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Evaluation of various glyphosate concentrations on DNA damage in human Raji cells and its impact on cytotoxicity

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ABSTRACT

Glyphosate is a highly used active compound in agriculturally based pesticides. The literature regarding the toxicity of glyphosate to human cells has been highly inconsistent. We studied the resulting DNA damage and cytotoxicity of various glyphosate concentrations on human cells to evaluate DNA damaging potential. Utilizing human Raji cells, DNA damage was quantified using the comet assay, while cytotoxicity was further analyzed using MTT viability assays. Several glyphosate concentrations were assessed, ranging from 15 mM to 0.1 μM. We found that glyphosate treatment is lethal to Raji cells at concentrations above 10 mM, yet has no cytotoxic effects at concentrations at or below 100 μM. Treatment concentrations of 1 mM and 5 mM induce statistically significant DNA damage to Raji cells following 30–60 min of treatment, however, cells show a slow recovery from initial damage and cell viability is unaffected after 2 h. At these same concentrations, cells treated with additional compound did not recover and maintained high levels of DNA damage. While the cytotoxicity of glyphosate appears to be minimal for physiologically relevant concentrations, the compound has a definitive cytotoxic nature in human cells at high concentrations. Our data also suggests a mammalian metabolic pathway for the degradation of glyphosate may be present.

1. Introduction

Since their inception in 1939 by the Swiss chemist Paul Muller, pesticides have become a global phenomenon and a standard approach to pest prevention (Aktar et al., 2009). The use of pesticides increased exponentially from 196 million pounds in 1960 to 632 million pounds in 1981, and in 2008 an estimated 516 million pounds were being used yearly (Fernandez-Cornejo and Nehring, 2014). It is estimated that if pesticides were banned for a year, the year-ending supplies of corn, wheat, and soybeans would decrease by 73% (Woodburn, 2000; Knutson, 1999). As a result, the use of these herbicides has become an integral part of the worldwide economy (Woodburn, 2000).

A critical component in the majority of pesticides and weed killers is the non-selective herbicide glyphosate. This chemical targets the shikimate pathway, which is crucial to the development and growth of plants (Amrhein et al., 2015). Glyphosate interrupts the function of the enzyme 5-enolpyruvylshikimate 3-phosphate synthase, which is responsible for catalyzing the reversible formation of 5-enolpyruvylshikimate 3-phosphate and inorganic phosphate from the organic molecules shikimate 3-phosphate and phosphoenolpyruvate (Padgette et al., 2015; Schönbrunn et al., 2001). By doing so, glyphosate halts the synthesis of the aromatic amino acids required for protein synthesis, thereby inhibiting plant growth.

Recently, there has been substantial debate regarding the non-toxic nature of glyphosate in humans (Delaplane, 2000; Horowitz and Amore, 2015; Gasnier et al., 2009). Glyphosate was labeled as a "probable carcinogen" by the IARC, and various studies have shown it to be cytotoxic at high concentrations (Gasnier et al., 2009; Mañas et al., 2009). These potential side effects are concerning due to glyphosate's extensive agricultural use worldwide. Despite being a topical treatment, there is evidence that glyphosate is absorbed into the soil and water, which causes great concern for consumer health (Peruzzo et al., 2008; Battaglin et al., 2009; Li et al., 2005). This concern has led to multiple studies of

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glyphosate cytotoxicity and carcinogenicity.

I*n vitro* studies have yielded inconsistent results regarding glyphosate's cytotoxic properties. In a study conducted by Gasnier et al., toxicity to HepG2 cells appeared at glyphosate concentrations as low as 5 ppm during a 24 h incubation period, and concentrations of 120 nM induced DNA damage after 24 h exposure (Gasnier et al., 2009). Koller et al. found that in TR146 cell lines, treatment with Roundup induced lower cell viability, while treatment with the active ingredient in Roundup, glyphosate, did not induce any significant change in cell viability (Koller et al., 2012). Li et al. found that at concentrations of 15 mM, 25 mM, and 50 mM, glyphosate did not decrease cell viability in epithelial cell lines RWEP-1, pRNA-1-1, and in normal cells (Li et al., 2013). Mañas et al. (2009) determined that in Hep-2 cells there was "no statistically significant clastogenic effects quantitatively detected in any glyphosate treatments." The extensive variation among the literature has made it difficult to accurately assess the health risk of glyphosate. Recently in 2015, the International Agency for Research on Cancer (IARC) concluded glyphosate induced significant genotoxic effects for both Glyphosate and its metabolite aminomethylphosphonic acid (AMPA). Although the Expert Panel reviewed the data and concluded glyphosate did not induce oxidative stress characteristic of carcinogenicity, there remains a substantial level of confusion with regards to the 'safe' nature of glyphosate (Brusick et al., 2016). Due to its high use in agricultural and consumer settings, continued research is important to ensure the protection of individuals exposed to the compound (De Roos et al., 2005; Eriksson and Ph, 1992).

The purpose of this study was to investigate the concentration-dependent nature of glyphosate DNA damaging potential in Burkitt's B Cell Lymphoma (Raji) cells using the comet assay and MTT viability assays (Braafladt et al., 2016; A. R. Collins, 2004; a R. Collins, 2004; Duty et al., 2003; Mañas et al., 2009; Olive and Banáth, 2006). We treated cells with concentrations of glyphosate ranging from 0.1 μM to 15 mM and measured resulting DNA damage and loss of cell viability after various lengths of exposure. We hypothesized that the discrepancies in past results may be, in part, due to the utilization of different treatment conditions across protocols. The use of a broad range of concentrations and incubation times allowed us to gain a more complete understanding of glyphosate's cytotoxic and carcinogenic effects in Raji human cells.

2. Materials and methods

2.1. Chemicals and reagents

Low melting agarose, Glyphosate (95% purity), MTT cell viability assay, and Propidium Iodide were purchased from Sigma-Aldrich, Inc. (Milwaukee, WI). Hydrogen Peroxide and L-glutamine was purchased from Fisher Scientific (Pittsburg, PA). Fetal Bovine Serum was purchased from Hyclone (Logan, UT). RPMI 1640 was purchased from Mediatech, Inc. (Manassas, VA).

2.2. Equipment

A Zeiss Axioscope fluorescence microscope was used to image all Comet experiments. TriTek CometScore Freeware v1.5 software was utilized to determine tail moment values.

2.3. Cell culture

Burkitt's Lymphoma (Raji) cells (ATCC CCL-86) were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations at 37 °C and 5% CO_2. Cells were cultured in RPMI 1640 (Mediatech, Inc. Manassas, VA) and supplemented with 10% FBS and 2 mM L-glutamine (Fisher Scientific. Pittsburg, PA). Media was replaced every 48 h. Cells utilized for experimentation were placed in exponential growth and had a minimum viability of 95% as determined by Trypan blue cell staining. Cells were authenticated by the University of Arizona Genetics Core in May 2016. Raji cells were utilized for this analysis because the replication time is 18 h long and allowed the assays to cover the entire cell cycle.

2.4. Compound preparation

Glyphosate was dissolved initially to a 50 mM stock concentration in PBS. This solution was then diluted further to create stocks of 25 mM, 15 mM, and 10 mM. These aliquots were diluted in PBS to the concentrations tested (5 mM, 1 mM, 100 μM, 10 μm, 1 μM, and 0.1 μM). Aliquots were stored in 15 mL conical vials at 4 °C. For use in MTT viability assays, glyphosate was diluted in cell culture RPMI media to the final test concentrations and stored at 4 °C.

2.5. Alkaline comet assay

Raji cells were incubated with either hydrogen peroxide, PBS, or glyphosate. The concentration and time points varied depending on the experimental run. Time intervals tested included 10, 20, 30, 40, 50, 60, 75, 90, 105, and 120 min. Concentrations of 0.1 μM, 1 μM, 10 μM, 100 μM, 1 mM, 5 mM, 10 mM, and 15 mM were tested at each of the time points. Cells were suspended at a concentration of 200,000 cells per 100 μL treatment. Once treated, cells were washed twice in 4 °C PBS, and then suspended at 200,000 cells per 100 μL of PBS. The cells were then prepared for the comet assay. Glyphosate treatment was conducted at 37 °C in a water bath.

Samples were prepared for comet analysis by following the methods described by Xiao et al. (2014). with slight modifications. In brief, samples were mixed with low melting point agarose and layered on double frosted microscope slides (Xiao et al., 2014). The slides were placed in alkaline lysis buffer for 60 min, rinsed with ddH_2O and then placed in alkaline electrophoresis buffer for 20 min. They were then electrophoresed for 30 min at 24 V and 400 mA. Following electrophoresis, slides were allowed to rest in ddH_2O for 15 min, then fixed in −20 °C 100% ethanol for 5 min and allowed to dry overnight. Slides were then stained with propidium iodide for 15 min, rinsed with ddH_2O, and imaged. All comets were scored using TriTek CometScore Freeware v1.5. Every experimental run tested a single concentration for multiple time points. Each time point contained a minimum of two slides as replicas. Approximately 50 comets were analyzed per slide, totaling 100 comets per time point per treatment concentration. Each concentration was replicated multiple times in order to ensure consistency. Comet Assay results are reported in terms of Tail Moment. Tail moment is defined as the product of the tail length and the percentage of DNA in the tail. These values are given as part of the output by the CometScore software and are widely reported for Comet analysis (Olive et al., 1990).

A similar protocol was utilized to test the effects of secondary glyphosate exposure at 1 mM and 5 mM concentrations. In these experiments, 200 μL additional glyphosate was added to the cells after 60 min of initial treatment, while 200 μL of PBS was added to the negative control.

2.6. Cell viability assay

Samples were prepared for the MTT cell viability assay by the methods described by Hamid et al. (2004). with slight modifications. Glyphosate treatments were diluted in Raji cell growth media to their final test concentrations. Raji cells were incubated in this prepared growth media for 24 h in a 96-well plate at 37 °C and 5%

CO_2. The 24 h time period was chosen because Raji cells divide every 18–24 h, which ensures the entire cell cycle was taken into account. After incubation, 10 μL of kit provided MTT reagent at a concentration of 5 mg/mL was added to each well. Following 3 h of incubation, 100 μL of DMSO detergent was added to each well. The plate was then incubated on a shaker at 4 °C for 2 h, and evaluated at a 570 nm absorbance.

2.7. Statistical analysis

Relationships between exposure time and tail moment were modeled statistically using a natural spline to account for non-linearity (Parang et al., 2000). The number of knots was selected based on Akaike Information Criteria (AIC) and parameters were estimated using least squares. *P*-Values <0.05 were considered statistically significant.

3. Results

3.1. Cell death at high concentrations of glyphosate

Glyphosate rapidly induced DNA damage and cell death in Raji cells following treatment at concentrations of 10 mM and 15 mM after only 30 min of treatment. Cells exposed at these

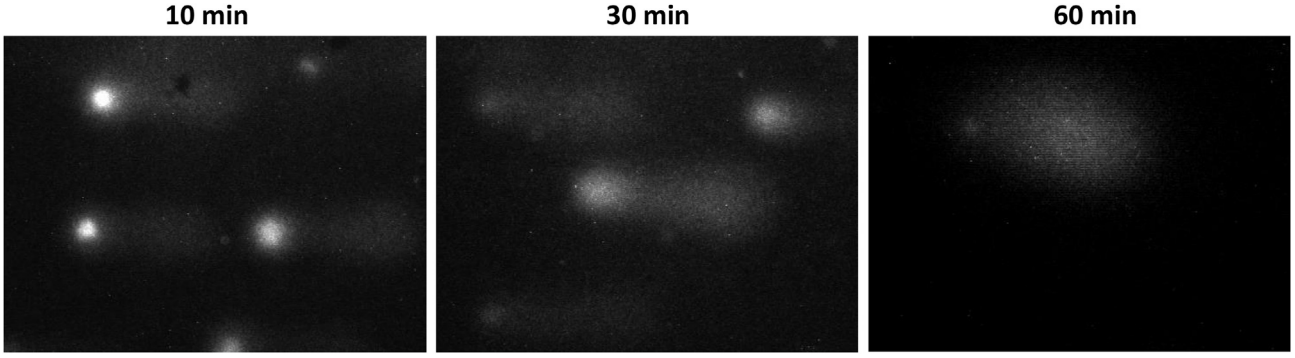

Fig. 1. Comet assay analysis of Raji cells exposed to 10 mM glyphosate. Cells at 10 mM and 15 mM concentrations underwent severe DNA damage and cell death soon after exposure. Dead cells were characterized by a loss of a defined comet head and a large, fragmented DNA tail. The extensive amount of damage at later time points made analysis impractical due to software restraints.

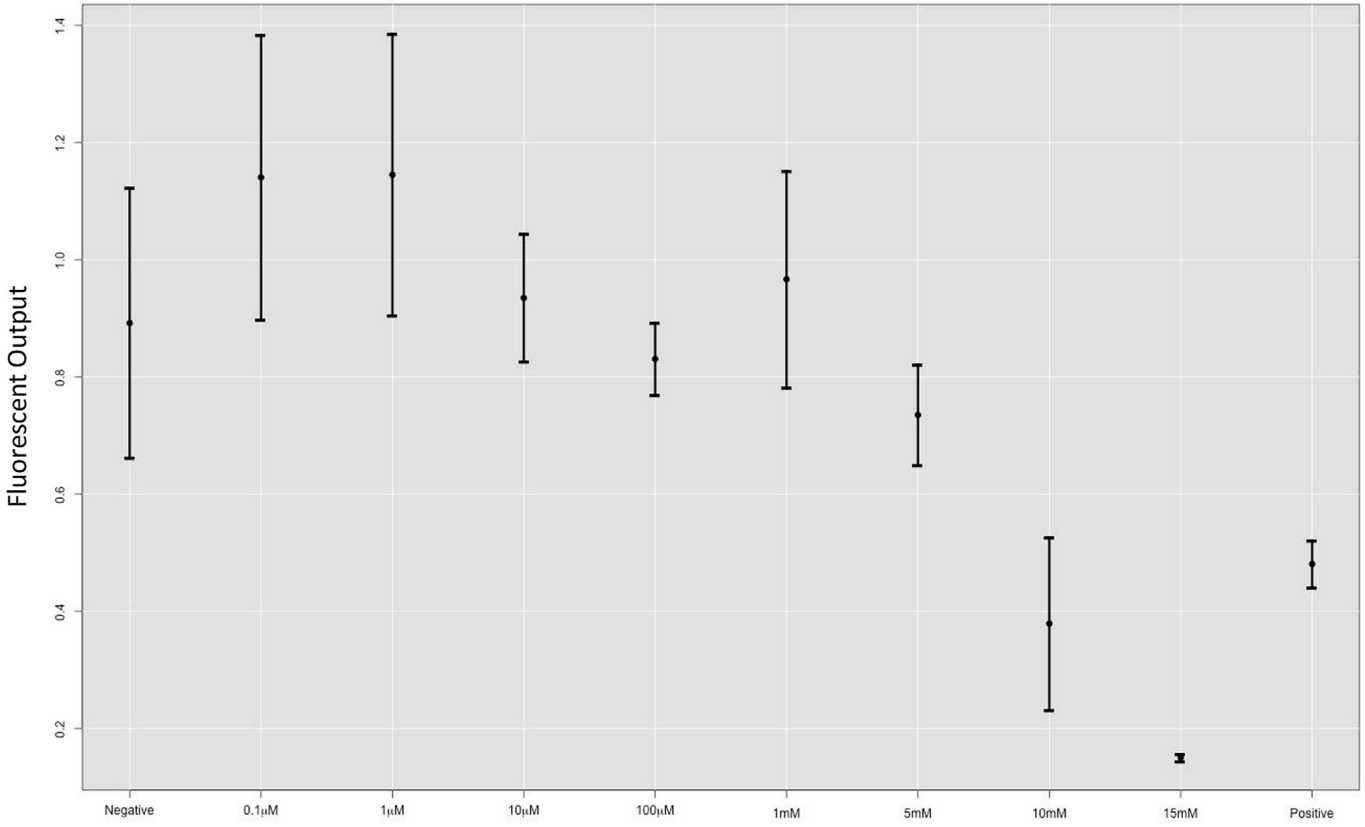

Fig. 2. MTT analysis of Raji cells exposed to various glyphosate concentrations. Hydrogen Peroxide (positive) was utilized as a control to measure thorough cell death, and cells suspended in cell growth media (negative) was utilized as a control for standard cell death as a result of treatment conditions. Following 24 h of incubation with glyphosate, there was a significant loss of cell viability following treatment with 10 mM and 15 mM glyphosate. Concentrations of 5 mM and lower did not have a significant loss of viability when compared to the negative control. This indicates the damage to Raji cells at 15 mM and 10 mM glyphosate was enough to sustain complete cell death, while concentrations at or below 5 mM sustained cell viability.

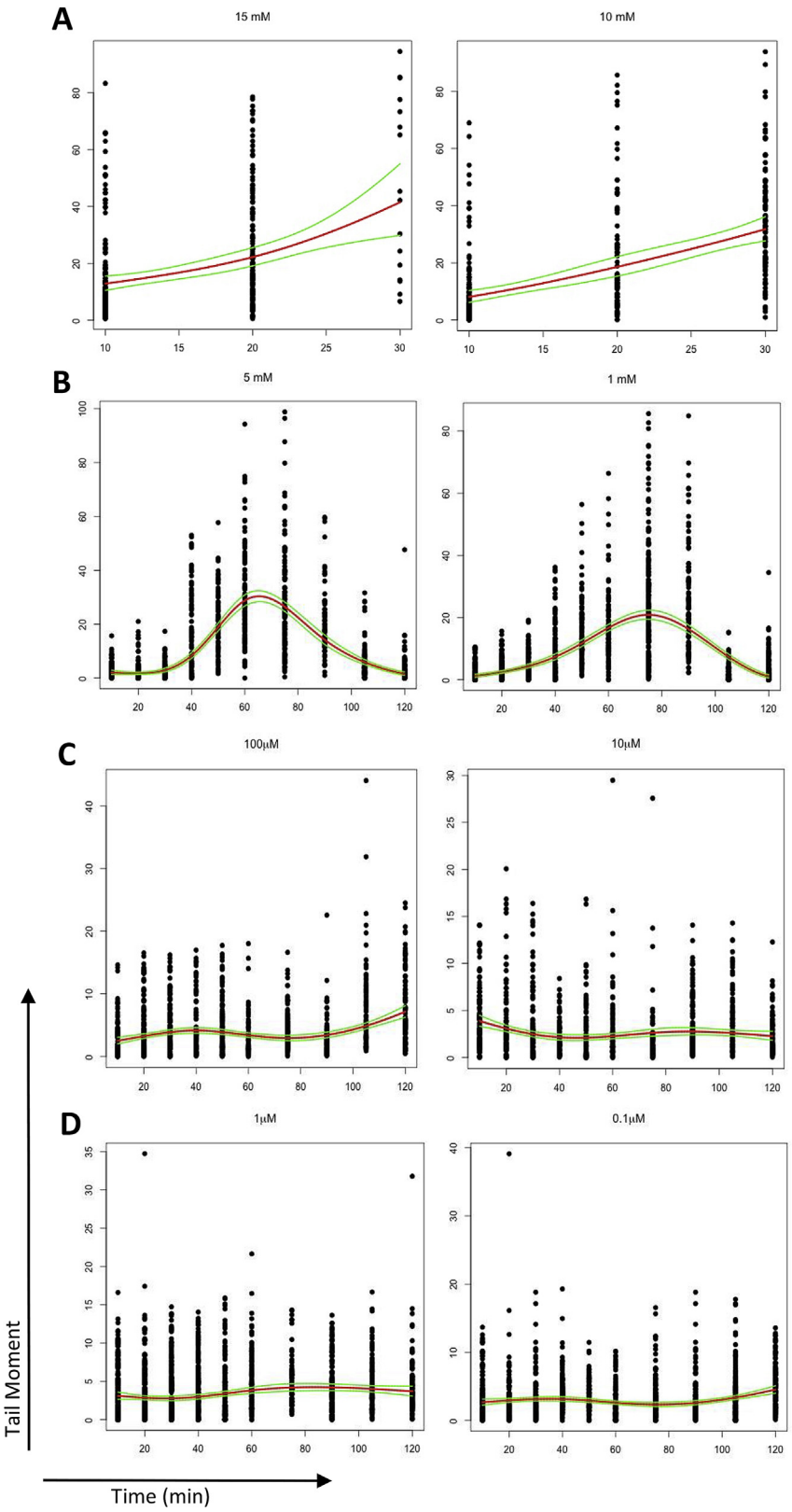

Fig. 3. *Tail Moment values of cells treated with various concentrations of glyphosate across 2 h of treatment.* Tail moment values (quantifiable measure of DNA damage) are listed on the y-axis (scale varies at different concentrations), and treatment times are labeled on the x-axis. Each concentration was individually evaluated and assigned a 95% confidence interval, which is displayed in green, while the mean value is shown in red. **A,** Cells exposed to glyphosate concentrations of 15 mM and 10 mM sustained severe DNA damage with tail moments above 25, which was indicative of cell death. Within 30 min of treatment, all cells had adopted the characteristic profile of a dead cell (Fig. 1). **B,** Raji cells exposed to glyphosate concentrations of 5 mM and 1 mM had statistically significant DNA damage after 60–75 min of treatment. This damage was not present in later time points and cells were able to recover full viability after 120 min of treatment. **C,** Cells treated with 100 μM and 10 μM of glyphosate did not show statistically significant DNA damage, and cells retained full viability throughout the full 120-min treatment. **D**, Physiologically relevant concentrations of glyphosate were exposed to Raji cells over a 120 min period and did not experience any significant DNA damage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

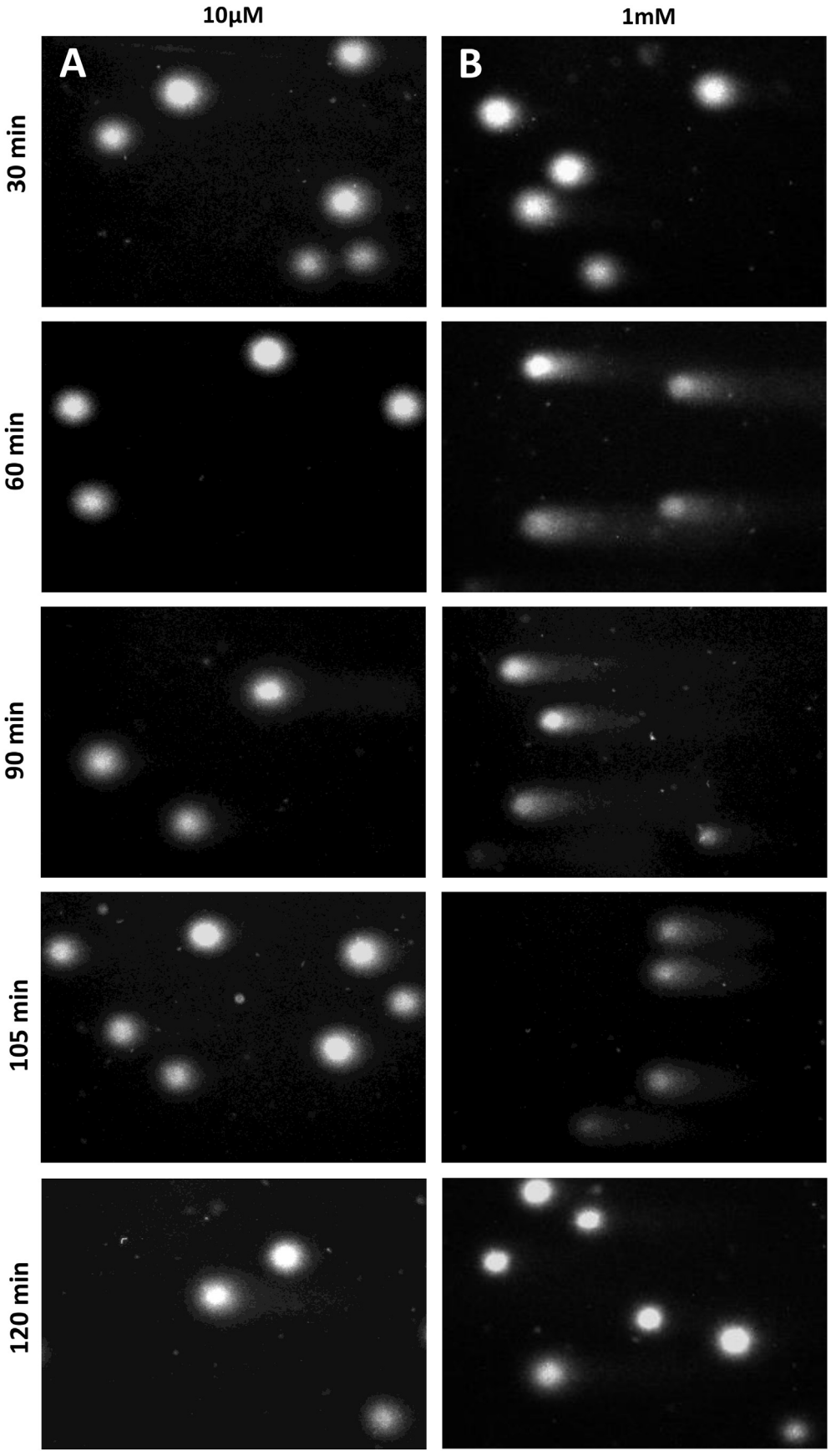

Fig. 4. *Raji cells treated with 1 mM and10 µM glyphosate concentrations experience different damaging events.* **A**, Following treatment with 10 µM glyphosate, Raji cells showed no signs of DNA damage and the 'head' of the cells stayed intact throughout the 2 h treatment time. **B,** After exposure to 1 mM glyphosate, cells started to show signs of damage after 60 min of treatment that subsided after 2 h. At the end of the 2 h incubation, cells were fully viable with no signs of severe DNA damage.

concentrations quickly adopted an apoptotic profile characterized by the lack of a clear head and the appearance of a long, rounded tail, as shown in Fig. 1. Tail moments were significant after just 10 min of glyphosate exposure. After 30 min, the damage was so extensive that comet analysis was unfeasible due to software restraints. To confirm that cell death had occurred, cell viability was quantified using MTT viability assays. Results, shown in Fig. 2, indicate a significant loss of cell viability after 24 h treatments with 10 mM and 15 mM concentrations of glyphosate. A comparison shown in Fig. 4 outlines the difference in appearance of cells that maintain viability to those who undertake severe DNA damage characteristic of cell death.

3.2. Minimal cytotoxicity at low, physiologically relevant concentrations of glyphosate

For concentrations of glyphosate at or below 100 μM, tail moments were not statistically significant at any time point as shown in Fig. 3. MTT analysis in Fig. 2 likewise showed no decrease of cell viability following glyphosate treatment at these concentrations. These findings indicate a lack of cytotoxicity to Raji cells at low treatment concentrations, suggesting that the risk of glyphosate exposure at standard physiological levels may be negligible.

3.3. DNA damage and cellular recovery at 1 mM and 5 mM concentrations

Cells exposed to 1 mM and 5 mM concentrations of glyphosate had significant tail moments after 40 min of glyphosate incubation. Tail moments reached a maximum following 60 min and 80 min of treatment for 5 mM and 1 mM concentrations of glyphosate, respectively. Interestingly, as shown in Fig. 3, a steady decrease in tail moment was observed in later time points and after 2 h of treatment, the DNA damage was no longer significant. The decrease in tail moment may suggest that the induced DNA damage was insufficient to trigger cell death, and that cells were able to recover from the damaging event. MTT analysis supported this hypothesis, showing no significant loss of cell viability after 24 h incubations at either concentration.

In order to further elucidate the comet analysis results at 1 mM and 5 mM, cells were treated again with glyphosate at these concentrations 1 h after initial treatment. There was a significant difference between cells receiving only primary treatment and cells receiving the additional treatment (Fig. 5). Raji cells exposed to the compound twice did not show the same pattern of recovery, with tail moments reaching levels above 20 for 1 mM and 25 for 5 mM glyphosate treatment. Meanwhile, cells with only primary exposure to the compound showed a decrease in DNA damage, with tail moments dropping from 15 to 5.8 for 1 mM and 23.67 to 6.74 for

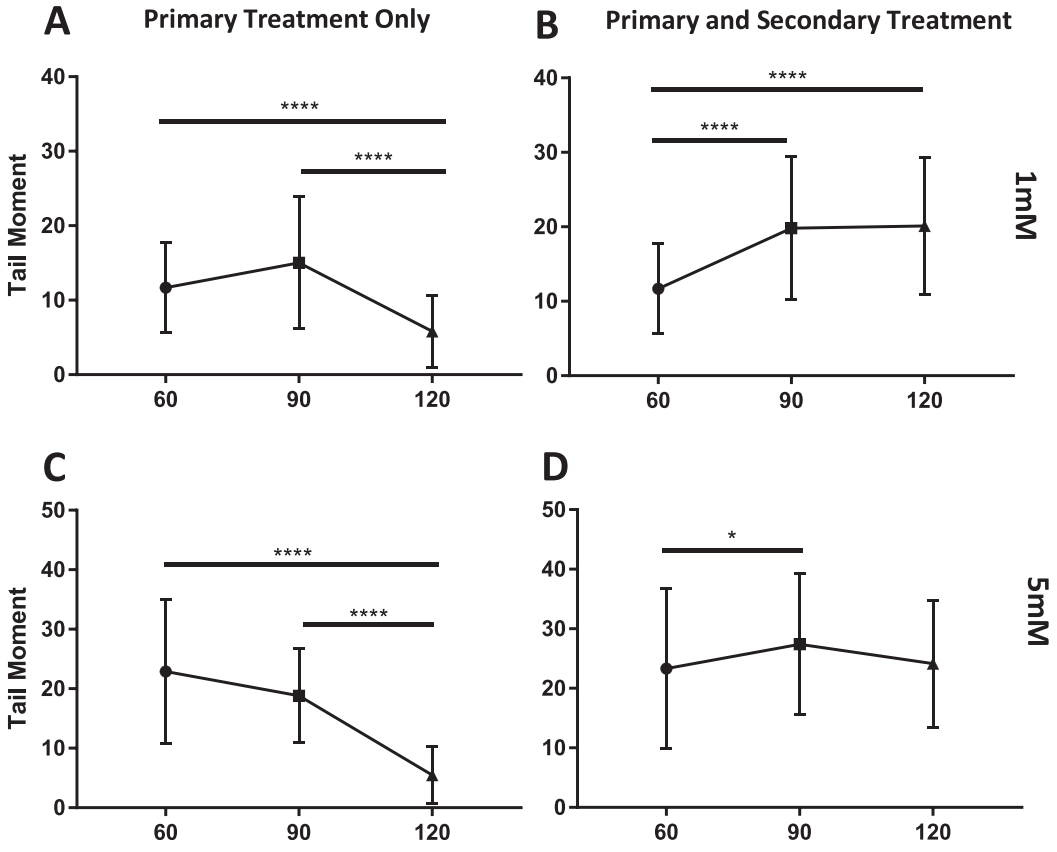

Fig. 5. Tail moments of Raji cells incubated with 1 mM and 5 mM glyphosate concentrations after primary and secondary exposure to the compound. **A,** Raji cells were evaluated after initial treatment with 1 mM glyphosate. Following the same pattern as previously reported, the cells underwent a damaging event (tail moment 15.01) that was later recovered. At the end of the 2 h incubation, cells had gained viability and maintained a low tail moment value of 5.82. **B,** Raji cells receiving both primary and secondary treatment of 1 mM glyphosate did not experience the same recovery as those only treated with primary glyphosate. At 60 min, Raji cells were again incubated with 1 mM glyphosate. Tail moments in this case were increased slightly and do not show the same decrease as primary only treated cells and maintained high tail moment values above 20. **C,** Cells treated with primary 5 mM glyphosate only showed a pattern of recovery in cell viability with tail moments dropping from 23.67 to 6.74. **D,** When Raji cells were incubated with 5 mM glyphosate primary and secondary treatment, there was no recovery observed and the tail moment increased from 23.67 to 28.03. These data suggest that after primary treatment, Raji cells may be metabolizing the compound and breaking it down to its less toxic metabolites.

5 mM treatments of glyphosate.

4. Discussion

These results show that the DNA damaging and cytotoxic potential of glyphosate is related to exposure length and treatment concentration, suggesting a dose-dependent relationship for glyphosate's cytotoxic effects. These data show that glyphosate induced significant DNA damage only when cells are exposed to concentrations several orders of magnitude larger than those attainable *in vivo*. Our data support the established evidence that glyphosate is "not genotoxic" in human cells at physiologically relevant concentrations (Solomon et al., 2009). While these data ultimately support glyphosate's classification as a potential carcinogen, they suggest that its effects are negligible when exposure is minimal. Our results do implicate the need for further studies of the physiological uptake and bioavailability of glyphosate for agricultural workers, who may be subject to extended exposure and are thus at higher risk. Furthermore, our studies at 1 mM and 5 mM suggest that cells initially damaged by glyphosate may have the ability to repair and regain viability if repeated exposure is not experienced.

Another important consideration obtained from this study is the utility of multiple time points in the comet assay. This aspect of the experimental design allowed for accurate assessment of the DNA-damaging event that took place. Our results show that incubation times used in the comet assay can affect results dramatically; the extent of DNA damage changed drastically across different incubation time points. The 1 mM concentration at 1 h, for example, showed that severe DNA damage occurred. Yet, at 2 h with the same treatment, no DNA damage was evident. If cells had only been evaluated at this time, results would suggest that there was no cytotoxic activity and the initial DNA damaging event would be missed. Cytotoxic activity might also be underestimated by standard viability assay in which the DNA damage is insufficient to induce cell death. Because of our analysis across multiple time points, we were able to observe both the DNA damaging event as well as the ensuing recovery. We recommend that in future utilizations of the comet assay or related assays measuring mutagenic or clastogenic events, incubation times be considered and evaluated.

5. Conclusion

Human cell exposure to glyphosate has minimal cytotoxicity and DNA damage at concentrations at or below 100 μM.

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Transparency document

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