Environmental Monitoring of *Glyphosate* and Assessment of its Combined Cytotoxicity with Adjuvants

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Abstract
Pesticide toxicology currently focuses mainly on two areas: long-term effects of given compounds and cocktail effects of chemicals, including combined effects of pesticide active ingredients with their adjuvants, as seen in the case of *glyphosate*-based herbicides. In this study surface water pollution in an agricultural region of Hungary by *glyphosate* was determined by ELISA method, and cytotoxic effects on *HEK293* and *NE-4C* cells by *glyphosate*, its formulated herbicide (ROUNDUP®) and adjuvant (polyethoxylated tallowamine, *POEA*) were compared. ROUNDUP and *POEA* were found to be equitoxic at short exposures (LC_{50}: 10-15 ng/ml in 6 hrs), while *glyphosate* occurred to be of 500-750-fold less toxicity.

Introduction
The role of pesticides in current industrial agriculture is to suppress damages in crop production by agricultural pests. To achieve such chemical protection, pesticides are used in formulations as mixtures of active ingredient(s) responsible for the main effect of the pesticide preparation with adjuvants added to improve physico-chemical properties, adsorption/penetration capability and other characteristics of the active ingredient(s) [1]. With the worldwide expansion of monoculture-based agriculture, overall pesticide consumption continuously grows not only affecting targeted crop yields, but also causing increasing chemical pressure on the environment. In addition, pesticide residues are often the source of chemical exposure as they enter the food chain upon agricultural practices.
Due to this environmental load and subsequent exposure to numerous non-target organisms, surveys on pesticide residues and their side-effects are expanding [2], and consequently pesticides are subject to strict registration processes specified in corresponding international recommendations [3, 4] and legal regulations [5, 6], and pesticide residues are strictly regulated through their maximal residue levels (MRLs) in food and feed set upon evidence-based scientific risk assessment [7, 8]. General pesticide toxicology research in the 70's was mostly focused on the acute effects. During the late 80's (with the development and extended use of the metabolic bacterial reverse mutation assay developed by Bruce Ames [9] and other microbial mutagenicity tests), attention turned towards mutagenicity, carcinogenicity, teratogenicity and epidemiological examination of pesticides, and toxicity requirements strictened towards candidate pesticide substances. Expanding knowledge justifies why active ingredients in plant protection products have to undergo regular re-assessment. During the past two decades, increasing interest has been expressed in fields, where sufficient knowledge was still lacking: the study of immunomodulant and endocrine disruptive effects [10].
Current toxicology focuses on two main areas: (*i*) long-term effects of chemicals at doses near the no observed effect level (NOEL) in exposures extending over long periods, even life-times, and (*ii*) effects of numerous compounds exerting toxicity in parallel, so called cocktail effect. The latter type includes the case of combined effects of active ingredients with their adjuvants. These adjuvants are considered „inert" in terms of the main effects of the pesticide

active ingredient, but they may cause substantial side-effects or increase side-effects of the active ingredients.

Glyphosate

Glyphosate is presently the largest selling herbicide active ingredient in the world, and its market continues to grow in line with restrictions/ban on other herbicides and the increase in the cultivation of *glyphosate*-tolerant (GT) transgenic crops [11]. By blocking the biosynthesis of essential aromatic amino acids though inhibiting the shikimic acid metabolic pathway and also inhibiting photosynthesis, *glyphosate* shows general phytotoxicity, allowing its broad pre-emergent herbicide applications. As a result of the long-term, intensive use, our surveys indicate glyphosate as a common contaminant in rivers and other surface waters [12-13]. Formulated preparations of *glyphosate* have been indicated to exert harmful biological effects, for example endocrine disruption [14-15] or teratogenicity [16], even under its no-effect level (NOEL) upon extended chronic exposures. The current evaluation by the UN International Agency for Research on Cancer (IARC) classified *glyphosate* as probably carcinogenic to humans (Group 2A), based on "limited evidence" in human experiments and "sufficient evidence" in animal-experiments [17]. In light of these adverse side effects, the recent re-approval of *glyphosate* has been claimed unacceptable [18]. As *glyphosate* has been reported as common surface water pollutant, and as substantial differences have been evidenced in the toxicity of *glyphosate* and its formulated herbicide products, attributed to side-effects of the adjuvants applied in formulation, our study aimed to evaluate the environmental occurrence of *glyphosate* in surface waters, and to comparatively assess cytotoxicity of *glyphosate*, its formulated herbicide preparation ROUNDUP® and common adjuvant (used also in ROUNDUP) polyethoxylated tallowamine (*POEA*).

Materials and Methods

Glyphosate and ROUNDUP were obtained commercially, *POEA* was provided by Lamberti SpA (Albizzate, Italy), chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). *Glyphosate* was determined in environmental samples by a commercial enzyme-linked immunosorbent assay (ELISA), cell viability was tested using the thiazolyl blue tetrazolium bromide (MTT) reduction assay detecting mitochondrial respiration intensity [19] on two cell types: a human embryonic kidney cell line expressing adenovirus-specific tumor antigen (*HEK293*) [20] and a mouse neuroectodermal stem cell line (*NE-4C*) [21]. *HEK293* cells were purchased from Sigma-Aldrich Co., *NE-4C* cells were kindly provided by the Institute of Experimental Medicine of the Hungarian Academy of Sciences.

Immunochemical analysis of glyphosate

Immunochemical analysis of *glyphosate* was performed using the ELISA kit by Abraxis LLC (Warminster, PA, USA), validated to determination of *glyphosate* in water (ground/surface/well water). Without sample extraction, water samples were derivatized (acetylated) prior to immunoanalysis, and then were pipetted onto the manufacturer-supplied 96-well microplates. Samples were incubated (30 min) on the microplate with *glyphosate*-specific antibodies, then IgG-specific second antibodies conjugated to a reporter enzyme were added, and upon further incubation (60 min) and washing, substrate (H_2O_2) and a chromophore were added, and optical density of the solutions was detected in a MULTISKAN ASCENT microplate reader (Labsystems, Finland). *Glyphosate* concentration was determined using analytical standard and sigmoid (logistic regression) calibration.

Cell culture work

Cell lines were stored at -196^{o}C in liquid nitrogen, and subsequently thawed in water bath, washed and cultured in buffer medium containing 10% fetal bovine serum. Cells were grown

at 37°C (under an atmosphere of 5% CO_2, 95% air) to 80% confluence, and prior to cytotoxicity measurement were passed through at least one passage. Upon being washed with serum-free medium buffer, cells were exposed to various chemicals for up to 24 hrs [22]. The MTT cytotoxicity tests were carried out in 96-well microplates. Cells were incubated with *glyphosate*, ROUNDUP and *POEA* at various concentrations for 2, 6 and 24 hrs in buffer medium with or without serum added [23] and after washing, MTT (0.1 mg/ml in buffer medium) was added, and color development was detected at 570 nm.

Results and Discussion

Determination of glyphosate

Using the Abraxis ELISA method, *glyphosate* was detected above the practical limit of detection (LOD) (0.12 ng/ml) in half of the surface water samples collected in the autumn period from a maize growing agricultural region of Hungary. *Glyphosate* concentrations showed a somewhat bimodal pattern: concentrations were either below (or in some cases slightly above) the LOD, or were found alarmingly high (0.542±0.003 to 0.984±0.003 ng/ml). In contrast, surface water samples collected in the spring period (before intensive pre-emergent herbicide applications) were found predominantly not to contain *glyphosate* above the LOD, possibly due to a dilution effect in standing water bodies or in rivers.

It has to be noted that the ELISA method is specific only to *glyphosate*, and it doesn't detect its primary metabolite, aminomethylphosphonic acid (AMPA). According to manufacturer's specifications, the cross-reactivity of the detection method for AMPA is slightly above 0.0001%. This means, analysis covers only the presence/absence of the parent compound (*glyphosate*) and not its official residue level, *glyphosate* and its metabolite(s).

Cytotoxicity measurements

ROUNDUP strongly suppressed cell viability, detectable even after 2 hrs of exposure, significantly dropped by 6 hrs, but with no further decay until 24 hrs. *POEA* caused similarly decreased cell viability above 5 ng/ml concentration, and the effect continuously increased from 2 to 24 hrs of exposure. LC_{50} values for ROUNDUP and *POEA* on *NE-4C* cells upon 6 hrs of exposition were found to be 15 and 10 ng/ml, respectively, but *POEA* caused more rapid cytotoxicity. In contrast, cytotoxicity of *glyphosate* (LC_{50}: 7.5% μg/ml in 6 hrs) was 500-750-fold lower. ROUNDUP is well known to be cytotoxic by inhibiting mitochondrial respiration (LC_{50}: 57.5 μg/ml) [22] and induced cell necrosis by a 15-fold increase in adenylate kinase release. The apoptotic effect of ROUNDUP was seen by an increase in caspase 3/7 activity by 6.29-8.24 times compared to the control level.

Conclusions

Glyphosate-based herbicides present dual hazards in terms of environmental contamination and cytotoxicity. In this study, *glyphosate* was found in half of the surface water samples from a maize growing region of Hungary, with contamination as high as 0.984 ng/ml in a bimodal pattern. *POEA*, the main adjuvant of *glyphosate*, was found cytotoxic above 1 ng/ml concentration on human cell lines after 2 to 24 hrs of exposure. The results evidenced the hazard of *glyphosate* and even more of its adjuvant *POEA* on human cell viability, underlying the necessity of toxicological risk assessment of pesticide formulating adjuvants for their combined effects with pesticide residues. Thus, risk assessment has to be extended to toxicological consequences of such parallel exposure.

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