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Mechanism of Neonicotinoid Toxicity: Impact on Oxidative Stress and Metabolism

Xu Wang,[1,2] Arturo Anadón,[1] Qinghua Wu,[3,4] Fang Qiao,[5] Irma Ares,[1] María-Rosa Martínez-Larrañaga,[1] Zonghui Yuan,[2,5,6] and María-Aránzazu Martínez[1]

[1]Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, 28040 Madrid, Spain; email: aanadon@ucm.es

[2]National Reference Laboratory of Veterinary Drug Residues (HZAU) and MAO Key Laboratory for Detection of Veterinary Drug Residues, Huazhong Agricultural University, Wuhan, Hubei 430070, China; email: yuan5802@mail.hzau.edu.cn

[3]College of Life Science, Yangtze University, Jingzhou 434025, China

[4]Center for Basic and Applied Research, Faculty of Informatics and Management, University of Hradec Králové, Hradec Králové 50003, Czech Republic

[5]MAO Laboratory for Risk Assessment of Quality and Safety of Livestock and Poultry Products, Huazhong Agricultural University, Wuhan, Hubei 430070, China

[6]Hubei Collaborative Innovation Center for Animal Nutrition and Feed Safety, Wuhan, Hubei 430070, China

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neonicotinoids, oxidative stress, ROS, toxicology, mechanisms, imidacloprid, thiamethoxam, clothianidin

Abstract

Thousands of tons of neonicotinoids are widely used around the world as broad-spectrum systemic insecticides and veterinary drugs. Researchers originally thought that neonicotinoids exhibited low mammalian toxicity. However, following their widespread use, it became increasingly evident that neonicotinoids could have various toxic effects on vertebrates and invertebrates. The primary focus of this review is to summarize the research progress associated with oxidative stress as a plausible mechanism for neonicotinoid-induced toxicity as well as neonicotinoid metabolism. This review summarizes the research conducted over the past decade into the production of reactive oxygen species, reactive nitrogen species, and oxidative stress as a

result of neonicotinoid treatments, along with their correlation with the toxicity and metabolism of neonicotinoids. The metabolism of neonicotinoids and protection of various compounds against neonicotinoid-induced toxicity based on their antioxidative effects is also discussed. This review sheds new light on the critical roles of oxidative stress in neonicotinoid-induced toxicity to nontarget species.

INTRODUCTION

As a systemic seed or in-furrow treatment to protect seedling crops from piercing-sucking and chewing insects, neonicotinoids are now registered and approved for use on hundreds of field crops in over 120 different countries (1–3). Furthermore, neonicotinoids such as imidacloprid (IMI) and nitenpyram (NIT) are also used for flea control on cats and dogs (4, 5). After more than 20 years of use, neonicotinoids currently dominate the insecticide market, with global annual sales in excess of $3.5 billion. The annual worldwide production of the active substance in neonicotinoids was estimated to be approximately 20,000 tons in 2010. These impressive figures can be attributed to their broad pest spectrum, the variety of application methods, and the relatively low associated risk to nontarget species (6, 7). The main regions of neonicotinoid use are Latin America, Asia, North America (75% of total use), and Europe (11% of total use) (8). IMI, thiamethoxam (TMX), thiacloprid (THI), NIT, acetamiprid (ACE), clothianidin (CLO), and dinotefuran (DIN) are the most commonly used neonicotinoids worldwide (7). TMX, IMI, and CLO accounted for almost 85% of the total neonicotinoid sales in crop protection in 2012 (8). IMI was one of the most widely used and was applied for over 140 agricultural crops, with approximately 14,000 tons of it produced annually in China alone (9–11). Sulfoxaflor (SUL), cycloxaprid (CYC), paichongding (IPP), and imidaclothiz are newly developed neonicotinoid-like insecticides (12–19). In recent years, SUL has been approved for use in China and the United States (7, 20). IPP and CYC are *cis*-neonicotinoids, which have been developed and tested in China and may soon be available on the Chinese market (21–24). The chemical structures of these systemic pesticides are presented in **Figure 1**.

Compounds are widely classified as neurotoxicants if they can disrupt normal cholinergic signaling, such as the nicotinic cholinergic agonist nicotine (25). Neonicotinoids are regarded as neurotoxicants because they act as agonists against nicotinic acetylcholine receptors (nAChRs) in insects and mammals (25). Compared to organophosphate pesticides (once widely used pesticides), neonicotinoids are thought to have reduced toxicity due to their presumed selectivity for insects over vertebrate nicotinic cholinergic receptors. Neonicotinoids selectively bind to insect nicotinic receptors with reduced action on the vertebrate nicotinic receptors (26, 27). Therefore, neonicotinoids might have lower neurotoxicity profiles for birds, fish, and mammals. However, recent studies suggest that the neurotoxicity induced by neonicotinoids should be given more attention (28). The fast-growing use of neonicotinoids in recent years has seen a concurrent dramatic increase in the number of acute neonicotinoid poisoning cases reported worldwide, such as in Taiwan (29). Alarmingly, reports of severe human toxicity attributed to neonicotinoids are emerging. THI, IMI, and ACE poisoning resulted from deliberate ingestion in humans, manifesting with neurotoxicities, such as status epilepticus, convulsions, and hypotension (28, 30–32). The absence of an effective antidote raises concern in this regard (31).

In spite of the original belief that neonicotinoids have low mammalian toxicity, there is increasing evidence that neonicotinoids could also cause a variety of toxic effects on animals and humans, such as neurotoxicity, immunotoxicity, hepatotoxicity, nephrotoxicity, and reproductive cytotoxic effects on vertebrates and invertebrates (25, 33–39).

IMI: imidacloprid
NIT: nitenpyram
TMX: thiamethoxam
THI: thiacloprid
ACE: acetamiprid
CLO: clothianidin
DIN: dinotefuran
SUL: sulfoxaflor
CYC: cycloxaprid
IPP: paichongding
nAChR: nicotinic acetylcholine receptor

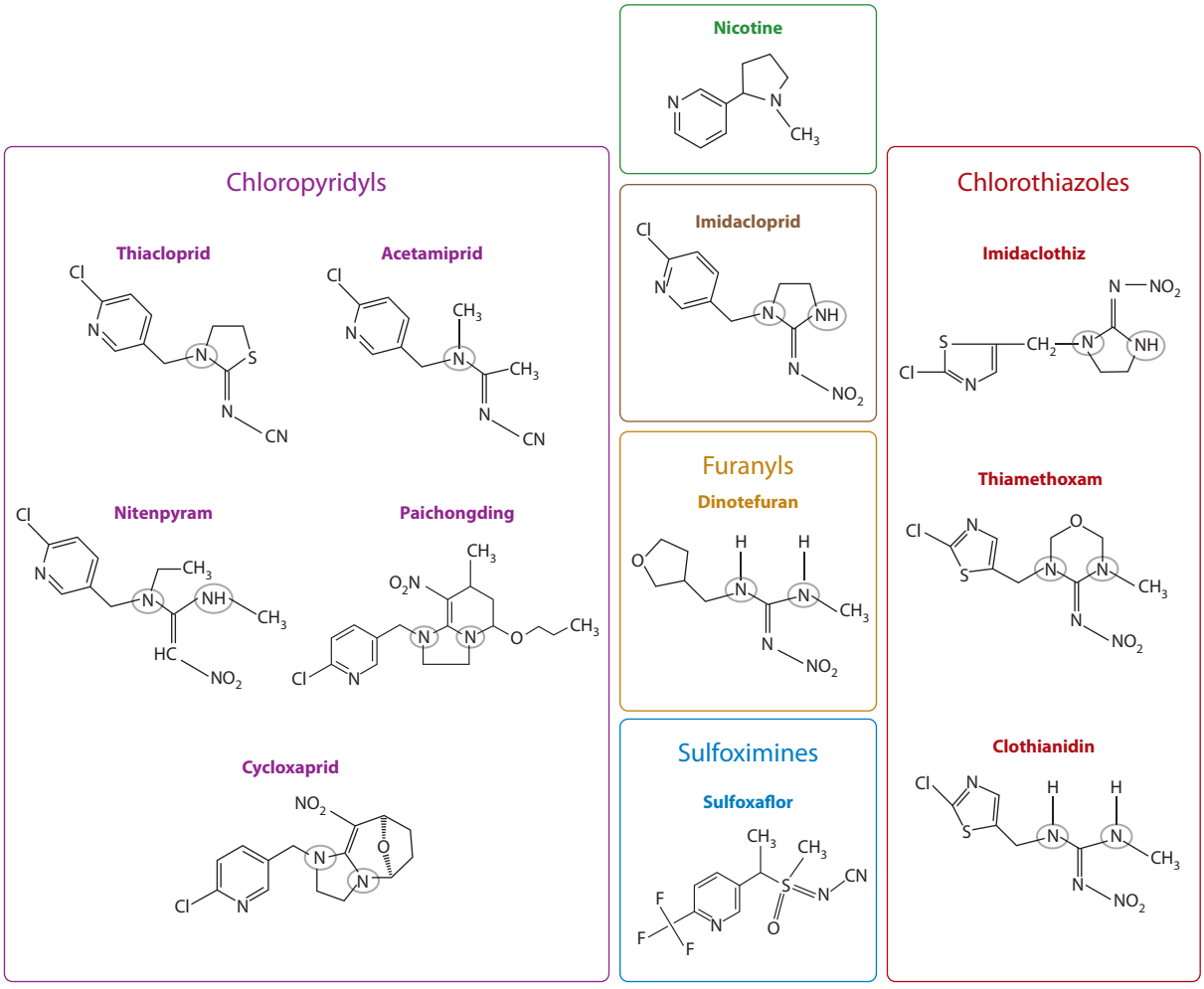

Figure 1

Chemical structures of some of the most important neonicotinoid insecticides. Neonicotinoids, agonists at nicotinic acetylcholine receptors, possess either a nitromethylene (CH-NO_2), nitroimine (N-NO_2), or cyanoimine (N-CN) group. Moreover, neonicotinoids, except sulfoximine insecticides, have at least one amine nitrogen. Imidacloprid is the first representative of the neonicotinoid insecticides (first-generation chloropyridyls). Other neonicotinoids include thiacloprid, acetamiprid, nitenpyram, paichongding, and cycloxaprid (first-generation chloropyridyls); imidaclothiz, thiamethoxam, and clothianidin (second-generation chlorothiazoles); dinotefuran (third-generation furanyls); and sulfoxaflor (fourth-generation sulfoximines).

Neonicotinoids such as IMI could change the concentrations of some kinds of hormones in animals. For example, when researchers exposed red munia (a small bird, commonly known as the strawberry finch) to IMI through the diet, plasma levels of triiodothyronine, thyroxine, and thyroid-stimulating hormone changed significantly, indicating that low-dose IMI exposure could affect thyroid homeostasis and reproduction (40). Concentrations of IMI of 45 and 90 mg/kg body weight (b.w.) resulted in significant decreases in 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD enzymatic activity and testosterone concentrations in the testis and plasma (41). When rats were treated with various concentrations of IMI for 90 days, the relative testosterone concentrations were lower in the treated groups than in the controls (42).

3β-HSD: 3β-hydroxysteroid dehydrogenase

17β-HSD: 17β-hydroxysteroid dehydrogenase

Neonicotinoids have been shown to induce reproductive toxicity in vertebrates. When rats were administered with 0.5, 2, and 8 mg/kg b.w. IMI for 90 days, researchers observed significant deteriorations in sperm motility in the highest group, epididymal sperm concentration in the middle and the highest groups, and abnormality in sperm morphology in the highest group (33). Najafi et al. (43) reported that IMI (112 and 225 mg/kg b.w.) exposure induced a histologically adverse effect on testicular tissue, spermatogenesis, and sperm viability and velocity. CLO treatment, at concentrations of 2, 8, and 24 mg/kg b.w., induced significant decreases in the weights of the epididymis, right cauda epididymis, and seminal vesicles of adult rats (44). At a concentration of 32 mg/kg b.w., CLO also significantly decreased the absolute weights of the right cauda epididymis and seminal vesicles, the epididymal sperm concentration, and the testosterone level when compared to the control group (45). A recent study documented that daily doses of CLO (10, 50, and 250 mg/kg b.w./day) for 4 weeks degenerated the seminiferous epithelia under an unpredictable chronic stress procedure dose dependently, suggesting that even low concentrations of CLO could become harmful under stress conditions, such as fasting (46). In a related bird study, CLO (1 and 50 mg/kg b.w.) affected the reproduction of the male quail through the fragmentation of germ cells and the inhibition or delay of embryonic development (47). In a study to examine the effect of ACE (30 mg/kg b.w.) on the reproductive function of male mice, ACE exposure resulted in damage to the seminiferous tubules and Leydig cells and the degeneration of the mitochondria and endoplasmic reticulum of Leydig cells (48). Furthermore, high-dose dietary exposure of SUL caused primarily limb contractures and reduced neonatal survival in rats (49).

The immunotoxic effects of neonicotinoids have received enormous interest as their general use has increased rapidly worldwide, and some of the principal findings in this regard are summarized below. Duzguner & Erdogan (50) reported in rats that exposure to 10 μM IMI upregulated mRNA transcription of the inflammatory cytokines tumor necrosis factor-α (TNF-α), IL-6, and IL-1β 2.5–5.2-fold in both the brain and the liver. Similarly, a rat study by the same group reported that after exposure to IMI (1 mg/kg b.w.) for 30 days, chronic inflammation was observed with an increase in proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, and interferon-γ in the liver and brain, indicating IMI induced proinflammatory cytokine production in the liver and central nervous system of nontarget organisms (51). When IMI (2.5, 5, and 10 mg/kg b.w.) was administered to mice over 28 days, a high dose of IMI specifically suppressed the cell-mediated immune response, including decreasing the response of delayed-type hypersensitivity and decreasing the stimulation index of T lymphocytes to phytohemagglutinin. These results suggest that IMI has immunosuppressive effects at doses >5 mg/kg b.w., and long-term IMI-exposure could be detrimental to the immune system (52). Gawade et al. (53) reported that IMI (10, 30, and 90 mg/kg b.w.) caused age-dependent adverse effects on the developing immunity of canine pups, which led to a compromised immune system when the pups were exposed to IMI in utero through dams, followed by exposure through lactation through weaning and subsequently by oral administration to young animals until puberty. Low doses (about 8.8 mg/kg b.w.) and high-exposure doses of IMI (about 53.4 mg/kg b.w.) have been shown to depress the T cell immune response and cellular immune response in adult partridges, respectively (54, 55). Mohany et al. (56) reported significant increases in the total leukocyte count, total immunoglobulins (Igs) (especially IgGs), and the hemagglutination of antibodies, as well as significant decreases in phagocytic activity, chemokine expression, and chemotaxis, after rats were subjected to 28 days of IMI exposure (0.21 mg/kg b.w.). Immunotoxicity has also been observed in TMX-treated animals. When mice were administered with TMX (43.5 and 87.1 mg/kg b.w.) for 28 days, they had hemosiderosis or extramedullary hematopoiesis along with mild congestion and depletion of lymphocytes in the spleen in both dosage groups. Significant dose-dependent decreases in the total leukocyte count and lymphocyte count were also noted (57). Similarly, in a study to evaluate the immunotoxicological potential of

ACE (27.5, 55, and 110 mg/kg b.w.) for 90 days in rats, administration of ACE was shown to significantly decrease the stimulation index of lymphocyte proliferation to B cell mitogen. Moreover, when rats were treated with 110 mg/kg of ACE, the nitrite production of macrophages, which is important for efficient inflammatory macrophage response, was suppressed (58).

Multiorgan toxicity induced by neonicotinoids has also been reported. For example, mice exposed to IMI (10 mg/kg b.w.) over 28 days displayed prominent histopathological alterations in the spleen and liver (52). In a 90-day oral toxicity study of IMI (5, 10, and 20 mg/kg b.w.) in female rats, a dose of 20 mg/kg b.w. led to significant increases in the relative body weights of liver, kidney, and adrenal glands and resulted in mild pathological changes in the brain, liver, and kidneys (59). When laying chickens were exposed to IMI (139 mg/kg b.w.), the activity of liver function enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) significantly increased followed by histopathological changes in the liver and kidneys (60). IMI-exposed rats had increased ALT, AST, and ALP activities and severe histopathological lesions in the liver, spleen, and thymus (56). When rats were given 80 mg/kg b.w. IMI daily by oral gavage for 28 days, significant histological changes such as swollen nuclei, varied size and shape of mitochondria, disrupted chromatin, and rough endoplasmic reticulum in the liver were noted (61). After rats were exposed to IMI (9 and 45 mg/kg b.w. for 4 weeks), significant increases in the levels of liver AST and plasma levels of AST, ALT, and ALP were observed (62). After oral administration of IMI (10 and 20 mg/kg b.w.) to rats for 60 days, the rats administered with the higher dosage showed marked dilation and congestion of the central vein, and a degeneration of hepatocytes was also observed (63). Yardimci et al. (39) recently documented that after exposure to 170 mg/kg b.w. IMI for 12 and 24 h, male and female rats displayed prooxidative and neurotoxic effects, predominantly in the kidneys of male rats after 24 h of exposure. Their results indicate that sex-, tissue-, and duration-specific effects of IMI in relation to its toxicity should be considered. In quail, only 0.62 mg/kg b.w. IMI lead to notable liver histological changes (64). Yeh et al. (32) reported that in humans, the ingestion of alcohol with an IMI-containing insecticide led to acute multiple organ failure, including oliguric kidney injury and acute lung injury within hours of ingestion.

Although TMX is not mutagenic either in vitro or in vivo, it has been reported to lead to an increased incidence of liver tumors in mice fed concentrations in the range of 500 to 2,500 mg/kg b.w. (TMX/diet) for 18 months (65). Similarly, THI poisoning can result in multiorgan toxicities including acute kidney injury and has actually caused fatal human toxicity when ingested heavily (31). In fish, NIT exposure induced DNA damage in zebrafish livers (66).

Oxidative stress, reactive oxygen species (ROS), and reactive nitrogen species (RNS) may play important roles in the induction of neonicotinoid-induced damage to lipids, DNA, and proteins in vertebrates and invertebrates. For this reason, the influence of oxidative stress, ROS, and RNS on neonicotinoid-associated neurotoxicity, immunotoxicity, hepatotoxicity, nephrotoxicity, and reproductive cytotoxic effects has been investigated (33, 50, 51, 56, 66–68). To date, several reviews on neonicotinoids have been published, including those that have focused on the insect resistance of neonicotinoids (8), the risk of neonicotinoids to ecosystem function and service (69), the impact of neonicotinoids on bees (70), the effects of neonicotinoids on vertebrate wildlife (71), enzyme-linked immunosorbent assays for the analyses of neonicotinoids (72), and the ecotoxicity of neonicotinoids to bees (73). In recent years, the toxicity and toxic mechanisms of neonicotinoids on nontarget organisms have attracted more and more attention, and some articles about the important role of oxidative stress in the various toxicities of neonicotinoids have been published. Therefore, it is prudent at this point to review the recent progress in research focused on the toxic mechanism of neonicotinoids. The scope of this review is primarily intended to summarize the evidence associated with neonicotinoid-induced toxicity and oxidative stress. The studies related

AST: aspartate aminotransferase

ALT: alanine aminotransferase

ALP: alkaline phosphatase

ROS: reactive oxygen species

RNS: reactive nitrogen species

to toxicity of neonicotinoids and oxidative stress in in vivo and in vitro conditions, respectively, are summarized in **Table 1** and **2**. The metabolic pathways, metabolizing enzymes, influential factors in the metabolism of neonicotinoids, and toxicity of neonicotinoid metabolites are also reviewed. In the future, as the application of neonicotinoids continues on an upward trend worldwide, neonicotinoids may pose a threat to more than just insects, and their toxicities to vertebrates and invertebrates should be investigated further. This review collates evidence reported over the past 10 years, which indicates that levels of oxidative stress, ROS or RNS generation, and antioxidase might correlate closely with various types of toxicity associated with neonicotinoids. Furthermore, information on the metabolism of neonicotinoids is summarized with a view to probing effective strategies for the application of antioxidants to inhibit neonicotinoid-induced toxicity.

HO•: hydroxyl radical

$O_2^{\bullet-}$: superoxide anion

NO: nitric oxide

iNOS: induced nitric oxide synthase

nNOS: neuronal nitric oxide synthase

eNOS: endothelial nitric oxide synthase

OXIDATIVE STRESS AND TOXICITY

Generation of Oxidative Stress, Reactive Oxygen Species, and Reactive Nitrogen Species

Oxidative stress occurs as a result of inadequate antioxidant defense or overproduction of free radicals and is initiated by ROS such as the hydroxyl radical (HO•), superoxide anion ($O_2^{\bullet-}$), and perhydroxyl radical and by RNS including nitric oxide (NO) and peroxynitrite (74–76).

Neonicotinoids may induce oxidative stress leading to ROS or RNS generation and related toxic effects (51, 67, 68). Recently, Ge et al. (68) reported that high concentrations of IMI (1.25 and 5 mg/L) could induce significant ROS production in zebrafish. Furthermore, even after 7 days of exposure, IMI (5 mg/L) could also lead to significant ROS generation. Similarly, exposure to NIT increased ROS production with increasing concentrations in the liver of zebrafish (66). Another study to evaluate the effects of IMI on antioxidant defense systems and digestive systems in earthworms investigated the ROS levels at different doses of IMI. The results indicated that with IMI doses of 2 and 4 mg/kg for 14 days, ROS generation was elevated significantly over the entire exposure period; at 0.66 mg/kg exposure, significant increases of ROS were recorded from day 1 to 7, whereas the low dose of 0.2 mg/kg did not induce ROS production. These results suggest that the balance of the activity of the antioxidant enzymes and the ROS levels was interrupted when the concentration of IMI was above 0.66 mg/kg, and the IMI influence led to dose- and time-dependent ROS generation (77).

Neonicotinoids may present their dangerous effects on animals in the form of NO generation (48). When rats were injected intravenously with 0.26 mg/kg b.w. IMI, NO levels in the plasma, brain, and liver increased significantly. Interestingly, the transcription of induced nitric oxide synthase (iNOS) in the liver increased significantly (6.54-fold), whereas both neuronal nitric oxide synthase (nNOS) and iNOS transcriptions were found to be downregulated in the brain (3.55-fold and 6.34-fold, respectively), suggesting that the elevated NO concentration in the brain might be due to the induction of endothelial nitric oxide synthase (eNOS) transcription (50). However, this assumption needed further study. When IMI was orally administered to rats by gavage for 30 days, IMI exposure caused oxidative stress and a significant increase in NO production in the brain and liver. This further study confirmed that IMI induced the mRNA transcription of the three isoforms of nitric oxide synthases (iNOS, eNOS, and nNOS) in the brain and two isoforms (iNOS and eNOS) in the liver (51). Zhang et al. (48) postulated that the toxic effects induced by ACE on the testis of male mice may be mediated by increasing oxidative stress, such as NO generation. Aydin (78) reported that THI increased the total NOx (NO_2 and NO_3) levels in polymorphonuclear leukocytes significantly when rats were exposed to THI.

Table 1 In vivo neonicotinoid-related oxidative stress studies

Species	Length of exposure	Dose	Objective	Results and conclusion	Reference
Rats					
Male Wistar rats	28 days	IMI (40 mg/kg b.w./day, orally) + NAC (2 g/L, orally, 7 days since the last day of IMI treatment)	Evaluate the recovery effect of NAC against IMI-induced oxidative stress and cholinergic transmission alteration in HPA axis of male rats	IMI showed toxic effects on the HPA axis. IMI caused a significant increase in MDA level, the activities of CAT, and SOD and decreased the activities of GST and total SH significantly. NAC decreased the levels of MDA, adrenal cholesterol, and GST activity and increased the activity of SOD and CAT in pituitary and adrenal glands significantly. NAC also decreased the AChE activity in hypothalamus and pituitary gland as compared to the IMI-treated group.	83
Male Wistar rats	28 days	IMI (45 and 90 mg/kg b.w./day, orally) CUR (100 mg/kg b.w./day, orally) IMI + CUR	Establish the toxic effects of IMI on the male reproductive system and the ameliorative effect of CUR	IMI decreased total epididymal sperm count, sperm motility, live sperm count, activities of 3β-HSD and 17β-HSD, and testosterone concentration in testis and plasma and increased sperm abnormalities, activities of gamma-glutamyl transpeptidase, LDH, and sorbitol dehydrogenase. IMI significantly increased MDA levels and decreased GSH levels and the activities of CAT, SOD, GPx, and GST. CUR could minimize the reproductive toxicity parameters and histopathological changes induced by IMI along with restoring the significant increase of ROS generation and MDA content, the significant decreases of GSH level, and the activities of CAT, GST, SOD, and GPx in testis.	41
Male and female Wistar rats	90 days	ACE (5.5, 11, and 22 mg/kg b.w./day, orally)	Assess the immunotoxicity of ACE	ACE decreased the lymphocyte proliferation and macrophage function at 22 mg/kg b.w./day. Generation of free radicals was involved in the functional impairment of macrophages.	58

(*Continued*)

Table 1 (*Continued*)

Species	Length of exposure	Dose	Objective	Results and conclusion	Reference
Male Wistar rats	90 days	IMI (0.5, 2, and 8 mg/kg b.w./day, orally)	Examine the toxic effects of IMI on the reproductive system	IMI at 2 and 8 mg/kg b.w. caused deterioration in sperm parameters, decreased testosterone and GSH levels (only 8 mg/kg b.w. dose affected GSH levels); increased apoptosis of germ cells, fragmentation of seminal DNA, and the depletion of antioxidants; and disturbed fatty acid composition. The reproductive NOAEL was 0.5 mg/kg b.w.	33
Male Wistar rats	28 days	IMI (45 and 90 mg/kg b.w./day, orally) CUR (100 mg/kg b.w./day, orally) IMI + CUR	Evaluate the neurotoxic effect and protective role of CUR against IMI	IMI led to meningeal congestion and degeneration changes in Purkinje cells in rat cerebellum and decreased spontaneous locomotor activity and pain threshold in rats. IMI significantly decreased the paw withdrawal threshold, increased MDA content and decreased the activities of SOD, GSH, GPx, and CAT. CUR caused a significant increase in the pain threshold values, protected from the decrease in AChE activity, and increased GSH level and CAT, SOD, and GPx activity.	36
Male and female Sprague-Dawley rats	12 and 24 h	IMI (170 mg/kg b.w.) alone or with piperonyl butoxide (100 mg/kg b.w.) or menadione (25 mg/kg b.w.), IP dosing	Assess the effects of the metabolic modulators, piperonyl butoxide, and menadione on IMI's adverse action in the liver and kidney	IMI displayed pro-oxidative and neurotoxic effects, predominantly in the kidney of male rats after 24 h of exposure. Total GSH decreased in the liver in male rats after 12 h of IMI exposure. Total GSH and TBARS increased in the kidney of male rats after 24 h of exposure. GST activity increased in female rats after 24 h of exposure. IMI-induced toxicity had sex-, tissue-, and duration-specific effects as well as oxidative stress.	39
Male Sprague-Dawley rats	4 weeks	IMI (80 mg/kg b.w./day) + vitamin C (10 mg/kg b.w./day, orally)	Assess the toxic effects of IMI on the liver and the protective effects of vitamin C	IMI led to significant pathological change in liver and decreased serum ALT, AST, and total protein. IMI significantly reduced GSH concentration in the liver, and vitamin C could significantly increase GSH level and protect against IMI-induced oxidative stress.	61

(*Continued*)

Table 1 (*Continued*)

Species	Length of exposure	Dose	Objective	Results and conclusion	Reference
Male Wistar rats	90 days	CLO (2, 8, and 24 mg/kg b.w./day, orally)	Determine the effect of subchronic exposure to low doses of CLO on the reproductive system in adult rats	CLO caused significant decreases in weights of epididymis, right cauda epididymis, and seminal vesicles and increased palmitic, linoleic, and arachidonic acids significantly in testis. CLO increased TBARS significantly in testicular tissue, and GSH level did not change significantly in all treated groups.	44
Male Wistar rats	90 days	CLO (2, 8, and 32 mg/kg b.w./day, orally)	Assess the deleterious effects of CLO on reproductive functions in developing male rats	CLO decreased the absolute weights of right cauda epididymis, seminal vesicles, testosterone level, body weight, epididymal sperm concentration and increased the abnormal sperm rates, docosapentaenoic, arachidonic, palmitic and palmitoleic acid. CLO resulted in a significant decrease in the GSH level in the testicular tissue.	45
Male Wistar rats	90 days	IMI (0.5, 2, and 8 mg/kg b.w./day, orally)	Assess the toxic effects of IMI on morphology, DNA fragmentation, antioxidant imbalance and apoptosis in the reproductive system	The three doses of IMI are capable of altering significant reproductive functions. All treated groups had increased lipid peroxidation, fatty acid concentrations, and rates of abnormal sperm. Apoptosis and fragmentation of seminal DNA were higher in rats treated at the two higher doses of IMI.	42
Wistar rats	30 days	IMI (1 mg/kg b.w./day, orally)	Evaluate oxidant and inflammatory responses to chronic exposure of IMI	IMI caused chronic inflammation and increased gene expressions of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 and IFN-γ in the liver and brain. IMI induced a significant increase in NO production in brain. IMI induced the mRNA transcription of iNOS, eNOS, and nNOS in brain and iNOS and eNOS in the liver. IMI significantly increased CAT activity in brain and increased MDA levels in plasma, brain, and liver. XO activity was elevated in liver and brain; MPO activity was increased only in the liver. GSH levels were significantly depleted in brain.	51

(*Continued*)

Table 1 (*Continued*)

Species	Length of exposure	Dose	Objective	Results and conclusion	Reference
Male albino rats	28 days	IMI (0.21 mg/kg b.w./day, orally) IMI (0.21 mg/kg b.w./day, orally) + TQ (1 mg/kg b.w. once every 7 days, orally)	Examine the impact of TQ on IMI-induced immunotoxicity	IMI induced a significant increase in total leukocyte counts; total immunoglobulins; the hemagglutination of antibodies; and ALT, AST, ALP, and MDA content and significant decreases in phagocytic activity, chemokine expression, and chemotaxis. TQ could ameliorate IMI toxicity by decreasing MDA level and enhancing immune efficiency.	56
Male Wistar rats	24 h and 30 days	THI (single acute dose of 112.5 mg/kg b.w.; subacute dose of 22.5 mg/kg b.w./day for 30 days)	Assess the effects on mammalian antioxidant-oxidant and inflammatory system responses	THI caused significant changes in the levels of AST and ALT. THI significantly decreased CAT, GPx, and GSH levels in lymphoid organs and significantly increased total NOx (NO_2 and NO_3) in polymorphonuclear leukocytes and TBARS levels in all lymphoid organs and the plasma.	78
Female Wistar rats	90 days	IMI (5, 10, and 20 mg/kg b.w./day, orally)	Establish the effect of IMI on ovarian morphology, hormones, and antioxidant enzymes	IMI decreased ovarian weight, and significant pathomorphological changes in follicles, antral follicles, and atretic follicles were observed at 20 mg/kg b.w./day. High doses of IMI produced significant alterations in the levels of luteinizing hormone, follicle-stimulating hormone, and progesterone. 20 mg/kg b.w./day significantly increased MDA content and significantly decreased SOD, CAT and GPx activities, and GSH content in ovary.	82
Female Wistar rats	2 h	IMI (10 μM equivalent to 2.6 mg/100 g b.w.), IV administration	Evaluate potential acute brain and liver toxicity	IMI increased mRNA transcription of the inflammatory cytokines TNF-α, IL-6, and IL-1β in both brain and liver and decreased anti-inflammatory mediator IL-10 mRNA in brain and liver. IMI increased the production of NO levels in liver and did not induce nNOS and iNOS in brain. The XO and MPO activities in liver and brain were elevated. MDA increased significantly in liver and plasma. CAT, SOD, and GPx activities responded differently to IMI administration. GSH level was significantly decreased in the liver and brain.	50

(*Continued*)

Table 1 (*Continued*)

Species	Length of exposure	Dose	Objective	Results and conclusion	Reference
Female Wistar rats	2 h	IMI (10 μM equivalent to 2.6 mg/100 g b.w.), IV administration	Evaluate potential acute brain and liver toxicity	IMI increased mRNA transcription of the inflammatory cytokines TNF-α, IL-6, and IL-1β in both brain and liver and decreased anti-inflammatory mediator IL-10 mRNA in brain and liver. IMI increased the production of NO levels in liver and did not induce nNOS and iNOS in brain. The XO and MPO activities in liver and brain were elevated. MDA increased significantly in liver and plasma. CAT, SOD, and GPx activities responded differently to IMI administration. GSH level was significantly decreased in the liver and brain.	50
Female Wistar rats	90 days	IMI (5, 10, and 20 mg/kg b.w./day); orally	Test the effect of IMI on oxidative stress and LPO in rats	IMI dose of 20 mg/kg b.w./day significantly decreased GSH level in liver and the activities of GPx, CAT, and SOD in liver and brain and significantly increased MDA content in brain and kidney. 10 mg/kg b.w./day may be considered as NOAEL through antioxidant enzymes and LPO in female rats.	81
Mice					
Male C57BL/6NCrSlc mice	4 weeks	CLO (0, 10, 50, and 250 mg/kg b.w./day, orally	Evaluate the combined effects of CLO and environmental stress on vertebrates	CLO resulted in seriously degenerated seminiferous tubules and inhibited the expression of GSH-Px4 in the testes.	46
Kunming male mice	35 days	ACE (30 mg/kg b.w./day) + vitamin E (20 mg/kg b.w./day, orally)	Examine the effect of ACE on the reproductive function of male mice and the protective effect of vitamin E	ACE exposure resulted in damage to the seminiferous tubules and Leydig cells and the degeneration of the mitochondria and endoplasmic reticulum of Leydig cells. ACE increased MDA and NO concentrations in the testes; reduced the activity of CAT, GPx, SOD; and activated p38. Vitamin E increased the concentrations of CAT, GPx, and SOD and prevented the elevation of p38 activity.	48
Male Swiss albino mice	24 h	IMI (14.976 mg/kg b.w., orally) + vitamin C (200 mg/kg b.w., orally) before and after IMI dosing	Evaluate the protective effects of vitamin C	IMI significantly increased MDA levels and CAT, SOD, GPx and GST activities and decreased GSH level. Vitamin C significantly decreased MDA level and the activity of CAT, GST, SOD, and GPx and significantly increased GSH activity.	67

(*Continued*)

Table 1 (*Continued*)

Species	Length of exposure	Dose	Objective	Results and conclusion	Reference
Birds					
Male and female red-legged partridge (*Alectoris rufa*)	10 and 25 days	IMI (8.8 and 44 mg/kg b.w./day, orally)	Evaluate toxic effects caused by the ingestion of IMI-treated seeds	The high dose killed all partridges. The low dose had no effect on mortality but reduced levels of plasma biochemistry parameters (glucose, magnesium, and LDH), produced changes in carotenoid-based integument coloration, reduced clutch size, delayed the first egg lay date, increased egg yolk vitamins and carotenoids, and depressed T cell immune response of chicks.	55
Male and female red-legged partridge (*Alectoris rufa*)	10 days	IMI (31.9–53.4 mg/kg b.w./day, orally)	Evaluate the lethal and sublethal effects caused by the ingestion of seeds treated with difenoconazole, thiram, or IMI	High dose of IMI produced mortalities of 58.3%. IMI caused sublethal effects, such as altered biochemical parameters, oxidative stress, and reduced carotenoid-based coloration. The high-exposure doses of IMI produced a decrease in cellular immune response in males. IMI reduced the size of eggs, fertilization rate, and chick survival and significantly decreased the GSH level and the activities of GPx in erythrocytes.	54
Male quail	30 days	CLO (0.02, 1, and 50 mg/kg b.w./day, orally)	Evaluate the deleterious effects of CLO on the reproductive functions of mature male quails	CLO induced lipid droplets in liver and caused DNA fragmentation in the testis with the increase of vacuolization in the seminiferous epithelia. CLO decreased the number of germ cells in a dose-dependent manner, indicating it probably affected the liver and reproductive functions through oxidative stress.	47
Male Japanese quail	3 and 6 weeks (3 weeks recovery)	IMI (0.62 mg/kg b.w.) + vitamin C (0.08 mg/kg b.w.) or + GSH (0.55 mg/kg b.w., orally)	Evaluate the histological changes in liver and testis of quail and the effect of vitamin C as a protective agent	IMI led to degenerative changes in liver, such as highly dilated portal spaces, large degenerated area, and faintly stained cells and nuclei, and degenerative changes in testis, such as bizarre cells, disappearance of spermatogenetic cells, thickened tunica albuginea, tubules devoid of sperm, and some pyknotic nuclei. GSH and vitamin C could protect against liver and testis damage induced by IMI.	64

(*Continued*)

Table 1 (*Continued*)

Species	Length of exposure	Dose	Objective	Results and conclusion	Reference
Fish					
Male and female zebrafish (*Danio rerio*)	7, 14, 21, and 28 days	IMI (0.3, 1.25, and 5 mg/L), dissolved in water	Assess the toxicity of IMI on zebrafish	IMI can induce oxidative stress and DNA damage in zebrafish. IMI led to dose- and time-dependent DNA damage. IMI (1.25 and 5 mg/L) induced excessive ROS production and markedly increased MDA content on the twenty-first day of exposure. SOD and GST activities were increased during early exposure but were inhibited toward the end of the exposure period.	68
Male and female zebrafish (*Danio rerio*)	7, 14, 21, and 28 days	NIT (0.6, 1.2, 2.5, and 5.0 mg/L), dissolved in water	Investigate oxidative stress, changes in the detoxifying system, and DNA damage	A significant dose-response relationship could be observed. NIT caused DNA damage in the exposed zebrafish livers. SOD and CAT activities were inhibited; ROS production, GST activity, DNA damage, and MDA content increased.	66
Earthworms					
Earthworm *Eisenia fetida*	7, 14, and 28 days	THI (1 and 3 mg/kg dry soil)	Test effects of THI on molecular biomarkers (GST, CarE, CAT, SOD, POD, and DNA damage)	THI led to significant DNA damage. THI inhibited the activities of GST, CarE, CAT, SOD, and POD.	34
Earthworm *Eisenia fetida*	14 days	IMI (0.2, 0.66, 2, and 4 mg/kg soil)	Test effects of IMI on antioxidant defense and digestive systems	IMI significantly increased ROS level, MDA content, and CAT and POD activities.	77

(*Continued*)

Table 1 (*Continued*)

Species	Length of exposure	Dose	Objective	Results and conclusion	Reference
Other species					
Land snail (*Helix aspersa*)	Single dose	IMI (21.84 μg/snail and 61.15 μg/snail; topical application inside shell cavity)	Evaluate the sublethal toxicity of IMI on biochemical biomarkers at 1, 3, and 7 days after treatment.	IMI caused a significant decrease in AChE activity as well as depletion of lipids and glycogen content. IMI significantly increased the activities of CAT and GST.	92
Honey bee (*Apis mellifera*)	24 and 48 h	TMX (51.16, 5.12, and 2.56 ng/bee)	Develop a set of enzyme biomarkers that could be used to assess bee health	TMX did not change activity of AChE, caused an increase for GST and CAT, and had differential effects for CarE isoforms with a decrease in CarE1 and CarE3 and an increase in CarE2. ALP and CarE3 displayed contrasting variations, but only at 2.56 ng/bee.	146
Terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea)	2 weeks	IMI (0, 2.5, 5, 10, and 50 μg/g dry food)	Assess the toxic effects of IMI in juveniles and adults (both sexes)	After 2 weeks of feeding on IMI-dosed food, weight gain (NOAEC 5 μg/g dry food) and feeding rate (NOAEC 10 μg/g) in juveniles and feeding rate (NOAEC <10 μg/g) and digestive gland epithelial thickness (NOAEC <10 μg/g) in adults were most affected. In juveniles, induction of GST activity and increase of total protein content was detected at 5 μg/g, whereas in adults, a reduction of GST was observed at 25 μg/g (NOAEC 10 μg/g).	9
Freshwater bivalve mussel (*Anodonta cygnea*)	Not reported	Not reported	Test the effect of ACE on the status of oxidative stress biomarkers, neurotoxicity, and metallothioneins	ACE induced the appearance of additional chromatographic metallothioneins' form. ACE decreased SOD activity and GSH content and increased the content of lipid and protein peroxidation products.	89

Abbreviations: ACE, acetamiprid; AChE, acetylcholinesterase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; b.w., body weight; CarE, carboxylesterase; CAT, catalase; CLO, clothianidin; CUR, curcumin; eNOS, endothelial nitric oxide synthase; GPx, glutathione peroxidase; GSH, glutathione; GSH-Px4, glutathione peroxidase 4; GST, glutathione *S*-transferase; HPA, hypothalamic-pituitary-adrenal; IFN-γ, interferon-γ; IMI, imidacloprid; iNOS, induced nitric oxide synthase; IP, intraperitoneal; IV, intravenous; LDH, lactate dehydrogenase; LPO, lipid peroxidation; MDA, malondialdehyde; NAC, *N*-acetyl-L-cysteine; NIT, nitenpyram; nNOS, neuronal nitric oxide synthase; NOAEC, no observed adverse effect concentration; NOAEL, no observed adverse effect level; POD, peroxidase; ROS, reactive oxygen species; SH, thiols content; SOD, superoxide dismutase; TBARS, thiobarbituric acid reacting substances; THI, thiacloprid; TMX, thiamethoxam; TNF-α, tumor necrosis factor-α; TQ, thymoquinone; XO, xanthine oxidase.

Table 2 In vitro neonicotinoid-related oxidative stress studies

Cell type	Length of incubation	Dose	Objective	Results and conclusion	References
CHO_{K1} cells	24 and 48 h	IMI (0.97– 500 μM)	Examine the cytotoxicity and genotoxicity of abamectin, chlorfenapyr, and IMI	IMI presented potential genotoxic effects on CHO_{K1} cells with significant inhibition of the activity of GST, GPx, and GR.	93
Bovine peripheral lymphocytes	2, 24, and/or 48 h	THI (30, 60, 120, 240, and 480 μg/mL)	Assess the potential genotoxicity of THI	THI decreased and increased the expression of bovine GSTM3 at the lowest and highest dose, respectively.	84
Supernatant fraction from the homogenates of liver from male Wistar rats	10 min	IMI (2, 5, 10, 20 and 40 mM) Antioxidants: dithiothreitol (3 mM), $ZnCl_2$ (100 mM), resveratrol (0.001, 0.1, 1, 5, 10, 100, and 1,000 μM), curcumin (0.001, 0.1, 1, 5, 10, 100 and 1,000 μM), ascorbic acid (10, 100, and 1,000 μM), or GSH (10, 100, and 1,000 μM)	Evaluate the effect of IMI on the activity of hepatic δ-ALA-D and the protective effect of some antioxidants	IMI inhibited the activity of δ-ALA-D, and GSH had the best antioxidant effect against δ-ALA-D inhibition caused by IMI, followed by curcumin and resveratrol.	37
Human T lymphocytes (Jurkat cell line)	24 h	IMI (0.2, 2, and 20 μM)	Assess the genotoxicity of IMI in relation to formulation, metabolic activation, and exposure level	No significant increase of intracellular ROS was noted because of an insufficient sensitivity of the ROS assay at the tested concentrations of IMI.	86

Abbreviations: δ-ALA-D, δ-aminolevulinate dehydratase; CHO_{K1}, Chinese hamster ovary; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; GSH, glutathione; GSTM3, glutathione *S*-transferase M3; IMI, imidacloprid; ROS, reactive oxygen species; THI, thiacloprid.

Furthermore, studies suggest that the progress of NO generation induced by neonicotinoids might show organ-dependent effects (39, 50).

The results of these studies indicate that the generation of ROS and RNS play important roles in the oxidative stress and related toxicities induced by neonicotinoids. Currently, it is thought that the oxidative stress induced by neonicotinoids may be dose dependent. Future research should identify what factors affect this dose-dependent behavior and quantify the dose threshold for all neonicotinoids for future risk assessment analyses.

Neonicotinoid-Mediated Oxidative Damage

Oxidative stress induced by neonicotinoids could increase the antioxidant defense system and lead to the damage of cellular macromolecules, such as DNA, lipids, and proteins (79). Following

oxidative stress, cell death can occur via apoptotic or necrotic mechanisms. During this process, DNA damage, enhanced lipid peroxidation, and protein damage may occur (**Tables 1** and **2**). A schematic representation of neonicotinoid-induced damage to DNA, lipids, and proteins is shown in **Figure 2**.

MDA: malondialdehyde

TBARS: thiobarbituric acid reacting substances

HPA: hypothalamic-pituitary-adrenal

Damage to lipids. The significant increase of lipid peroxidation can be attributed to the oxidative damage of cell membrane lipids. Lipid peroxidation can be measured by monitoring the changes in malondialdehyde (MDA) and thiobarbituric acid reacting substances (TBARS). As one part of TBARS, MDA is the most abundant individual aldehyde resulting from lipid peroxidation, and its level is a marker of lipid oxidation (41, 80).

Neonicotinoids can increase lipid peroxidation significantly. In a study of the acute neuro- and hepatotoxic effects of IMI on rats, IMI induced significant increases in the MDA content in the liver and plasma, suggesting that IMI caused oxidative damage in the liver in nontarget organisms (50). El-Gendy et al. (67) reported that the oral administration of 15 mg/kg b.w. of IMI in male mice could elevate MDA levels significantly. Kapoor et al. (81, 82) revealed that in female rats, only 20 mg/kg b.w./day of IMI produced a significant increase in the MDA content of the liver, kidneys, and ovaries in a 90-day study of IMI dosages of 5, 10, and 20 mg/kg b.w./day, suggesting there might be a dose threshold for IMI in leading to oxidative stress in vivo. Lonare et al. (36, 41) studied the effects of IMI on the neurotoxicity and male reproductive processes of rats and found that MDA production increased significantly when rats received orally administered IMI. In an evaluation of the effects of IMI on the reproductive system of developing male rats, the MDA content in the testis of rats increased significantly in all IMI-treated groups compared to the control group (42). Yardimci et al. (39) observed high oxidative toxicity and a significant increase of TBARS in the kidneys of male rats after IMI exposure. In research into IMI-induced immunotoxicity, MDA production increased significantly when rats were orally administered with IMI (56). IMI showed toxic effects on the hypothalamic-pituitary-adrenal (HPA) axis combined with a significant increase in the MDA level when 40 mg/kg b.w. of IMI was administered to rats daily by intragastric intubation for 28 days (83).

Researchers also investigated lipid peroxidation when treating earthworms, fish, and birds with neonicotinoids. The content of MDA increased significantly when earthworms were treated with IMI, whereas exposure to low doses did not result in similar MDA increases (77). A recent study showed that high concentrations of IMI in zebrafish markedly increased the MDA content (68). However, in a study to test the lethal and sublethal effects of treated seed ingestion by the red-legged partridge, both doses of IMI did not result in significant changes in TBARS levels in red blood cells, which may indicate that IMI toxicity may not only be dose dependent but also may vary between types of species (54).

Recently, Yan et al. (66) documented that NIT increased MDA content in zebrafish livers. Similarly, THI exposure in rats increased TBARS levels significantly in the spleen, thymus, bone marrow, polymorphonuclear leukocytes, and plasma (78). To determine the deleterious effects of CLO on the reproductive functions of developing male rats for 90 days, researchers quantified the concentration of TBARS in the testis samples and found that administration of CLO up to 32 mg/kg b.w. caused numerical but not statistically significant increases in the TBARS levels compared to the levels of control rats (45). However, another publication reported that CLO increased the TBARS levels of testicular tissue significantly at all doses when rats were exposed to low doses of CLO (44). The reason for the inconsistent results between the similar studies still remains unclear.

Therefore, it can be concluded that lipid peroxidation is a common phenomenon in the oxidative stress–related toxicity of neonicotinoids in vertebrates and invertebrates. Furthermore,

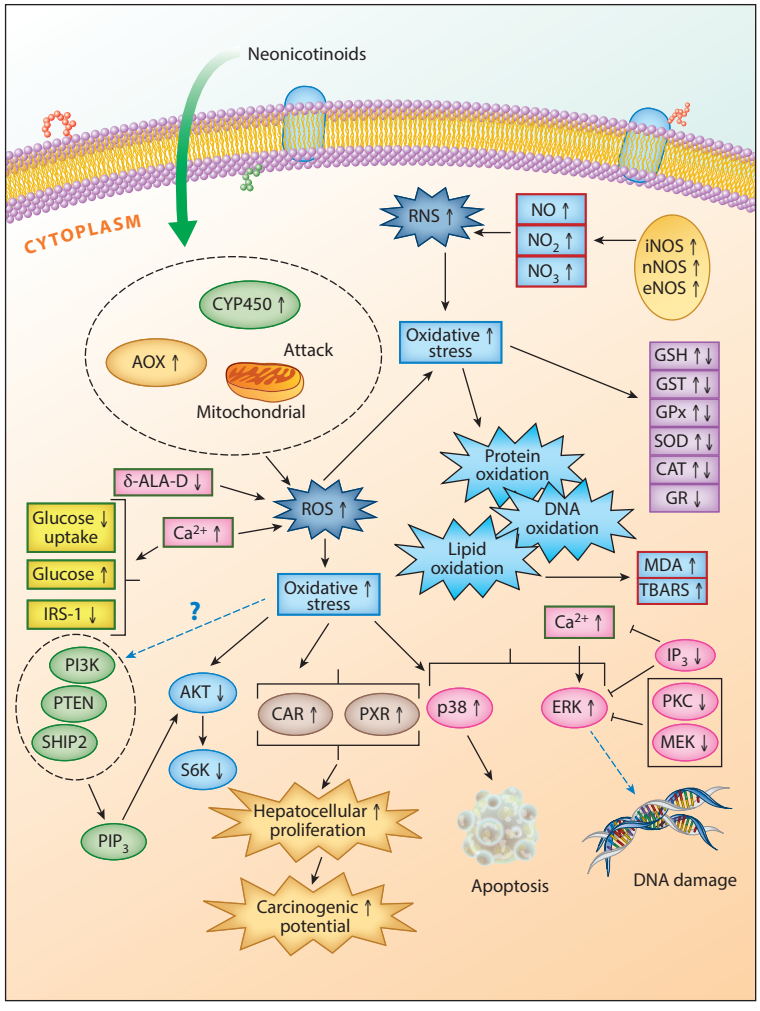

Figure 2

Oxidative stress–mediated mode of action proposed for neonicotinoids. Increased generation of ROS and RNS, as well as an alteration in the antioxidant status, may induce lipid, protein, and DNA oxidation, leading to various toxicities and apoptosis via ERK, p38, AKT, Ca^{2+}, and CAR/PXR pathways. Abbreviations: δ-ALA-D, δ-aminolevulinate dehydratase; AKT, protein kinase B; AOX, molybdo-flavoenzyme aldehyde oxidase; CAR, constitutive androstane receptor; CAT, catalase; CYP450, cytochrome P450; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal–regulated kinase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione *S*-transferase; iNOS, induced nitric oxide synthase; IP_3, inositol 1,4,5-trisphosphate; IRS-1, insulin receptor substrate-1; MDA, malondialdehyde; MEK, MAPK/ERK; nNOS, neuronal nitric oxide synthase; PI3K, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol-3,4,5-triphosphate; PKC, protein kinase C; PTEN, phosphatase and tension homolog; PXR, pregnane X receptor; RNS, reactive nitrogen species; ROS, reactive oxygen species; S6K, ribosomal S6 kinase; SHIP2, SH2-containing inositol phosphatase 2; SOD, superoxide dismutase; TBARS, thiobarbituric acid reacting substances.

lipid peroxidation may be dose dependent, and a dose threshold may exist during neonicotinoid-induced oxidative stress. These observations should be taken into account when considering the disparities in MDA levels induced by neonicotinoids at different doses, in different tissues, and in distinct species.

PC: protein carbonyl

Damage to DNA. The threat of oxidative damage is particularly significant to DNA. DNA damage can be measured by the alkaline comet assay or the cytokinesis-block micronucleus assay (34, 68, 84, 85). Furthermore, immunohistoplanimetry has also been used to analyze the frequency of DNA-fragmented germ cells in testis (47).

Costa et al. (86) revealed that high concentrations of IMI (20 μM) significantly increased the comet score and the frequency of micronuclei tested in human peripheral blood lymphocytes, whereas low doses of IMI (0.2 and 2 μM) did not. In a study to evaluate the negative effects of IMI on nontarget animals using zebrafish as the model animal, DNA damage and oxidative stress were shown to be dose and time dependent (68). Exposure of earthworms to THI increased DNA damage significantly according to the calculated comet assay olive tail moments. These results indicate that THI could be harmful to earthworms and that DNA damage could be used as one of the molecular biomarkers in the assessment of the risk of THI to the soil ecosystem environment (34). When researchers investigated DNA damage in bovine peripheral lymphocytes exposed to 30–480 μg/mL THI for 2, 24, and/or 48 h of incubation, THI concentrations of 120–480 μg/mL increased the frequency of DNA damage significantly, and THI failed to produce micronuclei (84). Kocaman et al. (85) documented that THI (75, 150, and 300 μg/mL) induced a significant increase in the cytokinesis-block micronucleus in human peripheral blood lymphocytes at all concentrations for 24 h; it also did so at 75 and 150 μg/mL for 48-h treatment periods in the absence of the S_9 mix and at all concentrations in the presence of the S_9 mix, indicating that THI, its metabolite (or metabolites), or both may act on DNA with the production of ROS that may cause DNA single-strand breaks. A study investigating the deleterious effects of CLO on the reproductive functions related to oxidative stress in mature male quails found that CLO administered at a dose of 50 mg/kg b.w. significantly increased the fragmented DNA in the seminiferous tubules with the increase of vacuolization in the seminiferous epithelia and decrease of the number of germ cells in a dose-dependent manner, suggesting that CLO might inhibit or delay embryo development (growth retardation) by the fragmentation of sperm DNA through oxidative stress (47). Other researchers investigating DNA damage in zebrafish found that NIT concentration had an obvious dose-response relationship with DNA damage and oxidative stress in the exposed zebrafish livers, which suggests that oxidative damage caused by NIT may be one of the underlining mechanisms of NIT-induced cell injury and DNA damage (66).

Like lipid peroxidation, damage to DNA during oxidative stress has also received much attention in terms of neonicotinoid toxicity effects. Additionally, a dose-dependent relationship between DNA damage and neonicotinoid concentration seems to be a common trend.

Damage to proteins. In addition to lipids and DNA, proteins are also major potential targets for oxidative damage, which results in the formation of protein carbonyls (PCs) (87). PCs represent a marker of global protein oxidation, as they are generated by multiple different ROS in blood, tissues, and cells (88). Although oxidative stress induced by neonicotinoids has been investigated, protein peroxidation has rarely been studied. One exception is a study conducted into the effects of ACE on the status of oxidative stress biomarkers, in which protein peroxidation products were reported when freshwater bivalve mussels *Anodonta cygnea* were treated with ACE (89).

Alterations in antioxidant status. Enzymatic antioxidant defense systems play a critical role in protecting cells from ROS such as $O_2^{\bullet -}$, $HO^{\bullet}$, and hydrogen peroxide (90). Superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione *S*-transferase (GST) are the primary antioxidant enzymes, and they serve as good redox biomarkers, as they are the first-line indicators of the antioxidant state through oxidation/reduction processes (90). As the most abundant intracellular antioxidant, glutathione (GSH) is involved in the protection of cells against oxidative damage and in various detoxification mechanisms (91). GSH also acts as a substrate and cosubstrate in many essential enzymatic reactions involving GPx, GR, and GST, and a decrease in the GSH level usually impairs cells' response to oxidants (78).

SOD: superoxide dismutase

CAT: catalase

GR: glutathione reductase

GPx: glutathione peroxidase

GST: glutathione *S*-transferase

GSH: glutathione

The alterations of the activities of antioxidant enzymes in oxidative stress are implicated as a mechanism of neonicotinoid neurotoxicity in vertebrates. After researchers exposed rats to IMI for 28 days, they observed meningeal congestion and degeneration changes in Purkinje cells in rat cerebellum, along with significant decreases in brain GSH levels and SOD activities at high doses of IMI. Furthermore, CAT and GPx activities at both doses of IMI were evident (36). IMI had a toxic effect on the HPA axis, the GST activity in hypothalamic tissues was perturbed, and pituitary SOD and CAT activities increased significantly in IMI-exposed rats. Contrarily, a decrease in the hypothalamic CAT activity was observed (83). Duzguner & Erdogan (50) reported that exposure to IMI caused inflammation in the brain, and that GPx activity was significantly elevated, indicating that IMI caused oxidative stress and inflammation in the central nervous system in nontarget organisms such as rats. Significant changes in the antioxidant enzyme activities were also evident when rats were exposed to IMI. CAT, SOD, and GPx were altered following IMI exposure, combined with chronic inflammation in the brain. Notably, significantly depleted antioxidant brain-GSH levels were detected (51).

Antioxidant enzymes are regarded as important mediators in the immunotoxicity reaction induced by the presence of neonicotinoids in vertebrates. Birds may still be at a high risk of poisoning by neonicotinoids through direct sources of exposure to coated seeds in autumn and winter. In male partridges, a high-exposure dose of IMI induced decreases in the cellular immune response, GSH levels, and activities of GPx in red blood cells (54). Furthermore, even a low dose of IMI could increase blood SOD activity and depress the T cell immune response (55). A study by Aydin (78) revealed that THI exposure in rats resulted in a significant decrease in CAT, GPx, and GSH levels in the lymphoid organs.

Antioxidant enzymes play a critical role in combating the reproductive toxicity of neonicotinoids in vertebrates. A study investigating the toxic effects of IMI on the reproductive system in male rats found that IMI treatments resulted in significant reproductive toxicity and a significant decrease in the GSH level, along with decreased activities of CAT, SOD, GPx, and GST (41). In male rats administered with varying doses of IMI, the levels of testosterone and GSH decreased significantly in the highest dose group (33). Also, CLO was detrimental to the reproductive organ system of male rats and resulted in a significant decrease in the level of GSH, suggesting CLO, even at low doses, could lead to an antioxidant imbalance in the reproductive organ system in vivo (45).

The antioxidant system plays a protective role in preventing liver and kidney damage when animals are administered with neonicotinoids. When rats were administered with IMI, GSH concentration decreased significantly, and significant histological changes in the liver were revealed (61). The total GSH level decreased in the livers of male rats after 12 h of IMI exposure, but total GSH increased in the kidneys of male rats after 24 h of exposure, and the GST activity in female rats increased after 24 h of exposure (39). El-Gendy et al. (67) revealed that the oral administration of IMI to rats elevated the activities of antioxidant enzymes in the liver, including CAT, SOD, GPx and GST, significantly and decreased the level of GSH significantly. In zebrafish exposed to

various IMI doses, IMI led to DNA damage in the liver in a dose- and time-dependent manner. Noticeable increases in SOD and GST activities during early exposure were observed, followed by decreases in their activities later, indicating that IMI can induce oxidative stress and DNA damage in zebrafish livers (68). Yan et al. (66) documented that when zebrafish were exposed to NIT, NIT induced DNA damage in the exposed zebrafish livers and dramatically inhibited SOD and CAT activities at most exposure times, with significantly increased GST activities observed for all treatment groups.

Interestingly, antioxidant enzymes show a threshold effect when animals are treated with neonicotinoids. After female rats were exposed to IMI, dosages of 5 and 10 mg/kg b.w./day did not produce changes in antioxidant enzyme levels in the liver, brain, kidney, or ovaries, whereas the highest dose of 20 mg/kg b.w./day induced significant changes in the SOD, CAT, GPx, and GSH levels in the liver and ovaries. These results support the existence of a threshold effect with regard to antioxidant enzymes in combating the oxidative stress induced by IMI (81, 82).

A misbalance in the enzymatic antioxidant defense systems was noted when researchers administered neonicotinoids to invertebrate animals. An investigation into the effects of IMI on the antioxidant defense and digestive systems in the earthworm found that doses of 0.66 and 2 mg/kg increased SOD activity significantly, whereas the highest dose of 4 mg/kg inhibited SOD activity markedly with prolonged exposure, and IMI dosages of between 0.2 and 4 mg/kg increased the activities of CAT and guaiacol peroxidase irregularly (77). Sublethal toxic doses of IMI (21.84 and 61.15 μg/snail) led to significant increases in the CAT and GST activities of treated snails, along with decreased acetylcholinesterase (AChE) activity (92). IMI also increased GST activity when administered through 5 μg/g dry food in juvenile *Porcellio scaber* (commonly known as woodlouse) and decreased GST activity significantly at 25 μg/g dry food in adults (9). Another recent study showed that the activities of GST, CAT, and SOD in earthworms were inhibited following exposure to THI (34). ACE also altered the status of oxidative stress biomarkers in *A. cygnea*, as attested to by the decrease in SOD activity and the GSH content in the gills and especially in the digestive gland (89).

A recent in vitro study suggested that antioxidant enzymes are involved in the genotoxicity of neonicotinoids. Exposure to 0.97–500 μM IMI presented potential genotoxic effects on Chinese hamster ovary (CHO_{K1}) cells, with significant inhibition of GST, GPx, and GR activity (93). Sauer et al. (37) revealed that in rat liver tissues, IMI inhibited the activity of δ-aminolevulinate dehydratase (δ-ALA-D), and GSH had the best antioxidant effect against IMI-induced δ-ALA-D inhibition, followed by curcumin (CUR) and resveratrol. Recently, an in vitro study found that THI exposure to bovine peripheral lymphocytes increased the frequency of DNA damage, led to unstable chromosome aberrations, and decreased the expression of bovine glutathione *S*-transferase M3 (GSTM3), even at low dosage levels. Furthermore, mRNA expression of GSTM3 increased at the higher concentrations of THI (84).

A misbalance of antioxidant status can be involved in the toxicities induced by neonicotinoids in vivo and in vitro. Furthermore, studies indicate that antioxidant enzymes as redox biomarkers are sensitive and may be considered as good biomarkers of the toxic effects of neonicotinoids.

AChE: acetylcholinesterase

CHO_{K1} cells: Chinese hamster ovary cells

δ-ALA-D: δ-aminolevulinate dehydratase

CUR: curcumin

GSTM3: glutathione *S*-transferase M3

Stress-Mediated Biological Response

Oxidative stress plays important roles in many biological responses and cell signaling pathways. Thus, significant changes in gene expression and the stimulation or inhibition of signal transduction usually results in many toxicological effects. The role of neonicotinoid-mediated oxidative stress in the induction of apoptosis and the respective cell signaling pathways has been studied widely in vivo (**Table 1**).

Apoptosis and cell signaling. ROS production results in oxidative stress, which can lead to apoptosis (94). Oxidative stress usually stimulates various cell signaling pathways involved in cell apoptosis (95). Apoptosis in cells can be detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling staining (96).

Investigations by Bal et al. (33, 42) revealed that when adult male rats were treated with IMI by oral gavage over a three-month period, apoptosis, along with oxidative stress, increased significantly in germ cells of the seminiferous tubules in the highest dose group when compared to the controls. This indicates that the adverse effect of IMI on the reproduction system in male rats appears to be due to the induction of oxidative stress in testis.

As nuclear receptors, the constitutive androstane receptors (CARs) and the pregnane X receptors (PXRs) are involved in the regulation of phase I–III metabolic enzymes responsible for the clearance of xenobiotics (97, 98). Furthermore, activation of the CAR/PXR signaling pathway has broad functions that include controlling liver regeneration and proliferation, inducing multiple detoxification enzymes, and regulating some antioxidant defenses, such as several GST members in vertebrates (99, 100). Additionally, oxidative stress could also induce CAR activity (99). LeBaron et al. (101) documented that the carcinogenic potential of SUL in rodents might be due to CAR/PXR nuclear receptor activation with subsequent hepatocellular proliferation. Considering that oxidative stress induced by neonicotinoids occurred in the liver, these authors suggested that a close relationship exists between the oxidative stress and liver carcinogenesis induced by neonicotinoids.

The p38 mitogen-activated protein kinase (MAPK) is phosphorylated in response to oxidative stress, which could block proliferation or promote apoptosis (102). When adult Kunming male mice were administered with ACE for 35 days, the p38 MAPK signaling pathway was activated in the testes of mice by increasing the concentration of the phospho-p38 protein, suggesting oxidative stress might be involved in the detrimental effects of ACE on testicular function (48).

Extracellular signal–regulated kinase (ERK p44/p42), belonging to the MAPK family, responds to a diverse array of extracellular stimuli, including neurotransmitters, hormones, growth factors, and several types of stress such as oxidative stress (103–105). Only 100 μM IMI and the metabolite of IMI, desnitro-IMI (DNIMI, 1 μM), induced significant induction of phospho-ERK (p44/p42) when mouse neuroblastoma N1E-115 cells were incubated with IMI (0.1–1,000 μM) and DNIMI (1 μM) for 30 min (105). Furthermore, DNIMI-induced ERK (p44/p42) activation in N1E-115 cells was not inhibited by the protein kinase A–selective inhibitor (2 μM) but was inhibited by the inhibitors of protein kinase C (PKC) and MAPK/ERK (MEK), respectively, indicating PKC and MEK might be involved in the activation of the ERK signaling pathway (105).

Ca^{2+} plays a crucial role in numerous cellular processes, Ca^{2+} cell homeostasis being one of many essential functions (106). Cellular proliferation, apoptotic processes, induction of oxidative stress, and physiological functions such as signal transduction are all part of the complex Ca^{2+} homeostasis process (107). A primary neurotoxic action of neonicotinoids is the alteration of AChE activity, resulting in overstimulation of nAChRs, which in turn leads to cholinergic effects and neurotransmission alteration (83). The Ca^{2+} influx that occurs owing to nAChR activation is subsequently amplified by the recruitment of intracellular Ca^{2+} stores (108). Ca^{2+} excitotoxicity could lead to the release of ROS (109). In IMI-exposed rats, plasma Ca^{2+} levels were 7.72 mg/dL on average in the controls, compared to 8.34 mg/dL in the IMI-exposed rats (50). Similarly, in a study to investigate Ca^{2+} mobilization and oxidative stress after chronic exposure to IMI in rats, an obvious increase in the Ca^{2+} level (8.62 mg/dL) was noted when the rats were treated with IMI (1 mg/kg b.w.) compared to the controls (8.19 mg/dL) (51). A study into the potential role of oxidative stress in the numerous biological and pathological processes induced by IMI in rats found that IMI caused significant elevation of the Ca^{2+} levels in hypothalamic and pituitary tissues but not

CAR: constitutive androstane receptor

PXR: pregnane X receptor

MAPK: mitogen-activated protein kinase

ERK: extracellular signal–regulated kinase

DNIMI: desnitro-IMI

PKC: protein kinase C

MEK: mitogen-activated protein kinase/extracellular signal–regulated kinase

in adrenal tissues. This indicates that IMI acted on the central nervous system as an agonist with the nAChRs, causing significant elevation of the Ca^{2+} levels, and that the toxic effects of IMI present a tissue-specific response (83). DNIMI, an IMI metabolite, could also activate nicotinic receptors via an intracellular Ca^{2+}-dependent mechanism (105). Furthermore, a rise in intracellular Ca^{2+} may impair the activation of insulin receptor substrate-1 (110–112). Based on the fact that IMI could increase oxidative stress and intracellular Ca^{2+}, oxidative stress induced by neonicotinoids might be involved in the control of Ca^{2+} homeostasis. However, a known thiolic antioxidant, *N*-acetyl-L-cysteine (NAC), showed a partial therapeutic potency against IMI toxicity, although it could not decrease the hypothalamic and pituitary Ca^{2+} content significantly, suggesting that NAC could not decrease intracellular Ca^{2+} concentrations by decreasing oxidative stress (83).

NAC: *N*-acetyl-L-cysteine

AKT: protein kinase B

S6K: ribosomal S6 kinase

Protein kinase B (AKT) is one of the major regulators of insulin signaling, and ribosomal S6 kinase (S6K) is the downstream target of AKT and is a feedback inhibitor of insulin signaling (113, 114). The AKT signal pathway can be depressed by oxidative stress (115). Kim et al. (111) reported on adipocytes (3T3-L1), hepatocytes (HepG2), and myotubes (C2C12) exposed to IMI (10 and 20 μM) for 4–6 days followed by treatment with insulin. The highest dosage of IMI reduced the glucose uptake stimulated by insulin in all the cell culture models, and treatment with IMI reduced the phosphorylation of AKT and S6K. These results indicate that IMI could induce insulin resistance by affecting the insulin signaling cascade, particularly upstream of AKT, in adipocytes, the liver, and muscle. Considering this, IMI exposure may contribute to the development of type 2 diabetes. Furthermore, these authors hypothesized that phosphoinositide 3-kinase, phosphatase and tension homolog, or SH2-containing inositol phosphatase 2 and phosphatidylinositol-3,4,5-triphosphate were involved in mediating the AKT signal pathway (111).

The signaling pathways, including the ERK, p38, AKT, Ca^{2+}, and CAR/PXR pathways, have been shown to be involved in the toxicity and apoptosis induced by neonicotinoids. These pathways were suggested to be closely correlated with the oxidative stress induced by neonicotinoids, indicating that more attention needs to be given to the signaling pathways in terms of research into the oxidative stress and toxicity induced by neonicotinoids.

Prevention of Neonicotinoid-Mediated Oxidative Stress

Vitamin C may protect against the neonicotinoid-induced oxidative stress (61, 67). As an important antioxidant, vitamin C can directly and rapidly scavenge free radicals, inhibit their formation, or both (116). The protective effect of vitamin C (200 mg/kg b.w.) might ameliorate oxidative damage induced by IMI (14.976 mg/kg b.w.) by decreasing MDA levels and altering the antioxidant defense system in mice liver. Furthermore, the protective effect of pretreatment with vitamin C against IMI-induced oxidative stress in mice liver is better than that of posttreatment (**Figure 3**) (67). Cotreatment with vitamin C (10 mg/kg b.w. orally) protected against the liver damage induced by IMI (80 mg/kg b.w. orally for 28 days) and increased GSH concentration significantly (61). Another study showed that vitamin C (0.08 mg/kg b.w.) could restore the histological changes in the liver and testis of quail caused by IMI exposure (0.62 mg/kg b.w.) (64). However, vitamin C treatment at 10, 100, and 1,000 μM was not able to protect against IMI-induced neurotoxicity in rats, as it could not restore the hepatic δ-ALA-D inhibition caused by IMI (20 mM) and therefore could not decrease the accumulation of the neurotoxic substrate (δ-aminolevulinic acid) of the enzyme δ-ALA-D (37). Low concentrations of resveratrol, a phytoalexin found in grapes and in foods such as peanuts, blueberries, and red wines, were effective at partially restoring enzyme activity and protecting cells from the oxidative effects of pesticides (37, 117, 118). In a study conducted by Sauer et al. (37), resveratrol (0.1, 1, 5, and 10 μM) restored the δ-ALA-D activity inhibited by IMI. CUR is also an antioxidant agent that can be isolated from ground rhizomes of *Curcuma longa* Linn.

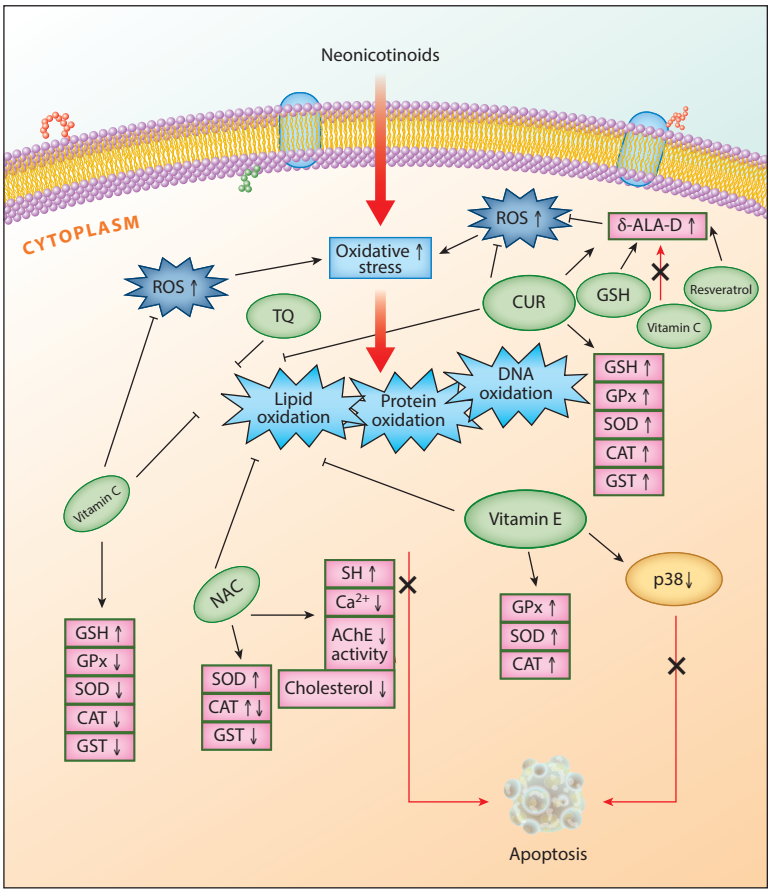

Figure 3

The preventive effects of different compounds including antioxidants and free-radical scavengers on neonicotinoid-induced oxidative stress. Use of different antioxidants such as vitamin C, vitamin E, resveratrol, CUR, GSH, TQ, and NAC significantly decreased MDA levels and improved the total antioxidant status, thus leading to the prevention of apoptosis and combating toxicity induced by neonicotinoids. Additionally, NAC significantly increased or decreased CAT activity in different tissues. Vitamin C could decrease MDA content and increase the reduction of GSH concentration. Resveratrol and GSH restored δ-ALA-D activity, whereas vitamin C was not able to restore the inhibition of δ-ALA-D induced by IMI. CUR and TQ restored the altered activity of the antioxidant system with a decrease in MDA concentration. Vitamin E decreased MDA and NO concentrations and increased the activity of antioxidant enzymes. NAC increased the activity of antioxidant enzymes, decreased the MDA levels of the pituitary and adrenal glands, and decreased the Ca^{2+} level. Abbreviations: δ-ALA-D, δ-aminolevulinate dehydratase; AChE, acetylcholinesterase; CAT, catalase; CUR, curcumin; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione *S*-transferase; IMI, imidacloprid; MDA, malondialdehyde; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species; SH, thiols content; SOD, superoxide dismutase; TQ, thymoquinone.

(turmeric) (119). CUR administered with IMI improved spontaneous locomotor activity and pain threshold values and prevented brain damage in rats, along with restoring the altered activity of the antioxidant system (36). Furthermore, CUR minimized the IMI-induced reproductive toxicity and histopathological changes in rat testis and led to significant restoration of ROS generation and MDA concentration. Moreover, increases of the GSH levels and the activities of CAT, GST,

SOD, and GPx in the testis were observed following coadministration of CUR in IMI-treated rats (41). A recent study showed that CUR could protect against IMI-induced neurotoxicity in rats, as CUR exhibits an antioxidant effect against the inhibition of hepatic δ-ALA-D caused by IMI and may therefore decrease the accumulation of δ-aminolevulinic acid of the enzyme δ-ALA-D (37). Compared to CUR, GSH (100 and 1,000 μM) had better antioxidant potency against the inhibition of IMI-induced δ-ALA-D (37). Thymoquinone (TQ) is an abundant component of black seed (*Nigella sativa*) oil extract and exerts antioxidant and anti-inflammatory properties (120, 121). TQ ameliorated IMI-induced immunotoxicity in rats and enhanced immune efficiency by decreasing oxidative stress, such as serum MDA levels (56). Zhang et al. (48) documented that vitamin E significantly protected male reproductive function against the increases of MDA and NO concentrations in the testes and against decreases in the activity of CAT, GPx, and SOD induced by the presence of ACE. As a known thiolic antioxidant, NAC could be a precursor for GSH synthesis as a cysteine supplier and could stimulate cytosolic enzyme activities involved in the GSH cycle (122). Researchers have reported protective effects of NAC (2 g/L) against oxidative stress and cholinergic transmission alteration in the HPA axis of male rats following subchronic exposure of IMI, and NAC could significantly restore the activity of hypothalamic and pituitary AChE. Additionally, NAC could aid in the restoration of hypothalamic, pituitary, and adrenal MDA levels and SOD and CAT activities, mainly in the adrenal gland, as well as hypothalamic and pituitary GST activity and total thiols content (83).

TQ: thymoquinone

6-CNA: 6-chloronicotinic acid

IMI-NO: nitrosoimine

NMI: (nitromethylene)imidazole

METABOLISM OF NEONICOTINOIDS

Metabolic Pathways

The metabolism of IMI has been documented in animals (35, 123, 124). The main IMI metabolites identified in mammals are 4-hydroxy-IMI, 5-hydroxy-IMI, 6-hydroxy-IMI (6-hydroxy nicotinic acid), 6-chloronicotinic acid (6-CNA), 2-imidazolidone, olefin, guanidine (DNIMI), and urea derivatives (123, 125, 126) (**Figure 4**). Two pathways have been identified by which IMI is metabolized by human cytochrome P450 (CYP450) isozymes: One is via imidazolidine hydroxylation and desaturation to give 5-hydroxyimidacloprid and the olefins, respectively, and the other is via nitroimine reduction and cleavage to yield the nitrosoimine (IMI-NO), DNIMI, and urea derivatives (124).

The metabolism of TMX and of CLO are closely related, with CLO serving as a principal intermediate in a major pathway for TMX in mammals. DIN and CLO differ only in that the tetrahydrofuranylmethyl moiety replaces the chlorothiazolylmethyl substituent, leading to many common metabolites and several unique to each compound (127, 128). The metabolic pathway of DIN is quite complex. DIN could be readily metabolized by *N*-demethylation, nitro reduction, tetrahydrofuran hydroxylations, and *N*-methylene hydroxylation and amine cleavage, indicating the intermediates during the metabolism process might be active as nicotinic agonists and iNOS inhibitors (127). CYC is transformed to the oxidation products assigned as isomers of hydroxyl addition at the 6-, 7-, 10-, or 11-position, and CYC is also metabolized to (nitromethylene)imidazole (NMI) followed by small amounts of the CYC-diol $(OH)_2$-CYC, nitroso-CYC, and amino-CYC (**Figure 5**) (11).

Metabolizing Enzymes

Researchers have documented that various enzymes are involved in the metabolism process of neonicotinoids. One study revealed that IMI was extensively metabolized oxidatively by

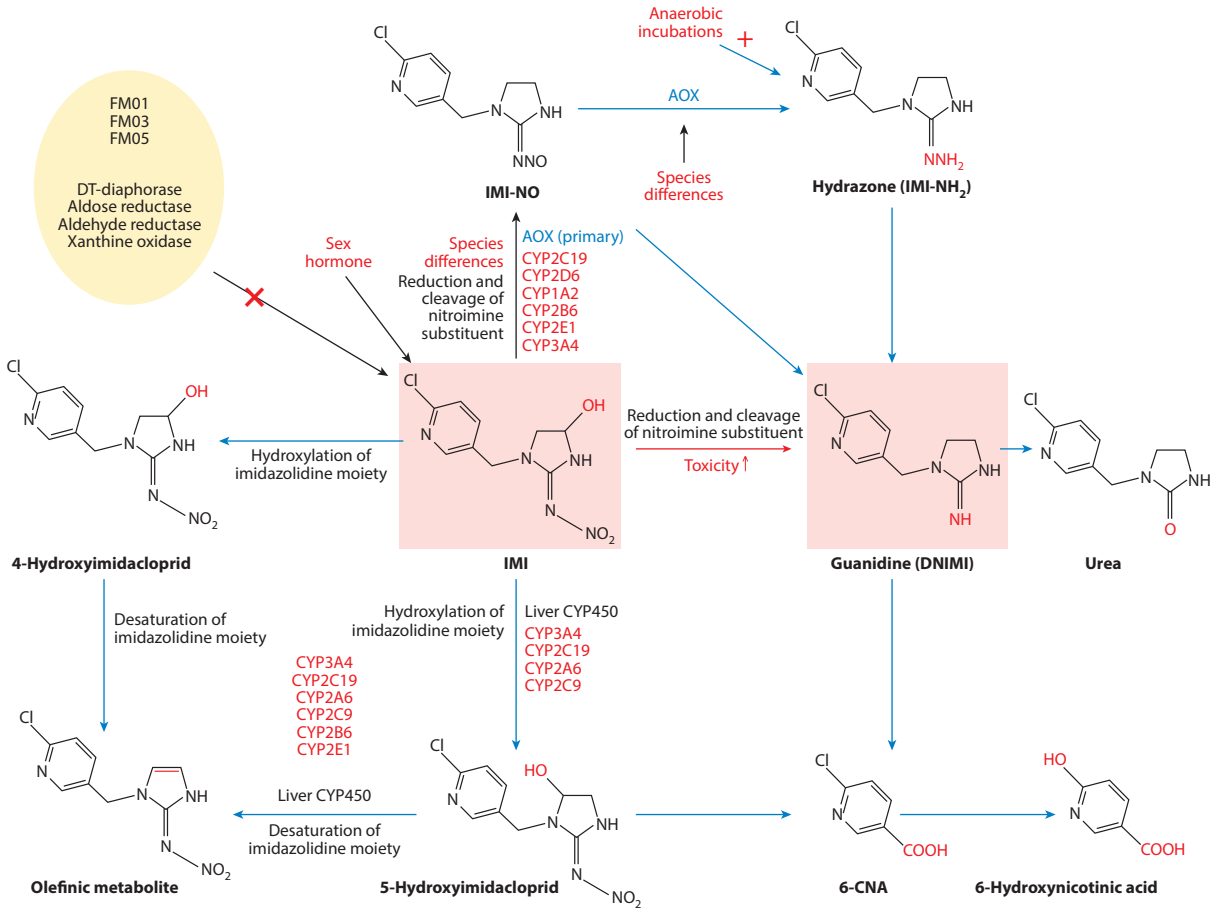

Figure 4

Metabolic pathways of IMI and effects of IMI on the metabolizing enzymes (35, 123–125, 129–132). Abbreviations: 6-CNA, 6-chloronicotinic acid; AOX, molybdo-flavoenzyme aldehyde oxidase; CYP450, cytochrome P450; DNIMI, desnitro-IMI; IMI, imidacloprid; IMI-NO, nitrosoimine.

cytochromes such as CYP3A4 and CYP2C19, whereas flavin monooxygenase isozymes (FMO1, FMO3, and FMO5) were not (124). Another study documented that IMI could be metabolized by human CYP3A4 with NADPH by imidazolidine hydroxylation and dehydrogenation to give 5-hydroxy-IMI and olefins, respectively, and by nitroimine reduction and cleavage to yield the IMI-NO, DNIMI, and urea derivatives (129). However, human CYP450 isozymes differ in selectivity for IMI imidazolidine oxidation versus nitroimine reduction (124). Further studies showed that IMI could be metabolized via aerobic nitroreduction by the molybdo-flavoenzyme aldehyde oxidase (AOX) (130, 131). Rabbit liver AOX is capable of reducing IMI to both IMI-NO and aminoguanidine metabolite (NNH_2). The addition of GSH (10 mM) for 10-min incubations with the electron donor substrate *N*-methylnicotinamide almost completely blocks the covalent binding of [^{3}H]IMI-NO to the partially purified AOX protein, indicating that rabbit AOX metabolically activates IMI-NO, forming both an irreversible inhibitor and a reactive intermediate that can bind covalently to protein (131). However, the understanding of the mechanism and toxicological relevance of IMI-NO inactivation of AOX is still limited. In vivo studies revealed that when

FMO: flavin monooxygenase

AOX: molybdo-flavoenzyme aldehyde oxidase

NNH_2: aminoguanidine metabolite

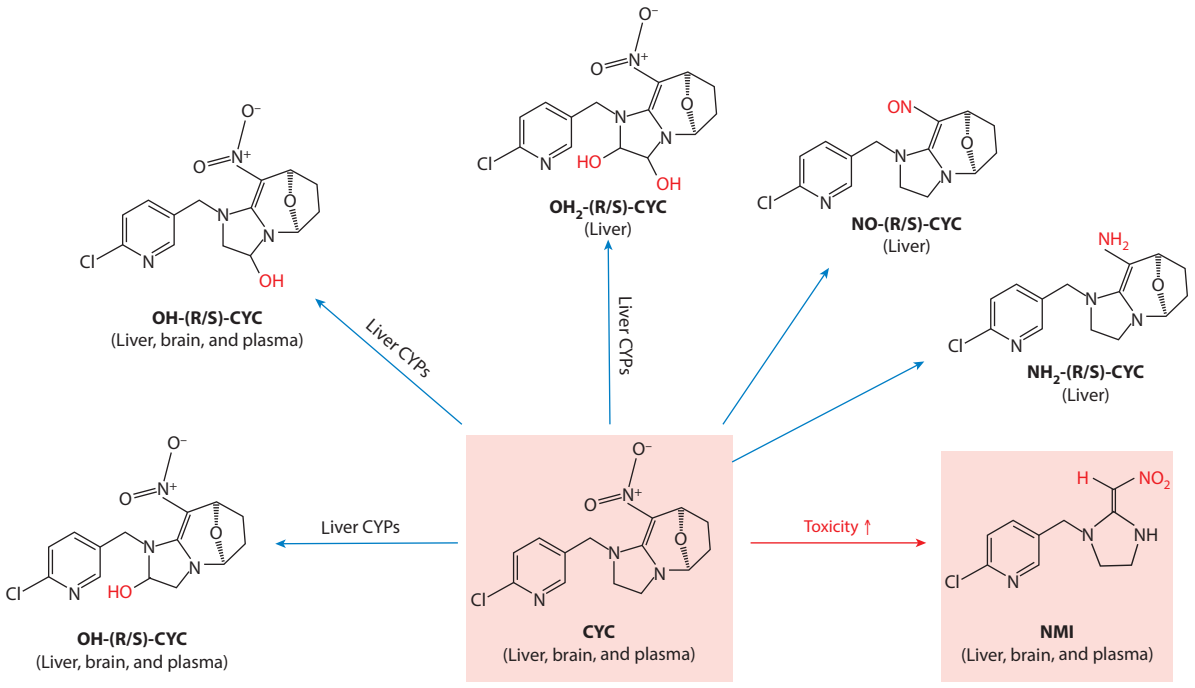

Figure 5

Metabolic pathways of CYC and effects of CYC on the metabolizing enzymes (11). Abbreviations: CYC, cycloxaprid; CYP, cytochrome; NMI, (nitromethylene)imidazole.

AOX-deficient DBA/2 mice were treated intraperitoneally with IMI (10 mg/kg b.w.), metabolism via CYP oxidation reactions was not appreciably affected, whereas the AOX-generated nitrosoguanidine metabolite (NNO) decreased by 30% with tungsten, 56% with hydralazine, and 86% in the AOX-deficient mice. The other IMI nitroreduction metabolite, DNIMI, decreased by 55%, 65%, and 81% with tungsten, hydralazine, and in the AOX-deficient mice, respectively, suggesting that the function of CYP oxidation reactions was not dependent on AOX and that AOX is the most important mouse IMI hepatic nitroreductase in vivo (132). AOX also reduces CLO to CLO-NNO and CLO-NNH_2 and reduces DIN to DIN-NNO and DIN-NNH_2 but does not reduce TMX to TMX-NNO or TMX-NNH_2 (133).

The metabolism of TMX, CLO, and DIN probably involves CYP450 enzymes for the NCH_3, NCH_2, and OCH_2 hydroxylations, AOX or CYP450 enzymes for the NNO_2 reduction, and GST for dechlorination (127). In the metabolism of TMX, TMX and desmethyl-TMX with CYP450 yield more formaldehyde (HCHO) than the other neonicotinoids such as CLO, DIN, ACE, NIT, and IMI. Furthermore, mouse CYP450 enzymes are more active than the rat or human equivalents in converting TMX or desmethyl-TMX to HCHO, which might be an alternative hypothesis for TMX hepatotoxicity (**Figure 6**) (128). Human CYP3A4 converts TMX to a more potent inhibitor of [^{3}H]IMI binding to the *Drosophila* nAChR, and the AOX system coupled with the *Drosophila* receptor strongly inactivates CLO, DIN, IMI, desmethyl-TMX, and TMX with some inactivation of NIT and nithiazine (134).

NNO: nitrosoguanidine metabolite

HCHO: formaldehyde

CYP450 pathways were largely involved in CYC metabolism in mice, and the tentatively assigned metabolites of CYC are five monohydroxy derivatives and a dihydroxy, nitroso, and amino modification (11).

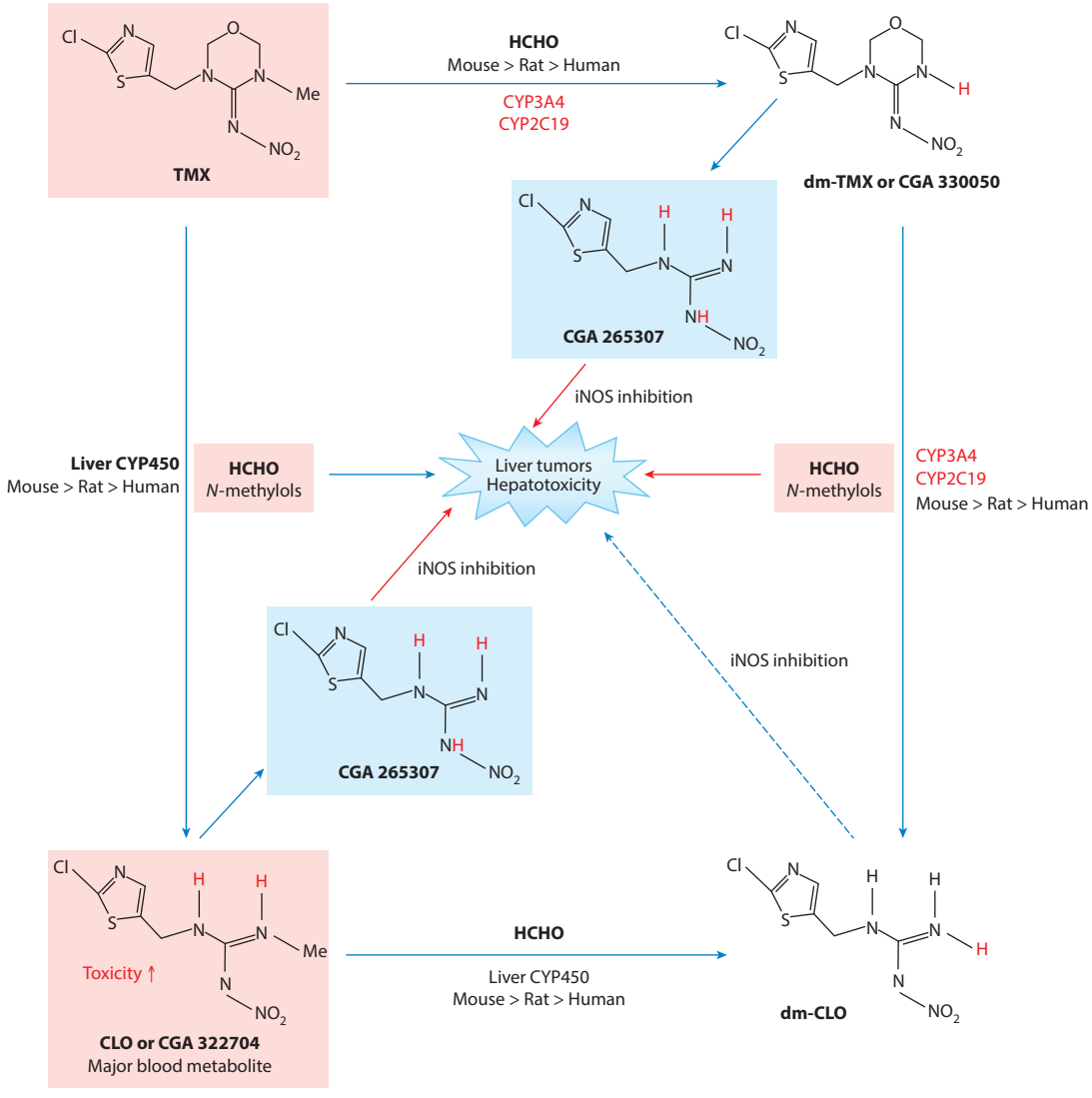

Figure 6

The key metabolic pathways of TMX and effects of TMX on the metabolizing enzymes (65, 128, 132, 136, 142). Abbreviations: CLO, clothianidin; CYP450, cytochrome P450; dm-CLO, desmethyl-CLO; dm-TMX, desmethyl-TMX; HCHO, formaldehyde; iNOS, induced nitric oxide synthase; TMX, thiamethoxam.

Other Factors Affecting the Metabolism of Neonicotinoids

The metabolism of neonicotinoids might be closely related to sex. In a study to investigate the effects of the metabolism modulators piperonyl butoxide (100 mg/kg b.w.) and menadione (25 mg/kg b.w.) on the toxicity of IMI (170 mg/kg b.w.), Arslan et al. (135) revealed that the CYP450-mediated metabolism of IMI and the genotoxicity of IMI is sex related.

TMX causes a significant increase in liver cancer in mice, but not rats, in chronic dietary feeding studies, suggesting that the species differences might exist in the toxicity of neonicotinoids (128, 136). Comparisons of the metabolism of TMX in rats and mice revealed that the concentrations of

IC_{50}: 50% inhibitory concentration

the two metabolites, CGA265307 and CGA330050, were 140- and 15-fold lower for CGA265307 and CGA330050, respectively, in rats than in mice following either a single oral dose or dietary administration of TMX for up to 50 weeks, indicating that metabolism differences might play a critical role in the toxicity of neonicotinoids in different species (136). Large species differences are observed in the IMI nitroreductive activity of liver cytosol, and rabbit and monkey give the highest levels of total metabolite formation. Human, mouse, cow, and rat also metabolize IMI rapidly whereas dog, cat, and chicken liver cytosols do not reduce IMI at appreciable rates (130). However, the rates of biokinetics, excretion, distribution, and metabolism of CLO were not markedly influenced by dose level and sex when male and female rats were exposed to CLO (5 and 250 mg/kg b.w.) (137).

With the tested temperatures (28, 30, and 33.5°C), THI (1, 5, 10, 15, and 20 mg/L) was shown to have no effects on early life stage parameters of zebrafish, except on the heart rate, which was probably due to an increased metabolism upon exposure to THI (138).

Toxicity of Neonicotinoids and Their Metabolites

Some metabolites of neonicotinoids have an equal or greater toxicity than their parent compounds, such as CLO, a metabolite of TMX (7). The main metabolites of IMI, such as 6-CNA and 2-imidazolidone, contributed drastically to the overall toxicity of IMI because of the noncovalent interactions of 6-CNA and 2-imidazolidone with biopolymers (125, 139). Although IMI shows an excellent safety profile, its metabolite DNIMI is over 300 times more potent than IMI to mammalian nAChRs [vertebrate $\alpha4\beta2$ 50% inhibitory concentration (IC_{50}) = 8.2 nM for DNIMI and 2,600 nM for IMI] and displays higher toxicity to mammals associated with agonist action at the nAChR in the brain than that of IMI (105, 132).

One of the main metabolites of CYC, NMI, was much more toxic than CYC because the in vitro binding affinity to the nAChRs of the three species studied (house fly, honeybee, and mouse) is 15–40-fold greater for NMI than CYC, determined by competitive inhibition of [^{3}H]NMI binding (11).

Another study revealed that the toxicity profile for X11719474, the primary metabolite of SUL, was limited to liver effects via the same mode of action as the parent, and overall, X11719474 was significantly less toxic than the parent according to genetic, acute, short-term rat and dog reproductive and developmental toxicity studies (140).

Critical analysis of the available toxicity results showed that the nitro-substituted compounds (CLO, DIN, IMI and its metabolites, TMX, and NIT) appeared to be the most toxic to bees, and the cyano-substituted neonicotinoids (ACE and THI) seemed to exhibit a much lower toxicity (73). However, ACE displayed slightly higher toxicity than IMI in a study to assess the influence of IMI and ACE on soil microbial activities according to their IC_{50} value and the thermodynamic parameters (141).

TMX is hepatotoxic and hepatocarcinogenic in mice but not rats, and its metabolite, desmethyl-TMX, is also hepatotoxic, whereas CLO is neither hepatotoxic nor hepatocarcinogenic (128). Green et al. (65) documented that the metabolite CGA330050 {3-(2-chloro-thiazol-5-ylmethyl)-[1,3,5] oxadiazinan-4-ylidene-*N*-nitroamine, 500 and 1,000 mg/kg diet} of TMX induced liver cancer in mice at a rate similar to TMX, suggesting that TMX is hepatotoxic and hepatocarcinogenic as a result of its metabolism to CGA330050. Furthermore, another metabolite, CGA265307 [*N*-(2-chloro-thiazol-5-ylmethyl)-*N'*-nitroguanidine] exacerbated the toxicity of CGA330050 in TMX-treated mice. A recent study revealed that mice yielded significantly more HCHO from TMX and desmethyl-TMX compared to rats or humans, suggesting that the production of HCHO and potentially *N*-methylols might be the candidate hepatotoxicants and hepatocarcinogens (128).

Considering the close relationships among the oxidative stress factors, CYP450, and iNOS and the fact that CGA265307 and desmethyl-CLO exacerbated the hepatic toxicity by inhibiting iNOS (128, 142), oxidative stress might be involved in TMX-induced toxicity.

In summary, the metabolites of neonicotinoids are numerous, and CYP450 and cytosolic enzymes play critical roles in their metabolism. Hormones from different species correlate with the metabolism of some neonicotinoids. The metabolites of neonicotinoids show different toxicities, with some exhibiting equal or greater toxicity than that of their parent compounds. The toxicity of neonicotinoids is attributed primarily to their action as nicotinic agonists, directly or as metabolites. Oxidative stress may be generated during the metabolism of neonicotinoids.

CONCLUSION

Thousands of tons of neonicotinoids are widely used as insecticides and veterinary drugs worldwide. Following significant increases in the use of neonicotinoids for the protection of crops, ornamentals and trees in horticulture, tree nurseries, agriculture, and forestry, the related toxicity effects on animals and humans are a growing cause for concern. Therefore, it is necessary to investigate the toxic effects and the toxicological mechanism of neonicotinoids to protect nontarget species, including humans, from injury.

Neonicotinoids show their toxicity as agonists at nAChRs in insects and mammals alike. However, oxidative stress may also be one toxicological mechanism for neonicotinoids, based on more than 10 years of studies. Interestingly, oxidative stress occurs in various species of animals, such as rats, mice, quail, and earthworms due to neonicotinoid-related toxicology. Various compounds, including free-radical scavengers, can efficiently combat neonicotinoid-induced damage, suggesting that the toxic effects of neonicotinoids might closely correlate with metabolism and oxidative stress. Most studies of oxidative stress induced by neonicotinoids (mainly IMI) have been carried out on in vitro models or in vivo animal studies (**Tables 1** and **2**). The studies of IMI, CLO, ACE, THI, NIT, and TMX clearly identified that oxidative stress played a critical role in their various toxicities, suggesting that oxidative stress might be one potential mechanism for other neonicotinoids, such as DIN, SUL, CYC, IPP, and imidaclothiz. However, the roles of oxidative stress in the toxic effects induced by other neonicotinoids are worthy of further investigation using in vitro models and in vivo studies because their use worldwide is growing fast.

Neonicotinoids were once believed to have low mammalian toxicity, including neurotoxicity. However, some studies identified that neonicotinoids showed a variety of potential toxic effects on animals and even humans, including hepatotoxicity, nephrotoxicity, and reproductive cytotoxicity (25, 33, 34, 36–39, 81). Interestingly, all these toxic effects were found to have some relationship with oxidative stress, indicating that oxidative stress might be one common phenomenon in toxicity induced by neonicotinoids. However, the relationship between the agonists of nAChRs and the generation of oxidative stress still remains unclear. Researchers knew oxidative stress might occur during the metabolism of drugs. However, a recent study revealed that IMI could induce oxidative stress in cells with few metabolic enzymes, such as CHO_{K1} cells (93), suggesting that the generation of oxidative stress might be independent of the metabolism of neonicotinoids in vitro. It is still not clear whether the generation of oxidative stress is a secondary effect or whether it has nothing to do with the agonists of nAChRs induced by neonicotinoids; this is worthy of further investigation.

The research summarized here indicates that IMI displays sex-, tissue-, and duration-specific effects in its toxicity (39). Similarly, the genotoxicity of IMI is sex related, as hormones control the CYP450-mediated metabolism of IMI (135). Furthermore, species differences should also be taken into account because of their different metabolism, such as in the case of TMX, which causes a significant increase in instances of liver cancer in mice, but not rats, in dietary feeding

studies (136). Therefore, it might not be suitable to infer the toxicity of neonicotinoids between different species or even different sexes. Considering that the degree of injury to cells might have a direct relationship with levels of oxidative stress (143, 144), it could be useful to assess the levels of oxidative stress and its mechanism of generation when studying the toxicity of various neonicotinoids.

nAChRs facilitate neurotransmission in the central and peripheral nervous systems (145). The mechanism of the toxicity of neonicotinoids is complex, but the major biochemical effects of neonicotinoids are mediated through their agonist activity on nAChRs (35). However, the ability of neonicotinoids to activate nAChRs does not seem to be the sole trigger for apoptosis or the various toxicities observed. ROS, RNS, oxidative stress, Ca^{2+} levels, and various signaling pathways also play critical roles in cellular apoptosis. However, few studies have examined the role of the agonists of nAChRs in neonicotinoid-induced oxidative stress, ROS generation, Ca^{2+} levels, and signaling pathways in vitro or in vivo. Although researchers have documented that neonicotinoids activate the ERK cascade through a primary action on nAChRs, the involvement of intracellular Ca^{2+} mobilization, possibly mediated by inositol 1,4,5-trisphosphate (105), and the relationship between Ca^{2+} level, the oxidative stress, and the signal integration pathways induced by neonicotinoids deserve further investigation to allow us to effectively understand the toxic effects of neonicotinoids.

To protect against neonicotinoid-induced oxidative stress, a variety of compounds have been evaluated for their antioxidative effects, including vitamin C, vitamin E, CUR, NAC, GSH, resveratrol, and TQ. These efforts underscore the urgency of finding a good antidote for neonicotinoids as a consequence of their increasing use worldwide. Further understanding of the role of oxidative stress as well as the metabolism of neonicotinoids in neonicotinoid-induced toxicity will shed new light on the use of antioxidants and scavengers of ROS or RNS. As thousands of tons of neonicotinoids continue to be applied worldwide, the search for highly effective antioxidants and efficient detoxification enzymes is of high priority to reduce the various toxic effects induced by neonicotinoids and circumvent future issues.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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