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Oxidative Stress and Metabolism: A Mechanistic Insight for Glyphosate Toxicology

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Abstract

Glyphosate (GLYP) is a widely used pesticide; it is considered to be a safe herbicide for animals and humans because it targets 5-enolpyruvylshikimate-3-phosphate synthase. However, there has been increasing evidence that GLYP causes varying degrees of toxicity. Moreover, oxidative stress and metabolism are highly correlated with toxicity. This review provides a comprehensive introduction to the toxicity of GLYP and, for the first time, systematically summarizes the toxicity mechanism of GLYP from the perspective of oxidative stress, including GLYP-mediated oxidative damage, changes in antioxidant status, altered signaling pathways, and the regulation of oxidative stress by exogenous substances. In addition, the metabolism of GLYP is discussed, including metabolites,

metabolic pathways, metabolic enzymes, and the toxicity of metabolites. This review provides new ideas for the toxicity mechanism of GLYP and proposes effective strategies for reducing its toxicity.

INTRODUCTION

Glyphosate (GLYP), *N*-(phosphonomethyl)-glycine, is a broad-spectrum systemic herbicide that acts on the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (1). GLYP accounted for approximately 48% of the global pesticide industry in 2016, making it the highest-selling herbicide (2). There are numerous GLYP-based herbicides (GBHs), but the commercial formulation is best known as Roundup. GBHs are commonly used in fallow fields, farmlands, and nonfarmland areas to control exposed weeds (3). In the United States, GBHs were used in 31% of corn acreage planted in 2005 and 92% of soybean acreage planted in 2006 (4). In non-GLYP-resistant crop fields, GBHs are primarily used as a burndown, nonresidual weed control treatment prior to the planting or emergence of a crop (5). However, GLYP also causes serious residual problems through irrigation and direct discharge into the water. In Lake Taihu, China, the maximum reported residual concentration of GLYP is 15.21 mg/L (6). In addition, GLYP residues have been found in food and even in the blood serum and urine of people (7). The abovementioned studies indicate that nontarget organisms may be harmed by exposure to GLYP.

GLYP has different toxic effects on different species. For example, it is harmful to bovine preimplantation development even at a very low concentration (8), damages swine ovarian and adipose stromal cell functions (9), and decreases the sperm concentration in rats (10). The viability and DNA integrity of fish sperm are also affected (11). GLYP exposure can also cause cognitive and psychomotor disorders and impair the brain redox balance, which leads to neurotoxicity (12–16). Moreover, GLYP may also cause varying degrees of damage to organs. After exposing rats to GLYP, the liver shows pathological damage and an inflammatory response with the elevated lipid peroxidation (LPO) and reduced enzymatic and nonenzymatic antioxidant activities (17). GLYP also increases the levels of plasma urea and creatinine (CRE) and uric acid in rats, resulting in kidney damage (18). In fish liver, GLYP exposure increases protein carbonylation (PC), glutathione *S*-transferase (GST), reduced glutathione (GSH), and ascorbic acid (ASA) levels and decreases superoxide dismutase (SOD) activity (19). GLYP exposure can also cause kidney damage in goldfish (20). In addition, whether GLYP is carcinogenic to humans has always been a highly controversial issue (21, 22).

Oxidative stress and metabolic changes are the important mechanisms that mediate GLYP's toxicity (23–25). GLYP significantly increases primary DNA damage and lowers thiobarbituric acid reactive substances (TBARSs) in rat liver (26). In the liver and muscle of fish, GLYP reduces enzymatic antioxidant activity while augmenting TBARS and PC levels (27). GLYP exposure also significantly reduces SOD, catalase (CAT), and GST activity and enhances LPO in caprine granulosa cells (28). In addition, GLYP adversely affects photosynthesis, carbon metabolism, and mineral nutrition and induces oxidative damage in plants (29, 30). The main metabolite of GLYP after microbial decomposition is aminomethylphosphonic acid (AMPA), which is rarely metabolized in mammals (31). The metabolism and elimination of GLYP have been evaluated in model animals, which are essential for predicting GLYP toxicity to humans. Studies performed in male Wistar rats have shown that the absorption half-life of oral GLYP is 2.29 h, and the elimination half-life is 14.38 h (31). Overall, although GLYP is not well absorbed when it enters the body, it is eliminated slowly, thus it may be distributed to all tissues and potentially cause damage. In addition, GLYP and AMPA also alter antioxidant status and cytochrome P450 (CYP450) enzymatic

activity. When searching for reviews about GLYP from the past decade, we found that there has been no review of GLYP detoxification from the perspective of oxidative stress. This review is the first comprehensive explanation of GLYP toxicity from the perspectives of oxidative stress and antioxidant capacity. GLYP-mediated oxidation state, regulation of oxidative stress by exogenous substances, in vivo and in vitro oxidative signaling pathways, and effects on GLYP metabolism are described in detail.

THE TOXICITY OF GLYP

Since the advent of GLYP, more and more studies have shown that GLYP causes a variety of toxic effects, including neurotoxicity, reproductive toxicity, and liver and kidney toxicity, which are related to oxidative stress. In addition, adjuvants added to commercial GLYP formulations also increase toxicity. Other scholars have pointed out that long-term exposure to GLYP may lead to chronic diseases such as asthma, osteoporosis, and infertility (32). In addition, the carcinogenicity of GLYP is still under debate. The studies related to the toxicity of GLYP are summarized in **Supplemental Table 1**.

Supplemental Material >

Neurotoxicity

Pesticide exposure has been linked to the development of neurodegenerative diseases (33). Acetylcholinesterase (AChE) is a sensitive biomarker for organophosphorus and urethane pesticide exposure (13). In addition, assessment of transaminase and alkaline phosphatase (ALP) activity in specific areas of the brain can also reflect impaired nerve conduction (34).

In one study, scholars orally treated female Wistar rats from the beginning of pregnancy to postnatal day (PND)21 with Roundup (corresponding to 0.65 or 1.30 g/L GLYP). By PND90, brain ALP activity was increased, and AChE and transaminase activities were decreased in the striatum and hippocampus (34). Similarly, after treating maternal rats during pregnancy and lactation (to PND15) with 3.6 g/L Roundup, there was glutamate excitotoxicity and oxidative stress in the hippocampus (35). When male Wistar rats were treated with 75 mg/kg body weight (b.w.) GLYP for 6 days, GLYP significantly reduced the content of dopamine, 5-hydroxytryptamine, and norepinephrine in the striatum, hypothalamus, hippocampus, and midbrain. This action can result in neurotoxicity or impaired cognitive behavior (36). CF-1 mice exposed to 50 mg/kg b.w. GLYP for 4 weeks presented with a disrupted redox balance in the brain (12). With regard to aquatic animals, zebrafish embryos were exposed to 50 μg/mL GLYP for 24 h. Loss of ventricular contour and reduction of the eye area were observed. Hence, GLYP promotes neurotoxicity in the forebrain and midbrain (15). For humans, after 24 h GLYP treatment (100 μM), the glucose uptake of nerve cells changed, resulting in nerve damage (16).

In general, exposure to GLYP leads to degenerative brain changes in nontarget organisms. These damages are associated with oxidative stress. However, most studies have employed mammalian and aquatic animals. Future studies in humans are required to assess potential GLYP-mediated neuropathology.

Reproductive Toxicity

Over the years, there have been efforts to determine whether GLYP potentially affects reproductive development. The quality of sperm and ovaries is an important factor in determining fertility.

A study treated male Sprague-Dawley rats with 5, 50, or 500 mg/kg b.w. GLYP for 5 weeks, after which there were reduced sperm counts and seminal vesicle and glandular weights in the 500-mg/kg b.w. group (37). Administration of 375 mg/kg b.w. GLYP to rats for 8 weeks decreased

sperm motility, sperm membrane integrity, and SOD activity (38). Similarly, GLYP reduced the testosterone level, sperm motility, and sperm count in male Wistar rats after 52 days of treatment at 5 mg/kg b.w. (39). However, male rats exposed to 2.5 or 15 mg/kg b.w. GLYP for 2 weeks did not present with significantly altered testes (40). The effect of GLYP on reproductive organs is dose and time dependent. Long-term exposure to high or low doses will damage the reproductive system.

In one study, the authors administered ICR mice with oral GLYP (25 mg/L solution administered through the daily drinking water) at the beginning of pregnancy. After 19 days, atretic follicles increased, while interstitial fibrosis and mature follicles decreased (41). Pregnant Wistar rats exposed to sublethal doses of Roundup (500 mg/kg b.w.) for 7 days exhibited decreased implantation site and corpus luteum numbers and a reduced implantation rate (42). For swine granulosa cells, GLYP can increase progesterone and nitric oxide (NO) secretion (9).

In short, GLYP can cause reproductive toxicity by reducing the sperm count, diminishing ovary weight, and so on. A relevant issue is whether GLYP exposure has any influence on human infertility. We suggest additional studies to collect relevant data.

Hepatotoxicity and Nephrotoxicity

GLYP can cause liver and kidney damage through oxidative stress (43). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and ALP activities serve as hepatotoxicity markers. Kidney injury can be determined by detecting changes in serum CRE and urea (44).

Adult male Wistar rats administered 50 mg/kg b.w. GLYP every other day for 15 days exhibited altered AST, ALT, and ALP. In addition, there was hepatic oxidative stress, evidenced by the increased levels of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and PC, and a significant decrease in SOD, GSH, nonprotein thiols, glutathione peroxidase (GPx), and vitamin C (45). One study investigated weaned hybrid piglets (Duroc × Landrace × Yorkshire) fed with 0, 20, or 40 mg/kg b.w. GLYP for 35 days. As the GLYP concentration increased, the liver gradually showed hepatocyte swelling, congestion, and local necrosis; ALP activity also increased linearly (46). Kalach 360 SL (KL) is an herbicide that contains 360 g/L GLYP. When female Wistar rats were exposed to KL (216 or 315 mg/mL) for 60 days, there were significantly increased plasma levels of CRE, urea, and uric acid and diminished CRE clearance. There was also tubular necrosis that caused distal tubular damage (18). GLYP also causes liver and kidney damage to aquatic organisms. Goldfish exposed to 0.2 mM GLYP for 90 days showed kidneys with a hyaline cast, as well as increased serum CRE and urine nitrogen levels. In the liver, ALT, AST, lactate dehydrogenase, and MDA levels were significantly increased, while SOD, GPx, and glutathione reductase (GR) activities were significantly decreased (20). Similar results were reported in catfish and carp (47, 48).

Based on these studies, GLYP can cause pathological damage to liver and kidney. Hepatotoxicity and nephrotoxicity can be reflected by blood analysis. Therefore, in future research, we can collect data on human liver and kidney indexes in areas where medicinal products are frequently used to analyze the toxicity of GLYP to humans.

Carcinogenicity

The carcinogenicity of GLYP has always been controversial. One study conducted in Swiss albino mice showed that the highest daily GLYP dose (1,460 mg/kg b.w.) significantly increased the incidence of malignant lymphoma (49). However, it is hard to conclude that GLYP would pose a cancer risk based on the extremely complex causes of lymphoma and the rare occurrence of an oral dose as high as 1,460 mg/kg b.w. In nine carcinogenicity studies on rats, there was no significant association between the incidence of tumors and GLYP exposure in any treatment group. As a

result, the European Food Safety Authority (EFSA) has suggested that the evidence linking GLYP exposure to cancer is very limited (49).

In an early study, neither GLYP nor the Roundup formulation was found to pose a human carcinogenic risk (50). Some scholars who summarized all relevant epidemiological peer-reviewed scientific literature evaluations before 2012 concluded that there is no significant correlation between the occurrence of prostate cancer, breast cancer, colorectal cancer, pancreatic cancer, cutaneous melanoma, leukemia, or glioma and GLYP exposure (22). Notably, GLYP exposure may cause multiple myeloma (MM) (51). However, a study of 27 subjects exposed to GLYP found no statistical correlation between GLYP exposure and monoclonal gammopathy of undetermined significance (MGUS) risk; MM often occurs with MGUS (52). Similarly, some scholars have proposed a link between GLYP exposure and non-Hodgkin's lymphoma (NHL), but when the statistical method was changed, the association was not confirmed (53). In summary, only the risk of toxicity from high doses or prolonged exposure to GLYP has been demonstrated. There is currently no evidence that GLYP is carcinogenic to animals or humans.

In short, the study of GLYP carcinogenicity is still in its infancy, with animal studies limited to mice and rats and human studies based on questionnaires. Given that cancer is often associated with DNA damage, we suggest that the carcinogenicity of GLYP can be further evaluated in terms of oxidative stress.

The Toxicity of Adjuvants

GLYP formulations have been reported to be more toxic than pure GLYP. These formulations contain GLYP (about 36–48%), water, salts, and adjuvants. GBHs are never used without adjuvants (43).

The main GBH adjuvant is the surfactant polyoxyethylene amine (POEA), which carries its own toxicity risk. For humans, POEA and its formulations have been classified as skin sensitizers (54). A study examined the toxicity of Atanor 48 (ATN) (in which the active ingredient is GLYP), GLYP, and POEA to zebrafish and showed that their lethal effects in descending order were POEA, ATN, and then GLYP (55). When examining the cytotoxicity of POEA and GLYP, POEA-treated A549 cells presented oxidative DNA damage, mitochondrial damage, apoptosis, and other phenomena that did not occur in the GLYP-treated A549 cells (56).

Cosmo-Flux 411F is an important adjuvant that is added at the time of spraying to control cocoa and poppy growth in Colombia (57). As for its toxicity, it was noted that honeybees acutely exposed to the mixture of GLYP and Cosmo-Flux 411F do not exhibit toxic effects. For mammals, this formulation only provides mild irritation to the skin and eyes, which can be relieved by washing. Furthermore, risk assessment of the GLYP formulation concluded that it posed no risk to bystanders. However, this formula is more toxic to aquatic animals [rainbow trout (*Oncorhynchus mykiss*), *Daphnia magna*, and others] compared to GLYP alone (58).

Overall, the addition of commercial adjuvant surfactants usually increases the toxicity of GLYP. The increased toxicity is usually related to DNA oxidation. The development of new adjuvant surfactants that are nontoxic and do not cause oxidative stress injury to reduce the toxicity of GBHs should be the focus of future research.

OXIDATIVE STRESS AND TOXICITY

Generation of Reactive Oxygen Species and Oxidative Stress

Oxidative stress is caused by excessive production of intracellular reactive oxygen species (ROS) or insufficient antioxidant defenses. GLYP-induced ROS generation plays critical roles in toxicity. Treating human renal proximal tubule cell line (HK-2) with 20, 40, or 60 μM GLYP for 24 h

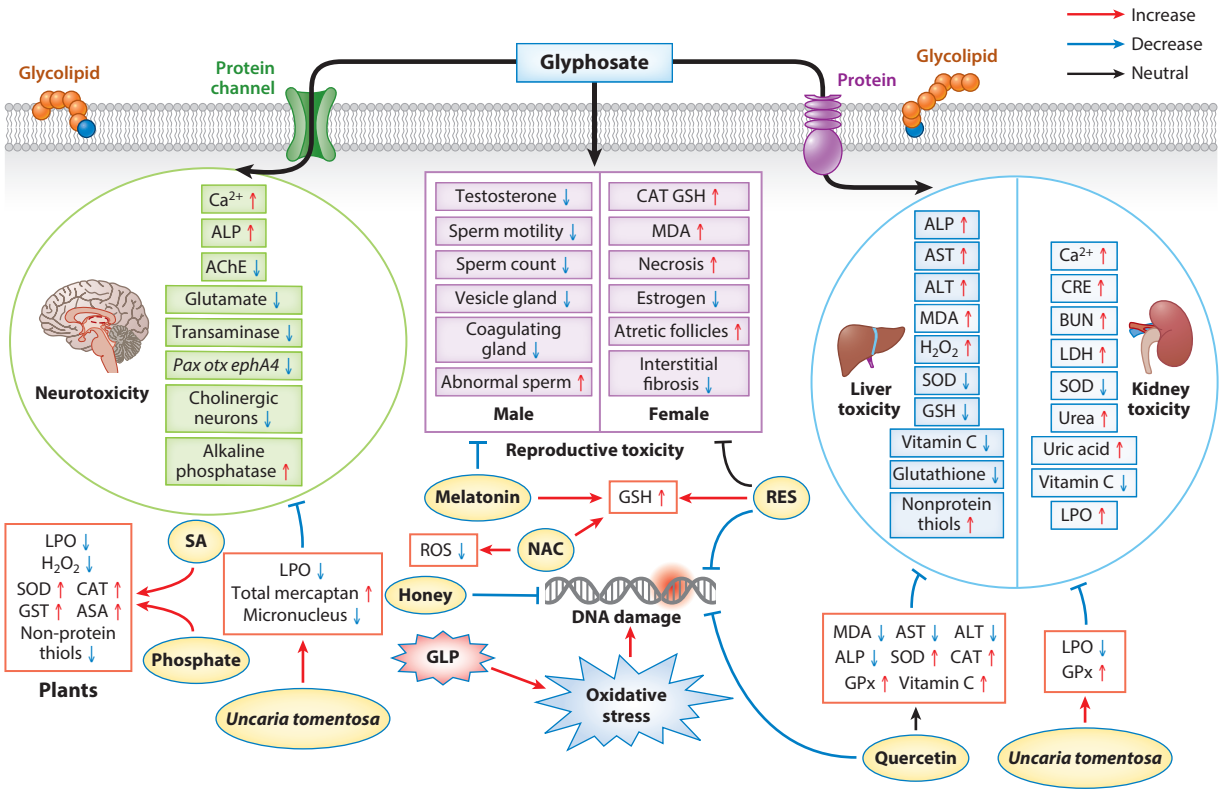

Figure 1

Antagonistic effects of exogenous substances on the toxicity and oxidative damage caused by GLYP. This herbicide can induce oxidative stress and various toxic effects. Many exogenous substrates, including dietary supplements, acids, and natural extracts, might diminish the toxicity caused by GLYP. Abbreviations: AChE, acetylcholinesterase; ALP, alkaline phosphatase; ALT, alanine transaminase; ASA, ascorbic acid; AST, aspartate transaminase; BUN, blood urea nitrogen; CAT, catalase; CRE, creatinine; GLYP, glyphosate; GPx, glutathione peroxidase; GSH, reduced glutathione; GST, glutathione *S*-transferase; H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; LPO, lipid peroxidation; MDA, malondialdehyde; NAC, *N*-acetylcysteine; RES, resveratrol; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase.

increased ROS production and reduced cell survival in a dose-dependent manner (59). In an in vivo study, the researchers administrated 0.0065, 0.065, or 0.28 g/L Roundup for 7 or 14 days to male or female prawns, respectively. In males, the genes of interest were downregulated in the presence of oxidative stress and ROS accumulation. By contrast, the same genes were overexpressed in females in response to oxidative stress (60). These data indicate that GLYP can alter the body's production of indicators related to oxidative stress (such as ROS) in a sex-dependent manner. Tomatoes were grown for 28 days with 0, 10, 20, or 30 mg/kg Roundup in the soil. The ROS content in the roots significantly increased (30). The imbalance between antioxidant defense and ROS production leads to oxidative stress, which eventually results in oxidative damage to DNA, lipids, and proteins. All of these findings are summarized in **Figure 1** and **Supplemental Table 2**.

Supplemental Material >

GLYP-Mediated Oxidative Damage

GLYP-mediated oxidative stress could destroy the oxidation and antioxidation balance to cause damage to lipids, DNA, and proteins and further lead to cell death and apoptosis.

Damage to lipids. LPO can be measured by monitoring changes in TBARSs and MDA. In male Wistar rats provided 0.1, 0.5, 1.75, or 10 mg/kg b.w. GLYP for 28 days, GLYP exposure significantly lowered TBARS levels in the liver and in plasma/serum (26). Pregnant Wistar rats exposed to 500 mg/kg b.w. Roundup for 7 days showed significantly elevated TBARS levels in the serum and liver (42). When hybrid jundiara fish (*Leiarius marmoratus* × *Pseudoplatystoma reticulatum*) were exposed to 1.357 mg/L GLYP at different times (6–96 h), there were significantly increased hepatic and muscular TBARS levels at all time points (27). Eyed-stage *O. mykiss* embryos exposed to sublethal Roundup concentrations (0.1 or 1 mg/L) for 3 weeks presented significantly decreased TBARS levels (61). In vitro, MDA increased significantly in human neuroblastoma SH-SY5Y cells exposed to 5 mM GLYP for 48 h (14). Similarly, TBARS values increased significantly in human lymphocytes (62).

In summary, LPO is a common consequence of GLYP-mediated oxidative stress. In addition, various studies have shown that GLYP-induced LPO appears to be dose and/or time dependent.

Damage to DNA. DNA damage caused by oxidative stress is involved in GLYP toxicity (63). The cell division block micronucleus test and alkaline comet test can determine DNA damage (64). Male Wistar rats treated with 0.1, 0.5, 1.75, or 10 mg/kg b.w. Roundup for 28 days showed a significant increase in primary DNA damage to hepatocytes and leukocytes at all tested concentrations (26). After exposing shrimp to 0.35, 0.70, 1.40, 2.80, or 5.60 mg/L GLYP for 96 h, the comet rate showed a significant time- and dose-dependent relationship (65). In vitro, mouse oocytes administered 500 μM GLYP for 14 h exhibited abnormal cell spindle morphology and double-stranded DNA breaks (66). In another study, A549 cells exposed to 50, 75, 100, or 125 mg/mL Roundup for 2 h presented DNA migration. Furthermore, the number of comet-positive cells significantly increased in a dose-dependent manner (67).

Based on these studies, the DNA damage is dose dependent and can occur at sublethal concentrations. Hence, the toxicity threshold of GLYP needs to be studied.

Damage to proteins. PC is a biomarker for protein damage in fish exposed to GLYP (68). Fish exposed to 1.357 mg/L GLYP for 6–96 h showed significantly elevated hepatic and muscle PC (27). Similarly, fish exposed to 0, 0.37, 0.75, 2.25, 4.5, or 7.5 mg/L GLYP for 96 h presented increased liver PC (19). In general, research on protein damage has focused on aquatic species. Thus, more research is needed on nonaquatic species.

Alterations in Antioxidant Status

GLYP mediates antioxidant status in plant and animal models. SOD represents the first line of defense against ROS (69). CAT, GR, and GPx constitute the first cellular defense against ROS. In addition, GST, ASA, vitamins, and other substances constitute a nonenzymatic antioxidant defense system (70).

In vivo animal studies. To investigate the relationship between GLYP toxicity, dose, and time in mice, subchronic (250 mg/kg b.w.) and chronic (500 mg/kg b.w.) groups were treated for 6 and 12 weeks. SOD activity was significantly reduced following subchronic as well as chronic exposure (71). When rats were orally treated by gavage with 0, 5, 50, or 500 mg/kg b.w. GLYP for 35 days, GSH, GPx, and total SOD activities decreased while MDA content in the small intestine increased (72). In weaned hybrid piglets (Duroc × Landrace × Yorkshire) fed with 0, 10, 20, or 40 mg/kg GLYP for 35 days, GLYP increased CAT and SOD activities in the duodenum (73). In the liver, the total antioxidant capacity (T-AOC) and CAT activities decreased and SOD activity increased at all concentrations. At 40 mg/kg, GPx activity was significantly higher compared with the other doses (10 and 20 mg/kg feed) (46).

When carp (*Cyprinus carpio*) were treated with 52.08 or 104.15 mg/L GLYP for 7 days, CAT, GPx, GR, T-AOC, and SOD activities were inhibited, and the contents of GSH were reduced in the gills (48). *Gammarus pulex* exposed to 10, 20, or 40 μg/L Roundup for 24 or 96 h showed decreased GSH, CAT, GPx, and AChE activities, with a temporary reduction in SOD activity (74). Similarly, in freshwater shrimp exposed to 0.35, 0.70, 1.40, 2.80, or 5.60 mg/L GLYP for 96 h, the SOD, CAT, and T-AOC levels decreased in a dose- and time-dependent manner, except for the 0.35 mg/L treatment (65). Prawns exposed to 0.0065, 0.065, or 0.28 mg/L Roundup for 7 or 14 days presented with changes in the expression of antioxidant genes such as *sod1*, *cat*, *gsh*, and *gpx*. These changes were greater in females compared with males (60). In summary, exposing whole organisms to GLYP causes damage to antioxidant enzymes. In addition to species, sex also affects the body's ability to resist oxidative damage caused by GLYP.

In vitro studies with animal cells. Caprine granulosa cells treated with 4.0 mg/mL GLYP for 72 h showed a significant decrease in SOD, CAT, and GST activities and enhanced LPO (28). When human L-02 hepatocytes were treated with 0, 60, 90, 120, 150, or 180 mg/L Roundup for 24 h, both GSH and SOD levels decreased while the MDA level increased (75).

Plant studies. Tomatoes were exposed to 10, 20, or 30 mg/kg GLYP for 28 days. SOD, CAT, and ascorbate peroxidase (APX) activities all increased in a dose-dependent manner in shoots. In roots, CAT and APX activities were the same as in shoots, but SOD activity was first increased and then inhibited (30). When *Hordeum vulgare* L. was treated with 30 mg/kg GLYP for 14 days, H_2O_2, SOD, CAT, APX, and GST were all elevated (76). When the floating aquatic plant species *Heteranthera dubia* was treated with 0, 1, 5, or 15 mg/L GLYP for 14 days, there were significant increases in SOD, CAT, guaiacol peroxidase, and APX activities (2). Plants and agricultural products can affect human health through enrichment of GLYP and in the food chain. Furthermore, plants are more exposed to GLYP than are animals. Hence, the use, dosage, and presence of GLYP residues in plants should also be studied.

STRESS-MEDIATED BIOLOGICAL RESPONSE

Many biological responses and cellular signaling pathways in plants and animals are affected by oxidative stress. In recent years, more and more studies have examined the role of GLYP-mediated oxidative stress in inducing apoptosis and autophagy and its respective cellular signaling pathways (**Figure 2** and **Supplemental Table 3**).

Supplemental Material >

Autophagy and Apoptosis

Microtubule-associated protein 1 light chain 3 (LC3), p62, and beclin-1 can monitor the formation of autolysosomes and autophagosomes (77). The mammalian target of rapamycin (mTOR) is an important autophagy regulator (78). Roundup (0, 50, 75, 100, or 125 μg/mL) transformed LC3-II, downregulated p62, and upregulated beclin-2. These alterations led to autophagy via AMPK/mTOR signaling in A549 cells (79). GLYP (0–160 μM) administration to a rat astroglioma cell line for 24 h increased the production of autophagy-related proteins and induced autophagy (80).

B cell CLL/lymphoma 2 (*Bcl-2*) is an antiapoptotic gene, while Bcl-2-associated X protein (*Bax*) is a proapoptotic gene (81). When human peripheral blood mononuclear cells (PBMCs) were treated with GLYP (0.01–5 mM) for 4 h, GLYP induced apoptosis through extrinsic and, in particular, intrinsic signaling pathways via caspase-8, caspase-9, and caspase-3 activation (82). A recent study in human alveolar cancer cells showed that Roundup increased Bax/Bcl-2, released

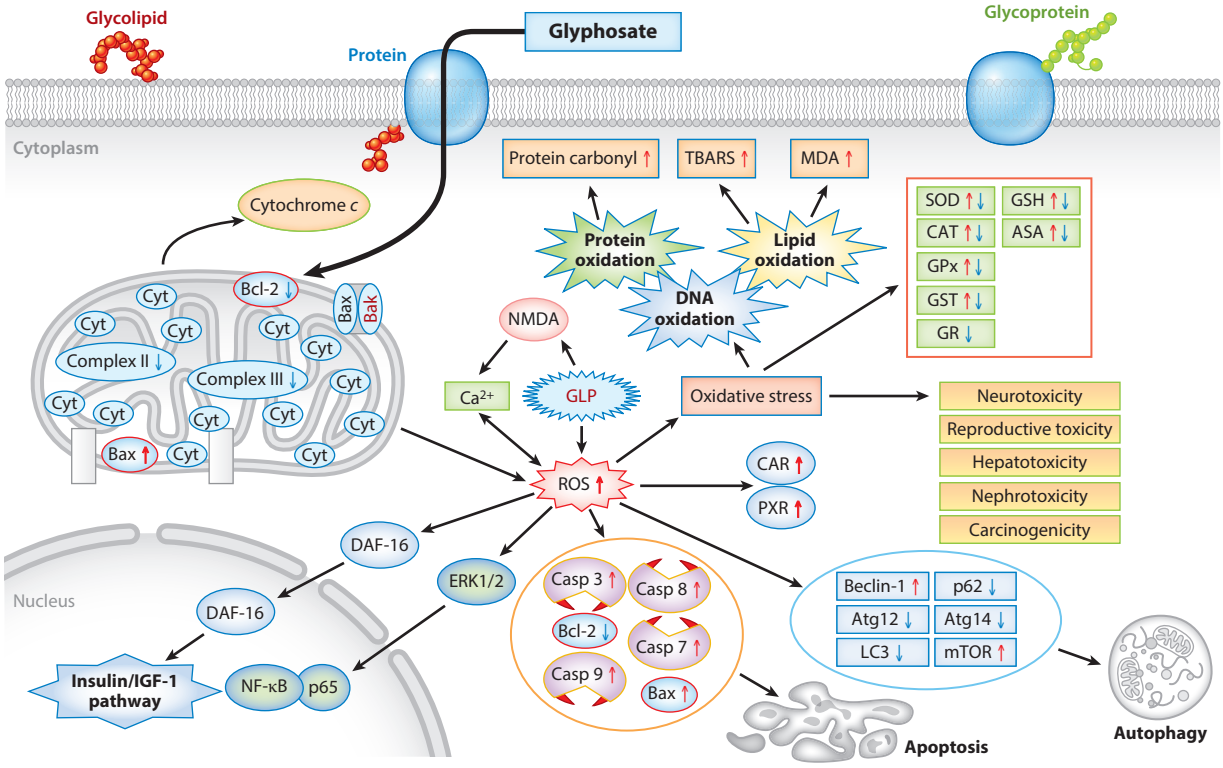

Figure 2

The oxidative stress–mediated mechanism proposed for GLYP. Increased ROS generation, as well as alterations in the antioxidant status, may induce lipid, DNA, and protein oxidative damage and subsequent apoptosis, autophagy, and/or mitochondrial damage. Toxicity and biological response may occur via caspases, mTOR, p62, ERK, NF-κB, CAR/PXR, and/or IGF-1 pathways. Abbreviations: ASA, ascorbic acid; Atg, autophagy related; Bax, Bcl-2-associated X protein; Bcl-2, B cell CLL/lymphoma 2; CAR, constitutive androstane receptor; CAT, catalase; Cyt, cytochrome *c*; DAF-16, FOXO transcription factor; ERK, extracellular signal-regulated kinase; GLYP, glyphosate; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione *S*-transferase; IGF-1, insulin-like growth factor 1; LC3, microtubule-associated protein 1 light chain 3; MDA, malondialdehyde; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NMDA, *N*-methyl-D-aspartate; PXR, pregnane X receptor; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance.

cytochrome *c* into the cell fluid, and then activated caspase-9/-3, leading to mitochondrial membrane collapse (67).

Apoptosis and autophagy overlap at different signal transduction stages in the same cell. To test the effect of GLYP on the mouse oocytes' maturation, the oocytes were cultured in culture medium supplemented with increasing concentrations of GLYP (50, 100, 200, or 500 mM) (66). The study revealed that mouse oocytes had significantly reduced rates of germinal vesicle breakdown and first polar body extrusion after treatment with 200 mM and 500 mM GLYP, while there was no significant variation in the 50 mM and 100 mM treatment groups. Hence, the 500 mM GLYP treatment was adopted for subsequent experiments. In mouse oocytes, administration of 500 mM GLYP increased Bax/Bcl-2. In addition, *Lc3*, *Atg14*, and *Mtor* gene expression was significantly decreased. These data indicate that GLYP activated autophagy and early apoptosis in oocytes (66). In SH-SY5Y cells, 5 mM GLYP or 10 mM AMPA significantly increased MDA,

ROS, and NO levels and caspase-3/-7 activity. *Bax*, *Bcl-2*, *Casp3*, *Casp7*, *Casp9*, *TNF*, and *NF-κB1*, among others, messenger RNA (mRNA) changed more than twofold. GLYP and AMPA induced cytotoxicity, oxidative stress through apoptosis, and autophagy pathways (14).

Inhibition of Mitochondrial Electron Transport Chain Transmission

GLYP can alter plant physiological responses, including photosynthesis and respiration (83, 84). After GLYP exposure, chlorophyll degradation and decreased photosynthetic electron transfer rate were related to the increase of ROS. The electrons produced by mitochondria can directly reduce oxygen (O_2), thereby generating ROS in mitochondria (85). One study determined the specific GLYP-induced ROS sites in duckweed (*Lemna minor* L.). Complex III of the mitochondrial electron transport chain generates ROS by transferring electrons from semiquinone to O_2, and GLYP can inhibit the activity of complex III (86). In a study using *Caenorhabditis elegans* to assess the potential toxicity mechanism of GLYP during acute exposure, it was found that mitochondrial activity was generally inhibited and H_2O_2 increased. This treatment also inhibited mitochondrial respiratory chain complex II activities. Inhibition of the electron transport chain decreases adenosine triphosphate (ATP) levels. Overall, GLYP may cause neurotoxicity by inhibiting complex II, reducing ATP levels, and increasing H_2O_2 production (87).

Cell Signaling Pathway

Several signaling pathways have been implicated in GLYP toxicity mechanisms.

Ca^{2+}. GLYP induced oxidative stress and affected intracellular Ca^{2+} homeostasis (88). The change in the intracellular Ca^{2+} concentration is related to the production of ROS (89). Treatment with 0.1 mM GLYP significantly induced HaCaT cell proliferation, reduced the intracellular Ca^{2+} concentration, and increased ROS production. In addition, Ca^{2+} inhibition can reduce Bax/Bcl-2 and thus inhibit apoptosis (90). As for neurotoxicity, after glutamate is released into the synaptic gap, glutamate uptake is reduced, and GLYP interacts with NMDA receptors, leading to the influx of Ca^{2+} ions into hippocampal cells (91). GLYP and AMPA can bind to NMDA receptors, so the NMDA receptor pathway in proximal tubule cells has been investigated. GLYP treatment increased expression of NMDAR1 (a subunit of the NMDA receptor) and intracellular Ca^{2+} and ROS levels, changes that led to apoptosis (59). Similarly, acute exposure to Roundup altered NMDAR and extracellular signal-regulated kinase (ERK) protein expression, the number of voltage-dependent calcium channels, Ca^{2+} influx, oxidative stress, and neuronal cell death in immature rats (35).

ERK/NF-κB signaling pathway. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) plays a key role in the inflammatory and immune responses. Its dysregulation can cause autoimmune diseases, chronic inflammation, and many types of cancer. NF-κB is also related to synaptic plasticity and memory. Researchers have studied NF-κB activity in the immature hippocampus. GLYP exposure inhibited the phosphorylation of p65 (part of NF-κB) and activated ERK1/2. These changes may be cellular responses to protect against GLYP-induced toxic events (91).

CAR/PXR signaling pathway. The constitutive androstane receptor (CAR) and pregnane X receptor (PXR), both of which are nuclear receptors, play an important role in regulating stage transcription of phase II metabolic enzymes and transporters. In weaned hybrid

Duroc × Landrace × Yorkshire piglets treated with various GLYP concentrations for 35 days, CAR mRNA expression only increased at the highest dose (40 mg/kg b.w.) (73).

Insulin/IGF-1 signaling pathway. FOXO transcription factor (DAF-16) is a *C. elegans* protein that is usually found in the cytoplasm. When there is adverse environmental stress, it will translocate to the nucleus, activate the transcription of a large number of genes, and increase the ability to resist stress (92). In *C. elegans*, DAF-16 regulates the stress response and is a major target of the insulin-like signaling pathway. GLYP treatment (4.8 mM for 16 h at 20°C) induced DAF-16 nuclear translocation and promoted the expression of antioxidant genes (92).

PREVENTION OF GLYP-MEDIATED OXIDATIVE STRESS

Numerous exogenous substances could exert a modulatory effect on GLYP-mediated oxidative stress.

Natural Extracts

Natural extracts can attenuate GLYP-induced oxidative stress.

***Uncaria tomentosa*.** *Uncaria tomentosa*, known as cat's claw, is a plant species with medicinal value. In the United States, it is used as a dietary supplement. *U. tomentosa* contains at least 50 constituents. For *Danio rerio*, treatment with 1.0 mg/L *U. tomentosa* extract prevented various GLYP-mediated toxicities: increased liver GPx activity, increased LPO, and decreased total thiols in the brain (63).

Resveratrol. Resveratrol (RES) is a type of natural phenol and a promising dietary compound with strong antioxidant properties (93, 94). Male Wistar rats were treated with 20 mg/kg b.w. RES and 375 mg/kg b.w. GLYP for 8 weeks. RES prevented the oxidative stress caused by GLYP by preventing LPO and increasing the GSH level (95). Similarly, 30 mg/kg b.w. RES protected sperm parameters, reduced GBH-induced LPO and DNA damage, improved antioxidant defense mechanisms, and regenerated damaged tissue in rat testes (38).

Quercetin. Quercetin is a flavonoid that is widely distributed in nature. It can exert its antioxidant capacity by reducing the generation of ROS (96). After intraperitoneally injecting mice with 50 mg/kg b.w. GLYP every 2 days for 15 days, there were marked changes: increased MDA, H_2O_2, and PC levels and elevated enzymatic (SOD, GPx, and CAT) and nonenzymatic antioxidant levels in the liver. There were also changes in serum AST, ALT, and ALP as well as DNA damage. The addition of 20 mg/kg b.w. quercetin administered daily by oral gavage significantly ameliorated all of these parameters, as well as the tissue structure of the liver (45).

***N*-acetylcysteine.** *N*-acetylcysteine is an *N*-acetylated derivative of L-cysteine. It can rapidly neutralize ROS. In addition, *N*-acetylcysteine has a regenerative effect on glutathione in the liver, which allows it to play a role in antioxidant stress (97). In Wistar rats treated with 160 mg/kg b.w. *N*-acetylcysteine and 375 mg/kg b.w. Roundup, the antioxidant attenuated the Roundup-mediated negative effects (98).

Melatonin. Melatonin is a methoxyindole hormone. Roundup disrupts pineal function and reduces melatonin production (99). Melatonin (10 mg/kg b.w.) administration with Roundup

(500 mg/kg b.w.) for 7 days improved reproductive parameters, reduced lesions at the implantation site, reduced serum TBARS and GSH levels, and corrected oxidative damage in female Wistar rats that was induced by GLYP (42).

Salicylic acid. As a lipophilic monohydroxybenzoic acid, salicylic acid (SA) stimulates the glutathione-ascorbate cycle in plants, helping to improve the efficacy of antioxidants and metal detoxification systems (100). For *H. vulgare* L., SA (100 μM) mitigated the effects of GLYP on H_2O_2 and LPO levels. It also reduced the ASA level and increased the activity of antioxidant enzymes (76).

Other Compounds

Other substances play a protective role on oxidative damage caused by GLYP.

Vitamins. Vitamin C and vitamin E abrogated GLYP-induced apoptosis of granulosa cells and oxidative stress of gastric mucosa. Vitamins C and E reduced oxidative stress–mediated apoptosis of granulosa cells, effectively reduced GLYP-mediated cytotoxicity, and prevented related fertility disorders (28).

Phosphate. In plants, both phosphate and GLYP use the same transporter. *H. dubia* treated with 0, 1, 5, or 15 mg/L GLYP for 14 days exhibited reduced MDA, H_2O_2, and soluble protein levels and antioxidant enzyme activity. Phosphate supplementation (at concentrations of 0, 50, or 100 mg/L) corrected these physiological responses (2). Similar results have been reported in willows (101). It is worth noting that the phosphate-mediated correction of GLYP-induced oxidative stress is limited. All of the antioxidant effects mentioned above are shown in **Figure 1**.

GLYP METABOLISM

Metabolic Pathways

GLYP acts by inhibiting EPSPS, an enzyme involved in the shikimate pathway (102). This pathway is found only in plants and bacteria—not in animals. The main GLYP metabolite is AMPA, which results from the cleavage of the C-N bond; it degrades slower than GLYP (103). Given that animals lack EPSPS, they are not direct targets of GLYP, and thus few studies have been conducted on GLYP metabolic pathways in animals. Notably, mammals contain gut bacteria that do have the shikimate pathway (104). However, the degradation rate of GLYP is low; its metabolites are usually discharged directly from the body with urine and feces (105). GLYP can also be degraded by photolysis, oxidation, and other pathways (106), but these processes do not involve the participation of specific enzymes. GLYP also can be acetylated, methylated, formylated, and nitrosylated (107). Other metabolites such as sarcosine are nontoxic. GLYP metabolism is shown in **Figure 3**.

Drug-Metabolizing Enzymes

Some studies have shown that GLYP alters CYP450 activity (108, 109). Healthy adult male and female Wistar rats were treated with 662 mg/mL GLYP for 90 days. In the females, hepatic 7-ethoxycoumarin *O*-deethylase (ECOD) (a marker for CYP-dependent enzymes) activity was 57% higher after GLYP treatment. In addition, ethoxyresorufin-*O*-deethylase (EROD, a CYP1A1/2-dependent enzyme) and 7-benzyloxyresorufin-*O*-debenzylase (BROD, a CYP2B-dependent

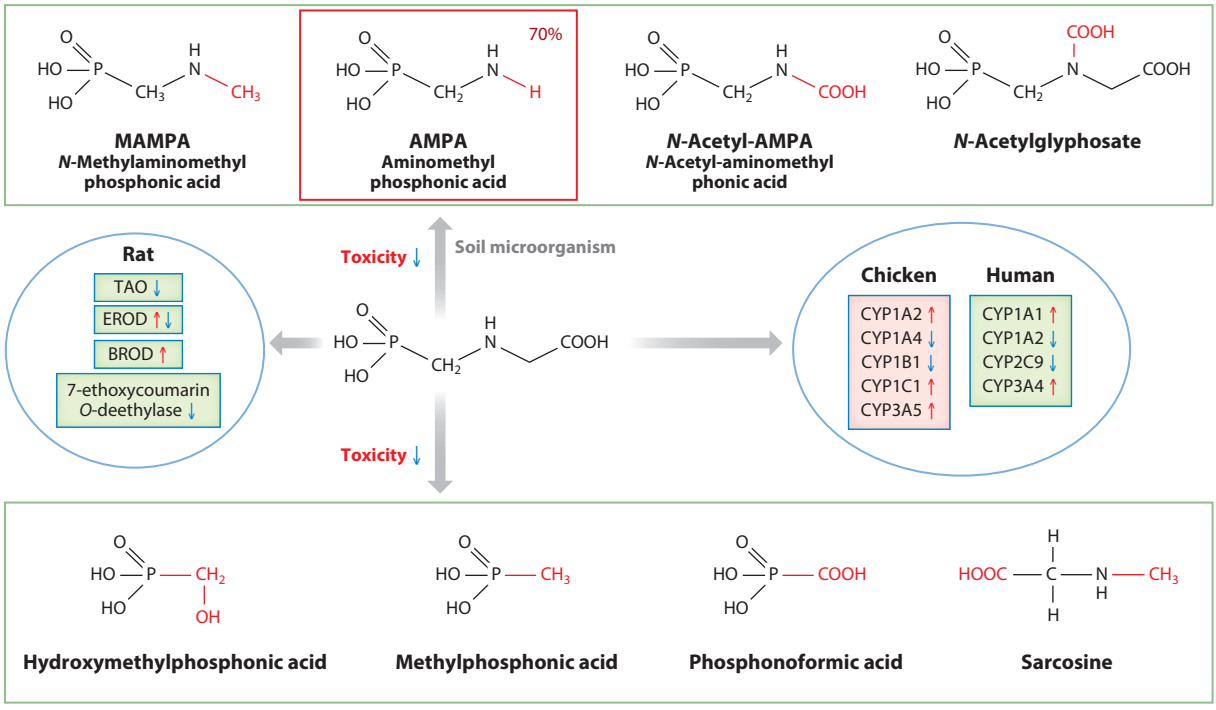

Figure 3

Effects of GLYP on metabolism, metabolites, and metabolizing enzymes. GLYP is metabolized by CYP450 enzymes or soil microorganisms. There has not been enough research on GLYP metabolism, although studies have revealed that its metabolites (shown at the bottom) are less toxic than the parent compound. Abbreviations: AMPA, aminomethylphosphonic acid; BROD, 7-benzyloxyresorufin-*O*-debenzylase; CYP, cytochrome P450; EROD, ethoxyresorufin-*O*-deethylase; GLYP, glyphosate; TAO, oleandomycin triacetate.

enzyme) activities were increased in liver microsomes. In males, hepatic ECOD activity was 58% lower after GLYP treatment. EROD and oleandomycin triacetate *N*-demethylase (a CYP3A-dependent enzyme) activities were decreased after GLYP treatment. The effects of GLYP on CYP450s were influenced by sex: In most cases, the metabolic capacity of male rats was higher than that of female rats (110). When chicken eggs were treated with 10 mg/kg b.w. GLYP or Roundup for 15 days, CYP1A2, CYP1A4, and CYP1B1 activities decreased in hepatic cytochromes and CYP3A5 increased. Moreover, there was decreased CYP1A4 activity in the duodenum and jejunum, increased CYP1A2 activity in the ileum, and elevated CYP3A5 and CYP1C1 mRNA expression in the jejunum after GLYP treatment (111). Roundup treatment specifically enhanced CYP3A4 (240–360%) and CYP1A2 (130–170%) activities in HepG2 cells. CYP3A4 was by far the major target of Roundup among the phase I metabolic enzymes measured (112). Moreover, GLYP had a strong inhibitory effect on the activity of CYP2C9 in human liver microsomes (113).

Oxidative Stress and Toxicity of GLYP and its Metabolite AMPA

According to the Food and Agriculture Organization, the accumulation of AMPA in the food chain makes it a potential toxicological problem (114). AMPA is reportedly less toxic than GLYP (24, 115). When PBMCs were exposed to GLYP and AMPA (0.01–5 mM) for 4 h, both molecules

activated caspase-3, caspase-8, and caspase-9 and caused chromatin condensation. AMPA exerted a lower effect on ROS compared with GLYP (82).

One study investigated the potential for ATN, GLYP, POEA, and AMPA to induce DNA damage in zebrafish larvae and Rtg-2 cells. Overall, the acute effects can be ranked in the following order: GLYP ≈ AMPA < ATN < POEA (55). Male and female guppies were exposed to different nominal concentrations of GLYP and AMPA for 96 h. Based on the LC_{50}, GLYP (68.78 and 70.87 mg/L, respectively) was more toxic than AMPA (180 and 164.3 mg/L, respectively). In the liver, the inflammatory response was more prominent in the reticulated male liver (116).

Willow plants were treated with either GLYP (0, 1.4, 2.1, or 2.8 kg/ha) or 0, 0.28, 1.4, or 2.8 kg/ha AMPA. Both molecules induced ROS, but only GLYP induced LPO. AMPA and GLYP affected photosynthesis via different mechanisms. Specifically, AMPA directly decreased the chlorophyll content, whereas GLYP increased ROS, a phenomenon that resulted in chlorophyll degradation (25).

Overall, in most cases AMPA is slightly less toxic than GLYP. When GLYP and AMPA cause oxidative damage, they sometimes do not trigger the same signaling pathway. Compared with GLYP, AMPA is more difficult to degrade and is more likely to cause toxicity due to residue accumulation. Therefore, more research is needed about its effects.

HUMAN EXPOSURE AND RISK

In 2015, the International Agency for Research on Cancer listed GLYP as a category 2A substance that may cause cancer in humans. Since then, the possible risks relating to human exposure to GLYP have attracted wide attention.

GLYP Exposure and Absorption

GLYP is a commonly used, low-toxicity pesticide (herbicide) with which many humans often come into contact. Additionally, human foods may contain small amounts of GLYP. It has been reported that 95% of beverages contain GLYP at concentrations ranging from 3.5 to 51 μg/kg (117). Maximal exposure to GLYP from the air is 1.04×10^{-6} mg/kg b.w. per day (118). For a 70-kg adult drinking 2 liters of water a day, the maximum daily GLYP intake is 2.25×10^{-5} mg/kg. Additionally, the US Environmental Protection Agency (EPA) estimates that dietary exposure of the general US population to GLYP is approximately 0.088 mg/kg b.w. per day (117). Judging from these data, the amount of GLYP humans are exposed to is below the acceptable daily intake (ADI) level set by the EPA.

The limited information available on the kinetics of GLYP in humans makes it difficult to interpret toxicological findings and to make a risk assessment for GLYP, topics that are still under debate. However, toxicokinetic variables are of major importance and should be considered in extrapolation of laboratory data to humans, since changes in various kinetic parameters either are easily determined experimentally or can be predicted with reasonable accuracy.

It has been reported that GLYP is slowly and poorly absorbed through the gastrointestinal tract in rats, having an absorption half-life of 2.29 h and a maximal plasma concentration of 4.62 μg/mL at 5.16 h after oral administration. The oral bioavailability of GLYP was 23.21% (31). The low bioavailability of GLYP may be caused by biliary excretion or GLYP degradation at the site of absorption. GLYP is poorly metabolized in rats. The metabolite AMPA represented 6.49% of the parent drug plasma concentrations (31).

Because toxicokinetic data on GLYP in humans are lacking, Zoller et al. (119) determined the fraction of GLYP and AMPA excretion in urine after consuming ordinary food with GLYP residue to estimate dietary GLYP intake. Twelve healthy adults, six males and six females,

participated in the study. Each participant was instructed to eat approximately 150 g of a falafel dish, corresponding to an intake of 196.8 μg of GLYP and 1.67 μg of AMPA (119). The low urinary excretion level identified in this study (approximately 1% of dietary exposure) suggests that intake estimations calculated from human urine data systematically underestimate the exposure. Further human toxicokinetic studies should be performed individually. In these studies, the determination of blood concentrations is necessary to improve human bioavailability data.

GLYP Exposure and Toxicity

The hazard quotient (HQ) is commonly used to evaluate toxicity. An HQ value below 1 indicates that toxicity is unlikely to occur. Reports have indicated that the HQ of GLYP is less than 1, meaning that it is unlikely to be toxic to humans (58). However, recent articles reviewing the relationship between GLYP exposure and both neurotoxicity and cancer have attracted much controversy. For example, the relationship between exposure to commonly used pesticides (paraquat, chlorpyrifos, GLYP, etc.) and Parkinson's disease has been studied. The results of the study showed that GLYP was the least toxic pesticide studied. Additionally, no statistically significant association between Parkinson's disease and GLYP exposure was identified (118). Nevertheless, a recent suicide incident garnered attention. A man attempted to commit suicide by taking 200 mL of GLYP and developed neurological symptoms such as an unstable gait 4 years after recovery. The man in question had no family history of Parkinson's disease and lived in a country with no medications that might cause Parkinson's disease (120). This phenomenon suggests that GLYP exposure may be related to Parkinson's disease onset and the dose may be critical.

As for the human carcinogenicity of GLYP, adult NHL and MM have been investigated, and no consistent evidence that exposure to GLYP results in NHL or MM has been found (121). Similarly, many researchers found no statistical association between GLYP and any subtype of cancer (122). In addition to Parkinson's disease and cancer, GLYP exposure has been suggested to increase the risk for intestinal disorders and a variety of emotional problems, including depression (119, 123, 124). In short, no clear evidence exists indicating that GLYP causes risk of diseases; however, improper or excessive use of GLYP may cause various toxic effects in humans.

GLYP Exposure and Regulations

GBHs have been sold in the United States since 1974. The EPA has agreed to the use of GBHs on a variety of food and nonfood crops, and more than 130 countries have registered such herbicides (125). However, in the United States, some regions of California and Florida have issued bans on GLYP. Similarly, French authorities banned the sale, distribution, and use of Roundup in early 2019. In May of 2019, it was announced that France would eliminate the use of GLYP by 2021, with limited exceptions, and some 20 mayors throughout the country have already banned GLYP in their municipalities (126). In December of 2019, the French Agency for Food, Environmental and Occupational Health and Safety decided that 36 GBHs would be withdrawn from the market and no longer permitted for use by the end of 2020. In addition, some individual countries (Portugal, Sri Lanka, and Vietnam) have introduced either legislation to ban or restrict private sales of GLYP or restrictions on spraying GLYP in public spaces.

Some regulatory agencies have set ADI and reference doses (RfDs) for GLYP. The EPA recommends the RfD of GLYP to be 1.75 mg/kg b.w. per day. The Joint Food and Agriculture Organization/World Health Organization Meeting on Pesticide Residues recommends the ADI to be less than 1 mg/kg b.w. per day. The ADI recommended by EFSA is 0.5 mg/kg b.w. (117). GLYP, however, is only approved in the European Union until 15 December 2022. To assess the

reauthorization of GLYP under the new political and legal landscape, the commission appointed four member states on 10 May 2019 to act jointly as rapporteurs in the next assessment of GLYP—this group of member states is known as the Assessment Group on Glyphosate (AGG). The AGG will assess the application dossier submitted by interested companies and will prepare a single draft renewal assessment report to EFSA in 2021 (127).

CONCLUSION

In summary, GLYP exposure to different nontarget organisms can cause a series of toxic reactions. Both oxidative stress and metabolism have distinct effects on toxicity, and the ability of antioxidants to modulate toxicity is also different. It is crucial to study the toxicity of GLYP and its mechanism to protect nontarget organisms from harm.

At present, the relationship between oxidative stress and drug toxicity is an important topic of research (128). Numerous studies have suggested that the toxicological effects of GLYP may be mediated via the induction of oxidative stress (30, 38, 44, 91). Many factors are related to oxidative stress. Previous studies have shown differences between plant and animal organs. In animals, GLYP affects the brain, liver, kidneys, and gills more than other organs. In plants, GLYP affects leaves, roots, and stems differently, even within the same plant (30). In addition, GLYP toxicity occurs in a time- and dose-dependent manner. In a reproductive toxicity study on male rats, we found that 5 or 25 mg GLYP/kg b.w. exposure for 14 days caused almost no toxicity, but when the rats were exposed to 5 mg GLYP/kg b.w. for 52 days, the testes and sperm were damaged (37, 38). Hence, further research is needed to determine the safe dose and duration of GLYP use.

Sex is also an important factor. In fish and crustaceans, many reports have indicated that females have more powerful antioxidant systems compared with males (60, 129–131). Female and male metabolic enzyme changes are also different (111). However, the exact reason for the sex difference remains unclear.

Exogenous substances can detoxify GLYP. The most common method of detoxification is to alter the organism's antioxidant properties, but the exact mechanism is not yet clear. Hence, we should find the key link of oxidative stress generation in future studies and use that information to find new antagonists.

Research on GLYP metabolism has focused on its main metabolite, AMPA, which is similar to GLYP in terms of mediating toxic effects and inducing oxidative stress. However, there have been few reports on the metabolic pathway of GLYP and the relationship between the GLYP metabolites produced and CYP450s. Therefore, the metabolism of GLYP needs to be further studied.

In general, with the widespread use of thousands of GLYP-based products around the world, understanding the metabolic characteristics of GLYP, as well as finding effective antidotes and safe adjuvants, should be the focus of GLYP research. This review elucidates the toxic mechanism of GLYP from oxidative stress and its metabolism, which helps to reduce the effects of GLYP on animals and humans.

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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