Glyphosate and Oxidative Stress:

ECHA's superficial approach neglects existing hazards

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Oxidative stress occurs when the production of 'reactive oxygen species' (ROS) exceeds the capacity of antioxidant defence mechanisms to deactivate them. ROS are highly reactive molecules derived from molecular oxygen (O2) normally generated during essential intracellular processes, e.g., energy conversion. The defence mechanisms in place ensure that these ROS are inactivated and the cells are not damaged.

Xenobiotics, including pesticides, can release ROS or interfere with the molecular defence processes of ROS inactivation, resulting in oxidative stress. Oxidative stress has been linked to both the causes and consequences of several diseases (Aghadavod et al. 2016, Kamceva et al. 2016, Qureshi et al. 2016, Sayanthooran et al. 2016, Turkmen 2017, Vakonaki et al. 2016), including cancer (Smith et al. 2016, Hecht et al. 2016, Kakehashi et al. 2013, Li et al., 2016, Perse 2013, Prasad et al. 2016, Toyokuni 2016) and damage to the nervous system (Martínez Leo and Segura Campos 2019, Singh and Devasahayam 2019, Yaribeygi et al. 2018). It is well-documented that all forms of inflammations lead to the release of ROS and cause neoplastic transformation (Yu et al. 2022). Oxidative stress can also be involved in reproductive failures (Archibong et al. 2018, Kovacic and Jacintho 2001, Santini et al. 2018).

In contrast to biomarkers for liver and kidney function (specific enzymes and other blood components measured in serum or plasma samples), biomarkers of oxidative stress are not part of the spectrum of parameters of OECD test guidelines. Therefore, the assessment of oxidative stress – although closely related to the potential damages mentioned above – depends exclusively on the results of studies conducted by the scientific community and subsequently published in scholarly journals. Therefore, it is obsolete to belittle these studies as "non-standard", "non-guideline" or "non-GLP" studies (e.g. ECHA 2022, p. 43-44). "Standard" studies (following OECD guidelines) just do not cover this important mechanism.

Though multiple biomarkers exist, measuring oxidative stress can be difficult due to redundant pathways of a highly interconnected system. The most commonly used biomarkers are increased antioxidant enzyme activity, depletion of glutathione (GSH) or increases in lipid peroxidation. An additional option to confirm the occurrence of oxidative stress consists of combining exposure to the test compound with subsequent or concomitant administration of antioxidants with the expectation that the effect of oxidative stress would be diminished.

Molecular oxygen is essential to the proper function of a cell. During the course of normal oxidative phosphorylation, between 0.4 and 4% of all oxygen consumed is converted into the free radical superoxide ($^{\bullet}O_2$). This $^{\bullet}O_2$ can be converted into other ROS and reactive nitrogen species (RNS) and is normally eliminated by antioxidant defences. $^{\bullet}O_2$ molecules are quickly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 is then either detoxified to H_2O and O_2 by

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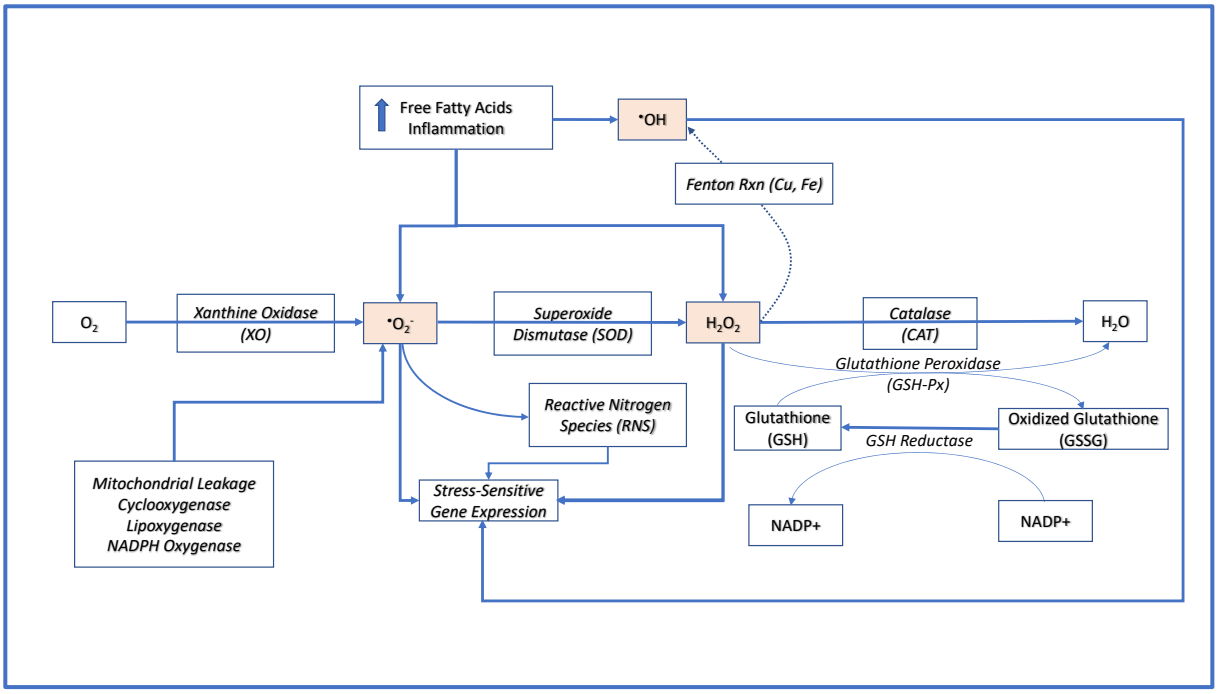
glutathione peroxidase or diffuses into the cytosol and is detoxified by catalase (CAT). However, in the presence of reduced transition metals such as copper (Cu) or iron (Fe), H_2O_2 can be converted to the highly reactive hydroxyl radical ($^{\bullet}OH$). These linkages are illustrated in the figure below.

Figure 1. Exogenous and endogenous stimuli leading to ROS generation and activation of stress-sensitive gene expression (modified from Evans et al. 2002).



The three ROS in the cell ($^{\bullet}O_2$, $^{\bullet}OH$, H_2O_2) can be measured directly, changes in the activity of the major enzymes (XO – xanthine oxidase, SOD, CAT, GSH peroxidase, GSH reductase) can be measured, as well as changes in GSH or glutathione disulfide (GSSG), changes in gene expression, changes in nitrogen oxide (NO) or changes in other enzymes (e.g. cyclooxygenase). In most studies two or more of these endpoints of this system were measured in animals or cells exposed to chemicals to see if they have changed due to the chemical exposure.

According to Regulation 1107/2009 (Article 8) industry is obliged to submit, and EU authorities are obliged to comprehensively assess, scientific publications describing potential health and environmental effects of pesticide active ingredients - see also Guidance document by EFSA[4]. It goes without saying that oxidative stress needs to be part of these considerations, because it represents a "blind spot" in the OECD test guidelines.

As it will be shown here for glyphosate, using ECHA's Opinion dated 30 May 2022 (ECHA 2022) as the most recent example, oxidative stress was taken into account during the assessment, but in a very superficial way, resulting in serious deficiencies with regard to the assessment of the potential hazards and their underlying mechanisms. EFSA has endorsed ECHA's opinion in its assessment.[5]

The problem starts at the numerical level. Since the last assessment report in 2015, only 11 new papers were included, whereas another 21 describing original results of studies conducted in

[4] https://doi.org/10.2903/j.efsa.2011.2092
[5] https://doi.org/10.2903/j.efsa.2023.8164

vertebrates or in *in vitro* systems were omitted.[6] In these 21 studies published between 2016 and April 2022 glyphosate of analytical grade purity was used. In addition, a considerable number of studies using glyphosate-based herbicides (GBH) are available. And since May 2022 (the month when ECHA published its opinion) further papers investigating oxidative stress of glyphosate and/or GBH have been published.

Neglecting the available studies ECHA's risk assessment committee (RAC) nevertheless concluded in its Opinion that glyphosate may induce oxidative stress (emphasis added), but denied any relevance for germ cell mutagenicity and carcinogenicity either by not mentioning it at all (Section on "Mechanistic studies from public literature", ECHA Opinion p.54/55; ECHA 2022), or verbally minimizing its potential significance ("As discussed in the germ cell mutagenicity section, the evidence that glyphosate induces mutations is very weak.", ECHA Opinion, p. 75; ECHA 2022).

While there is a controversy of its own, whether glyphosate is genotoxic or not, the failure to acknowledge glyphosate-induced oxidative stress as a potential mechanism for carcinogenicity and DNA damage is in contradiction to the experts' view of the International Agency on Research on Cancer (IARC) who identified "strong evidence" for genotoxicity and oxidative stress as mechanisms of glyphosate carcinogenicity (IARC 2015).

Mitochondria, ROS, calcium and cancer

As mentioned above, oxidative stress is induced by excess ROS generated during processes running in intracellular organelles, particularly mitochondria. In the past, mitochondria were just considered the intracellular "power plant". Today, however they are seen as information processing systems that – in communication with other cell compartments – sense and respond to both endogenous and environmental inputs, integrate information, and produce output signals (Picard and Shirihai 2022). According to these authors, the mitochondria's output signals are deeply involved in physiological regulation; disturbances can change this physiological regulation into pathological processes which may become irreversible (genetic damage, epigenetic changes, tumorigenesis, neurodegeneration).

ROS cause DNA damage, but they are also able to promote oncogenes, inhibit tumour-suppressor genes and stimulate carcinogenesis via epigenetic alterations. Different (secondary) signalling molecules affected by ROS participate in these processes. This includes transcription factors NFκB[7] and PDK-1[8], involved in cell proliferation in general, and protein kinase B involved in cancer cell proliferation in particular (Miyata et al 2017). At moderate concentrations ROS can activate the cancer cell survival signalling cascade (protein kinase B is part of it), while at high concentrations ROS can cause cancer cell apoptosis (Aggarwal et al. 2019). Thus, it critically depends upon ROS levels and their interplay with other signalling molecules, whether tumorigenesis is augmented or apoptosis (cell death) is elicited.

However, it is not ROS alone that interact with intracellular signalling. A mutual regulation of the signalling pathways of ROS and calcium (Ca^{2+}) is well established (Hempel and Trebak, 2017). These authors emphasize that coordinated surges of ROS and Ca^{2+} are necessary to initiate apoptosis, while "localized sublethal changes in both ROS and Ca^{2+} levels fine-tune signalling cascades that maintain proliferative and metastatic signals".

[6] of these 21 studies, 6 were conducted in mammals, 7 *in vitro* (including 2 in bird eggs), 2 in amphibians, 4 in fish, and 2 were epidemiological studies

[7] Nuclear factor κB

[8] 3-phosphoinoinositide-dependent kinase-1

Increased ROS levels result in oxidative damage to macromolecules, including DNA (Klauning et al. 2011). It is a peculiarity of tumour cells that they tolerate higher levels of ROS than normal cells or may even be promoted under these conditions. Ray et al. (2012) point out that "aberrant regulation of proliferation and apoptosis" by ROS are "essential in tumorigenesis". If the interplay between ROS and Ca^{2+} is altered, this can result in resistance to apoptosis and in signalling cascades promoting proliferation and metastasis (Hempel and Trebak,2017).

In other words, even if ROS are not directly generated from chemicals themselves (as a result of their metabolic degradation), the interference of these chemicals with endogenous ROS generation and calcium signalling cascades can result in tumorigenesis, tumour promotion, and other irreversible damage. Thus, only part of the adverse effects of ROS is related to genetic damage. They are also able "to interact with any biological molecule (e.g., protein, lipids, DNA), triggering the formation of new radical species able to propagate and amplify cellular damage" (Antonucci et al. 2021).

Therefore, indicators of oxidative stress associated with exposure to chemicals should always be considered a warning sign of carcinogenic, genotoxic or neurodegenerative potential. In the light of the complex processes briefly touched upon here, it would be scientifically flawed to reduce ROS-elicited tumorigenesis to genetic damage *per se*. Furthermore, we know that Ca^{2+} and ROS are key actors of non-linear processes, hence contradicting expectations of linear dose-response relationships for carcinogenic effects elicited through oxidative stress. This has particular significance for the carcinogenicity assessment of glyphosate because a main argument of the authorities' conclusion why glyphosate should not be considered a carcinogen is that the numerous tumours seen in the glyphosate carcinogenicity studies did not follow simple dose-dependence across studies (AGG 2021 Volume 1, pp.256-298).

Glyphosate, ROS and calcium signalling

As it will be shown below, there is plenty of evidence that glyphosate and GBH increase markers of oxidative stress indicating an excess of ROS. In addition, glyphosate or GBH exposure is associated with increased Ca^{2+} levels (see de Batista et al. 2023 for review).

Here we consider glyphosate (active ingredient) only because this is the focus of ECHA's hazard assessment.

The first report of uncoupling of mitochondrial oxidative phosphorylation by glyphosate (resulting in ROS increase) was published more than 40 years ago (Bababunmi et al. 1979). In 2015, the authorities (RMS Germany 2015) acknowledged that uncoupling of oxidative phosphorylation by glyphosate was demonstrated in this and another study (Lopes et al. 2014).

Although oxidative stress was at least discussed in the carcinogenicity sections of the 2015 assessments (Renewal Assessment Report of 2015, Harmonised Classification and Labelling report, ECHA Opinion), it was not even mentioned in the carcinogenicity section of the 2021 draft Renewal assessment report (AGG, 2021), despite of additional studies published since 2015. A number of studies had been submitted during the public consultation (Gao et al. 2019; Liu et al. 2022a, 2022b, Tang et al. 2020, Eaton et al. 2022). But they were dismissed in ECHA's Opinion with the umbrella statement that "RAC considers the data from the studies to be equivocal due to deficiencies in reporting …" (ECHA 2022, p. 46), without specification for any of these studies what "deficiencies in reporting" were meant.

According to our review, study design and results are well described in the studies where ROS biomarkers were significantly increased. Specifically, this relates to evidence of oxidative stress:

- in mice at 400 mg/kg given orally for 28 days (Gao et al. 2019),
- in rats at 2 and 50 mg/kg via dietary exposure for 2 months (Liu et al. 2020a) and for 4 months (Liu et al. 2020b),
- in rats at 50 and 500 mg/kg given orally for 35 days (Tang et al. 2020).

It should be noted that in two of these studies, "proof of concept" was provided by combining the exposure to glyphosate with "antidots" to oxidative stress, i.e. antagonists for receptors involved in the generation of oxidative stress (Gao et al. 2019, Liu et al. 2020b). This combined administration of glyphosate and receptor antagonists ameliorated or prevented the oxidative stress induced by glyphosate. Remarkably, kidney tumour incidences (adenomas and/or carcinomas) were significantly increased in male mice in the majority of the mouse carcinogenicity studies (three out of five). It should be noted that it is well-known that ROS play a major role in the development and progression of renal tumours (Shanmugasundaram and Block 2016).

Liu et al. (2020b) analysed glyphosate concentrations in serum and target tissues, thus providing direct evidence of glyphosate exposure – quality marker of study design lacking in most guideline studies.

In addition to the peer-reviewed scientific studies initially ignored by ECHA and EFSA, and submitted during the public consultation in November 2021, further *in vitro* and *in vivo* studies have been published, demonstrating oxidative stress after exposure to glyphosate:

- in mice (Lu et al. 2022),
- pigs (Xing et al. 2022), and
- human cell lines (Mehtiyev et al. 2022).

Furthermore, recent epidemiological studies show an increase in oxidative stress markers in humans is associated with increasing urinary levels of glyphosate and/or its main metabolite AMPA[9] (Chang et al., 2023, Eaton et al. 2022, Makris et al. 2022, Sidthilaw et al. 2022).

Chang et al. (2023) – using data of the Agricultural Health Study, a study considered by ECHA and EFSA of highest quality – draw the explicit conclusion that the findings "contribute to the weight of evidence supporting an association between glyphosate exposure and oxidative stress in humans and may inform evaluations of the carcinogenic potential of this herbicide." Their findings show clear dose-response for 8-OHdg (8-Hydroxydesoxyguanosin) and MDA. The excessive formation of 8-OHdg is one of the most important markers concerning carcinogenicity elicited by oxidative stress (Wu et al. 2004).

Glyphosate disturbs epigenetic processes

Essentially, epigenetics is defined as a functional modification to the DNA that does not involve an alteration of the base sequence. According to Meaney (2010, p. 57), "the essential features of epigenetic mechanisms are (a) structural modifications to chromatin either at the level of the histone proteins or the DNA, (b) regulation of the structure and function of chromatin, (c) effects on gene expression, and (d) that these effects occur in the absence of any change in nucleotide sequence. The functional byproduct of the epigenetic modifications is that of a change in gene transcription."

As mentioned above, ROS can stimulate carcinogenesis (and other pathological processes) also via epigenetic alterations. Therefore, it is plausible, that glyphosate – via ROS-generation – could cause

[9] aminomethylphosphonic acid

epigenetic changes. Two recent reviews (Rossetti et al. 2021, Bukowska et al. 2022) have summarized the existing knowledge on glyphosate's epigenetic effects.

It should be noted that epigenetic modifications – similar to those of oxidative stress – are not covered by OECD guideline studies, but they have a serious hazard potential, because they can cause the development of diseases long after exposure to the chemical hazard (Bukowska et al. 2022). Contrary to a statement made in ECHA's opinion that considers epigenetic modifications as "mostly reversible" (ECHA 2022, p. 44), "epigenetic marks could be maintained over time and be transmitted transgenerationally in second, third and fourth generations" (Rossetti et al. 2021). Bukowska et al. (2022) point out: "Even carcinogens, which do not exhibit genotoxic, apoptotic and cytotoxic potential to the cell may still be implicated in carcinogenesis in an epigenetic manner by direct influence gene expression during transcription, translation, and post-translational events."

Of the 15 studies summarized by Bukowska et al. (2022), only three were taken into consideration by ECHA (2022), and these three were dismissed because of "no clear dose-response" (Kwiatkowska et al. 2017, Wozniak et al. 2021) or "one dose only" was investigated (Duforestel et al. 2019). Instead of dismissing these studies using oversimplified arguments, ECHA in its alleged "weight of evidence"-approach should have performed a comprehensive review of all existing evidence taking into account the potential non-linearity of ROS effects that can lead to epigenetic alterations (see above).

Conclusions

AGG (2021) in its alleged "proof" of lack of dose-dependence made simple comparisons of tumour incidences across strains with no adjustments for any of the restrictions recommended by OECD (2012) for historical control data (HCD)[10]. In contrast, Portier (2020) used pooled logistic regression analyses of tumour incidences within the same strains of rats or mice as a method to adjust for between-study variations. This proper mathematical approach already demonstrated across-study consistency for many of the statistically significant increases seen. The complexity of the roles of ROS and Ca^{2+} as part of carcinogenic mechanisms (Miyata et al. 2017, Hempel and Trebak 2017) supports the demand to refrain from simplistic dose-response-comparisons across studies used by ECHA to dismiss observed increases in tumour incidences.

In the four *in vivo* studies mentioned here (Gao et al. 2019; Liu et al. 2022a, 2022b, Tang et al. 2020) the same species (rats or mice) and same route of exposure (oral) were used as in the guideline studies of carcinogenicity. Significant increases in biomarkers of oxidative stress were demonstrated at lower doses and shorter exposure times than in the carcinogenicity studies. Therefore, it is plausible to assume that oxidative stress was present in dose groups with significantly increased tumour incidences of the carcinogenicity studies. This applies for instance to the kidney tumours seen in males of three out of the five mouse studies. Gao et al. (2019) demonstrated that glyphosate caused oxidative stress in kidneys of male mice – the same sex as kidney tumours were seen in the carcinogenicity studies, and they experimentally identified the underlying mechanism. A "weight of evidence approach" that is worth its name requires a synoptic consideration of the effects seen in the carcinogenicity studies and the mechanistic evidence eventually available only in the scientific literature – something ECHA failed to do.

[10] Restrictions were recommended by OECD (2012) for the use of HCD, because they are frequently used as an additional tool to assess tumor effects. Scientifically, however, the same restrictions apply to inter-study comparisons of tumor incidences in general to avoid bias due to variation in environmental conditions and/or genetic background. The major restrictions require that only studies should be compared which were conducted in the same strain of animals, in laboratory and within a 5-year window of time.

As outlined here, oxidative stress does not need to be generated from chemicals directly but can result from the interference of the compound with the endogenous ROS cycle. While guideline studies do not contain endpoints covering this aspect, oxidative stress has been demonstrated extensively for glyphosate in academic studies. ECHA's risk assessment committee failed to use an appropriate weight of evidence approach by matching the existing mechanistic evidence with the statistically significant increases in tumour incidences seen in the carcinogenicity studies. Instead of performing a comprehensive integrated review of existing evidence, ECHA overlooked a large number of studies and dismissed others based on their isolated consideration using oversimplified arguments.

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