INTRODUCTION
Endosulfan (6,7,8,9,10,10-hexachloro - 1,5,5a,6,9,9a-hexahydro - 6,9-methano - 2,4,3-benzodioxathiepin-3-oxide) is an organochlorine pesticide (OCP), which along with aldrin, dieldrin, endrin, chlordane, and heptachlor belongs to the group of chlorinated cyclodienes. As a commercial pesticide it comprises of two isomers (α- and β-endosulfan) at a ratio of 70:30 (Maier-Bode, 1968). It is widely used in a variety of crops, including cotton, cereals, fruit trees and plantation crops, such as tea and coffee. In 2005, the European Union excluded endosulfan from the common list of allowed phytosanitary products (2005/396/EC). Despite its prohibition in the European Union as a plant protectant since 2005 (Commission Decision 2005/864/EC), plant protection products containing endosulfan are still used in a number of countries worldwide. The Persistent Organic Pollutants Review Committee (POPRC) at its meetings in Geneva in 2009 reviewed and adopted a revised draft risk profile on endosulfan by which it agrees that the POP characteristics of the chemical warrant global action. Endosulfan is con-

Original Article
Endosulfan-induced lipid peroxidation in rat brain and its effect on t-PA and PAI-1: ameliorating effect of vitamins C and E
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ABSTRACT — Endosulfan provokes systemic toxicity in mammals and induces reactive oxygen species (ROS) and lipid peroxidation (LPO). The brain is susceptible to LPO and several studies implicate ROS and LPO in CNS diseases. Tissue plasminogen activator (t-PA) has been accredited with plasminogen-dependent roles in the CNS, as well as plasminogen-independent functions. The aim of this study was to investigate the activities of t-PA and its inhibitor, plasminogen activator inhibitor-1 (PAI-1) in the adult rat brain, after subchronic endosulfan treatment. Furthermore, the potency of vitamins C and E to attenuate these effects was explored. Endosulfan was administered in Wistar rats either alone or with vitamin C and/or vitamin E. The induced oxidative stress was manifested by induction of LPO as determined by higher malondialdehyde levels. This was accompanied by elevation of t-PA and PAI-1 activities. Vitamins E and C, both well-known for their antioxidant properties, substantially acted in a preventive way and protected the brain from these effects.

Key words: Antioxidants, Reactive oxygen species, Oxidative stress, Extracellular proteolysis, Endosulfan, Neurotoxicity

INTRODUCTION
Endosulfan (6,7,8,9,10,10-hexachloro - 1,5,5a,6,9,9a-hexahydro - 6,9-methano - 2,4,3-benzodioxathiepin-3-oxide) is an organochlorine pesticide (OCP), which along with aldrin, dieldrin, endrin, chlordane, and heptachlor belongs to the group of chlorinated cyclodienes. As a commercial pesticide it comprises of two isomers (α- and β-endosulfan) at a ratio of 70:30 (Maier-Bode, 1968). It is widely used in a variety of crops, including cotton, cereals, fruit trees and plantation crops, such as tea and coffee. In 2005, the European Union excluded endosulfan from the common list of allowed phytosanitary products (2005/396/EC). Despite its prohibition in the European Union as a plant protectant since 2005 (Commission Decision 2005/864/EC), plant protection products containing endosulfan are still used in a number of countries worldwide. The Persistent Organic Pollutants Review Committee (POPRC) at its meetings in Geneva in 2009 reviewed and adopted a revised draft risk profile on endosulfan by which it agrees that the POP characteristics of the chemical warrant global action. Endosulfan is con-
sidered a worldwide persistent organic pollutant, which bioaccumulates and biomagnifies in food chains (Weber et al., 2010). Therefore, humans and animals are still exposed to it.

Exposure to OCPs and endosulfan has been associated with oxidative stress in man (Pathak et al., 2010) and also in rats. In fact, results on rats suggest that endosulfan may cause more oxidative damage than malathion (Kayhan, 2008). Tests on isolated rat hepatocytes have shown the effects of endosulfan on antioxidant parameters. The activities of antioxidant enzymes like superoxide dismutase, glutathione peroxidase and glutathione-S-transferase were decreased. The same treatment reduced the level of antioxidant glutathione and increased the level of lipid peroxidation (LPO), indicating the induction of oxidative stress in the cells (El-Shenawy, 2010). In addition, in vitro tests on rat liver suggest a synergistic effect of endosulfan with other pesticides, such as chlorpyriphos, on LPO (Chebab et al., 2009). Subchronic studies have implicated the oxidative damage pathway in the mechanism of endosulfan toxicity on the male reproductive system (Zhu et al., 2002), as well as in the immune system (Pal et al., 2009) of the rat.

Tissue-type plasminogen activator (t-PA) is a serine-protease that cleaves the zymogen plasminogen to generate the active protease plasmin, thus initiating a potent proteolytic cascade. Its activity is modulated by the serpins plasminogen activator inhibitor-1 (PAI-1) and neuroserpin, which bind t-PA and render it inactive. Traditionally, research on the t-PA/plasmin system has been focused on its role in haemostasis and fibrin clot degradation. However, recent data indicate different functions, among which is the role of t-PA in the central nervous system (CNS). Tissue-type PA is expressed in the CNS by both neurons and microglia (Yepes and Lawrence, 2004). Functioning as a protease and/or as a modulator of cell signalling, it has been implicated in the development and pathophysiology of the nervous system, i.e. in neuronal plasticity and reorganization, microglia activation, regulation of the permeability of the neurovascular unit, as well as neuronal cell death (Tsirka, 1996; Yepes and Lawrence, 2004; Gravanis and Tsirka, 2005; Melchor and Strickland, 2005).

Plasmin and t-PA are mediators of neuronal degeneration (Tsirka et al., 1996); t-PA activates plasmin or matrix metalloproteinases (MMPs) and provokes degradation of extracellular matrix (ECM) components, such as laminin. Such activity disrupts the neuron-ECM interaction and induces neuronal cell death (Chen and Strickland, 1997). The activation of cellular receptors establishes the neuro-modulatory activity of t-PA; such are the NMDA receptor, or the LDL receptor-related protein (LRP). Both in vitro and in vivo studies indicate that t-PA acts as a direct modulator of NMDA receptor and NMDA receptor-mediated signalling (Nicole et al., 2001; Benchenane et al., 2007; Zhang et al., 2009a, 2009b).

The high concentration of unsaturated lipids in the brain and the basically aerobic metabolism in this organ, make this tissue very susceptible to peroxidative damage. The objective of the present study was to investigate the possible oxidative stress induced by subchronic endosulfan intoxication in the rat brain and correlate this effect with the modulation of brain t-PA and PAI-1 activities. Additionally, we studied the possibility to diminish, or even annul, this modulation by the administration of two vitamins with well known antioxidant properties, namely vitamin E and vitamin C.

**MATERIALS AND METHODS**

**Materials**

The active rat t-PA and PAI-1 ELISA Kits were products of Molecular Innovations, Novi, MI, USA. Triton X-100, Trizma base and other chemicals were purchased from Merck KGaA, Darmstadt, Germany. Endosulfan technical grade (7:3 ratio, 70% α isomer/30% β isomer, 97.8% purity) was purchased from Makhteshim Chemical Works Ltd., Beer Sheva, Israel.

**Animals**

For the purposes of the 6-week study, sixty male Wistar rats were used. The animals were 2.5 months old and had an average body weight of 267 g at the onset of the study; they were housed in polystyrene cages at 22-24°C with a controlled 12 hr light. They were fed and watered *ad libitum* and treated in accordance with the guiding principles of the European Community Council Directive (89/609/EEC) for the care and use of laboratory animals (Committee on Care and Use of Laboratory Animals, 1996).

The animals were divided randomly into 12 groups, each comprising of 5 individuals. The addition of vitamins in the diet or the water received by the animals was calculated given that a 280 g animal consumes approximately 30 g of dry food/day and drinks water at the rate of 10-12 ml/100 g b.w./day. Group 1 (control) were fed a standard commercial diet. Group 2 received the same diet enriched with 200 ppm vitamin E. Group 3 animals received the standard diet but vitamin C was added in their water so that they were provided with 500 mg/kg/day. Group 4 received the standard diet plus vitamin E and vitamin C at doses as mentioned above. Group 5 received...
the control diet but additionally the animals were treated with 1.5 mg/kg/day of endosulfan. For this purpose the pesticide was dissolved in olive oil (vehicle) and administered orally via gastric intubation. Groups 6, 7 and 8 were treated as groups 2, 3 and 4 respectively, but endosulfan was administered at the same dose as in group 5. Groups 9, 10, 11 and 12 were treated as 5, 6, 7 and 8 respectively, but the endosulfan dose was 10 mg/kg/day (Table 1). The oral LD<sub>50</sub> values for technical endosulfan range from 7 to 121 mg of endosulfan/kg depending upon species, sex, formulation tested, vehicle used and nutritional status of the animal (WHO, 1984). Dosing was therefore determined taking into account the above mentioned high variability, as well as, the range that is usually tested when implementing subchronic toxicity experimental designs (Silva and Gammon, 2009).

All rats were on the experimental diets for 6 weeks before they were sacrificed; for this purpose the animals were euthanized by decapitation under deep anaesthesia by diethyl ether. The brains were excised immediately, the stem and cerebellum were removed, and the remaining tissues were rinsed in phosphate buffered saline, pH 7.4, vacuum packaged and stored at -80°C until analyzed.

**Brain homogenate preparation**

Brain tissue samples were thawed at 37°C, minced and homogenized in 0.1 M Tris-HCl (pH 7.4), 1% Triton X-100 (50 mg tissue/ml) with a motor-driven Kinematica homogenizer. The homogenates were centrifuged at 5,000 g (4°C) for 20 min and the supernatants were filtered through 1.2 μm Millipore filters. Filtered supernatants were used for the ELISA assays.

**Functionally active t-PA activity assay**

Functionally active (PAI-1-free) rat t-PA was determined using the active rat t-PA ELISA Kit according to manufacturer’s instructions. Briefly, 0.1 ml homogenate was added in each PAI-1-coated well and unbound proteins were washed away after appropriate washing steps. PAI-1-bound t-PA was captured by polyclonal anti-murine t-PA primary antibody. Excess antibody was washed away and bound antibody was then reacted with the horseradish peroxidase (HRP)-conjugated secondary antibody. Following an additional washing step, 3,3’ ,5,5’-tetramethylbenzidine (TMB) substrate was used for color development. The amount of color detected at 450 nm is directly proportional to the concentration of active t-PA in the sample.

**Functionally active PAI-1 activity assay**

Functionally active (PA-free) rat PAI-1 was determined using the active rat PAI-1 ELISA Kit according to manufacturer’s instructions. Briefly, 0.1 ml homogenate was added in each u-PA-coated well and unbound proteins were washed away. u-PA-bound PAI-1 was captured by anti-murine PAI-1 primary antibody. Following washes bound antibody was then reacted with the HRP-conjugated secondary antibody. Following an additional washing step, TMB substrate was used for color development. The amount of color detected at 450 nm is directly proportional to the concentration of active PAI-1 in the sample.

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**Table 1.** Presentation of the animal groups as divided for our experiments and the respective administration of endosulfan, vitamin E and vitamin C

<table>
<thead>
<tr>
<th>No</th>
<th>Group name</th>
<th>Endosulfan</th>
<th>Vitamin E</th>
<th>Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>E</td>
<td>-</td>
<td>200 ppm</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>-</td>
<td>500 mg/kg/day</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>E+C</td>
<td>1.5 mg/kg/day</td>
<td>200 ppm</td>
<td>500 mg/kg/day</td>
</tr>
<tr>
<td>5</td>
<td>Endo1.5</td>
<td>1.5 mg/kg/day</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Endo1.5+E</td>
<td>1.5 mg/kg/day</td>
<td>-</td>
<td>500 mg/kg/day</td>
</tr>
<tr>
<td>7</td>
<td>Endo1.5+C</td>
<td>1.5 mg/kg/day</td>
<td>200 ppm</td>
<td>500 mg/kg/day</td>
</tr>
<tr>
<td>8</td>
<td>Endo10</td>
<td>10 mg/kg/day</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Endo10+E</td>
<td>10 mg/kg/day</td>
<td>-</td>
<td>500 mg/kg/day</td>
</tr>
<tr>
<td>10</td>
<td>Endo10+C</td>
<td>10 mg/kg/day</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Endo10+E+C</td>
<td>10 mg/kg/day</td>
<td>200 ppm</td>
<td>500 mg/kg/day</td>
</tr>
<tr>
<td>12</td>
<td>Endo10+E+C</td>
<td>10 mg/kg/day</td>
<td>200 ppm</td>
<td>500 mg/kg/day</td>
</tr>
</tbody>
</table>
Malondialdehyde assay in brain extracts

To estimate the malondialdehyde (MDA) concentration in the samples, we followed the respective method described in detail by Botsoglou et al. (1994). In brief, 1 g brain tissue was extracted with 5% aqueous trichloroacetic acid (10 ml), in the presence of 0.8% butylated hydroxytoluene in hexane (5 ml). After centrifugation, 2.5 ml of the bottom trichloroacetic acid layer was mixed with 1.5 ml of 0.8% aqueous 2-thiobarbituric acid and following incubation for 30 min at 70°C the reaction mixture was submitted to third-derivative spectrophotometry against blank reaction mixture. The concentration of MDA in brain extracts was calculated using a calibration curve constructed by plotting values of peak height at 521.5 nm versus known concentrations of MDA in the final reaction mixtures.

Statistical analysis

The values of the three outcome variables (t-PA in ng/ml of extract, PAI-1 in ng/ml of extract and MDA in ng/g of tissue) were compared among the study groups using non-parametrical statistical methodology. The reason for selecting non-parametrical over parametrical methodology, in this case ANOVA, was that two of the respective assumptions (homogeneity of the variances and normality of the probability distributions of the responses for each treatment level) could not be satisfied for any of the three outcome variables, even after attempting several numerical transformations of the values of the variables. Therefore, the comparisons of median values of the three outcome variables among all treatment groups were made using a Kruskall-Wallis non-parametric test. Whenever this test indicated an overall significant difference, pairwise comparisons among pairs of groups were made using Mann-Whitney non-parametric test, in order to identify which group medians were significantly different. For all statistical test significance was assessed in reference to a 0.10 level of significance. An alpha of 0.10 was used because we were concerned that the small sample size combined with the use of the appropriate non-parametrical statistical tests would result to hypothesis tests with a very high probability of a type II error. Therefore, we decided to increase slightly alpha (which is the probability of a type I error) in order to gain a decrease in beta (the probability of a type II error). Nevertheless, the p-values themselves are provided for the readers to assess the results irrespectively of significance cut-offs. All analyses were conducted using SPSS for Windows version 15.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

In the present study we evaluated the active t-PA and PAI-1 levels in the rat brain after a 6-week administration of endosulfan at two different doses. Additionally, we estimated the potentially protective effect of vitamin E and vitamin C, two vitamins with well-established antioxidant properties. To estimate the endosulfan induced oxidative stress, we also evaluated the concentration of MDA, a main lipid peroxidation product, in the brains of the subjects.

The concentration of MDA in the brains of the control group was evaluated at the average of 188.71 ng/g of tissue. Both vitamins E and C induced a reduction of MDA concentration in the brain to 88% and 75% of the control level, respectively (Fig. 1). The combination of the two vitamins proved to have an even stronger anti-oxidant effect, with the level of MDA significantly dropping as low as 81.96 ng/g of tissue, which is just the 43% of the control value (P = 0.008).

The effect of endosulfan administration on lipid peroxidation in the brain is shown in Fig. 2. Both groups that received endosulfan had an increased MDA concentration. The animals that received 1.5 mg of endosulfan/kg/day showed an increase in MDA concentration in brain homogenates up to an average of 217.10 ng/g of tissue (an up-regulation of 15%), but this was not statistically significant (P = 0.151). However, the high endosulfan dose (10 mg/kg/day) significantly increased brain MDA up to 253.98 ng/g of tissue, i.e. an up-regulation of 35% compared to controls (P = 0.095).

The protective role of vitamins E and C against the effect of endosulfan on MDA is shown in Figs. 3 and 4. Administration of the vitamins either individually or in combination effectively inhibited the elevation of MDA concentration by endosulfan, irrespectively of the dose of endosulfan administered. Indeed, the MDA levels in the brains of endosulfan-treated animals that were on a vitamin-supplemented diet were significantly lower compared to the animals that received endosulfan alone. Specifically, MDA in the groups Endo1.5+E and Endo1.5+C had an average concentration of 148.46 ng/g of tissue (P = 0.056) and 168.29 ng/g of tissue (P = 0.032) respectively, while MDA in group Endo1.5+E+C dropped to 108.85 ng/g of tissue (P = 0.008), (Fig. 3). The same pattern was observed in the animals that received the higher dose of endosulfan (10 mg/kg/day). The individual vitamins and their combination were able to inhibit the effect of the pesticide, reducing MDA concentration in groups Endo10+E and Endo10+E+C by 20.67% (P = 0.016) and 31.98% (P = 0.008) respectively, com-
Endosulfan effect on lipid peroxidation, t-PA and PAI-1 in rat brain

Fig. 1. Mean values, standard deviation and medians of the concentration of MDA in rat brain, expressed in ng/g of brain tissue. The charts present the groups that received vitamin E, vitamin C and the vitamin combination. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.

Fig. 2. Mean values, standard deviation and medians of the concentration of MDA in rat brain, expressed in ng/g of brain tissue. The charts present the groups Endo1.5 and Endo10 that received endosulfan at the doses of 1.5 mg/kg/day and 10 mg/kg/day. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.

Fig. 3. Mean values, standard deviation and medians of the concentration of MDA in rat brain, expressed in ng/g of brain tissue. The charts present the groups Endo1.5+E, Endo1.5+C and Endo1.5+E+C that received vitamin E, vitamin C and the vitamin combination as protection against endosulfan. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.

Fig. 4. Mean values, standard deviation and medians of the concentration of MDA in rat brain, expressed in ng/g of brain tissue. The charts present the groups Endo10+E, Endo10+C and Endo10+E+C that received vitamin E, vitamin C and the vitamin combination as protection against endosulfan. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.
levels of active t-PA in the rat brain. Although vitamin C did not alter significantly t-PA concentration, vitamin E and the combination of the two vitamins induced an increase of active t-PA concentration (P = 0.008). Specifically, while the average t-PA value in the brain extracts of the control animals was 4.15 ng/ml, vitamin E elevated it up to 7.24 ng/ml, an increase of 74.4% (P = 0.067), while the combination of the two vitamins had an even stronger effect (7.65 ng/ml, an increase of 84.3%, P = 0.07) (Fig. 5).

Fig. 5. Mean values, standard deviation and medians of the concentration of t-PA in rat brain, expressed in ng/ml of brain extract. The charts present the groups that received vitamin E, vitamin C and the vitamin combination. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.

Fig. 6. Mean values, standard deviation and medians of the concentration of t-PA in rat brain, expressed in ng/ml of brain extract. The charts present the groups Endo1.5 and Endo10 that received endosulfan at the doses of 1.5 mg/kg/day and 10 mg/kg/day. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.

Fig. 7. Mean values, standard deviation and medians of the concentration of t-PA in rat brain, expressed in ng/ml of brain extract. The charts present the groups Endo1.5+E, Endo1.5+C and Endo1.5+E+C that received vitamin E, vitamin C and the vitamin combination as protection against endosulfan. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.

Fig. 8. Mean values, standard deviation and medians of the concentration of t-PA in rat brain, expressed in ng/ml of brain extract. The charts present the groups Endo10+E, Endo10+C and Endo10+E+C that received vitamin E, vitamin C and the vitamin combination as protection against endosulfan. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.
Endosulfan effect on lipid peroxidation, t-PA and PAI-1 in rat brain

Endosulfan intake, even at low concentration, increased active t-PA levels in brain extracts up to 5.9 ng/ml (P = 0.008), which corresponds to a 42.2% increase compared to controls (Fig. 6). Vitamins C and E failed to inhibit the endosulfan effect on t-PA, even at the low dose of the pesticide. Groups Endo1.5+C and Endo10+C maintained the high levels of active t-PA concentration induced by endosulfan (7.68 ng/ml and 10.84 ng/ml respectively). However, vitamin E reduced t-PA level close to that of the controls (5.58 ng/ml in group Endo1.5+E and 6.56 ng/ml in group Endo10+E). Interestingly, the combination of the two vitamins dramatically reduced t-PA level (Figs. 6, 7 and 8); in groups Endo1.5+E+C and Endo10+E+C the t-PA activity was 2.47 ng/ml and 1.30 ng/ml respectively (Figs. 7 and 8).

Dietary supplementation of either vitamin C or E, or their combination, had no effect on active PAI-1 levels in rat brain extracts (Fig. 9). However, endosulfan mediated an increase of the concentration of active PAI-1 in a dose-dependent manner (Fig. 10). The low dose did not affect significantly active PAI-1 (P = 0.151), while the high dose increased it by 28% (P = 0.016). This effect of the pesticide was reversed by vitamin E but not vitamin C. Specifically, vitamin E reduced significantly the PAI-1 level of the Endo1.5+E group at 6.03 ng/ml (P = 0.016), while vitamin C had a statistically non-significant effect (7.23 ng/ml). The combination of the two vitamins did not affect significantly PAI-1 level (Fig. 11).

Similar results were observed in the groups treated with the higher dose of endosulfan: the group Endo10+E had a significantly lower average of PAI-1 (7.44 ng/ml, P = 0.1), while the Endo10+C group had no significant effect on it (8.05 ng/ml). Once again, the combination of the vitamins proved to be the most effective in terms of inhibiting the endosulfan induced increase of PAI-1 concentration (6.56 ng/ml which is 23.36% lower than the control group, P = 0.006). (Fig. 12).

DISCUSSION

The brain is rich in lipids and especially in polyunsaturated fatty acids, which are very sensitive to peroxidation. The antioxidant defences of the organ include, among others, dietary free-radical scavengers such as ascorbate and α-tocopherol (Warner et al., 2004). Given the high aerobic metabolism in this tissue which consumes a large quantity of oxygen, this scavenging system is moderately adequate to protect against oxidative damage. Moreover, it can become inadequate against any oxidative insult imposed by external factors or neurodegenerative diseases (Halliwell, 2006). Various degenerative diseases involve mechanisms related to such a damage of the neuronal cells (Floyd, 1999). In some of these diseases the PA/plasminogen system is involved (Zhang et al., 2009a). On the other hand, there is a plethora of data on endosulfan toxicity, part of which refers to the impact of the pesticide on the brain tissue and CNS in general (Silva and Gammon, 2009). It is noteworthy that after a single administration of endosulfan (5 mg/kg p.o.s.), the pesticide is traced in various organs of the rat, brain included, as soon as 1 hr after later (Chan et al., 2005). It has been suggested that oxidative pathways could be involved in the mechanism of endosulfan toxicity (Zhu et al., 2002; Pal et al., 2009).

In the present study, we induced subchronic endosulfan intoxication in male rats and investigated the possible oxidative stress the pesticide may cause in the rat brain by measuring the MDA concentration in homogenates of the organ. Furthermore, we specifically investigated the impact of the pesticide on the activities of t-PA and PAI-1 and examined the possible alleviation of this impact via the supplementation of vitamin E and/or vitamin C.

Subchronic endosulfan intoxication induced LPO in the rat brain as determined by the increased MDA concentration (Fig. 2). These results are in accordance with data showing that acute, subchronic or chronic intoxication by endosulfan induces LPO and alters the glutathione redox status in rat cerebral tissues (Hincal et al., 1995). In our study we observed a dose-dependent increase in rat brain MDA levels, following administration of endosulfan via gastric intubation. This complies with previous reports that organochlorine pesticides induce oxidative damage which is considered to be one of the mechanisms of their toxicity. Specifically endosulfan induces free radical lipid peroxidation and oxidative damages in various organs in the rat. Literature includes such results referring not only to the brain, as mentioned before, but additionally to the liver (Chebab et al., 2009), kidney and gonadal tissues (Kayhan, 2008), testes (Zhu et al., 2002), blood serum (Pal et al., 2009), blood plasma, erythrocytes and lung (Bebe and Panemangalore, 2003).

In general, tocopherols are chain-breaking antioxidants that compete with the substrate for the chain peroxyl radicals. On the other hand, vitamin C may act as an antioxidant through various mechanisms, among which are oxygen scavenging, hydroperoxide reduction and stabilization of free radicals (Frankel, 1996). In our study, both vitamins E and C induced a reduction of MDA concentration in the brain (12% and 25% respectively, compared to controls). The combination of the two vitamins had a stronger anti-oxidant effect, with the level of MDA concentration dropping to 57% compared to controls (Fig. 1). This is of no surprise, since tocopherols and ascorbic acid
are reported to exhibit a synergistic effect during LPO: they can mutually reinforce one another by regenerating their oxidized forms, thus multiplying their antioxidant capacity (Kiokias et al., 2008).

Previous studies have indicated a significant attenuation of the oxidative stress markers occurs with the administration of α-tocopherol. Moreover, in the same studies, vitamin E attenuated histopathological findings in endo-

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**Fig. 9.** Mean values, standard deviation and medians of the concentration of PAI-1 in rat brain, expressed in ng/ml of brain extract. The charts present the groups that received vitamin E, vitamin C and the vitamin combination. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.

**Fig. 10.** Mean values, standard deviation and medians of the concentration of PAI-1 in rat brain, expressed in ng/ml of brain extract. The charts present the groups Endo1.5 and Endo10 that received endosulfan at the doses of 1.5 mg/kg/day and 10 mg/kg/day. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.

**Fig. 11.** Mean values, standard deviation and medians of the concentration of PAI-1 in rat brain, expressed in ng/ml of brain extract. The charts present the groups Endo1.5+E, Endo1.5+C and Endo1.5+E+C that received vitamin E, vitamin C and the vitamin combination as protection against endosulfan. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.

**Fig. 12.** Mean values, standard deviation and medians of the concentration of PAI-1 in rat brain, expressed in ng/ml of brain extract. The charts present the groups Endo10+E, Endo10+C and Endo10+E+C that received vitamin E, vitamin C and the vitamin combination as protection against endosulfan. In comparison with control group, statistically significant differences (P < 0.1) are indicated with an asterisk.
sulfan treated male rats (Jalili et al., 2007a) and reduced oxidative stress in the rat heart (Jalili et al., 2007b). In our experiments, the administration of vitamin E, vitamin C or their combination in adult rats were able to protect the brain from oxidative stress caused by endosulfan, as indicated by MDA measurements. Regardless of the dose of the pesticide, the preventive administration of vitamins resulted in MDA concentrations that were even lower than the control values (Figs. 3 and 4). It is noteworthy that vitamin E provided a more potent protection than vitamin C. The lipid soluble vitamin E is freely distributed in cell membranes, and is more effective at scavenging free radicals in the lipid-rich phase of the neuronal cells (McCay, 1985). On the other hand, vitamin C, being water soluble, is more effective in aqueous systems (Porter et al., 1989). Nevertheless, the combination of the two vitamins had an even stronger effect, a result that is in accordance with the above mentioned data.

We furthermore investigated the possible impact of endosulfan administration on two major parameters of the fibrinolytic system, t-PA and its inhibitor, PAI-1. When localized extracellular matrix degradation and remodelling takes place during various pathophysiological events, the PA/plasmin system is frequently implicated. Such events include CNS diseases, where oxidative stress is known to play a pivotal role (Vassalli et al., 1991; Melchor and Strickland, 2005; Tsantarliotou et al., 2008). To our knowledge, there was no published data with respect to a possible effect of endosulfan on any fibrinolytic agent of the brain in vivo. Nevertheless, given that in vitro experiments showed modulation of t-PA by ROS (Radha et al., 2008; Noguchi et al., 2001), it was interesting to explore the possible impact of endosulfan-mediated LPO on t-PA activity in the brain. Indeed, regardless the dose, endosulfan intake resulted in a significant increase of active t-PA levels (Figs. 6 and 10). We have previously observed a similar effect regarding t-PA activity in rat brain as a result of CCl4 intoxication -a recognized model to induce LPO- as well as in rabbit spermatozoa (Kokoli et al., 2008a, 2008b). These results are in agreement with previous reports which clearly indicate interplay between ROS production, LPO, and plasminogen activator activity. In particular, ROS and LPO increase plasminogen activator activity in human fibroblasts (Radha et al., 2008) and in rat hepatocytes (Noguchi et al., 2001).

Eventually, we evaluated the antioxidant potency of vitamins C and E or their combination, on untreated or endosulfan treated brains. Although vitamin C did not alter t-PA concentration in control brains, vitamin E and the combination of the two vitamins induced a significant increase of t-PA (Fig. 5). These results show that scavenging of free radicals, which are products of basal brain metabolism, by antioxidants, has a positive impact on the levels of active (PAI-1-free) t-PA. Interestingly, simultaneous administration of the two vitamins alleviated the effect of endosulfan on t-PA activity. The combination of the vitamins was equally effective regardless of the dose of endosulfan. It appears therefore that although vitamins C and E can each one sufficiently inhibit the elevation of MDA in the brains of endosulfan-treated rats, their synergistic action is required for the inhibition of endosulfan-mediated t-PA induction. Any apparent contradiction between the effect of the vitamins on basal t-PA activity and the endosulfan-induced t-PA activity can be justified, given the differences in the oxidant/antioxidant load of the tissues in each case. We would like to emphasize that in this experiment vitamins were administered simultaneously with endosulfan, in order to scavenge the free radicals that are expected to be generated upon endosulfan metabolism, and thus prevent oxidative damage.

The inhibitory effect of PAI-1 on t-PA proteolytic activity is demonstrated via direct binding to t-PA, which renders it inactive. In our experiments, no vitamin supplementation to healthy animals had any significant impact on active PAI-1. It appears that the antioxidant effect of these vitamins on t-PA activity is not mediated via interruption of the latent t-PA/PAI-1 complex but rather through other mechanisms that target protein expression. On the other hand, both doses of endosulfan increased PAI-1 concentration, as shown in Fig. 10. This effect of the pesticide was inhibited by vitamin E and the combination of the two vitamins, but not by vitamin C, as it was expected according to the data on MDA and active t-PA level. To our knowledge there is no literature clarifying the mechanism by which endosulfan may affect active t-PA or PAI-1 concentration. Nevertheless, previous studies have shown that oxidative stress in general is capable of affecting the transcription of both genes in rat liver causing an increase in blood plasma t-PA and PAI-1 concentrations (Noguchi et al., 2001). A similar effect of oxidative stress is reported with respect to u-PA gene transcription in human RC-K8 lymphoma and H69 lung carcinoma cells (Kiguchi et al., 2001). The observed elevation of t-PA and PAI-1 activity after endosulfan administration alters the equilibrium with respect to proteolysis of extracellular matrix elements in the area. The altered enzymatic activity could contribute to any regeneration process initiated due to tissue damaging.

The concomitant increase in both active (PAI-1-free) t-PA and active (t-PA-free) PAI-1 is in accordance to previous observations in other experimental systems.
(Oszajca et al., 2008; Rekkas et al., 1991). A possible explanation for this apparently conflicting observation is that brain exposure to endosulfan-induced ROS may increase the levels of active t-PA by inducing dissociation of the t-PA/PAI-1 inactive complex, which resides in the extracellular milieu. Such an effect would result in the simultaneous elevation in the active (free) forms of both proteins. On the other hand, one cannot rule out the possibility that endosulfan induces t-PA production, which is followed by a secondary induction of PAI-1 synthesis, thus maintaining an equilibrium in the proteolytic activity of the t-PA/PAI-1 system in a spatial and temporal manner.

In conclusion, the present study suggests that subchronic endosulfan intoxication induces oxidative stress in the adult rat brain, as indicated by elevation of MDA levels in the organ. Additionally, an elevation of t-PA and PAI-1 activities is observed, possibly attributed to the induced oxidative stress. Vitamin E, either alone or in combination with vitamin C can substantially act in a preventive way and moderate the effect of endosulfan on lipid peroxidation and t-PA/PAI-1 activities in the adult rat brain.

REFERENCES


Endosulfan effect on lipid peroxidation, t-PA and PAI-1 in rat brain


