

Original article

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Mechanism of acute endosulfan intoxication-induced neurotoxicity in Sprague-Dawley rats

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The purpose of this study was to investigate the molecular mechanism underlying oxidative and inflammatory neuronal cell death induced by endosulfan, a pesticide belonging to the chemical family of organochlorines. The cortical and hippocampal tissues derived from Sprague-Dawley (SD) rats treated with endosulfan exhibited increased intracellular accumulation of reactive oxygen species and oxidative damages to cellular macromolecules such as depletion of glutathione, lipid peroxidation, and protein carbonylation. Conversely, the expression of antioxidant enzymes including γ -glutamylcysteine ligase (GCL), superoxide dismutase (SOD), and heme oxygenase-1 (HO-1) was markedly reduced in the brain tissues exposed to endosulfan. Moreover, during endosulfan-induced neuronal cell death, mRNA expression of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) was elevated, which seemed to be mediated by the activation of nuclear factor-kappa B (NF- κ B) by phosphorylation of p65 subunit. These results suggest a new molecular mechanism underlying the endosulfan-induced acute neurotoxicity via induction of oxidative stress and pro-inflammatory responses.

KEY WORDS: *endosulfan; inflammation; neurotoxicity; oxidative stress*

Endosulfan (6, 7, 8, 9, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide) is an organochlorine pesticide developed in Germany in 1956. Organochlorine pesticides are organic compounds containing at least one covalent bond with chloride atom. They are classified according to their chemical structures and toxicity into four groups: cyclodienes (hexachlorocyclopentadienes such as aldrin, dieldrin, endrin, and heptachlor), hexachlorocyclohexanes (HCHs such as lindane), dichlorodiphenyltrichloroethanes (DDTs), and hexachlorinatedbenzenes (HCBs) (1). Endosulfan is a cyclodiene derivative and has one double bond with α and β isomers at a ratio of 64-67 % and 29-32 %, respectively. The α isomer usually exerts higher toxicity than the β isomer. The use of cyclodienes has been prohibited due to their continuous high toxicity to mammals. However, endosulfan, a type of ester compound that is quickly and easily hydrolysed in the body, is still widely used as an agricultural pesticide and mite insecticide, particularly in some developing countries (2).

As it shows a wide range of acute, subchronic, and chronic toxicities in insects and mammals including humans, it is strictly controlled by the government in many countries. In October 2010, the POP Surveillance

Committee reported the risk management evaluation on endosulfan including neurotoxicity and recommended its use be restricted throughout the world. Endosulfan is an extremely toxic organophosphate insecticide and acute poisoning results in a 30 % or higher death rate (3). When it is taken orally, various clinical manifestations appear: rhabdomyolysis, metabolic and respiratory acidosis, liver toxicity, kidney toxicity, aspiration pneumonia, thrombopenia, hypernatremia, hyperpotassemia, CNS stimulation, status epilepticus, cardiovascular collapse, respiratory failure, and death (2-4). Due to high lipid solubility of endosulfan, it is quickly absorbed and redistributed in the body, particularly in the brain. It induces convulsions in a short time, which then last for a long time. It also causes neural disorders, reduces the convulsion threshold, and stimulates CNS, which results in convulsions, status epilepticus, and respiratory failure lasting for several days (3).

The acute neurotoxicity of endosulfan, as is the case with other cyclodiene pesticides, is caused mainly by its neural over-excitability. Endosulfan is known to bind at a picrotoxin binding site, at the chloride ion channel of γ -aminobutyric acid (GABA) receptor, to function in a similar manner to picrotoxin and act as a non-selective antagonist of GABA (5). Accordingly, the influx of chloride ions into the nerve cells is blocked and uncontrollable excitation develops. The disturbance of the GABA neurotransmitter system is associated with ischemic and

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convulsion-related brain damages and free radical-mediated responses have been reported to play an important role in this process (6). Cyclodiene insecticides strongly inhibit sodium-potassium ATPase and calcium-magnesium ATPase, which are essential for calcium transport through the cell membrane. As a result, intracellular calcium is accumulated and calcium-induced neurotransmitters are liberated to transmit excitations in the CNS. In addition, other organochlorine pesticides have been reported to cause neurotoxicity by oxidative stress (7-11), mitochondrial dysfunction, protein aggregation, and apoptosis (7).

Oxidative stress and damages develop when balance between the generation of reactive oxygen species (ROS), which are spontaneously produced during the *in vivo* metabolic process, and the antioxidant defence system to defend against them collapses to a pro-oxidant state. The imbalance between ROS formation and antioxidant capacity occurs in various physiological and pathological conditions such as inflammation, aging, cancer, drug-induced toxicity, and drug addiction (12). Although the neurotoxic mechanism of endosulfan is not yet fully understood, except for its GABA antagonistic and ATPase-inhibiting functions, the study outcomes of organochlorine pesticides from *in vitro* cell cultures (13, 14) and *in vivo* animal models (15) imply that the oxidative damages may play an important role in the acute neurotoxicity of endosulfan. Therefore, in this study the neurotoxic mechanism of endosulfan was investigated focusing on the oxidative stress by analysing ROS levels, antioxidant defence capacity, and oxidative damages to critical macromolecules such as proteins and lipids.

MATERIALS AND METHODS

Materials

Endosulfan ($C_9H_6Cl_6O_3S$, $\alpha+\beta=2+1$) was purchased from Sigma, Inc. (St. Louis, MO, USA) upon the approval of the Daegu Regional Environmental Office (Daegu, Korea).

Experimental animals

All animal experiments were conducted in accordance with the NIH guideline and Care and Use of Laboratory of Animals of the Daegu Haany University. Eight-week old male Sprague-Dawley rats (SD rats) with a body weight of 250-270 g were obtained from Hyochang Science, Inc. (Daegu, Korea) and were adapted for two days before their use. They were maintained at 22-24°C temperature, 40-60% humidity, and a 12-hour day and night lighting cycle. SD rats were divided into five groups, orally administered increasing doses of endosulfan (0, 5, 10, 25, and 50 mg kg⁻¹ dissolved in corn oil, n=6), and general behaviour changes as well as survival were monitored up to 24 hours. Particularly, a neurotoxic sign of status epilepticus with

prolonged or repeated seizures was defined when the active part of a tonic-clonic seizure lasted for 5 min or longer; a second seizure occurred without recovering from the first one; or repeated seizures were those that lasted for at least 30 min. As endosulfan-treated SD rats (less than 20%) started to experience status epilepticus at a dose of 25 mg kg⁻¹ and did not survive at higher doses (≥ 50 mg kg⁻¹) until 24 h, 25 mg kg⁻¹ was selected for additional mechanistic studies (0 and 25 mg kg⁻¹, n=6). For the time-dependent molecular analysis (0, 0.5, 1, 2.5, and 5 h), another set of SD rats was divided into five groups (n=3) and endosulfan (25 mg kg⁻¹) was given orally.

Western blot analysis

The experimental animals were anaesthetised by intraperitoneal injection of Zoletil® (30 mg kg⁻¹, Virbac, France) with Roupun® (10 mg kg⁻¹, Bayer, Germany) and sacrificed by decapitation. The tissues from cerebral cortex, hippocampus, midbrain, and cerebellum were excised and homogenised using the radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, MA, USA) containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After centrifugation (15,000 g, 20 min), supernatants were used for the experiments and protein concentrations were quantified by bicinchoninic acid (BCA) reagents (Thermo Scientific, IL, USA). Electrophoresis (180 V, 2 h) was performed using 50 µg of protein at 12% SDS-PAGE and proteins were transferred (300 mA, 3 h) to the polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was incubated with 5% skim milk in phosphate buffered saline plus 0.1% Tween-20 (PBST) for an hour to exclude non-specific bands and then further treated with primary antibody diluted with 1:500 and 1:1000 ratio (2% skim milk in PBS) at 4°C overnight. The membrane was washed three times with the PBST solution and reacted with a secondary antibody in 3% skim milk PBS at room temperature for an hour. After washing three more times with the PBST solution for 10 min each, the enhanced chemiluminescence (ECL) reagent (Amersham Bioscience, NJ, USA) was added to measure the protein expression levels. The band intensity was obtained using an image analyser (Chemiluminescent Immunoblotting Imaging, UVP, USA).

Dichlorodihydrofluorescein-diacetate (DCF-DA) staining

To measure intracellular ROS, the experimental animals were sacrificed and brain tissues were excised. Ice-cold phosphate buffer solution (20 mM, pH 7.4) was added to homogenise the samples using a homogeniser, after which centrifugation was conducted at 12,000 g for 30 min to collect the supernatant. Samples from each brain area (100 µL) were mixed with 10 µL of 50 mM 2',7'-DCF-DA (Invitrogen, CA, USA) and incubated for 10 min at room temperature. The fluorescent intensity was measured using Molecular Device's Gemini XS fluorometer

with an excitation at the 485 nm and emission at the 585 nm wavelengths.

Measurement of endogenous antioxidant glutathione (GSH)

The intracellular GSH amount in the brain tissues was measured using the ApoGSH Glutathione Colorimetric Detection Kit (BioVision, CA, USA). After the experimental animals were anaesthetised by intraperitoneal injection of Zoletil® (30 mg kg⁻¹) with Roumpun® (10 mg kg⁻¹) and sacrificed by decapitation, the brain tissues were excised and homogenised with ice-cold 20 mM phosphate buffer solution, and then centrifuged at 12,000 g for 30 min. Reaction mixtures with 20 µL NADPH generation mixture, 20 µL glutathione reductase, and 120 µL glutathione reaction buffer were prepared and kept at room temperature for 10 min. Samples (20 µL) were then added and incubated at room temperature for additional 10 min. Afterwards, 20 µL of the substrate solution was added and reacted at room temperature for five minutes. The optical intensity was measured at 405 nm using a microplate reader (EMax®, Molecular Device).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

To separate mRNA, TRIzol® (Bioscience Technology, NJ, USA) was added on the excised brain tissues and homogenised using a homogeniser. The homogenate samples were then mixed with chloroform and centrifuged at 12,000 g for 15 min to separate the supernatant that contains mRNA. mRNA was precipitated with isopropyl alcohol, washed with 75 % ethanol, dried, and dissolved in RNase-free deionised distilled water. mRNA was further converted to cDNA using M-MLV reverse transcriptase (Promega, WI, USA). The amount of cDNA was amplified through the PCR reaction to compare the relative expression levels using specific DNA sequence primers (Table 1). The

reaction mixtures were subject to electrophoresis using 1.5 % agarose gel, stained with ethidium bromide (EtBr), and visualised under UV lamp-equipped GelDoc System (TCP-20.M, Vilber Lourmat, France).

Measurement of malonedialdehyde (MDA) formation

MDA levels were measured using the BIOXYTECH LPO-586 Colorimetric Kit (Oxis Research, OR, USA). After the experimental animals were anaesthetised by intraperitoneal injection of Zoletil® (30 mg kg⁻¹) with Roumpun® (10 mg kg⁻¹) and sacrificed by decapitation, the brain tissues were excised and homogenised with ice-cold 20 mM sodium phosphate buffer solution, and then centrifuged at 12,000 g for 30 min. The 200 µL supernatant was added into the polypropylene tube and mixed with 650 µL of the R1 reagent (*N*-methyl-2-phenylindole in acetonitrile) by vortex. R2 reagent (150 µL), methanesulfonic acid, was then added to the reaction mixture and incubated at 45°C for 60 min. After centrifugation at 15,000 g for 10 min, the supernatant was collected and the absorbance at 540 nm was monitored using a microplate reader.

Measurement of protein carbonylation

To measure protein carbonylation, OxyBlot Protein Oxidation Detection Kit (Milipore, MA, USA) was used. After the experimental animals were anaesthetised by intraperitoneal injection of Zoletil® (30 mg kg⁻¹) with Roumpun® (10 mg kg⁻¹) and sacrificed by decapitation to excise the brain tissues, RIPA buffer was added to extract protein samples. After homogenisation and centrifugation (12,000 g, 30 min), 15 µL of the supernatant was collected and incubated with 5 µL of 12 % SDS and 10 µL of 2,4-dinitrophenyl hydrazine (DNPH) at room temperature for 15 min. Thereafter, 7.5 µL of a neutralisation reagent and 20 µL of a buffer solution were then added and reaction mixtures were electrophoresed at room temperature for an hour using 10 % SDS-PAGE. Protein samples were further

Table 1 Lists of specific sequences for each primer

Primer		DNA sequence	Gene bank accession number
GAPDH	sense	5'-GCC AAG GTC ATC CAT GAC AAC-3'	XM_011241212
	antisense	5'-AGT GTA GCC CAG GAT GCC CTT-3'	
MnSOD	sense	5'-TGA CCT GCC TTA CGA CTA TG-3'	NM_013671
	antisense	5'-CGA CCT TGC TCC TTA TTG AA-3'	
CuZnSOD	sense	5'-CCA TCA ATA TGG GGA CAA TAC AC-3'	NM_011434
	antisense	5'-ACA CGA TCT TCA ATG GAC AC-3'	
HO-1	sense	5'-ACT TTC AGA AGG GTC AGG TGT CC-3'	NM_010442
	antisense	5'-TTG AGC AGG AAG GCG GTC TTA G-3'	
TNF-α	sense	5'-AAA TGG GCT TTC CGA ATT CA-3'	NM_013693
	antisense	5'-CAG GGA AGA ATC TGG AAA GGT-3'	
IL-1β	sense	5'-CAA ATC TCG CAG CAG CAC A-3'	NM_008361
	antisense	5'-TCA TGT CCT CAT CCT GGA AGG-3'	

transferred to PVDF membrane, which was then incubated with 1 % BSA in PBST solution for an hour to reduce non-specific bindings. The primary antibody was added and left to react at room temperature for an hour and the membrane was washed two times with PBST for 15 min each. The second antibody was reacted at room temperature for an hour, after which was washed again with PBST two times for 15 min each. The membrane was developed using the ECL reagent and images were taken by an image analyser (Chemiluminescent Immunoblotting Imaging).

Statistical analysis

SPSS Version 16.0 (IBM Co., NY, USA) was used for the statistical processing of the study results. To evaluate the statistical significance of the data between two groups with or without endosulfan administration in each brain region of the cerebral cortex, hippocampus, midbrain, and cerebellum, Student t-test was performed. The data were regarded as statistically significant when *p* value was less than 0.05.

RESULTS

Endosulfan-induced neuronal cell death and formation of ROS

To evaluate neurotoxicity induced by endosulfan, SD rats were orally administered endosulfan at various doses (0, 5, 10, 25, and 50 mg kg⁻¹) and observed at different time points up to 24 hours. Five hours after the administration of 25 mg kg⁻¹ or higher doses, status epilepticus responses were observed and at 50 mg kg⁻¹ rats did not survive. To investigate the type of neuronal cell damage and death,

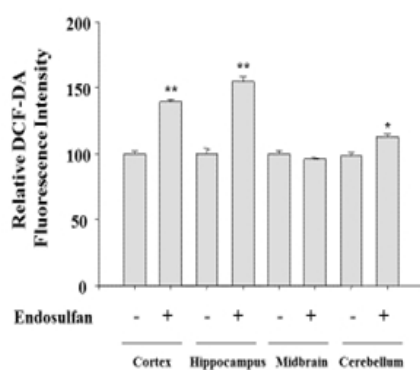


Figure 1 Endosulfan-induced intracellular accumulation of ROS. Endosulfan (25 mg kg⁻¹) was orally administered to SD rats and intracellular ROS levels were determined based on the peroxide-sensitive DCF fluorescence, as described in the Materials and Methods. Data are represented as mean \pm S.D. (*n*=3). Significantly different between groups: **p*<0.05 and ***p*<0.01 vs. vehicle-treated control group. Y axis: Relative DCF-DA Fluorescence Intensity (% of Vehicle-treated Control)

DNA fragmentation, which is a representative index of apoptosis, was measured by *in situ* TUNEL staining. In the experiment group to which 25 mg kg⁻¹ of endosulfan was administered, the number of cells with positive TUNEL staining increased compared with the vehicle-treated control group (data not shown).

To elucidate the molecular mechanisms of neuronal cell death induced by endosulfan, the formation of ROS in brain tissues was examined using the DCF-DA fluorescent dye. After the oral administration of 25 mg kg⁻¹ endosulfan, DCF-DA fluorescence intensity increased to 139.5 \pm 1.85 %, 154.8 \pm 3.79 %, and 113.01 \pm 1.64 % in the cerebral cortex, hippocampus, and cerebellum, respectively when compared with the vehicle-treated control group (Figure 1), which suggests the generation of ROS in the brain by oral administration of endosulfan.

Endosulfan-induced depletion of reduced form of GSH

To further investigate the oxidative damages caused by endosulfan treatment, the endogenous antioxidant GSH levels were examined. When 25 mg kg⁻¹ of endosulfan was orally administered, the amount of GSH in the cerebral cortex and hippocampus decreased to 82.3 \pm 3.11 % and 77.7 \pm 2.45 %, respectively compared with the vehicle-treated control group (Figure 2A). The endosulfan-induced depletion of GSH seemed to be mediated by the inhibiting protein expression of γ -glutamylcysteine ligase (GCL), a critical enzyme in the rate-determining step of GSH biosynthesis. Endosulfan treatment dose-dependently decreased GCL levels, particularly in the cerebral cortex and hippocampus (Figure 2B).

Endosulfan-induced oxidative damages to lipids and proteins

In this study, the MDA and 4-HNE levels were measured as lipid peroxidation indexes. When 25 mg kg⁻¹ of endosulfan was orally administered, MDA formation in the cerebral cortex and the hippocampus increased to 140.72 \pm 7.07% and 131.73 \pm 9.33%, respectively compared with the vehicle-treated control group (Figure 3A). Moreover, the levels of another lipid peroxidation marker, 4-HNE, were up-regulated in the cerebral cortex and hippocampus as assessed by the western blot analysis (Figure 3B). In another experiment, we also confirmed that oral administration of endosulfan (25 mg kg⁻¹) induced protein oxidation as the protein carbonylation in the cerebral cortex, hippocampus, and cerebellum was evident in the endosulfan-treated group (Figure 4).

Effect of endosulfan on the expression of antioxidant enzymes

To further verify the effect of endosulfan on the intracellular antioxidant defence system, the mRNA expression levels of superoxide dismutase (SOD) and heme oxygenase-1 (HO-1), which are representative antioxidant enzymes, were measured. In all the areas of brain tissues,

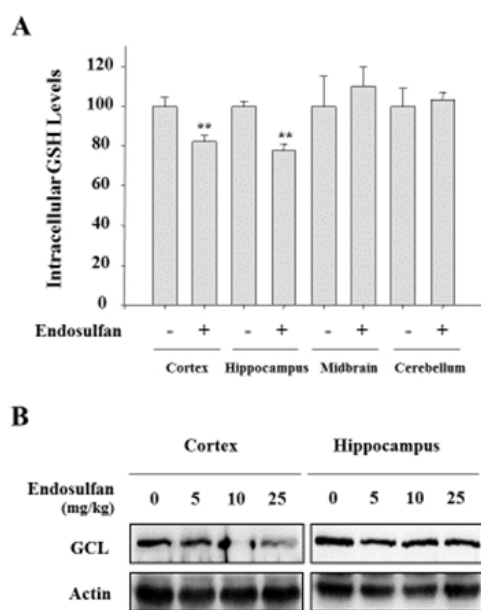


Figure 2 Endosulfan-induced depletion of GSH levels
A. SD rats were exposed to 25 mg kg⁻¹ endosulfan and glutathione (GSH) levels in the brain tissues were determined using a commercially available ApoGSH glutathione colorimetric detection kit following the procedure described in the Materials and Methods. Data are represented as mean ± S.D. (n=3). Significantly different between two groups: **p<0.01, vehicle control vs. endosulfan groups in each brain area. The GSH levels of vehicle-treated control group in the cortex, hippocampus, midbrain, and cerebellum are 6.135±0.281, 7.443±0.193, 6.614±1.004, and 5.544±0.500 μmol g⁻¹, respectively. **B.** The protein expression of γ-glutamylcysteine ligase (GCL) in the brain tissues of SD rats treated with different doses of endosulfan (5, 10, and 25 mg kg⁻¹) was assessed by the Western blot analysis. Y axis: Intracellular GSH Levels (% of Vehicle-treated Control)

the mRNA levels of MnSOD were not much altered by endosulfan treatment; however, the mRNA expression of CuZnSOD significantly decreased after the oral intake of 25 mg kg⁻¹ endosulfan (Figure 5A). In addition, the measurement of the mRNA expression level of HO-1, which also exhibits antioxidant and anti-inflammatory activities, showed that it decreased in the cerebral cortex and the hippocampus after the endosulfan intake (Figure 5B).

Endosulfan-induced pro-inflammatory responses

In another experiment to investigate the inflammatory reactions induced by endosulfan, the mRNA expression of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), which are representative inflammatory cytokines, was measured. The oral administration of endosulfan (25 mg kg⁻¹) increased mRNA levels of TNF-α and IL-1β, particularly in the cerebral cortex and the hippocampus, which was confirmed by RT-PCR (Figure 6A). Among various upstream transcription factors involved in the up-regulation of inflammatory cytokines, the phosphorylation levels of p65, a subunit of NF-κB, time-dependently increased in the cerebral cortex and the hippocampus after the oral

administration of endosulfan (Figure 6B). This seemed to be mediated by the activation of upstream extracellular signal-regulated kinase 1/2 (ERK1/2). The phosphorylation of ERK1/2 showed a similar activation pattern as p65 via phosphorylation (Figure 6C).

DISCUSSION

To investigate the acute neurotoxic molecular mechanism of endosulfan, an array of biochemical indexes and signal transduction were examined such as ROS formation, GSH depletion, lipid peroxidation, protein oxidation, and expression of antioxidant enzymes and pro-inflammatory mediators. According to previous studies, the median lethal dose (LD₅₀) of endosulfan in rats is about 80 mg kg⁻¹ regardless of their gender and age (16) and 30 mg kg⁻¹ is the lowest fatal dose. As a preliminary study to investigate the type of neuronal cell death caused by endosulfan, TUNEL staining was performed. TUNEL

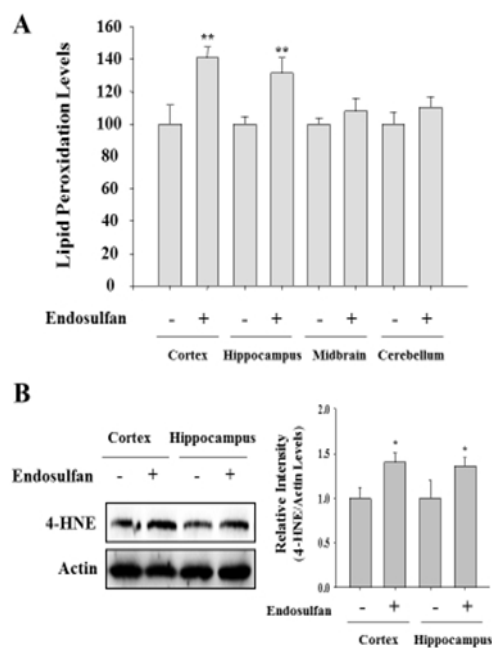


Figure 3 Endosulfan-induced oxidative damage to lipids
A. SD rats were exposed to 25 mg kg⁻¹ endosulfan and lipid peroxidation was determined by measuring the levels of malonaldehyde (MDA) formed using commercially available colorimetric assay kit Bioxytech LPO-586 and following the procedure described in the Materials and Methods. Data are represented as mean ± S.D. (n=3). Significantly different between two groups: **p<0.01, vehicle control vs. endosulfan groups in each brain area. The MDA levels of vehicle-treated control group in the cortex, hippocampus, midbrain, and cerebellum are 1.054±0.126, 1.084±0.050, 1.037±0.040, and 1.138±0.083 nmol mg⁻¹, respectively.
B. Lipid peroxidation was determined by measuring the expression of 4-hydroxynonenal (4-HNE) in the western blot analysis. Actin levels were measured for the confirmation of the equal amount of protein loading. Quantitative data are indicated as mean ± S.D. (n=3). Significantly different between groups: *p<0.05 vs. vehicle-treated control group. Y axis: Lipid Peroxidation Levels (% of Vehicle-treated Control)



Figure 4 Endosulfan-induced oxidative damage to proteins SD rats were exposed to 25 mg kg⁻¹ endosulfan and protein oxidation was measured by comparing protein carbonylation using a commercially available OxyBlot protein oxidation detection kit as described in the Materials and Methods.

staining is a method for detecting apoptotic cell death by combining enzyme-tagged dUTP at the 3'-OH of fragmented DNA into 180-200 bp units by the action of endonuclease activated during the apoptosis process. After the oral intake of endosulfan, brain damages were induced dose-dependently and, particularly, the group that was given the 25 mg kg⁻¹ dose showed a significant increase in the cell numbers with positive TUNEL staining. However, there were fewer TUNEL-positive apoptotic cells at 50 mg kg⁻¹ than at 25 mg kg⁻¹ implying that other types of cell death such as necrosis may be involved in the higher doses of endosulfan-induced neurotoxicity.

To confirm a possible involvement of ROS in the endosulfan-induced neuronal cell death, the amount of intracellular accumulation of ROS was measured using DCF-DA fluorescent reagent. DCF is not fluorescent out of cells, but it is highly lipid soluble to easily enter into the cells. Then the DCF-DA is hydrolysed by an esterase to form DCFH which reacts with peroxide to form DCF, a fluorescent substance. When 25 mg kg⁻¹ of endosulfan was orally administered, the DCF-DA fluorescence intensity in the cerebral cortex and the hippocampus increased, which confirms the intracellular formation of ROS. ROS excessively formed by exogenous noxious stimuli is known to induce reversible or irreversible oxidative damages to the cellular macromolecules such as genes, proteins, and lipids. The most representative index of these oxidative damages is the depletion of GSH, an intracellular endogenous antioxidant material. GSH is known to have strong reducing power and an important role in the antioxidant defence mechanism. It detoxifies toxic materials from outside, removes oxygen free radicals, and functions as an oxidation-reduction buffer (17) to maintain neuronal redox functions. In the brains of patients with diverse neurological disorders, GSH has been reported to be depleted (17). In this study, when 25 mg kg⁻¹ of endosulfan was orally administered, the amount of intracellular GSH significantly decreased. Furthermore, this change seemed to be mediated by the down-regulation of GCL, a rate-limiting enzyme in the biosynthesis of GSH.

Besides depletion of GSH, lipid peroxidation is regarded as another indicative marker of oxidative stress. ROS attack lipids that consist of cell membranes highly reactive to continuous oxidation reactions. Unsaturated lipids in the membrane have multiple double bonds, which are vulnerable to oxidative damages. The lipid hydroperoxyl radicals take hydrogen atoms from the double bond of the nearby unsaturated lipid to make them alkyl radicals, which then combine with oxygen to form new hydrogen peroxide radicals, otherwise known as a chain oxidation reaction (18). At this moment, various by-products such as unsaturated aldehyde are formed. Their high reactivity makes them function as mutagens that inhibit enzyme activities or form a cross-link with other macromolecules.

We also observed that the oral administration of endosulfan induced lipid peroxidation as measured by the increased formation of MDA and 4-HNE. Furthermore, when highly reactive ROS and proteins interact, protein is oxidised to increase the carbonylation levels. When 25 mg kg⁻¹ of endosulfan was orally administered, an increase in protein carbonylation, a protein oxidation index, was also confirmed. In line with this notion, in the case of hexachlorocyclohexane insecticides, excessive ROS formation, increased oxidative damages, and decreased antioxidant defence capacity have been reported to mediate their toxicity in the liver (19, 20), testis (21), and heart (22). In addition, in cultured immune cells (23) and serum samples from scabies patients (24), lindane induced ROS generation and/or subsequent oxidative damages. In other

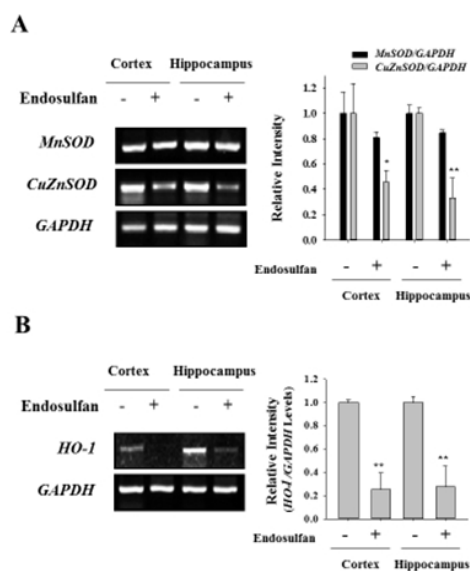


Figure 5 Effect of endosulfan on the mRNA expression of antioxidant enzymes

Total RNA from endosulfan-treated SD rats was extracted with TRIzol reagent ($n=3$ per each group). The mRNA levels of superoxide dismutase (SOD) (A) and heme oxygenase-1 (HO-1) (B) from the brain tissues of SD rats were determined by RT-PCR. GAPDH levels were measured as an equal loading control. Quantitative data are indicated as mean \pm S.D. ($n=3$). Significantly different between groups: * $p < 0.05$ and ** $p < 0.01$ vs. vehicle-treated control group.

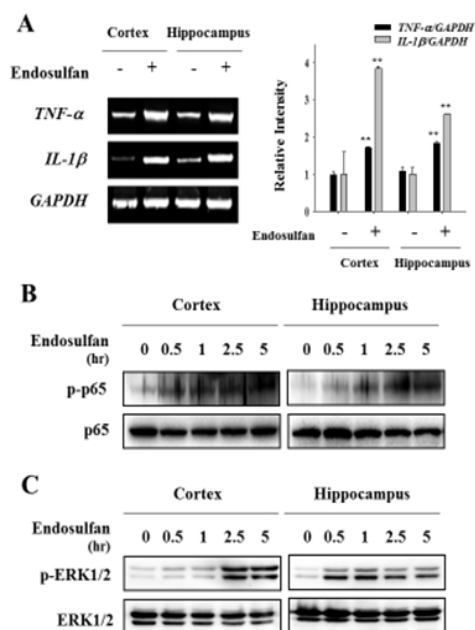


Figure 6 Endosulfan-induced pro-inflammatory responses
A. Effect of endosulfan on the mRNA expression of tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) were measured by RT-PCR using specific primers. Total RNA was isolated from endosulfan-treated SD rats using TRIzol reagent. GAPDH levels were measured as an equal loading control. Quantitative data are indicated as mean \pm S.D. ($n=3$). Significantly different between groups: ** $p<0.01$ vs. vehicle-treated control group.
B. Effect of endosulfan on the activation of NF- κ B was examined by measuring phosphorylation of p65, an active subunit of NF- κ B dimers. Cortical and hippocampal tissue samples were prepared from endosulfan (25 mg kg⁻¹)-treated rats. Western blot analysis was performed using phospho-p65 (p-p65)-specific antibody. The protein levels of p65 were measured for the confirmation of the equal amount of loading. **C.** Effect of endosulfan (25 mg kg⁻¹) on the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) was assessed by western blot analysis using specific antibodies for the phosphorylated (p-ERK1/2) and unphosphorylated (ERK1/2) forms. The unphosphorylated form of ERK1/2 was measured as control.

studies, the chronic use of organochloride insecticide resulted in lipid peroxidation due to deterioration of the antioxidant defence system in the brain (9-11).

To counteract oxidative damages, there are various enzymatic and non-enzymatic antioxidant defence systems in the body to remove the excessively formed ROS. The representative antioxidant enzymes include SOD, catalase, and HO-1 (25, 26). The measurement of the mRNA expression of representative antioxidant enzymes showed that CuZnSOD and HO-1 levels decreased when 25 mg kg⁻¹ of endosulfan was orally administered. SOD is an enzyme that converts superoxide anions to hydrogen peroxide. It has two types such as CuZnSOD and MnSOD with similar functions, however CuZnSOD is mainly found in the cytoplasm and MnSOD is exist in the mitochondria. The catalyst activation varies according to the types of cells and tissues. Hydrogen peroxide is then degraded to water during the GSH redox cycle, in which catalase and glutathione peroxidase (GPx) are involved. HO-1 is an enzyme that is

involved in the rate-determining step of heme degradation process and its reaction products include carbon monoxide, biliverdin, and free ion (Fe²⁺). The HO-1 levels increase in various types of cells as a defence mechanism against stress such as UV, hydrogen peroxide, hypoxia, and GSH depletion (26, 27). Carbon monoxide inhibits pro-inflammatory mediators and inflammatory enzymes by interacting with heme or metal ion moieties of the enzymes to change their structures and activities. Biliverdin is transformed to bilirubin by biliverdin reductase and these two molecules have strong antioxidant functions, thereby mediating cellular protection by HO-1.

In human neuroblastoma SH-SY5Y cells, endosulfan isomers and their major metabolites induced intracellular accumulation of ROS and lipid peroxidation by suppressing activities of antioxidant enzymes (13, 14). In Wistar albino rats, endosulfan significantly increased the levels of tissue MDA and plasma 8-OHdG, an indicator of oxidative DNA damage, which were effectively restored by a treatment with melatonin (28). Moreover, the antioxidant GSH contents in the liver and kidney tissues and the antioxidant capacity of the plasma decreased by endosulfan intoxication (28).

In the neuronal cell death process, these oxidative damages and inflammatory reactions in the glia both play important roles. Various inflammatory cytokines are secreted from the activated glia due to oxidative stress and consequently, neuronal cells respond sensitively to external stimuli. When SD rats were orally administered endosulfan in this study, both the oxidative stress and the mRNA expression of TNF- α and IL-1 β increased. To investigate the underlying molecular mechanisms of the aforementioned inflammatory responses, the activation of NF- κ B was observed. Cytokine expression is known to be controlled by the activation of NF- κ B, which is a representative redox-sensitive transcription factor. The NF- κ B activation is affected by the intracellular redox status, external oxidants, and internal antioxidant systems. When NF- κ B is activated, diverse biological functions such as the immune and inflammatory reactions, stress, cell proliferation, cell death, and developmental process are mediated (29, 30). The non-activated NF- κ B stays in the cytoplasm and attaches to the inhibitory protein I κ B α . Once I κ B α is stimulated, it is phosphorylated and degraded due to κ B kinase (IKK). NF- κ B then moves into the nucleus, attaches to the κ B site, and controls the expression of the inflammation-mediating cytokines, cytokine receptors, cell adhesion molecules, and growth factors (29, 30).

The activation mechanisms of NF- κ B are not yet fully understood, but the IKK activation is known to be mediated by the NF- κ B-inducing kinase (NIK), mitogen-activated protein kinase/ERK kinase kinase (MEKK), 90-kDa ribosomal S6 kinase (p90 RSK), atypical protein kinase C (PKC), and transforming growth factor beta-activated kinase 1 (TAK1) (31). NF- κ B consists of the heterodimer of the Rel family proteins [Rel A (p65), Rel B, p50, p52,

c-Rel, v-Rel, and Rel B]. When the phosphorylation levels of I κ B α or p65 increase, the movements of NF- κ B into the nucleus are promoted (31). Upstream kinases such as ERK1/2 and p38 MAPK are known to be involved in the phosphorylation of I κ B α and p65. In this study, endosulfan activated NF- κ B through the increase in the phosphorylation level of p65, which particularly seemed to be mediated by the activation of ERK1/2. In RAW 264.7 macrophage cells, endosulfan stimulated the production of nitric oxide (NO) and expression of proinflammatory cytokines such as TNF- α , interleukin-1 β (IL-1 β), and IL-6 via transactivation of NF- κ B (32). In addition, organochlorine insecticides such as dieldrin, endosulfan, heptachlor, and lindane activated the Raf, MEK1/2, extracellular-signal-regulated kinases1/2 (ERK1/2), c-Jun, and PKC pathways in HaCaT cells but did not alter the activity of p38 MAPK (1). Particularly, *N*-acetylcysteine, a representative antioxidant agent significantly reduced the phosphorylation of ERK1/2; nevertheless, Raf and MEK1/2 were not much affected.

CONCLUSION

In summary, endosulfan increased intracellular ROS levels in the cerebral cortex and the hippocampus to deplete endogenous antioxidant GSH, reduced the expression levels of antioxidant defence enzymes such as GCL, SOD, and HO-1, and triggered oxidative damages in lipids and proteins. During these processes, the production of pro-inflammatory cytokines was stimulated by NF- κ B activation and neuronal cells were damaged and underwent apoptosis. This suggests oxidative and/or inflammatory stress plays an important role in endosulfan-induced neurotoxicity in SD rats. However, further studies are needed to elucidate the possible interactions between oxidative and inflammatory responses and key molecular mediators in this process.

REFERENCES

- Ledirac N, Antherieu S, d'Uby AD, Caron JC, Rahmani R. Effects of organochlorine insecticides on MAP kinase pathways in human HaCaT keratinocytes: key role of reactive oxygen species. *Toxicol Sci* 2005;86:444-52. doi: 10.1093/toxsci/kfi192
- Han BG, Lee JH, Lee KW. Effect of seizure on prognosis in acute endosulfan intoxication. *J Korean Soc Clin Toxicol* 2009;7:77-82.
- Moon JW, Moon JM, Lee BK, Roo HH. Prognostic predictors of endosulfan intoxication. *J Korean Soc Emerg Med* 2009;20:185-91.
- Kim SK, Cho SH, Cho NS. A case of endosulfan intoxication occurring in groups. *J Korean Soc Emerg Med* 2004;15:409-12.
- Lawrence LJ, Casida JE. Stereospecific action of pyrethroid insecticides on the gamma-aminobutyric acid receptor-ionophore complex. *Science* 1983;221:1399-401. PMID: 6310756
- Krogsgaard-Larsen P. GABA and glutamate receptors as therapeutic targets in neurodegenerative disorders. *Pharmacol Toxicol* 1992;70:95-104. doi: 10.1111/j.1600-0773.1992.tb00436.x
- Kanthasamy AG, Kitazawa M, Kanthasamy A, Anantharam V. Dieldrin-induced neurotoxicity: relevance to Parkinson's disease pathogenesis. *Neurotoxicology* 2005;26:701-19. doi: 10.1016/j.neuro.2004.07.010
- Bagchi M, Ghosh S, Bagchi D, Hassoun E, Stohs SJ. Protective effects of lazaroid U74389F (16-desmethyl tirilazad) on endrin-induced lipid peroxidation and DNA damage in brain and liver and regional distribution of catalase activity in rat brain. *Free Radic Biol Med* 1995;19:867-72. PMID: 8582661
- Numan IT, Hassan MQ, Stohs SJ. Protective effects of antioxidants against endrin-induced lipid peroxidation, glutathione depletion, and lethality in rats. *Arch Environ Contam Toxicol* 1990;19:302-6. doi: 10.1007/BF01054969
- Sahoo A, Samanta L, Chainy GB. Mediation of oxidative stress in HCH-induced neurotoxicity in rat. *Arch. Environ Contam Toxicol* 2000;39:7-12. doi: 10.1007/s002440010073
- Srivastava A, Shivanandappa T. Hexachlorocyclohexane differentially alters the antioxidant status of the brain regions in rat. *Toxicology* 2005;214:123-30. doi: 10.1016/j.tox.2005.06.005
- Sies H. Oxidative stress: from basic research to clinical application. *Am J Med* 1991;91:31S-8S. doi: 10.1016/0002-9343(91)90281-2
- Enhui Z, Na C, MengYun L, Jia L, Dan L, Yongsheng Y, Ying Z, DeFu H. Isomers and their metabolites of endosulfan induced cytotoxicity and oxidative damage in SH-SY5Y cells. *Environ Toxicol* 2014; doi: 10.1002/tox.22066 [Epub ahead of print]
- Jia Z, Misra HP. Reactive oxygen species in in vitro pesticide-induced neuronal cell (SH-SY5Y) cytotoxicity: role of NFkappaB and caspase-3. *Free Radic Biol Med* 2007;42:288-98. doi: 10.1016/j.freeradbiomed.2006.10.047
- Hincal F, Gürbay A, Giray B. Induction of lipid peroxidation and alteration of glutathione redox status by endosulfan. *Biol Trace Elem Res* 1995;47:321-6. PMID: 7779565
- Saiyed H, Dewan A, Bhatnagar V, Shenoy U, Shenoy R, Rajmohan H, Patel K, Kashyap R, Kulkarni P, Rajan B, Lakkad B. Effect of endosulfan on male reproductive development. *Environ Health Perspect* 2003;111:1958-62. doi: 10.1289/ehp.6271
- Aoyama K, Watabe M, Nakaki T. Regulation of neuronal glutathione synthesis. *J Pharmacol Sci* 2008;108:227-38. doi: 10.1254/jphs.08R01CR
- Adibhatla RM, Hatcher JF. Lipid oxidation and peroxidation in CNS health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxid Redox Signal* 2010;12:125-69. doi: 10.1089/ARS.2009.2668
- Anilakumar KR, Nagaraj NS, Santhanam K. Reduction of hexachlorocyclohexane-induced oxidative stress and cytotoxicity in rat liver by *Emblia officinalis* gaertn. *Indian J Exp Biol* 2007;45:450-54. PMID: 17569287
- Videla LA, Tapia G, Varela P, Cornejo P, Guerrero J, Israel Y, Fernández V. Effects of acute gamma-

- hexachlorocyclohexane intoxication in relation to the redox regulation of nuclear factor-kappaB, cytokine gene expression, and liver injury in the rat. *Antioxid Redox Signal* 2004;6:471-80. doi: 10.1089/152308604322899530
21. Saradha B, Vaithinathan S, Mathur PP. Lindane alters the levels of HSP70 and clusterin in adult rat testis. *Toxicology* 2008;243:116-23. doi: 10.1016/j.tox.2007.09.029
 22. Ananya R, Subeena S, Kumar DA, Kumar DT, Kumar MS. Oxidative stress and histopathological changes in the heart following oral lindane (gamma hexachlorohexane) administration in rats. *Med Sci Monit* 2005;11:BR325-9. PMID: 16127354
 23. Olgun S, Misra HP. Pesticides induced oxidative stress in thymocytes. *Mol Cell Biochem* 2006;290:137-44. doi: 10.1007/s11010-006-9178-7
 24. Oberoi S, Ahmed RS, Suke SG, Bhattacharya SN, Chakraborti A, Banerjee BD. Comparative effect of topical application of lindane and permethrin on oxidative stress parameters in adult scabies patients. *Clin Biochem* 2007;40:1321-4. doi: 10.1016/j.clinbiochem.2007.07.011
 25. Venarucci D, Venarucci V, Vallese A, Battilà L, Casado A, De la Torre R, Lopez Fernandez ME. Free radicals: important cause of pathologies refer to ageing. *Panminerva Med* 1999;41:335-9. PMID: 10705716
 26. Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 2003;24:449-55. doi: 10.1016/S1471-4906(03)00181-9
 27. Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* 1989;86:99-103. PMID: 2911585
 28. Omurtag GZ, Tozan A, Sehirli AO, Sener G. Melatonin protects against endosulfan-induced oxidative tissue damage in rats. *J Pineal Res* 2008;44:432-8. doi: 10.1111/j.1600-079X.2007.00546.x
 29. Liang Y, Zhou Y, Shen P. NF-kappaB and its regulation on the immune system. *Cell Mol Immunol* 2004;1:343-50. PMID: 16285893
 30. Xiao W. Advances in NF-kappaB signaling transduction and transcription. *Cell Mol Immunol* 2004;1:425-35. PMID: 16293211
 31. Bowie A, O'Neill LA. Oxidative stress and nuclear factor-kB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* 2000;59:13-23. PMID: 10605930
 32. Han EH, Hwang YP, Kim HG, Jeong HG. Inflammatory effect of endosulfan via NF-kappaB activation in macrophages. *Biochem Biophys Res Commun* 2007;355:860-5. PMID: 17261270

Mehanizam akutne neurotoksičnosti u Sprague-Dawley štakora izazvane trovanjem endosulfanom

Istražen je molekularni mehanizam koji dovodi do smrti neurona potaknute oksidativnim i upalnim procesima uzrokovanim organoklornim pesticidom endosulfanom. U tkivima korteksa i hipokampusa Sprague-Dawley (SD) štakora tretiranih endosulfanom uočena su oksidativna oštećenja staničnih makromolekula, poput smanjene razine glutationa, lipidne peroksidacije i karbonilacije proteina, te povećane unutarstanične akumulacije reaktivnih kisikovih spojeva. Isto tako, u moždanom tkivu nakon izlaganja endosulfanu značajno je smanjena ekspresija enzimskih antioksidansa, uključujući i γ -glutamilstein ligazu (GCL), superoksidnu dismutazu (SOD) i hem oksigenazu-1 (HO-1). Tijekom endosulfanom izazvane smrti neurona povećala se i ekspresija mRNA pro-upalnih citokina poput čimbenika nekroze tumora- α (TNF- α) i interleukina-1 β (IL-1 β), što je čini se bilo posredovano aktivacijom nuklearnoga faktora kapa B (NF- κ B) putem fosforilacije podjedinice p65. Navedeni rezultati upućuju na novi molekularni mehanizam koji stoji iza akutne neurotoksičnosti izazvane endosulfanom putem indukcije oksidativnoga stresa i pro-upalnih odgovora.

KLJUČNE RIJEČI: *endosulfan; upala; neurotoksičnost; oksidativni stres*