Original Article

Histological Evaluation of The Effect of Tamoxifen on The Retinas of Adult Female Albino Rats and The Potential Protective Capacity of Melatonin Versus Lutein

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ABSTRACT

Introduction: Tamoxifen is the most frequently prescribed therapy for estrogen positive breast cancer. The duration of a therapeutic course might range between five to ten years.

The purpose of this study is to examine histological reactions to Tamoxifen on retinas of adult female albino rats and the potential beneficial impact of melatonin versus lutein.

Material and Methods: Sixty adult female albino rats were randomly divided into 6 groups. Control group (I), melatonin administered group (II) rats obtained daily doses of melatonin (10 mg/kg), Lutein administered group (III) rats obtained lutein daily (50 mg/kg), Tamoxifen administered group (IV) rats obtained daily Tamoxifen (5mg/kg), Tamoxifen and melatonin treated group (V), Tamoxifen and lutein treated group (VI) rats received the aforementioned doses. At termination of the study (4 weeks), the animals were anesthetized, sacrificed, and retinal samples were collected for histological, immunohistochemical, and electron microscopic examination.

Results: Tamoxifen was found to be deleterious to retinal tissue. Both total retinal thickness and number of ganglion cells showed an extremely significant reduction. There was a significant rise in GFAP and Caspase-3 immunohistochemical reactivity. Ultrastructural alterations revealed degeneration of pigmented epithelium, distortion of photoreceptors, degeneration of outer and inner nuclear layer cells with small shrunken nuclei, intercellular gap expanding, and mitochondrial degeneration. Ganglion cells have notched, irregular nuclei and degraded mitochondria. Coadministration of Lutein exhibited significantly retinal tissue protection than melatonin.

Conclusion: Lutein has a greater protective effect on retinal tissue than melatonin against the potentially harmful effects of Tamoxifen. Patients on long term or high dose tamoxifen therapy were recommended to have regular follow up for possible retinal changes. More studies were recommended on the protective effects of lutein on patients receiving tamoxifen therapy. Various antioxidants in the market should be taken after medical consultation to take benefit of them and avoid their hazardous effects.

Received: 13 July 2023, **Accepted:** 16 August 2023

Key Words: Lutein, melatonin, retina, tamoxifen.

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ISSN: 1110-0559, Vol. 47, No. 3

INTRODUCTION

Breast cancer is the second principal cause of cancer-related mortality among women worldwide. It is more common in women than in men. Women have 100 times the number of instances[1]. Sex, aging, estrogen, family history, gene mutations, and an unhealthy lifestyle are all risk factors that can raise the possibility of getting breast cancer[2]. The estrogen receptor is a primary major target for chemotherapy as over 70 percent of breast cancers tumors are ER-positive. Selective estrogen receptor modulators (SERMs) are chemicals that function as either estrogen receptor agonists or antagonists. Tamoxifen (TAM), one of the most widely recognized SERMs, has been administered in treating breast cancer for over thirty years. Tamoxifen is prescribed for the management of all stages of breast cancer[3].

Tamoxifen is the most frequently prescribed selective estrogen receptor modulator (SERM) used in the management of hormone-receptor-positive, early-stage breast cancer in women and men after surgery, chemotherapy, and radiation to minimize the chance of cancer recurrence. It is also employed in the treatment of terminal or metastatic hormone-receptor-positive conditions. Tamoxifen is additionally prescribed to minimize the probability of breast cancer in women who have not been diagnosed but are at high risk for this deadly malignancy[4].

DOI: 10.21608/ejh.2023.222464.1920

Tamoxifen is a nonsteroidal anti-estrogen that functions as an antagonist in the neurosensory retina as well as the retinal pigment epithelium (RPE). Ocular symptoms have been observed to occur at frequencies ranging from 0.9% to 11%[5]. Melatonin (N-acetyl-5-methoxytryptamine) is a hormonally active, evolutionary-old serotonin derivative. It is the pineal gland's major neuroendocrine secretory product[6]. Melatonin is best known for its ability to regulate circadian rhythms and reduce the pigmentation of vertebrate skin. It additionally acts as a free radical scavenger, triggering complex antioxidative and DNA repair processes as well as having immunomodulatory, thermoregulatory, and anticancer characteristics[7]. Melatonin, as a cytoprotective molecule, reverses inflammatory damage[8].

Melatonin is produced in both the retina and the pineal gland, where it acts as an intrinsic neuromodulator. It may act as a beneficial factor in ophthalmic disorders including photokeratitis, cataracts, retinopathy of prematurity, ischemia/reperfusion injury, and human refractory central serous chorioretinopathy. Furthermore, earlier studies have shown that melatonin has a preventive impact against retinal glaucoma, uveitis, and type 2 diabetes damage[9]. Melatonin has been proposed as an important adjuvant with chemotherapeutic drugs to boost their efficacy in breast cancer patients[10].

Lutein (3R,3'R,6'R-, -caroten-3,3′-diol) is an oxygenated component of carotenoids (oxycarotenoids). It is unable to be generated in mammals as a result of a deficiency in the carotenoid synthesis enzyme. Its existence in human tissues is solely due to dietary sources. As a result, optimal carotene consumption in the diet is critical[11]. It is abundant in leafy green vegetables, including spinach, kale, broccoli, and zucchini[12].

The distribution of lutein throughout tissues is comparable to that for various carotenoids, but it is found exclusively in the center of the retina, together with zeaxanthin, and is referred to as macular pigments, which safeguard the eye from oxidative injury and boost visual function[13]. This implies that Lutein may be involved in retinal function and viability[14]. Many basic and clinical research investigations have indicated that lutein has anti-oxidative and anti-inflammatory properties in the eye, implying that it may be beneficial for both the avoidance and management of ocular disorders such as age-related macular degeneration, diabetic retinopathy, retinopathy of prematurity, myopia, and cataract[15]. The aim of this work is to investigate the histological impact of Tamoxifen on the retina of adult female albino rats and the possible protective effect of melatonin versus lutein.

MATERIAL AND METHODS

Drugs

Tamoxifen is marketed in the form of tablets 20mg manufactured by AMRIYA Pharmaceutical Industries, Amriya, Alexandria City, Egypt.

Melatonin 5 mg tablets were manufactured by Nature's Bounty, Inc. in the USA, FDA treats melatonin as a dietary supplement. It was obtained in Egypt by the health shop company.

Lutein 40 mg soft gels were manufactured by Nature's Bounty, Inc. in the USA, FDA approved, NDC number 74312049026 and obtained in Egypt by the health shop company.

Animals

60 adult albino female rats ranging between 150 and 200 grams were selected in the present research. They were confined in six sanitary stainless-steel cells in a well-ventilated space. They had unrestricted access to food and water. To keep every rat in a typical and healthy environment, constant monitoring and cleanliness were utilized. We monitored the behavior and general well-being of the animals. The rats were classified equally into 6 main groups at random as follow:

Group I (Control): served as the control for the other groups, each received 1 ml of distilled water.

Group II (melatonin-treated): Melatonin was administered to each animal at a dosage of 10 mg/kg/ day aqueous solution orally by gastric tube for 4 weeks[16].

Group III (lutein-treated): each got lutein orally at a dosage of 50 mg/kg/ day as an oily solution for 4 weeks[15].

Group IV (tamoxifen-treated group): each was administered tamoxifen orally as a 5 mg/kg/day aqueous solution[17].

Group V (tamoxifen and melatonin-treated group): each was given tamoxifen by the same dose, route, and period of group IV and oral melatonin by the previous doses and period as in group II.

Group VI (tamoxifen and lutein-treated group): each was given tamoxifen by the same dose, route, and period of group IV and oral lutein at the previous doses in group III.

At the termination of the study (4 weeks), animals from all groups were anesthetized with inhaled diethyl Ether 1.9% (0.08 ml / Liter of container volume)[18], sacrificed, and both eyeballs were dissected. Histological, immunohistochemical, and transmission electron microscopy examinations were performed on specimens.

Histological and Immunohistochemical studies

All rat eyes were fixed in a 10% buffered formalin solution, dehydrated in increasing ethanol concentrations, and embedded in paraffin. For staining, serial paraffin sections of 5-6 μm thickness were cut. These sections were stained with:

1. Haematoxylin and Eosin (H&E): used to examine the histological morphology of different groups[19].
2. Immunohistochemical staining

A-Anti-glial fibrillary acidic protein (GFAP): Glial cell reactivity was evaluated. The sections were then incubated with a primary rabbit polyclonal anti-GFAP antibody (1: 1000, 4°C, overnight; DAKO, Carpinteria, California, USA), and then with a biotinylated goat antirabbit secondary antibody from DAKO. Negative controls were left out of the primary antibody. Malignant glioma was a positive control[20].

B-Caspase-3 (apoptosis marker): Anti-Caspase-3 (rabbit polyclonal antibody from Thermo Science in Fermont, California) was used at a concentration of 1/200. Ready-to-use rabbit polyclonal antibody (CAT-No. RB-3425-R2) was used as the primary antibody for caspase-3. Counterstaining was done with hematoxylin. As a positive control for caspase-3, normal lymphoid tissue was served. Negative controls were left out of the primary antibody[21].

3-Transmission Electron Microscopic Analysis (TEM)

The eyeballs were fixed in phosphate buffer containing 1% glutaraldehyde and 4% paraformaldehyde for 1 hour. Each eyeball's anterior section was removed, and the posterior segments were sliced into thin sections, dehydrated, and then inserted in epoxy resin after being subsequent fixed with 1% osmium tetroxide. Uranyl acetate and lead citrate were employed to stain ultrathin retinal sections (70-90 nm) produced on copper grids. The ultrastructure of the tissues was examined using a JEM-1400plus electron microscope at the Electron Microscopy Unit, Faculty of Science, Alexandria University[22].

Morphometric analysis

All quantitative data were acquired using Image J's Image analyzer program version K1.45. We compared the means of retinal thickness, ganglion cell count, mean area percentage of GFAP expression, and mean intensity of caspase-3 expression in experimental groups to the control, and in group VI (Tamoxifen plus lutein treated) to group V (Tamoxifen plus melatonin treated).

Statistical analysis

SPSS version 16.00 (Chicago, Illinois, United States) was applied for the statistical analysis. The mean standard deviation was selected to express all data. To compare groups, a one-way analysis of variance (ANOVA) and the least significant difference (LSD) post-hoc test were performed. $p < 0.05$ was regarded as significant.

RESULTS

Histological Results

a-LM examination of hematoxylin and eosin: retinal sections stained with hematoxylin and eosin from the control group I, the melatonin-treated group II, and the lutein-treated group III revealed normal histological morphology with well-organized retinal layers. The retinal thickness of Groups II and III did not differ significantly from the control group ($P > 0.05$) (Table 1, Histogram 1). The photoreceptor layer (PRL) was composed of rods and cones with vertically striated processes. The outer nuclear layer (ONL) contained deeply stained nuclei of rods and cones. Between the outer and inner nuclear layers, the outer plexiform layer (OPL) appeared as a thin, pale area. INL contained bipolar nerve cells with large, pale nuclei. The inner plexiform layer (IPL) was visible as a light reticular area. The ganglion cell layer consisted of one layer of cells with large vesicular nuclei (Figures 1A,1B,1C) and (Figures 2A,2B,2C). The ganglion cell count of groups II and III did not differ significantly from the control group ($P > 0.05$) (Table 1, Histogram 2).

The tamoxifen-treated group (group IV) demonstrated a statistically significant reduction in the total thickness of the retina ($P < 0.001$) (Table 1, Histogram 1). Several photoreceptor processes exhibited focal loss, degradation, and an irregular appearance. The outer layer of plexiform was extremely thin and damaged. There was a reduction in INL and ONL with significant vacuolation of their cells resulting in the expansion of intercellular gaps. Some cells had small, dark nuclei, while others had dissolved nuclei (cell ghosts). There was thinning of the IPL and vacuolation between its fibers. GCL demonstrated extensive vacuolation surrounding shrunken nuclei. Some ganglion cells were lost, and glial cells were observed with their flattened, darkly stained nuclei (Figures 1D, 2D). Ganglion cell count morphometric analysis indicated a statistically significant decline when contrasted with the controls ($P < 0.001$) (Table 1, Histogram 2).

Tamoxifen plus melatonin treatment (group V) exhibited a significant decline in total retinal thickness ($P < 0.05$) (Table 1, Histogram 1). The retinal layers were arranged in a regular pattern. There were gaps between the nuclei of the ONL. Some vacuolation and dark nuclei were visible in the inner nuclear layer. Some ganglion cells were still lost, while vacuolations were seen in others. The GCL still contained small, dark glial cells (Figures 1E, 2E) Ganglion cell count morphometric analysis significant decline compared to the control group ($P < 0.05$) (Ttable 1, Histogram 2).

Tamoxifen plus lutein treatment (group VI) exhibited an insignificant variation in the total thickness of the retina ($P > 0.05$) (Table 1, Histogram 1). The retinal morphology was almost normal. The photoreceptor layer exhibited a striated pattern with outer and inner segments. ONL and INL seemed to be normal. Both IPL and OPL preserved their reticular appearance. Some ganglion cells had large nuclei that were all euchromatic, but some still had small nuclei that were stained darkly (Figures 1F, 2F). An insignificant change in ganglion cell count morphometric analysis was found when compared to the control group ($P > 0.05$) (Table 1, Histogram 2).

b- Toluidine blue-stained semi-thin sections of Group I, Group II, and Group III retinal sections revealed typical photoreceptor layers and densely packed dark nuclei of the outer nuclear layer. The INL showed larger and paler nuclei. The OPL and IPL appeared as pale, reticular areas.

GCL showed one layer of large vesicular nuclei. Intact nerve fiber layers and the inner limiting membrane were observed (Figures 3A,3B, 3C). Group IV (Tamoxifen-treated group) revealed destroyed photoreceptors in the photosensitive layer. The ONL showed irregularity and loss of nuclei. Some INL nuclei were lost, while others were tiny and pycnotic. There was increased intercellular space. The GCL showed loss of the nuclei, and some nuclei were small and darkly stained (Figure 3D). Group V (Tamoxifen and Melatonin-treated group) demonstrated some enhancements in retinal structure. The PRL appeared normal as in control group. Among the nuclei of the ONL, there were empty gaps. The inner nuclear layer showed some vacuolation and dark nuclei and also the IPL showed some vacuolation. The GCL showed a loss of nuclei (fig. 3E). Group VI (Tamoxifen and Lutein-treated group) showed retinal sections with almost normal retinal architecture, almost like the control (Figure 3F).

Immunohistochemical Results

a-GFAP (glial fibrillary acidic protein): Group I (control group), Group II (melatonin treated group), and Group III (lutein treated group) revealed negative immune reactions in all retinal layers except for a slight positive cytoplasmic reaction present in the form of brown granular reaction in the Müller cells, ganglion cells, and nerve fiber layers (Figures 4A,4B, 4C). The morphometric analysis of Groups II and III revealed an insignificant change in the area percentage of GFAP expression relative to the control group ($P > 0.05$) (Table 1, Histogram 3).

Group IV (Tamoxifen-treated group) revealed a highly positive cytoplasmic immunological response. The extent of brown staining through the entire retinal thickness increased markedly. It responded strongly positive to immunological stain. A high immunological response in the outer and inner nuclear layers was detected (Figure 4D). Morphometric analysis revealed a significant increase in the area % of GFAP expression relative to the control group ($P < 0.001$) (Ttable 1, Histogram 3).

Group V (Tamoxifen and Melatonin-treated group) showed a positive immunological response. The positive cytoplasmic reaction was seen in almost all retinal layers, but it was weaker than in the tamoxifen-treated group (Figure 4E). Morphometric analysis showed a highly significant rise in the area percentage of GFAP expression relative to the control group ($P < 0.001$) (Table 1, Histogram 3).

Group VI (Tamoxifen and lutein-treated group) revealed a negative immunological reaction in the outer limiting membrane of retinal sections. The outer nuclear layer and the outer plexiform layer both showed a very mild positive immunological response. There was a negative immunological reaction in the inner nuclear layer, except for Muller cells. The inner plexiform layer, ganglion cell layer, nerve fiber layer, and inner limiting membrane manifested a weak positive immunological reaction (Figure 4F). Morphometric analysis revealed no statistically significant differences in the area% of GFAP expression with respect to the control group ($P > 0.05$) (Table 1, Histogram 3).

b-Anti-Caspase-3 antibody: Group I (control group), Group II (melatonin-treated group), and Group III (lutein-treated group) showed negative cytoplasmic immune expression for caspase-3 (Figures 5A,5B,5C). Group II and Group III morphometric analysis showed insignificant differences in caspase-3 immune reactivity with respect to the control group ($P > 0.05$) (Table 1, Histogram 3).

Group IV (Tamoxifen-treated group) showed a strongly positive cytoplasmic immune reaction in the outer, inner nuclear, and ganglion cell layers in the form of brown granular reaction. Strong immunological reactions were also observed in the photoreceptor inner segments. The extent of staining along the entire retinal thickness increased significantly (Figure 5D). Morphometric analysis showed a highly significant increase in caspase-3 reactivity compared to the control group ($P < 0.001$) (Table 1, Histogram 3).

Group V (Tamoxifen and Melatonin-treated group) showed a moderately positive cytoplasmic immune reaction compared to the control group (Figure 5E). Morphometric analysis revealed a significantly substantial rise in caspase-3 reactivity relative to the control group ($P < 0.001$) (Table 1, Histogram 3).

Group VI (Tamoxifen and Lutein-treated group) exhibited the same negative immunological response as the control group (Figure 5F). Morphometric analysis revealed an insignificant difference in caspase-3 immune reactivity in contrast to the control group ($P > 0.05$) (Table 1, Histogram 3).

Electron microscopic Results

Group I (control group) and Group III (lutein-treated): Transmission electron microscopic analysis of rat retinal sections from the control group (group I) and lutein-treated (group III) revealed a pigmented epithelial cell having a large oval euchromatic nucleus with a regular normal nuclear membrane and an intact Bruch's membrane. The nuclear membrane appears normal. The cytoplasm contained Golgi bodies, rough endoplasmic reticulum, smooth endoplasmic reticulum, and free ribosomes. There were basal membrane infoldings and intact mitochondria. Apical microvilli protruded between photoreceptor outer segments and were pigmented with melanin (Figure 6A). The photoreceptors were densely packed, with outer segments (OS) and inner segments (IS), with mitochondria (M) at the base of their cilia (Figure 6B). The outer nuclear layer looked like it had cells with clustered heterochromatin and sparse cytoplasm with normal mitochondria. There is minimal intercellular space between the cells (Figure 6C). Bipolar cells with euchromatic, spherical nuclei were found in the inner nuclear layer (Figure 6D). Amacrine cells had an electron-lucent cytoplasm and a euchromatic nucleus. The inner plexiform layer had nerve fibers with

intact mitochondria (Figure 6E). The ganglion cell layer had large, spherical euchromatic nuclei with a normal trilaminar nuclear membrane and normal mitochondria (Figure 6F).

Group II (Melatonin-treated): The analysis of melatonin-treated rat retinal sections (Group II) by transmission electron microscopy showed normal pigmented cells with a large oval euchromatic nucleus. There were basal membrane infoldings and intact mitochondria. The apical microvilli between the photoreceptor's outer segments appeared normal. Melanin pigments were present. Normal Golgi bodies were observed in the cytoplasm (Figure 7A). With their lamellar discs and connected cilium, the photoreceptors' outer segments appeared normal (Figure 7B). Normal-appearing outer nuclear layer cells contained condensed heterochromatin. There were expanded intercellular gaps to some degree (Figure 7C). The inner nuclear layer included euchromatic, spherical nuclei (Figure 7D). Amacrine cells appeared normal with their euchromatic nuclei. Besides, Muller cell processes appeared between the nuclei (Figure 7E). The ganglion cell had an intact nuclear membrane and a euchromatic nucleus. Mitochondria were present and normal in the cytoplasm (Figure 7F).

Group IV (Tamoxifen-treated): retinal sections from rats treated with Tamoxifen (Group IV) revealed pigmented cells with small oval nuclei that had shrunk, degenerating mitochondria with broken cristae, and degenerating and vacuolating areas. In certain areas, the apical microvilli were damaged or even lost (Figure 8A). The photoreceptor layer exhibited degenerating lamellar discs and degeneration areas (Figure 8B). The outer nuclear layer exhibited degeneration, shrunken nuclei, and expanded intercellular gaps (Figure 8C). The inner nuclear layer exhibited damaged nuclei, damaged nuclear membranes, and degraded mitochondria with presence of cell remnants (Figure 8D). An amacrine cell with a shrunken, irregular nucleus and degenerating mitochondria (Figure 8E). The ganglion cells had irregularly notched nuclei and degraded mitochondria (Figure 8F).

Group V (Tamoxifen and Melatonin-treated): A transmission electron microscopy analysis of rat retinal sections from the Tamoxifen and Melatonin-treated group (Group V) revealed a pigmented cell with an irregular nucleus. Some mitochondria were normal, but others had degenerated cristae while Golgi's body was degenerating. In the cytoplasm, degeneration and vacuolations were still visible. The apical microvilli had zones of degeneration (Figure 9A). The photoreceptor layer exhibited degenerated lamellar discs and degeneration. Inner-segment mitochondria were small and degenerating (Figure 9B). The outer nuclear layer had degenerated nuclei. A portion of the inner plexiform layer exhibited degeneration (Figure 9C). The inner nuclear layer had both normal and degenerating nuclei with cell remnants. The nucleus of the Muller cell was irregularly shaped (Figure 9D). Amacrine cells with irregularly notched nuclei and degenerating mitochondria were observed (Figure 9E). Normal euchromatic nuclei and degenerating mitochondria were observed in the ganglion cell (Figure. 9F).

Group VI (Tamoxifen and Lutein-treated): A transmission electron microscopy study of rat retinal slices from the group treated with Tamoxifen and Lutein (Group VI) revealed a picture almost identical to the control. The pigmented cell with its large, oval, euchromatic nucleus was observed. The normally appearing nuclear membrane, mitochondria, and melanin pigments were observed but the apical microvilli showed some destruction (Figure 10A). The photoreceptors' outer and inner segments (OS and IS), which had mitochondria (M) and cilia linking the inner and outer segments, appeared normal (Figure 10B). The outer nuclear layer cells included vacuoles and condensed heterochromatin in their sparse cytoplasm. There was little intercellular space between the cells (Figure 10C). The inner nuclear layer was characterized by euchromatic, spherical nuclei with a typical nuclear membrane. A portion of the Muller cell looked to have a compacted heterochromatic nucleus (Figure 10D). The cytoplasm of amacrine cells was electron-lucent and had a euchromatic nucleus. The inner plexiform layer contains intact nerve fibers (Figure 10E). The ganglion cell layer displayed a large euchromatic nucleus (Figure 10F).

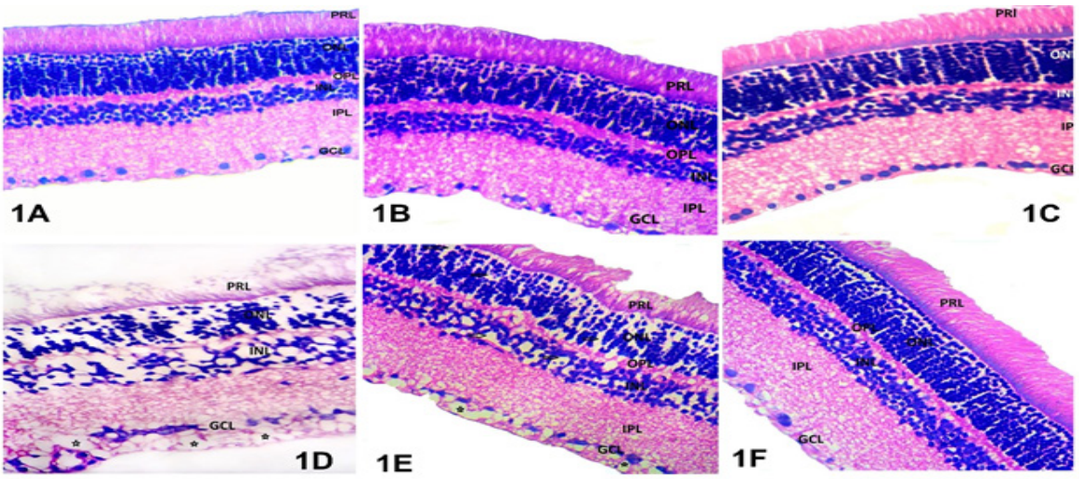


Fig. 1: (1A) control group (I) Photoreceptors are visible as vertical striations (PRL). ONL contains typical, densely packed dark photoreceptor nuclei, a narrow, pale zone created by the nerve outer plexiform layer (OPL), and the large, pale nuclei of bipolar nerve cells. The IPL is represented by the pale reticular region. One cell layer with big vesicular nuclei (GCL). (1B) Melatonin-treated (groupII) retinal layers show their typical appearance. The photoreceptor layer (PRL), the outer and inner nuclear layer (ONL-INL), the outer and inner plexiform layer (OPL-IPL), and the ganglion cell layer (GCL). (1C) Lutein treatment (group III) demonstrates normal retinal layers. The photoreceptor layer (PRL), ganglion cell layer (GCL), outer and inner nuclear layer (ONL-INL), and inner plexiform layer (IPL) are all visible. (1D) Tamoxifen treated (group IV) shows photoreceptor degeneration (PRL). Vacuolations and loss of outer nuclear layer nuclei (ONL). The inner nuclear layer (INL) was characterized by small, darkly stained nuclei, vacuolations, and nuclear loss. The Ganglion cell layer demonstrated extensive clear areas (*). Reduced thickness of the outer plexiform layers (arrow). (1E) Tamoxifen and Melatonin treated (group V) demonstrating normal retinal layers. There are still some gaps between the outer and inner nuclear layers' nuclei (arrow). In the ganglion cell layer, there was vacuolation, and some nuclei were lost (*). The outer plexiform layer is still thin and vacuolated (OPL). (1F) Tamoxifen and Lutein treated (group VI) showing normal retinal architecture. All layers appeared with their regular patterns and appeared normal. The ganglion cell layer showed some vacuolations (*). (Hx. & E. x 200)

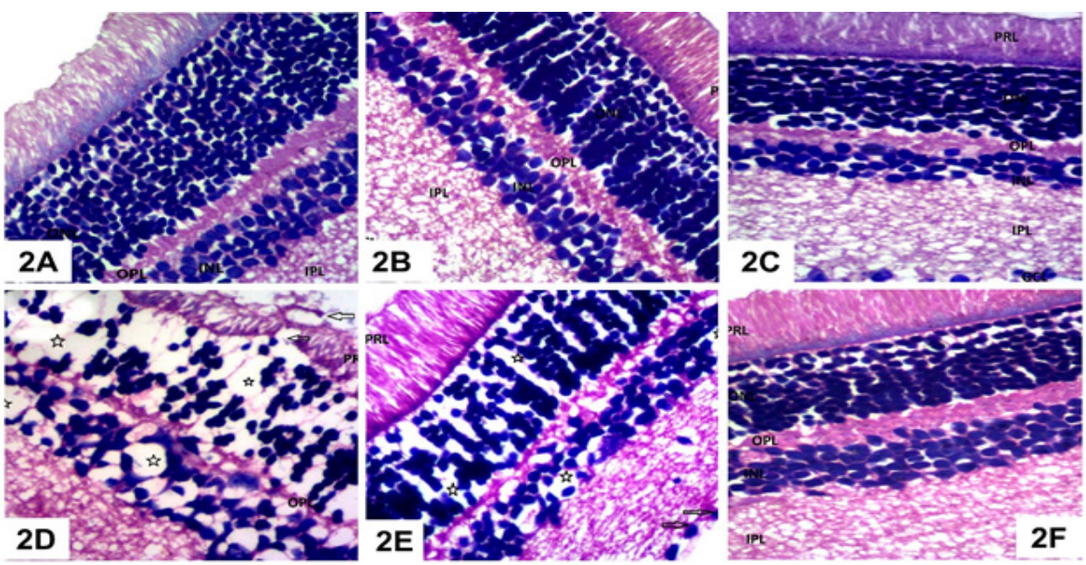


Fig. 2: (2A) Control group (I) revealed the outer nuclear layer (ONL), composed of darkly stained nuclei of rods and lighter nuclei of cones. Inner Nuclear Layer (INL) nuclei were lightly stained and densely packed. Note the outer and inner plexiform layers (OPL-IPL). (2B) Melatonin treated (group II) demonstrates normal retinal layers. Vertical striations were visible as photoreceptors (PRL). The dark nuclei of the ONL were visible. (INL) revealed nuclei with light staining. Large, faintly stained nuclei could be seen in the Ganglion cell layer (GCL). (2C) Lutein treated (group III) shows normal retinal layers. The photoreceptor layer (PRL), ganglion cell layer (GCL), outer and inner nuclear layer (ONL-INL), and inner plexiform layer (IPL) are all visible. (2D) Tamoxifen treated (group IV) demonstrating irregular and focal loss of photoreceptors (PRL) and a damaged outer limiting membrane (arrow). Vacuolations and loss of nuclei in the outer and inner nuclear layers (*), along with thinning and destruction of outer plexiform layer (OPL), were seen. (2E) Tamoxifen and Melatonin treated (group V). The photoreceptors appear to have an intact and regular outer limiting membrane (PRL). The outer and inner nuclear layers still have some gaps between their nuclei (*). Small and relatively darkly stained ganglion cells are still present (arrow). (2F) Tamoxifen and Lutein treated (group VI) showing normal photoreceptors (PRL) and an intact outer limiting membrane. The outer nuclear layer (ONL) appeared normal with its closely packed dark nuclei. The inner nuclear layer (INL) showed larger and lighter nuclei with no spaces or vacuolations. The outer and inner plexiform layers (OPL, IPL) appeared normal. (Hx. & E. x 400)

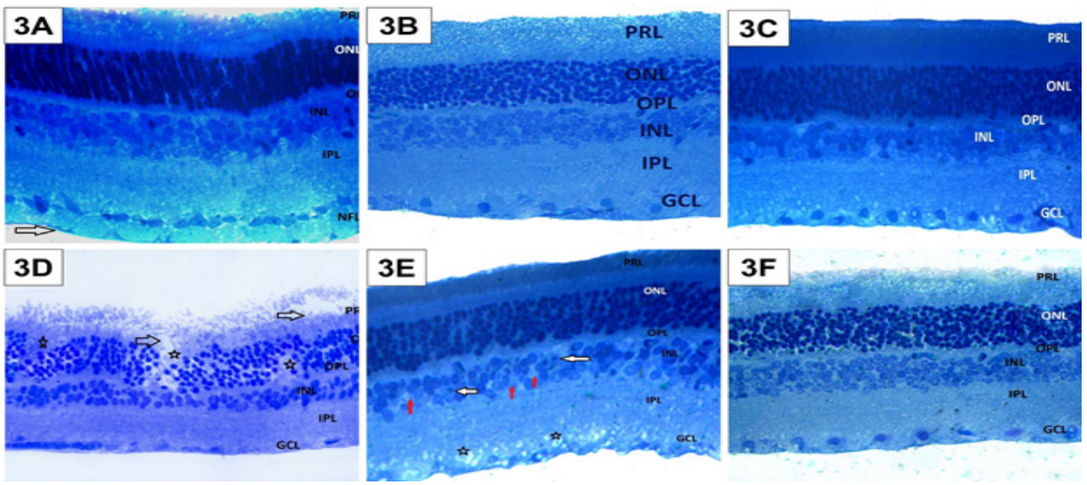


Fig. 3: (3A) Control group reveals normal photoreceptor layer (PRL). Normally arranged dark nuclei of outer nuclear layer (ONL). Lightly stained nuclei of inner nuclear layer (INL). Normal outer and inner plexiform layers (OPL), (IPL). Ganglion cell layer with large lightly stained nuclei (GCL). Normal nerve fiber layer (NFL). Intact inner limiting membrane (arrow). (3B) Melatonin treated (group II) shows normal retinal layers. Photoreceptor layer (PRL), Outer nuclear layer (ONL), Outer plexiform layer (OPL), Inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL). (3C) Lutein treated (group III) shows the normal appearance of the different retinal layers. Ganglion cell layer (GCL), photoreceptor layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and photoreceptor layer (PRL). (3D) Tamoxifen treated (group IV) shows loss of some photoreceptors in the PRL (arrow). ONL shows loss of nuclei (star). Widening of intercellular spaces between INL cells. GCL shows loss of some nuclei, and some are small pycnotic. (3E) Tamoxifen and Melatonin treated (group V) shows regularly arranged retinal layers. Normal photoreceptor layer (PRL). ONL shows some spaces between the cells. Inner nuclear layer is characterized by devoid spaces (red arrow) and dark nuclei (white arrow). The GCL shows some vacuolation (star) and loss of nuclei. (3F) Tamoxifen and Lutein treated (group VI) shows normal retinal layers almost like the control. Photoreceptor layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) are all components of the retina. (Toluidine blue *200)

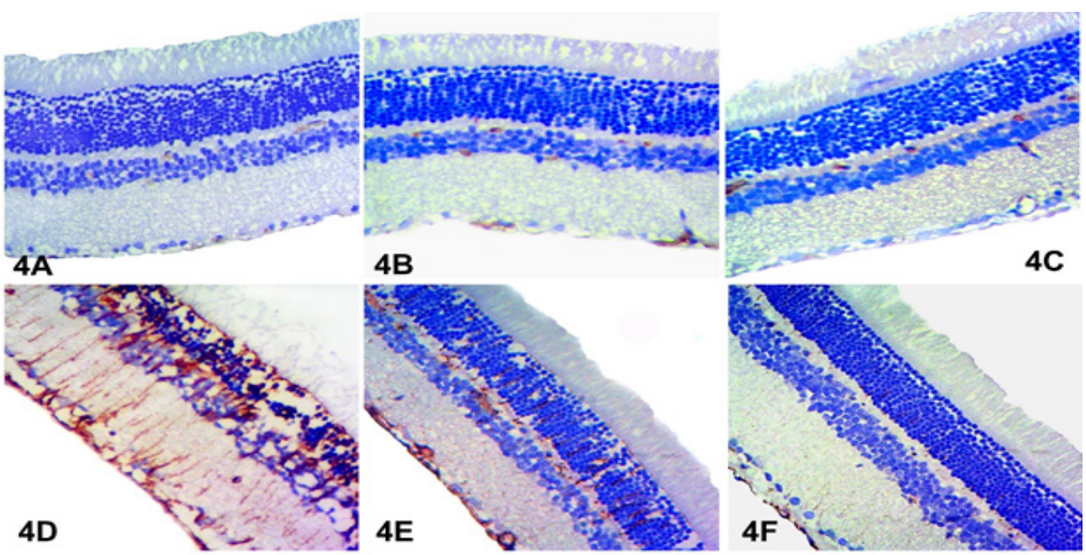


Fig. 4: A section of control retina (4A) demonstrates a weak typical immune response in the Müller and nerve fiber layer. (4B) Melatonin treated (group II) exhibiting a normal immunological response in the Müller and nerve fiber layers. (4C) Lutein treated (group III) shows an immune reaction just like the control group. (4D) Tamoxifen treated (group IV) showing a strongly positive immune reaction throughout the whole retinal thickness. (4E) Tamoxifen and Melatonin treated (group V) immunological responses were detected in the whole retinal thickness. (4F) Tamoxifen and Lutein treated (group VI) exhibit some positive responses in the outer plexiform layer, nerve fiber layer, and inner limiting membrane. (GFAP * 200)

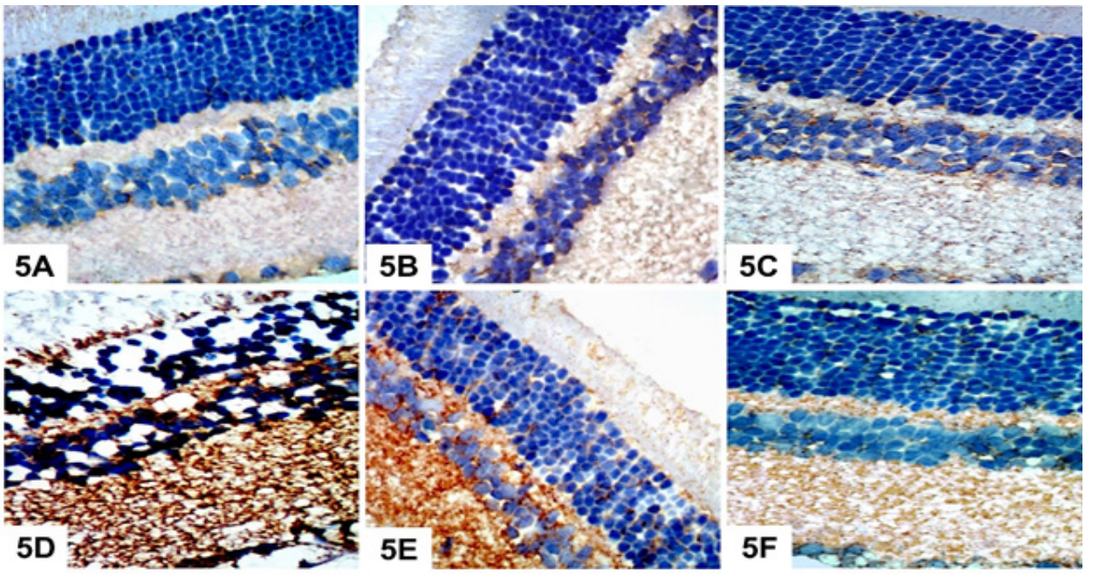


Fig. 5: A section of the retina (5A) exhibiting a negative immunological reactivity in all retinal layers in the control group. (5B) Melatonin treatment (II) results in a negative immune response. (5C) The immunological response to lutein (III) was negative. (5D) Tamoxifen treatment (IV) elicits an extremely positive immunological response. (5E) Tamoxifen and Melatonin treatment (V) results in a moderately positive immunological response. (5F) Tamoxifen and Lutein treatment results in a negative immune response. (Caspase-3*400)

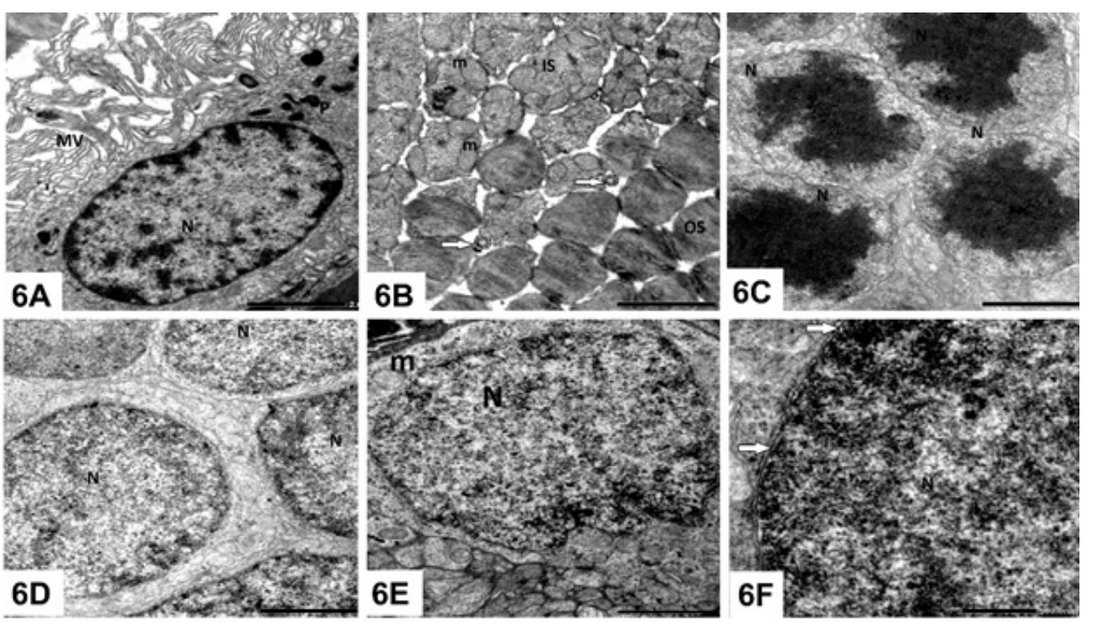


Fig. 6: An electron micrograph of the control group's retina (6A) A normal pigmented epithelium (RPE) with a euchromatic nucleus (N), apical microvilli (MV), melanin pigment (P), basal membrane infoldings (arrow), and mitochondria (m). (TEM * 4000) (6B) Photoreceptor layer (PRL) showing normally packed photoreceptors with their inner segments (IS) containing normal mitochondria (m) and the basal body of the photoreceptor cilia (arrow) and their outer segments (OS). (TEM *4000) (6C) Outer nuclear layer (ONL) appears with packed cells. Condensed chromatin nuclei (n). (TEM*4000) (6D) Normal cells with euchromatic nuclei (N) in the inner nuclear layer (INL). (TEM*4000) (6E) Amacrine cell with euchromatic nuclei (N) and electron-lucent cytoplasm (m). Normal nerve fibers were seen in parts of the IPL. (TEM*4000) (6F) Ganglion cell with intact nuclear membrane and euchromatic nucleus (N) and mitochondria (m). (TEM *12000)

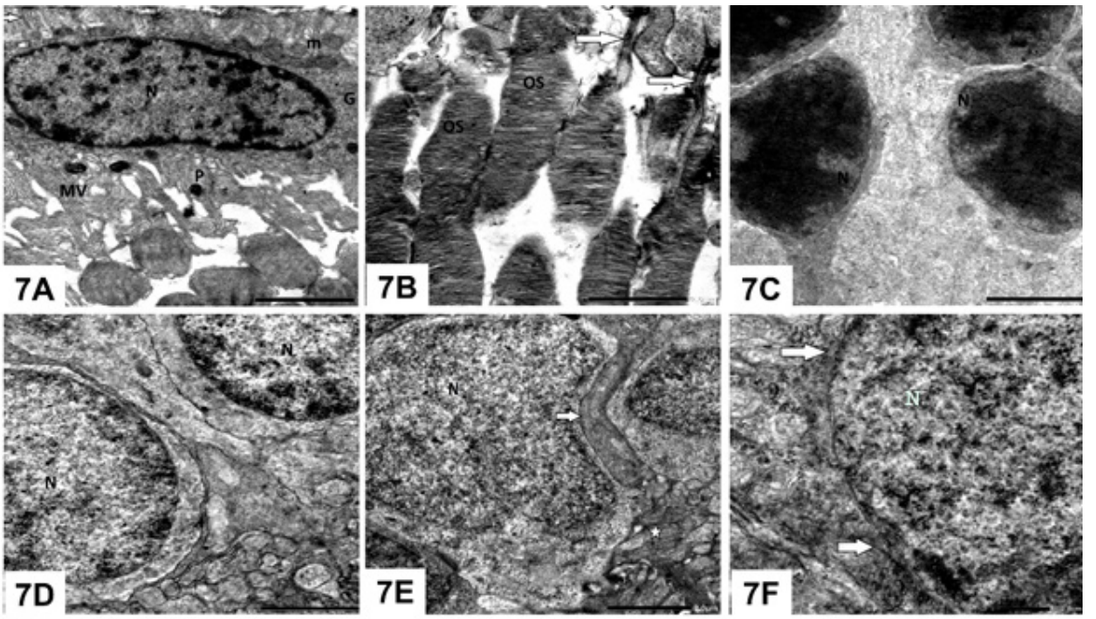


Fig.7: Group III (Melatonin-treated) retina. (7A) Normal RPE with a euchromatic nucleus (N), apical microvilli (MV), melanin pigment (P), basal membrane infoldings (arrow), mitochondria (m), and Golgi body (G). (TEM*4000) (7B) Normal photoreceptor outer segments with parallel lamellar discs (OS) and cilia linking the inner and outer segments (arrow). (TEM*4000) (7C) Outer nuclear layer (ONL) with condensed chromatin (N). There are expanded intercellular gaps to some degree. (TEM*4000) (7D) Inner nuclear layer (INL) appearing normal with euchromatic nuclei (N). (TEM*4000) (7E) Amacrine cell appearing normal with its euchromatic nucleus (N). Besides, muller cell process appeared between the nuclei (arrow). (TEM*4000) (7F) Ganglion cell with normal mitochondria (m), intact nuclear membrane, and euchromatic nucleus (N). (TEM*12000)

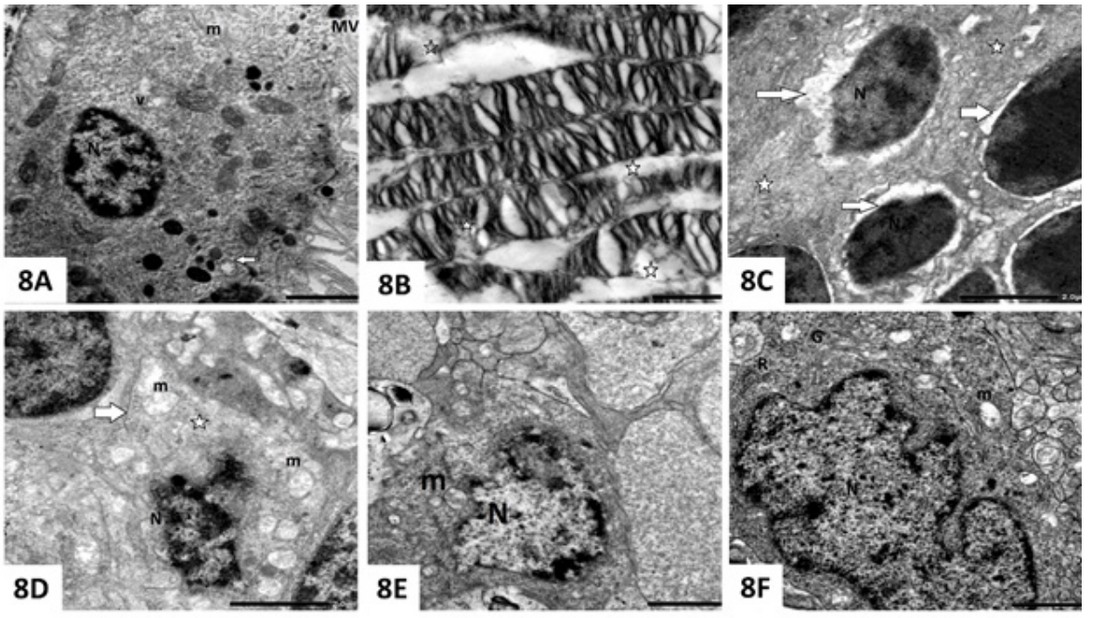


Fig. 8: Group IV (Tamoxifen treated) retina. (8A) RPE with a condensed heterochromatin and a tiny, shrunken nucleus (N). Degenerated mitochondria (arrow) exhibiting cristae disintegration. Some mitochondria have irregular cristae (m). Vacuolation (v) developed in the cytoplasm and damaged microvilli (MV). (TEM*4000) (8B) Photoreceptor layer (PRL) with degenerating photoreceptors Irregularly degenerating lamellar discs Areas of necrosis appeared (star). (TEM*6000) (8C) Outer nuclear layer (ONL) with shrunken degenerated nuclei (N). increased perinuclear cistern (arrow). increased intercellular spaces (star). (TEM*4000) (8D) Inner nuclear layer with a damaged nucleus (N) and mitochondrial degeneration (m). Cell membrane destruction (arrow). A cell remnant (star) appears. (TEM*4000) (8E) Amacrine cell with an unevenly shaped nucleus (N) and mitochondrial degeneration (m). (TEM*4000) (8F) The nucleus (N) of a ganglion cell is unevenly notched and mitochondrial damage (m). The Golgi apparatus (G) has been irregularly destructed. The rough endoplasmic reticulum (R) ,Part of the inner plexiform layer was visible, including degenerating mitochondria in nerve fibers and necrotic areas (star). (TEM*4000)

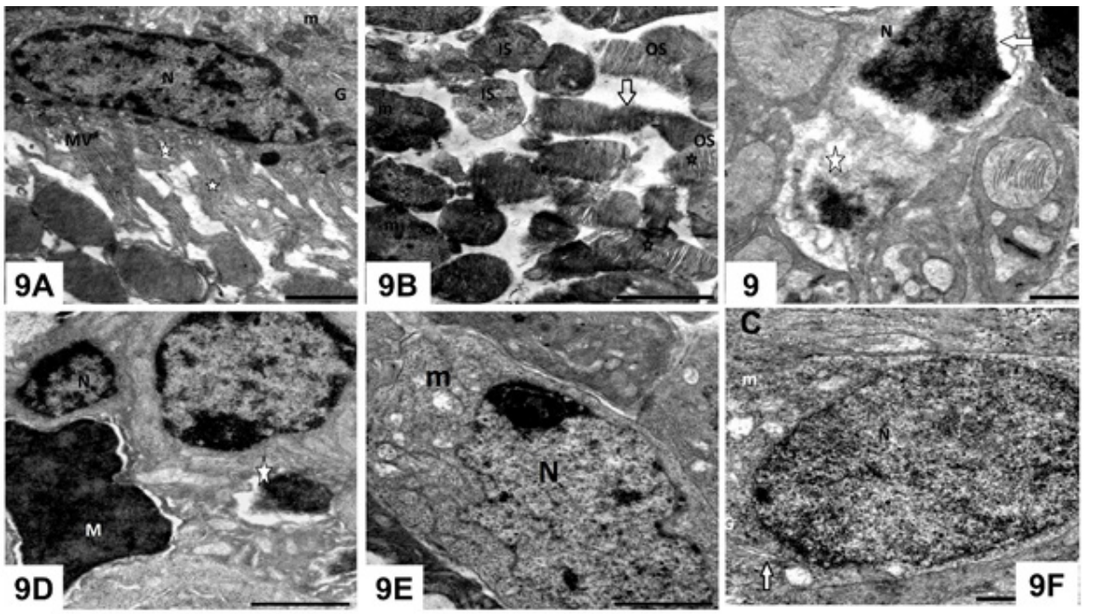


Fig. 9: Group V (Tamoxifen and Melatonin) (9A) Retinal pigmented epithelium (RPE) with an irregular nucleus (N). Degenerated mitochondria (m). Degenerated Golgi (G). normal apical microvilli (mv), degenerated microvilli (star). (TEM*4000) (9B) Photoreceptor layer (PRL) with some degenerated photoreceptors (arrow) and some normal photoreceptors (star). Inner segments (IS) with small, degenerating mitochondria (m). (TEM*4000) (9C) Outer nuclear layer (ONL) with shrunken degenerated nuclei (N). Increased perinuclear cistern (arrow). Remnant of a cell (star). (TEM*6000) (9D) INL has a shrank nucleus (N). A cell remnant (star) appeared. Muller cell with a distorted nucleus (M). (TEM*4000) (9E) Amacrine cell with an uneven nucleus (N) and mitochondrial degeneration (m). (TEM*4000) (9F) Normal euchromatic nucleus (N) in a ganglion cell. mitochondrial breakdown (m). The Golgi apparatus (arrow) has been irregularly deconstructed. (TEM*4000)

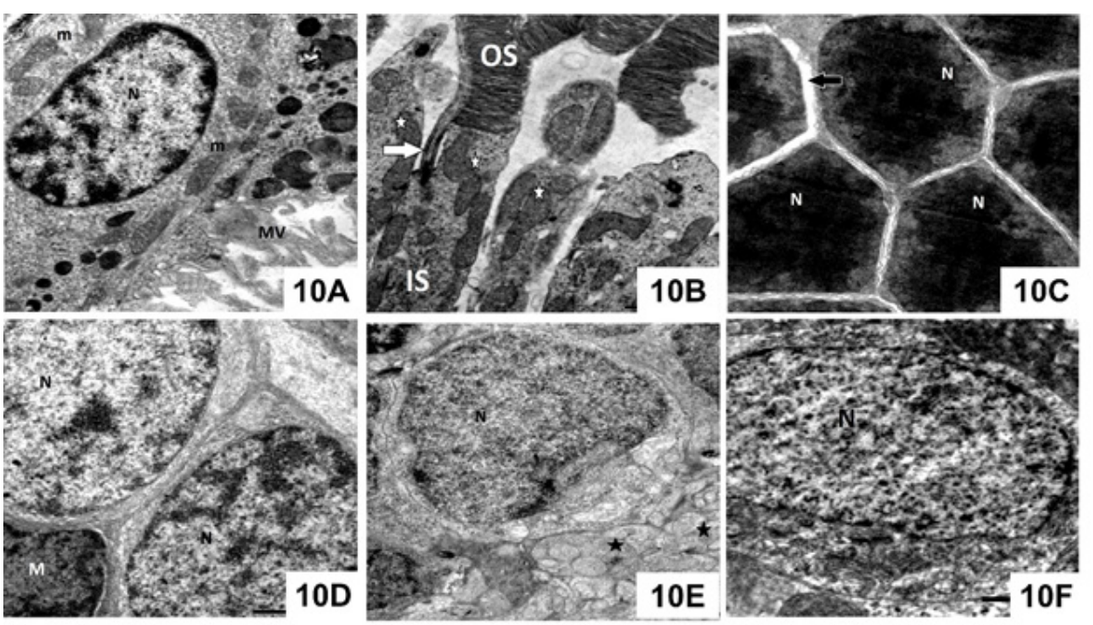
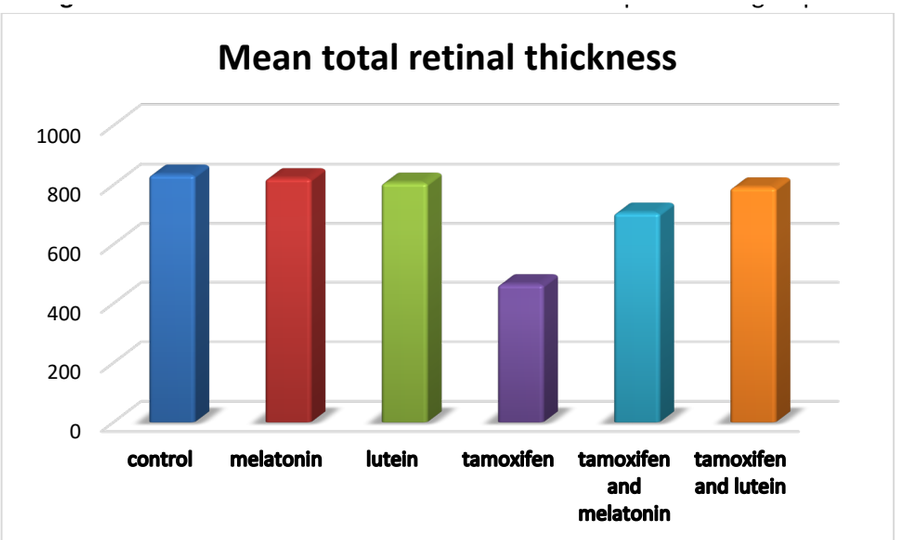


Fig. 10: Group VI (Tamoxifen and Lutein) (10A) RPE with a large nucleus (N). normal mitochondria (m), melanin pigments (P), and destroyed apical microvilli (mv). (TEM*4000) (10B) PRL appears normal. Inner segments (IS) containing normal mitochondria (star). The cilium's L.S. (arrow) connects the inner and outer segments (OS). (TEM*4000) (10C) ONL with normal heterochromatic nuclei (N) and scanty cytoplasm. Perinuclear space is increased in one cell (arrow). (TEM*4000) (10D) INL with normal euchromatic rounded nuclei (N). Part of the Muller cell appeared normal (M). (TEM*4000) (10E) Normal amacrine cell with a euchromatic nucleus (N) and light cytoplasm. Part of the inner plexiform tissue appears normal (*). (TEM*4000) (10F) A normal ganglion cell with a typical euchromatic nucleus (N). (TEM*4000)

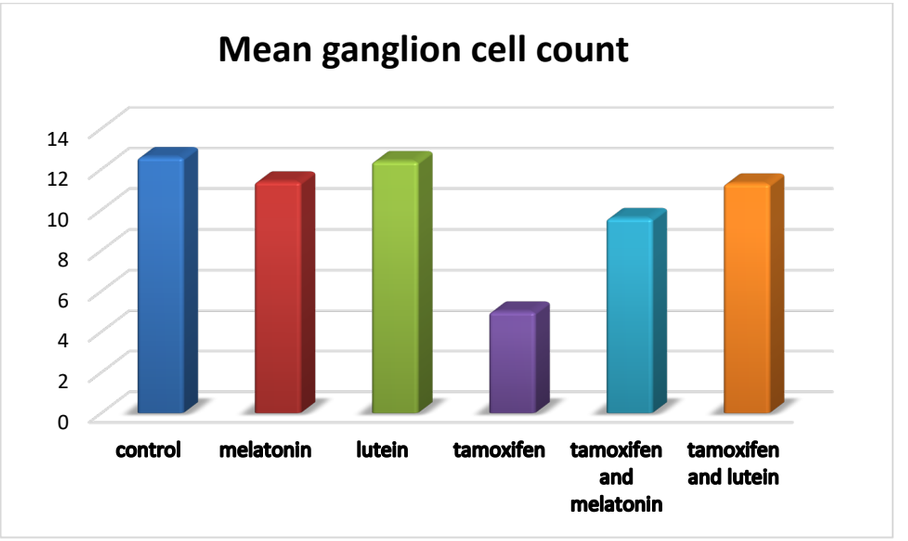
Table 1: Effect of different treatments on mean of total retinal thickness, ganglion cell count and immune reactivity of GFAB, Caspase-3 of all groups of rats

	G I	G II	G III	G IV	G V	G VI	*P- value*
Total retinal thickness	835.8 ± 46.1	823.2 ± 37.2	808.6 ± 32.4	465.9 ± 32	707.9 ± 89.7	792.5 ± 48.8	PII= 0.545 PIII=0.176 PIV=0.000** PV=0.003* PVI=0.068 P°=0.015*
Ganglion cell count	12.6 ± 1.8	11.4 ± 3	12.4 ± 2.9	5 ± 1.7	9.6 ± 1.6	11.3 ± 0.9	PII= 0.351 PIII=0.916 PIV=0.000** PV=0.002* PVI=0.071 P°=0.009*
Area % of GFAB	1.6 ± 0.2	2 ± 0.9	1.9 ± 0.6	20.3 ± 4	3.9 ± 0.9	2.0 ± 0.4	PII= 0.285 PIII=0.278 PIV=0.000** PV=0.000** PVI=0.059 P°=0.000**
Caspase-3 immune reactivity	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	11.3 ± 3.3	1.9 ± 0.5	0.4 ± 0.2	PII= 0.79 PIII=0.086 PIV=0.000** PV=0.000** PVI=0.081 P°=0.000**

P value < 0.05 = Significant. (*) PIII= *P value* of GIII compared to control
P value < 0.001 = Highly significant. (**) PIV= *P value* of GIV compared to control
P° = *P value* of GVI compared to GV PV= *P value* of GV compared to control
PII= *P value* of GII compared to control PVI= *P value* of GVI compared to control

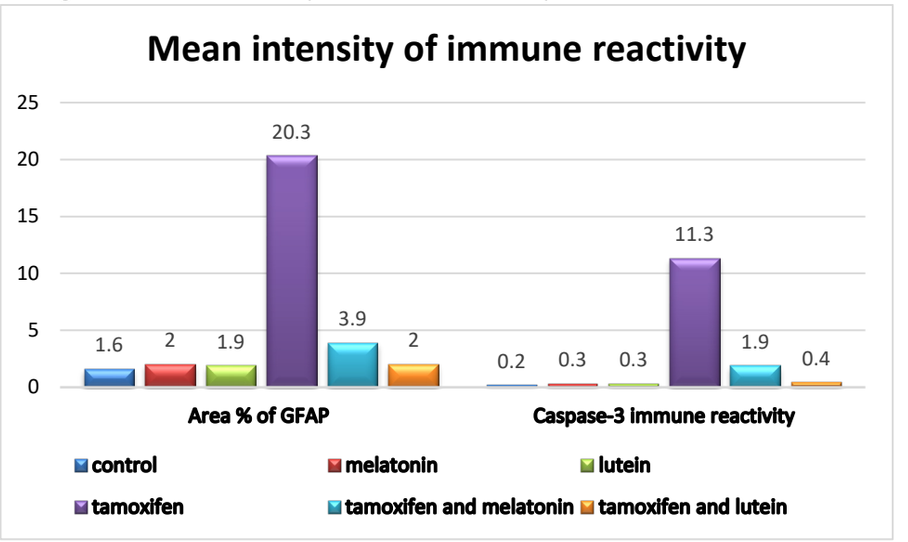


Histogram 1: Mean total retinal thickness in different experimental groups.



Histogram 2: Mean ganglion cell count in different experimental groups.

Histogram 3: Mean intensity of immune reactivity



Histogram 3: Mean intensity of immune reactivity

DISCUSSION

Breast cancer is the highest anticipated cancer in women and the second leading cause of cancer-related mortality in women, after lung cancer[23]. Surgery, radiation, hormone treatment, regular chemotherapy, as well as targeted therapy are routinely combined. Targeted therapy affects molecular targets on tumor membranes with tiny therapeutic molecules and immunological materials[24]. Thus, targeted therapy offers a less toxic, site-specific breast cancer treatment. Breast cancer (BC) targeted therapy focuses on estrogen receptor (ER). Selective estrogen receptor modulators (SERMs), which compete with estrogen for ER binding in breast tissues, are one of the most efficient therapies. Tamoxifen (TAM) the most popular SERM, promotes cancer cell death by downregulating ERs[4].

Since there are significant gender disparities in optical anatomy and pathology, particularly in the retina, this study focused on female rodents. According to mice research, males and females have different retinal structures, and females of reproductive age had better retinal function than males and older females[25,26].

Tamoxifen therapy significantly reduced retinal thickness in this research. Crisóstomo *et al*. (2020) found that tamoxifen patients had thinner choroid and overall retinal thickness than controls, indicating retinal structural changes[27]. Tamoxifen significantly reduced ganglion cell numbers. Bolukbasi *et al*. (2019) found that tamoxifen reduced ganglion cell layer thickness and linked this to choroidal vascular alterations[28]. In this study, tamoxifen produced localized photoreceptor loss, degeneration, and an uneven appearance, with a decrease in INL/ONL thickness. INL and ONL cell vacuolation increased intercellular gaps. These alterations may be attributed to estrogen's neuroprotective activity loss and tamoxifen's apoptotic effects[29].

Tamoxifen inhibits phospholipases C, D, and protein kinase C, which trigger apoptosis. Caspases 3, 8, and 9 are activated[30,31]. Tamoxifen inhibits the electron transport chain and induces mitochondrial dysfunction, resulting in reactive oxygen species (ROS) that combine with polyunsaturated fatty acids to create lipid peroxidation byproducts. ROS can change cellular integrity and permeability by interacting with micro or macro-biostructures. Tamoxifen intoxication causes iron ions to become more reactive and aids in the formation of hydroxyl radicals, the most active reactive oxygen species (ROS) which react with most cellular elements[32]. Tamoxifen's interaction with cholesterol epoxide hydrolase may also produce neuronal and vascular cellular stress[33].

Anti-caspase-3 immunohistochemical labeling showed strong immune responses in the outer, inner nuclear layers, and ganglion cell layers. Photoreceptor inner segments showed a strong positive immunological response. Staining spreads throughout the retina. El-Kashef and El-Sheakh (2019) found that tamoxifen-induced liver damage increased caspase-3 expression and hepatic apoptosis[34]. Tamoxifen increases nitric oxide production causing oxidative stress and activation of caspase-3[35]. Caspase-dependent apoptosis was present along with TAM-induced autophagic cell death[36].

The tamoxifen-treated group had a significantly stronger immunological reactivity to GFAP than the control group in immunohistochemical staining. Staining spread throughout the retina. Microglia and astroglia control the normal activation of glial cells and release of pro-inflammatory cytokines and chemokines to protect neural tissue. Chronic glia activation can damage neural tissue and cause neurotoxicity[37,38]. Müller cells (retinal glial cells) release cytokines that damage retinal neurons and blood vessels[39]. Doshi *et al*. (2014) linked Muller cell dysfunction to tamoxifen retinopathy symptoms[40]. Tamoxifen's glutamate uptake suppression produced neuronal and vascular damage in Muller cells[41].

Transmission electron microscopy showed that rats who were given Tamoxifen had pigmented cells with shrunken small oval nuclei, damaged mitochondria with broken cristae, degeneration, and vacuolations. Apical microvilli have been damaged or absent and the lamellar discs of photoreceptors degraded. The outer nuclear layer nuclei were damaged, and intercellular gaps widened. The mitochondria, nuclear membranes, and inner nuclear layer nuclei have all been damaged. In cell culture, Cho *et al*. (2012) discovered vacuoles in the retinal pigmented epithelium and photoreceptor cytoplasm 3 hours after tamoxifen injection. After 18 hours, most of the cells were dead, with enlarged cell bodies and detached from culture tubes[41].

Muller cells had irregular nuclei and degraded mitochondria. Astrocytes had flat nuclei and numerous Golgi bodies with degraded mitochondria. Tamoxifen produced sporadic apoptotic astrocyte cell death in adult mice[42].

Ganglion cells exhibited degraded mitochondria and irregular nuclei. This is consistent with previous research that indicated tamoxifen-induced ganglion cell damage and reduced complex thickness[43].

Antioxidant co-administration with conventional drugs for breast cancer patients is important[44]. This study examined the retinal effects of melatonin or lutein with tamoxifen. Melatonin which produces by pineal glands regulates circadian rhythm, reproduction, immunological response, and hemostasis. Melatonin contains oncostatic and pro-apoptotic characteristics that alter tumor growth and survival[45].

In this work melatonin plus tamoxifen improved retinal tissue, but not sufficiently. Retinal layers were regular while their nuclei had empty gaps. The inner nuclear layer showed vacuolation and dark nuclei. Some ganglion cells were lost, and others were vacuolated. Tamoxifen and melatonin reduced retinal thickness and ganglion

cell count relative to the control group. Caspase-3 and GFAP staining elicited a mild immunological response. Melatonin provides protection through receptor-independent antioxidants and MT1/MT2 receptors. In mice with experimental nonexudative age-related macular degeneration, it improves visual function and retinal structure[46]. It inhibits mitochondrial cell death, astrocytic activation, and microglial activation[47]. Melatonin protects avascular retinal ganglion neurons, as well as bipolar, horizontal, and amacrine neurons[48]. Melatonin protects newborn rats from oxidative neuronal damage and apoptosis in hemolytic and hyperbilirubinemic conditions[49]. Melatonin reduces the levels of these cytokines in hypoxic microglial cells. Melatonin shields developing RGCs from oxidative, inflammatory, and apoptotic stress[50]. Melatonin safeguarded the neuronal retina and retinal microvasculature from the effects of STZ on mitochondrial dynamics and Ca2+ buffering[51].

On the other hand, some authors established that melatonin could damage retinal cells. After transient high-intensity illumination, it disrupts some photoreceptors. The aging retina may lose photoreceptor cells as a consequence of chronic exposure to natural or artificial light and melatonin[52]. At larger doses, intravitreal melatonin decreased the mean number of retinal ganglion cells (RGC) and caused retinal edema and RGC loss[53].

Lutein, a plant-produced carotenoid, can only be accumulated in animals through food (dark green leafy vegetables). It acts as a free radical scavenger, preserves photoreceptors from phototoxicity, and prevents retinal injury[54].

The tamoxifen and lutein group had better retinal tissue than the tamoxifen group. Normal retinal architecture and thickness. The photoreceptor layer was striated. INL and ONL were normal. A previous study showed that lutein protects photoreceptors. Lutein increased rod and cone cell rhodopsin and opsin expression in retinitis pigmentosa mice. Photoreceptor survival and ONL thickness increased[55].

The ganglion cell count was the same in the control and tamoxifen and lutein-treated groups. In rats, lutein protects retinal ganglion cells. Lutein inhibited p38 MAPK and c-Jun and hence reduced NMDA-induced RGC apoptosis. Bcl-2 levels increased while Bax, caspase-3, and cytochrome c levels declined[56].

Lutein and tamoxifen co-administration did not affect GFAP staining. Lutein reduces retinal Müller cell gliosis and GFAP expression in retinitis pigmentosa due to its anti-inflammatory properties[55].

Tamoxifen and lutein treatment did not significantly alter immune histochemical staining with anti-caspase 3. AbuBakr *et al*. (2018) found that lutein reduced caspase-3 in charred rats' livers, kidneys, and lungs[57]. Lutein's neuroprotective effects reduced cell loss and apoptosis. It may prevent hypoxia-induced apoptosis and autophagy by boosting glial cell survival and reducing autophagosome formation. Lutein's chemical structure helps cells communicate via intercellular gap junctions[58]. Lutein may prevent apoptosis by inhibiting caspases 3, -8, and -9 and promoting Bcl-2 synthesis. It reduced apoptotic death, mitochondrial dysfunction, and antioxidant defense, protecting dopaminergic neurons[59].

CONCLUSION

Our findings showed that tamoxifen produced histological changes in retinae. When combined with tamoxifen, lutein was more effective in protecting retinal tissue than melatonin. It is a highly potent neuroprotective agent. More research on the application of lutein in the treatment of patients receiving long-term tamoxifen therapy, such as breast cancer patients, is strongly recommended.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التقييم النسيجي لتأثير عقار تاموكسيفين على شبكية العين عند إناث الجرذان البيضاء البالغة والقدرة الوقائية المحتملة للميلاتونين مقابل اللوتين

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المقدمة: عقار تاموكسيفين هو العلاج الأكثر شيوعًا لسرطان الثدي الإيجابي المستجيب للإستروجين. قد تتراوح مدة الدورة العلاجية بين خمس إلى عشر سنوات.

الغرض من هذه الدراسة هو فحص التفاعلات النسيجية لتاموكسيفين على شبكية العين عند إناث الجرذان البيضاء البالغة والتأثير المفيد المحتمل للميلاتونين مقابل اللوتين.

المواد وطرق البحث: تم تقسيم ستين أنثى بالغة من الجرذان البيضاء بشكل عشوائي إلى ٦ مجموعات. المجموعة الضابطة (I) ، مجموعة الجرذان (II) المعالجة بالميلاتونين حصلت على جرعات يومية من الميلاتونين (١٠ مجم / كجم) ، مجموعة الجرذان (III) المعالجة باللوتين والتي حصلت على اللوتين يوميًا (٥٠ مجم / كجم / يوم) ، المجموعة المعالجة بالتاموكسيفين (IV) حصلت الجرذان يوميا علي عقار تاموكسيفين (٥ مجم / كجم) , ومجموعة معالجة بالتاموكسيفين و الميلاتونين معا (V) ومجموعة جرزان المعالجة بالتاموكسيفين واللوتين معا (VI) وقد تلقت الجرعات المذكورة أعلاه. في نهاية الدراسة (٤ أسابيع) تم تخدير الحيوانات والتضحية بها ، وتم جمع عينات شبكية العين للفحص النسيجي والكيميائي المناعي والمجهري الإلكتروني.

النتائج: وجد أن عقار تاموكسيفين ضار بأنسجة الشبكية. أظهر كل من سمك الشبكية الكلي وعدد الخلايا العقدية انخفاضًا كبيرًا للغاية. كان هناك ارتفاع كبير في تفاعل GFAP و Caspase-٣ المناعي. كشفت نتائج المجهر الاللكتروني عن انخفاض عدد الخلايا المصطبغة ، وتشوه المستقبلات الضوئية ، وانخفاض عدد خلايا الطبقة النووية الخارجية والداخلية مع نوى صغيرة منكمشة ، واتساع الفجوة بين الخلايا ، وتنكس الميتوكوندريا. الخلايا العقدية لديها نوى غير منتظمة وميتوكوندريا متدهورة. أظهر التناول المتزامن للوتين حماية أنسجة شبكية أكبر بكثير من الميلاتونين.

الخلاصة: لوتين تأثير وقائي أكبر بكثير على أنسجة الشبكية من الميلاتونين ضد الآثار الضارة المحتملة لتاموكسيفين. المرضى الذين يتناولون جرعة طويلة أو عالية من عقار تاموكسيفين أوصوا بمتابعة منتظمة لتغيرات الشبكية المحتملة. تمت التوصية بإجراء مزيد من الدراسات حول التأثيرات الوقائية للوتين على المرضى الذين يتلقون علاج تاموكسيفين. يجب تناول مضادات الأكسدة المختلفة الموجودة في السوق بعد استشارة طبية للاستفادة منها وتجنب آثارها الخطرة.