Redox imbalance caused by pesticides: a review of OPENTOX-related research

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Pesticides are a highly diverse group of compounds and the most important chemical stressors in the environment. Mechanisms that could explain pesticide toxicity are constantly being studied and their interactions at the cellular level are often observed in well-controlled in vitro studies. Several pesticide groups have been found to impair the redox balance in the cell, but the mechanisms leading to oxidative stress for certain pesticides are only partly understood. As our scientific project “Organic pollutants in environment – markers and biomarkers of toxicity (OPENTOX)” is dedicated to studying toxic effects of selected insecticides and herbicides, this review is focused on reporting the knowledge regarding oxidative stress-related phenomena at the cellular level. We wanted to single out the most important facts relevant to the evaluation of our own findings from studies conducted on in vitro cell models.

KEY WORDS: antioxidants; apoptosis; glyphosate; in vitro; neonicotinoids; organophosphates; oxidative stress; pyrethroids; reactive oxygen species

Over the years, population growth and changes in food consumption patterns have challenged agricultural production to meet the demand for food and quality standards. This has led to an increased use of pesticides. Pesticides are defined as “any substance or mixture of substances of chemicals or biological ingredients intended for repelling, destroying or controlling any pest, or regulating plant growth” (1). There is a wide range of pesticide types, including insecticides, herbicides, rodenticides, and fungicides. Because of their recognised potential to adversely affect untargeted biological systems, they have been studied extensively for their toxicity and associated risks (2, 3).

For the last two decades, toxicological research has been focused on oxidative stress as a possible mechanism of pesticide toxicity, but the precise mechanisms by which pesticides affect human metabolism at the cellular level are still unclear. Their toxic effects usually depend on the chemical structure of a pesticide, dose received, and time of exposure (4). Many of them are believed to be mediated by the regulation of apoptosis and redox signalling (5). Pesticides have been shown to induce apoptosis by activating signalling pathways mediated by mitochondria and DNA damage as well as through activation of death receptors (6).

As our scientific project “Organic pollutants in environment – markers and biomarkers of toxicity (OPENTOX)”, financed by the Croatian Science Foundation (HrZZ), is dedicated to studying the toxic effects of two major pesticide classes with three subgroups each: (A) insecticides (organophosphates, neonicotinoids, and pyrethroids) and (B) herbicides (triazines, organophosphates, allelopathic compounds) (7), this review is focused on reporting the knowledge regarding oxidative stress-related phenomena at the cellular level. Although the amount of information on this issue is remarkable, we selected only those groups (or single compounds) which are covered by our project, as we wanted to single out the most important facts relevant to the evaluation of our own findings from studies conducted on in vitro cell models.

REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

Reactive oxygen species (ROS) are products of normal cell metabolism and metabolism of cells affected by xenobiotics. Their effects in the cell can be beneficial or harmful, depending on their concentration (8). Many of the ROS are free radicals, such as hydroxyl, peroxyl, superoxide, or nitric oxide, with one or more unpaired electrons, which makes them unstable and reactive. Seeking stability, radicals attack nearby molecules to obtain another electron, but in the process damage the structure and function of the attacked molecules (9). ROS interact with receptors, second messengers, and transcription factors that alter gene expression and influence cell growth and survival.

ROS attack three targets in the cell: proteins, DNA, and membrane lipids (10). The brain is particularly vulnerable to oxidative injury due to its high oxygen consumption, low
antioxidant defence, and high content of polyunsaturated fatty acids, which are easily oxidised. Lipid peroxidation can alter membrane fluidity, inactivate membrane-bound receptors or enzymes, and impair normal cell function and membrane permeability (9). Dopaminergic cells are particularly sensitive to ROS, because dopamine metabolism creates hydrogen peroxide and superoxide radicals (11). Dopaminergic neurons mainly develop after birth, which makes the developing nervous system highly sensitive to pesticides (12, 13).

Under normal conditions the levels of ROS and antioxidants is balanced. Their imbalance, manifested in an excess of ROS or lack of antioxidants or both, is what causes oxidative stress (8, 14).

**INSECTICIDES**

*Organophosphates*  
Organophosphorus pesticides (OP) are widely used in agriculture due to their high effectiveness and a relatively short half-life in the environment. However, they are generally more toxic to vertebrates than other classes of insecticides (15), as they inhibit the activity of acetylcholinesterase (AChE), which causes accumulation of acetylcholine (ACh) at the neuronal synapses and neuromuscular junctions and results in convulsions, paralysis, and death (16). OPs also affect DNA and RNA synthesis, signal transduction pathways, and expression of different transcription factors, and cause oxidative stress, as described above (17-20). One such OP that causes oxidative stress even at low concentrations is chlorpyrifos (21). It also disturbs neurotransmission (22), inhibits the replication of cells in the nervous system (23), and disrupts neuronal differentiation (24). A study on PC12 cells, which are used as a standard model for neural cell differentiation, showed immediate increase in ROS after chlorpyrifos exposure (25). Because a developing nervous system is less capable of scavenging free radicals than adult, oxidative stress is a likely mechanism by which chlorpyrifos damages immature brain. Lee et al. (26) reported that chlorpyrifos inhibited mitochondrial activity in PC12 cells, which led to excessive ROS formation, cytochrome c release from mitochondria, and activation of apoptotic cell death. In this process, mitogen-activated protein kinase (MAPK) signalling played a crucial role in dopaminergic cell death. Moreover, exposure to chlorpyrifos altered the expression of proteins involved in antioxidant defence.

In SH-SY5Y cells paraoxon, parathion, phenyl saligenin phosphate, tri-ortho-tolyl phosphate, and triphenyl phosphate induced similar forms of time-dependent cell death. Their cytotoxicity manifested itself in nuclear condensation, budding, fragmentation, and caspase-3 activation (27).

A similar effect was seen after chlorpyrifos exposure (28). In a study of Raszewski et al. (5) chlorpyrifos induced death in SH-SY5Y cells by down-regulating anti-apoptotic Bcl-2 and Bcl-xL and increasing caspase-3 activity. These changes point to mitochondrial dysfunction and consequent apoptosis. Mitochondrial membrane potential is commonly used to evaluate mitochondrial function as an indicator of cell health. The stability of mitochondrial membrane preserves the dynamic equilibrium of intracellular free calcium concentrations, and their decline may also lead to apoptosis (29). A recent study (30) showed higher free Ca²⁺ concentrations, higher plasma membrane potential, and lower mitochondrial transmembrane potential in HepG2 cells after a 24-hour exposure to chlorpyrifos. In another *in vitro* study (31) chlorpyrifos-ethyl induced lipid peroxidation and disturbed the activity of antioxidant enzymes in erythrocytes, which suggests an involvement of ROS in the toxic effects of OP pesticides.

Oxidative stress was also observed in human erythrocytes after exposure to malathion *in vitro* (32). In this study the increased levels of malondialdehyde (MDA) pointed to lipid peroxidation. It was reduced by pretreatment with vitamins C and E but only where malathion levels were low (25 μmol L⁻¹ and 75 μmol L⁻¹).

*Neonicotinoids*  
Neonicotinoid insecticides are neurotoxicants that act as nicotinic acetylcholine receptor (nAChR) agonists in insects and mammals. Compared to OPs, they are considered less toxic to vertebrates due to preference for insect receptors (10). Today, they make one third of the global insecticide market. Among them imidacloprid was the world’s top selling insecticide in 2010 (33). In spite of the original belief that neonicotinoids are only mildly toxic to mammals, there is increasing evidence of a variety of toxic effects on animals and humans (34-37). *In vitro* studies reported that imidacloprid disrupted the glutathione redox cycle by affecting its components glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione-S-transferase (GST) in Chinese hamster ovary cells (CHO K1) (33) and to activate the ERK cascade via nAChRs and intracellular calcium mobilisation in mouse N1E-115 neuroblastoma cells (38), which may affect the functioning of the neurons. The effects of imidacloprid were also studied in human lymphocytes and HepG2 cells after four- and 24-hour exposure to concentrations corresponding to the acceptable daily intake (ADI), residential exposure level (REL), and occupational exposure level (OEL) (39). The results showed that the applied imidacloprid concentrations did not trigger significant lipid peroxidation nor did they affect the total antioxidative capacity of lymphocytes or HepG2 cells (39).

On the other hand, a number of *in vivo* animal models have reported oxidative stress as neonicotinoids’ mechanism of action (40-43). So far, it is still not clear whether
Table 1. An overview of in vitro studies of oxidative stress induced by pesticides

<table>
<thead>
<tr>
<th>Pesticide type</th>
<th>Cell type / cell line</th>
<th>Antioxidant status and oxidative stress</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos</td>
<td>human neuroblastoma</td>
<td>Increased ROS level and concentration of MDA</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>(SH-SY5Y)</td>
<td>Increased level of cytosolic cytochrome c</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased level of cleaved caspase-9, caspase-3, and PARP</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>MAPK activation</td>
<td></td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>rat pheochromocytoma</td>
<td>Increased level of cytosolic cytochrome c and increased level of cleaved caspase-9, caspase-3, and PARP</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>cell line (PC12)</td>
<td>Increased ROS generation</td>
<td></td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>rat pheochromocytoma</td>
<td>Increased ROS generation, Increased concentration of MDA</td>
<td>(25)</td>
</tr>
<tr>
<td></td>
<td>cell line (PC12)</td>
<td>Significant decrease of mitochondrial complex I activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Altered expression of antioxidant enzymes (CuZnSOD, MnSOD)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAPK activation, apoptosis</td>
<td></td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>human neuroblastoma</td>
<td>Down-regulation of Bcl-2 and Bcl-xL</td>
<td>(5)</td>
</tr>
<tr>
<td>Chlorpyrifos+</td>
<td>(SH-SY5Y)</td>
<td>Increased caspase 3 activation</td>
<td></td>
</tr>
<tr>
<td>Cypermethrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>hepatocellular carcinoma</td>
<td>Increased concentration of free intracellular Ca(^{2+}) and plasma membrane potential</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td>(HepG2)</td>
<td>Decrease in mitochondrial transmembrane potential</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Slight apoptosis</td>
<td></td>
</tr>
<tr>
<td>Chlorpyrifos-ethyl</td>
<td>erythrocytes</td>
<td>Increased concentrations of MDA and GPX activity</td>
<td>(31)</td>
</tr>
<tr>
<td>Malathion</td>
<td>erythrocytes</td>
<td>Decreased activity of SOD and CAT</td>
<td></td>
</tr>
<tr>
<td>α-cypermethrin</td>
<td>hepatocellular carcinoma</td>
<td>Oxidative stress biomarkers were not significantly altered</td>
<td>(39)</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>(HepG2)</td>
<td></td>
<td></td>
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<tr>
<td>Imidacloprid</td>
<td>human lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abamectin</td>
<td>Chinese hamster ovary</td>
<td>Significant inhibition of GST and GPX activity</td>
<td>(33)</td>
</tr>
<tr>
<td>Chlorfenapyr</td>
<td>(CHO-K1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>mouse neuroblastoma</td>
<td>Activated MAPK/ERK signalling cascade at low concentrations</td>
<td>(38)</td>
</tr>
<tr>
<td>Desnitro-imidacloprid</td>
<td>mouse neuroblastoma</td>
<td>Calcium mobilization</td>
<td></td>
</tr>
<tr>
<td>Fenvalerate</td>
<td>(imidacloprid metabolite)</td>
<td>Increased TBARS levels</td>
<td>(51)</td>
</tr>
<tr>
<td>and its metabolite</td>
<td>mouse neuroblastoma</td>
<td>Decreased activity of antioxidant enzymes CAT, SOD, GR and GST</td>
<td></td>
</tr>
<tr>
<td>p-chlorophenyl isovaleric acid (p-CPIA)</td>
<td>erythrocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifenthrin</td>
<td>erythrocytes</td>
<td>Enhanced lipid peroxidation</td>
<td>(52)</td>
</tr>
<tr>
<td>Atrazine</td>
<td>rat pheochromocytoma</td>
<td>Decreased CAT activity and GSH levels</td>
<td>(66)</td>
</tr>
<tr>
<td></td>
<td>cell line (PC12)</td>
<td>Increased ROS level, lipid peroxidation, activity of GPX and GR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induced apoptosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-regulation of mRNA expression of Bax, p53, caspase-3, caspase-9 and down-regulation of Bcl-2</td>
<td></td>
</tr>
</tbody>
</table>
oxidative stress is a secondary effect or has nothing to do with the nAChR agonists. As the application of neonicotinoids continues to grow, further research of their toxicity to vertebrates and invertebrates is absolutely necessary.

**Pyrethroids**

Pyrethroids are synthetic analogues and derivatives of natural insecticides pyrethrins obtained from the flowers of pyrethrum (Chrysanthemum cinerariaefolium) (44). Pyrethroids are extensively used in agriculture and indoors. They were meant to replace restricted or banned organophosphates but were also found to cause adverse effects (45). Because of their lipophilicity, they tend to act on biological membranes. They react with voltage-gated sodium nerve channels and prolong the time during which the channels are open (46). Because the structure and function of voltage-gated sodium channels are very similar between insects and mammals, mammals may also be affected by this mechanism of action.

It is likely, however, that voltage-gated sodium channels are not the only pyrethroid target (47). Pyrethroids were found to indirectly generate superoxide radicals, reactive nitrogen species such as peroxynitrite, nitric oxide, and hydroxyl radicals, causing damage consistent with oxidative stress (48, 49), whose likely target are erythrocytes because of high content of polyunsaturated fatty acids in cell membranes and elevated concentrations of oxygen and haemoglobin (50). In a study of Prasanthi et al. (51) another common insecticide, fenvalerate, induced oxidative stress in erythrocytes in vitro through lipid peroxidation and inhibition of antioxidant enzymes. Similar results in erythrocytes were observed after bifenthrin (52) and lambda-cyhalothrin exposure (53).

Cypermethrin is a class II synthetic pyrethroid pesticide that crosses the blood-brain barrier, affects the central nervous system, and impairs motor function (54). Although considered the safest pesticide, studies have shown its connection to developmental neurotoxicity, oxidative stress, and apoptosis (55-57). Cypermethrin seems to generate reactive oxygen and nitrogen species and reduce the antioxidant levels through its metabolites mediated by the cytochrome P450 2E1 (54).

**HERBICIDES**

**Triazine herbicides**

Triazine herbicides inhibit electron transport in photosynthesis and have been used as selective herbicides in agriculture for more than 50 years. They include asymmetrical triazines or triazinones (metribuzin) and symmetrical triazines. The major commercial symmetrical triazines are further divided into chloro-s-triazines (atrazine, propazine, terbutylazine), thiomethyl-s-triazines (ametryn, terbutryn), and methoxy-s-triazine (prometon) (58). Recent years saw a growing concern about the toxicity and environmental persistence and mobility of triazines and their metabolites (59). Atrazine has been used extensively, mainly due to its low cost and ease of application and is the most common contaminant of groundwater and surface water. Because of its persistence in the environment and toxicity for wildlife and possible effects on human health, it was banned in the EU in 2004 (60). Even so, it remains a significant environmental and biological hazard (61). The connection between atrazine and oxidative stress was observed in different in vivo and in vitro studies. Song et
al. (62) detected genotoxic effects of atrazine through the formation of ROS, while Zhang et al. (63) reported enhanced lipid peroxidation and activity of antioxidant enzymes in male Wistar rats exposed to atrazine. Oxidative stress induced by atrazine was also detected in two bacterial strains (64) and adult female zebrafish (65). In addition, atrazine was found to induce apoptosis in PC12 cells by altering the expression of p53, caspase-3, and caspase-9 (66) and to cause apoptosis-related neurodegenerative damage in the nerve cells (61).

Unlike for atrazine, in vitro research has produced very limited knowledge about the oxidative stress caused by other triazine herbicides. We investigated the in vitro effects of a four-hour exposure to terbuthylazine concentrations of 8.00, 0.80, and 0.58 ng mL⁻¹ in whole peripheral blood, isolated lymphocytes, and HepG2 cells (67), which is comparable with current reference values set by the European Commission in 2011 (68). ROS levels in plasma were significantly increased by all terbuthylazine concentrations, and in lymphocytes by the concentrations of 0.80 and 0.58 ng mL⁻¹, while no effect was observed in HepG2 cells. The activities of GPX in whole blood and of superoxide dismutase (SOD) in erythrocytes dropped, while in HepG2 cells and lymphocytes they rose possibly in response to oxidative/antioxidative disequilibrium. Significantly increased lipid peroxidation was only observed in plasma at the highest tested concentration (67).

In vivo studies showed that exposure to metribuzin was associated with lipid peroxidation and impaired antioxidant activity in crayfish (69) and oxidative stress in goldfish (70). Common carp in their embryo-larval stages were also affected by terbutylazine and metribuzin through oxidative stress (71).

**Glyphosate**

Glyphosate is a non-selective, broad-spectrum, systemic organophosphorus herbicide for all plant types. It interferes with the production of the aromatic amino acids phenylalanine, tyrosine, and tryptophan, which are essential for plant growth (72). Since the pathway operates only in plants and microorganisms, glyphosate had long been considered safe for humans (73). Recently, however, the WHO and IARC changed its classification to probably carcinogenic (group 2A) to acknowledge doubts about its safety at low doses (74).

The effects of low doses of glyphosate on HepG2 cells were also tested in our research study (75). The results showed no increase in the ROS levels after four- and 24-hour exposure, indicating that ROS was efficiently removed by antioxidant defences. This was also confirmed by lower total antioxidant capacity (TAC). GPX activity dropped significantly after the four-hour treatment with the ADI concentrations while the OEL concentrations lowered it only after the 24-hour treatment.

Glyphosate was also found to adversely affect HaCaT cell adhesion potential, trigger hydrogen peroxide production and chromatin condensation, disrupt the cytoskeleton, and eventually induce apoptosis (76). The induction of apoptosis was confirmed in a variety of cell cultures (77-79).

Research has shown that ingredients added to commercial glyphosate formulations may influence the cytotoxicity of glyphosate and other herbicides (80-82). Coalova et al. (83), for example, showed that the addition of an adjuvant (alkyl-aryl-polyglycol ether) to glyphosate formulation increased its toxicity to HepG2 cells. The adjuvant increased ROS production, catalase activity, and glutathione concentrations. Moreover, this glyphosate formulation activated caspase 3/7 and the apoptosis pathway. An earlier study (84) has shown that glyphosate-based formulations can be responsible for oxidative damage to human epidermal cells (HaCaT).

4-hydroxyphenylpyruvate dioxygenase inhibitors

Recent bans of various agrochemicals in many European countries have created a demand for new effective compounds. Their development is mostly focused on targeting enzymes. One such enzyme is 4-hydroxyphenylpyruvate dioxygenase (HPPD), as it catalyses the initial steps in the tyrosine degradation pathway (85). In plants, this enzyme regulates growth, and its inhibition impairs photosynthesis, followed by leaf bleaching (86). In animals, it regulates blood tyrosine levels, and its inhibition results in blood tyrosine accumulation (87).

There are three main classes of commercial HPPD-inhibiting herbicides that specifically target a variety of broadleaf weeds without affecting the crops, whose application rate and toxicity is low, and which can be used for pre- and post-emergence treatment (88). These are isoxazoles, pyrazoles, and triketones (89). So far, the triketone herbicide family has not been proven unsafe for the environment and human health and is considered “eco-friendly” due to its “natural” origin and rapid degradation. Yet even “natural” products can have toxic effects on the environment. Beside leaf bleaching, these herbicides can trigger oxidative stress that disrupts cell metabolism. A toxicity study of sulcotrione (90) showed up-regulation of the genes inducing ROS and antioxidant enzymes in the fava bean cells (*Vicia faba*), followed by increased lipid peroxidation. Some pathogens, in turn, such as *E. coli* DH5-α (91) and *Pantoea ananatis* (92), showed resistance to oxidative stress caused by mesotrione, while human liver cancer HepG2 cells were only mildly affected by tembotrione (93).

Because these chemicals are relatively new, knowledge about their in vitro or in vivo effects is still very modest, and their extensive use and the formation of many
degradation products call for intensive research and real risk assessment.

Conflicts of interest

None to declare.

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Oksidacijsko-reduksijska neravnoteža uzrokovana pesticidima: pregled istraživanja vezanih uz OPENTOX

Pesticidi su raznovrsna skupina spojeva i glavni izvor stresa u okolišu. Mehanizmi kojima se nastoji objasniti toksičnost pesticida kontinuirano se istražuju, a njihove interakcije na staničnoj razini često se promatraju u sklopu kontroliranih in vitro istraživanja. Za nekoliko skupina pesticida utvrđeno je da narušavaju oksidacijsko-reduksijsku ravnotežu u stanići, a mehanizmi koji vode do nastanka oksidacijskoga stresa za pojedine su pesticide još uvijek nedovoljno poznati. Budući da je naš znanstveni projekt "Organska zagađivala u okolišu-markeri i biomarkeri toksičnosti (OPENTOX)" posvećen istraživanju toksičnih učinka odabranih insekticida i herbicida, ovaj pregledni rad usmjeren je na prikaz spoznaja koje se odnose na promjene uzrokovane stresom na staničnoj razini. Željeli smo izdvojiti najvažnije činjenice koje su bitne za procjenu vlastitih rezultata istraživanja provedenih na in vitro staničnim modelima.

KLJUČNE RIJEČI: antioksidansi; apoptoza; glifosat; in vitro; neonikotinoidi; organofosfati; oksidacijski stres; piretroidi; reaktivni kisikovi spojevi