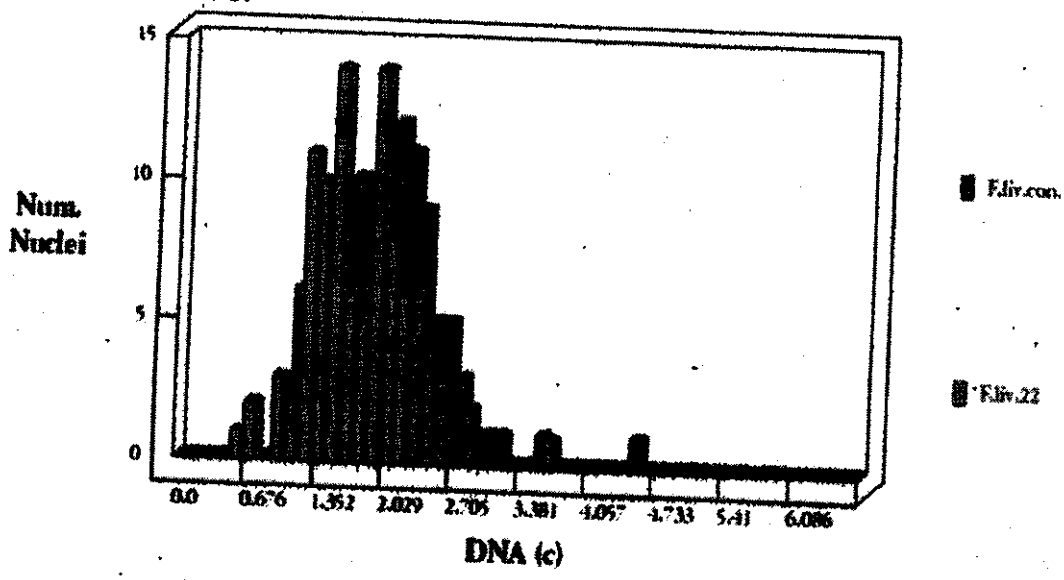
Range	Tot. Cells	% Cells	DNA Index
All	109	100%	1.000
5cER	0	0.000%	-
<1.5c	22	20.183%	0.646
1.5c-2.5c	71	65.138%	1.007
2.5c-3.5c	14	12.844%	1.372
3.5c-4.5c	2	1.835%	2.023
>4.5c	0	0.000%	-

Histogram (2) & Table (2): DNA Ploidy of rat liver treated with tamoxifen.



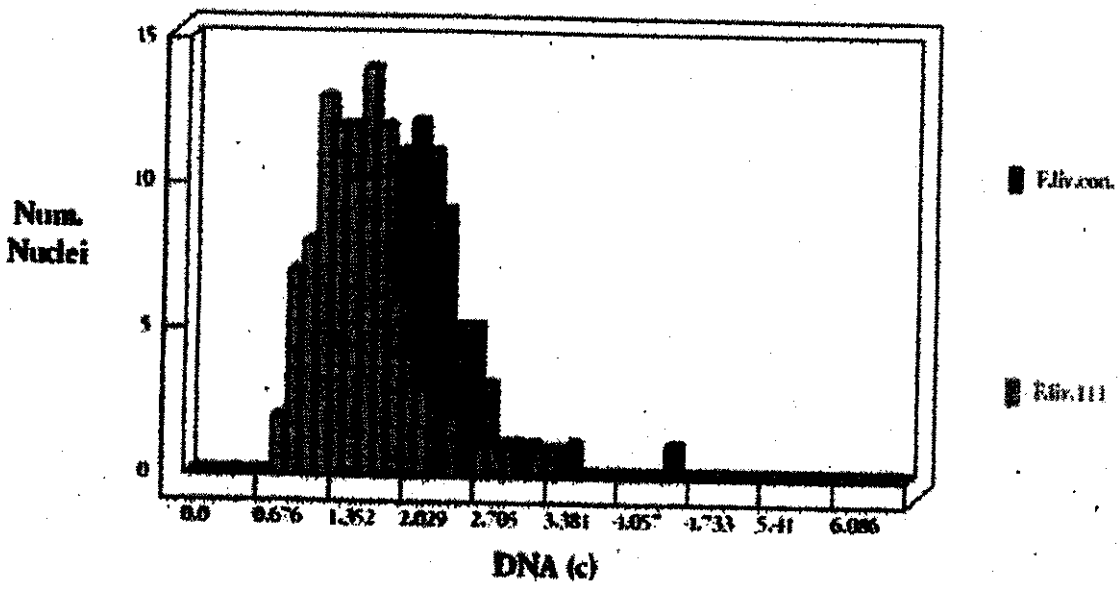
Range	Tot. Cells	% Cells	DNA Index
All	107	100.0%	0.572
5cER	0	0.0%	-
<1.5c	100	93.458%	0.553
1.5c-2.5c	7	6.542%	0.851
2.5c-3.5c	0	0.0%	-
3.5c-4.5c	0	0.0%	-
>4.5c	0	0.0%	-

Histogram (3) & Table (3): DNA Ploidy of rat liver treated with tamoxifen along with vitamin C.



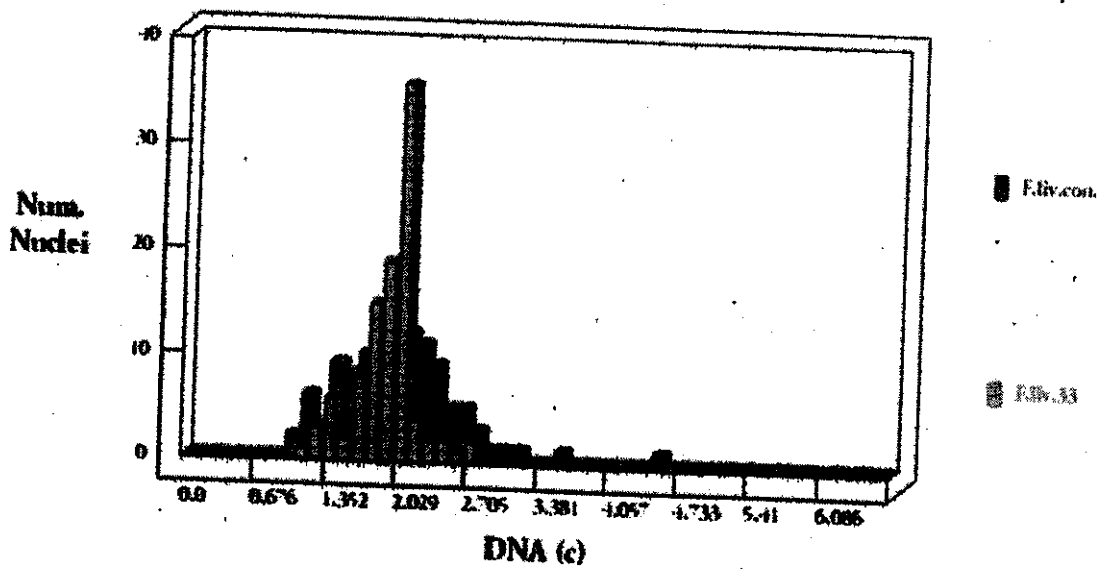
Range	Tot. Cells	% Cells	DNA Index
All	98	100.0%	0.899
5cER	0	0.0%	-
<1.5c	31	31.633%	0.630
1.5c-2.5c	60	61.224%	0.979
2.5c-3.5c	6	6.122%	1.338
3.5c-4.5c	1	1.02%	1.849
>4.5c	0	0.0%	-

Histogram (4) & Table (4): DNA Ploidy of rat liver treated with tamoxifen along with vitamin E.



Range	Tot. Cells	% Cells	DNA Index
All	100	100.0%	0.819
5cER	0	0.0%	-
<1.5c	43	43.0%	0.626
1.5c-2.5c	51	51.0%	0.902
2.5c-3.5c	6	6.0%	1.504
3.5c-4.5c	0	0.0%	-
>4.5c	0	0.0%	-

Histogram (5) & Table (5): DNA Ploidy of rat liver treated with tamoxifen along with vitamin C and vitamin E.



Range	Tot. Cells	% Cells	DNA Index
All	101	100.0%	0.981
5cER	0	0.0%	-
<1.5c	10	9.901%	0.681
1.5c-2.5c	87	86.139%	0.999
2.5c-3.5c	4	3.96%	1.332
3.5c-4.5c	0	0.0%	-
>4.5c	0	0.0%	-

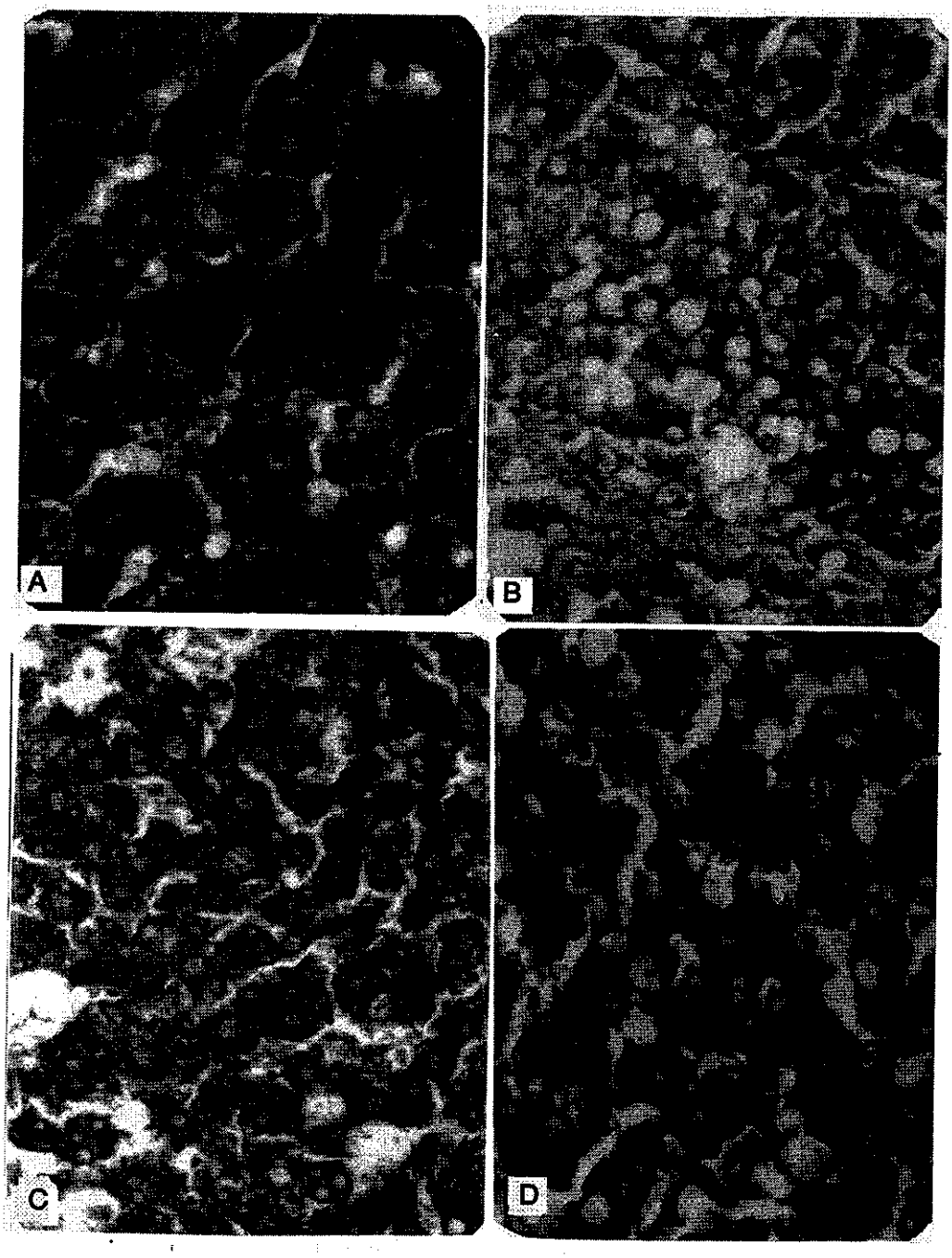


Fig. (4): Section of the liver of a rat showing greenish blue protein content in the cytoplasm of hepatocytes (A): control. (B): Treated with tamoxifen: showing marked diminution of protein content. (C): Treated with tamoxifen along with vitamin E showing moderate improvement of protein content. The same results were obtained from the group of rats treated with Vitamin C along with tamoxifen. (D): Treated with tamoxifen along with combination of vitamin C and vitamin E showing marked improvement in protein content. (Bromophenol blue stain x 400)

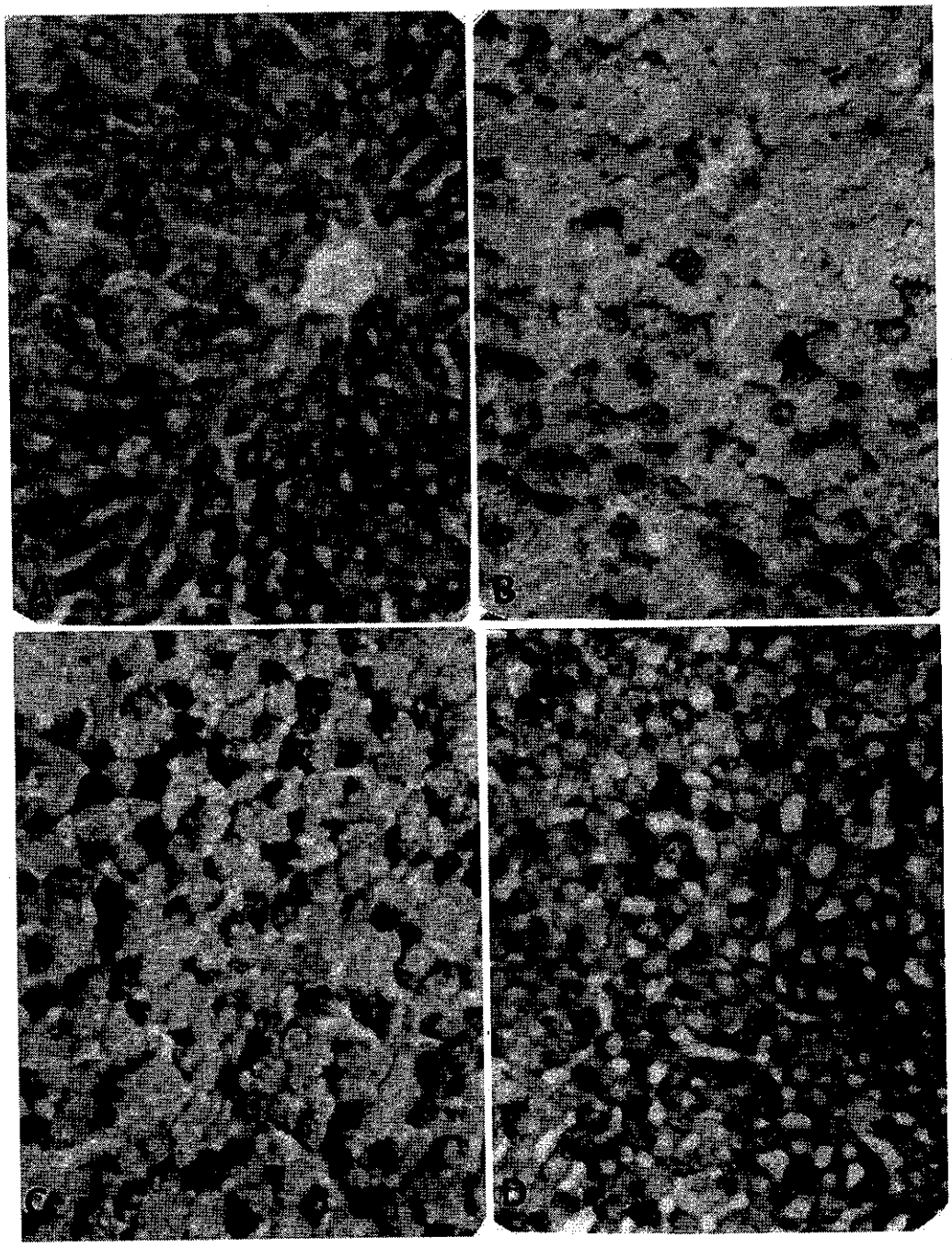


Fig. (5): Section of the liver of a rat showing PAS+ ve materials in the cytoplasm of hepatocytes (A) control. (B): Treated with tamoxifen: showing decreased stainability of PAS + ve materials. (C): Treated with vitamin E along with tamoxifen showing mild improvement in PAS +ve materials. The same results were obtained from the group of rats treated with Vitamin C along with tamoxifen. (D): Treated with tamoxifen along with vitamin C and vitamin E showing increased PAS+ ve materials. (PAS reaction x 400).

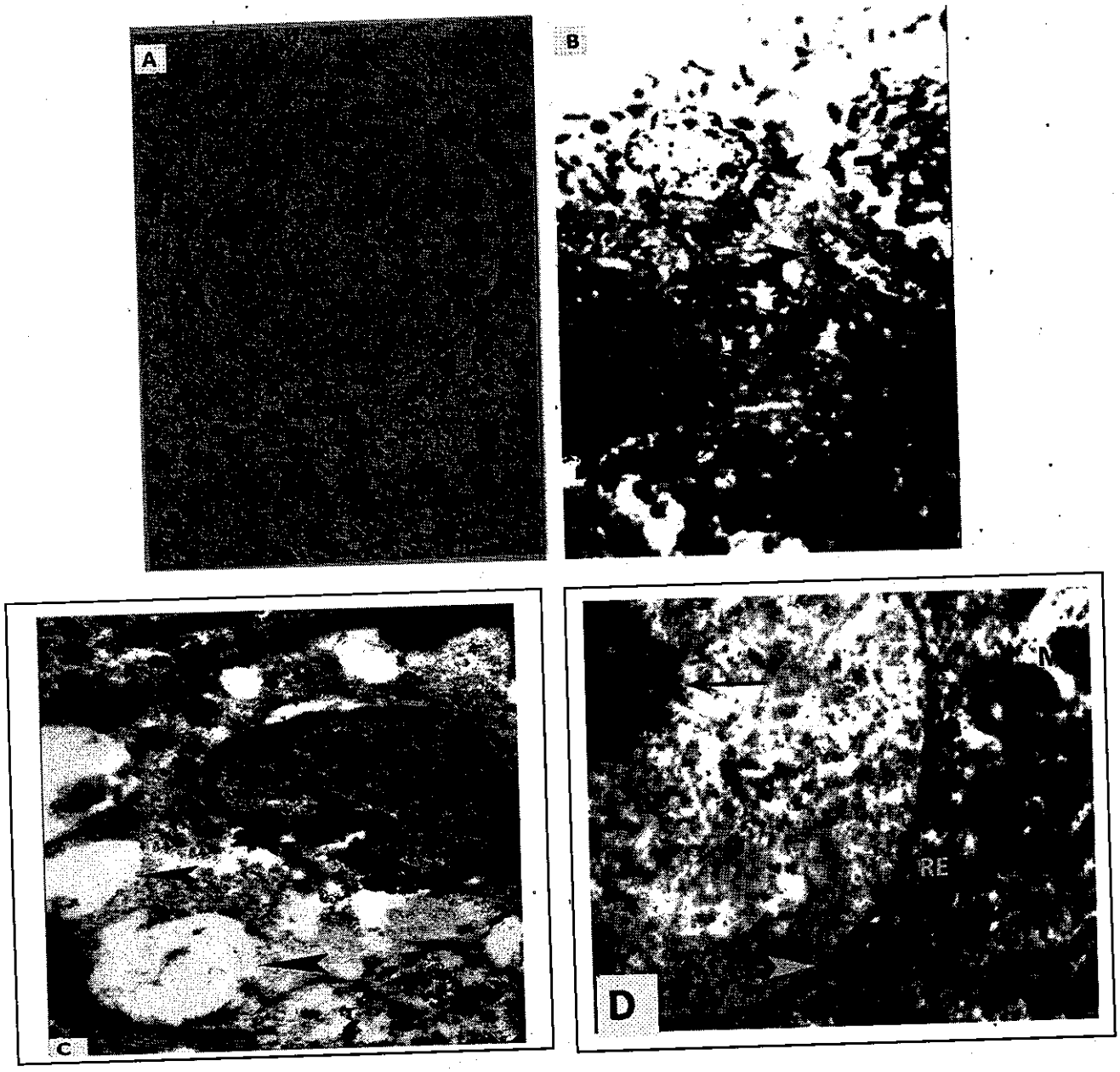


Fig. 6: (A): Photoelectron micrograph of a control adult albino rat (x8000), (B): Electron micrograph of hepatocytes treated with tamoxifen showing swollen mitochondria with dense matrix, partial clumping of nuclear chromatine (arrow) and nuclear shrinkage (arrow head) (x6000). (C): Another field of electron micrograph of hepatocytes treated with tamoxifen showing dilatation of endoplasmic reticulum with no obvious ribosomes (arrow head) the nuclear membrane is corrugated. (arrow) (x 10000). (D): treated group with a combination of vitamin C & E along with tamoxifen showing well-defined nucleolus (arrow) and nuclear envelope (arrow head). Also seen normal-shaped rough endoplasmic reticulum (RE), although mitochondria (M) are still dilated. (x 6000) .

Discussion

Tamoxifen is a triphenyl ethylene derivative commonly used in the treatment of breast cancer (Kennel et al., 2003 and Mati & Chen, 2003).

Tamoxifen is known to have varied biological effects ranging from complete estrogen antagonism to pure estrogen agonism dependeing upon its concentrations, the sex of animals and target organ (Williams, 1984). In humans and rats tamoxifen is predominantly antiestrogenic with residual estrogenic activities (Furr and Jordan, 1984).

Rat liver is an organ with especial sensitivity of developing tumors after exposure to many chemicals and drugs (Maronpot *et al.,* 1995). The rat at which the liver tumors develop is known to be strongly influenced by tamoxifen's promoting effect on hepatocyte proliferation where sustained proliferation has also been associated with chronic cell death (Carthew *et al.,* 1996).

The microscopical appearance of liver in rats receiving 20mg/kg b.w. of tamoxifen by an oral route for two weeks was characterized by vacuolar degeneration and hydropic degeneration. Results of this work go in agreement with Hirsimaki *et al,* (1993) who noticed that the treatment of rats with tamoxifen at dose level of 45 mg/kg b.w. for two weeks caused vacuolar degeneration in the liver of rats. In controversy Kasahara *et al.,* (2002) stated that no pathological changes could be noticed in the liver of rats treated with tamoxifen at dose level of 20 mg/kg b.w. for two weeks. Pathological altered cell foci and placental form of glutathione s-transferase (GST-P) positive foci were observed in the liver after 12 weeks. Treatment for 52 weeks resulted in the formation of liver hyperplastic nodules that strongly expressed GST-P. According to Badawy *et al,* (2002) the treatment of rabbit with tamoxifen at dose level of 14mg/kg b.w. daily for 60 days induced histopathological changes in the testis in the form of vacuolar degeneration of spermatogenic cells, atrophied and collapsed seminiferous tubules with asospermia.

Vacuolation observed in the present study may be due expansion of the mitochondrial intermembrane space and extension of the outer mitochondrial membrane (Higgins *et al.*, 2003) consistent with ultrasturctural changes observed in the present study. Vacuolation may be due to disturbance of ionic milieu of the cell with consequent retention of water and sodium leading to cellular swelling (Jaarsma *et al.,* 2001 and Wiedemann *et al.,* 2002).

Tamoxifen was associated with higher risk of development of non-alcoholic steatohepatitis only in overweight and obese women (Bruno *et al.,* 2005). Adjuvant tamoxifen increases the incidence of fatty liver in patients with breast cancer (Liu et al., 2006). In the present work fatty changes were observed in the liver after treatment of rats with tamoxifen only. Fatty change observed in the present work may be due to damage in rough endoplasmic reticulum confirmed by electron microscopic changes observed in the present work, impaired protein synthesis and inhibition of lipoprotein manufacture. The latter is involved in the transport to hepatic triglycerides to extrahepatic tissue and its inhibition results in accumulation of fat in the cytoplasm (Deboyser *et al.*, 1989). According to Marzouk (1995) mitochondria are known to contain many of the enzymes necessary for the metabolism of triglycerides (i.e. fatty acid oxidases). This leads to another explanation that the fatty changes observed in the present work may be due to mitochondrial damage.

Tamoxifen decreases hepatic triglyceride secretion, and it accumulates electrophoretically in mitochondria, where it impairs β-oxidation and respiration. Tamoxifen also inhibits topoisomerases and mitochondrial DNA synthesis and progressively depletes hepatic mitochondrial DNA in vivo. These combined effects could decrease fat removal from the liver, thus causing hepatic steatosis despite the secondary down-regulation of hepatic fatty acid synthase expression (Larosche *et al.*, 2007).

In the present work the treatment of rats with tamoxifen only induced foci of necrosis, signs of degenereation in the form of, karyolysis, karyorhexis and fibrosis of hepatocytes. Displastic cells could be noticed. These results were in agreement with Hirsimaki *et al.* (1993) they noticed that the treatment of rats with tamoxifen only induced area of hepatic necrosis and apoptosis. In controversy Coe *et al,* (1992) reported that subcutaneous injection of tamoxifen alone at dose level of 0.1mg (5mg) did not cause degenerative changes or neoplastic lesions in Armenian hasmster. Also Sauvez *et al.* (1999) they found that the treatment of rats with tamoxifen induced biliary proliferation and peribiliary fibrosis and degeneration of hepatocytes. Coinciding with Smith *et al.* (2000), they reported that tamoxifen revealed tissue damage and carcinogenic changes in rats by an oral route.

The hepatic fibrosis observed in the present study may be due to increased level of malondialdehyde (MDA) and decreased production of superoxide dismutase (SOD) and glutathione peroxidase in the liver cells. Oxidative stress plays a role in the development of hepatic fibrosis and degeneration (Duthie *et al.,* 1995). Accordin to Hu *et al.* (2003) one of the proposed pathways for the metabolic activation of tamoxifen involves oxidation to 4- hydroxytamoxifen which may futher oxidized to an electrophilic quinone methide and may affect cytochrome P-450. However, Badawy *et al.* (2002) they reported that the administration of tamoxifen caused the production of reactive oxygen species (ROS) which can damage the cellular elements. Oxidative modifications of DNA, protein and lipid by ROS play a role in ageing and disease.

In the present work the pathological changes observed in the liver of rats due to oral route of tamoxifen may be due to lipid peroxidation and free radicals. Free radical may propagate damage in the endoplasmic reticulum and oxidation of membrane component of the liver cells consistent with ultrastructural changes observed in the present work. Oxidation has been shown to be associated with apoptosis (Programmed cell death) (Mohan *et al.,* 2003).

The effects of tamoxifen may be neutralized by radical scavenger antioxidants such as vitamin C and /or vitamin E (Babu et al., 2000).

In the present work the oral administration of vitamin C prior to administration of tamoxifen showed some improvement in pathological changes in comparison with group of rats subjected to tamoxifen only. According to Okolie and Iroanya (2003) the supplementation of vitamin C led to marked reduction of histopathological degeneration in tissues by toxic agents. Vitamin c exerted antioxidant action and free radical scavenger (Barros *et al.,* 2004). Coinciding with Sharma and Slocum (1999), who reported that vitamin C adverse some pathological changes induced in liver of rats treated with tamoxifen. According to Sharma *et al,* (2003) ascorbic acid reduced the level of alpha hydroxyt-amoxifen substantially (68.9%) when exposure of endometrial explanted culture to 100 micro M tamoxifen and 1mM ascorbic acid.

The treatment of rats with vitamin E conditioned the adverse effect of tamoxifen in liver of rats. According to Custodio *et al.* (1994) oral administration of antioxidant such as vitamin E has a high protective capacity of vitamin against lipid peroxidation. Also Inal and Kahraman (2000) they reported that vitamin E exerted the antioxidant action and can interfer with the production of reactive oxygen species and other reactive oxygen species scavengers such as glutathione peroxidase, superoxide dismutase, xanthine. and increase glutathione in cells.

In the present work the treatment of rats with vitamin E prior to administration of tamoxifen showed marked diminution of vacuolar degeneration and fatty changes. Crewe *et al.* (2002) found that the vitamin E may be possible therapeutic agent with potential applications against pathological states due to reactive oxygen species. Coinciding with Babu *et al.* (2000) they demonstrated that vitamin E adverse some pathological changes induced in tissue of rats treated with tamoxifen. Vitamin E also decreased dimethyl valeronitrile induced phospholipid peroxideation. Also Mohan *et al.* (2003) reported that the pretreatment of rats with vitamin E

inhibitedt apoptosis by acting a quite upstream in the apoptosis cascade at the mitochondrial level as well as down stream at the caspase.

In the present work the treatment of rats with combination of the two vitamins (vitamin C and vitamin E) before an oral route of tamoxifen showed more improvement in the pathological changes. Results of this work go in agreement with Prasad *et al.* (1994) who reported that a mixture of vitamins (vitamin C and vitamin E) were more effective in reducing the effect of tamoxifen on tissue damage and they were more effective in reducing growth of human melanoma cells. In the previous studies of Babu *et al.* (2000) showed that the combined effect of tamoxifen, vitamin C and vitamin E encumber the abnormalities investigated by tamoxifen.

The measurement of DNA ploidy has the advantages of being precise, rapid and quantitative, (Filipe *et al.*, 1991). Image cytometry for DNA quantification has become an established technique in the field of analytical cellular pathology, used as an important parameter providing significant information about the biological behavior of tumors (Cohen, 1996).

Concerning ploidy results, the treatment of rats with tamoxifen only resulted in decreased nuclear DNA content, 93.54% of the examined cells contained DNA <1.5c i.e hypoploidy and low proliferation index. 6.54% of the examined cells contained diploid DNA value. These results go in agreement with (Phillips, 2001 and Cardoso *et al*,. 2003) who reported that, the mechanism by which tamoxifen causes liver cancer in rats is through accumulation of DNA damage, caused by adduct formation between tamoxifen and hepatocytes DNA. According to Süzme *et al.* (2001), tamoxifen injections induced DNA aneuploidy, but did not stimulate proliferation in the liver as estimated by S-phase fraction. Friedlander *et al.* (1984) found that the normal human somatic cell contains 46 chromosomes which is referred diploid (2n), the gametes contain one set of chromosomes (23) referred to as haploid. While a cell with fewer or more than 46 chromosomes is described as hypoploid or hyperploid respectively. Also Umenoto *et al.* (2001) found that DNA adduct is formed when chemical carcinogen or their metabolites bind covalently with DNA. On the other hand Sierens *et al.* (2001) and Kasahara *et al.* (2003) found that tamoxifen has demonstrated genotox activity in rat liver causing unscheduled DNA synthesis and hepatic aneuploidy. They added that tamoxifen causes hepatic tumors through a genotoxic mechanism. Moreover Dragan *et al.* (1998) found that the treatment of rats with tamoxifen resulted in a shift of DNA from tetraploid to diploid. Marrero et al. (1996) reported that the cellular DNA content is abnormal at an early stage in dysplasia and may even predate it. Increasing value of abnormal DNA content is related to the severity of dysplasia. Also Carthew *et al.* (1997) explained that the endogenous DNA damage was not generated by estrogen receptor mechanisms but by microsomal cytochrome p-450 mediated redox cycling of catechol estrogen.

In the present work the treatment of rats with vitamin C along with tamoxifen showing improvement in DNA content as compared to group of rats treated with tamoxifen only. According to Nefic (2001) vitamin C (ascorbic acid) is an antioxidant that can scavenge free radicals and protect cellular macromolecules, including DNA, from oxidative damage induced by different agents. Some studies indicated that vitamin C is much more than just an antioxidant; it regulates the expression of some genes participating in apoptosis or DNA repair processes (Konopacka, 2004). Also,vitamin C provides high ability to decrease the number of aneuploid DNA value. Tarin *et al.*, (1998) reported that the DNA aneuploid and diploid were highly increased in mouse treated with some toxic agents and decreased DNA aneuploid after administration of vitamin C. They added that mixture of vitamins C and vitamin E induced more improvement in DNA content.

In the present work the treatment of rats with tamoxifen only showed marked diminution in protein content. These results disagreement with Kulesar and Gergely (1991) stated that tamoxifen caused protein synthesis in healthy and in

injured liver. These results were in agreement with Gong *et al.* (1999) who reported that the tamoxifen or 4-hydroxytamoxifen caused decrease in mRNA and protein levels depending on time and dose. Also Divi *et al.* (2001) noticed that tamoxifen induces the formation of hepatic enzyme altered foci that have lost the capacity to metabolize the drug to DNA binding species. Tamoxifen induced modified mitochondrial DNA or tamoxifen modified protein. On the other hand Matin *et al.* (1987) showed that subcellular fractionation of mouse liver showed that 82% of the antiestrogen binding protein was associated with the rough endoplasmic reticulum where it was confined to the membranous component. The antiestrogen binding protein was also present in smooth endoplasmic reticulum, nuclei and cytosol. High affinity of protein was recorded in tissue of mouse treated with tamoxifen.

In the present work the treatment of rats with vitamin C and/or vitamin E prior to administration of tamoxifen produced more improvement in protein content. These results are in agreement with Sierens *et al.* (2001) who stated that the antioxidant species may act in vivo to decrease damage of protein content in tissues. However Sharma *et al.* (2003) noticed that the antioxidants vitamin C & vitamin E play an important role in stimulating intercellular signals indirectly for activation of gene responsible for protein synthesis related to DNA repair.

Results of the present work showed that the oral administration of rats with tamoxifen produced marked diminution in mucoploysaccharides content. Results of this work go in agreement with Kulesar and Gergely (1991) who found that tamoxifen caused moderate glycogen loss in liver lesion. According to Hirsimaki *et al.* (1993) the amount of smooth endoplasmic reticulum appeared to be increased in some cells. after administration of tamoxifen at the dose level of 45mg/kg at total period of 52 weeks.

Depletion of glycogen that was observed in the present study was most probably consequent to hydropic and fatty degeneration manifested in this work, or due damaging effect of tamoxifen on the cytoplasmic organelles and the associated enzymes. However Poop and Cattley (1991) reported that the decrease in mucopolysaccharides content in tissues may be due to disturbed role of Golgi apparatus which is responsible for synthesis of polysaccharides.

In the present work the treatment of rats with tamoxifen only showed swollen mitochondria with dense matrix. According to Hirota (1997) tamoxifen induced mitochondrial disappearance of cristae. However, Hoyta *et al.* (2000) stated that high concentration of tamoxifen (100micro M) caused mitochondrial depolarization. Also Andreassen *et al.* (2000) reported that mitochondrial dysfunction can lead to energy deficiency, ionic imbalance, elevated reactive oxygen species (ROS) and oxidative damage. The mitochondrial vacuolation and swelling represent an accelerated form of mitochandrial damage caused by high level of mutant superoxide dismutase accumulation (Wang *et al.*, 2002).

The liver of rats treated with tamoxifen only showed dilated endoplasmic reticulum with no obvious attached ribosomes. According to Hirota (1997) tamoxifen induced damage of granular endoplasmic reliculae. The dilatation of endoplasmic reticulum may be the cause of vacuolation of the cytoplasm observed by light microscope in the liver of rats treated with tamoxifen only.

The dilatation of rough endoplasmic reticulum was considered by Robbin *et al.* (1984) to be reaction to cell injury. Detachment of ribosomes most probably reflected a disturbance in protein synthesis confirmed by histochemical changes observed in the present work. According to Traynor and Hall (1981) the increase of protein catabolism is a major effect of the body's response to stress.

Conclusion

Tamoxifen treatment induces liver damage that was performed by histopathological, histochemical and ultrastructural changes. These changes may be due to the production of reactive oxygen species (ROS) which could damage the cellular elements. The using of vitamin C and/ or vitamin E

ameliorate the harmful effects of tamoxifen. This protection may be due antioxidant action which can interfere with production of reactive oxygen species scavengers such as glutathione peroxidase, superoxide dismutase, xanthine and increase glutathione in cells.

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دور فيتامين ج، هـ فى تحسين الأضرار الناتجة عن التاموكسفين على الكبد في الجرذان البيضاء (دراسات هستولوجية وهستوكيميائية ودراسة خلوية دقيقة)

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قسم الباثولوجى – المركز القومى للبحوث

يعتبر التاموكسفين من الأدوية التى تستخدم فى علاج سرطان الثدى وقد أتضح من الدراسات السابقة أن معالجة الجرذان لمدة طويلة بهذا العقار يؤدى إلى سرطان فى كبد الجرذان البيضاء.

تهدف هذه الدراسة إلى تقييم التأثيرات الهستولوجية والهستوكيميائية والفحص بالمجهر الإلكترونى التى يحدثها احد أدوية علاج سرطان الثدى وهو التاموكسفين على كبد أنثى الجرذان البيضاء.

وقد استخدم فى هذا البحث عدد 70 من إناث الجرذان البيضاء تتراوح أوزانهم مابين 130-160 جرام وقد تم توزيع الجرذان كالآتى :

- المجموعة الأولى : وهى المجموعة الضابطة وهى مكونة من 10 جرذان.
- المجموعة الثانية : تم معالجة جرذان هذه المجموعة بعقار التاموكسفين عن طريق الفم بجرعة مقدارها 20ملجم/ كجم لمدة أسبوعين.
- المجموعة الثالثة: وقد تم معالجتها بفيتامين ج بجرعة مقدراها 0.01 جرام/100 جرام من وزن الجسم قبل معالجتها بعقار التاموكسفين بحوالى 15 دقيقة وقد تمت المعالجة بعقار التاموكسفين عن طريق التجريع اليومى عن طريق الفم.
- المجموعة الرابعة : وقد تم معالجتها بفيتامين هـ بجرعة مقدارها 100 مجم / كجم قبل معالجتها بعقار التاموكسفين بحوالى 15 دقيقة لمدة أسبوعين.
- المجموعة الخامسة: وقد تم معالجتها بفيتامين ج بجرعة مقدارها (0.01جرام) وفيتامين هـ بجرعة مقدارها (100ملجم/كجم) عن طريق الفم قبل معالجتها بالتاموكسفين لمدة أسبوعين

تم إعداد قطاعات شمعية صبغت بالهيماتوكسلين والإيوسين وتم إعدادها للفحص الهستولوجى. وقد تم إعداد صبغات للفحص الهستوكيميائى وهى طريقة فولجن لفحص حامض الداى أكسي ريبونيوكليك وكذلك قياس كميته عن طريق جهاز تحليل الصورة وطريقة البيرأيوديك شف لعديدات التسكر المخاطية وطريقة البروموفينول الأزرق لتبيان كمية البروتين. واظهرت نتائج الفحص بالمجهر الإلكترونى تجمعات جزئية من الكروماتين النووى وانكماش واضح فى أنوية الخلايا ، بالإضافة إلى تمدد ملحوظ في كل من الميتوكوندريا والشبكة الإندوبلازمية الخشنة في خلايا كبد الجرذان التي تم معالجتها بعقار التاموكسفين.

كما أدى معالجة الجرذان بالتاموكسفين إلى حدوث التغيرات الهستولوجية فى كبد الجرذان مثل ظهور فراغات دهنية وفجوات وتغيرات هيستولوجية فى خلايا الكبد ووجود إتساع فى الجيوب الدموية مع ظهور بعض التجمعات الكروماتينية فى الخلايا، وكذلك ظهور بعض من الأنسجة الليفية.

وقد أظهرت الدراسة الهستوكيميائية مايلى :

أظهر الفحص للحمض النووى الداى أكسى ريبونيوكليك عن طريق جهاز تحليل الصورة إلى نقص فى محتوى الحامض النووى الداى أكس ريبونيو كليك. وكذلك نقص فى كمية عديدات التسكر المخاطية وفى كمية البروتين في خلايا كبد الجرذان التي تم معالجتها بعقار التاموكسفين، كما أظهر الفحص بالمجهر الألكترونى أن معالجة الجرذان بفيتامين د وهـ قد أدى إلى تقليل نسبة تأثير عقار التاموكسفين على الكبد.

ومن الممكن أن ننصح باستخدام فيتامين ج و هـ وهى مواد طبيعية مضادة للأكسدة للمرضى المصابين بالأورام السرطانية للوقاية من تأثير العقارات المستخدمة فى العلاج وخصوصاً عقار التاموكسفين مما له من تأثير واضح على الكبد.