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RESEARCH ARTICLE

CURCUMIN SUPPRESSES ENDOSULFAN TOXICITY IN RABBIT'S CORNEA

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ABSTRACT

The aim of the present study was to evaluate the change in corneal protein and oxidative stress state after endosulfan-administered in rabbits and the effect of topical curcumin. The treatment scheme for the different groups was as follows: control group, endosulfan group, Endosulfan+40 µmol/L curcumin, endosulfan+ curcumin 80µmol/L and Endosulfan + curcumin 160 µmol/L. All animals groups decapitated after 6 weeks then eyes were inoculated and corneas were isolated for determination of protein content, SDS-PAGE, catalase (CAT), superoxide dismutase (SOD),Na^+-K^+-ATPase, and the malondialdehyde (MDA) level activities were measured in the cornea. The results indicated that for endosulfan group a significant increase ($p < 0.05$) in total protein content accompanied by shift of the corneal protein peaks towards high molecular weight. Also a significant increase ($p < 0.05$) in catalase and MDA activities was observed. Topical application of curcumin was useful in reducing oxidative stress induced by endosulfan.

INTRODUCTION

Pesticides represent one of the classes of chemicals that are intentionally released into the environment precisely because of their recognized potential to adversely affect biological systems. Among them, chlorinated organic pesticides are chemicals responsible for environmental contamination. Many of the chlorinated organic pesticides are highly toxic and considered potential risks to human health (Kullman and Matsumura, 1996; US Department of Health and Human Services, 1990). Endosulfan (1,2,3,4,7,7-hex- achlorobicyclo-2,2,1-heptene-2,3-bis-hydroxy methane-5,6 sulfite) is a cyclodiene insecticide used extensively throughout the world to control the insect pests of a wide range of crops including cereals, tea, coffee, cotton, fruits, oil seeds and vegetables (Guerin, 2001; Lee *et al*., 1995) making it an environmental contaminant and a public health hazard. Endosulfan is not dissolved easily in water but sticks to particles or soil. Breaking down slowly, it may accumulate in non-target species, such as cattle, fish, birds and even humans that are exposed to it by different routes of exposure such as inhalation, ingestion or dermal contact (Mor and Ozmen, 2003; Naqvi and Vaishnavi, 1993; Wan *et al*., 2005). A number of recent studies provided evidence for the induction of dose- and time-dependent oxidative stress by organochlorine pesticides in different organs (Bachowski *et al*., 1998; Bagchi *et al*., 1993; Bagchi *et al*., 1995; Dorval *et al*., 2003; Hassoun and Stohs, 1996; Hincal *et al*., 1995). In accordance, organochlorine pesticide endosulfan was shown to induce alterations in the activities of enzymes involved in oxidative stress and lipid peroxidation (Bebe and Panemangalore, 2003; Dorval and Hontela, 2003) and resultant declines in cell viability (Dorval *et al*., 2003), increased lipid peroxidation (Panday *et al*., 2001), depressed oxygen consumption and red blood cell count (Anusha *et al*., 1994) in different species.

Curcumin, a constituent of turmeric is an effective antioxidant and is known to induce the enzymes of glutathione linked detoxification pathways in rats; curcumin seems to prevent oxidative damage. Curcumin, a plant polyphenol, possesses diverse antioxidant and anti-inflammatory properties. It significantly decreases lipid peroxidation, increases intracellular antioxidant, regulates antioxidant enzymes, and scavenges hyperglycaemia (Renu and Mamta, 2011). In addition, curcumin is shown to inhibit the pro-inflammatory transcriptional factor, NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), and prevent up-regulation of VEGF mRNA (vascular endothelial growth factor of messenger ribonucleic acid) and micro vascular angiogenesis. The antioxidant capacity, a measure of the total protective antioxidant mechanisms (both for preventing the production of free radicals and for repairing oxidative damage), of curcumin has been considered to be mediated via its beneficial effects on the antioxidant defence system, the scavenging of free radicals and/or via preventing lipid peroxidation and it is at least 10 times more active as an antioxidant than vitamin E (Jin Dai and Mumper, 2010). Emoto *et al*., 2013 indicated that curcumin inhibited N-methyl-N-nitrosourea-induced photoreceptor cell apoptosis by suppressing DNA oxidative stress. The present study evaluates the changes in corneal protein and oxidative

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stress state after endosulfan-administered in rabbits and the effect of topical curcumin to minimize these changes.

MATERIALS AND METHODS

Animals and treatment

New Zealand male rabbits (n = 30) weighing 2–2.5 kg were randomly selected from the animal house facility at the Research Institute of Ophthalmology, Giza, Egypt. The animals were maintained in a standard 12-h light/dark cycle with free access to water and balanced diet. The experimental protocol was approved by the local ethical committee that applies ARVO (The Association for Research in Vision and Ophthalmology) statements of using animals in ophthalmic and vision research. The doses of endosulfan was calculated according to the rabbits' body weights measured weekly and administered by gavage between 09 and 10 a.m., using a plastic oral gavage affixed to a glass syringe. The experiment lasted for 6 weeks. Endosulfan was administered orally in a sublethal dose of 1.0 mg/kg/day in corn oil (Mor and Ozmen, 2010). Eyes were treated with curcumin 40, 80, or 160 µmol/L topically two times a day (Kim *et al*., 2010). The treatment scheme for the different groups was as follows: Control group, Endosulfan group, Endosulfan+40 µmol/L curcumin, Endosulfan+curcumin 80µmol/L and Endosulfan+ curcumin 160 µmol/L. All animals groups decapitated after 6 weeks then eyes were inoculated and corneas were obtained from rabbits via cutting through the ora serata. The corneas from all animal's groups were weighed and homogenized using cell homogenizer (type Tübingen 7400, Germany), in a 10- fold volume of 20 mM ice-cold tris-HCl buffer,pH7.4.The homogenate was centrifuged for 20 minutes at 10,000 rpm in a bench centrifuge (Awel centrifuge MS 20, France). The resultant supernatant was used for the following measurements.

Protein analysis

Corneal protein content was determined according to the method of Lowry *et al*. (1951). The developing color was measured with a spectrophotometer (type UV-visible recording 240 Graphical, Shimatzu, Japan) at 750 nm. Protein composition of the cornea was analyzed by SDS-PAGE according to its molecular weight by the method of Laemmli (1970) using 3% stacking gel and 10% separating gel. The gel was scanned using scanner model SG-700 Imaging Densitometer (Bio Rad).

Enzyme and metabolite assays

Catalase was assayed spectrophometrically by estimating the rate of decomposition of hydrogen peroxide**,** H_2O_2 (Beers *et al*., 1952). SOD was assayed by monitoring the inhibitory effect of the enzyme on the auto-oxidation of epinephrine (Misra and Fridovich 1989). The assay of Na^+-K^+-ATPase was carried out colorimetrically by estimating the amount of inorganic phosphate, P_i liberated upon incubation of the tissue homogenates with disodium ATP (Hesketh *et al*., 1979). The P_i was determined colorimetrically using ammonium molybdate.

MDA was estimated colorimetrically based on its reaction with thiobarbiturate to yield a pink-coloured complex that absorbs strongly at 532 nm (Gutteridge and Wilkins 1982).

Statistical analysis

Statistical analysis was performed using Student´s t-test. The results were expressed as the mean ± standard deviation (SD). Statistical significance was assumed at a level of $p < 0.05$ (Snedecore and Cochran 1976).

RESULTS

Protein analysis

The total protein concentration of the control cornea was 26.4 ± 0.7 mg/g tissue (Fig. 1). This value was significantly increased in corneal endosulfan group to 64.6 ± 1 ($p < 0.05$) with percentage change of 145% with respect to the control. After treatment with curcumin 40, 80µmol/L, the protein content decreased to 54.1± 0.6 and 43.6 ± 0.8 mg/g tissue, respectivel. After 160 µmol/L of curcumin, the protein content showed progressive reduction nearly to the control value (28.7±0.6mg/g tissue)

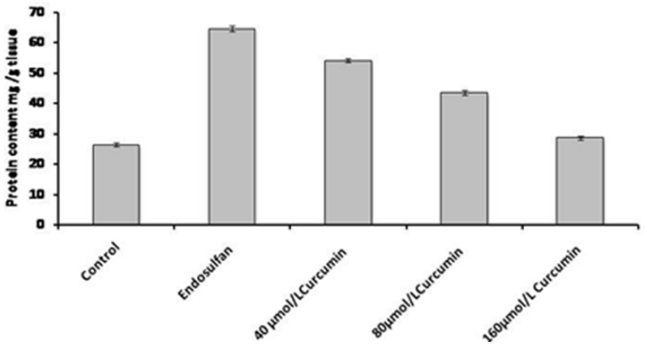

Figure 1. Protein content of rabbit cornea for control group, endosulfan group and curcumin treated groups to 40,80 and 160 µmol/L.

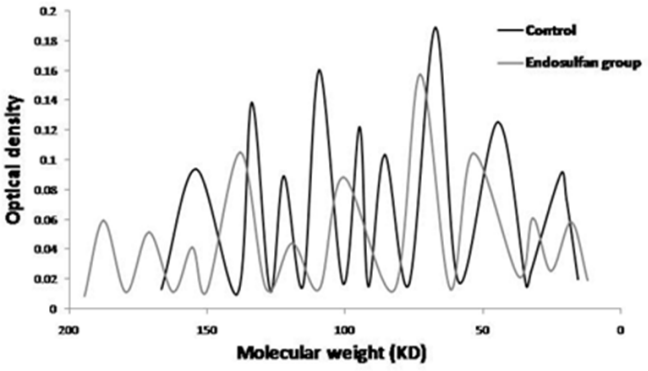

Figure 2. SDS-PAGE pattern of rabbits´corneal protein for control vs. corneal endosulfan group

Figure (2) illustrated the electrophoresis pattern of normal rabbit's cornea and endosulfan group. The normal pattern was characterized by 9 fractions represent the different proteins from cornea with a molecular weights range of (167-16 KDa).

The pattern of rabbit's cornea from endosulfan group appeared in 10 peaks with a molecular weights range of (187-13 KDa). Also it revealed a significant decreased in the intensity of different bands and the peaks were shifted towards high molecular weight.

Figure (3) illustrated the electrophoresis pattern of normal rabbit's cornea, endosulfan group and curcumin (40µmol/L) group. The electrophoretic pattern was little shifted towards lower molecular weight compared to endosulfan group but it was significant different than the control group.

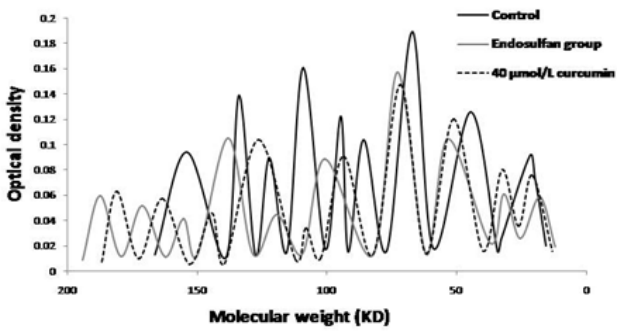

Figure 3. Electrophoretic pattern of rabbits´corneal protein for control vs. corneal endosulfan group and treated with 40 µmol/L curcumin group

After eyes were treated with 80 µmol/L curcumin, the electrophoretic pattern was shifted towards lower molecular weight with a range of (175-6 KDa) as shown in Figure (4). There is a diffuse of the last two peaks bands.

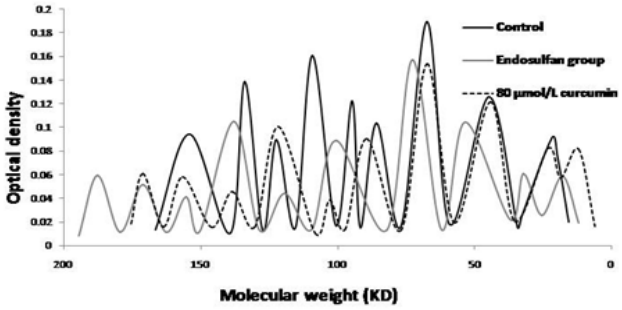

Figure 4. SDS-PAGE pattern of rabbits' corneal protein for control vs. corneal endosulfan group and treated with 80 µmol/L curcumin group

Figure (5) illustrated the electrophoresis pattern of normal rabbit's cornea, endosulfan group and rabbits treated with 160 µmol/L curcumin after administrated a dose of endosulfan 1.0 mg/kg/day in corn oil. The profile of treated group revealed the decrease of peaks to 9 fractions and the similarity to the normal profile in molecular weight but little difference in intensity of some peaks.

Enzyme and metabolite assays

Table 1 illustrated Catalase activities, SOD activities, Na^+-K^+-ATPase and MDA for control corneas, endosulfan and treated with all doses of curcumin groups. Catalase activity was significantly increased ($p<0.05$) in the cornea of rabbits given endosulfan. After treatment with curcumin, there is dose response relationship appeared as decrease the effect of endosulfan in line with increase the dose of curcumin. When it reached 160 µmol/L the catalase activities matched the control value.

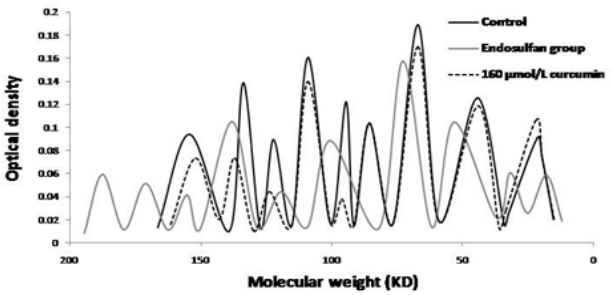

Figure 5. SDS-PAGE pattern of rabbits´corneal protein for control vs. corneal endosulfan group and treated with 160 µmol/L curcumin group

Table 1. Catalase activities, SOD activities, Na^+-K^+-ATPase and MDA for control corneas, endosulfan and treated with all doses of curcumin groups

	Catalase activities (Moles of H_2O_2 decomposed/min/g tissue)	SOD activities (Units/min/mg wet tissue)	Na^+-K^+-ATPase (µmolePi/hr/mg protein)	MDA (moles/g wet tissue)
Control	32.6±2	0.23±0.01	630±4	10±0.4
Endosulfan	41.5±2*	0.16±0.01*	420±6*	20.7±2*
Curcumin 40µmol/L	39.7±1*	0.17±0.02*	500±3*	18.1±1*
Curcumin 80µmol/L	36.1±2	0.20±0.03	510±4*	15.3±0.9*
Curcumin 160 µmol/L	35.2±3	0.22±0.04	620±7	12.1±0.9

*Statistically significant ($P< 0.05$)

SOD activity was significantly decreased ($p<0.05$) in the cornea of rabbits given endosulfan and the same phenomena for the group treated with 40µmol/L of curcumin. For the two groups treated with 80 and 160 µmol/L of curcumin, there were no significant differences in SOD activity compared to control. Na^+-K^+-ATPase activities were significant decrease ($p<0.05$) for all groups except which treated with 160 µmol/L equal to 620±7 µmolePi/hr/mg protein that was matched control value. MDA were significant increase ($p<0.05$) for all groups except which treated with 160 µmol/L equal to 12.1±0. moles/g wet tissue that match control value.

DISCUSSION

Endosulfan, an organochlorine pesticide, is a broad spectrum contact insecticide widely used in pest control. There is a global concern over the acute toxicity of endosulfan. The health and ecological hazards caused by exposure to endosulfan has been a global concern. Endosulfan persists in the environment and bioaccumulates in animals and plants, leading to instances of food contamination and eventually dietary exposure in humans. Xenobiotic chemicals such as

pesticides may affect the metabolism of natural chemicals and modify their antioxidant potential with concomitant exposure, and also trigger molecular events that could increase their health risk (Yang 2006).

A number of studies provided evidence for the capacity of organochlorine pesticides to induce oxidative stress (Ozdem *et al*., 2011). Moreover, endosulfan-induced oxidative stress was reported in rainbow trout, Oncorhynchus mykiss (Dorval and Hontela, 2003; Dorval *et al*., 2003). In accordance, in the present study, endosulfan administration for 6 weeks caused a significant increment in MDA which is as an indicator of lipid peroxidation. SOD activity was significantly reduced, and CAT activity was significantly increased. The cause of increased MDA is the increase in lipid damage by the oxygen (O_2) radicals arising from the decrease in SOD activity. As for the decrease in SOD activity and the increase in CAT activity, one possibility is that of the H_2O_2 produced apart from the SOD, thereby providing a balance mechanism for the organism.

Oxidative stress is a result of one of three factors: (1) an increase in ROS, (2) an impairment of antioxidant defense systems or (3) a default to repair oxidative damage. ROS have no specific targets; they can attack lipids (mainly unsaturated lipids), proteins, peptides, amino acids, nucleic acids, carbohydrates and low-molecular weight compounds, with variable kinetics of reaction producing various derivatives dependent on the nature of the ROS and the target molecule (Guetens *et al*., 2002; Landar *et al*., 2006). Although ROS is essential for physiological functions, including cell growth, proliferation, differentiation and apoptosis, their excess may have a detrimental influence on cellular components. It can cause oxidative modifications of biomolecules, including lipids, proteins, carbohydrates and nucleic acids, leading to cellular and tissue damage. Accumulation of ROS produced by external factors and normal cellular metabolism was associated with the development of several diseases, including: cancer, diabetes, autoimmune disorders, neurodegenerative diseases and aging (Birben *et al*., 2012; Kryston *et al*., 2011). Also, the cornea was shown to be affected by the accumulation of ROS as well as due to malfunctions in corneal antioxidant defense mechanisms, leading to structural and functional changes in this tissue. The level of ROS in normal corneas is regulated by antioxidant defense mechanisms, however results of some studies suggest disturbance in the level of transcripts and/or activities of different antioxidant enzymes in animal exposed to pesticides (Bebe and Panemangalore 2003). A decreased activity of extracellular superoxide dismutase (SOD) corneas was recorded when compared to normal samples. SOD is an important enzyme responsible for the dismutation of superoxide to oxygen and hydrogen peroxide. Alterations in the activity of SOD may lead to an increased amount of superoxide radicals (Olofsson *et al*., 2007; Zelko *et al*., 2002).

Ocular oxidative stress leads to changes in electrolyte balance in the cornea, as consequence of depression of Na^+-K^+-ATPase activity in cornea tissue. Na^+-K^+-ATPase functions to maintain electrolyte balance in the cornea (Whikehart 2004). The enzyme is inhibited by many factors such as free radicals, H2O2 and changes in membrane lipid composition. Consequently the pattern of Na^+-K^+-ATPase in the cornea of the endosulfan group are strongly indicative of the toxicity of endosulfan. In the present study, the change in corneal protein after induction of endosulfan was evaluated. The results indicated elevated levels of corneal protein percentage above the baseline of the control (145%). These results suggested that, the administration of endosulfan caused corneal hypoxia. This process was associated with an enhanced formation of blood vessels containing blood component, leading to elevated levels of protein content of the cornea and therefore changing in its SDS-electrophoresis pattern. In accordance, Atif *et al*. (2005) reported significant increments in formation of protein carbonyls in liver, kidney and gill of endosulfan-exposed freshwater fish, demonstrating a direct damage to proteins or chemical modification of amino acids as a result of oxidative stress.

Curcumin is a polyphenol extracted from the rhizome of the plant Curcuma longa. It appears to be nontoxic, and it has anti-oxidative, anti-inflammatory, and anti-carcinogenic properties through the blockage of several signal pathways. Though the exact reaction mechanism and target molecule of curcumin is unknown, the substance has been shown to promote blockage of the signal pathway associated with protein kinase C, phospholipase A2, and arachidonic acid. In our study, endosulfan toxicity was inhibited in the 80 and 160 µmol/L curcumin treatment groups, but not in the 40 µmol/L treatment group. Being a lipophlilic molecule, curcumin interacts with cellular membrane and is subsequently transported inside the cell. In a previous study, quantitative cellular uptake and intracellular localization of curcumin were reported in normal and tumor cells (Kunwar *et al*., 2008). It revealed that a linear dependency on the uptake was observed with treatment concentration of curcumin, and curcumin is located both in the cell membrane and the nucleus. Although it was the experiment with spleen lymphocyte, not corneal cell, we could analogize the pharmacologic activity of curcumin to the corneal cells. Further study is required to investigate quantitative uptake of curcumin in the corneal cells.

In summary, our results indicate that topical curcumin reduces experimental corneal toxicity due to endosulfan related to the inhibition of ROS. These findings might help in the treatment of various corneal diseases, and our results might indicate the basic dosage required in the clinical application of curcumin. We could not investigate the other effects of topical curcumin on the cornea, and our study had a short follow-up period. Hence, the safety and longevity of the treatment effect remain unknown. Further studies are required to investigate these factors.

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