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Effect of pesticides on cell survival in liver and brain rat tissues

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ABSTRACT

Pesticides are the main environmental factor associated with the etiology of human neurodegenerative disorders such as Parkinson's disease. Our laboratory has previously demonstrated that the treatment of rats with low doses of dimethoate, zineb or glyphosate alone or in combination induces oxidative stress (OS) in liver and brain. The aim of the present work was to investigate if the pesticide-induced OS was able to affect brain and liver cell survival. The treatment of Wistar rats with the pesticides (i.p. 1/250 LD50, three times a week for 5 weeks) caused loss of mitochondrial transmembrane potential and cardiolipin content, especially in substantia nigra (SN), with a concomitant increase of fatty acid peroxidation. The activation of calpain apoptotic cascade (instead of the caspase-dependent pathway) would be responsible for the DNA fragmentation pattern observed. Thus, these results may contribute to understand the effect(s) of chronic and simultaneous exposure to pesticides on cell survival.

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1. Introduction

Human aging is associated with a progressive loss of nigrostriatal neurons and an exponential increase in the prevalence of Parkinson's disease (PD) (Singh and Dikshit, 2007). The incidence of this neurodegenerative disease has shown a significant increase over the past few decades. PD is characterized by a loss of dopaminergic neurons in the midbrain substantia nigra (SN), and it is the second most common neurodegenerative illness, following Alzheimer's disease (Samantaray et al., 2007; Gorman et al., 1996; Honig and Rosenberg, 2000). The etiology of PD has long been thought to involve multiple variables such as age, heredity and environmental factors (Nielsen et al., 2006; Patel et al., 2006; Aldridge et al., 2003; Paolini et al., 2004; Zhang et al., 1999; Dauer and Przedborski, 2003). Epidemiological studies have linked exposure to pesticides with the incidence of this neurological disorder. Humans are exposed to complex mixtures of toxic compounds every day in residential and occupational environments (Di Monte, 2003; Liu et al., 2003). This exposure particularly includes pesticides, which are used sometimes indiscriminately in large amounts and which cause progressive pollution. Agrochemicals are continuously required for global food production and linger as residues in food from vegetable and animal origins, as well as in the air and water (John et al., 2001; Thiruchelvam et al., 2002; Bolognesi and Morasso, 2000). Many of the most-used pesticides in our country and all over the world exert their toxic effects via oxidative stress (OS) mechanisms. They lead to the generation of oxygenated and/or nitrogenated reactive species (ROS/RNS) that affect both the antioxidant levels and the activity of the scavenging enzyme system (Sharma et al., 2005a; Banerjee et al., 1999; Beuret et al., 2005; Barlow et al., 2005).

The central nervous system, specially SN, is highly sensitive to free radical damage for reasons such as a high oxidative metabolic rate, a high ratio of membrane surface area to cytoplasmic volume, high levels of unsaturated lipids (mainly polyunsaturated fatty acids, or PUFAs), high iron levels, increased free radical generation derived from dopamine metabolism, and inefficient scavenging mechanisms (De Zwart et al., 1999; Erikson et al., 1997; Abuja and Albertini, 2001; Chong et al., 2005). A higher density of resting microglia in the SN (compared to other brain regions) might be another reason why dopamine-containing neurons are extremely vulnerable to OS (Gao et al., 2003).

We have previously demonstrated that simultaneous intoxication with agrochemicals affects the brain, liver, kidney, and plasma, causing a marked OS (Astiz et al., submitted). In the present work, we aimed to investigate whether the OS induced by pesticide administration was able to induce damage that would

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eventually lead to cell death. It is well known that decrease in cell survival is a hallmark of neurodegenerative diseases (Singh and Dikshit, 2007; Kanthasamy et al., 2005; Honig and Rosenberg, 2000). We studied three of the most commonly used pesticides worldwide: zineb (Z), glyphosate (G), and dimethoate (D), either alone or in combination. Zineb (zinc ethylene-bis-dithiocarbamate) is a widely used contact fungicide used to control pests in carrots, onions, citric fruits, and potatoes (Fitsanakis et al., 2002; Heikkila et al., 1976). Glyphosate (N-phosphonomethyl-glycine) is a systemic herbicide used to control undergrowth before seeding in corn, soybean, vine, etc. (Daruich et al., 2001; Le Couteur et al., 1999), while dimethoate (O,O-dimethyl-S-methyl-carbamoyl-methyl phosphorodithioate) is an organophosphorus insecticide of systemic action extensively used in the pest treatment in onions, tomatoes, and citric fruits, among others (Sharma et al., 2005a).

Results from this investigation may contribute to the understanding of the effect(s) of chronic and simultaneous exposition to pesticides on cell survival, and they may be of clinical interest in the evaluation of the degree of damage to which humans are involuntarily exposed due to environmental pollution.

2. Materials and methods

2.1. Chemicals

All chemicals used were of reagent grade and were obtained from Sigma Chemical Co. (CA, or Buenos Aires, Argentina) or Merck Laboratories (Darmstadt, Germany). Organic solvents were from Carlo Erba (Milan, Italy). Pesticides employed were purchased from local commercial sources and were of analytical grade; zineb, dimethoate, and glyphosate were obtained as a gift from Instituto Nacional de Tecnologia Agropecuaria (INTA, Castelar, Argentina).

2.2. Animals and treatments

Male Wistar rats weighing 190±20 g with specific pathogen-free status were used. Upon arrival, they were allowed to acclimatize for a week before the start of the experiment. The rats were maintained at a controlled temperature (25±2 °C) and with a normal photoperiod of 12 h darkness and 12 h light. They were fed with standard Purina chow from Ganave Co. (Santa Fe, Argentina) and given water ad libitum. Clinical examinations and body weight evaluations were performed every week during the experiment. The animals were randomly divided into nine groups of four rats each. These groups included control rats without any treatment (C), injected i.p. with polyethylene-glycol 400 (PEG-400) (V), 15 mg zineb/kg body weight (b.w.) in PEG-400 (Z), 10 mg glyphosate/kg b.w. in PEG-400 (G), or 15 mg dimethoate/kg b.w. in PEG-400 (D). We also included groups with combined treatments injecting the drugs in the same vehicle and using the same administration route: 15 mg zineb/kg b.w.+10 mg glyphosate/kg b.w. (Z+G), 15 mg zineb/kg b.w.+15 mg dimethoate/kg b.w. (Z+D), or 10 mg glyphosate/kg b.w.+15 mg dimethoate/kg b.w. (G+D). A group receiving the mixture of the three agrochemicals simultaneously (Z+G+D) was also analyzed. All the animals were injected three times a week for five weeks. The doses used were consistent with those reported in previous works from other laboratories (Nielsen et al., 2006; Patel et al., 2006; John et al., 2001; Sharma et al., 2005a; Beuret et al., 2005; Sharma et al., 2005b; Bagchi et al., 1995; Sivapiriya et al., 2006). Animal maintenance and handling procedures were in accordance with the NIH guide for the care and use of laboratory animals (National Reseach Council, 1985). This model was developed to simulate sub-chronic exposure to low doses of pesticide mixtures such as those incorporated from the general environment (Cory-Slechta, 2005).

2.3. Sample collection

At the end of the treatments, animals were killed by decapitation. Livers (L) were taken out, washed, weighed, and quickly homogenized in HEPES 50 mM pH 7.4 with CHAPS 5 mM, dithiotreitol 5 mM, and aprotinin 10 μg/ml, in a proportion of 300 μl buffer to 50 mg tissue. Brains were dissected to obtain the cerebral cortex (CC) (ventromedial areas directly connected with SN) and substantia nigra, using the Paxinos & Watson Atlas (1998). CC and SN samples were washed, weighed, and homogenized as described above. The cytosolic fraction for caspase and calpain measurements was prepared by homogenate centrifugation at 20,000*g* for 15 min at 4 °C as recommended by the manufacturer of the Caspase-3 Assay kit (CASP-3-C, Sigma Co.). All the samples were stored at −80 °C until assayed.

Mitochondrial fractions were prepared from all tissues by homogenization with HEPES 10 mM, pH 7.5 containing mannitol 200 mM, sucrose 70 mM, and EGTA 1 mM followed by a combination of low- (600*g*) and high-speed (11,000*g*) centrifugation procedures as recommended by the manufacturer of the isolation kit (MITO-ISO1, Sigma Co.). Samples of mitochondrial suspensions were treated with glutaraldehyde (2% W/V in buffer phosphate 50 mM pH 7.0), then impregnated in osmium tethroxide, and finally included in epoxyde-polymer for electron microscopy processing in order to check purity and membrane integrity (data not shown).

DNA samples were obtained and purified according to the Qiagen kit protocol, after homogenization of 25 mg of tissue with 180 μl of the sample buffer provided by the manufacturer.

2.4. Analytical methods

To asses the integrity of the inner mitochondrial membrane (IMM), we tested the electrochemical proton gradient ($\Delta\Psi$) using a membrane potential-sensitive probe (JC-1), following the method of Reers et al., 1991. (MITO-ISO1, Sigma Co.). The membrane potential of energized mitochondria (negative inside) promoted a directional uptake of JC-1 into the matrix and the formation of JC-1 aggregates. A fluorescence emission at 590 nm is directly related to the transmembrane potential. The results were expressed as percentage of intact IMM compared to control.

The integrity of the outer mitochondrial membrane (OMM) was measured by determining the cytochrome c (Cyt_c) oxidase activity, in the presence and absence of the detergent n-dodecyl β-D-maltoside. The ratio of the activities provides an indication of membrane integrity (CYTOCOX1, Sigma Co.).

Milli- (m-) and micro- (μ-) calpain activities were measured in the cytoplasmic fractions by the method of Botha et al. (2004). The assay involves the hydrolysis of whole ultra-pure casein (Sigma, Chem. Co.) by calpain activity and the subsequent detection of trichloroacetic acid (TCA)-soluble peptidic fragments at 280 nm. Assays were performed by adding 5 mM and 500 μM of $CaCl_2$ to evaluate milli- and micro-calpains, respectively. Caspase-3 activity was measured by a colorimetric assay kit (CASP-3-C), based on the hydrolysis of the synthetic peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA) by caspase-3. The resulting p-nitroaniline (p-NA) released was monitored at 405 nm. Three controls were used for each caspase-3 colorimetric assay: inhibitor-treated cell lysate control (to measure the non-specific hydrolysis of the substrate), caspase-3 positive control (using commercial caspase-3, 5 μg/ml provided by the kit manufacturer), and a reagent blank as negative control. Moreover, in order to protect the caspases and calpains activities in the tissue homogenates from non-specific proteolysis, the serine protease inhibitor aprotinin was added to the homogenization buffers as recommended by the manufacturer of the assay kit.

Total mitochondrial glutathione was measured by an adaptation of the Ellman's method using the purified mitochondrial suspension as sample (Ellman, 1959; Anderson and Meister, 1984).

DNA fragmentation patterns were also analyzed using the DNA laddering technique described by Sambrook et al. (1989). Samples were electrophoretically separated on agarose gel (1.5%) after genomic DNA extraction, using the Qiagen DNeasy blood and tissue extraction kit (Cat. 69506).

Protein content was determined according to the method of Lowry et al. (1951).

2.5. Lipid analysis

Mitochondrial cardiolipin (CL) content was quantified by means of phosphorous measurement using the method of Chen et al. (1956). Colorimetric reactions were performed on lipid extracts previously obtained by the method of Folch et al. (1957). Samples were separated by high-performance thin layer chromatography (HPTLC) on pre-coated silica gel plates (10 × 20 cm) from Whatman Schleicher and Schuell (Maidstone, England). The mobile phase was chloroform:methanol:ammonium hydroxide (65:25:4; by volume). Spots were visualized using iodine vapor, coomassie brilliant blue R-250 reagent (Nakamura and Handa, 1984), or charring procedure ($FeCl_3$–H_2SO_4) (Kritchevsky et al., 1972), with similar final results. The peroxidation of mitochondrial membrane lipids was assayed by measuring the thiobarbituric acid-reactive substances (TBARS) using the method of Yagi (1976) with tetraethoxypropane as standard. Lipid peroxides were expressed as nmol malondialdehyde (MDA)/mg protein.

2.6. Statistical analysis

The results were expressed as the mean±standard error of four independent experiments. They were statistically analyzed by one-way analysis of variance (ANOVA) followed by a Tukey multiple comparison test, and were considered different with respect to control data at two levels of significance: $^{*}P<0.05$ and $^{**}P<0.01$. Linear and non-linear correlation coefficients were calculated electronically using Sigma Plot Statistical Software (8.0) from Sigma Chem. Co. (St. Louis, MO).

3. Results

Exposure to pesticide, alone or in combination, did not affect animal behavior, water consumption, body weight, or the rate of body weight gain (data not shown). Also, no clinical signs of toxicity were observed during the entire experimental period. A veterinary observes for the presence of miosis, mouth smacking, salivation, or lacrimation, then the rats were placed in an open field for observation of tremors or gait abnormalities (Moser et al., 2006); there were no significant differences between controls and treated animals. Also, as we could find no differences between experimental data from either the control group (C) and the group treated with the vehicle (PEG-400) (V), the results from the PEG-injected animals were omitted for simplicity.

Because, mitochondrial membrane potential is usually altered during an early stage of programmed cell death, we first studied the state of mitochondrial membranes. Modification of membrane potential may play a crucial role in extrinsic and intrinsic apoptotic pathways. Fig. 1 shows the integrity of IMM, expressed as a percentage of control in the liver and in both brain regions, SN and CC. We observed a significant decrease in the percentage of intact IMM, especially in those groups treated with the mixture of the three pesticides. This finding reflects a loss of mitochondrial inner membrane potential ($\Delta\Psi$). OMM integrity assays (Fig. 2) were also expressed as a percentage of control. In this case, we found lower values only in the SN from the group treated with Z, G, and D in combination. In L and CC, no significant changes were observed (data not shown).

One of the main events involved in apoptotic induction is the release of mitochondrial proteins such as cytochrome c. The dissociation of Cyt_c from the IMM involves the perturbation of its association to acidic phospholipids, primarily cardiolipin (Petrosillo et al., 2003) localized mainly in the IMM. For that reason we measured the CL levels in the mitochondrial fraction from intoxicated animals (Fig. 3), detecting a 60% reduction in both brain regions and a drop of almost 30% in liver preparations. We also found a clear dependence between the decrease in CL levels and the inner mitochondrial membrane potential. Statistical analysis of this dependence shows clearly that when the concentration of CL decrease, the percentage of IMM integrity was decreased too, in a fashion that seems to follow a polynomial quadratic function ($r^2 = 0.92$) rather than a linear behavior ($r^2 = 0.62$) (Fig. 4). This finding probably means that the damage of the IMM is aggravated in a non-proportional way during the loss of mitochondrial cardiolipin (exponentially increased from mild to severe stages of CL depletion). Interestingly, these changes were observed in animals treated with pesticide mixtures. CL is composed by 4 acyl chains that are predominantly PUFAs. It is well known that OS is involved in lipid peroxidation and that CL is situated near to the main reactive species source (the mitochondrial transport chain). Taking into account these facts we determined the degree of lipid peroxidation by means of the thiobarbituric acid-reactive substances. An increase in MDA levels (the main end product of lipid peroxidation) was likely produced in our experimental model (Fig. 5). Mitochondrial fractions from both liver and brain obtained from the group treated with the combination of Z, G and D exhibited a marked increase in the production of MDA levels (TBARS) compared to control.

Alteration of the mitocondrial membranes integrity may produce protease-dependent cascade activation(s). For that

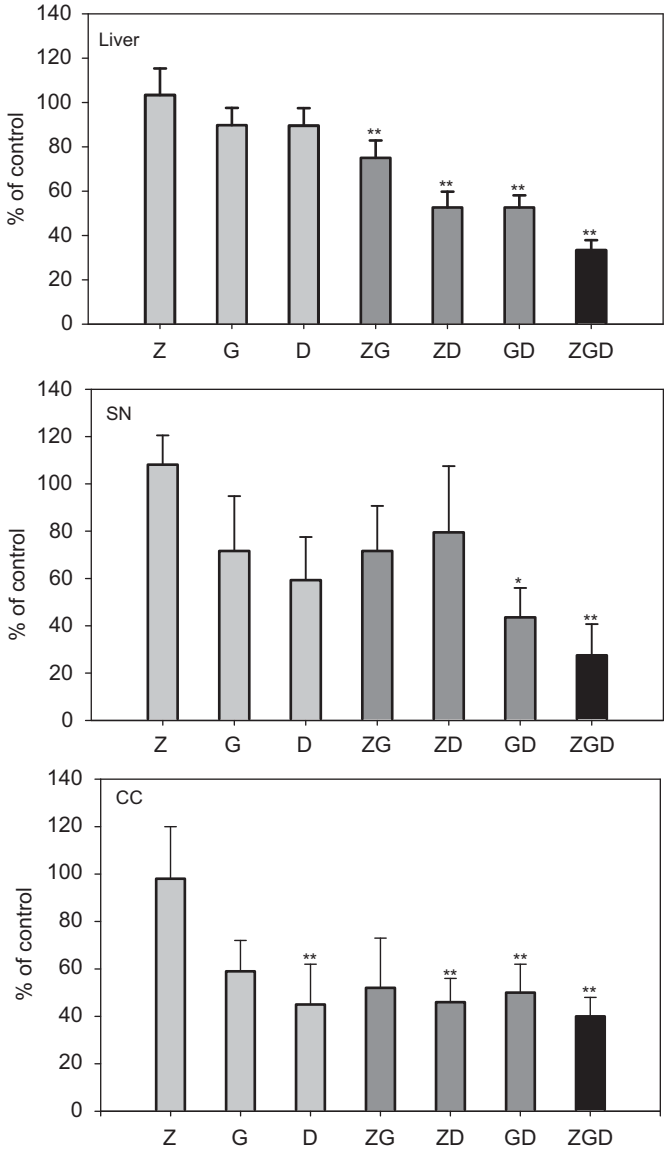

Fig. 1. Liver, substantia nigra (SN) and cerebral cortex (CC) inner mitochondrial membrane integrity from the groups exposed to one (grey bars), two (dark grey bars), or three (black bar) drugs. Animals were treated as described above. C, control or vehicle-injected group; Z, i.p. injected with zineb; G, glyphosate; D, dimethoate; or the indicated combinations of those drugs. Results are expressed (% of control) as the mean ± SD of four independent determinations. Significantly different from control data: $^{*}P<0.05$ or $^{**}P<0.01$.

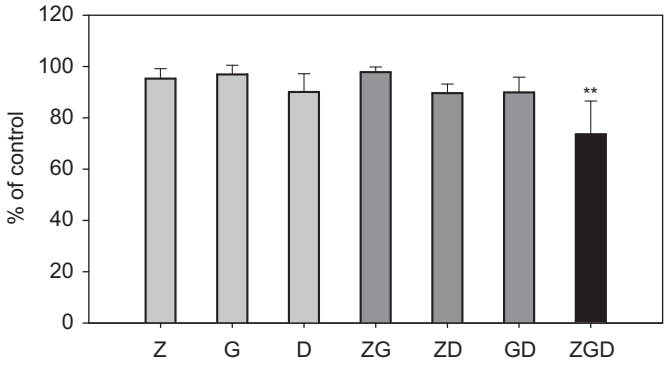

Fig. 2. Outer mitochondrial membrane integrity in the substantia nigra (SN) from the groups exposed to one (grey bars), two (dark grey bars) or three (black bar) drugs. Animals were treated as described above. C, control or vehicle-injected group; Z, i.p. injected with zineb; G, glyphosate; D, dimethoate; or the indicated combinations of those drugs. Results are expressed (% of control) as the mean ± SD of four independent determinations. Significantly different from control data: $^{*}P<0.01$.

reason we determined the activities of the main known proteolytic system involved in cell survival. The activity of the key effector caspase (caspase-3) was unaltered in all the experimental groups and in all tissues analyzed (data not shown); however, calpain activity was clearly modified. Measurements of milli- and micro-calpain activities in CC and SN demonstrated a change in both isoenzymes determined in those groups treated with the combination of two or three pesticides. Table 1 shows the results obtained for the activity of m-calpain, which was higher in SN than in the other tissues assayed. Similar results were obtained for μ-calpain activity (data not shown).

It is well known that the activation of the caspase-dependent cascade is crucially linked to the energetic and redox cellular status (Orrenius, 2004). In connection with this, the content of reduced glutathione (GSH) measured in mitochondrial fractions from liver and brain regions were lower in the groups treated with the mixtures of pesticides (ZGD) than in control group, suggesting that at least the redox status is clearly altered in both tissues investigated (Fig. 6).

Finally, we analyzed DNA fragmentation patterns by the isolation of genomic DNA and its separation on an agarose gel. The aim of this experiment was to investigate the impact of the previously observed changes on the integrity of genomic DNA. Fig. 7 shows representative patterns observed for liver and brain samples. After ethidium bromide treatment, a typical (although non apoptotic) pattern of DNA damage was observed. The fragmentation becomes more evident when pesticides were administered in combination than in single exposures.

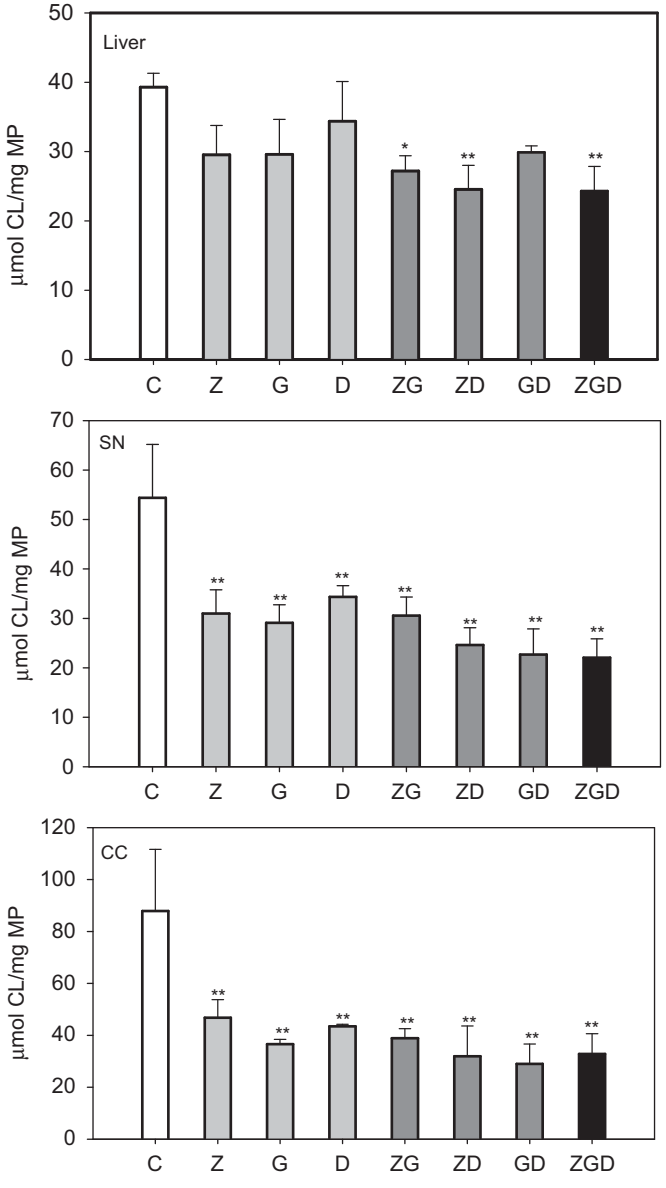

Fig. 3. Liver, substantia nigra (SN) and cerebral cortex (CC) mitochondrial cardiolipin content from control (white bar) group, exposed to one (grey bars), two (dark grey bars) or three (black bar) drugs. Animals were treated as described above. C, control or vehicle-injected group; Z, i.p. injected with zineb; G, glyphosate; D, dimethoate; or the indicated combinations of those drugs. Results are expressed (μmol of cardiolipin/mg of mitochondrial protein) as the mean ± SD of four independent determinations. Significantly different from control data: $^{*}P<0.05$ or $^{**}P<0.01$.

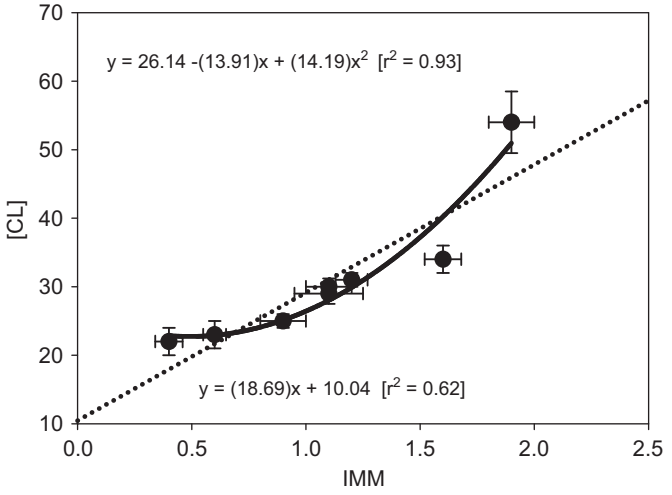

Fig. 4. Correlation of mitochondrial cardiolipin content and IMM. Data was processed electronically using the statistical software described in Section 2. Linear regression was performed by least minimal square method and was shown by a dotted line. Non-linear fittings were assayed by means of different exponential and polynomial adjustments. The curve indicated in solid line provided the best correlation coefficient.

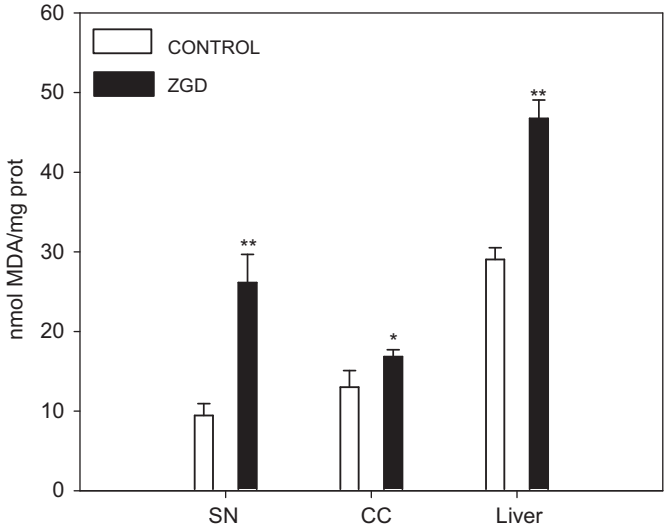

Fig. 5. Mitochondrial TBARS levels in the substantia nigra (SN), cerebral cortex (CC) and liver from control (white bar) group, exposed to three (black bar) drugs. Animals were treated as described above. C, control or vehicle-injected group and the group treated with the combinations of three drugs. Results are expressed (nmol MDA/mg of protein) as the mean ± SD of four independent determinations. Significantly different from control data: $^{*}P<0.05$ or $^{**}P<0.01$.

4. Discussion

Previous works from this laboratory have demonstrated that pesticide treatment evokes an oxidative stress in rat liver and brain by causing both an increase in ROS/RNS production and a decrease in antioxidant defense ability (Astiz et al., submitted). This effect was more evident in those groups treated with pesticide mixtures. OS has been implicated in the pathogenesis of neurodegenerative disorders such as PD and AD (Halliwell and Gutteridge, 1999; Stewart and Heales, 2003; Chong et al., 2005). Furthermore, the loss of mitochondrial function is associated with many human neurodegenerative disorders. The brain is particularly dependent upon mitochondrial energy to maintain normal physiology (Pope et al., 2008). During the past decade, the role of mitochondria in the apoptotic process has been the focus of cell death research. Mitochondrial membrane potential ($\Delta\Psi$) is lost during an early stage of apoptosis, and many mitochondrial proteins are released into the cytosol during the first steps of programmed cell death (Ricci et al., 2003; Von Ahsen et al., 2000;

Table 1
Activity of mili- (m-) calpain in liver (L), cerebral cortex (CC) and substantia nigra (SN) from control and treated rats.

Treatments	m-Calpain activity (mUI/mL)		
	L	CC	SN
C	9.2±0.30	3.6±0.26	0.91±0.09
Z	9.0±0.14	4.3±0.05**	1.14±0.01**
G	8.4±0.08	4.4±0.20**	1.17±0.05**
D	9.7±0.70	4.0±0.10*	1.40±0.09**
ZG	10.4±0.40	3.9±0.01	1.18±0.06**
ZD	9.9±0.41	4.0±0.04**	1.43±0.04**
GD	10.0±0.14	4.0±0.12**	1.43±0.07**
ZGD	10.2±1.32	4.0±0.04*	1.63±0.04**

Results were expressed as the mean±SD of four independent assays (mUI/ml homogenate). Values significantly different with respect to control group were indicated as $^{*}P<0.05$ or $^{**}P<0.01$.

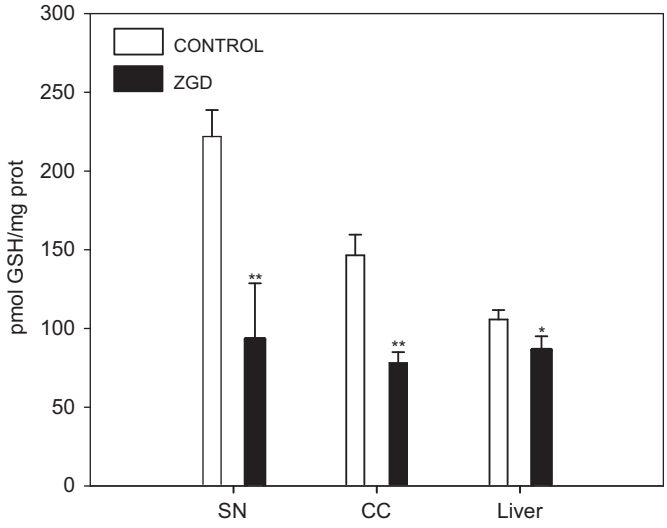

Fig. 6. Mitochondrial GSH levels in the substantia nigra (SN), cerebral cortex (CC) and liver from control (white bar) group, exposed to three (black bar) drugs. Animals were treated as described above. C, control or vehicle-injected group and the group treated with the combinations of the three drugs. Results are expressed (pmol GSH/mg of protein) as the mean±SD of four independent determinations. Significantly different from control data: $^{*}P<0.05$ or $^{**}P<0.01$.

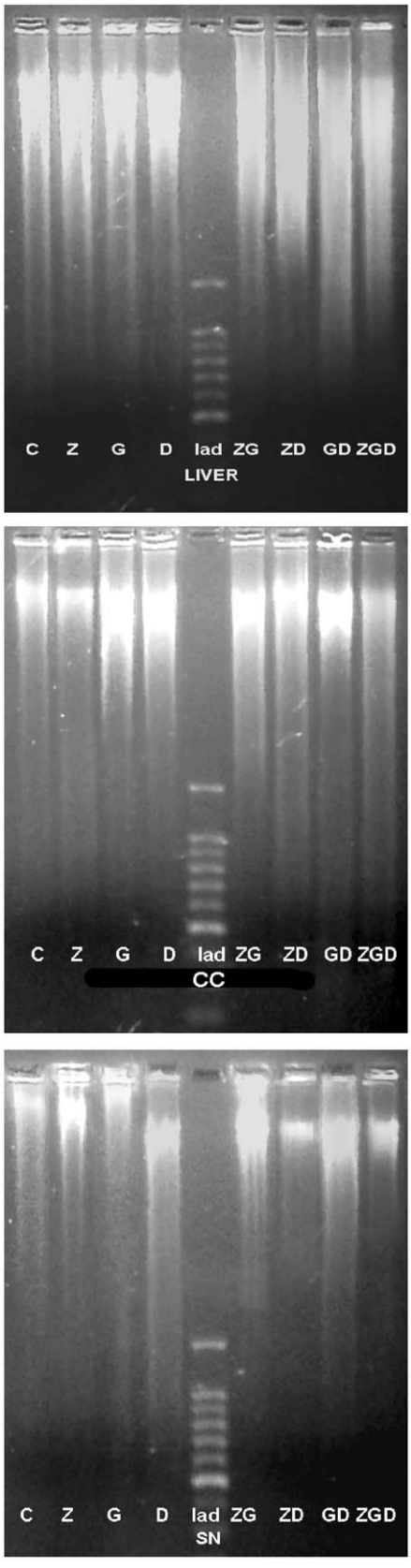

Fig. 7. Liver, cerebral cortex (CC) and substantia nigra (SN) DNA analysis in agarose gel electrophoresis. Samples were revealed by ethidium bromide. From left to right, C (control group), exposed to Z, G, and D alone, 100 pb control ladder, exposed to ZG, ZD, GD, and ZGD. Animals were treated as described above. C, control or vehicle-injected group; Z, i.p. injected with zineb; G, glyphosate; D, dimethoate; and the indicated combinations of drugs.

Jackson et al., 2002). In our experimental system, we demonstrated alterations in the integrity of the IMM involved in the loss of $\Delta\Psi$ for all tissues studied and in all treated groups, with decreases near 70% of control. However, the alteration of the OMM was observed only for the most sensitive tissue (SN), and with the combination of the three pesticides (almost a 20% decrease respect to control group). It is interesting to observe that the magnitudes of the damages associated to OMM and IMM were significantly different. A possible explanation for this fact may be the different proportion of the lipid contents (especially CL) in both membranes (Iverson and Orrenius, 2004; Shidoji et al., 1999). The OS status established by pesticide treatment should be, in consequence, more destructive for the IMM than for the external membrane. At the same time, it is possible to assume that during a chronic exposure some (still unknown) mechanism of reparation and/or adaptation may be accomplished on the external membrane. This question deserves more investigation to clarify the differences observed; however, from these results it is apparent that, at least in our experimental model, the reduction in OMM integrity is less important than the damage on the IMM to modify the cellular survival route. In addition, our results are in agreement with other studies which have demonstrated that the apoptotic pathway started without OMM disruption (Petrosillo et al., 2003; Von Ahsen et al., 2000).

Release of cytochrome c from mitochondria appears to be a central event in the induction of the apoptotic cascade that ultimately leads to programmed cell death (Petrosillo et al., 2003). The mechanism underlying Cyt_c release from mitochondria is not fully understood, but it appears to be largely mediated by ROS (Fernandez-Checa, 2003). Cyt_c is a water-soluble basic protein that specifically binds to the acidic phospholipid cardiolipin. This glycerolipid is abundant in the outer leaflet of the IMM (Iverson and Orrenius, 2004; Shidoji et al., 1999). The binding of Cyt_c to CL is very tight, apparently irreversible, and stoichiometric. CL is necessary not only for Cyt_c function, but also for almost all mitochondrial protein complexes (Paradies et al., 2000; Paradies et al., 2002; Pfeiffer et al., 2003). CL is a dimeric phospholipid with 4 acyl side chains intimately associated with the electron transport chain, the major site for ROS production (Jackson et al., 2002). It is also particularly rich in unsaturated fatty acids and, as a consequence, is highly susceptible to oxidative attack. The high degree of CL unsaturation is maintained by a constant remodeling process integrated between the mitochondria and the endoplasmic reticulum (Esposti, 2002; van der Berg et al., 1993; Hatch et al., 1993; Schlame and Rüstow, 1990). Changes in CL content have also been reported to trigger the release of Cyt_c from mitochondria during the initiation of the apoptotic process, due to oxidative damage or to the alteration of its biosynthetic pathway (Petrosillo et al., 2001; Shidoji et al., 1999). In fact, many authors have established a strong linear correlation between the decrease of CL levels and Cyt_c release (Petrosillo et al., 2001, 2003; Ostrander et al., 2001; McMillin and Dowhan, 2002; Ott et al., 2002). We demonstrated that CL concentrations were significantly decreased in the mitochondrial fraction from the liver and brain from all treated groups compared to the control, being more significant in the brain (60% for both regions) than in the liver (approx. 30%). This finding may be explained by the differential peroxidizability of the fatty acyl composition between the liver and brain CL. In mammalian tissues such as the liver, heart, kidney, and skeletal muscle, the predominant fatty acyl chain form of CL is tetralinoleoyl-CL [CL-$(18{:}2n\text{-}6)_4$]; however, brain CL contains highly oxidizable molecular species such as 20:4 n-6 and 22:6 n-3 (Kagan et al., 2006; Bayir et al., 2007). This could render brain CL more susceptible to peroxidation due to the higher double-bound index of their fatty acyl side chains. This explanation is also in good agreement with the analysis of lipid peroxide formation in the mitochondria from these tissues. We observed higher levels of TBARS in mitochondria from animals treated with the combination of the three drugs, suggesting that peroxidation may be involved in the observed decrease of CL levels. Our finding is also in agreement with similar results obtained by other authors (Shidoji et al., 1999; Petrosillo et al., 2001; Sen et al., 2006; Kirkland et al., 2002).

From this scenario, we expected to obtain changes in the activity of caspase-3, which is a typical effector for the programmed cell death pathway. However, there were no significant changes in either the liver or brain regions. In contrast, the activities of milli-calpains were increased in both brain regions, especially in the groups treated with pesticide mixtures. In recent years, it has become increasingly clear that multiple mechanisms of cell death, as well as cross-talk among different death pathways, contribute to neurodegeneration. The striking similarity between the substrates for caspases and calpains raises the possibility that both families of proteases contribute to the structural deregulation and functional loss of neurons in neurodegenerative conditions (Raynaud and Marcilhac, 2006). Caspases have been reported to regulate calpain activity through the modification of calpastatin activity (endogenous calpain inhibitor) by proteolytic cleavage. Several recent studies also suggest that calpains could cleave endogenous caspases such as caspase-3, -7, -8, and -9. Although numerous cross-talks exist between caspases and calpains, the exact signaling pathway linking both proteases families that eventually leads the cell to death is still unclear (Chong et al., 2005; Chen et al., 2006; Chua et al., 2000; Wang et al., 1998; Bizat et al., 2003a). In the central nervous system, these two proteolytic systems may be interdependent, making it difficult to take a general view of their complex in vivo interactions (Samantaray et al., 2007). From previous experimental evidence reported for other laboratories, we can assume that in our experimental model the calpains should be interfered with caspase activation by (probably) the cleavage of initiator and/or effector members of this group of proteases. Moreover, the predominant activation of calpains instead of caspases is in agreement with other findings reported for experimental models of chronic – but not acute – injury (Bizat et al., 2003a; Bizat et al., 2003b). In addition, ATP levels (Eguchi et al., 1997) and redox cellular status (Orrenius, 2004) appear to be key factors in determining the activation of caspase-dependent pathways. In relation to this, results from Orrenius's group indicated that a variety of environmental toxicants influence the rate of apoptosis by increasing the level of oxidized glutathione (GSSG). GSSG by itself is sufficient to inhibit pro-caspase-3 processing; suggesting that formation of active caspase-3 is dependent upon the maintenance of thiol redox status within the cell (Orrenius, 2004). We observed that GSH levels are lower in the liver and brain regions from the groups treated with the mixtures of pesticides, indicating that the redox status would be altered in those tissues.

The pesticide treatment (especially in those groups receiving two or three drugs) induces DNA fragmentation which is one of the criteria currently used to define cell death (by necrosis or apoptosis). A laddering pattern was difficult to detect in all tissues analyzed. In addition, the fragmentation observed was more evident in the liver, in which m-calpain activities were not significantly modified. This finding may be probably related to the magnitude of the exposure to pesticides (the liver was the first target organ for pesticides and could receive higher doses than brain). In relation to this, it was reported by other laboratory (Rami et al., 2000) that calpain activation is directly involved in DNA fragmentation. So, the major changes observed in DNA fragmentation of liver may suggest a direct effect of the pesticides rather than an indirect action involving previous

calpain activation. The main conclusion obtained is that DNA was extensively damaged as a consequence of the experimental treatment. However, this question deserves more investigation since, at least in the liver, DNA fragmentation is likely to occur via a combination of factors other than caspase or calpain activation, as Griffiths et al. (2002) have previously suggested.

The intimate mechanism(s) by which the agrochemicals here studied provoked cellular damage are far away to be elucidated. This is mainly due to the almost complete absence of experimental data concerning this question. However, it was reported that glyphosate reduced the uptake of cysteine en HeLa cells in culture evoking a decrease of glutathione biosynthesis (Hultberg, 2007), while dimethoate probably acts as inducer of P450 enzymes in liver (Sharma et al., 2005a, b; Sivapiriya et al., 2006) which in turn may lead to a raise of the reactive species generation. In addition, the increased risk for Parkinson′s disease linked to occupational and residential dithiocarbamate exposure may be related to various effects on neurotransmitter metabolism such as inhibition of dopamine β-hydroxylase activity, increased catechol oxidation, and decreased uptake of glutamate (Fitsanakis et al., 2002). All these evidence are in agreement with our results since they justify, at least in part, the OS induced by the treatment(s) and the subsequent disruption of DNA integrity.

Another important question to be addressed is the real degree of human being's exposure to these kinds of drugs. Unfortunately there are no concise reports regarding this problem. Measurements of daily human intake of glyphosate and other agrochemicals via food, drinking water, or absorption by inhalation or skin contact are completely unknown (Mensink and Janssen, 1994; Daruich et al., 2001). Recent regulations of the European Union established the maximum residual limits (MRLs) for dimethoate (plus omethoate), glyphosate, and dithiocarbamates (zineb) in several foods between the range 0.01 to 5 mg/kg depending on the product considered (Reg. EC No. 839/08 entry into force by Sept/2008). However, this data is difficult to extrapolate to the real amount of pesticides that living organisms are exposed to during long periods of feeding. So, more investigation on this area should be necessary to compare sub-chronical experimental exposition to that really achieved by human populations. Data on the occurrence of residual pesticides in biota and environmental abiota are actually scarce (Daruich et al., 2001). The situation becomes worse considering that agriculturists usually combine the commercial herbicide glyphosate with zineb, and the systemic insecticide dimethoate to obtain better results in productivity. The practice of creating these cocktails could produce a potentiation of their individual properties and could increase their levels of damage such as those we investigated in our experiments.

In conclusion, our investigation demonstrated that (a) the associations of agrochemicals were able to produce more damage than individual administration; (b) the induced OS condition generated by pesticide treatment is enough to modify the biomarkers of redox status in liver and brain tissues and that this may be linked to the activation of routes involved in cell survival; (c) the damage is greater in the SN, the cerebral region implicated in PD pathogenesis, probably due to its higher sensitivity to OS; and (d) the pesticide-induced OS condition could be responsible for the activation of calpains rather than caspases. These results may contribute to the clarification of the etiological role of pesticides in human neurodegenerative diseases, and are relevant to the identification of putative therapeutic targets. In addition, results may be relevant in order to protect the general population exposed daily to dietary residues of pesticides in food and water, and also for workers exposed during handling and application of these agrochemicals.

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