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Changes in glutathione-redox balance induced by hexachlorocyclohexane and lindane in CHO-K1 cells

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1. The basal cytotoxic effect of the organochlorine pesticides hexachlorocyclohexane and lindane on CHO-K1 cultures was assessed at fractions of their lethal doses as determined by the neutral red incorporation (NRI) assay ($NRI_{6.25}$, $NRI_{12.5}$ and NRI_{25}). The sulphur-redox cycle enzymes glutathione peroxidase, glutathione reductase and glutathione *S*-transferase, and total and oxidized glutathione were evaluated at several points during the standard growth curve of the cultures.

2. After incubation with each compound for 24 h, both glutathione peroxidase and reductase showed a substantial increase at the lowest exposure doses ($NRI_{6.25}$) – more significantly for lindane than for 1,2,3,4,5,6-hexachlorocyclohexane (HCH) – and dropped at higher doses of both compounds. The reduced and oxidized glutathione content was greatly diminished at the lower exposures, whereas the total glutathione content was higher at $NRI_{12.5}$ values.

3. Changes in cell membrane integrity were assessed for a wide range of pesticide concentrations with the lactate dehydrogenase release assay and lipid peroxidation. Membrane leakage and peroxide production were significantly enhanced at concentrations of HCH 50 $\mu g\,ml^{-1}$, although this effect was not significant at lindane concentrations $< 200\,\mu g\,ml^{-1}$.

4. Lipid peroxidation increased with exposure to HCH at concentrations as low as $NRI_{6.25}$, whereas in the case of lindane, this increase was only significant at doses of NRI_{25} and above.

Introduction

Lindane, the γ-isomer of the 1,2,3,4,5,6-hexachlorocyclohexane (HCH), is an effective organochlorine insecticide widely used on fruit and vegetable farming, seed treatment and forestry, as well as for animal health in the treatment of head and body lice and scabies (ATSDR 1992). Unlike HCH isomer mixtures, lindane is widely used for agricultural and clinical purposes in industrialized countries, making the assessment of the possible risks such use involves a concern to be addressed. As in other organochlorine insecticides, the most usual route for human exposure is oral ingestion of food containing the insecticides, although cases of dermal and inhalatory exposures have also been reported (Loffler and van Bavel 2000, Nordt and Chew 2000, Klein *et al.* 2001). Acute and/or chronic responses to

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these pesticides include neurotoxicity and the effects on the cardiovascular, gastrointestinal, immune, and muscle and skeletal systems (Videla *et al.* 1990, ATSDR 1992, Arisi *et al.* 1994). Histological and biochemical alterations of the liver such as hypertrophy, steatosis and necrosis (Junqueira *et al.* 1988), and impairment of reproductive function in male animals have been described after chronic exposure (Samanta *et al.* 1999). There is growing evidence that most of these processes may be the result of free-radical intermediates induced by exposure to environmental contaminants (Junqueira *et al.* 1988, Videla *et al.* 1990, Arisi *et al.* 1994, Samanta and Chainy 1997, Samanta *et al.* 1999).

In vivo experimental studies have shown that the HCH and lindane hepatotoxicity may be due to oxidative stress associated with lipid peroxidation (Videla *et al.* 1990, Hassoun *et al.* 1993, Arisi *et al.* 1994, Samanta and Chainy 1995). Similar mechanisms have been reported in reproductive organs such as the testis and placenta as well as in foetuses (Hassoun and Stohs 1996, Chitra *et al.* 2001, Sujatha *et al.* 2001) and the immune system (Koner *et al.* 1998). More controversial reports have likewise been published on effects on cerebral tissue (Arisi *et al.* 1994, Sahoo *et al.* 2000). Agrawal *et al.* (1991) suggested that HCH produces significant changes in the glutathione redox system in rat erythrocytes, leading to oxidative membrane damage, although Junqueira *et al.* (1994) did not observe alterations in pro-oxidant/antioxidant status.

Lipid peroxidation destroys membrane integrity, causing cell dysfunction and ultimately cell death (Zaman and Pardini 1996). Junqueira *et al.* (1988) reported morphological evidence of hepatic cell injury in lindane-treated rats, which appeared to increase with time and be associated with enhanced formation of thiobarbituric acid reactants. Moreover, in one of the rare studies on cultured cells, Verma *et al.* (1992) suggested that the primary action of biologically active HCH isomers is to disrupt the organization of the plasma membrane, affecting cell viability. Hassoun and Stohs (1996) and Hassoun *et al.* (1993), in turn, described increased DNA single-strand breaks in foetal and placental tissues and in the liver, respectively. However, unlike other organic pesticides such as the herbicide paraquat (Palmeira *et al.* 1994), in the substances addressed in the present study the source of the reactive oxygen species (ROS) that have deleterious effects on many cellular processes is not yet known.

The ROS species induced by lindane and HCH may, like the paraquat ROS, be neutralized by the cellular antioxidant system, comprising both mitochondrial Mn-containing and cytosolic Cu- and Zn-containing superoxide dismutases (SODs) (Bagley *et al.* 1986), as well as glutathione peroxidase and glutathione reductase, which are instrumental in maintaining the balance between the reduced and oxidized forms of glutathione (Krall *et al.* 1991). Although superoxide anion radicals can be rapidly removed, the hydroxyl-free-radical (OH), formed by the Fenton reaction, reacts efficiently with polyunsaturated fatty acids and other lipid membrane components, being the source of various deleterious lipid peroxidation species (Samanta and Chainy 1997). Involvement in pathological processes occurs when free-radical production exceeds the tissue free-radical-scavenging capacity (Zaman and Pardini 1996). Despite information on lipid peroxidation induced by HCH and lindane in experimental animals (Hassoun and Stohs 1996, Junqueira *et al.* 1997, Samanta and Chainy 1997), more information about its role in cellular cytotoxicity or its effect on free-radical-scavenging enzymes is necessary.

This study compares the dose–response effects of HCH and lindane on membrane permeability and glutathione content and interconversion in Chinese hamster ovary cells (CHO-K1) in order to ascertain whether ROS are involved in the toxicity of these pesticides in mammalian cells.

Materials and methods

Chemicals

Culture medium Hams' F12, gentamicine sulphate (10 mg ml^{-1}), neutral red dye (NR), reduced and oxidized glutathione (GSSG) and glutathione reductase were from Sigma Chemical Co. (St Louis, MO, USA). Hexachlorocyclohexane isomer mixture and the γ-isomer (lindane) were acquired from Alltech Associates (Deerfield, IL, USA). Foetal calf serum was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). CytoTox 96® LDH-releasing kit was from Promega (Madison, WI, USA). All other reagents were of standard laboratory grade.

Cell culture and media

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC CCL 61); 10^4 cells cm^{-2} were plated in monolayer in 22.1-cm^2 polystyrene tissue culture dishes with Hams' F12 supplemented with 25 mm Hepes buffer (pH 7.4), 10% heat inactivated foetal calf serum and gentamicine (30 µg ml^{-1}) at 37 °C. Cells were counted in an improved Neubauer haemocytometer and viability was determined by exclusion of Trypan blue. Cells were grown to 65% confluence (day 3, mid-log phase) and then pulsed with different concentrations of organochlorine insecticides dissolved in 1% dimethylsulfoxide (DMSO). Control wells were pulsed with a similar volume of DMSO solution. After 24 h, cells were washed with phosphate-buffered saline (PBS) and medium was replaced. Cell extracts were used to determine sulphur-redox species and the enzymes involved in glutathione metabolism.

Determination of cytotoxicity

The cytotoxicities of lindane and HCH were estimated in CHO-K1 cells using the neutral red incorporation (NRI) assay described by Borenfreund and Puemer (1985). Cells were grown up to 65% confluence and then pulsed with different concentrations of lindane and HCH (ranging from 0.1 to 500 µg ml^{-1}). After 24 h, cells were washed and medium replaced with fresh medium, containing 40 µg ml^{-1} NR dye. After 3 h, cells were washed with PBS and fixed with 0.5% formaldehyde and 1% $CaCl_2$ in distilled water. Colour was developed with 0.2 ml 1% acetic acid, 59% ethanol and water. The absorbance at 540 nm of each plate was determined in an automatic ELISA reader and curves were fitted using Sigma Plot (v.2.1, Microsoft®) for non-linear regression using probit/log curves to obtain the concentrations of each insecticide equivalents to their correspondent fractions of NRI ($NRI_{6.25}$, $NRI_{12.5}$, NRI_{25}, NRI_{50}).

Alteration of membrane permeability induced by HCH and lindane insecticides was assessed in CHO-K1 cultures. Membrane status was analysed by measuring the lactate dehydrogenase (LDH) released to the culture medium by CHO-K1 cells exposed to different concentrations of these insecticides in a short period (as short as 4 h). LDH release was spectrophotometrically estimated using the CytoTox 96 kit (Promega), following the manufacturer's manual. One hundred per cent of LDH release was determined adding 1 mg ml^{-1} SDS to control-column wells.

Lipid peroxidation assay

Malondialdehyde (MDA) measurement was used as indicator of lipid peroxidation in CHO-K1 cells after exposure to lindane and HCH. Cells were grown in 24-well plates using medium supplemented with 10% foetal calf serum. After cell adherence was achieved, the medium was replaced with 0.5 ml serum-free medium containing the insecticide at final concentrations ranging from 6.25 to 50 µg ml^{-1}. After 4-h exposure, the medium was collected, centrifuged and 250 µl of the supernatant was transferred to a test tube for the lipid peroxidation assay. The reaction mixture contained 250 µl sample, 100 µl 7% sodium dodecyl sulfate (SDS), 1 ml 0.1 N HCl, 150 µl 1% phosphotungstic acid and 500 µl 0.67% thiobarbituric acid (TBA). The mixture was mixed thoroughly and heated at 95 °C for 60 min in the dark. After cooling in an ice bath, 1 ml *n*-butanol was added, and the mixture was shaken vigorously. After centrifugation at 1200*g* for 10 min, the organic layer was analysed for MDA by spectrofluorometry at 350 nm (ex.) and 590 (em.). MDA standards and blanks (cells exposed to 1% DMSO) were also assayed.

Glutathione content

Intracellular total glutathione was determined spectrophotometrically using the method described by Akerboom and Sies (1981). CHO-K1 monolayers from 22.1-cm^2 polystyrene Petri dishes were harvested from the plastic surface with a trypsin/EDTA solution, washed twice with PBS, pH 7.4, and disrupted by sonication with 1 M cold perchloric acid (PCA) containing 2 mM EDTA. One hundred microlitres of the extracts were neutralized with 70 µl KOH and 70 µl MOPS 0.3 M before measuring glutathione. The protocol for total glutathione determination was as follows: 730 µl 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 200 µl neutralized cell extracts, 50 µl 4 mg ml^{-1} NADPH dissolved in 0.5% $NaHCO_3$ and 20 µl 1.5 mg ml^{-1} 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) in 0.5% $NaHCO_3$ were combined to a final volume of 1 ml. Reactions were started with 6 U glutathione reductase and measured at 412 nm for 1 min at 25 °C. Slopes of each determination were interpolated from a calibration curve expressing the total content of glutathione as nmol mg^{-1} total protein.

GSSG was assayed in freshly acidified cell extracts using the method described by Anderson (1985). CHO-K1 cultures were harvested and disrupted as above. PCA-treated extracts were then centrifuged and neutralized with 2 µl 2-vinylpyridine Fluka Chemie (Buchs, Switzerland) and 824 µl 25% triethanolamine. The reaction mixture contained in a 1 ml final volume: 650 µl sodium phosphate buffer, 0.1 M, pH 7.5, that included 6.3 mM EDTA, 50 µl 4 mg ml^{-1} NADPH, 100 µl DTNB 6 mM, 200 µl cell extracts and 1 U glutathione reductase. Similarly to total glutathione, GSSG was estimated by interpolating the slopes of each assay to a standard curve, expressing GSSG content as nmol mg^{-1} total protein.

Enzyme assays

The glutathione reductase mixture contained, in a final volume of 1 ml, 500 µl potassium phosphate buffer, 0.2 M, pH 7.0, with 2 mM EDTA, 50 µl of a 2 mM NADPH solution dissolved in 10 mM HCl-Tris, pH 7.0, 50 µl 20 mM GSSG, 300 µl distilled water and 100 µl freshly isolated cellular extract. The absorbance rate of the reaction mixture at 340 nm was measured after incubation at 30 °C for 1 min. One unit of glutathione reductase activity is defined as the amount of enzyme reducing 1 µmol GSSG min^{-1} mg^{-1} soluble protein.

Total glutathione peroxidase was assayed as described by Wendel (1981). Included in a 1-ml final volume was 500 µl potassium phosphate buffer 0.25 M, pH 7.0, containing 2.5 mM EDTA and 2.5 mM sodium azide, 100 µl 10 mM reduced glutathione, 100 µl 2.5 mM NADPH in 0.1% $NaHCO_3$ solution and 100 µl 2.4 U ml^{-1} freshly prepared glutathione reductase in PBS. One hundred microlitres of cell extracts were added and incubated at 37 °C. The assay was started by adding 100 µl 12 mM *tert*-butyl peroxide (Sigma) and monitoring the absorbance rate at 366 nm during 1 min at 37 °C. One unit of glutathione peroxidase is defined as the amount of enzyme that oxidizes 1 µmol reduced glutathione min^{-1} mg^{-1} soluble protein. Protein was assayed according to Bradford (1976).

Glutathione *S*-transferase was measured spectrophotometrically according to Habig *et al.* (1974). In a 1-ml plastic cuvette, the following reagents were added: 800 µl sodium phosphate buffer, 0.2 M, pH 6.5, 50 µl chlorodinitrobenzoic acid (Sigma) 20 mM dissolved in 95% ethanol, 50 µl reduced glutathione 20 mM, and 100 µl enzymatic extract. Enzymatic activity was assayed at 25 °C for 1 min at 340 nm. One unit of GST activity is defined as the amount of enzyme consuming 1 µmol GSH min^{-1} mg^{-1} protein.

Results

HCH and lindane cytotoxicities were determined using the neutral red incorporation (NRI) assay, which monitors lysosomal cell function. Both organochlorine compounds were added to CHO-K1 cultures during the mid-log phase (day 3) and incubated for 24 h, after which cell viability was analysed by the above technique. The probit/log curves yielded the NRI values shown in table 1. HCH was 2.5–3.4 times more toxic than lindane in CHO-K1 cells. On the grounds of the high reliability of the NRI assay, fractions of the NRI_{50} ($NRI_{6.25}$, $NRI_{12.5}$ and NRI_{25}) were used to determine the biochemical parameters assayed here.

The metabolic effects of HCH and lindane on the sulphur-redox cycle were measured after exposure to the agents for 24 h in the presence of foetal calf serum in the culture medium. Table 2 summarizes total glutathione and GSSG content, determined in the mid-/late log phase after exposure to HCH and lindane. Total glutathione was found to increase significantly ($p < 0.01$) at HCH and lindane concentrations equivalent to their $NRI_{12.5}$, but remained unchanged with respect to control cells at higher and lower concentrations. Unlike total glutathione,

Table 1. Basal cytototoxicities of HCH and lindane on CHO-Kl cells using the neutral red uptake (NRI) method.

	Insecticide concentrations ($\mu g\,ml^{-1}$)				
Insecticide	$NRI_{6.25}$	$NRI_{12.5}$	NRI_{25}	NRI_{50}	r^2
HCH	5.9 ± 0.6	10.5 ± 0.9	46.9 ± 1.1	55.7 ± 1.2	0.97
Lindane	19.4 ± 0.8	33.3 ± 2.2	105.4 ± 1.9	144.1 ± 1.7	0.97

Cells were grown until mid-log phase and were then exposed to different concentrations of HCH and lindane for 24 h. NRI values were calculated by the probit/log approach. Results are the average ± SEM of three independent experiments.

Table 2. Intracellular glutathione (total and oxidized forms) content in 24-h HCH and lindane-treated CHO-Kl cells.

		Glutathione content ($nmol\,mg^{-1}$ protein)		
Insecticide	Glutathione	$NRI_{6.25}$	$NRI_{12.5}$	NRI_{25}
HCH	total	25.82 ± 1.45	40.80 ± 4.90	26.10 ± 1.56
	GSSG	0.18 ± 0.01***	0.22 ± 0.02*	0.26 ± 0.01**
	GSSG/total	0.007	0.0054	0.01
Lindane	total	25.80 ± 2.73	49.22 ± 1.58***	30.77 ± 3.73
	GSSG	0.18 ± 0.03*	0.16 ± 0.01***	0.17 ± 0.04*
	GSSG/total	0.007	0.0032	0.0055

CHO-K1 cultures were grown up to 65% confluence (day 3) and then incubated with fresh medium containing concentrations of HCH and lindane equivalent to its $NRI_{6.25}$, $NRI_{12.5}$ and NRI_{25}. Each value is the average ± SEM of three independent experiments. *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$ with respect to control $23.48 \pm 0.67\,nmol\,mg^{-1}$ protein (total glutathione) and with respect to 0.32 ± 0.05 (GSSG), respectively, obtained in control cultures.

Table 3. Glutathione peroxidase, reductase and *S*-transferase activities in CHO-Kl cells exposed to different sublethal concentrations of HCH and lindane.

	$NRI_{6.25}$	$NRI_{12.5}$	NRI_{25}
Glutathione peroxidase activity			
HCH	235.96 ± 6.04***	231.10 ± 13.71**	177.56 ± 7.04**
Lindane	686.13 ± 27.41***	324.40 ± 0.12***	321.07 ± 33.91**
Glutathione reductase activity			
HCH	48.07 ± 1.81‡‡	36.03 ± 1.46	33.30 ± 0.26
Lindane	158.86 ± 5.24‡‡‡	59.76 ± 1.41‡‡‡	42.07 ± 5.06
Glutathione *S*-transferase activity			
HCH	38.60 ± 1.67	$30.23 \pm 0.88^{+++}$	$20.56 \pm 1.58^{+++}$
Lindane	39.73 ± 2.63	$22.33 \pm 2.41^{++}$	$51.21 \pm 0.19^{+++}$

Cells were grown until mid-log phase and then equivalent concentrations to their $NRI_{6.25}$, $NRI_{12.5}$ and NRI_{25} were added for 24 h to the cultures. Each value is the average ± SEM of three independent experiments. *** $p \leq 0.001$, ** $p \leq 0.01$ with respect to $129.19 \pm 9.68\,nmol\,min^{-1}\,mg^{-1}$ protein obtained in control cultures. ‡‡‡ $p \leq 0.001$, ‡‡ $p \leq 0.01$ with respect to $34.19 \pm 1.33\,nmol\,min^{-1}\,mg^{-1}$ protein obtained in control cultures. $^{+++}p \leq 0.001$, $^{++}p \leq 0.01$ with respect to $42.05 \pm 1.81\,nmol\,min^{-1}\,mg^{-1}$ protein obtained in control cultures.

GSSG levels showed significant ($p < 0.01$) depletion at HCH and lindane concentrations equivalent to their $NRI_{6.25}$.

Large increases in glutathione peroxidase activities are associated with exposure to HCH and lindane (table 3). Total glutathione peroxidase activity at HCH and lindane concentrations equivalent to their $NRI_{6.25}$ was 1.8- and >5.3 times

Table 4. Membrane permeability assessment using LDH released to culture medium after 4-h HCH and lindane exposed CHO-K1 cells.

	LDH released (%)					
Concentration ($\mu g\,ml^{-1}$)	6.25	12.5	25	50	100	200
HCH	n.d.	n.d.	0.41 ± 0.60	5.06 ± 1.26	31.22 ± 1.07	44.72 ± 0.47
Lindane	n.d.	n.d.	n.d.	0.83 ± 1.03	6.12 ± 0.46	18.15 ± 1.33

CHO-K1 cultures were grown up to 65% confluence (day 3) and then incubated for 24 h with fresh medium containing different concentrations of HCH and lindane insecticides. Incubation media were removed to fresh plates and analysed for LDH content using the CytoTox96®(Promega) assay. Each value represents the average ± SEM of three independent experiments. n.d., Not detected.

higher, respectively, than recorded for the control culture. At higher levels of exposure to HCH, glutathione peroxidase activity remained high. Most significantly, glutathione peroxidase induction persisted in lindane-exposed cells at insecticide concentrations equivalent to the NRI_{25}.

Glutathione reductase remained almost unchanged at pesticides concentrations equivalent to their NRI_{25}, although it did respond to exposure to lindane at the $NRI_{12.5}$ and $NRI_{6.25}$, and to HCH at $NRI_{6.25}$ (table 3). On the contrary, the activity of glutathione *S*-transferase, one of the enzymes involved in Phase II detoxification in mammals, was only induced at the highest concentration of lindane, remaining unchanged or declining at the lowest concentrations of lindane and at all concentrations of HCH (table 3).

Since lipid peroxidation is one of the causes of cell membrane disruption and necrosis in organisms (Chitra *et al.* 2001), membrane permeability and lipid peroxidation were studied in CHO-K1 cells exposed to lindane and HCH. The two compounds induced similar modifications in membrane permeability, as measured by LDH release from the cells, at concentrations equivalent to their $NRI_{6.25}$, $NRI_{12.5}$ and NRI_{25}, releasing significant quantities of LDH into the medium at HCH concentrations $> 25\,\mu g\,ml^{-1}$ and lindane concentrations $> 50\,\mu g\,ml^{-1}$. In any event, LDH release was 2.5–5.0 times higher in cells exposed to HCH than in cells exposed to lindane (table 4).

The reaction of peroxides with thiobarbituric acid is a classical method to measure lipid peroxidation in biological samples (Hiroski *et al.* 1979). Figure 1 shows that both HCH and lindane prompted a significant increase ($p < 0.001$) in peroxides at concentrations equivalent to NRI_{25}. Therefore, owing to the greater toxicity of HCH, lipid peroxidation occurs at lower concentrations than with lindane. In any event, lipid peroxidation followed a pattern similar to the behaviour observed for LDH release (figure 1A, B).

Discussion

Several reports have documented the role of lindane and HCH in producing oxidative stress in experimentally exposed animals (Verma *et al.* 1992, Junqueira *et al.* 1997, Sahoo *et al.* 2000, Sujatha *et al.* 2001). Oxidative stress is thought to be the primary cause of lipid peroxidation (Videla *et al.* 1990, Hassoun *et al.* 1993, Samanta and Chainy 1995), DNA damage (Hassoun and Stohs 1996), morphological alterations in several tissues (Videla *et al.* 1990, Junqueira *et al.* 1997) and, finally, carcinogenic processes (Iscan *et al.* 2002). On the basis of lindane being an

animal liver carcinogen, the US Environmental Protection Agency (1994) has classified this compound as Group B2 (possible human carcinogen of low to medium carcinogenic hazard).

Unlike other pesticides such as the herbicide paraquat, where the source of ROS is well established (Smith *et al*. 1979), in the case of HCH isomers the origin of these active species is not clear. Junqueira *et al*. (1988) reported that the increase in superoxide anion content in rat cells exposed to lindane is time- and dose-dependent. These latter authors suggested that both cytochrome P450 induction and the increase in microsomal generation of superoxide anion occur in parallel with the increase of cellular lipid peroxidation (Junqueira *et al*. 1997).

Several parameters, including sulphur redox-cycle enzymes and total and oxidized glutathione levels, were analysed in the present study to assess the toxicity of NRI_{50} fractions of lindane and HCH isomers in CHO-K1 cells.

Untreated control cells showed high glutathione peroxidase activity during the CHO-Kl mid-log phase, the period of maximum growth rate, with activity declining in the late and stationary phases (Bayoumi *et al*. 2000). Since oxygen consumption in aerobic cells peaks coinciding with maximum ROS production, the role of glutathione peroxidase, together with superoxide dismutase and catalase, are likely related to membrane disruption (Meister and Anderson 1983). Furthermore, glutathione reductase activity was found to peak at 48 h in the subculture, thereby maintaining a high reduced to oxidized glutathione ratio during CHO mid-log phase.

The exposure of mid-log CHO-Kl cultures to NRI_{50} fractions of lindane and HCH for 24 h produced substantial changes in the antioxidant metabolism. Our results show that exposure to these compounds prompted a large increase in total glutathione peroxidase activity. The highest glutathione peroxidase activity (more than fivefold) was found for lindane, the less toxic of the two compounds, with a milder effect on membrane permeabilization. Similar findings have been reported previously for cyclodiene insecticides (Bayoumi *et al*. 2001) and may explain the culture resistance to this compound. The induction of glutathione peroxidase is associated with overexpression of SOD, the enzyme controlling superoxide anion disruption and cell homeostasis. Lee and Ho (1994) reported that CHO cells exhibiting glutathione peroxidase activities 10-fold higher than human HFW fibroblasts are 10 times more resistant to xenobiotics. Warner *et al*. (1993) provided evidence of enhanced resistance to several xenobiotics in a human SOD-transfected CHO cell line. These findings agree with the experiments reported by Cutler (1985) for mammals, which correlated the enzymatic antioxidant defence system with the life span of several organisms.

Owing to high glutathione peroxidase activity and the significant induction of glutathione reductase by the insecticides at the lowest NRI_{50} fraction, it is not unreasonable to find glutathione levels within the normal range. Thus, at the lowest NRI_{50} fraction, total glutathione levels remained unchanged, whereas GSSG concentrations fell significantly. These results may be explained as the antioxidant mechanism response to weak damage induced by the insecticides. However, glutathione peroxidase and glutathione reductase activity declined at the highest concentrations in the series, concurrent with a significant increase in lipid peroxidation which in turn affected plasma membrane integrity and caused LDH leakage (figure 1). Similar findings have been reported for CHO-K1 cells exposed to cyclodiene (Bayoumi *et al*. 2001). In a study on V79 cells, Verma *et al*. (1992)

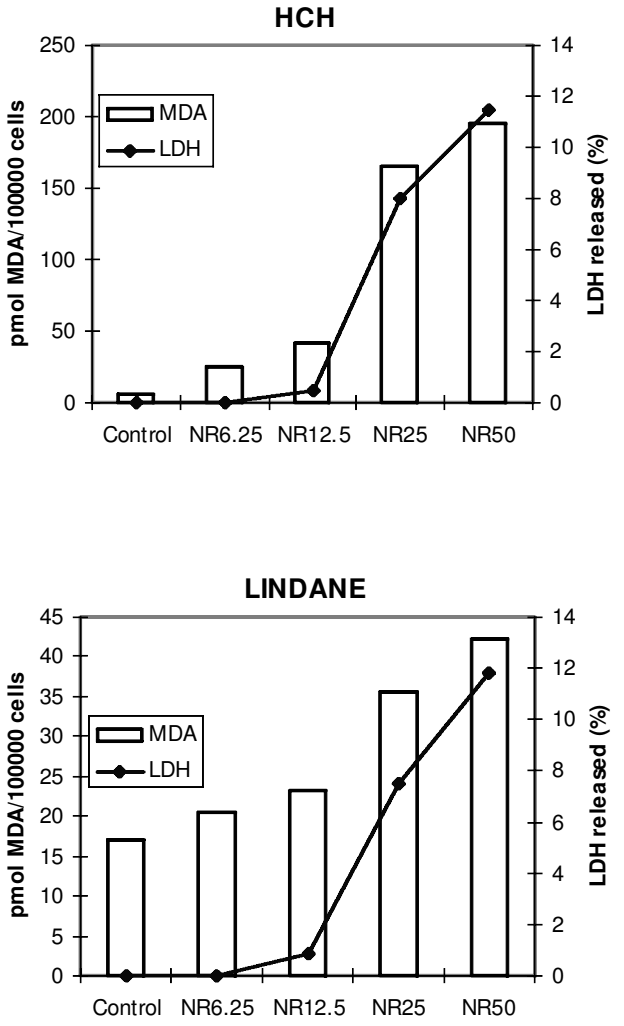

Figure 1. Comparison of plasma membrane integrity using the LDH-releasing assay with lipid peroxidation induced by HCH (A) and lindane (B) after a 4-h exposure. CHO-K1 cultures were grown up to 65% confluence (day 3) and then incubated with fresh medium containing different concentrations of HCH and lindane. Incubation media were removed to fresh plates analysed for LDH content using the CytoTox96 ® (Promega) assay or the lipid peroxidation using the thiobarbituric method. Each value is the average of three independent experiments. The ordinate scales are different in the two panels.

suggested that HCH isomers disrupted the plasma membrane, affecting cell viability. Bagchi *et al.* (1995) reported that exposing neuractive PC12 cells to various pesticides produced a significant increase in lipid peroxidation and membrane leakage at concentrations as low as 100 nM. These results were similar to findings for rat liver and brain tissue treated with these pesticides, thereby demonstrating that consistency between the pathological events observed *in vitro* and *in vivo*.

The results of the present study suggest that lindane and the HCH isomer mixture induced substantial increases in both total glutathione peroxidase and glutathione reductase activities in CHO-Kl cultures after exposure for 24 h at the

lowest doses ($NRI_{6.25}$) as a defence mechanism against oxidative stress. The release of LDH into the culture medium is indicative of dose–response membrane leakage, which may be the result of lipid peroxidation owing to the oxidative stress induced by these compounds at higher doses (NRI_{25} and higher).

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References

Agrawal, D., Sultana, P. and Gupta, G. S., 1991, Oxidative damage and changes in the glutathione redox system in erythrocytes from rats treated with hexachlorocyclohexane. *Food Chemistry Toxicology*, **29**, 459–462.

Akerboom, T. P. M. and Sies, H., 1981, Assay of glutathione, glutathione disulfide and glutathione mixed disulfides in biological samples. *Methods in Enzymology*, **77**, 373–382.

Anderson, M. E., 1985, Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology*, **113**, 548–555.

Arisi, A. C., Simizu, K., Kogake, M., Bainy, A. C., Silva, M. A., Barros, S. B., Boveris, A., Videla, L. A. and Junqueira, V. B., 1994, Brain and liver lipid peroxidation levels following acute and short-term lindane administration in the rat. *Toxicology Letters*, **74**, 61–68.

ATSDR Agency for Toxic Substances and Disease Registry, 1992, *Toxicological Profile for α, β, δ and γ-hexachlorocyclohexane*. Draft (Atlanta: US Public Health Service, US Department of Health and Human Services).

Bagchi, D., Bagchi, M., Hassoun, E. A. and Stohs, S. J., 1995, *In vitro* and *in vivo* generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. *Toxicology*, **104**, 129–140.

Bagley, A. C., Krall, J. and Lynch, R. E., 1986, Superoxide mediates the toxicity of paraquat for Chinese hamster ovary cells. *Proceedings of the National Academy of Sciences, USA*, **83**, 3189–3193.

Bayoumi, A. E., García-Fernández, A. J., Ordóñez, C., Pérez-Pertejo, Y., Cubría, J. C., Reguera, R. M., Balaña-Fouce, R. and Ordóñez, D., 2001, Cyclodiene organochlorine insecticide-induced alterations in the sulfur-redox cycle in CHO-K1 cells. *Comparative Biochemistry and Physiology*, **130[C]**, 315–323.

Bayoumi, A. E., Pérez-Pertejo, Y, Ordóñez, C., Reguera, R. M., Cubría, J. C., Balaña-Fouce, R. and Ordóñez, D., 2000, Alterations on polyamine content and glutathione metabolism induced by different concentrations of paraquat in CHO-K1 cells. *Toxicology in Vitro*, **14**, 211–217.

Borenfreund, E. and Puemer, J. A., 1985, Toxicity determined *in vitro* by morphological alteration and neutral red absorption. *Toxicology Letters*, **24**, 119–124.

Bradford, M., 1976, A rapid sensitive method for quantification of microgram quantities of protein utiIizing the principle of protein–dye binding. *Analytical Biochemistry*, **72**, 248–254.

Chitra, K. C., Sujatha, R., Latchoumycandane, C. and Mathur, P. P., 2001, Effect of lindane on antioxidant enzymes in epididymis and epididymal sperm of adult rats. *Asian Journal of Andrology*, **3**, 205–208.

Cutler, R. G., 1985, Antioxidants and longevity of mammalian species. *Basic Life*, **35**, 15–73.

Habig, W. H., Pabst, M. J. and Jakoby, W. B., 1974, Glutathione *S*-transferase. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry* **249**, 7130–7139.

Hassoun, E. and Stohs, S. J., 1996, TCDD, endrin and lindane induced oxidative stress in fetal and placental tissues of C57BL/6J and DBA/2J mice. *Comparative Biochemistry and Physiology*, **115[C]**, 11–18.

Hassoun, E., Bagchi, M., Bagchi, D. and Stohs, S. J., 1993, Comparative studies on lipid peroxidation and DNA-single strand breaks induced by lindane, DDT, chlordane and endrin in rats. *Comparative Biochemistry and Physiology*, **104[C]**, 427–432.

Hiroski, O., Nobuko, O. and Kunio, Y., 1979, Assay for lipid peroxids in animal tissues by TBA reaction. *Analytical Biochemistry*, **95**, 351–358.

Iscan, M., Coban, T., Cok, I., Bulbul, D., Eke, B. C. and Burgaz, S., 2002, The organochlorine pesticide residues and antioxidant enzyme activities in human breast tumors: is there any association? *Breast Cancer Research and Treatment*, **72**, 173–182.

JUNQUEIRA, V. B., BAINY, A. C., ARISI, A. C., AZZALIS, L. A., SIMIZU, K., PIMENTEL, R., BARROS, S. B. and VIDELA, L. A., 1994, Acute lindane intoxication: a study on lindane tissue concentration and oxidative stress-related parameters in liver and erythrocytes. *Journal of Biochemical Toxicology*, **9**, 9–15.

JUNQUEIRA, V. B., KOCH, O. R., ARISI, A. C., FUZARO, A. P., AZZALIS, L. A., BARROS, S. B., CRAVERO, A., FARRE, S. and VIDELA, L. A., 1997, Regression of morphological alterations and oxidative stress related parameters after acute lindane-induced hepatotoxicity in rats. *Toxicology*, **117**, 199–205.

JUNQUEIRA, V. B., SIMIZU, K., VAN HALSEMA, L., KOCH, O., BARROS, S. B. and VIDELA, L. A., 1988, Lindane-induced oxidative stress. I. Time course of changes in hepatic microsomal parameters antioxidant enzymes, lipid-peroxidative morphological characteristics. *Xenobiotica*, **18**, 1297–1304.

KLEIN, R. G., SCHMEZER, P., AMELUNG, F., SCHROEDER, H. G., WOESTE, W. and WOLF, J., 2001, Carcinogenicity assays of wood dust and wood additives in rats exposed by long-term inhalation. *International Archives of Occupational and Environmental Health*, **74**, 109–118.

KONER, B. C., BANERJEE, B. D. and RAY, A., 1998, Organochlorine pesticide-induced oxidative stress and immune suppression in rats. *Indian Journal of Experimental Biology*, **36**, 395–398.

KRALL, J., SPERANZA, M. J. and LYNCH, R. E., 1991, Paraquat resistant HeLa cells increased content of glutathione peroxidase. *Archives of Biochemistry and Biophysics*, **286**, 311–315.

LEE, T. C. and HO, I. C., 1994, Differential cytotoxic effects of arsenic on human and animal cells. *Environmental and Health Perspectives*, **102**, 101–105.

LOFFLER, G. and VAN BAVEL, B., 2000, Potential pathways and exposure to explain the human body burden of organochlorine compounds: a multivariate statistical analysis of human monitoring in Wurzburg, Germany. *Chemosphere*, **40**, 1075–1082.

MEISTER, A. and ANDERSON, M. E., 1983, Glutathione. *Annual Reviews of Biochemistry*, **52**, 711–760.

NORTH, S. P. and CHEW, G., 2000, Acute lindane poisoning in three children. *Journal of Emergencies Medical*, **18**, 51–53.

PALMEIRA, C. M., MORENO, A. J. and MADEIRA, V. M., 1994, Metabolic alterations in hepatocytes promoted by the herbicides paraquat, dinoseb and 2,4-D. *Archives of Toxicology*, **68**, 24–31.

SAHOO, A., SAMANTA, L. and CHAINY, G. B., 2000, Mediation of oxidative stress in HCH-induced neurotoxicity in rat. *Archives of Environmental Contamination and Toxicology*, **39**, 7–12.

SAMANTA, L. and CHAINY, G. B. N., 1995, Hexachlorocyclohexane-induced changes in lipid peroxidation, superoxide dismutase and catalase activities and glutathione content in chick liver. *Indian Journal of Experimental Biology*, **33**, 131–333.

SAMANTA, L. and CHAINY, G. B. N., 1997, Comparison of hexachlorocyclohexane-induced oxidative stress in the testis of immature and adult rats. *Comparative Biochemistry Physiology*, **118[C]**, 319–327

SAMANTA, L., SAHOO, A. and CHAINY, G. B. N., 1999, Age-related changes in rat testicular oxidative stress parameters by hexachlorocyclohexane. *Archives of Toxicology*, **73**, 96–107.

SMITH, L. L., ROSE, M. S. and WYATT, I., 1979, *The Pathology and Biochemistry of Paraquat: Oxygen Free Radicals and Tissue Damage* (London: CIBA Foundation), pp. 321–341.

SUJATHA, R., CHITRA, K. C., LATCHOUMYCANDANE, C. and MATHUR, P. P., 2001, Effect of lindane on testicular antioxidant system and steridogenic enzymes in adult rats. *Asian Journal of Andrology*, **3**, 135–138.

USEPA US ENVIRONMENTAL PROTECTION AGENCY, 1994, Technical Background Document to Support Rulemaking Pursuant to the Clean Air Act-Section 112(g). Ranking of Pollutants with Respect to Hazard to Human Health. EPA450/3-92-010 (Research Triangle Park: Emissions Standard Division, Office of Air Quality Planning and Standards).

VERMA, S. P., RASTOGI, A. and LIN, P. S., 1992, Hexachlorocyclohexane pesticides reduce survival and alter plasma membrane structure of Chinese hamster V79 cells. *Archives of Biochemistry and Biophysics*, **298**, 587–593.

VIDELA, L. A., BARROS, S. B. and JUNQUEIRA, V. B., 1990, Lindane-induced liver oxidative stress. *Free Radicals in Biology and Medicine*, **9**, 169–179.

WARNER, B., PAPES, R., HEILE, M., SPITZ, D. and WISPE, J., 1993, Expression of human Mn SOD in Chinese hamster ovary cells confers protection from oxidant injury. *American Journal of Physiology*, **264**, L598–L605

WENDEL, A., 1981, Glutathione peroxidase. *Methods in Enzymology*, **77**, 325–333.

ZAMAN, K. and PARDINI, R. S. 1996, An overview of the relationship between oxidative stress and mercury and arsenic. *Toxic Substance Mechanisms*, **15**, 151–181.