The Possible Ameliorating Effect of Nanocurcumin on Experimentally Induced Retinotoxicity in Adult Male Albino Rat

Original Article

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ABSTRACT

Introduction: Retina is an organ that is very sensitive to getting hurt because of its high oxygen requirement and high polyunsaturated fatty acid content. Cisplatin is used as an effective chemotherapy in different type of cancer but it reduced the cellular viability and increased the reactive oxygen species (ROS) generation.

Aim of Work: The goal of this work was to investigate the role of nano curcumin particles in preventing the Cisplatin (CP) - induced retinotoxicity in adult male albino rats.

Material and Methods: 30 adult male albino rats divided into three groups as following; group I was the control group, group II was the Cisplatin-treated group (single intraperitoneal injection of CP 7 mg/kg), and group III was the nano curcumin -treated group (single intraperitoneal of CP 7 mg/kg + oral nano curcumin 50 mg/kg/14 d).

Results: Cisplatin (CP) single injection caused histological changes in the retinal tissue and decreased the expression of eNOS level and elevated the caspase-3 as well as the TNF-α in retinal tissue of albino rats. By colorimetric analysis, reduction in the total antioxidant and elevation in MDA levels were also seen. Co-administration of nano curcumin particles with CP restored the normal histological structure of the retina of rats and significantly improved the cellular antioxidant capacity of the retina resulting in retinal protection against the retinotoxicity and apoptotic cell death caused by the CP therapy.

Conclusion: Cisplatin has severe destructive impacts on the retina and co- administration of nano curcumin particles have a protective role on Cisplatin- induced retinotoxicity.

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Key Words: Cisplatin; immunohistochemical; nanocurcumin; total antioxidant capacity.

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INTRODUCTION

Cancer is a wide world problem which represents one of the leading causes of death[1]. Cisplatin is a commonly used chemotherapeutic agent as it works well for a variety of malignancies, including sarcoma, cerebral lymphoma, and small cell lung cancer[2]. However, it is related to different adverse effects as nephrotoxicity, ototoxicity and suppression of bone marrow[3,4].

Many mechanisms explained the toxic effects of CP involving its binding to cellular DNA forming DNA complexes that disturb its replication and transcription and activates apoptosis in cells[5]. In addition, it causes damage of cellular organelles especially mitochondria through inflammation and oxidative stress[6].

Curcumin is water-insoluble natural product of turmeric root[7]. It posses antioxidant, anti-inflammatory and anti-carcinogenic properties[8]. Curcumin has been reported to decrease lipid peroxidation, increase intracellular antioxidant, regulate antioxidant enzymes, and scavenge oxygen free radicals[9]. The hydrophobic nature of curcumin decreases its water solubility and bioavailability[10].

Nanoformulation of curcumin into nanoparticles enhances its bioavailability and reduces the required dose to reach the desired therapeutic effect[11]. The nanoparticles can elevate the antioxidant levels, decrease the inflammatory cytokines production and inhibit the different cancer cells as medulloblastoma, and cancer of pancreas[12].

MATERIALS AND METHODS

Drugs

- Cisplatin: was obtained in a vial form from Mylan Pharmaceutical Company, France. Each vial contains 50mg\50ml Cisplatin.
- Nanocurcumin: was supplied by the Egypt Nanotechnology of the Photo Electronics Communication Centers in shape of yellow powder.

Preparation of Curcumin Nano emulsion

At Cairo University's Faculty of Medicine's Biochemistry and Molecular Biology Department .

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Nano emulsion form of curcumin was prepared using an ethanolic extracts of raw powder of curcumin longa roots. It was prepared by adding 200g raw powder of the roots of tested plant to 800 ml of 98 percent ethanol at a high speed mixture. At room temperature, Evaporation of liquid phase was done. The produced powder was maintained in a clean containers at the room temperature until it was used to make the nano emulsion. By mixing phase of oil of ethanolic extracts in the aqueous phase, an oil water curcumin nano-emulsion was created. Tween 80 (depending on phase of oil), and maltodextrin (4%and 10%) that was melted in a phosphate buffer solution and stirred at least for 15 minutes with a high -speed (20000 rpm) magnetic mixers to be hydrated completely. End result was an emulsion with a 20% ethanolic extracts of curcumin[13].

Nano-emulsion specifications

By the transmission electron microscope (Figure 1), the nanoparticles were spherical in shape, 27.3- 41.8 nm in diameter and freely water soluble

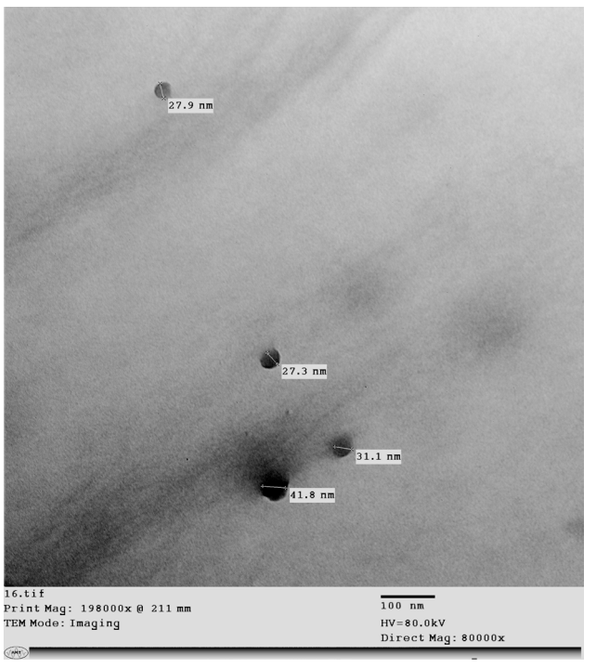


Fig. 1: Transmission electron microscopy (TEM) image of the nanocurcumin shows the particles are spherical in shape and less than 50 nm in diameter.

Animals

Before the study, the Instituional Animal Care and Use Committee (CU-IACUC) gave its consent (NO. CU III F 27 19). All animals that were used in this study were cared for according to the committee's recommendations. All study was performed in compliance with guidelines set forth by the Animal Care and Use Committee.

Thirty Wistar adult male albino rats, weighing approximately 200 gm± 20, were purchased from the animal house at Cairo University's Faculty of medicine. Rats were kept in conventional laboratory and environmental circumstances and were given free access to food and drink.

Experimental design

The rats were divided into three groups, 10 rats each as follow; control group (I) received no medication, Cisplatin-treated group (II) received a single intraperitoneal injection of Cisplatin (CP) at a dose of (7mg/kg)[14], Cisplatin and nanocurcumin- treated group (III) received single intraperitoneal injection of CP at a dose of 7mg/kg) + nanocurcumin (orally at a dose of 50mg/kg using orogastric gavage once daily for consecutive 14 days)[15].

The rats were sacrified under phenobarbital anesthesia on the 14th day of CP therapy. The eyeballs and stump of optic nerve were enucleated with a curved round ended scissor. Next, the top of nasal portion of the sclera was punctured 1.5mm thick behind limbus using a gauge Hamilton needle attached to a syringe. Behind the lens, the vitreous portion of the eye balls was injected with 10% formalin and left for 24 hours[16]. The retinas of each rat were removed and tissue samples were obtained for biochemical and histological analysis. Tissue samples were preserved in glass vials and frozen at minus 80o C for biochemical study.

Light microscopic study

The tissue specimens were fixed in a 10% neutral buffered formalin solution and washed. Next, dehydration was done in ethanol in progressive grades. The specimens were immersed in melted paraffin, sectioned at a thickness of 5-µm, 1-2 mm from the optic disc and stained with hematoxylin and eosin[17].

Immunohistochemical stain for Caspase- 3 was used for detection of apoptosis in the retinal tissue using anti-Caspase-3 antibody, polyclonal rabbit antibodies (ab2302, Abcam, Cambridge, UK, 1: 1000).

Immunohistochemical stain for Tumor necrosis factor-alpha (TNF- α) was used for detection of inflammation in the retinal tissue using Polyclonal rabbit antibody (ab6671, Abcam, Cambridge, Massachusetts,USA).

Histomorphometric study

Morphometric image J program image analyzer was used for quantitative analysis. The image analyzer was initially calibrated automatically to translate the image analyzer program's measurement units (pixels) into actual measurement units (µm). At magnification of 400 in each retinal image, ten non-overlapping microscopic fields were randomly chosen for the evaluation of the following features:

Total retinal layer thickness, in hematoxylin and eosin-stained sections, were assessed.

Area percentage of Caspase- 3 and TNF-α positive immunoreactions, in immunohistochemical sections, were also assessed.

Biochemical study

At Cairo University's Faculty of Medicine's Biochemistry and Molecular Biology Department, the following parameters were assessed;

Assessment of MDA and Total anti-oxidant level by colormetry

The data were given in nanomoles per gramme of protein

Assessment of endothelial nitric oxide synthetase (eNOS) by Western blot assay.

Cells were lysed for total protein extraction. The bicinchoninic acid (BCA) technique was utilized to detect the protein concentration. The data were given in nanomoles per milligramme of protein

Statistical analysis

Graph Pad Prism Version (7) was utilized to code and enter data. The mean and the standard deviation were applied to describe the data. When comparing more than two groups, one way analysis of variance (ANOVA) was used, along with Turkeys multiple comparison test.

RESULTS

Histological results

Sections of the control group (I) stained in Hematoxylin and Eosin exhibited the arrangement of the ten layers of retina from outward inwards as: retinal pigment epithelium (RPE), photoreceptor layer (Ph), outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), nerve fiber layer (NFL) and inner limiting membrane (Figure 2).

In retinal tissues of Cisplatin-treated group (II), a marked increase in retinal thickness and extensive damage of the photoreceptor layer with loss of striation were seen. Marked increase in cell density was noticed in both inner nuclear layer (INL) and outer nuclear layer (ONL) with separation of latter was observed. Ganglion cell layer (GCL) exhibited disorganization and irregular cells distribution with a dilated congested blood vessel. Also, displaced nuclei were detected in both IPL and OPL (Figures 3,4). In the retinal tissues of group III, an obvious restoration of the normal architecture was observed (Figure 5).

The immunohistochemical reaction of Caspase- 3 and TNF- α in retinal tissues are shown in (Figures 6-11). A negative immunoreaction for Caspase- 3 and TNF- α in control group I. However, Caspase- 3 and TNF- α were significantly expressed as dark brown granules in GCL and INL retinal layer in group II. The apparent reduction in Caspase- 3 and TNF- α immnuoreaction in group III was marked observed.

Histomorphometric results

mean value of total retinal thickness significantly increased in group II when compared to control and group III. With the use of nanocurcumin, there was a non-significant increase in total retinal thickness as compared to control group.

The mean value of the area percentage of Caspase-3 and TNF- α showed a significant increase in group II in comparison with groups I and III. With the use of nanocurcumin, there was a non- significant difference in mean of the area % of Caspase- 3 and TNF- α as compared to group I (Tables 1, 2 and Bar Charts 1, 2, 3).

Biochemical results

The retinal tissues in group II exhibited a significant increase in the mean value of MDA level as compared to groups I and III. There was a non- significant change between nanocurcumin - treated group and control group.

The retinal tissues in group II showed a significant decrease in mean value of total antioxidant level in comparison with groups I and III. With the use of nanocurcumin, there was a non-significant change as compared to control group.

The mean value of endothelial nitric oxide synthetase (eNOS) in retinal tissue exhibited a significant decrease in group II as compared to that of the groups I and III. There was a significant reduction in eNOS expression in group III when compared to control group (Figure 12, Tables 3, 4 and Bar Charts 4, 5, 6).

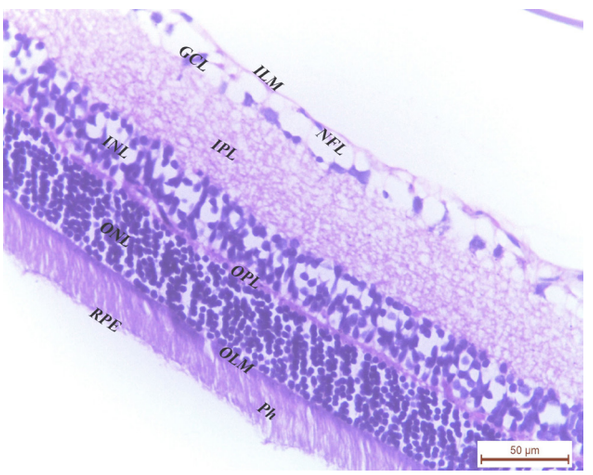


Fig. 2: Photomicrograph of the retinal section in control group (I) showing the arrangement of ten layers from outward inward; retinal pigment epithelium (RPE), photoreceptor layer (Ph), outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), nerve fiber layer (NFL) and inner limiting membrane (ILM) (H&E, 400).

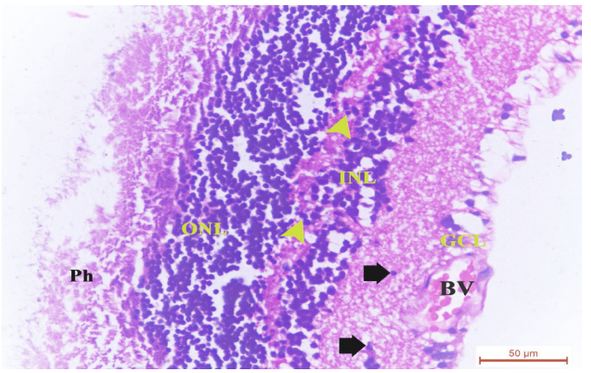


Fig. 3: Photomicrograph of retinal section in Cisplatin- treated group (II) showing thickening of the retina. The photoreceptor layer (Ph) reveals fragmentation with loss of striation appearance. An increase in cell density also is seen in both inner nuclear layer (INL) and outer nuclear layer (ONL). The outer plexiform layer (arrow heads) and the inner plexiform layer (arrows) exhibit displaced nuclei. Ganglion cell layer (GCL) exhibits irregular cell distribution with a dilated blood vessel (BV) (H&E, 400).

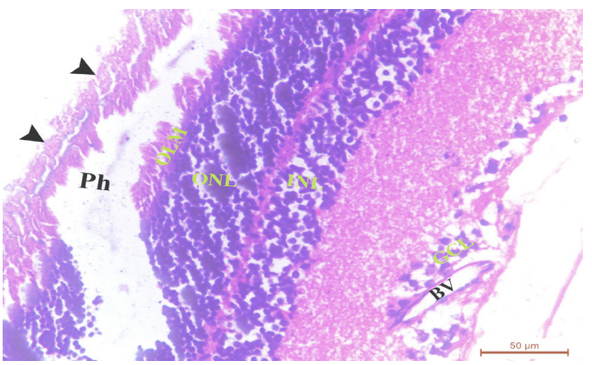


Fig. 4: Photomicrograph of the retinal section in Cisplatin- treated group (II) showing marked increase in retinal thickness. The photoreceptor layer (Ph) reveals degeneration and separation. An increase in cell density is seen in both inner nuclear layer (INL) and outer nuclear layer (ONL). Ganglion cell layer (GCL) exhibits disorganization of cells with a dilated blood vessel (BV) (H&E, 400).

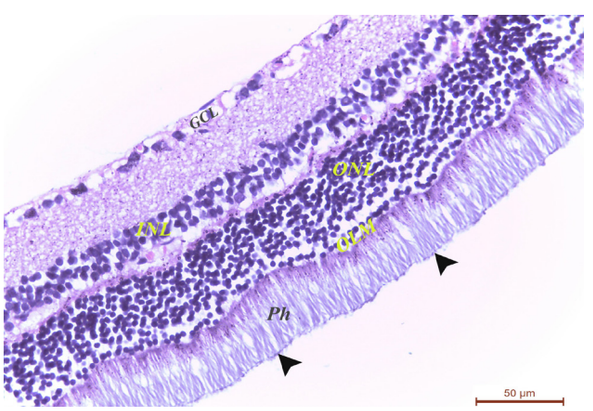


Fig. 5: Photomicrograph of the retinal section in curcumin nanoparticles-treated group (III) showing intact RPE (arrow heads). The striation appearance of the photoreceptor layer (Ph) is preserved. The outer limiting membrane (OLM) is also preserved. Outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) are almost normal (H&E, 400).

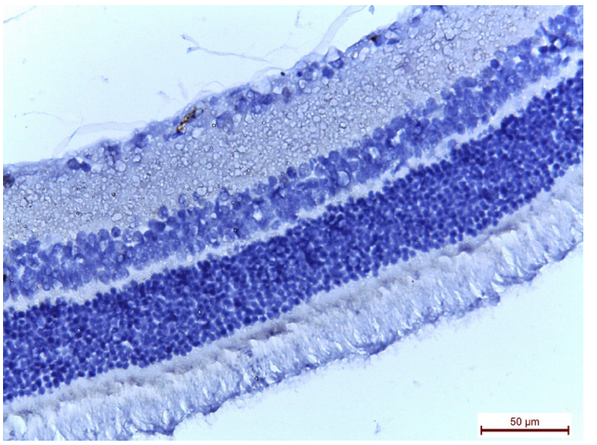


Fig. 6: Caspase- 3 immunohistochemical staining of control group showing negative reaction (Caspase-3 x 400).

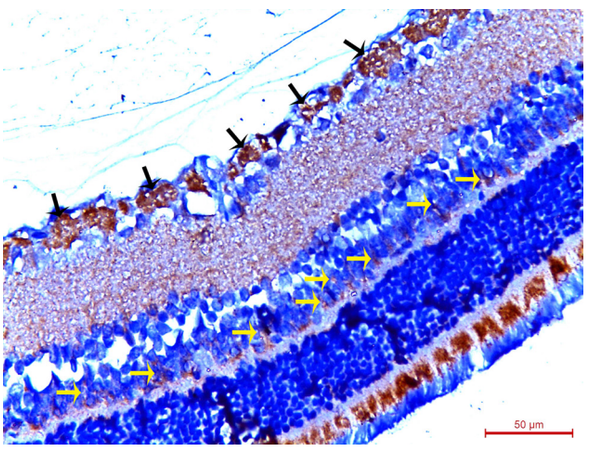


Fig. 7: Caspase- 3 immunohistochemical staining of Cisplatin- treated group(II) exhibiting marked increase cytoplasmic reaction in GCL (black arrow) and INL (yellow arrows) (Caspase-3 x 400).

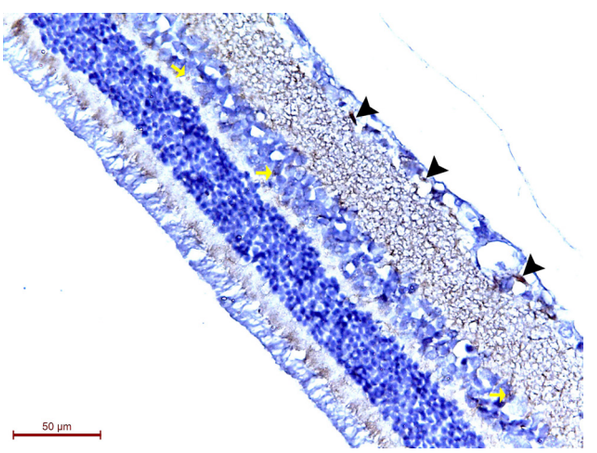


Fig. 8: Caspase- 3 immunohistochemical staining of curcumin nanoparticles - treated group (II) showing apparent reduction cytoplasmic reaction in GCL (arrow heads) and INL (arrows) (Caspase-3 x 400).

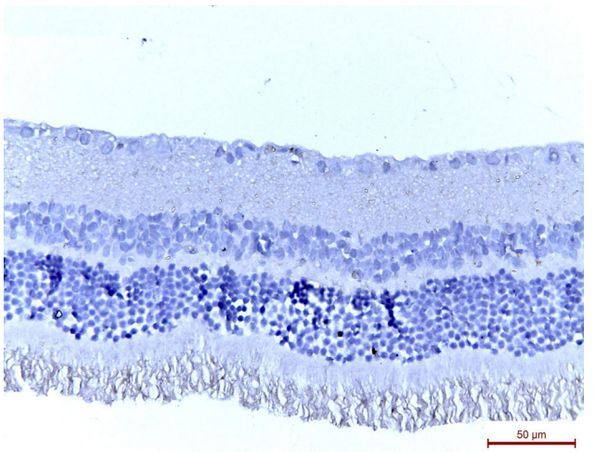


Fig. 9: TNF- α immunohistochemical staining of control group showing negative reaction (TNF- α x 400).

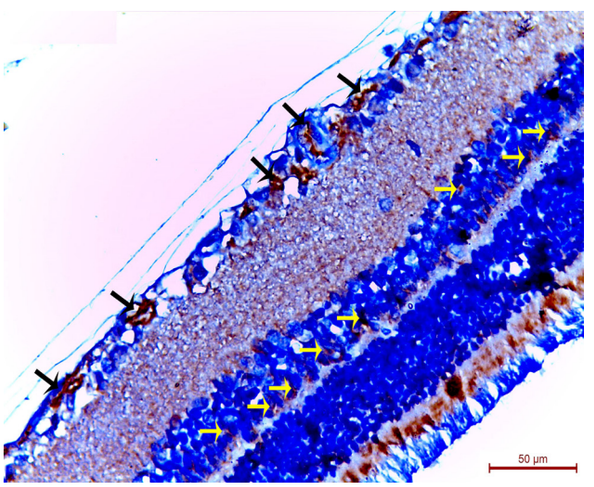


Fig. 10: TNF- α immunohistochemical staining of Cisplatin- treated group (II) exhibiting marked increase cytoplasmic reaction in GCL (arrow heads) and INL (arrows) (TNF- α x 400).

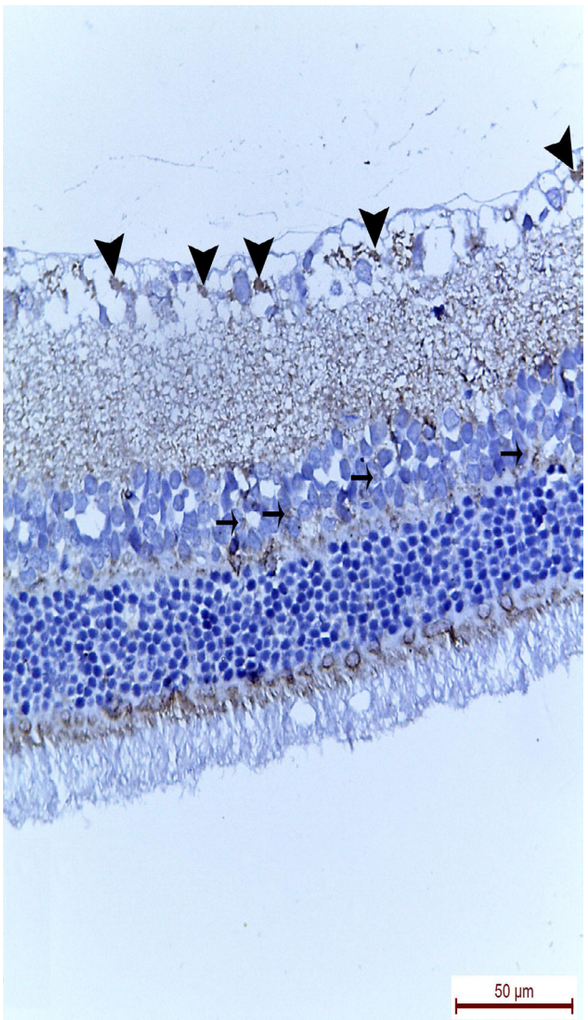


Fig. 11: TNF- α immunohistochemical staining of curcumin nanoparticles - treated group (III) showing apparent reduction cytoplasmic reaction in GCL (arrow heads) and INL (arrows) (TNF- α x 400).

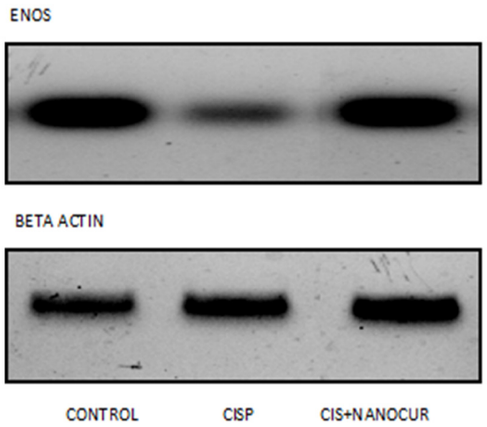


Fig. 12: Representative western blot analysis for endothelial nitric oxide synthetase (eNOS) protein expression.

Table 1: Multiple comparisons of *P* values for retina thickness (µm), area percentage (%) of Caspase- 3, area percentage (%) of TNF- alpha between different experimental groups of rats.

	group	*P* value
Retina thickness (µm)	Control vs CP- treated	<0.001
	Nanocurcumin- treated	0.121
	CP vs Nanocurcumin- treated	0.022
Caspase- 3 (%)	Control vs CP- treated	<0.001
	Nanocurcumin- treated	0.073
	CP vs Nanocurcumin- treated	0.020
TNF- alpha (%)	Control vs CP- treated	<0.001
	Nanocurcumin- treated	0.096
	CP vs Nanocurcumin- treated	0.020

P 0.05 ≤ is considered significant.

Table 2: Mean± SD for retina thickness (µm), area percentage (%) of Caspase- 3, area percentage (%) of TNF- alpha between different experimental groups of rats.

Groups		Retinal thickness (µm)	Caspase- 3 (%)	TNF- alpha (%)
Control	Mean± SD	137.8± 0.83	0.0004± 0.0007	0.0005± 0.0007
Cisplatin- treated	Mean± SD	239.5± 1.26*	3.500± 0.292*	3.1000± 0.2022*
Nanocurcumin-treated	Mean± SD	140.4± 0.65#	0.103± 0.142#	0.1210± 0.0348#

* *P*0.05 ≤, significant difference compared to that of the control group.

*P*0.05≤, significant difference compared to that of the CP- treated group.

Table 3: Multiple comparisons of *P* values for MDA (nmol\g), Total anti-oxidant (mmol\g), eNOS levels between different experimental groups of rats.

	groups	*P* value
MDA	Control vs CP- treated	<0.001
	Nanocurcumin- treated	0.970
	CP vs Nanocurcumin- treated	<0.001
Total anti-oxidant	Control vs CP- treated	<0.001
	Nanocurcumin- treated	0.083
	CP vs Nanocurcumin- treated	<0.001
eNOS	Control vs CP- treated	<0.001
	Nanocurcumin- treated	<0.001
	CP vs Nanocurcumin- treated	<0.001

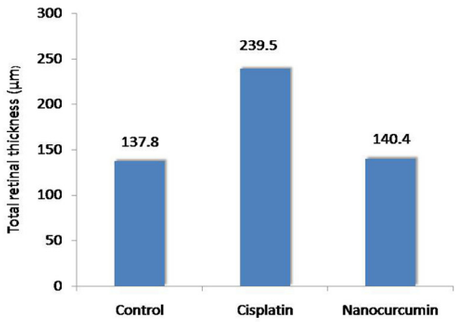
P 0.05 ≤ is considered significant.

Table 4. Mean± SD for MDA (nmol\g), Total anti-oxidant (mmol\g), eNOS levels between different experimental groups of rats.

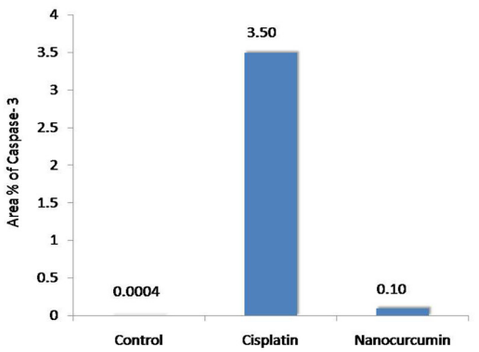
Groups		MDA (nmol\g)	Total anti-oxidant (mmol\g)	eNOS
Control	Mean± SD	13.2± 0.7	26.5± 1.0	1.04± 0.02
Cisplatin- treated	Mean± SD	29.9± 1.5*	12.3± 1.5*	0.26± 0.02*
Nanocurcumin-treated	Mean± SD	13.5± 0.8#	25.3± 0.9#	0.92± 0.02*#

* *P*0.05 ≤, significant difference compared to that of the control group.

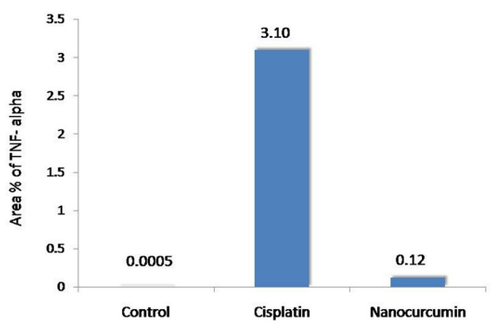
*P*0.05 ≤, significant difference compared to that of the CP- treated group.



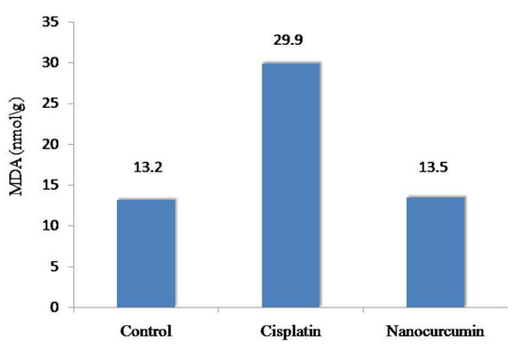
Bar Chart 1: Mean values of total retinal thickness (µm) in different groups of rats.



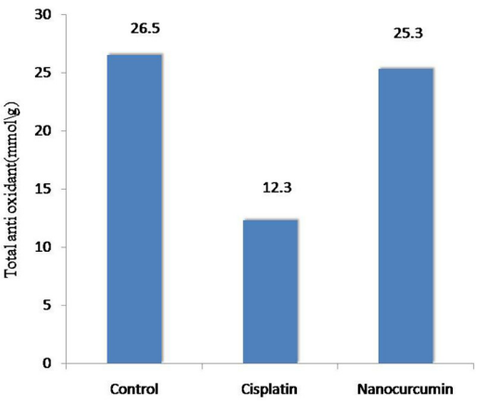
Bar Chart 2: Mean values of area percentage of Caspase- 3 in different groups of rats.



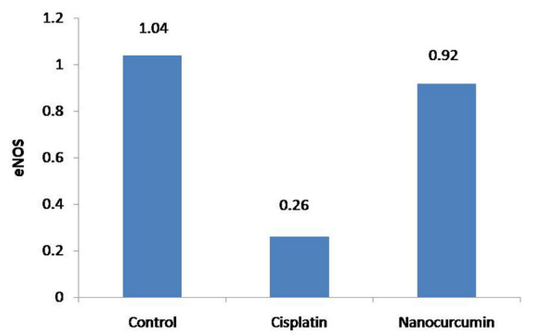
Bar Chart 3: Mean values of the area percentage of TNF- alpha in different rats' groups.



Bar Chart 4: The mean values of the MDA level in the retina (nmol\g) in different rats' groups.



Bar Chart 5: Mean values of the total antioxidant level in the retina (mmol\g) in different rats' groups.



Bar Chart 6: Mean values of eNOS level in the retina in different groups of rats.

DISCUSSION

Cisplatin (CP) is a chemotherapeutic medication that can be used to treat a variety of cancer. Retinotoxicity is one of the most dangerous adverse effects that limits its use. CP may enhance the production of reactive oxygen species resulting in retinal oxidation and degeneration which are causes of retinotoxicity[18]. Polat *et al*[14] attributed the ocular toxicity induced by CP to its high ability to penetrate the central nervous system.

In the present work, single i.p injection of CP showed several histological alterations in the form of extensive damage of photoreceptor layer with loss of its striation appearance, marked increase in cellular density of both ONL and INL and dilated congested blood vessels. Similar findings were recorded by Gul Bykalir *et al*[19]. Moreover, these histological changes were attributed to the oxidative stress, the reactive oxygen species (ROS) and severe inflammation caused by CP[20]. Also, a marked increase in total thickness of the retina was detected in this work and could be attributed to inflammation, edema and vascular congestion that is induced by Cisplatin as supposed by Sunar *et al*[21]. Whereas, Findik *et al*[22] recorded that on 3rd day of single Cisplatin administration, the retinal sections appeared more acidophilic with a slight increase of the retinal thickness.

The present work showed restoration of normal structure of the retina with the use of nanocurcumin with a significant reduction in total thickness of the retina when compared to CP- treated group in agreement with Khadrawy *et al*[15]. This findings might be attributed to the ability of nanocurcmin to elevate total anti-oxidant capacity, decrease the oxidative stress and suppress inflammation.

In this study, Cisplatin showed significantly expressed Caspase- 3 immunoreaction that mainly were found in both GCL and INL. This is supported by Yazici *et al*[23] who mentioned that Cisplatin has more toxic effects on the inner layers than the outer. CP might cause suppression of mitochondria respiration and calcium efflux from it. High intracellular calcium levels induce cytochrome c and free radicals production which stimulate Caspase 9 after that Caspase 9 stimulates Caspase 3[2]. With the use of nanocurcumin in the present work, apparent reduction of Caspase- 3 immunoreaction was detected as supported by Sarawi *et al*[24]. Nanocurcumin could prevent the free radical generation and calcium influx[15].

The current study recorded a high expression of TNF- α reaction with Cisplatin administration mainly in GCL and INL that indicated the intense inflammation this is supported by Yazici *et al*[23]. The overgeneration of ROS induced by CP could accelerate the production of inflammatory cytokines[25]. Moreover, this work demonstrated that the nanocurcumin significantly decreased the high TNF- α level induced by CP. Curcumin nanoparticles could inhibit calcium efflux from mitochondria and the ROS generation and thereby inhibiting the inflammatory cytokines production[15].

The present study demonstrated an elevation in MDA and reduction in total antioxidant levels after Cisplatin injection in agreement with Sunar *et al*[21]. CP could induce the oxidative damage of cellular proteins and lipids by over generation of reactive oxygen radicals. Lipid peroxidation leads to disturbance of lipid bilayer cell membrane and leakage of enzymes into the blood[26]. Disturbance of the anti-oxidant mechanism accumulate the ROS which activates the pro-apoptotic process and ultimately leads to cell death[27]. With the use of nanocurcumin in this study, a significant reduction in MDA and elevation in total anti-oxidant levels were recorded. The protective action of nanocurcumin might be reffered to its potency to quench free radicals and inhibiting lipid peroxidation as supposed by Sarawi *et al*[24].

The eNOS is produced by capillaries endothelium of the choroid that is important for the function and integrity of the endothelium and share in flow of blood to the globe[28]. In the present work, Cisplatin administration showed a reduction in eNOS levels this is supported by Abdelkader *et al*[29] who attributed these findings to over generation of free radicals and oxidative stress. Moreover, nanocurcumin has elevated the low eNOS level that is induced by Cisplatin in this work. These results corroborated previous findings[30]. Curcumin could counter balance the effect of ROS, restored the antioxidant and subsequently increase the eNOS expression[31].

Nanocurcumin has an ameliorating effect because it can scavenge the superoxide and hydroxyl radicals and induce the cytoprotective and antioxidant enzymes as mentioned by Motterlini *et al*[32]. So, nanocurcumin could reduce the retinal damage induced by CP through decreasing the lipid peroxidation, adjusting the oxidative system and improving the histological changes in retina.

CONCLUSION

Regardless of the importance of Cisplatin as chemotherapeutic agent, it has several harmful effects on the retinal structure and function. Co- administration of nanocurcumin possess a potential retino-protective effect against Cisplatin intoxication. So it's highly recommended to use nanocurcumin with Cisplatin to protect against its toxic effect.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير المحسن المحتمل للنانوكوركومين على تسمم شبكية العين المستحدثة تجريبيا فى ذكور الجرذان البيضاء البالغة

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مقدمه: يستخدم السيسبلاتين كعلاج كيماوي فعال و لكن يصاحب ذلك العديد من الآثار الجانبية مثل تسمم شبكية العين. فشبكية العين عضو شديد الحساسية لاستهلاكه المزيد من الاكسجين و احتوائه على المزيد من الاحماض الدهنية الغير مشبعة. ال إن- استيل سيستاين و جزيئات النانوكوركومين لديهم تأثيرات مضادة للاكسدة، مضادة للالتهاب ومقاومة للاستماتة.

الهدف من البحث: وقد صممت الدراسة الحالية لبحث التغيرات الشكلية ،التركيبة و الوظيفية التى قد تحدث في شبكية العين مع السيسبلاتين و التأثير الوقائي لجزيئات النانوكوركومين.

المواد والطرق: تم استخدام ثلاثون فأرا ذكرا أبيضا في هذه الدراسة. و قد قسمت الفئران الى ثلاث مجموعات متساوية, المجموعة الاولى (مجموعة ضابطة) اعطيت الغذاء المعتاد، المجموعة الثانية (مجموعة معالجة بالسيسبلاتين) تم حقنها بالسيسبلاتين بجرعة واحدة فى اول يوم من التجربة ثم تركها لمدة اربعة عشرا يوما والمجموعة الثالثة (مجموعة معالجة بالسيسبلاتين و بالنانوكوركومين) تم حقنها بالسيسبلاتين بجرعة واحدة فى اول يوم من التجربة ثم اعطائها النانوكوركومين لمدة اربعة عشرا يوما . تم وزن كل فأر و استخراج العينين و تم فصل الشبكية و معالجتها لدراستها هستولوجيا و هستوكيميائية مناعية و بيوكيميائيا.

النتائج: أظهرت المجموعة المعالجة بالسيسبلاتين زيادة ملحوظة في سماكة شبكية العين، أضرار جسيمة، مع فقدان مظهر التحززات المتوازية للمستقبلات الضوئية. و قد أظهرت كل من الطبقة النووية الخارجية و الداخلية تصلباز. علاوة على ذلك، أوضحت طبقة الخلايا العقدية اضطراب الخلايا و إحتقان الأوعية الدموية. بالاضافة الى زيادة صبغة كاسباس٣ وعامل نخر الورم ألفا في الطبقة النووية الداخلية و طبقة الخلايا العقدية.

من ناحية اخرى, قد ظهر تحسن نسيجي واضح في شبكية العين المعالجة بالسيسبلاتين مع استخدام النانوكوركومين.

الاستنتاج: اظهر النانوكوركومين عن تأثير محسن ضد سمية السيسبلاتين فى الجرذان.