

## Reactive oxygen species in *in vitro* pesticide-induced neuronal cell (SH-SY5Y) cytotoxicity: Role of NFκB and caspase-3

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### **Abstract:**

Oxidative stress has been implicated in pesticide-induced neurotoxicity, based on its role in the cascade of biochemical changes that lead to dopaminergic neuronal cell death. We have, therefore, examined the role of oxidative stress caused by the pesticides endosulfan and zineb in human neuroblastoma cells (SH-SY5Y) in culture. Upon treatment with 50-200 μM concentrations of either of these pesticides, SH-SY5Y cells generated both superoxide anion and hydrogen peroxide in a dose- and time-dependent manner. Mixtures of the pesticides significantly enhanced the production of these reactive oxygen species compared to individual pesticide exposures. Pesticide treatment decreased superoxide dismutase, glutathione peroxidase, and catalase activities in SH-SY5Y cells. Additionally, these pesticides induced lipid peroxide (thiobarbituric acid reactive products) formation in these cells. While both pesticides individually (at 100 μM) increased caspase-3 activity, cells exposed to a mixture of the pesticides exhibited significantly low levels of this enzyme, probably due to excessive necrotic cell death. Furthermore, exposure to these pesticides increased nuclear NFκB activity. Taken together, these findings suggest that the cytotoxicity of endosulfan and zineb, both individually and in mixtures may, at least in part, be associated with the generation of reactive oxygen species with concomitant increased expression of NFκB.

## Abbreviations

AD, Alzheimer's disease;  
ANOVA, analysis of variance;  
caspase, cysteinyl-aspartate-specific proteinase;  
CAT, catalase;  
DA, dopamine;  
DCF, 2',7'-dichlorofluorescein;  
DCF-DA, 2',7'-dichlorofluoroscine diacetate;  
DMSO, dimethylsulfoxide;  
EBDC, ethylene bisdithiocarbamate;  
EN, endosulfan;  
GPX, glutathione peroxidase;  
GR, glutathione reductase;  
GSH, reduced glutathione;  
HBSS, Hanks' Balanced Salt Solution;  
HE, hydroethidine;  
MDA, malondialdehyde;  
 $O_2^{\bullet -}$ , superoxide anion;  
PBS, phosphate-buffered saline;  
PD, Parkinson's disease;  
pNA, *p*-nitroaniline;  
ROS, reactive oxygen species;  
SOD, superoxide dismutase;  
TBA, thiobarbituric acid;  
ZB, zineb

**Keywords:** Pesticides | Neuronal cell | Endosulfan | Zineb | Reactive oxygen species | Oxidative stress | Caspase-3 | NF $\kappa$ B | Superoxide dismutase | Catalase | Glutathione peroxidase | Free radicals

## Article:

### Introduction

Parkinson's disease (PD) is typically considered an aging-related neurodegenerative disorder characterized by degeneration of the nigrostriatal system [1] and [2]. Although the etiology of idiopathic PD is not known, most studies strongly suggest that environmental factors, particularly pesticide exposure, may play an important role in the pathogenesis of this disorder [3], [4] and [5]. In a proportional mortality study, increased incidence of PD mortality was observed in rural California counties with high use of agricultural pesticides [6]. Our laboratory has demonstrated that mice exposed to endosulfan (an organochlorine cyclodiene pesticide) and zineb (zinc ethylene bisdithiocarbamate (EBDC) fungicide) as juveniles and re-exposed at 8 months of age showed loss of dopamine (DA) in striatum. Mixtures of these pesticides also caused significantly increased levels of alpha-synuclein, a major component of

Lewy bodies and a hallmark of neurodegenerative diseases such as PD and Alzheimer's disease (AD). In addition, we have demonstrated that mice exposed to these pesticides during critical period of brain development have enhanced susceptibility to the same chemicals later in life [7]. These pesticides are widely used in agriculture and, in some countries, in public health [8]. In humans with endosulfan poisoning, overstimulation of the central nervous system is the major characteristic [9], [10], [11], [12], [13], [14] and [15]. Studies in animals have shown changes in neurotransmitter levels and alterations in neurobehavioral processes after exposure to endosulfan [16] and [17]. EBDCs have been implicated as potential risk factors for the PD phenotype [18],[19] and [20]. Maneb (manganese EBDC) appears to possess potent dopaminergic activity and is well known to enhance the toxic effects of MPTP in mice [21]. Exposures of mice to manebe were also shown to produce selective nigrostriatal DA system neurotoxicity, including loss of striatal DA and degeneration of cell bodies of DA neurons in the substantia nigra pars compacta [22], [23] and [24]. Zineb was reported to cause reduction of high affinity dopamine- and tyrosine hydroxylase positive- neurons in mesencephalic striatal primary co-culture [25]. Recently, our laboratory has demonstrated that exposure to endosulfan and zineb caused both, apoptosis and necrotic cell death in SH-SY5Y neuroblastoma cells [26]. However, the mechanism(s) involved in neuronal cell death caused by these pesticides is not known.

Several hypotheses have linked environmental toxicant (particularly pesticide) exposure, with caspases (cysteine/aspartate-specific proteinases) in the induction of cytotoxicity. Thus, caspase-3 was suggested to play a critical role in both, upstream and downstream pathways in dieldrin-induced apoptosis in dopaminergic PC12 cells [27] and [28]. Caspase-3 activation was also observed with high concentrations of heptachlor, an organochlorine insecticide [29]. Because apoptosis and necrosis could be induced by ROS through the activation of caspase-3-like proteases [30], we investigated the role of caspase-3 and oxidative stress in pesticide-induced cytotoxicity in SH-SY5Y cell *in vitro*.

An alternative hypothesis postulates that oxidative stress, which has been implicated in the pathogenesis of neurodegenerative diseases such as PD and AD, plays an important role in pesticide-induced neurotoxicity [31], [32], [33], [34], [35], [36], [37], [38] and [39]. It has been suggested that many pesticides are capable of inducing oxidative stress by overwhelming or modulating cellular drug metabolizing systems [40],[41] and [42]. Oxidative stress has been thought to be a key aspect of Mn-EBDC-induced neurotoxicity [43] and [44]. Oxidative stress-inducing effects of endosulfan have been reported in rats [45], human cell lines [46] and [47], and fish [48], [49] and [50]. However, the involvement of oxidative stress in neuronal cell exposure to combinations of these types of pesticides is not known. Because NF $\kappa$ B (an eukaryotic transcription factor) is activated consequent to oxidative stress [51], and plays a key role in the regulation of several genes involved in pathogen responses and cellular defense mechanisms during programmed cell death [52], we explored the process(es) of pesticide-induced oxidative stress as a possible mechanism of pesticide induced neuronal cell cytotoxicity using SH-SY5Y cell cultures.

In the present study, we examined endosulfan and zineb individually and in combination for their potential to stimulate oxidative stress in human neuroblastoma cells (SH-SY5Y) *in vitro*. We also monitored the fate of expression of NFκB and Caspase-3 in SH-SY5Y cells upon exposure to these pesticides. We report here that exposure of SH-SY5Y cells to endosulfan and zineb augmented production of superoxide and hydrogen peroxide and decreased the levels of certain antioxidant enzymes. We also show that exposure to these pesticides altered caspase-3 activity in the cells and increased NFκB activity in the cell nucleus. These new findings add to the growing body of knowledge documenting that reactive oxygen species generated during the metabolism of environmental chemicals may be involved in the degeneration of dopaminergic neurons in idiopathic PD.

## **Materials and methods**

### Cell culture and treatment

RPMI-1640 medium with phenol red was used for culturing the cells. The complete medium was prepared by adding 10% fetal bovine serum, 1% MEM non-essential amino acids, 1% penicillin/streptomycin, 1% l-glutamine and 1% HEPES to the RPMI-1640. All these chemicals and media were purchased from Sigma-Aldrich (St. Louis, MO). Human neuroblastoma SH-SY5Y cells were purchased from ATCC (Manassas, VA) at 23 passages and seeded in 75 cm<sup>2</sup> flasks. They were grown for 4–5 days until 90% confluence in a 95% air, 5% CO<sub>2</sub> humidified incubator at 37°C, harvested using 0.25% trypsin (Sigma-Aldrich, St. Louis, MO) and seeded into 96-well microtiter plates ( $3 \times 10^4$  per well) or 75 cm<sup>2</sup> flasks ( $3 \times 10^6$ ). The cells were then allowed to grow at 37°C in 5% CO<sub>2</sub> for 1–2 days prior to treatment.

### Preparation of pesticides

Endosulfan and zineb were obtained from Chem Service (West Chester, PA). 100 mM stock solutions of endosulfan and zineb were prepared using 100% dimethylsulfoxide (DMSO). These stock solutions were serially diluted with incomplete RPMI-1640 medium to prepare 4×-working solutions.

### H<sub>2</sub>O<sub>2</sub> Quantitation by DCF-DA assay

Conversion of nonfluorescent chloromethyl-DCF-DA (2',7'-dichlorofluorescein diacetate (Molecular Probes, Inc., Eugene, Oregon) to fluorescent DCF was used to monitor intracellular H<sub>2</sub>O<sub>2</sub> production. DCF was quantitated on a Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation, Sunnyvale, California) using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. Briefly, SH-SY5Y cells were seeded onto 96-well plates (Corning Costar), allowed to adhere for at least 24 h, and then incubated in Hanks' Balanced Salt Solution (HBSS) containing 5 μM chloromethyl-DCF-DA (diluted from a 5 mM DMSO solution) for 30 min at 37°C in the dark to allow dye loading into the cells. Subsequently, excess dye was removed by aspiration and the cells were treated with phenol-free RPMI

containing 0, 50, 100 or 200  $\mu\text{M}$  of zineb or endosulfan separately in order to obtain a dose response for each pesticide exposure. The reaction was initiated upon addition of pesticides to cells and allowed to proceed by incubation at  $37^\circ\text{C}$  up to 20 h. A cell sample without DCF-DA was included as a negative control. The generation of peroxides was measured and reported as relative fluorescence intensity.

#### $\text{O}_2^{\cdot-}$ Quantitation by Hydroethidium assay

Hydroethidine (HE), a sodium borohydride-reduced derivative of ethidium bromide, was used to detect  $\text{O}_2^{\cdot-}$ , which converts it to ethidium bromide causing an increase in red fluorescence. The increase in fluorescence was measured on a Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation, Sunnyvale, California) using an excitation wavelength of 510 nm and an emission wavelength of 590 nm. SH-SY5Y cells were seeded onto 96-well plates (Corning) and allowed to adhere for at least 24 h. Cells were then stained with 5  $\mu\text{M}$  HE for 30 minutes prior to addition of pesticides to initiate the reaction.  $\text{O}_2^{\cdot-}$  generation was measured as relative fluorescence intensity.

#### Antioxidant and other enzyme assays

##### Preparation of cell extracts for enzyme assays

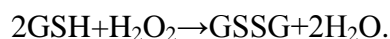
Cells ( $8 \times 10^6$  grown as mentioned above) were incubated for 16 h with various concentrations of the test pesticides, pooled into a tube, pelleted by centrifugation at  $300g$  at  $4^\circ\text{C}$  for 5 min, washed once with phosphate buffered saline (PBS) and resuspended in 120  $\mu\text{l}$  of PBS. This suspension of cells was freeze-thawed twice, centrifuged at  $20,000g$  at  $4^\circ\text{C}$  for 10 min and the supernatant collected for various enzyme assays.

##### Superoxide dismutase (SOD)

Total SOD levels in treated and untreated cell extracts were measured on an automated oxidative stress analyzer (OxyScan<sup>TM</sup>; Oxis Health Products Inc., Portland, OR, USA) using quantitative, colorimetric assay test kits (SOD-525<sup>TM</sup>) obtained from Bioxytech®. A 50  $\mu\text{l}$  sample was used for measuring the SOD activity (units/ml of sample) using the method of Nebot [53]. Specific activity of SOD was reported as units/mg protein.

##### Glutathione peroxidase (GPX)

Total GPX levels in treated and untreated cell extracts were measured on an automated oxidative stress analyzer (OxyScan<sup>TM</sup>; Oxis Health Products Inc., Portland, OR, USA) using quantitative, colorimetric assay test kits (Oxyscan<sup>TM</sup> GPX-340<sup>TM</sup> Kit) obtained from Bioxytech®. GPX catalyzes the reduction of various organic hydroperoxides and  $\text{H}_2\text{O}_2$  using reduced glutathione (GSH) as donor.



By coupling this reaction with GR (glutathione reductase), the specific activity of this enzyme can be calculated by measuring the consumption of NADH in the reaction spectrophotometrically at 340 nm [54]. A 50  $\mu\text{l}$  sample was used to measure the GPX activity (units/ ml of sample). Specific activity of GPX was reported as units/mg protein.

#### Catalase (CAT)

Catalase activity was determined by the kinetic assay adopted from Beers and Sizer [55]. The measurement of CAT activity is based on the quantification of hydrogen peroxide breakdown; thus, we define one unit of CAT as the amount of enzyme required to decompose 1  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  per minute at 25°C. The rate of decrease in absorbance at 240 nm was measured on a Shimadzu UV-visible spectrophotometer at 25°C. The concentration of  $\text{H}_2\text{O}_2$  were determined on the basis of a molar extinction coefficient of  $43.6 \text{ M}^{-1} \text{ cm}^{-1}$  at 240 nm [55]. The specific activity was reported as units/ mg protein.

#### Lipid peroxidation

Lipid peroxides were detected as malondialdehyde (MDA) reacting with thiobarbituric acid (TBA) (Oxi-Tek, Zeptometric, Buffalo, NY) to form a 1:2 adduct (colored complex, TBARS) measurable by spectrofluorometric analysis. Briefly, following 16 h incubation with pesticides, cells ( $2 \times 10^7$ ) were collected by centrifugation at 300g at 4°C for 5 min, and resuspended in 200  $\mu\text{l}$  of PBS. This cell suspension was sonicated on ice for three 5-sec intervals at setting 7.0 (Fisher Scientific Sonic Dismembrator F550, USA). Whole homogenates was used to quantify lipid peroxides using the TBARS assay. Cell homogenates or standard (MDA) were mixed with 100  $\mu\text{l}$  of sodium dodecyl sulfate in test tubes and swirled to mix. Then, 2.5 ml of a TBA/Buffer reagent mix was added by pouring down the side of each tube. The mixtures were incubated in a boiling water bath for 60 min. Marbles were placed on the tops of tubes during the incubation period to avoid excessive evaporation of the reaction mixture. After cooling the tubes on ice, the reaction mixture was centrifuged at 3000g for 15 min. The fluorescence in the supernatants was read by a Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation, Sunnyvale, California) with an excitation wavelength of 530 nm and an emission wavelength of 550 nm. The concentrations of TBARS were calculated using MDA as a reference standard. The quantities of TBARS were expressed in terms of amount (pmol) per mg protein.

#### Caspase-3 activity

Caspase-3 activity was measured by a colorimetric assay [56]. The release of *p*-nitroaniline (pNA) from the substrate (Ac-DEVD-pNA) upon cleavage by caspase-3 was detected by following the absorbance at 405 nm. Fifty-microliter cell extracts were incubated with the reaction buffer (CaspACE™ Assay system colorimetric, Promega Corporation, Madison) for 4 h at 37°C. The absorbance of pNA was measured at 405 nm against the reagent blank devoid of recombinant caspase-3. The caspase-3 activity was expressed in terms of absorbance at 405 nm [56]. The assay was run in triplicate and repeated 5 times.

## Quantitation of NFκB by ELISA

The DNA-binding capacity of NFκB was assayed using the Trans-AM NFκB p50 transcription factor assay kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions.

Quantitative analysis of NFκB/p50 was performed using ELISA. For this assay, nuclear extracts of SH-SY5Y cells were prepared using the Nuclear Extraction Kit (Active Motif) according to the manufacturer's protocol. According to the manufacturer, this Trans-AM detection ELISA kit is 10-fold more sensitive and 40-fold faster than the electromobility shift assay. The assay was done in triplicate and absorbance read at 450 nm with reference taken at 650 nm. The assay was repeated 5 times.

## Protein analysis

Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard (BioRad, Hercules, CA).

## Statistical analysis

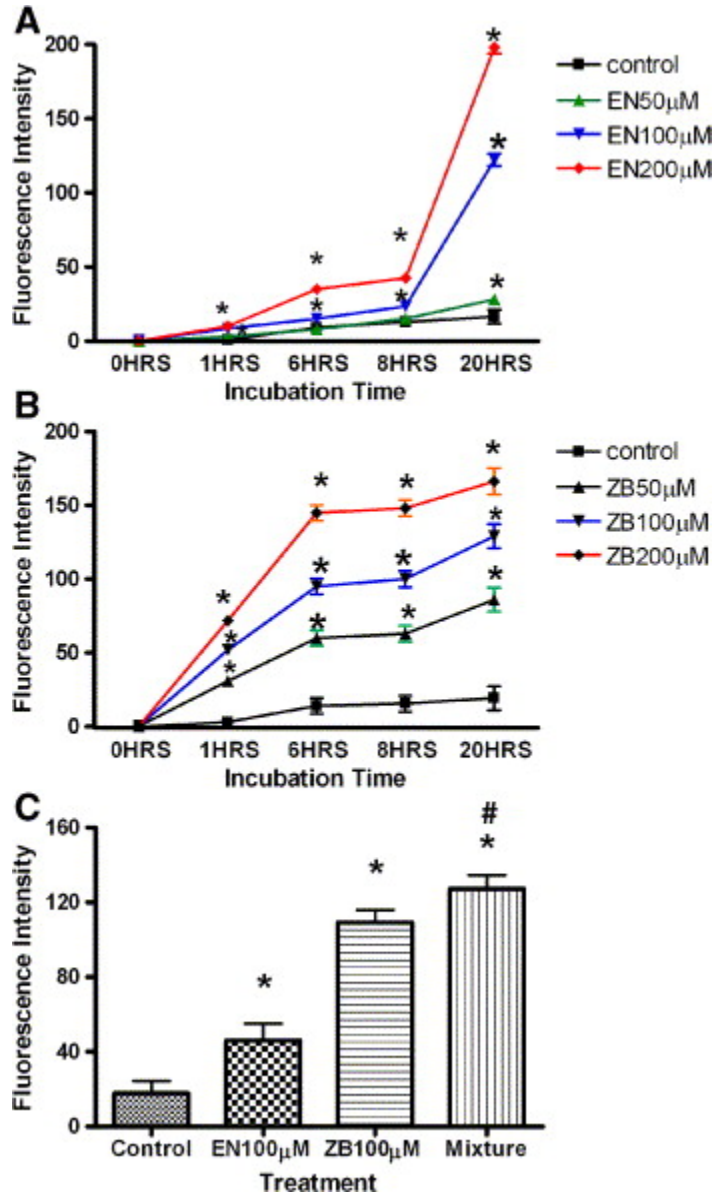
Analysis of variance (ANOVA) was used with general linear models (GLM) procedure of the SAS system (Version 8.2, SAS Institute Inc., Cary, NC) for statistical analysis of the data. Further statistical analyses for post hoc comparisons were performed using the Tukey–Kramer test. Standard residual plots were used to assess model adequacy. All differences of  $p \leq 0.05$  were considered as significant.

## Results

### Effects of pesticide on hydrogen peroxide generation

Intracellular production of H<sub>2</sub>O<sub>2</sub> in SH-SY5Y cells following pesticide exposure was analyzed by using DCF-DA dye assay as mentioned above and previously described [57]. SH-SY5Y cells were treated with 50, 100 or 200 μM of zineb or endosulfan for 1, 6, 8 or 20 h and assessed for DCF accumulation. As shown in Fig. 1, H<sub>2</sub>O<sub>2</sub> generation by SH-SY5Y cells treated with endosulfan (Fig. 1A) or zineb (Fig. 1B) at various concentrations for different time periods followed both, dose- and time-dependent responses to chemical exposure. There was a significant increase in H<sub>2</sub>O<sub>2</sub> production in cells exposed to as little as 50 μM endosulfan for 20 h. Higher concentrations (100 or 200 μM) of this pesticide caused a significant increase in this ROS (Reactive Oxygen Species) production as early as 1 h within exposure compared to control ( $p \leq 0.05$ , Fig. 1A). Similarly, cells treated with as little as 50 μM of zineb exhibited a significant increase in endogenous H<sub>2</sub>O<sub>2</sub> production within 1 h exposure time ( $p \leq 0.05$ , Fig. 1B). Based on these results, all subsequent studies were designed to investigate endosulfan- and zineb-mediated oxidative stress using 100 μM concentrations of each of the pesticides individually and in mixtures for 16 h. As shown in Fig. 1C, the pesticide mixture invoked significantly higher levels

of H<sub>2</sub>O<sub>2</sub> production ( $p \leq 0.05$ ) by the cells than did either pesticide individually or control ( $p \leq 0.05$ ).

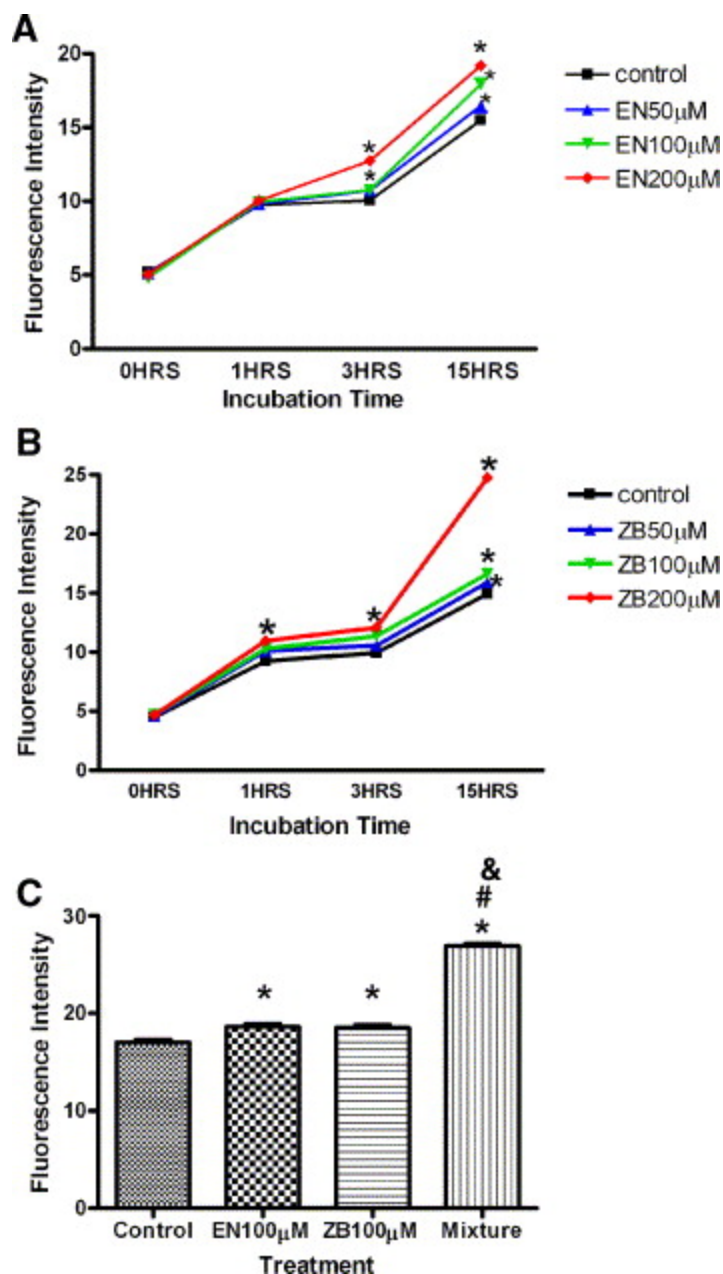


**Fig. 1.** Intracellular hydrogen peroxide production in SH-SY5Y cells following exposure to endosulfan (EN) and/or zineb (ZB). SH-SY5Y ( $3 \times 10^4$ ) cells were seeded in 96-well plates and incubated with 5  $\mu$ M of DCF-DA for 30 min before indicated concentration of pesticides was added and further incubated for various time periods. Generation of DCF was measured by a spectrofluometric analysis at excitation wavelength 492 nm and emission wavelength 527 nm. Turkey's HSD post hoc test for each treatment indicated difference ( $p \leq 0.05$ ) compared to control (\*) or compared to EN (#). The data are presented as the means  $\pm$  SEM.  $N = 8$ . (A) Cells exposed to indicated concentrations of EN. (B) Cells exposed to indicated concentrations of ZB. (C) Cells exposed to EN (100  $\mu$ M) or ZB (100  $\mu$ M) individually or in a mixture for 16 h.



## Effects of pesticides on superoxide generation

Superoxide anion ( $O_2^-$ ) production in pesticide-treated SH-SY5Y cells was monitored by the HE dye assay as mentioned above. Figs. 2A and B show the amount of  $O_2^-$  generated as a function of time and concentration of endosulfan and zineb exposure, respectively. SH-SY5Y cells treated with as little as 50  $\mu$ M zineb or endosulfan for 15 h showed a significant increase in  $O_2^-$  production when compared to control ( $p \leq 0.05$ , Fig. 2). Exposure to 100 or 200  $\mu$ M endosulfan also caused a significant increase in this free radical production as early as 3 h ( $p \leq 0.05$ , Fig. 2A). However, zineb had little effect on  $O_2^-$  levels in cells exposed to 50 or 100  $\mu$ M concentrations for 3 h ( Fig. 2B). Cells treated with 200  $\mu$ M zineb exhibited a significant increase in superoxide anion production at all time periods tested when compared to control ( $p \leq 0.05$ , Fig. 2B). Experiments using a mixture of endosulfan and zineb (100  $\mu$ M each) were performed to examine if there was an additive or synergistic effect. As shown in Fig. 2C, the pesticide mixture caused significant augmentation of superoxide anion production by SH-SY5Y cells ( $p \leq 0.05$ ) over levels detected in control or individual pesticide-treated cells. In fact, the mixture of endosulfan and zineb caused more than an additive effect of the individual pesticides on superoxide anion generation when levels were corrected for basal (solvent control) amounts. Thus, there was stimulation in superoxide anion production of  $\sim 9\%$  by each of the pesticides and  $> 57\%$  increase by the mixture as compared to control. These data indicate that exposure to multiple pesticides simultaneously can cause synergistic effects on superoxide anion production by neuronal cells.



**Fig. 2.** Intracellular superoxide anion production in SH-SY5Y cells following exposure to EN and/or ZB. SH-SY5Y ( $3 \times 10^4$ ) cells were seeded on 96-well plates and incubated with 5  $\mu$ M of hydroethidium (HE) for 30 min before indicated concentration of pesticides was added and further incubated for various time periods. Generation of ethidium bromide was measured by a spectrofluometric analysis at excitation wave length 510 nm and emission wave length 590 nm. Turkey's HSD post hoc test for each treatment indicated difference ( $p \leq 0.05$ ): compared to control (\*) or compared to EN (#), or compared to ZB (&). The data are presented as the means  $\pm$  SEM.  $N = 8$ . (A) Cells exposed to indicated concentrations of EN. (B) Cells exposed to indicated concentrations of ZB. (C) Cells exposed to EN (100  $\mu$ M) or ZB (100  $\mu$ M) individually or in a mixture for 16 h.

## Effects of pesticides on antioxidant enzyme levels

Intracellular levels of antioxidant enzymes such as SOD, CAT and GPX in SH-SY5Y cells following exposure to endosulfan or zineb individually and in mixtures for 16 h were measured by spectrophotometric analysis. The results are presented in Table 1. SH-SY5Y cells exposed to 100  $\mu$ M zineb showed a significant decrease in the specific activities of SOD (27%), CAT (35%) and GPX (31%) when compared to cells treated with vehicle control ( $p \leq 0.05$ ). Cells exposed to 100  $\mu$ M endosulfan also exhibited a significantly lower CAT (37%) activity ( $p \leq 0.05$ ) than control cells although there were no significant effects on SOD and GPX levels (Table 1). Exposure to a mixture of 100  $\mu$ M each of endosulfan and zineb significantly ( $p \leq 0.05$ ) decreased the activities of SOD (39%), CAT (52%) and GPX (42%) in SH-SY5Y cells as compared to controls.

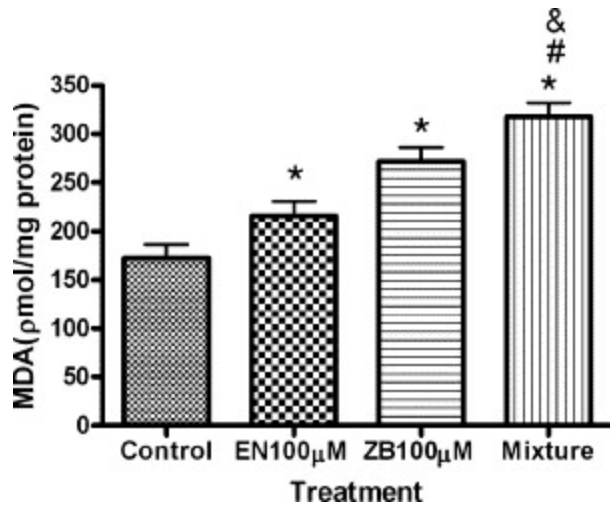
**Table 1.** Change in superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) in SH-SY5Y cells following exposure to EN, ZB and their mixtures

Treatment	SOD (U/mg protein)	Catalase (mU/mg protein)	GPX (mU/mg protein)
Control	5.2 $\pm$ 0.4	2.7 $\pm$ 0.2	63.1 $\pm$ 2.9
EN 100 pM	4.6 $\pm$ 0.4	1.7 $\pm$ 0.2 <sup>a</sup>	57.7 $\pm$ 3.2
ZB 100 pM	3.8 $\pm$ 0.4 <sup>a</sup>	1.7 $\pm$ 0.2 <sup>a</sup>	43.6 $\pm$ 3.2 <sup>a</sup>
EN + ZB	3.2 $\pm$ 0.4 <sup>a,b</sup>	1.3 $\pm$ 0.2 <sup>a</sup>	36.4 $\pm$ 3.2 <sup>a,b</sup>

Specific Activity of SOD, catalase and GPX in SH-SY5Y following exposure to endosulfan, zineb and their mixture for 16 hrs. The specific activities of enzyme in cells homogenate treated with pesticides were assayed as described in the Materials and Methods. Turkey's HSD post hoc test for each treatment indicated difference ( $p \leq 0.05$ ): a = data compared to control; b = compared to EN.  $N = 5$ . Results are expressed as the mean  $\pm$  SEM. Treatments with same letters are not different from each other ( $p > 0.05$ ).

## Effects of pesticides on lipid peroxide levels

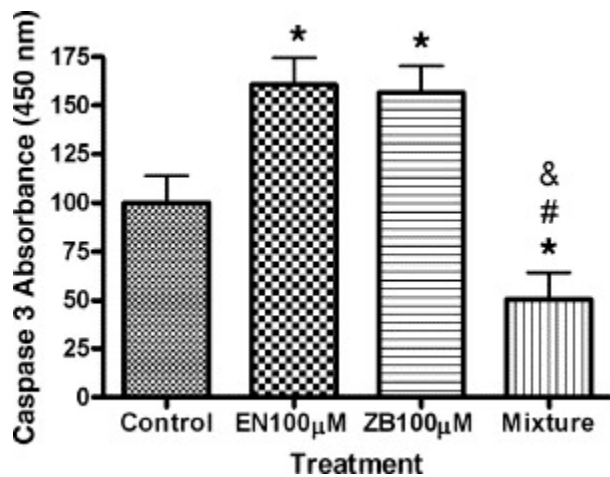
The levels of MDA, an indicator of lipid peroxidation, in SH-SY5Y cells following exposure to endosulfan or zineb individually and in mixtures for 16 h were measured using TBARS assay. Fig. 3 illustrates that cells exposed to 100  $\mu$ M each of endosulfan and zineb or a mixture of both for 16 h exhibited a significant increase in MDA levels compared to vehicle control cells. Furthermore, MDA levels in pesticide mixture treated cells were significantly higher than those in individual pesticide treated cells ( $p \leq 0.05$ ).



**Fig. 3.** Production of lipid peroxides in SH-SY5Y following exposure to endosulfan, zineb and their mixture for 16 h. Lipid peroxidation was evaluated using a TBARS assay kit as described in the Materials and methods. TBARS are expressed as pmol malondialdehyde (MDA)/mg protein. Turkey's HSD post hoc test for each treatment indicated a significant difference ( $p \leq 0.05$ ) compared to control (\*), compared to EN (#), or compared to ZB (&).  $N = 5$ . Results are expressed as the means  $\pm$  SEM.

#### Effects of endosulfan and zineb on caspase-3 activity

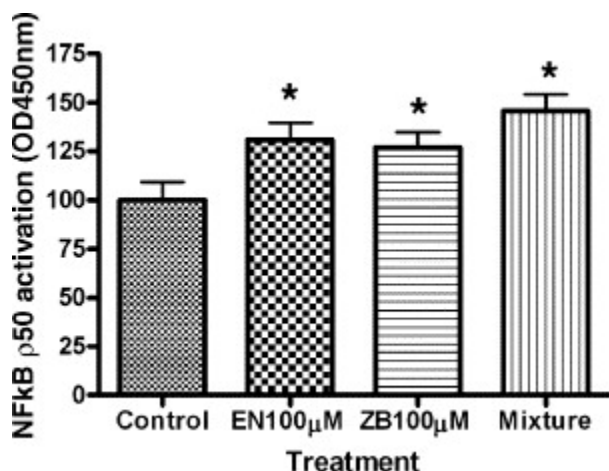
Because ROS has been implicated in activation of caspase-3 [58], we measured caspase-3 activity in SH-SY5Y cells after 16 hours incubation with 100  $\mu$ M each of endosulfan and zineb individually or in mixture. As shown in Fig. 4, cells exposed to endosulfan or zineb were found to have significantly higher levels of caspase-3 activity compared to controls ( $p \leq 0.05$ ) by 60% and 56%, respectively. However, the mixture of pesticides (100  $\mu$ M endosulfan + 100  $\mu$ M zineb) caused a significant decrease in caspase-3 activity to 50% of that measured in controls. This anomaly will be discussed later.



**Fig. 4.** Caspase-3 activity in SH-SY5Y cells in SH-SY5Y following exposure to endosulfan, zineb and their mixture for 16 h. Caspase-3 activity was measured by a colorimetric assay as described in the Materials and Methods. The caspase-3 activity was expressed in terms of absorbance at 405 nm. Turkey's HSD post hoc test for each treatment indicated difference ( $p \leq 0.05$ ) compared to control (\*), compared to EN (#), or compared to ZB (&).  $N = 5$ . Results are expressed as the means  $\pm$  SEM.

Effects of endosulfan and zineb on NF $\kappa$ B p50 subunit activity

Several transcription factors are known to be modulated by ROS [51]. To support the evidence that cells exposed to pesticides undergo oxidative stress, we monitored the activity of NF $\kappa$ B, a ubiquitous transcription factor, as an indicator of oxidative stress. NF $\kappa$ B expression was monitored by ELISA as mentioned above. As shown in Fig. 5, cells exposed to 100  $\mu$ M each of endosulfan and zineb individually or in combination expressed significantly higher levels of NF $\kappa$ B compared to vehicle treated control cells ( $p \leq 0.05$ ). However, when cells were exposed to a mixture of 100  $\mu$ M each of endosulfan and zineb, no significant differences were observed in the levels of the NF $\kappa$ B p50 transcription factor as compared to cells treated with individual pesticides ( $p > 0.05$ ).



**Fig. 5.** NF $\kappa$ B p50 subunit expression in pesticide exposed SH-SY5Y following exposure to endosulfan, zineb and their mixture for 16 hrs. NF $\kappa$ B activation was measured using TransAM NF $\kappa$ B p50 kit as described in the Materials and methods. Turkey's HSD post hoc test for each treatment indicated difference ( $p \leq 0.05$ ) compared to control (\*).  $N = 5$ . Results are expressed as the mean  $\pm$  SEM.

## Discussion

Accumulated evidence supports oxidative stress as one of the important pathways leading to neuronal cell death in PD [31], [32], [33], [34], [35], [36], [37], [38] and [39]. Known neurotoxic agents, such as MPTP, paraquat, rotenone have been shown to induce apoptosis through cellular signaling mechanisms [59], [60],[61], [62], [63] and [64]. In the present study, we show that

neuronal cells (human neuroblastoma SH-SY5Y) exposed to individual pesticides (endosulfan and zineb) exhibited increased production of hydrogen peroxide as well as superoxide anion in a dose-dependent manner. The mixture of pesticides caused more than additive effects in production of superoxide anion. Furthermore, cells treated with pesticides showed decreased levels of the antioxidant enzymes SOD, GPX, and CAT. Pesticides also induced lipid peroxide (TBA reactive products) formation in SH-SY5Y cells. These results indicate that the cytotoxic action of these pesticides in SH-SY5Y cells is mediated, at least in part, by an oxyradical mechanism involving overproduction of ROS and downregulation of certain key antioxidant enzymes such as SOD, GPX, and CAT. Both events would lead to oxidative stress in the pesticide-exposed cells. The increased activity of NF $\kappa$ B in the nucleus seems to be a consequence of oxidative stress. Because SH-SY5Y cells are a human catecholaminergic neuroblastoma cell line which has been proposed to be a suitable *in vitro* model of human dopaminergic neurons [65], the results of this study provide *in vitro* evidence that oxidative stress is involved in pesticide-induced neuronal cell death.

Hydrogen peroxide can induce both apoptotic and necrotic forms of cell death [66] and [67]. High concentrations of hydrogen peroxide are known to cause rapid cell death with no evidence of apoptosis [66]. Moderate concentrations of hydrogen peroxide induce DNA cleavage and are associated with morphologic evidence of apoptosis [68]. Hydrogen peroxide has also been shown to induce neuronal cell death with more or less necrotic and/or apoptotic characteristics depending on concentrations used [69] and [70]. Our present study shows that hydrogen peroxide generation from SH-SY5Y cells treated with zineb or endosulfan at various concentrations for different time periods (1–20 h) follows a dose- and time- dependent response to chemical treatment. A mixture of pesticides augmented hydrogen peroxide generation ( $p \leq 0.05$ ) to levels above those observed in response to individual pesticide treatment ( Fig. 3). In earlier studies, we have demonstrated that exposure of SH-SY5Y cells to endosulfan and zineb at similar doses causes both apoptosis and necrosis and that, at higher doses ( $> 100 \mu\text{M}$ ) or in mixtures necrosis predominates [26]. The accumulation of hydrogen peroxide production as demonstrated here can be speculated to have a correlation with the increased number of apoptotic and necrotic cells reported earlier [26]. It is interesting to note that cells exposed to endosulfan and zineb concurrently generated more superoxide anion than the total amount of this free radical cumulatively generated upon individual pesticide treatment. Although superoxide anion lacks the ability to penetrate lipid membranes, it can react with hydrogen peroxide in the presence of  $\text{Fe}^{3+}$  to generate the more reactive hydroxyl radicals which damage lipid, DNA and protein [71] and [72]. The present study does not delineate the exact nature of ROS causing apoptosis/necrosis in neuronal cells. However, it is of interest to note that there is indeed augmentation of ROS production upon exposing cells to these pesticides.

To minimize oxidative damage to cellular components, cells have adaptive mechanisms to increase antioxidant defenses [73]. Antioxidant enzymes including isoforms of SOD, GPX, and CAT, have been found to be inducible in transgenic mice in response to oxidative stress inflicted

by exposure to certain pesticides [74]. The antioxidant enzyme systems that were monitored in the present study namely SOD, CAT, and GPX are enzymes known to protect against the ROS produced during the metabolism of various drugs and toxicants. Results of this study show that SH-SY5Y cells exposed to 100  $\mu$ M zineb alone or in mixture with 100  $\mu$ M endosulfan showed a significant decrease in the specific activities of SOD, GPX and CAT when compared to cells treated with solvent control ( $p \leq 0.05$ ). These results are consistent with a recent report of decreased SOD and GPX in lungs of endosulfan-exposed rats [75]. However, in the same report the authors noted increased liver GPX in the same rats. These results suggest that different cell types may show a differential response in GPX enzyme activity following endosulfan exposure. Reduced levels of GPX and CAT activity following endosulfan exposure have also been reported in a fish model [48],[49] and [50]. Inhibition of Cu, Zn SOD activity by *N-N'*-diethyl-dithiocarbamate was observed in human umbilical vein endothelial cells [76]. Hence, our data on lowered levels of antioxidant enzymes are consistent with the above reports.

Oxidative stress occurs when the rate at which the ROS are generated exceeds the capacity of the cell to remove them by antioxidants. Because the antioxidant enzymes studied here are part of a vital defense mechanism against ROS-induced tissue damage, the increased production of ROS with a concomitant reduction of antioxidant enzyme activities could lead to oxidative stress, including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation, leading to spontaneous apoptosis in pesticide-exposed cells [77], [78] and [79]. Lipid peroxidation is one of the major outcomes of free radical-mediated injury that directly damages membranes and contributes to DNA damage [80], [81] and [82]. Accumulation of MDA is regarded as reliable biomarker of age and of the degree of oxidative stress [83] and [84]. Results of this study show, that cells exposed for 16 hours to 100  $\mu$ M endosulfan, zineb or their mixture had a significant increase in MDA level compared to vehicle control treated cells ( $p \leq 0.05$ ). There are a number of reports documenting that both endosulfan and metal-EBDC pesticides induce lipid peroxidation in various cell systems both, *in vivo and in vitro* [45], [74], [85], [86], [87], [88] and [89]. Hence, our data are in accord with those reports showing both endosulfan and zineb to be potent neurotoxicants, exposure to which could lead neuronal cells, such as SH-SY5Y cells to suffer oxidative stress.

To determine if the caspase pathways are involved in endosulfan and zineb-induced apoptosis in SH-SY5Y cells, we examined the caspase-3 activity in these cells. Caspase-3 appears to play an important role in developmentally regulated cell death in the brain [90], [91] and [92]. Activation of caspase-3 is a necessary step for neuronal apoptosis following exposure to neurotoxins such as MPP<sup>+</sup>[93]. Activation of caspase-3 has been implicated in PD and AD [94], [95], [96], [97] and [98] This suggests that caspase-3 may act as a common downstream effector of cell death in many neurodegenerative disorders. Results of the present study show that exposure of SH-SY5Y cells to 100  $\mu$ M endosulfan or zineb for 16 hours results in a significant increase in caspase-3 activities. However, a mixture of endosulfan and zineb at 100  $\mu$ M each decreased caspase-3 activity, suggesting that necrosis replaces apoptosis as the

dominant form of cell death in SH-SY5Y cells under these conditions. Because most, if not all, chemicals tested seem to be capable of causing apoptosis at low doses and necrosis at higher doses [66], [99] and [100], and our earlier study demonstrated high necrotic cell death in cells exposed to mixtures of chemicals, the lower level of caspase-3 activity observed in the presence of mixtures of chemicals can be attributed to predominately necrotic processes.

ROS have also been thought to serve as common messengers in activation of NFκB [101] and [102] which is suggested to be primarily an oxidative stress-responsive transcription factor [103]. Activation of NFκB has been implicated as an important pathway in the regulation of many genes that code for mediators of the immune and acute phases of the inflammatory response [104], [105], [106] and [107]. The inactive form of NFκB is present in the cytoplasm as a heterodimer of 50 kDa DNA-binding (p50) and 65 kDa DNA-binding (p65) subunits bound to an inhibitory (IκB) monomer. The activation of NFκB in response to oxidative stress involves release of the inhibitory subunit (IκB) from the cytoplasmic complex and translocation of the DNA binding subunits to the nucleus where they bind to specific DNA sequences [73], [108] and [109]. The p50 homodimers are the most common dimers found in NFκB signaling. In this study, exposure to 100 μM endosulfan, zineb or their mixture for 16 hours significantly increased expression of NFκB in the nucleus of SH-SY5Y cells when compared to vehicle controls ( $p \leq 0.05$ ; Fig. 5). Hence, our results confirmed that the oxidative stress induced by pesticide exposure to cells contributes, at least in part, to the activation of NFκB. The mechanism(s) by which NFκB activation induces cells death is not well understood. JNK/SAPK signaling pathway has been suggested to be one of the apoptosis inducing pathways [110] and [111]. Blocking activation of JNK/SAPK protected against PC12 dopaminergic neuronal cell apoptosis [112]. NFκB may also function through the activation of various kinases including MAPK and Bcl-2 induced pathway [113], [114] and [115]. Bcl-2 family members such as Bax, Bcl-xl and Bad were affected following exposure to paraquat and maneb in transgenic mice [74]. MAPK activity was decreased in neuronal stem cells following endosulfan exposure [116]. Thus, examination of Bcl-2 and MAPK activity might shed some light and help to fully understand pesticide induced neuronal cell death.

Predicting the effects of these pesticides and pesticide mixtures in humans on the basis of our *in vitro* experiments is tempting but is a long shot. Therefore, until further studies are performed, it is difficult to extrapolate the concentrations of pesticides employed in the current *in vitro* studies (100 μM each) to the “real world value”. There is likelihood that occupational hazards may cause certain individuals to be exposed to high levels of these pesticides. Concomitant or sequential exposure of certain chemicals may also increase bioavailability. These chemicals may also interact while crossing cell membranes (hepatic, renal), when binding to plasma proteins, or at the receptor level [117] and [118]. Furthermore, duration of exposure, cumulative effects of chemicals and age of the individuals may, at least in part, contribute to the level of tissue damage that occurs. Thus, estimating actual levels of these chemicals directly acting on neuronal cells from an epidemiological exposure study is also difficult to predict. A critical limitation is the



lack of general scheme for assigning intracellular exposure to certain chemical based only on plasma concentration. The assumption that plasma protein binding may restrict neuronal cell uptake is not tenable. Other potential limitations include: hepatic clearance, chemical inhibition or induction of transporter proteins and receptors for reuptake of chemicals. Therefore, these findings with *in vitro* exposure cannot be extended to entire body systems. However, the results presented here are still important because they demonstrate the possible pathway of neuronal cells death to these xenobiotics. Our findings suggest that cytotoxicity of endosulfan and zineb, both individually and in mixtures may, at least in part, be associated with the generation of reactive oxygen species with concomitant increased expression of NF $\kappa$ B in SH-SY5Y human neuroblastoma cells.

Further more, the effects of pesticides observed in SH-SY5Y cells may not have the same effects on primary neurons or *in vivo* situations and the data presented here should be used with caution. Nevertheless, the *in vitro* cell culture models to date have provided reasonably accurate forecast the mechanisms of action of various drugs and xenobiotics occurring *in vivo*. Such predictive models deserve credit because they may ultimately provide more cost-effective and expeditious screening of chemicals for toxicity and drug development studies. Traditionally, most toxicological studies are performed on *in vitro* cells models, such as reported here, to determine the mechanism of action of drugs and other chemicals. Moreover, we believe the results reported here using SH-SY5Y cells may have potential relevance to the “real world” disease problems of neurons because the human neuroblastoma clone SH-SY5Y was derived from a human sympathetic ganglion [119] and it is generally classified as dopaminergic neurons [120]. These cells are known to retain catecholaminergic phenotype. Previous studies have shown that SH-SY5Y cells contain high level of dopamine beta hydroxylase and levels of tyrosine hydroxylase [121], both of which are key enzyme in catecholaminergic neurotransmitter synthesis. In addition to synthesizing these enzymes, SH-SY5Y cells express dopamine receptors [122], muscarinic M1, M2 [123] and 5-HT [124] receptors. Therefore, human neuroblastoma (SH-SY5Y) cells possess many properties as dopaminergic cells and are used as a cultured cell model for numerous disease processes associated with the nervous system. SH-SY5Y cells have also been employed for investigating the toxicities of diverse pesticides such as dithiocarbamates, organ chlorines and organophosphorus compound [125], [126] and [127] because they can express the appropriate surface receptors, neurotransmitters, and morphological and biochemical characteristics of neurons.

In summary, the results of the present study demonstrate that neuronal cells (SH-SY5Y) exposed to endosulfan and zineb individually or in a mixture increase the production of hydrogen peroxide as well as superoxide anion, decrease SOD GPX, and CAT enzyme levels and increase lipid peroxide levels. It was also demonstrated that exposure to these pesticides altered caspase-3 activity in these cells and increased the active NF $\kappa$ B levels in nucleus. We believe this is the first report demonstrating that pesticide-induced oxidative stress may play a key role in neuronal activation of the redox-sensitive transcription factor NF $\kappa$ B and caspase-3 resulting in cell death.

This holds tremendous implication for the derivation of risk assessment guidelines for human exposure to pesticides.

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