In vitro cytotoxicity and genotoxicity of single and combined pesticides used by Bolivian farmers

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Abstract
We previously showed that farmers in Bolivia are exposed to many pesticides, some at elevated levels, and that this was associated with increased risk of genetic damage. To improve the understanding of possible mixture effects, the cytotoxicity and genotoxicity of pesticides were studied in vitro using human liver HepG2 cells. The studied pesticides were 2,4-D, chlorpyrifos, cypermethrin, glyphosate, methamidophos, paraquat, profenofos, and tebuconazole. Three mixtures (U1, U2, and U3) were based on profiles of urinary pesticide metabolites and one mixture on the most frequently used pesticides (S1). The results showed that paraquat and methamidophos were the most cytotoxic pesticides (EC50 ≤ 0.3 mM). Paraquat, chlorpyrifos, cypermethrin, and the U1, U2, and U3 mixtures, which contained a large proportion of either chlorpyrifos or tebuconazole, significantly increased intracellular ROS levels. Most pesticides activated DNA damage signaling through proteins Chk1 and H2AX. Strongest responses were elicited by paraquat, profenofos, chlorpyrifos, cypermethrin, and the S1 mixture, which contained 25% paraquat. Comet assay revealed significant increases of DNA damage in response to paraquat, cypermethrin, and U2 and S1 mixtures, which contained high levels of cypermethrin and paraquat, respectively. In summary, we showed that the tested pesticides, alone or in mixtures, in general induced oxidative stress and that most pesticides, and especially paraquat and cypermethrin, were genotoxic in HepG2 cells. We could also show that mixtures dominated by these two pesticides displayed a marked genotoxic potency, which agreed with our previous population studies.

KEYWORDS
comet assay, DNA damage response, gene expression, glyphosate, mixtures, paraquat

INTRODUCTION

Pesticide exposure is a health hazard that might lead to acute and chronic health effects, including neurotoxicity, endocrine disruption, and cancer. It has been estimated that around 25 million people are poisoned by pesticides globally each year, leading to around 300,000 deaths annually (Jeyaratnam, 1990, Jors et al., 2018). The general population is mainly exposed through the diet, but usually at low levels. The populations with the risk of being highly exposed to pesticides are primarily farmers and sprayers, and especially so in low-
middle-income countries where there is little public concern for proper handling and protection (Jeyaratnam, 1990; Jors et al., 2018). It has also been shown that farmers’ family members, although not being active as farmers, can be exposed to increased levels of pesticides (Alvarado-Hernandez et al., 2013; Ruíz-Guzman et al., 2017).

One of the main health concerns for chronic exposures to pesticides is the development of cancer, and several pesticides have been classified as being or probably being human carcinogens by the International Agency for Research on Cancer (IARC, group 1 or 2A). These include organochlorines and arsenical insecticides that are now being banned globally, but also current and frequently used organophosphate insecticides and herbicides, such as glyphosate and malathion (IARC, 2017). Glyphosate or glyphosate-containing products are currently restricted or banned in several countries after reviews from IARC and the European Food Safety Authority (EFSA) concluded that there was an association between exposure and increased risk of various cancers, including non-Hodgkin lymphoma (NHL) (EFSA, 2015; IARC, 2017). However, other international regulatory agencies, including WHO/FAO and the European Chemicals Agency (ECHA), have concluded that glyphosate is unlikely to pose a carcinogenic risk to humans (ECHA, 2017; WHO/FAO, 2016).

2,4-dichlorophenoxy acetic acid (2,4-D), which is one of the most used herbicides, estimated at 150,000 tons per year globally, is classified as a possible human carcinogen by IARC (group 2B) (IARC, 2017). The studied pesticides were the organophosphate insecticides chlorpyrifos, methamidophos, and profenofos, the organophosphate herbicide glyphosate, the pyrethroid insecticide cypermethrin, the herbicides 2,4-D and paraquat and, the azole fungicide tebuconazole. The mixtures tested were based on data from Bolivian farmers: three on typical profiles of UPMs found in each of the three communities included in our previous studies, and one on the most frequently used pesticides across all communities (Barron Cuenca et al., 2019, Barron Cuenca et al., 2020).

2 | MATERIALS AND METHODS

2.1 | Pesticides

The pesticides 2,4-dichlorophenoxy acetic acid (2,4-D, CAS No. 94-75-7), chlorpyrifos (CAS No. 2921-88-2), cypermethrin (CAS No. 52315-07-8), glyphosate (CAS No. 1071-83-6), methamidophos (CAS No. 10265-92-6), paraquat (CAS No. 75365-73-0), profenofos (CAS No. 41198-08-7), and tebuconazole (CAS No. 107534-96-3) were all PESTANAL™ analytical standard, purity ≥90% and purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Dimethyl sulfoxide (DMSO, ≥99.7%, from Sigma Aldrich) was used for diluting all pesticides except glyphosate and paraquat which were diluted in deionized sterile water. All pesticides were stored at 4°C in darkness.

2.2 | Cell culture and exposure

The human hepatocellular carcinoma HepG2 cell line was procured from the American Type Culture Collection (Rockville, MD). Cell culture was performed in a Corning® T-75 flasks with minimum essential medium (MEM), supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), all from Gibco by Life Technologies, Stockholm, Sweden. Cells were maintained in a humified atmosphere at 5% CO₂ in an incubator at 37°C. HepG2 cells were exposed to different concentrations of the pesticides and their mixtures ranging from 0.001 to 1 mM for 3, 6, or 24 h depending on the method applied. For the mixtures, concentrations of the individual
pesticides reflected the proportions of UPMs and an applied survey (Barron Cuenca et al., 2020). DMSO at 0.1% was used as a negative control in all experiments.

2.3 | Cell viability assay

Cell viability was measured using Alamar blue™ (Invitrogen, Carlsbad, CA) and by seeding 1 × 10⁵ cells/ml in 96-well plates. After 24 h of incubation, cells were exposed to different concentrations of pesticides in a final volume of 200 μl Triton X-100 (1%, Sigma-Aldrich) was used as a positive control. After exposure, 20 μl of 10% Alamar blue was added, and cells were incubated for 2 h at 37°C. Immediately, the plates were placed in a microplate reader (Tecan Infinite F200) for reading the fluorescence at 560 nm excitation and 590 nm emission.

2.4 | ROS assays

Intracellular reactive oxygen species (ROS) production was measured through the dichloro-fluorescein diacetate (DCFH-DA) assay (Wang & Joseph, 1999) and the MitoSOX assay (Wojtala et al., 2014). 2.5 × 10⁵ cells/ml or 1 × 10⁵ cells/ml were seeded in black and clearbottom 96-well plates (Corning, New York, NY) for DCFH-DA respectively MitoSOX assay. After 24 h incubation, the medium was removed, and cells were exposed to pesticides. For the DCFH-DA assay, after exposure, cells were washed twice with ice-cold PBS and scraped into HBSS again and fluorescence intensity was measured at 510/590 nm excitation/emission wavelengths. A 1 h exposure to tert-butyl hydroperoxide (1 mM, Sigma-Aldrich) was used as a positive control. For the MitoSOX assay, after exposure, cells were washed with HBSS and incubated with 5 μM MitoSOX Red reagent (M36008, Thermo Fisher Scientific, Waltham, MA) in HBSS for 15 min. Subsequently, cells were washed with HBSS again and fluorescence intensity was measured at 510/590 nm excitation/emission wavelengths (Tecan Infinite F200). Cells were subsequently lysed in Complete-Lysis M buffer (Roche) and total protein was measured by Bradford assay using Coomassie protein assay reagent (Thermo Fisher Scientific). Fluorescence values were subsequently normalized to protein concentrations.

2.5 | RNA purification and quantitative real-time PCR

Cells were seeded at 1.5 × 10⁵ cells/ml in a 6-well plate for 24 h of incubation. After exposure, the RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for extracting total RNA following the manufacturer’s protocol. High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) was used to generate cDNA according to the manufacturer’s protocol and using 1 μg RNA. Next, quantification of gene expression was performed in duplicates using Maxima™ SYBR® Green qPCR Master Mix (Thermo Fisher Scientific) with detection on a QuantStudio 5 Real-Time PCR System (Applied Biosystems). The reaction cycles used were 95°C for 10 min and then 40 cycles at 95°C for 15 s and 60°C for 1 min followed by melting curve analysis. Primers used were: SOD1 forward GAGGAGAGGAAAGTAAATGG and reverse GATTTAAGTGAGCCTGC, CAT1 forward AGAGGAAATCCTCAGACACATC and reverse CAGCTTGAGAGTATGTAAC, GPX1 forward CTACTTATCGAAGATGTGCC, GPX3 forward CTTATACGAGATGTGCC and reverse CAGAAATCTCTGTCTTTG, HMOX1 forward CAACAAAGTGCAAGTTCCTG and reverse TCAGTTCCATACGGTAAAG, CDKN1A forward CAGCATGACAGATTCTCACC and reverse CAGGGTATGCTACATGAGAG, and GAPDH (housekeeping gene) forward ACAGCGCACGGCTAAC and reverse TTAGGGATAGCTTCTTACC. Relative gene expression quantification was based on the comparative threshold cycle method (2^ΔΔCt).

2.6 | Western blotting

Cells were seeded in 6-well plates at 1.5 × 10⁵ cells/ml and allowed to grow for 24 h. Western blotting was performed following protocol as described (de Oliveira Galvao et al., 2020). Exposure to camptothecin (10 μM, Sigma-Aldrich) was used as a positive control. In short, after exposure, cells were washed twice with ice-cold PBS and scraped into IPB-7 buffer (20 mM triethanolamine-HCl pH 7.8; 0.7 M NaCl; 0.5% Igepal CA-630; 0.2% sodium deoxycholate) supplemented with Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) and PMSF (Sigma-Aldrich). Subsequently, the lysate was sonicated for 10 s on ice and centrifuged at 16,060g for 20 min at 4°C. Protein concentrations were measured, and samples (25 μg) were subjected to standard SDS-PAGE. Separated proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) by wet electro-blotting, and proteins were detected using a monoclonal primary antibody against Chk1 phosphorylated at Ser-317 (#2344, Cell Signaling Technology, Danvers, MA), H2AX phosphorylated at Ser-139 (#2577, Cell Signaling Technology, Danvers, MA), and the endogenous control Cdk2 (sc-163, Santa Cruz Biotechnology, CA). Proteins were detected using the WesternBright™ ECL chemiluminescence kit (Advansta, San Jose, CA) and band densitometry analysis was performed using ImageJ version 1.48 (National Institute of Health, USA).

2.7 | Mini-gel comet assay

The mini-gel alkaline version of the comet assay was performed as described previously (Di Buchianico et al., 2017; Singh et al., 1988). Cells were seeded in 24-well plates at 0.6 × 10⁵/ml for 24 h. After exposure, cells were washed with PBS and collected by trypsinization. Cells exposed to hydrogen peroxide (25 μM, Sigma-Aldrich) for 5 min on ice was used as a positive control. Shortly, cells were embedded in 0.7% low-melting agarose on precoated (0.3% agarose) microscope slides with three mini-gels per slide. Once the agarose hardened, the
slides were placed into lysis buffer (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, pH 10) for 60 min on ice at dark conditions. Slides were washed by dipping in PBS, then placed in a cold alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH >13) for 40 min on ice and in the dark. After incubation, electrophoresis was run at 1.15 V/cm (29 V) in cold alkaline buffer. Slides were neutralized by washing twice for 5 min in 0.4 M Tris, pH 7.8 and then twice for 5 min in deionized H2O. Slides were dried overnight in darkness and then fixed for 5 min in methanol followed by staining for 15 min in cold SYBR-green TAE-solution (1:10.000, Life Technologies). From two different slides (duplicate) 100 comets in total were scored in a fluorescence microscope (Leica DMLB, Houston, TX) and using the Comet assay IV software (Perceptive Instruments, Suffolk, UK).

2.8 | Statistical analysis

At least three independent experiments were done for all the experiments and mean values ± SE were determined. EC50 values for the Alamar Blue assay were determined by nonlinear regression. Differences between exposures and controls were analyzed by one-way ANOVA or Kruskal–Wallis’ test followed by Dunnett’s, respectively Dunn’s test with p-value adjustment for multiple comparisons. A p-value <.05 was considered statistically significant. All analyses were performed using the GraphPad Prism 8 (GraphPad Software LLC).

3 | RESULTS AND DISCUSSION

3.1 | Choice of pesticides and design of mixtures

In our previous studies, the exposure to pesticides was assessed in three agricultural communities in Bolivia: Sapahaqui, La Paz

| Pesticide       | Type     | IARCa  | US EPAb | WHOc |
|-----------------|----------|--------|---------|------|
| 2,4-D           | Herbicide| 2B     | D       | II   |
| Chlorpyrifos    | Insecticide| NL    | E       | II   |
| Cypermethrin    | Insecticide| NL    | C       | II   |
| Glyphosate      | Herbicide| 2A     | Not likely | U   |
| Methamidophos   | Insecticide| NL    | Not likely | Ib |
| Paraquat        | Herbicide| NL     | C       | II   |
| Profenofos      | Insecticide| NL    | E       | II   |
| Tebuconazole    | Fungicide| NL     | C       | II   |

aIARC classification: 2A, probably carcinogenic to humans; 2B, possibly carcinogenic to humans; NL, not listed.
bUS EPA classification: C, possible human carcinogen; D, not classifiable; E, evidence of noncarcinogenicity in humans.
cWHO hazard classification: Ib, highly hazardous; II, moderately hazardous; U, unlikely to be acutely hazardous to humans.

FIGURE 1 Design of pesticide mixtures. Overview of the four mixtures used in the study. Percentages reflect the relative proportions of the included pesticides in each mixture and were based on previous measurements of UPMs (U1, U2, and U3) or information from a survey (S1) applied to Bolivian farmers. In all experiments, the sum of concentrations of the individual pesticides were used.
(community 1), Villa Bolivar, Cochabamba (community 2) and Villa 14 de Septiembre, Cochabamba (community 3) \( (n = 297; \) Barron Cuenca et al., 2019; Barron Cuenca et al., 2020). The most frequently used pesticides were identified by a survey, and UPMs were measured to assess exposure levels. Based on this information, eight pesticides were chosen to be studied as single compounds and mixtures (Table 1). Mixtures U1, U2, and U3 were based on UPM data for four pesticides from the three communities (Figure 1). These pesticides had the highest detection frequency and found at the highest levels as urinary metabolites. Cypermethrin was used as a representative for all the measured pyrethroids (bifenthrin, cyfluthrin, cypermethrin, and permethrin). Mixture S1 represented a combination of the six most frequently used pesticides for all communities based on the survey (Figure 1). In all experiments, the sum of concentrations for the individual pesticides were used for the mixtures. A similar approach has been used to study in vitro genotoxic effects of pesticide mixtures found in the diet of the French population (Graillot, Takakura, et al., 2012).

![Assessment of cellular viability. HepG2 cells were exposed to different concentrations of single pesticides (a) or mixtures (b) for 24 h and viability assessed by Alamar Blue. DMSO control = 100%. Data show mean ± SE, \( n = 3-6 \). *\( p < .05 \), **\( p < .01 \), ***\( p < .001 \), ****\( p < .0001 \) compared to control by one-way ANOVA followed by Dunnett’s test](image-url)
3.2 | Induction of cytotoxicity and oxidative stress

Most pesticides caused a concentration-dependent reduction of cell viability after 24 h (Figure 2a). Due to a lower solubility compared to the other pesticides, glyphosate was only tested up to 0.07 mM, which did not cause a reduced cell viability. The most potent pesticides were paraquat, methamidophos, and tebuconazole with EC50 values at 0.25, 0.30, and 0.42 mM, respectively. Similar EC50 values after 24 h exposure were previously found for paraquat in human lung A549 cells (Mitsopoulos & Suntres, 2010), methamidophos in HepG2 cells (Ramirez-Vargas et al., 2017), but lower for tebuconazole in HepG2 cells (ca., 100 μM) (Knebel et al., 2018). We found published EC50 values based on similar methods as used here and after 24 h exposure for profenofos in rat adrenal pheochromocytoma PC12 cells at 54 μM (Lu & Yu, 2014), cypermethrin in human intestinal Caco-2, human neuroblastoma SH-SY5Y and HepG2 cells ranging between 34 and 95 μM (Romero et al., 2015), and chlorpyrifos in HepG2 cells at 900 μM (Zhou & Li, 2018). For the mixtures, it was only U3 that induced a significant reduction of cell viability at the highest dose of 1 mM (to 74%, p < .0001, Figure 2b). Due to limited solubility, mixture S1 was only used at up to 0.2 mM. EC50 values were not determined for the mixtures since none of them caused >50% reduction in viability.

Induction of intracellular ROS and oxidative stress have been proposed as important mechanisms for the toxicity of most of the pesticides included in this study. Our results showed that the

![Graphs showing relative ROS production for single pesticides and mixtures](image)

**FIGURE 3** Assessment of intracellular ROS. HepG2 cells were exposed to different concentrations of single pesticides (a) or mixtures (b) for up to 24 h and intracellular ROS levels measured using the MitoSOX assay. Data show mean ± SE, n = 3 *p < .05, ***p < .001, ****p < .0001 compared to control by one-way ANOVA followed by Dunnett’s test.
MitoSox-based assay detected significantly increased levels of ROS production in response to chlorpyrifos, paraquat, and tebuconazole at least at one concentration 24 h post exposure (>1.8-fold, \( p < .05 \); Figure 3a). In contrast, the DCFH-based ROS assay could not detect increased levels in response to any of the pesticides or mixtures (Figure S1). While DCFH-DA is commonly used to detect intracellular ROS such as \( H_2O_2 \) (Hempel et al., 1999), MitoSOX Red specifically detects production of mitochondrial superoxide (Wojtala et al., 2014), an important source of intracellular ROS (Brand et al., 2004). Our results thus suggest that chlorpyrifos, paraquat and tebuconazole primarily induced production of mitochondrial ROS in HepG2 cells. In agreement, chlorpyrifos has previously been shown to induce apoptosis on neuronal cells by disrupting mitochondrial function and ROS production (Lee et al., 2012; Singh et al., 2018). Similarly, paraquat and tebuconazole are known to induce mitochondrial superoxide production in mammalian cells (Ben Othmene et al., 2020; Mukhopadhyay et al., 2007). As can be seen in Figure 3b, the three mixtures based on typical UPM profiles among Bolivian farmers all significantly induced mitochondrial ROS production at the highest concentration (3.5–4.4-fold, \( p < .0001 \)) and probably because the composition of these mixtures were dominated by either chlorpyrifos (U1 and U2) or tebuconazole (U3). No effects were observed for the survey based S1 mixture although it was composed of 25% paraquat.

Gene expression analysis of the oxidative stress-responsive genes superoxide dismutase 1 (SOD1), catalase 1 (CAT1), glutathione peroxidase 1 (GPX1), and heme oxygenase 1 (HMOX1) showed that only HMOX1 expression was induced, 3.4- (\( p < .0001 \)) respectively 2.6-fold (\( p = .09 \)), in response to 0.1 mM paraquat at 6 and 24 h, and 2.0-fold (\( p < .05 \)) by 0.1 mM profenofos at 24 h (Figure 4). HMOX1, which is redox regulated through Nrf2, exhibits its anti-oxidative effects through the oxidation of heme and production of the ROS scavenger biliverdin, and plays a critical role in protection against many inflammatory disorders (Wu et al., 2019). Through its regulation by Nrf2, induction of HMOX1 should be considered as a marker of more general intracellular oxidative stress (Loboda et al., 2016), similar to the DCFH assay. Although the DCFH assay was negative, this suggest

**FIGURE 4** Gene expression analysis of oxidative stress response genes. Gene expression levels of SOD1, CAT1, GPX1, and HMOX1 were measured by qRT-PCR in HepG2 cells after 6 h (a) and 24 h (b) exposure to different concentrations of single pesticides. Data show mean ± SE, \( n = 3–4 \). *\( p < .05 \), ****\( p < .0001 \) compared to control by one-way ANOVA followed by Dunnett’s test.
that paraquat and profenofos induced intracellular oxidative stress. None of the mixtures affected the expression levels of these genes (Figure S2). Several studies have shown that mixtures of pyrethroids induce oxidative stress and neurotoxicity in a dose additive manner (Cao et al., 2011; Romero et al., 2015; Wolansky et al., 2009). No such conclusions could be made on the mixtures used here.

**FIGURE 5** Determination of DNA damage signaling. Levels of pChk1 and γH2AX were measured by western blot in HepG2 cells after 6 h (a) and 24 h (b) exposure to different concentrations of single pesticides. Cdk2 was used as loading control and 10 μM camptothecin as a positive control. Top panels show representative blots and bottom panels show densitometry data normalized to DMSO control (= 1) and loading control. Data show mean ± SE, n = 3–4. *p < .05, **p < .01, ***p < .001 compared to control by Kruskal–Wallis followed by Dunn’s test.
3.3 Activation of DNA damage signaling

It has previously been shown by us and others that activation of DNA damage signaling through phosphorylation of checkpoint kinase 1 (Chk1) at Ser-317 (pChk1) and H2A histone family member X (H2AX) at Ser-139 (γH2AX) in HepG2 cells are good markers for genotoxic potency of environmental pollutants (Dreij et al., 2017; Kopp et al., 2019). Both proteins are phosphorylated by phosphatidylinositol 3-kinase related kinases in response to a wide range of DNA damage, including DNA adducts, single- and double-strand breaks (Marechal & Zou, 2013).

Levels of pChk1 and γH2AX were assessed at 6 and 24 h post-treatment in response to three concentrations of single pesticide (all <EC50). The results showed a similar time- and concentration-dependent activation for the two proteins. For pChk1, paraquat, profenofos, and cypermethrin induced levels >2.5-fold with \( p < .05 \) at one or two concentrations at 6 h (Figure 5a). Levels of pChk1 were basically maintained for cypermethrin 0.01 mM at the late time point, but increased for paraquat and profenofos, up to 10-fold (\( p < .01 \)) and 5-fold (\( p < .01 \)), respectively compared to DMSO control. For γH2AX, a stronger response was in general observed at 24 h compared to the early time point (Figure 5b). Paraquat and profenofos induced a clear time- and concentration-dependent increase of γH2AX, up to >10-fold compared to control at 24 h (\( p < .05 \)). The highest concentration of tebuconazole and cypermethrin (0.1 mM) also caused increased levels of γH2AX, about 6-fold, but due to some variability no statistical significance was observed (\( p > .05 \)). To the authors’ knowledge, only one previous study has reported DNA damage signaling through pChk1 in response to a pesticide. Huang et al. (2015) showed that the herbicide atrazine activated Chk1 in a concentration-dependent manner in normal human breast epithelial cells (MCF-10A). In accordance with our results, the activation of Chk1 by atrazine was also associated with increased levels of γH2AX (Huang et al., 2015).

The induction of γH2AX in response to pesticides in vitro has been studied extensively (Graillot, Takakura, et al., 2012; Hershman et al., 2017). However, few data are available on the pesticides studied here. Previous studies have demonstrated that paraquat and cypermethrin are capable of inducing γH2AX in mammalian cells (Huang et al., 2016; Mao et al., 2011) and glyphosate induced γH2AX in human lymphocytes in vitro (Suarez-Larios et al., 2017).

Based on the results from the single pesticides, which in general showed a stronger DNA damage response at 24 h post-treatment, the mixtures were tested at 24 h (Figure 6). The results showed that the lowest concentration of U2 and the highest concentration of S1 were the only conditions that increased the activation of pChk1 compared to control (4- and 5-fold, respectively). Similar to the single pesticides, a stronger response was observed for γH2AX compared to pChk1 in response to the mixtures. U2, U3, and S1 displayed a clear
concentration-dependent increase of γH2AX. The two latter mixtures caused an almost 40-fold increase compared to control (p < .01 – .001). The high potency of S1 is probably due to the presence of paraquat in that mixture (25%). The high potency of U3 is more difficult to explain since the major constituent 2,4-D (61%) neither induced pChk1 nor γH2AX on its own. Similarly, higher levels of γH2AX were observed in HepG2 cells exposed to an equimolar pesticide mixture consisting of λ-cyhalothrin, iprodione, procymidone, cyprodinil, and fluodioxonil compared to what would be expected from the levels induced by the individual pesticides (Grailiot, Takakura, et al., 2012). The authors concluded that this could be due to interaction effects on the metabolism of the pesticides, which also could be a possible explanation here for U3, although different pesticides were included in the mixture tested. Current risk assessment approaches for pesticide mixtures are based on the assumption of additivity, although a recent systematic review suggested that nonadditive effects might occur (Martin et al., 2020). If the S1 and U3 mixtures induced additive or nonadditive effects on γH2AX were not studied here, but the presented results motivate further investigation.

In addition, effects on gene expression of CDKN1A, the gene coding for the negative cell cycle regulator protein p21, were assessed (Figure 7). In response to DNA damage, the expression of CDKN1A is typically induced in a p53-dependent manner (El-Deiry et al., 1994). In agreement with the effects on pChk1 and γH2AX, paraquat, and profenofos of the single pesticides and S1 of the mixtures significantly induced gene expression of CDKN1A compared to control (>2-fold, p < .05 – .0001, Figure 7a,b,d). In agreement with the results obtained with paraquat, human lung A549 cells exposed to 0.25 mM paraquat displayed increased expression of growth arrest and cell cycle-related genes including CDKN1A (Mitsopoulos & Suntres, 2010). Together these results suggested that some of the pesticides are genotoxic and that γH2AX could be a sensitive marker for assessing genotoxicity also for pesticides (Dreij et al., 2017; Kopp et al., 2019). Furthermore, the mixture experiments suggested that the effects of pesticide mixtures cannot always be predicted by what is known about the constituents.

3.4 | Induction of DNA damage

Based on the results from assessing levels of pChk1, γH2AX, and CDKN1A; paraquat, cypermethrin, tebuconazole, and profenofos were
identified as the most genotoxic pesticides in HepG2 cells. For comparison, induction of DNA strand breaks was measured by the Comet assay in response to these four pesticides at 6 and 24 h and the four mixtures at 24 h. All pesticides and mixtures displayed a trend of concentration-dependent increase of DNA damage at one or both time points. However, based on statistical analysis, only the highest concentration of paraquat (at both time points) and cypermethrin (at 24 h) induced significantly higher levels of DNA damage than DMSO control (up to 34-fold, \( p < .05 \)– .01, Figure 8a). All pesticides but tebuconazole displayed maintained levels of DNA damage at 24 h compared to 6 h, indicating a continuous formation of DNA damage during this time. This was also in agreement with the western blot data. Paraquat has been shown to induce base modifications and strand breaks as a consequence of oxidative DNA damage in vitro and in vivo (Dusinska et al., 1998; Tokunaga et al., 1997). Similarly, cypermethrin has been shown to induce DNA damage and chromosomal damage in vitro and in vivo (Amer et al., 1993; Patel et al., 2006).

For the mixtures, U2 and S1 induced significant levels of DNA damage at their highest respectively two highest concentrations (up to 26-fold, \( p < .05 \), Figure 8b). This agreed with the \( \gamma H2AX \) data for these two mixtures. In contrast, the high levels of \( \gamma H2AX \) induced by U3 were not associated with a significant increase of DNA damage although being 15-fold compared to DMSO control (\( p = .08 \)). The lack of significance was probably due to the variation of Comet assay data. Based on the DNA damage signaling and Comet assay data, mixture U1 was the least genotoxic mixture. Notably, both U1 and U2 had high proportions of chlorpyrifos and cypermethrin (Figure 1) but displayed marked differences in genotoxicity. However, in U1 their relationship was roughly 80:20 while it was closer to 50:50 in U2, suggesting that this ratio was of importance. In support, Sultana Shaik et al. (2016) observed synergistic induction of DNA strand breaks

![FIGURE 8](image-url)
from equimolar binary mixtures of profenofos, endosulfan, and chlorpyrifos in human peripheral blood lymphocytes. How differences in mixture compositions determine the type and level of toxic effects of mixtures is something that warrants further investigation.

Measurement of DNA damage among the farmers in our previous study showed that farmers in community 3, represented here by mixture U3, had the highest levels of DNA strand breaks and micronuclei, followed by community 1 and 2 (Barron Cuenca et al., 2019). Here we observed some discrepancies. Although the highest induction of γH2AX was caused by U3, a nonsignificant but 15-fold increase in DNA damage was also found. These discrepancies can be due to the limited number of pesticides that were included in the mixture used for in vitro testing, that the composition was based on UPM data which might not reflect true exposure levels, and that we are comparing in vitro genotoxicity data in a liver cell line with human data from peripheral blood samples. However, the results of in vitro experiments give some insight into the different mechanisms involved in cellular and genetic toxicology and thus lay foundations for further experiments at the molecular level as well as in vivo conditions. The applicability of using HepG2 cells as an alternative in vitro model to test the genotoxicity of pesticides, as has been suggested for other compounds (Dreij et al., 2017; Kopp et al., 2019), needs to be better studied before any conclusions can be made.

3.5 Relevance of used concentrations

The concentrations used here, in the sub mM range and corresponding to 0.1–50 mg/L, are likely higher than what is found in tissue for the general population. Few data are available that could be directly compared. In maternal plasma samples from pregnant women living in an agricultural community in California, up to 1.4 mg/L of chlorpyrifos was found (Huen et al., 2012) and in pesticide spray workers in northern Egypt, up to 35 mg/L (2.7 mg/kg body weight) of profenofos was found (Shalaby & Abdou, 2020). A recent review on human exposure to glyphosate reported that up to 200 μg/L has been found in maternal serum samples from Thai women (Gillezeau et al., 2019; Kongtip et al., 2017). Intentional or unintentional poisoning is however associated with higher levels. In cases of chlorpyrifos, paraquat, or glyphosate poisoning by ingestion, serum levels at 5 mg/L >100 mg/L, and 10 mg/L, respectively, have been reported (Cho et al., 2019; Gil et al., 2008; Martinez et al., 2004). Based on this, future in vitro studies should apply lower doses and study designs that better mimic a more chronic exposure.

4 CONCLUSIONS

Here we confirm and expand on the in vitro toxicity for many commonly used pesticides in Bolivia and globally. We could confirm the role of oxidative stress as an important mechanism of toxicity for chlorpyrifos, paraquat, and tebuconazole. Based on our results on activation of DNA damage signaling and induction of DNA strand breaks we also found that paraquat, profenofos, and cypermethrin were among the most genotoxic pesticides of those tested. Similar to our previous population study (Barron Cuenca et al., 2019), we show that certain pesticides seem to be drivers for the toxic effects of pesticide mixtures. The mixture containing paraquat was clearly the most genotoxic mixture studied here. Paraquat has been banned or its use restricted in many countries due to its toxicity but is still used in Bolivia. Clearly, efforts should be made to reduce its use in Bolivia and globally by implementing more stringent policies to protect and educate farmers by developing well-targeted training programs for pesticide retailers and farmers on pesticide use, personal protective device use, as well as pesticide management and law. Additionally, policy implementers have to be motivated and have the necessary resources to carry out their mandate and make sure that the pesticide legislations are followed.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Barron Cuenca: Investigation, formal analysis, visualization, writing - original draft, writing - review and editing. de Oliveira Galvão: Investigation, formal analysis, writing - original draft, Writing - review and editing. Endirlik: Investigation, formal analysis, writing - review and editing. Tirado: Funding acquisition, writing - review and editing. Dreij: Funding acquisition, conceptualization, formal analysis, writing - original draft, Writing - review and editing.

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