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Genotoxic Potential of Glyphosate Formulations: Mode-of-Action Investigations

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A broad array of in vitro and in vivo assays has consistently demonstrated that glyphosate and glyphosate-containing herbicide formulations (GCHF) are not genotoxic. Occasionally, however, related and contradictory data are reported, including findings of mouse liver and kidney DNA adducts and damage following intraperitoneal (ip) injection. Mode-of-action investigations were therefore undertaken to determine the significance of these contradictory data while concurrently comparing results from ip and oral exposures. Exposure by ip injection indeed produced marked hepatic and renal toxicity, but oral administration did not. The results suggest that ip injection of GCHF may induce secondary effects mediated by local toxicity rather than genotoxicity. Furthermore, these results continue to support the conclusion that glyphosate and GCHF are not genotoxic under exposure conditions that are relevant to animals and humans.

KEYWORDS: Glyphosate; genotoxicity; mode of action

INTRODUCTION

The potential genotoxicity of glyphosate has been tested in a wide variety of in vitro and in vivo assays. No genotoxicity was observed in standard assays conducted according to international guidelines and Good Laboratory Practice (GLP) Standards. These assays are described briefly in Williams et al. (*1*), and the results have led to the conclusion that glyphosate does not pose a risk for the production of heritable or somatic mutations in humans (*1*–*6*). The original Roundup formulation and subsequent glyphosate-containing herbicide formulations (GCHF) have also been evaluated for genotoxic responses in several assays. Although a number of studies conducted according to international guidelines and GLP Standards show that these materials are not genotoxic (*1*), a few other studies have reported positive effects.

Apparent evidence of DNA adducts in the liver and kidneys of CD-1 mice was reported (*7*) when a formulation that was identified as "Roundup" (30.4% glyphosate, purchased from Monsanto, Italy) was administered intraperitoneally (600 mg/kg) using dimethyl sulfoxide (DMSO)/olive oil as a vehicle. However, no DNA adducts were observed following intraperitoneal (ip) injection of isopropylamine salts of glyphosate. In contrast, ip injection of CD-1 mice with analytical grade glyphosate or the same "Roundup" formulation resulted in an increased incidence of alkali-labile sites in DNA from liver and kidney (*8*). The effects reported in the latter study (*8*) were observed at 300 mg/kg with glyphosate and at 900 mg/kg for GCHF, including a dramatic increase in the number of 8-hydroxydeoxyguanine (8-OHdG) residues in DNA from liver cells after treatment with glyphosate but not the GCHF; opposite results were found in the kidney. All of these changes were observed only under unrealistic exposure conditions (very high dose levels administered by an irrelevant route of exposure for an agricultural herbicide).

To better understand the significance of these results (*7, 8*), four separate but inter-related assays were undertaken to determine if high-dose ip administration produces toxicity that may be responsible for the observed changes via secondary effects, rather than direct genotoxicity, and whether a more relevant (oral) route of exposure produces the same toxic responses as those seen with ip administration. The first assay was performed to understand the relevance of findings reported by Bolognesi et al. (*8*) by investigating the degree of liver and kidney toxicity that occurred under the dosing conditions used by those investigators. Similarly, another assay was conducted to understand the relevance of findings reported by Peluso et al. (*7*); this assay also examined whether the vehicles used in their studies (DMSO/olive oil) contributed to the hepatic

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Table 1. Overall Study Experimental Design

study/test material	GCHF[a] dose (mg/kg)	route of dosing	necropsy: h after dosing	group size	evaluations conducted			
					CC[b]	pathology	NMO[c]	8-OHdG[d]
			Group 1					
isotonic saline	0	ip[e]	24	10	+	O[f], G[g]	+	−
isotonic saline	0	ip	24	8	+	O, G	+	+
GCHF in isotonic saline	600	ip	24	8	+	O, G	+	−
isotonic saline	0	ip	4	10	+	O, G	−	−
GCHF in isotonic saline	600	ip	4	10	+	O, G, M[h]	+	+
			Group 2					
DMSO[i]/OO[j]	−	ip	24	10	+	O, G, M	+	−
GCHF/DMSO/OO	600	ip	24	10	+	O, G, M	+	−
			Group 3					
GCHF/DMSO/OO	600	ip	24	10	+	O, G	−	−
GCHF without glyphosate/DMSO/OO	600	ip	24	10	+	O, G	−	−
			Group 4					
isotonic saline	0	ip	24	10	+	O, G, M	+	+
GCHF in isotonic saline	900	ip	24	10	+	O, G, M	+	+
isotonic saline	0	oral	24	10	+	O, G, M	−	−
GCHF/DMSO/OO	600	oral	24	10	+	O, G, M	−	−

[a] GCHF, glyphosate-containing herbicide formulation. [b] CC, clinical chemistry. [c] NMO, NADPH menadione oxidoreductase mRNA. [d] 8-OHdG, 8-hydroxydeoxyguanidine. [e] Intraperitoneal injection. [f] Organ weights obtained. [g] Gross pathology examination. [h] Microscopic pathology examination. [i] Dimethylsulfoxide. [j] Olive oil.

and renal toxicity. A third assay was performed to investigate the relationship of glyphosate and the other GCHF ingredients to the marked toxicity observed in the second study. Finally, a fourth assay was conducted to determine if the marked toxicity observed in the studies using ip administration of the GCHF/DMSO/olive oil mixture was also produced after oral administration, the more relevant route of exposure for herbicides.

MATERIALS AND METHODS

The assay design and the parameters evaluated by group are outlined in **Table 1**. Each assay was conducted at the same laboratory by the same group of investigators. The sex and strain of the animals used, animal housing and handling procedures, in-life observations, dosing methods (oral by gavage or ip injection), animal sacrifice procedures, and analytical procedures were the same in all of the assays.

Animals. Male Crl:CD-1(ICR)BR mice were obtained from Charles River Laboratory (Raleigh, NC). The animals, 8−10 per group, were 7–8 weeks of age at the start of the studies. Following 3−10 days of acclimatization, the mice were computer randomized by body weight and were then allocated to dosing groups so that individual animal body weights were within ±20% of the group mean.

Housing. The mice were housed individually in stainless steel cages with wire mesh bottoms. Food (Certified Rodent Diet no. 5002, PMI Feeds, Inc., St. Louis, MO) and water (public water supply, St. Louis, MO) were available ad libitum. Animal room temperature and relative humidity were targeted to be within 64–74 °F and 30–70%, respectively. A 12/12 h light/dark cycle was observed. Animal housing and husbandry were performed in accordance with the provisions of the *Guide for the Care and Use of Laboratory Animals* (*9*).

In-Life Observations. Mortality checks were conducted at least once daily during the assays. The mice were also observed daily at 6−7 h postdosing and/or at the time of terminal sacrifice for overt signs of toxicity. Nonfasted body weights were taken prior to randomization, on the morning of dosing, and just prior to sacrifice.

Test Materials. The following materials were used in these assays: (a) the formulated herbicide product (Roundup, Monsanto Co., St. Louis, MO) that was the same GCHF reported to be used by Peluso et al. (*7*) and Bolognesi et al. (*8*) and that contained an isopropylamine salt (IPA) of glyphosate (∼30% by weight) and an alkyl sulfate surfactant; (b) the same GCHF minus the IPA glyphosate; (c) 1% DMSO in olive oil (DMSO/OO) (both from Sigma Chemical Co., St. Louis, MO); and (d) isotonic saline solution (Phoenix Scientific, Inc.).

Dosing Methods. The mice received the appropriate test or control GCHF by ip injection or orally by gavage. In each case, a single dose was administered at a volume of 10 mL/kg of body weight. The test material was administered as a suspension in DMSO/OO or as a solution in isotonic saline. Vehicle control groups received DMSO/OO or isotonic saline only.

Scheduled Sacrifice. All animals were sacrificed by CO_2 asphyxiation at 4 ± 0.5 h or 24 ± 2 h after dosing. This was the same sacrifice schedule used by Peluso et al. (*7*) and Bolognesi et al. (*8*). Blood was collected from the posterior vena cava into serum microvette clot tubes. All sacrificed animals were necropsied, and the livers and kidneys were removed, observed grossly, rinsed in saline, blotted dry, and weighed. Sections from the left lateral and median lobes of the liver and sections from each kidney (hilus, cortex, and medulla from the right kidney; pelvis, cortex, and medulla from a longitudinal section of the left kidney) were placed in cassettes and retained in 10% neutral-buffered formalin for microscopic evaluation. Five-micrometer histological sections were prepared from the formalin-fixed tissues, stained with hematoxylin and eosin, and examined microscopically. The remainder of the liver and kidneys was divided for the various assays, snap frozen in liquid nitrogen, and stored until analyzed.

Clinical Chemistry. The collected serum was analyzed for alanine aminotransferase (ALT/SGPT), aspartate aminotransferase (AST/SGOT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and sorbitol dehydrogenase (SDH). A Hitachi clinical analyzer was used for these analyses.

Other Liver and Kidney Analyses. *8-Hydroxydeoxyguanosine (8-OHdG).* Frozen liver and kidney tissues were transferred on dry ice for analysis to the Medicinal Chemistry and Pharmacognosy group at the University of Illinois, Chicago. Tissue samples were weighed, placed in cell-lysing buffer, and homogenized. The sample solution was centrifuged, and the nuclei pellet was retained. RNA contaminants were digested with RNases and washed away from the DNA pellet. Next, a mixture of DNase and phosphodiesterase was added to hydrolyze the DNA to nucleosides, which were purified using C_{18} solid-phase extraction. Deoxyguanosine was quantified by UV absorbance at 260 nm. 8-OHdG was measured using LC-MS-MS in multiple-reaction-monitoring mode with [$^{13}C_{10}$,$^{15}N_5$]-8-OHdG as internal standard.

NADPH Menadione Oxidoreductase (NMO) mRNA. Total RNA was isolated from frozen tissues and quantified using a UV spectrophotometer at 260 nm. Four hundred nanograms of RNA was reverse transcribed into cDNA using reverse transcriptase and amplified by real-time Polymerase Chain Reaction (PCR) using AmpliTaq Gold DNA polymerase and PCR primers and fluorescent dye-labeled probes specific for each mRNA, as described elsewhere (*10, 11*). The first doubling cycle at which product may be detected above a threshold level (C_T, the cycle threshold) was determined by real-time RT-PCR. C_T values were converted to relative expression levels in individual

Table 2. Terminal Body Weights and Organ Weights for CD-1 Mice (Groups 1 and 4) Following Intraperitoneal Administration of Isotonic Saline or GCHF[a] in Isotonic Saline

dose group (8 or 10 mice/group)	body wt (g)	liver wt (g)	liver-to-body wt ratio (×100)	kidney wt (g)	kidney-to-body wt ratio (×100)
			Group 1		
isotonic saline control, 4 h sacrifice	30.5 ± 1.4	1.61 ± 0.19	5.27 ± 0.42	0.519 ± 0.039	1.70 ± 0.12
GCHF (600 mg/kg), 4 h sacrifice	30.3 ± 1.3	1.55 ± 0.22	5.12 ± 0.66	0.502 ± 0.053	1.66 ± 0.20
isotonic saline control, 24 h sacrifice	32.5 ± 3.2	1.91 ± 0.24	5.88 ± 0.39	0.578 ± 0.065	1.78 ± 0.14
GCHF (600 mg/kg), 24 h sacrifice	29.5 ± 2.4[b]	1.53 ± 0.22[c]	5.23 ± 0.81	0.505 ± 0.057[b]	1.71 ± 0.15
			Group 4		
isotonic saline control, 24 h sacrifice	32.3 ± 1.3	1.65 ± 0.17	5.12 ± 0.43	0.546 ± 0.063	1.69 ± 0.17
GCHF (900 mg/kg), 24 h sacrifice	31.6 ± 1.0	1.43 ± 0.14[b]	4.52 ± 0.36[c]	0.442 ± 0.086[b]	1.40 ± 0.27[c]

[a] Glyphosate-containing herbicide formulation. [b] Statistically significantly different from control, $p \leq 0.05$. [c] Statistically significantly different from control, $p \leq 0.01$.

animals by adjusting for expression levels of the housekeeping gene cyclophilin, average expression levels in control animals, and the doubling of product that occurs at each PCR cycle. Detailed calculation methods are described elsewhere (*12*). Group means of individual animal relative expression levels were also calculated.

Statistical Analyses. Results are presented as the mean ± standard deviation (SD) for the number of animals indicated. Comparisons between respective control and treated animals were made with Student's *t* test or Dunnett's multiple-comparison test (*13, 14*). These were used to evaluate mRNA expression of NMO, 8-OHdG levels, and body weights. Fisher's exact test (*15*) was used to evaluate the incidences of microscopic lesions. Terminal body weights, absolute organ weights, organ/body weight ratios, and clinical chemistry data were evaluated by a decision-tree statistical analysis that, depending on the results of tests for normality and homogeneity of variances (Bartlett−Box test) (*16*), used either parametric (Dunnett's test and linear regression) (*17*) or nonparametric [Kruskal–Wallis (*18*), Jonckheere's (*19*), and/or Mann–Whitney (*20*)] test routines to detect group differences and analyze for trend. Grubbs' test (*21, 22*) was used to identify outliers for cell proliferation and for 8-OHdG. Due to assay variability, Grubbs' test was not run on results from the NMO reductase mRNA analyses. All tests were evaluated at $p < 0.05$ and $p < 0.01$.

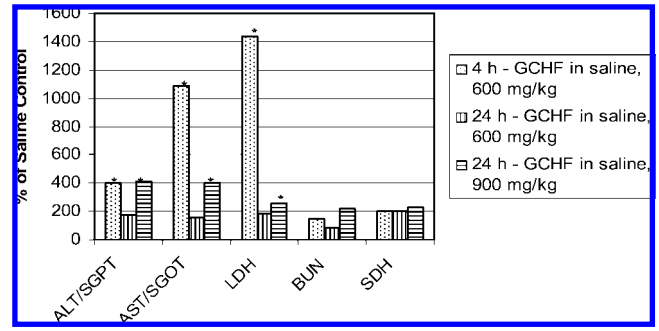

Figure 1. Comparison of clinical chemistry values for CD-1 mice following intraperitoneal administration of GCHF for 4 and 24 h at 600 or 900 mg/kg. The values presented are shown as the percentage of clinical chemistry values for the treatments with herbicide formulation compared to saline controls. ALT/SGPT, alanine aminotransferase; AST/SGOT, aspartate aminotransferase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; SDH, sorbitol dehydrogenase. An asterisk indicates statistically significant difference from control, $p \leq 0.05$.

RESULTS

Evaluation of Toxicity Following Intraperitoneal Injection of the GCHF in Saline. Terminal body weights were unaffected in treated animals sacrificed 4 h after dosing (**Table 2**). The body weights of animals given the GCHF at 600 mg/kg were statistically significantly reduced (9% below control mean) at the 24 h time point. However, in a separately run experiment (see **Table 1** for a description of the overall testing program), the highest dose group, 900 mg/kg, sacrificed 24 h after dosing, no such decrease was observed. The reason for the decreased body weights in the 600 mg/kg animals is unknown. Absolute liver and kidney weights were decreased (13–20 and 13–19%, respectively) in both the 600 and 900 mg/kg dose groups compared to control groups at the 24 h time point. Also, statistically significant reductions in liver and kidney-to-body weight ratios were observed at the 900 mg/kg dose level.

Intraperitoneal injection of the GCHF resulted in several statistically significant changes in clinical chemistry values. Four hours after a dose of 600 mg/kg, substantial increases in clinical chemistry values were observed, most notably ALT, AST, and LDH (406, 1087, and 1433% of controls, respectively) (**Figure 1**). Most of these values returned to near control levels by 24 h postdosing in mice treated with 600 mg/kg. However, statistically significant elevations in ALT, AST, and LDH (218−410% of controls) were still observed at 24 h in other mice given a 900 mg/kg dose.

No microscopic alterations in liver or kidney were observed in the 600 mg/kg dose group mice sacrificed 4 h after treatment. The only notable histopathology finding from the 600 mg/kg dose group sacrificed at 24 h was the deposition of fibrin/amorphous material on the capsule of the kidneys in three mice. This lesion was also observed in one mouse from the saline control group. There were no abnormal findings in the liver.

Several microscopic changes occurred in the kidneys and livers of mice given the GCHF at the 900 mg/kg dose level. Renal changes consisted of vacuolization of cortical tubules in three of the treated mice. Degeneration and necrosis also occurred in the medulla of the kidneys from one mouse. Acute inflammation of the renal capsule and deposition of amorphous material on the renal capsule occurred in a different mouse. Hepatic changes included a generalized increase in hepatocellular vacuolization, subcapsular necrosis, and subcapsular hepatocellular vacuolization; these lesions occurred in 6 of 10, 4 of 10, and 5 of 10 mice, respectively. None of these kidney and liver lesions occurred in any of the control animals.

Evidence of oxidative stress was observed in the kidneys of animals given the GCHF at a dose of 900 mg/kg but not at 600 mg/kg. A statistically significant increase in NMO (relative expression level of 2.66 ± 0.79 versus 1.02 ± 0.20 or 261% of controls) was seen in the kidney of the 900 mg/kg group of animals (**Figure 2**). There was no statistically significant increase in 8-OHdG, although the level in kidneys of mice given an ip injection of the GCHF at 900 mg/kg was 143% of the control value [mean degree of oxidation ($\times 10^5$) was 0.28 ± 0.05 for control and 0.40 ± 0.28 for treated].

Evaluation of Toxicity Following Intraperitoneal Injection of DMSO/OO and the GCHF/DMSO/OO Mixture. The ip administration of DMSO/OO alone did not produce any significant evidence of toxicity. Terminal body weights and liver and kidney weights, as well as organ-to-body weight ratios, were unchanged for animals sacrificed at 24 h when treated animal

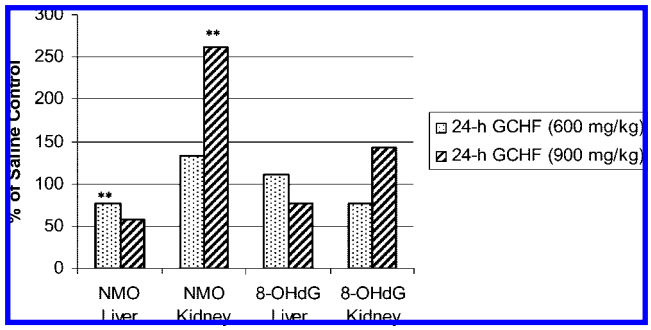

Figure 2. Evaluation of expression of NADPH menadione oxidoreductase (NMO) and 8-hydroxydeoxyguanosine (8-OHdG) in the liver and kidney of CD-1 mice exposed for 24 h to GCHF at 900 and 600 mg/kg by intraperitoneal administration. Values are presented as the percent of saline control. A double asterisk indicates statistically significant difference, $p \leq 0.01$.

were compared to control animals (**Table 3**). The DMSO/OO vehicle did not alter clinical chemistry values (**Figure 3**), and the only notable microscopic finding was the occurrence of renal capsule fibrosis in three animals. There was no statistically significant increase in liver or kidney NMO values (liver relative expression level of 1.44 ± 1.86 versus 1.04 ± 0.29 or 138% of controls and kidney relative expression level of 1.03 ± 0.24 versus 1.03 ± 0.26 or 100% of controls, **Figure 4**).

In contrast to the findings for DMSO/OO alone, the ip administration of the GCHF/DMSO/OO mixture at 600 mg/kg produced a significant effect. Whereas liver weights were unaffected in these animals (group 2, **Table 3**), absolute and relative liver weights were reduced (14 and 13%, respectively, below controls) in another group of mice (group 3, **Table 4**) given the same test material. Significant decreases in absolute and/or relative kidney weights were observed in both groups of animals (group 2, 11 and 9%, **Table 3**; and group 3, 19 and 18%, **Table 4**, respectively). Dramatic, statistically significant increases in clinical chemistry values were also observed. Serum ALT, AST, LDH, BUN, and SDH levels in treated animals were 151−1065% (**Figure 3**). In addition, pathology examinations revealed several changes in the capsule or subcapsular tissue in both livers and kidneys. The changes included deposition of fibrin and an amorphous material on the capsule of livers and kidneys, inflammation, and hemorrhage involving the renal capsule. The deposition of the fibrin/amorphous material on the surface of the liver was accompanied by necrosis of hepatocytes immediately subjacent to the capsule along with acute inflammation in subcapsular regions. In addition, there was vacuolization of hepatocytes in most subcapsular regions. Oxidative stress was also observed (**Figure 4**) in the kidneys of animals given the GCHF/DMSO/OO mixture as indicated by a statistically significant increase in NMO (relative expression level of 3.09 ± 1.53 versus 1.03 ± 0.24 or 300% of controls; liver was 0.48 ± 0.22 versus 1.04 ± 0.29 or 46% of controls).

Comparison of Toxicity Produced by the GCHF/DMSO/OO Mixture with and without Glyphosate. The toxicity produced by the GCHF/DMSO/OO mixture was further evaluated by directly comparing effects produced by that mixture to those observed after administration of a surfactant/DMSO/OO mixture. This "formulation blank" contained all of the same components of the GCHF in DMSO/OO except glyphosate. Reductions in absolute and relative organ weights were very similar for both test materials (**Table 4**). For example, reductions in absolute liver weights for animals given the test material with and without glyphosate were 14 and 15%; for kidney weights, the values were 19 and 22%, respectively. Similar results were obtained for serum clinical chemistry values. As seen in **Figure 5**, serum enzyme levels from mice given the GCHF blank were generally comparable to values from animals given the test material with glyphosate. Gross necropsy evaluations showed the presence of a white particulate material adhering to the surface of tissues (i.e., liver, kidneys, spleen, and small intestines) in the peritoneal cavity in a majority of animals in both treated groups (data not shown).

Evaluation of Toxicity Following Oral Administration of the GCHF/DMSO/OO Mixture. Administration of the GCHF/DMSO/OO mixture by the oral route of exposure produced essentially no evidence of toxicity. Terminal body weights and absolute and relative kidney weights were unaffected by oral treatment with the GCHF at 600 mg/kg in DMSO/OO for animals sacrificed at 24 h when treated animals were compared to control animals (**Table 5**). Although absolute and relative hepatic weights were statistically significantly decreased, the changes were considered to be of little or no consequence due to their small magnitude (7.6 and 6.8% below controls, respectively). Serum chemistry parameters were not elevated for oral exposure in comparison with ip exposure (**Figure 6**), and no histopathological lesions were observed in the liver or kidneys (data not shown).

DISCUSSION

Evaluation of Toxicity Following Intraperitoneal Injection of the GCHF in Saline. Bolognesi et al. (*8*) reported that a single 900 mg/kg dose of the GCHF administered intraperitoneally produced DNA damage, as evidenced by the induction of DNA single-strand breaks and 8-OHdG. The levels of DNA single-strand breaks were statistically significantly increased in both liver and kidneys (2.5- and 2.3-fold, respectively) from animals sacrificed 4 h after administration of the GCHF. By 24 h postdosing, there were no statistically significant differences in the levels of DNA strand breaks, although the values for livers and kidneys from treated animals remained numerically elevated above control levels (1.5- and 1.6-fold, respectively). The reported increases in the numbers of 8-OHdG residues were somewhat more pronounced. Levels of 8-OHdG were significantly increased (2.7-fold) only in kidneys 4 h after dosing; by 24 h, increases in both liver and kidneys (2.8- and 3.1-fold) were observed, although only the kidney value was statistically significantly different from controls.

The work described herein demonstrated that the ip injection of the GCHF in saline resulted in significant toxicity. Four hours after a single 600 mg/kg dose, substantial increases in clinical chemistry values were observed. Most of these values returned to near control levels by 24 h postdosing. However, statistically significant elevations were still observed at 24 h in mice given a 900 mg/kg dose. Although clinical chemistry values were not evaluated at 4 h in the 900 mg/kg group, results from the 600 mg/kg group at this time point indicate that substantial elevations occurred in the higher dose group. Histopathological lesions noted in the livers (hepatocellular vacuolization, subcapsular vacuolization, and necrosis) and kidneys (cortical tubule vacuolization, medullary necrosis, and acute capsular inflammation) of mice given the 900 mg/kg dose also indicate significant organ toxicity. The statistically significant increase in NMO observed in the kidneys of animals given the GCHF at a dose of 900 mg/kg was evidence of oxidative stress. These data provide a strong indication that the dosing conditions used by Bolognesi et al. (*8*) produced marked hepatic and renal toxicity. The induction of DNA damage in liver and kidneys produced under

Table 3. Terminal Body Weights and Organ Weights for CD-1 Mice (Group 2) Following Intraperitoneal Administration of Isotonic Saline, DMSO[a]/OO[b], or GCHF[c] in DMSO/OO

dose group (8 or 10 mice/group)	body wt (g)	liver wt (g)	liver-to-body wt ratio (×100)	kidney wt (g)	kidney-to-body wt ratio (×100)
isotonic saline control, 24 h sacrifice	30.0 ± 1.1	1.63 ± 0.24	5.42 ± 0.74	0.533 ± 0.045	1.77 ± 0.12
DMSO/OO, 24 h sacrifice	29.7 ± 1.3	1.54 ± 0.14	5.19 ± 0.31	0.522 ± 0.060	1.75 ± 0.15
GCHF (600 mg/kg) in DMSO/OO, 24 h sacrifice	29.6 ± 0.9	1.54 ± 0.09	5.20 ± 0.24	0.476 ± 0.039	1.61 ± 0.13[d]

[a] Dimethly sulfoxide. [b] Olive oil. [c] Glyphosate-containing herbicide formulation. [d] Statistically significantly different from control, $p \leq 0.05$.

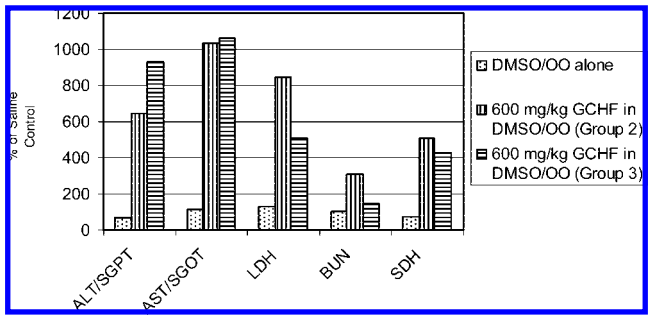

Figure 3. Clinical chemistry values for CD-1 mice following intraperitoneal administration of GCHF in DMSO/OO (600 mg/kg, 24 h) or DMSO/OO only. The values presented are shown as the percentage of clinical chemistry values for the treatments (GCHF in DMSO/OO or DMSO/OO only) compared to saline controls. ALT/SGPT, alanine aminotransferase; AST/SGOT, aspartate aminotransferase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; SDH, sorbitol dehydrogenase.

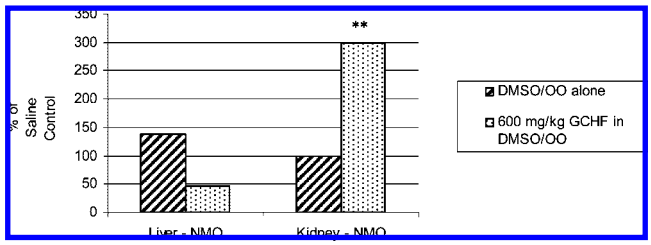

Figure 4. Evaluation of expression of NADPH menadione oxidoreductase (NMO) in the liver and kidney of CD-1 mice exposed for 24 h to GCHF at 600 mg/kg in DMSO/OO and to DMSO/OO alone by intraperitoneal administration. Values are presented as the percent of saline control. A double asterisk indicates statistically significant difference, $p \leq 0.01$.

conditions of substantial organ toxicity via an ip route of exposure is of doubtful biological relevance.

By definition, an increase in 8-OHdG is not an indicator of interaction with DNA, but rather is an event that occurs secondarily to oxidative effects. It should be noted that the increases in 8-OHdG reported by Bolognesi et al. (*8*) (in one group of three mice) were not observed in the present study. There was no statistically significant increase in 8-OHdG in either liver or kidney, and the highest value observed (kidneys, 900 mg/kg dose group) in any treated group was only 143% of the control value. The reason for this discrepancy is not apparent. Certainly the sample size used in this study (two groups of five mice each) should have been sufficient to reproduce effects that Bolognesi et al. (*8*) reported in a single group of three mice. Therefore, because of the more robust nature of the present investigation, the previous report is not considered to be sufficient to conclude that high-dose ip administration of the GCHF causes oxidative damage to DNA.

Evaluation of Toxicity Following Intraperitoneal Injection of DMSO/OO, GCHF, and the GCHF/DMSOO/OO Mixture. Peluso et al. (*7*) reported that the GCHF, when administered in a DMSO/OO mixture, induced DNA adduct formation in the liver and kidneys of mice injected intraperitoneally at doses of 400−600 mg/kg. The present study investigated the potential for different components of this GCHF to produce hepatic and renal toxicity. The administration of the DMSO/OO vehicle only produced no apparent adverse affects 24 h after treatment. As discussed above, the GCHF administered in a saline vehicle at 600 mg/kg produced only slight increases in some clinical chemistry values at 24 h after dosing. In contrast, administration of the GCHF at 600 mg/kg in a DMSO/OO vehicle produced marked toxicity at 24 h after treatment as evidenced by dramatic increases in clinical chemistry values (**Figure 3**). In addition, pathology examinations of the animals that received the 600 mg/kg GCHF/DMSO/OO mixture revealed deposition of fibrin on the capsule of livers and kidneys, renal inflammation and hemorrhage, and hepatocellular inflammation and necrosis. From these data it is clear that the unusual combination of GCHF, DMSO, and OO is required to produce the substantial toxicity observed.

Comparison of Toxicity Produced by the GCHF/DMSO/OO Mixture with and without Glyphosate. To assess the contribution of glyphosate to the GCHF toxicity observed, another assay was performed in which the toxicity produced by the injection of the GCHF/DMSO/OO mixture was directly compared to that observed following administration of a "formulation blank," consisting of DMSO/OO mixed with the components (primarily a surfactant system) of the GCHF except glyphosate. These two test materials produced essentially the same severe, adverse effects. The results support the conclusion that substantial toxicity is caused by the surfactant/DMSO/OO mixture used by Peluso et al. (*7*) and that glyphosate contributes little, if anything, to the adverse effects observed.

As noted above, Peluso et al. (*7*) reported that the GCHF/DMSO/OO mixture induced a dose-dependent formation of DNA adducts in the liver and kidneys of mice injected intraperitoneally at doses of 400, 500, and 600 mg/kg. The relative adduct levels (RAL, expressed as adducts per 10^9 nucleotides) reported at these dose levels were 8, 15, and 17, respectively, in liver and 19, 22, and 30, respectively, in kidneys. The significance of these RALs should not be taken at face value, however, and should instead be assessed within the context of the formation of endogenous adducts that arise from natural metabolic processes and environmental factors.

For example, upper range RAL values for several types of normal endogenous adducts have been reported to be 70–2100 cyclic adducts/10^9 nucleotides in human liver and 1400 alkylated bases/10^9 nucleotides in human lung (*23*), and levels of oxidized bases arising from natural oxidants are much higher still (e.g., 700–23000 oxidized bases/10^9 in human white blood cells). Therefore, the levels of adducts reported by Peluso et al. (*7*) (8−17 and 19−30 adducts/10^9 nucleotides in liver and kidney, respectively) are very low compared to typical levels of endogenous adducts, and their biological relevance is consequently suspect, especially considering the fact that the adducts were produced after relatively large doses of a complex test material mixture that was injected directly into the intraperitoneal cavity.

Another consideration in evaluating the significance of the study conducted by Peluso et al. (*7*) is that the identification of

Table 4. Terminal Body Weights and Organ Weights for CD-1 Mice (Group 3) Following Intraperitoneal Administration of Isotonic Saline, GCHF[a] in DMSO[b]/OO[c], or GCHF without Glyphosate in DMSO/OO

dose group (8 or 10 mice/group)	body wt (g)	liver wt (g)	liver-to-body wt ratio (×100)	kidney wt (g)	kidney-to-body wt ratio (×100)
isotonic saline control, 24 h sacrifice	29.7 ± 1.2	1.88 ± 0.17	6.33 ± 0.44	0.547 ± 0.057	1.84 ± 0.19
GCHF (600 mg/kg) in DMSO/OO, 24 h sacrifice	29.4 ± 1.8	1.61 ± 0.15[d]	5.49 ± 0.42[d]	0.444 ± 0.062[d]	1.51 ± 0.20[d]
GCHF (without glyphosate) in DMSO/OO, 24 h sacrifice	29.3 ± 1.8	1.60 ± 0.13[d]	5.46 ± 0.40[d]	0.429 ± 0.059[d]	1.47 ± 0.23[d]

[a] GCHF, glyphosate-containing herbicide formulation. [b] Dimethyl sulfoxide. [c] Olive oil. [d] Statistically significantly different from control, $p \leq 0.01$.

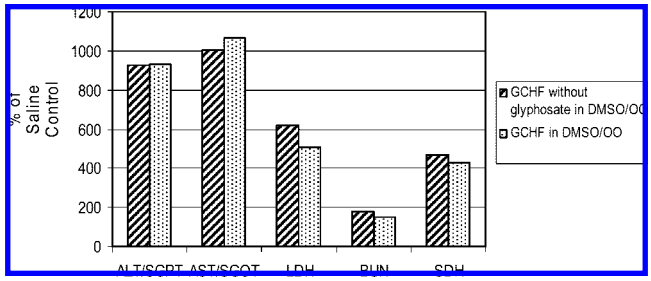

Figure 5. Comparison of clinical chemistry values for CD-1 mice following intraperitoneal administration of GCHF (600 mg/kg, 24 h) in DMSO/OO and GCHF without glyphosate (600 mg/kg, 24 h) in DMSO/OO. The values presented are shown as the percentage of clinical chemistry values for the treatments compared to saline controls. ALT/SGPT, alanine aminotransferase; AST/SGOT, aspartate aminotransferase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; SDH, sorbitol dehydrogenase.

adducts was not performed. It is known that the ^{32}P-postlabeling methodology used can label certain compounds and mimic the behavior of DNA adducts. For example, the labeling of bile salts causes them to appear as adduct-like spots (*24*). There are also cases of adducts having been formed from endogenous metabolites arising from normal metabolic processes, including cases in which treatment has induced increases in adducts derived from endogenous metabolites (*23, 25*). In addition to the potential to label endogenous compounds, it is possible that one or more of the formulation/test material ingredients might be capable of becoming labeled and therefore have an adduct-like appearance. Finally, it is conceivable that adducts were derived from lipid peroxidation products, induced by an oxidative toxic response caused by the dosing regimen used. The inclusion of appropriate controls or the characterization of adducts could have tested these possibilities, but it does not appear that these were done. In the absence of these controls, it cannot be definitively concluded that DNA adducts were actually produced or if adducts were produced that reflect covalent DNA binding of formulation components or metabolites of formulation components.

Comparison of Toxicity Following Intraperitoneal and Oral Administration of the GCHF/DMSO/OO Mixture. In contrast to the marked hepatic and renal toxicity observed following ip injection of the GCHF/DMSO/OO mixture, there was no evidence of adverse effects following oral administration. This is best illustrated by the sharp contrast between the occurrence of numerous histopathological lesions and large increases in serum enzyme levels (**Figure 6**) following ip injection compared to the complete lack of such effects after oral dosing. Oral ingestion is a more relevant route of exposure for the general population that consumes agricultural products and/or food derived from such products following the agricultural use of herbicides. Because the human consumption of glyphosate in food occurs only at extremely low levels (*1, 26*) and oral administration of the GCHF in mice produced no adverse effects at levels exceeding this intake by several orders of magnitude, it is concluded that results from studies using ip injection have no real significance for human risk assessment.

Summary and Conclusions. In a series of four inter-related assays as described herein, it was determined that high-dose ip administration of a GCHF produced significant liver and kidney toxicity. This suggests that methodology involving ip injection of GCHF may induce secondary effects mediated by local toxicity rather than genotoxicity. Importantly, there was no evidence of adverse effects following oral administration of GCHF. The experimental methods (high-dose, ip injection) used by Bolognesi et al. (*8*) and Peluso et al. (*7*) also produced marked hepatic and renal toxicity from exposure to a GCHF. Furthermore, the location and nature of the lesions resulting from this exposure scenario (i.e., direct injection into the intraperitoneal cavity) indicate that they, too, are most likely responses to local deposition of the GCHF rather than systemic toxicity. Thus, these experimental conditions (*7, 8*) do not assess the potential in vivo genotoxicity of the GCHF from the perspective of real-life exposure scenarios, and the DNA findings reported should not, therefore, be considered to constitute convincing evidence of relevant genotoxic activity for glyphosate or glyphosate formulations. The occurrence of severe hepatic and renal toxicity under these extreme conditions strongly indicates, instead, that effects on DNA, such as strand breaks and the formation of oxidized bases, if they do occur, may represent a secondary effect related to toxicity. Furthermore, current results indicate that some combination of the surfactant/DMSO/OO mixture is responsible for the effects reported by Peluso et al. (*7*) and, thus, such effects would not occur under actual use conditions of the GCHF. The large increases in 8-OHdG reported by Bolognesi et al. (*8*) were not reproduced here. Because of the more robust nature of the present investigation, the previous studies do not appear to provide sufficient evidence to conclude that high-dose ip administration of glyphosate causes oxidative damage to DNA. Oral administration, a route of exposure most relevant to the general human population, did not produce the hepatic or renal toxicity that occurred after ip injection. Thus, effects on DNA that are secondary to cytotoxicity would not occur following a more

Table 5. Terminal Body Weights and Organ Weights for CD-1 Mice (Group 4) Following Oral Administration of Isotonic Saline or GCHF[a] in DMSO[b]/OO[c]

dose group (8 or 10 mice/group)	body wt (g)	liver wt (g)	liver-to-body wt ratio (×100)	kidney wt (g)	kidney-to-body wt ratio (×100)
isotonic saline control, 24 h sacrifice	32.2 ± 1.2	1.71 ± 0.12	5.32 ± 0.37	0.555 ± 0.043	1.72 ± 0.11
GCHF (600 mg/kg) in DMSO/OO, 24 h sacrifice	31.8 ± 1.3	1.58 ± 0.13[d]	4.96 ± 0.34[d]	0.558 ± 0.050	1.76 ± 0.19

[a] Glyphosate-containing herbicide formulation. [b] Dimethyl sulfoxide. [c] Olive oil. [d] Statistically significantly different from control, $p \leq 0.05$.

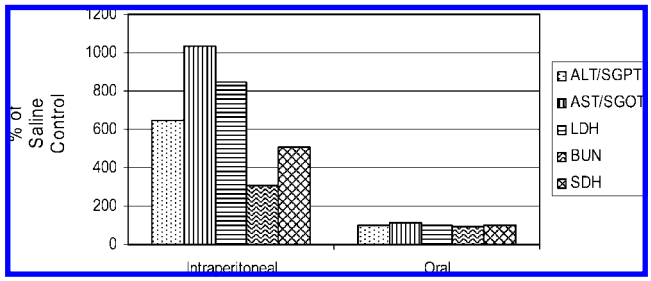

Figure 6. Comparison of clinical chemistry values for CD-1 mice following oral and intraperitoneal administration of GCHF in DMSO/OO (600 mg/kg, 24 h). The values presented are shown as the percentage of clinical chemistry values for the treatments compared to saline controls. ALT/SGPT, alanine aminotransferase; AST/SGOT, aspartate aminotransferase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; SDH, sorbitol dehydrogenase. Statistical analyses were not performed.

likely actual exposure scenario, that is, dietary intake. The results from these studies continue to support the conclusion that glyphosate and GCHF are not genotoxic under exposure conditions that are relevant to humans.

ABBREVIATIONS USED

8-OHdG, 8-hydroxydeoxyguanosine; ALT/SGPT, alanine aminotransferase; AST/SGOT, aspartate aminotransferase; BUN, blood urea nitrogen; DMSO/OO, DMSO in olive oil; GCHF, glyphosate-containing herbicide formulations; GLP, Good Laboratory Practice; ip, intraperitoneal; IPA, isopropylamine; LDH, lactate dehydrogenase; NMO, NADPH menadione oxidoreductase; PCR, Polymerase Chain Reaction; SD, standard deviation; SDH, sorbitol dehydrogenase

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