



Preventive Role of quercetin against Non-enzymatic Peroxidation and Oxidative stress in Brain Mitochondria induced by Endosulfan in Rat

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Abstract

Endosulfan (END) is still in use in north-Africa although it has large environmental ubiquity and toxicity. The non-enzymatic peroxidation and redox status in rat brain mitochondria after END toxicity have not been sufficiently investigated. In the present study, we first investigated the pro-oxidant effect of END on brain mitochondria and its effect upon lipid peroxidation in this organelle. Gavages of END into rats at a dose of 4 mg/kg induced oxidative stress in brain mitochondria, so it provoked a statistically significant reduction of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) levels and proteins. Significant increase in malondialdehyde (MDA) levels – an indicator of lipid peroxidation – was observed in neuronal mitochondria. Second, the protective effect of quercetin (QE) (5mg/kg) against endosulfan-induced non-enzymatic peroxidation in the same organelle was also investigated. Indeed, the pretreatment of rats with QE protected brain mitochondria from oxidative stress and membrane modification. This treatment conserves the integrity of mitochondrial membranes following the inhibition of lipid peroxidation. Thus, QE works through the prevention of mitochondrial membrane perforation and the antioxidant defense system of brain mitochondria.

Keywords: Endosulfan, brain mitochondria, redox status, quercetin, lipid peroxidation, preventive effect.

Introduction

Endosulfan is an organochlorine insecticide belonging to the cyclodiene subgroup. It is composed of a mixture of two stereoisomers: α and β -endosulfan, in the ration of 70/30. This compound has been widely used for its broad spectrum insecticide/acaricide since its introduction in the 1950s, but little information is available concerning the volumes of production. As an indication, it was estimated that 10,000 metric tons were produced worldwide in 1984^{1,2}. Nowadays, this rate remains comparable³. Subsequently, END has shown a large environmental ubiquity, persistence and toxicity^{4,5}. As a result, it is now banned for sale and use in Europe (EU Directive 2005/864/EC) and has been proposed to be listed for a global ban under the Stockholm Convention on Persistent Organic Pollutants³. Nevertheless, END is still in use in several countries, including north-Africa, where it has been detected in fruits and vegetables at 1.20 mg/kg⁶. However, higher levels have been found, reaching 4 ppm in tomatoes harvested in Jijel (Algeria, unpublished results).

Exposure to endosulfan mainly occurs through ingestion of contaminated food, but also happens via inhalation or dermal contact⁴. Due to its liposolubility⁷, this xenobiotic accumulates in human tissues⁸, including brain⁹, where it is able to cross the blood-brain barrier. Exposure of the unborn and the new-born is also observed since END is able to cross the placental barrier¹⁰

and is lactationally transferred through the lipid phase of breast milk¹¹. Overstimulation of the central nervous system is the major characteristic of END poisoning. The most important reported symptoms are the induction of epileptic seizures, focal motor seizures, unconsciousness, agitation, disorientation and an increase in anxiety¹². The neurological damages resulting from END poisoning can be permanent. This xenobiotic induces neurotoxicity by binding and blocking the Cl⁻ channel linked to the γ -amino-butyric acid (GABA_A)-receptor at synapses, resulting in uncontrolled excitation¹³. Increase in serotonin concentrations in the cerebrum and midbrain regions have been also enlightened after oral administration in male rats¹⁴. In addition, it has been shown that END exerts oxidative stress *in vivo* when administered at low doses, in several tissues, including cerebral tissue¹⁵.

The oxidative stress, as a pathological condition, is defined as a state of imbalance reached when the production of potentially destructive reactive oxygen species (ROS), that are products of normal and aberrant cell metabolism, exceeds the capacity of detoxification systems, leading to cell damage induced by the interaction of ROS with cellular constituents. Mitochondria are considered to be the major ROS producers, the free radicals being formed during the enzymatic cascades of the oxidative phosphorylation¹⁶.

As the brain is particularly susceptible to oxidative stress because of its high O₂ consumption, its lipid-rich constitution and its limited amount of antioxidant capacity^{17, 18} and because substantial evidence that mitochondrial dysfunction and oxidative damage may play a critical role in the pathogenesis of neurodegenerative disease such as Alzheimer's and Parkinson's disease or multiple sclerosis^{19, 20}, we were interested in assessing the pro-oxidant activity of END and its implication upon non-enzymatic per oxidation in brain mitochondria and to determine if quercetin (QE), a well-known anti-oxidant compound, could exert a protective effect *in vivo* against sub-acute END toxicity. Indeed, according to Heijnen et al. and Kebieche et al. works^{21, 22}, QE is the most active scavenger of ROS and reactive nitrogen species due to the presence of two pharmacophores within the molecule. More, it is one of the most widely distributed polyphenolic flavonoids, highly abundant in food and beverage sources (broccoli, lettuce, apples, tomatoes, onions, tea and coffee). The estimation of daily consumption has been set at about 25-50 mg/day²³, and QE has been shown to be safe and effective at relatively low dosages²⁴.

To achieve this aim, END was orally administered for one week to a first group of rat while and a second group was treated with END in association with QE, after that malondialdehyde (MDA), glutathione (GSH), proteins levels as well as superoxide dismutase (Mn-SOD) and catalase (CAT) activities are assessed in the rat brain mitochondrial matrix and compared to a control group.

Material and Methods

Chemicals: The majority of chemicals were procured from Sigma Aldrich, Germany. Endosulfan was purchased from Pharmacia, St. Quentin Yvelines, France.

Animal maintenance: Male Wistar *albinos* rats (body weight 220–280g) were originally from the Pasteur Institute in Algiers, Algeria, were used in these experiments. Rats were bred in our animal facility in stainless metallic cages. The room housing threats was temperature controlled (average of 22 °C, 50–60% relative humidity) and kept under a daily 12h light/dark cycle. Rats were fed food and water *ad libitum*. Rats were adapted for 1 week before the indicated treatments. All experimental assays were carried out in conformity with international guidelines for the care and use of laboratory animals.

Animal treatment protocol: The animals were grouped as follows: *Group 1, control rats:* rats were administered 1 ml of olive oil *per os* daily for 6 days. *Group 2, Endosulfan treated:* rats were administered 1 ml of endosulfan at 4 mg/kg in olive oil, *per os* daily for 6 days. *Group 3, preventative group:* rats were administered 1 ml of QE (5 mg/kg) + endosulfan (4 mg/kg) in olive oil, *p.o* daily for 6 days.

Preparation of mitochondria matrix fraction: Mitochondrial matrix was prepared by applying the method described by

Rustin et al.²⁵: Brains were quickly removed and perfused with 0.86% cold saline to completely drain all the red blood cells, chopped into small pieces and placed into ice-cold isolation buffer for mitochondria (10 mM Tris-HCl, pH 7.4, 250 mM Sucrose, 0.5 M Methylene diamine tetra acetic acid (EDTA) and 0.5% bovine serum albumin). After being homogenized, the homogenate was centrifuged first at 2000 rpm for 20 min to eliminate cell membrane fragments. The supernatant obtained was centrifuged at 10,000 rpm for 10 min at 4°C. Mitochondrial pellets were washed twice with and then resuspended in isolation buffer. The mitochondrial matrix was extracted from freshly prepared mitochondria by freezing and defrosting with repeated homogenization in order to burst mitochondria. After centrifugation at 10,000 rpm for 10 min, the supernatant was the source of CAT, SOD, GSH and MDA. Protein estimation was performed by Lowry method²⁶.

Biochemical evaluation of MDA, GSH, Proteins, CAT and SOD in rat brain mitochondria: MDA levels in the mitochondria were evaluated as follows²⁷. First, 0.5 ml of mitochondria matrix fraction was added to 0.5 ml of trichloroacetic acid (TCA) (20%) and 1 ml of thiobarbituric acid reactive substances (TBARS) (0.67%). The mixture was placed in boiling water. The tubes were moved to an ice-bath, received 4 ml of n-butanol and were centrifuged at 3000 rpm for 15 min. The optical density of the supernatant was then assessed at 532 nm (UV mini 1240 UV-vis spectrophotometer SHIMADZU, China). MDA amounts are expressed as nmol/g of brain and were calculated using a standard curve prepared under the same conditions with a solution of 1, 1, 3, 3-tetraethoxypropane that produces MDA after hydrolysis.

Levels of GSH were assessed using Ellman assay²⁸. Then, 50 µl of the cytosol fraction were diluted in 10 ml of phosphate buffer (0.1 M, pH 8.0). Twenty microliter of 5, 5'-dithiobis 2-nitrobenzoic acid (0.01 M) (DTNB) was added to 3 ml of the mixture dilution. After 15 min, the absorbance of thionitrobenzoic acid (TNB) produced after oxidation of GSH by DTNB was evaluated at 412 nm against a blank prepared by TCA (5%) under the same conditions. The GSH amounts were calculated using a standard curve of GSH, and were expressed in mmol/g.

Mitochondrial CAT assessment was performed by Clairborne²⁹ method. This assay is based on the disappearance of H₂O₂ at 25°C in the presence of mitochondrial enzyme source. Briefly, the assay mixture contained 1 ml of phosphate buffer (KH₂PO₄ 0.1 M, pH 7.2), 0.975 ml of freshly prepared H₂O₂ (0.091 M) and 0.025 ml of the enzymatic source. The absorbance was measured at 240 nm each minute, for 2 min. The enzymatic activity was calculated in terms of IU/mg of protein.

Mitochondrial Mn-SOD assessment was performed by Beauchamp and Fridovich³⁰ technique. The assay mixture contained 2 ml of reactive milieu (cyanide of sodium 10⁻² M, NBT solution 1.76x10⁻⁴, EDTA 66 mM, methionin 10⁻² M and riboflavin 2 µM, pH 7.8) and 5 mL of the enzymatic source

(Mitochondrial matrix). This solution mixture was exposed to a 15W lamp for 10 min to induce the photoreaction of riboflavin and O₂. The reduction of NBT by a superoxide anion on formazan was measured Spectrophotometrically at 560nm. The enzymatic activity was calculated in terms of IU/mg of protein.

Statistical analysis: The numerical and graphical results are presented as mean \pm standard error (SE). The significance of the difference between two treatment groups was verified by the Student's t-test. The degree of statistical significance was set at a level of $p < 0.05$. Statistical calculations were carried out using the Statviews 4.5 statistical package (Abacus Concept,Int) and the Excel 6.0 (Microsoft,Inc.).

Results and Discussion

Mitochondria, as a primary site of cellular energy generation and oxygen consumption represent itself a likely target for END poisoning. Therefore, the objective of the current study was planned with an aim to investigate the effect of acute END exposure on homeostasis redox in mitochondria brain in rats, oxidative stress generation and its implication in non-enzymatic lipid peroxidation.

In this study, assessment of in vivo lipid peroxidation in brain mitochondria through MDA levels in control group, END-treated group and preventive group were significantly increased ($p < 0.05$) in brain mitochondria (0.150 ± 0.016 nmol/g) in the END-treated group compared to the normal control (0.124 ± 0.002 nmol/g). However, no significant difference was recorded between the normal group and the preventive group (0.121 ± 0.011 nmol/g), (figure-1). Lipid peroxidation at the level of the mitochondria impairs mitochondrial metabolism and induction of the Mitochondrial Pore Transition Permeability (PTP). In previous studies, cytochrome c which is bound to the inner mitochondrial membrane by an association with the anionic phospholipid cardiolipin has been shown to be released via oxidation of cardiolipin during apoptosis which precedes its release to the cytosol³¹.

In present study, GSH level, such as non-enzymatic antioxidant, was significantly reduced ($p < 0.01$) in brain mitochondria in END-treated group (0.068 ± 0.043 nmol/g) compared to the control group (0.435 ± 0.147 nmol/g). In the same time, there was no different between normal group and preventive group (0.344 ± 0.074 nmol/g), (figure-2). Regarding the decrease of this tripeptide, which constitutes the first line of defense against free radicals, is possibly due to its action against the ROS generation in the END-treated group but the increase of GSH level in preventive group may be due to *de novo* GSH synthesis or GSH regeneration following ROS neutralization by the quercetin. The previous studies have showed that GSH is a potentially important link between oxygen radical production and mitochondrial damage, because of the scavenging activity of this tripeptide against accumulation of oxygen radicals and its decrease in the brains of parkinsonian patients^{22,32,33}.

Proteins assessment in brain mitochondria is shown in figure-3. The global value of these proteins was significantly decreased in END-treated group ($p < 0.01$) compared to the proteins level in control group (454.212 ± 37.780 μ g/g; 707.372 ± 30.27 μ g/g of tissue) successively. Moreover, these reduces values observed in the intoxicated group with END are normalized in the preventive group (727.372 ± 73.37 μ g/g of brain tissue). Assessment of antioxidant enzymes, CAT and Cu/Zn-SOD is shown in Table-1. The administration of END to animals caused a highly significant ($p < 0.01$) decrease in CAT and Cu/Zn-SOD levels (150.84 ± 35.9 UI/mg and (0.994 ± 0.299 UI/mg) respectively when compared to the control group (352.4 ± 23.5 UI/mg and 1.932 ± 0.325 UI/mg) successively. On the other hand, the treatment of animals with QE (5mg/kg) and END normalized the cellular content of these antioxidant enzymes in brain mitochondria (260.34 ± 32.4 UI/mg and 1.636 ± 0.359 UI/mg in order, compared to the levels contained in normal controls. Because of their high reactivity and short life, ROS, in this study, have been analysed indirectly in vivo by measuring the changes in antioxidant enzymes including SOD and CAT. SOD catalyses the dismutation of O₂ Radicals into H₂O₂ and O₂. In parallel, CAT decomposes H₂O₂ into O₂ and H₂O. The reduction of these antioxidant enzymes explains the intense production of superoxide anion in the respiratory chain following the probable alteration in its electron transport system. This abnormality in the rate of different antioxidants might have resulted from intense ROS generation induced by endosulfan administration in brain mitochondria^{34,35}, which in turn might have caused an increase in malondialdehyde, as a result of enhanced lipid peroxidation. Thus, acute organochlorine exposure has the potential to disturb cellular antioxidant defence system and alterations in lipid membrane of brain mitochondria. In previous studies, researchers have largely demonstrated, through different mechanisms, alterations in the redox status induced by environmental stressors. Indeed, environmental toxicants can attack the mitochondria directly causing intense production of ROS which can therefore deplete antioxidant defences and mediates other redox reactions that promotes oxidative stress³⁶. Environmental stressors can also induce oxidative stress by mediating a variety of reactions through different metabolic pathways such as those mediated by detoxifying enzymes. These phenomena have a crucial impact on the mitochondria damage and its integrity in the cell³⁷⁻³⁸. It is conceivable therefore that regardless of whether oxidative stress or mitochondrial damage represents the initial insult, these toxic mechanisms may both contribute to neuronal degeneration via changes in glutathione levels³⁸⁻⁴⁰. In the current study, the treatment of animals with QE maintained the normal content of GSH, SOD and MDA. Therefore, QE showed really antioxidant properties against ROS observed during animal exposition to endosulfan brain injury and traduced consequently its cytoprotective effects. In our previous studies, the same results were obtained when rats were administered by QE to prevent hepatotoxicity against oxidative stress induced by epirubicin²².

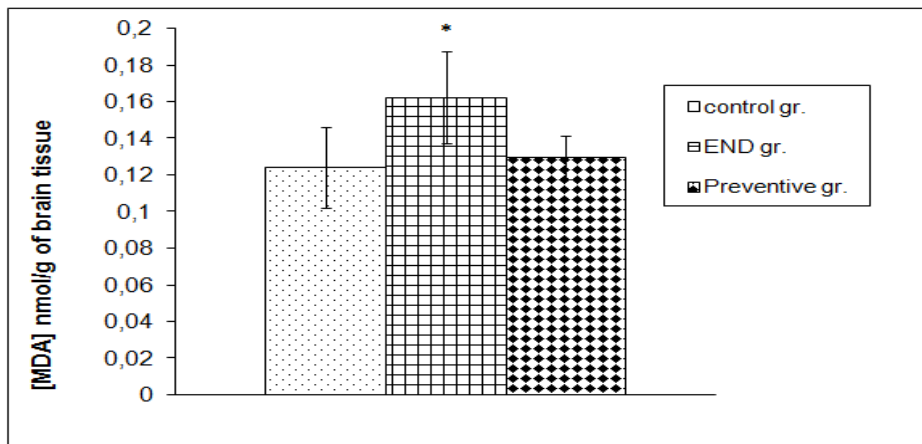


Figure-1

Effect of endosulfan treatment on brain mitochondria level of MDA, in rats and preventive role of QE. Values are mean \pm S.E. (n=5). * $P < 0.05$ as compared to normal control

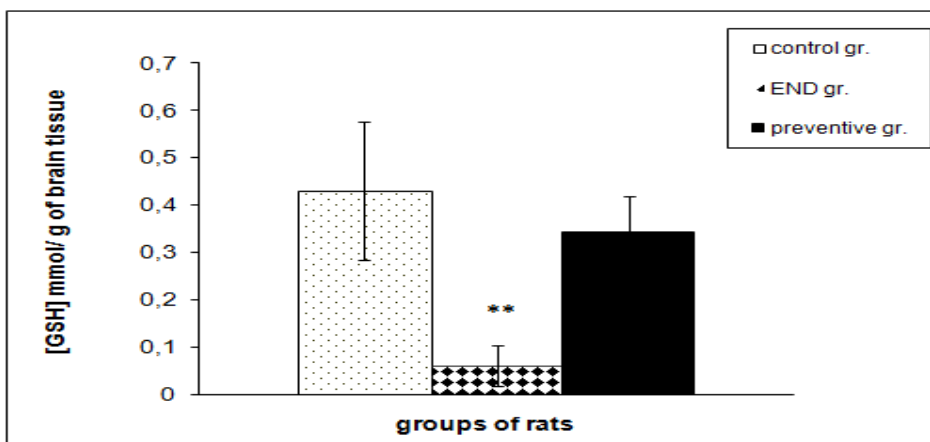


Figure-2

Effect of endosulfan treatment on brain mitochondria level of GSH, in rats and preventive role of QE. Values are mean \pm S.E. (n=5). ** $P < 0.01$ as compared to control group

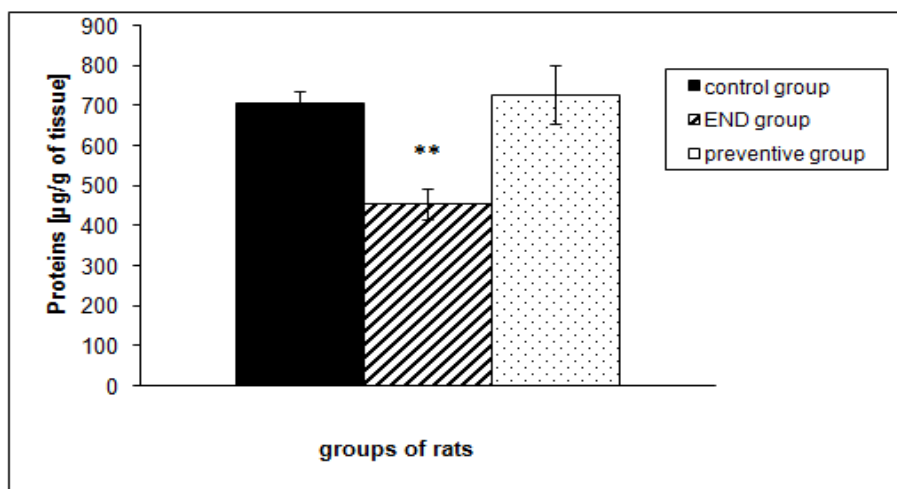


Figure-3

Effect of endosulfan treatment on brain mitochondria level of proteins, in rats and preventive role of QE. Values are mean \pm S.E. (n=5). ** $P < 0.01$ as compared to control group

Table-1
Enzymatic activities of SOD and CAT in brain mitochondria in different rat groups

Parameters Rats	Antioxidases activity	
	SOD	CAT
Control group (1ml of olive oil)	352.4 ± 23.5	1.932 ± 0.325
Endosulfan group (4mg/kg in olive oil)	150.84 ± 35.9 **	0.994 ± 0.299*
Preventive group (END4 mg/kg+ QE 5mg/kg in olive oil)	260.34 ± 32.4	1.636 ± 0.359

Values are mean ± S.E. (n=5). ** P< 0.01 and * P< 0.05 as compared to control group

Conclusion

In summary, this is in vivo experiment to demonstrate that END has oxidative stress and non-enzymatic peroxidation effect in experimental rats. Redox status imbalance was showed by increasing of antioxidant systems (SOD, CAT, GSH) and intense peroxidation indicated by MDA elevation in rats receiving END. QE has a protective effect against the damage following END brain injury in rats. The oxidative stress and lipid peroxidation were normalized and brain mitochondria were significantly protected following QE administration in association with the pesticide. However, further investigations are essential to elucidate the precise mechanisms of END, its impact upon mitochondrial integrity and respiratory function. The mechanisms of QE prevention against brain injury of END must also be deeply investigated.

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