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RESEARCH ARTICLE

Oxidative stress and brain mitochondria swelling induced by endosulfan and protective role of quercetin in rat

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Abstract The neurological damages resulted by endosulfan poisoning is not completely elucidated, especially in cellular organelles such as mitochondria. In the present study, the pro-oxidant effect of endosulfan on brain mitochondria was first investigated. Gavages of endosulfan into rats at the dose of 2 mg/kg induced oxidative stress in this organelle since it provokes a significant reduction of catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH) level. In addition, a significant increase in mitochondria swelling and malondialdehyde (MDA) levels were observed in neuronal mitochondria, indicating clearly an intense peroxidation within mitochondria. Second, the protective effect of quercetin (QE) (10 mg/kg) against endosulfan-induced oxidative stress in mitochondria was also assessed. Indeed, the pretreatment of rats with QE protects brain mitochondria from oxidative stress, lipid peroxidation, and mitochondria swelling induced by endosulfan. The activities of antioxidant enzymes and the mitochondrial content of GSH and MDA were returned to control values. Thus, although endosulfan can have neurotoxic effects in brain rats, this toxicity can be prevented by quercetin.

Keywords Endosulfan · Brain mitochondria · Pro-oxidant effect · Mitochondrial swelling · Quercetin · Rat

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Introduction

Endosulfan (END) is an organochlorine insecticide belonging to the cyclodiene subgroup. This compound has been widely used for its broad spectrum insecticide/acaricide since its introduction in the 1950s. Exposure to endosulfan mainly occurs through ingestion of contaminated food, but also happens via inhalation or dermal contact (WHO 2002; Silva and Gammon 2009). This xenobiotic induces neurotoxicity in insects by binding and blocking the Cl^- channel linked to the γ-amino-butyric acid ($GABA_A$) receptor at synapses, resulting in uncontrolled excitation (Silva and Beauvais 2010). Subsequently, END is a persistent organic pollutant and has shown a large environmental ubiquity, persistence, and toxicity (ATSDR. 2013; Sunitha et al. 2012). As a result, it is now banned for sale and use in Europe and has been proposed to be listed for a global ban under the Stockholm Convention on Persistent Organic Pollutants (POPRC-4 2008). Nevertheless, endosulfan is still in use in several countries, including North Africa, where it is detected in fruits and vegetables at 1.20 mg/kg (Zerouali et al. 2005). However, higher levels have also been found, reaching 4 mg/kg in tomatoes harvested in Jijel (Algeria, unpublished results).

Human organism produces oxygen free radicals and other reactive oxygen species (ROS), as by-products through numerous physiological and biochemical processes, such as cellular metabolism (respiratory burst and enzyme reactions) which confers free radicals to the cellular environment. Adding to that is the human exposure to pollutants such as polycyclic aromatic hydrocarbons and pesticides. The most common reported cellular free radicals are superoxide ($O_2^{\cdot}$), hydroxyl ($OH^{\cdot}$), and nitric monoxide ($NO^{\cdot}$). Even some other molecules like hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are not free radicals; they are reported to generate free radicals through various chemical reactions (Halliwell 2006; Uttara et al. 2009; Kebieche et al. 2009).

Overproduction of free radicals can cause an imbalance in cellular redox status producing oxidative damage to biomolecules (lipids, proteins, and DNA). At the same time, antioxidants, such as glutathione, arginine, citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C, vitamin A, and polyphenols, help to regulate the generated ROS. The antiradical system is further supported with antioxidant enzymes, superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase, that exert synergistic actions in removing free radicals (Mytilineou et al. 2002; Uttara et al. 2009).

The brain is particularly susceptible to oxidative stress because of its high O_2 consumption, its lipid-rich constitution, and its limited amount of antioxidant capacity (Halliwell 2006; Ng et al. 2009). There is substantial evidence that oxidative damage and mitochondrial dysfunction play a central role in different cell death pathways, leading to either apoptosis or necrosis which is the origin of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Cassarino and Bennett 1999; Emerit et al. 2004; Uttara et al. 2009). This report describes the impact of endosulfan on the brain mitochondria redox status and mitochondrial permeability transition pore (MPTP) and determines also if lipid-soluble antioxidants, such as quercetin (QE), are useful to protect endosulfan toxicity in rats.

Materials and methods

Chemicals

The majority of chemicals were procured from Sigma-Aldrich, Germany. Assay kits for enzymes were purchased from Biomerieux, and endosulfan was purchased from Pharmacia, St. Quentin in Yvelines, France.

Animal maintenance

Male albino Wistar rats (body weight 220–280 g), 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 the rats was temperature controlled (average of 22 1 C, 50–60 % relative humidity) and kept under a daily 12 h light/dark cycle. Rats were fed food and water ad libitum. Fasted rats were deprived of food for at least 16 h, but were allowed free access to water. 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 (p.o.) daily for 6 days. *Group 2*, *endosulfan-treated*: Rats were administered 1 ml of endosulfan at 2 mg/kg in olive oil per os (p. o.) daily for 6 days. *Group 3*, *preventative group*: Rats were administered 1 ml of QE (10 mg/kg)+endosulfan (2 mg/kg) in olive oil *p.o*. daily for 6 days.

Preparation of mitochondria matrix fraction

Mitochondrial matrix (stroma) was prepared by applying the method described by Fan et al. (2005) and Rustin et al. (1994). Briefly, brains were quickly removed and washed 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 at 750 g for 10 min. Next, the supernatant was centrifuged at 10,000 rpm for 10 min at 4 °C. Mitochondrial pellets were washed twice with isolation buffer and then resuspended in the same buffer solution. 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 catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA). Protein estimation was performed by the method of Lowry et al. (1951).

Biochemical evaluation of MDA, GSH, CAT, and SOD in rat brain mitochondria

MDA levels in the mitochondria were evaluated using the method of Ohkawa et al. (1979). MDA amounts are expressed as nanomoles per gram 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 assay (1959). The GSH amounts were calculated using a standard curve of GSH and were expressed in millimoles per gram. Mitochondrial CAT assessment was performed by using the method of Clairborne (1985). This assay is based on the disappearance of H_2O_2 at 25 °C in the presence of mitochondrial enzyme source. Mitochondrial Mn-SOD assessment was performed by Beauchamp and Fridovich (1971) technique. The enzymatic activity was calculated in terms of international unit per milligram of protein.

In vitro mitochondria swelling essay

The assessment of mitochondria swelling is realized upon mitochondrial suspension according to the method of Kristal et al. (1996), with modification. Briefly, a mitochondrial suspension is used at 1 mg/ml and incubated in total volume of 1.8 ml of breathing buffer added to 10.8 μl of succinate (6 mM). After 1 min of incubation, 18 μl of different concentrations of endosulfan (0–200 to 300–400 μM), associated or not with QE (200 μM), is added to induce mitochondria swelling. A decrease of the absorbance at 540 nm is monitored by spectrophotometry (UV–vis mini 1240 spectrophotometer SHIMADZU, China) every minute during 5 min.

Statistical analysis

The numerical and graphical results are presented as mean± 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

Assessment of in vivo lipid peroxidation in brain mitochondria

Levels of MDA, the last product of lipid peroxidation caused by oxidative stress, were assessed in brain mitochondria of different groups of rats. MDA values were significantly increased ($P<0.01$) in brain mitochondria (0.185±0.015 nM/g) in the endosulfan-treated group compared to the normal control (0.109±0.009 nM/g). However, no significant difference was recorded between the normal group and the preventive group (0.113±0.010 nM/g) (Fig. 1).

Assessment of antioxidant enzymes in brain mitochondria, CAT, and Cu/Zn-SOD

The administration of endosulfan caused a highly significant ($P<0.001$) decrease of CAT and Cu/Zn-SOD (1.270±0.66 and 140.637±19.184 IU/mg), respectively, in rats when compared to the control group (2.018±0.083 and 335.307± 19.184 IU/mg) successively. On the other hand, the protective treatment of animals with QE (10 mg/kg) and endosulfan normalized clearly the cellular content of these antioxidant enzymes in brain mitochondria (1.819±0.015 and 290.835± 18.998 IU/mg) in order compared to the levels contained in normal controls (Figs. 2 and 3).

GSH evaluation

The GSH-reduced levels were significantly decreased ($P<0.01$) in brain mitochondria (0.088±0.09 mM/g) compared to the control group (0.475±0.029 mM/g). At the same time, there was no different between normal group and preventive group (0.433±0.074 mM/g) (Fig. 4).

Mitochondria swelling essay

This in vivo essay showed a proportional relationship between elevation of mitochondria swelling and endosulfan concentrations (Fig. 5) with strong correlation ($r=0.98$). By contrast, when endosulfan (200 μM) is associated with quercetin

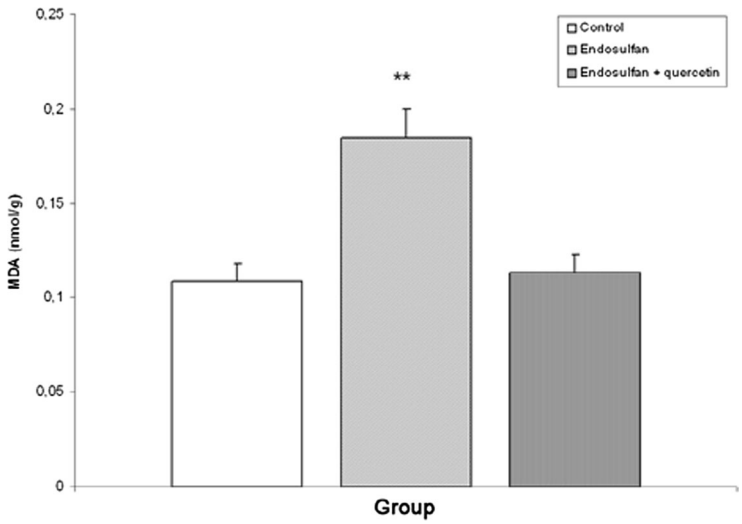

Fig. 1 Effect of endosulfan treatment on brain mitochondria level of MDA in rats and protective role of QE. Values are mean±S*E* ($n=5$). $^{**}P<0.01$ as compared to normal control

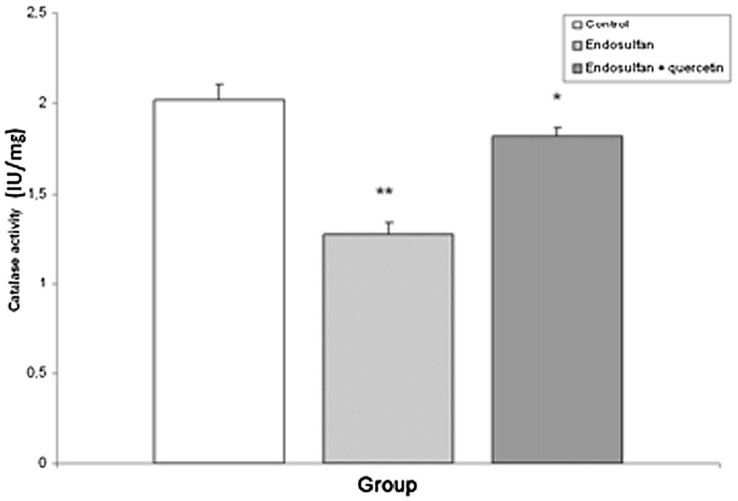

Fig. 2 Effect of endosulfan treatment on brain mitochondria level of CAT in rats and preventive role of QE. Values are mean±SE (n=5). **P<0.01, *P<0.05 as compared to normal control

(200 μM) in the same tube, mitochondria swelling was very low and reduced by half (56 %) (Fig. 6).

Discussion

The objective of the current study was planned with an aim to investigate the effect of acute endosulfan exposure on homeostasis redox in rat brain mitochondria, oxidative stress generation, and its implication in lipid peroxidation and mitochondrial swelling. GSH depletion can enhance oxidative stress and may also increase the levels of excitotoxic molecules; both types of action can initiate cell death in distinct neuronal populations (Jaswinder and Christopher 1997; Uttara et al. 2009). In the present study, the mitochondrial preparation from the rat brain treated with endosulfan demonstrated significant decrease in mitochondria GSH uptake, and on the other hand, mitochondrial GSH was increased when the animals were treated preventatively with QE. This result may be due to de novo GSH synthesis or GSH regeneration following ROS neutralization by the phenolic compound. In this study, because of their high reactivity and short life, the ROS has been analyzed indirectly in vivo by measuring the changes in antioxidases including SOD and CAT. Reduced activity of SOD and CAT was observed in mitochondria when endosulfan was administered to rats. This abnormality in the rate of different antioxidants might be the result of intense ROS generation induced by endosulfan administration in brain mitochondria, which in turn might cause an increase in malondialdehyde, as a result of enhanced lipid peroxidation (Silva and Beauvais 2009). Thus, environmental toxicants can directly attack the mitochondria, inducing the generation of ROS, which can further induce the depletion of antioxidant defenses and mediate other oxido-reduction reactions that promote mitochondrial damage, ROS formation, and

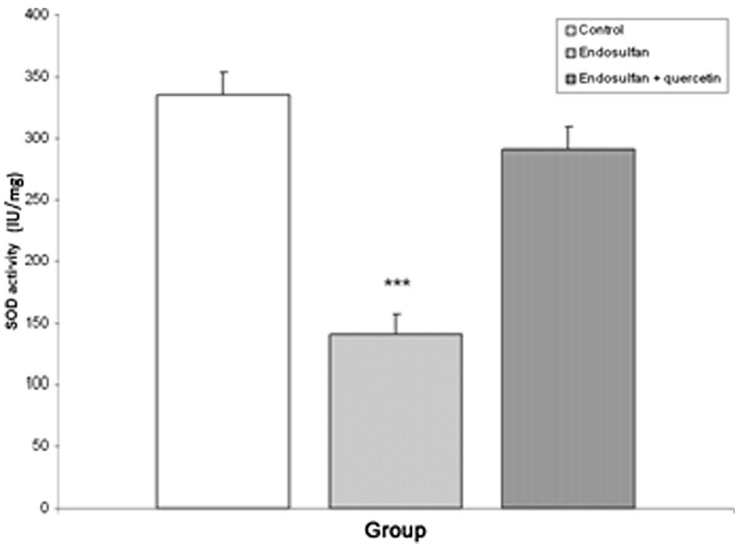

Fig. 3 Effect of endosulfan treatment on brain mitochondria level of SOD in rats and preventive role of QE. Values are mean±SE (n=5). ***P<0.001 as compared to normal control

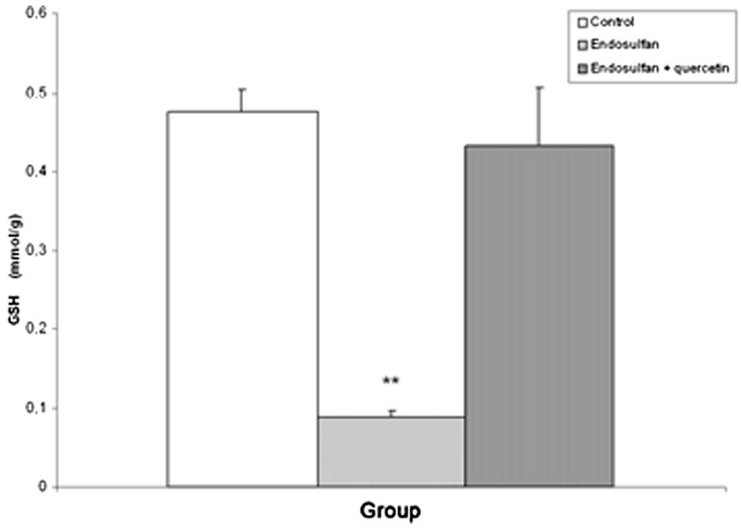

Fig. 4 Effect of endosulfan treatment on brain mitochondria level of GSH in rats and preventive role of QE. Values are mean±SE ($n=5$). **$P<0.01$ as compared to normal control

depletion of antioxidant molecules in the cell (Kaur et al. 2007; Ahmed et al. 2008; Franco et al. 2009). In mitochondria, lipid peroxidation impairs its metabolism and causes induction of the mitochondrial pore transition permeability (MPTP). Indeed, the results of the present study showed increase mitochondria swelling in rats that received endosulfan only, probably was induced by MPTP induction. Several authors have reported that apoptosis induced by environmental toxicants is widely associated with alterations in homeostasis redox which include both the depletion of antioxidant defenses such as GSH, SOD, and CAT and the increase accumulation of ROS which exerts a direct damage upon brain mitochondria (Shi et al. 2004; Assefa et al. 2005). Previous studies have showed that there is a link between GSH level and oxygen radical production and mitochondrial damage because of the scavenging activity of this tripeptide against accumulation of ROS and its decrease in the brains of parkinsonian patients (Di Monte et al. 1992; Sechi et al. 1996). The results of other studies have also shown that depletion of GSH contributes to neuronal degeneration (Merad-Boudia et al. 1998; Franco et al. 2009). Furthermore, this study was designed to elucidate whether quercetin can protect the brain against oxidative stress and avoid deficits of brain mitochondria. Indeed, the treatment of END-treated animals with QE has maintained normal content of GSH, SOD, and MDA in brain mitochondria and normalized lipid peroxidation that keeps mitochondrial integrity intact despite the apparent neurotoxicity in rats treated only with endosulfan. Previous studies have reported that QE has a powerful antioxidant and cytoprotective effects when it is used to prevent endothelial apoptosis caused by oxidants (Choi et al. 2003) and oxidative stress induced by alloxan in rat pancreas (Kebieche et al. 2011). QE is a more effective antioxidant nutrient than other antioxidants such as vitamin C, vitamin E, and β-carotene, and it can chelate ions of transition metals, including iron, thus preventing the Fenton reaction (Rice-Evans et al. 1995; Ferrali et al. 2000).

In conclusion, this is in vivo experiment to demonstrate that endosulfan has oxidative stress and mitochondria swelling effect in experimental animals. Also, this study has showed that QE can be cytoprotective agent against the damage following endosulfan brain injury in rats. However,

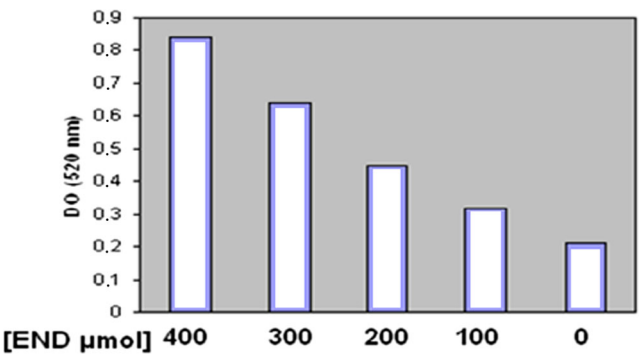

Fig. 5 Effect of endosulfan on brain mitochondria swelling according to its concentration. Optical density values are the mean of three consecutive measurements

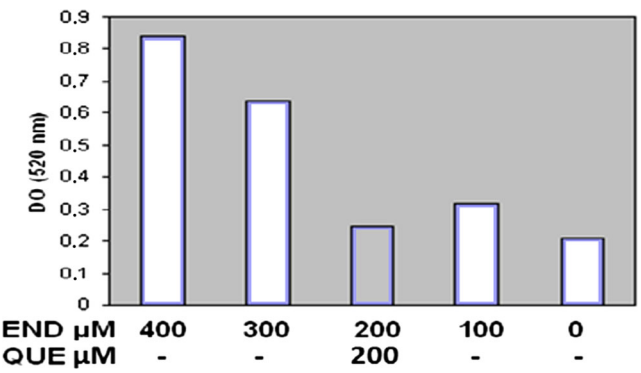

Fig. 6 Preventive effect of quercetin (median tube) associated to endosulfan treatment on brain mitochondria swelling in rats. Optical density values are the mean of three consecutive measurements

further investigations are essential to elucidate the precise mechanisms of endosulfan injury upon brain cells and if there is an obvious possibility to be excitotoxic molecule. Recently, much attention has been focused on the protective biochemical functions of naturally occurring antioxidants such as flavonoids to prevent neurodegenerative diseases in men. It would be interesting, thus, to determine the mechanism by which QE protects brain mitochondria against ROS and normalizes its swelling and regulates MPTP opening to prevent the incidence of neurodegenerative diseases in the general population.

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